Cellular Subclasses in Human Leukemic Hemopoiesis

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Abstract

Cellular organization and communication in leukemic hemopoiesis may be compared with its counterpart in normal hemopoiesis. Results obtained using cell culture methods have provided some support for the view that leukemic hemopoiesis, like normal hemopoiesis, may involve 3 levels of differentiation: leukemic stem cells, committed leukemic progenitors, and more mature cells. Evidence is also beginning to emerge that leukemic populations may be regulated by messages from the environment in a manner analogous to normal hemopoiesis. The apparent similarities between leukemic and normal hemopoiesis raise the possibility that the target cell for leukemic transformation is the normal pluripotent stem cell. The development of culture methods for the production of leukovirus-like particles from human leukemic cells provides a possible first step toward the direct identification of leukemic target cells.

Indrotuction

The purpose of this paper is to discuss leukemic hemopoiesis in relation to its normal counterpart. The approaches being used to investigate leukemic hemopoiesis are analogous to those that have, over the last decade, provided information about cellular organization and communication in the normal hemopoietic system. These approaches, principally using developmental methods based on colony formation either *in vivo* or in culture, have led to a generally accepted view of interrelationships among normal hemopoietic progenitors (1, 2). The earliest identified class is the pluripotent stem cells, whose proliferative potential includes sufficient capacity for self-renewal to maintain the system (3). The next position in hemopoietic lineage is occupied by populations of progenitor cells each committed to a specific differentiation pathway such as granulopoiesis, erythropoiesis or megakaryocytopoiesis. These committed progenitor cells, while probably lacking sufficient proliferative potential for self-maintenance, permit subpopulations of cells within the different pathways of differentiation to expand independently of each other, giving rise to a variety of functional cells in appropiate numbers.

Each stage in hemopoietic differentiation represents a potential site of regulation. It has been proposed (4) that distinct classes of "managerial" cells with specialized regulatory functions coexist with the classes of cells subject to their control. A defect in the function of one such managerial cell class has been detected in genet-

ically anemic *Sl/Sl^d* mice (5). Recently developed cell culture methods have greatly extended the study of regulatory mechanisms. Extensive studies on granulopoiesis in culture have shown that diffusible substances derived from another coexistent cell class can promote the production of granulocytes (6, 7). Although extrapolation from events observed in cell culture to the corresponding events *in vivo* must be done with great caution, the analytical power of cell culture techniques provides justification for their use to study regulation.

This background of conceptual and methodological information about normal hemopoiesis makes it feasible to examine leukemic hemopoiesis. Specifically, one can ask whether or not some of the organizational and regulatory features of normal hemopoiesis persist in human leukemia. Some evidence that this is the case, based on results obtained using cell culture methods, will be summarized below.

Heterogeneity in leukemia

Leukemic cell populations might be considered to retain features of normal hemopoiesis if they were, like normal hemopoietic populations, composed of heterogeneous cell classes interrelated as cell lineages and responsive to regulatory messages. Initial evidence in favor of this view came from the application to leukemic cell populations of methods for growing granulopoietic colonies in cell culture. Reports from a number of laboratories indicated that marrow specimens from patients with apparently similar clinical forms of leukemia behaved very differently in culture. For example, in some patients with acute myelogenous leukemia (AML), colony formation was greatly diminished or absent, while in other patients it exceeded normal levels (8, 9). Thus, cell populations from different leukemic patients showed heterogeneous behaviour in cell culture. In addition, it was evident that marrow from some patients with acute leukemia contained cells capable of differentiation in culture and, like their counterparts in normal marrow, were dependent for growth in culture on the presence of appropriate stimulatory factors (see, for example, refs. 10, 11). Thus, these initial observations were compatible with the view that analogies might be found between leukemic and normal hemopoiesis.

More clear-cut evidence for heterogeneity in leukemic cell populations has emerged from studies of leukemic cells in liquid cultures (12, 13). Large quantities of morphologically identified blasts may be obtained from the peripheral blood of patients; repeated experiments can be done on cells from a single source by storage of the cell populations at –70 °C in 5 % dimethyl sulfoxide. When such cells are placed in fluid cultures in appropriate media, little change in cell number is usually observed over a period of many days. Nonetheless, active proliferation is occurring; when cultures are pulse-labelled at different times with ^{3}H-thymidine, extensive increases are observed in the incorporation of the label into acid-insoluble material (12). This incorporation is associated with increased numbers of labelled cells as detected by autoradiography. The increase in ^{3}H-thymidine incorporation is sensitive to ionizing radiation and survival curves have been obtained with parameters characteristic of mammalian cell proliferation (Fig. 1) (14). Recent studies based on the technique of limiting dilution indicate that the proliferative subpopulation detected by ^{3}H-thymidine incorporation is a minority one, con-

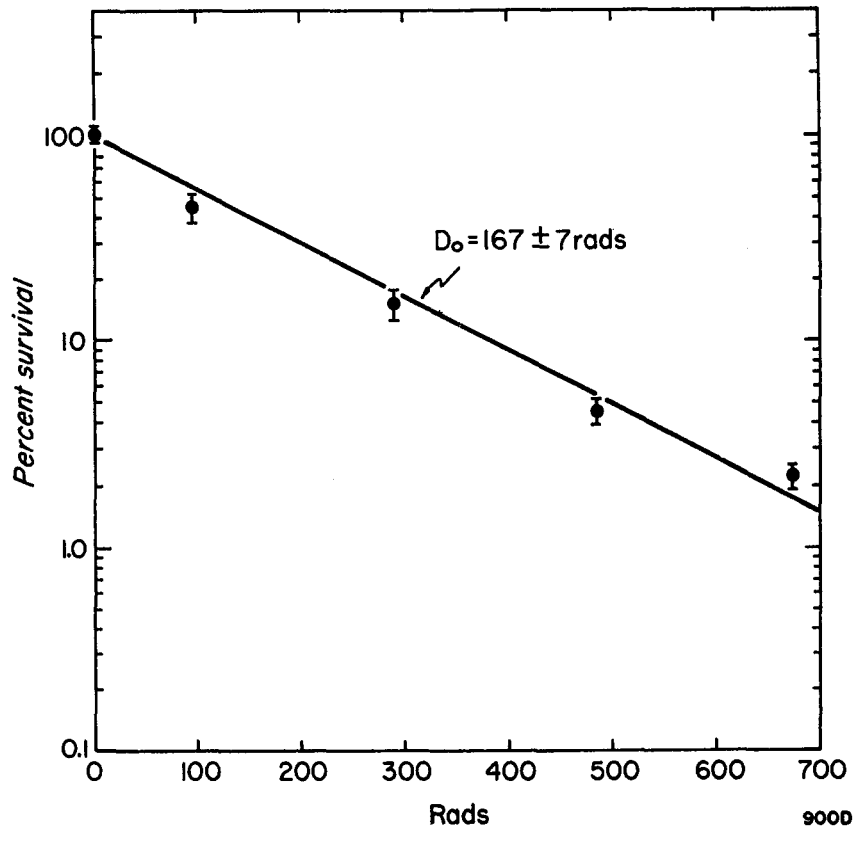

Fig. 1: Radiation survival curve for the incorporation of ^{3}H-thymidine by cells in liquid culture, derived from a patient with AML. Ordinate: percent survival of ^{3}H-thymidine incorporation over 45 min by 10^{5} cells per ml in 3 ml liquid cultures, 4 cultures per point. The cells were exposed to ^{3}H-thymidine 8 days after irradiation and initiation of the cultures. Abscissa: dose of ^{137}Cs gamma radiation. The D_0 value is the dose required to reduce the percent survival to 37 % of the initial value. The errors indicated are standard errors.

sisting of between 1:100 and 1:1000 of the total cells (14). If it is assumed that at least a portion of the proliferating subpopulation consists of leukemic cells (15), then these results are compatible with the view that a minority population of leukemic cells is able to proliferate and contribute to the numbers of a larger leukemic population with a modified capacity for proliferation. Such a situation would be analogous to that found in normal hemopoietic differentiation where a minority population of stem cells proliferates and gives rise to a large population of progeny, most of which have lost stem cell properties.

Regulation in populations of leukemic cells

Two of the major properties of stem cells are the capacity for extensive proliferation including self-renewal, and the capacity to give rise to cells with different characteristics. Approaches to the identification of cells with these properties in a minority subpopulation in leukemia were outlined above. The third major property of stem cells is their sensitivity to control mechanisms (16). Some evidence for cellular interactions of a regulatory nature has also been obtained from studies of leukemic peripheral blood cells in fluid cultures. When the usual synthetic culture

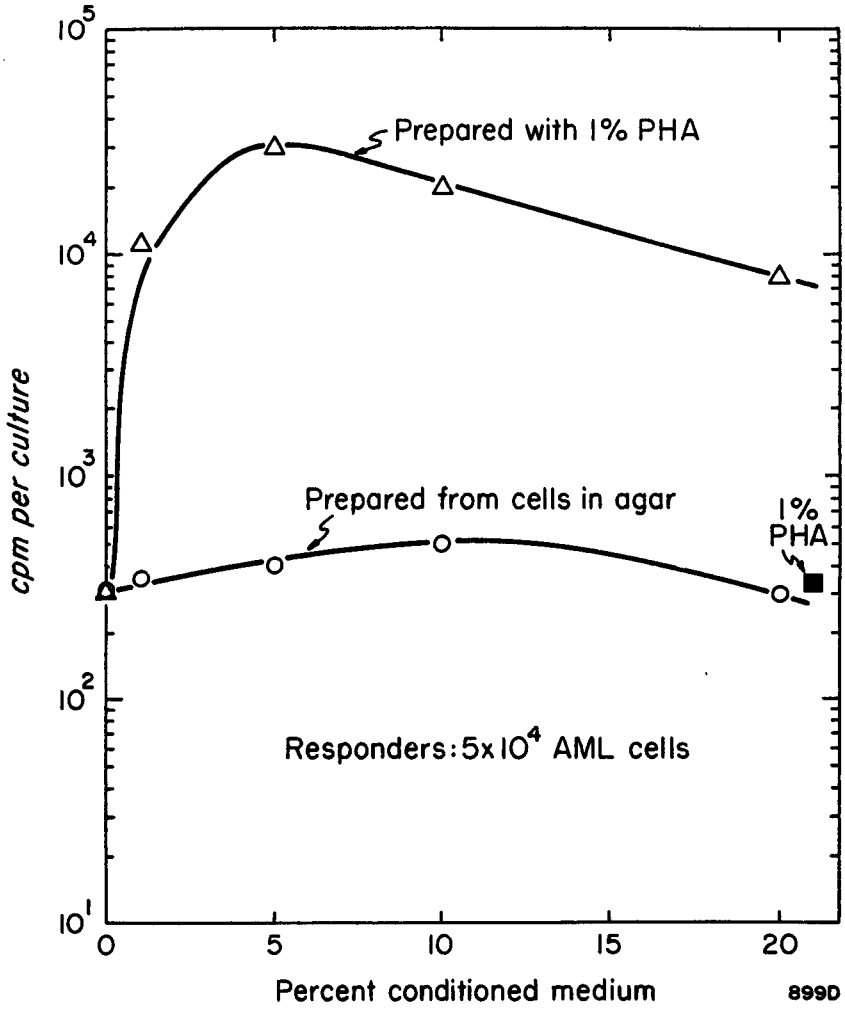

Fig. 2: Stimulation of ^{3}H-thymidine incorporation into AML cells in liquid culture by various concentrations of leukocyte-conditioned medium. Triangles: an active conditioned medium prepared in liquid culture in the presence of 1 % phytohemagglutinin (PHA) (12). Circles: a conditioned medium prepared from cells immobilized in agar, in the absence of PHA (47); this conditioned medium showed little or no activity when assayed on these particular responder cells. Closed square: the same conditioned medium as for the results shown as circles, but with 1 % PHA added to the cultures of responder cells together with the conditioned medium.

medium supplemented with fetal calf serum was supplemented further by the addition of supernatants from cultures of normal or leukemic leukocytes, incorporation of ^{3}H-thymidine into peripheral blood cells from some patients increased at a more rapid rate. Although considerable patient-to-patient variation has been observed, it is usually possible under conditions of limiting dilution to demonstrate that the leukemic populations contain cells that proliferate only in the presence of an active leukocyte-conditioned medium (Fig. 2). Using cell separation methods, preliminary evidence has been obtained that leukemic populations not only contain cells capable of responding to growth-promoting factors, but also another class of cells capable of producing these growth-promoting factors, either spontaneously or in response to phytohemagglutinin (13). Thus, evidence is available that the third major stem cell property, sensitivity to control mechanisms, may be retained by leukemic stem cells. This implies that a basic feature of normal

granulopoiesis in culture, regulation by cellular interaction, is also found in leukemic populations.

Committed progenitor cells in leukemia

Progenitor cells committed to granulopoiesis can be detected readily by their capacity to form granulopoietic colonies in culture (6). The granulopoietic progenitors detected using this assay in the marrow or blood of patients with leukemia might belong either to the leukemic population or to a co-existing normal population. Convincing evidence for the existence of leukemic committed progenitors has been obtained for patients with chronic myelogenous leukemia (CML). The Philadelphia chromosome has been identified in colonies formed by cells from some but not all patients with Ph^+ CML (11, 17–20). Analogous evidence is available for acute myelogenous leukemia (AML) in that characteristic chromosomal abnormalities found in direct marrow preparations have been identified in pooled cultures (21) and in individual colonies (10, 11) derived from the marrow or peripheral blood of leukemic patients. These results indicate that at least some committed progenitors found in leukemic cell populations are of leukemic origin. In some patients, therefore, leukemic stem cells give rise to progeny as detected by the culture assay for granulopoietic progenitors. Although the differentiation processes occurring in cultures derived from leukemic granulopoietic progenitors may be abnormal (10, 22, 23), these progenitors are, like their normal counterparts, dependent for their growth in culture on diffusible factors derived from either normal or leukemic cells (8–11). We conclude that, in addition to cellular heterogeneity in leukemia of the kind to be expected if leukemic stem cells are present, evidence for parent-to-progeny lineage relationships also exists and that leukemic committed granulopoietic progenitors respond in culture to factors similar to those that regulate differentiation in culture by their normal counterparts.

Erythropoietic differentiation from pluripotent leukemic stem cells has been demonstrated in CML on the basis of chromosomal evidence (24–26). Analogous though less extensive evidence is also available for acute leukemia (27, 28). Colony techniques are now available for human erythropoietic progenitors (29, 30); as these are applied to cells from patients with leukemia it will become possible to investigate the potential for differentiation of leukemic erythropoietic progenitors in a manner similar to the studies already carried out on leukemic granulopoietic progenitors (10, 11, 21).

Diffusible regulators of cell growth in culture

Granulopoietic colony formation in culture is dependent on the presence of a suitable source of certain diffusible factors. These factors, collectively termed colony stimulating activity (CSA) have been studied extensively in the serum and urine of patients with leukemia. Studies on sera have been complicated by the presence of inhibitors (31) and even when these were removed, correlation of CSA serum levels with clinical status was not observed (32). These CSA measurements have generally been complicated by the use of mouse marrow cells rather than human marrow cells in the assay procedure. Evidence is available that CSA

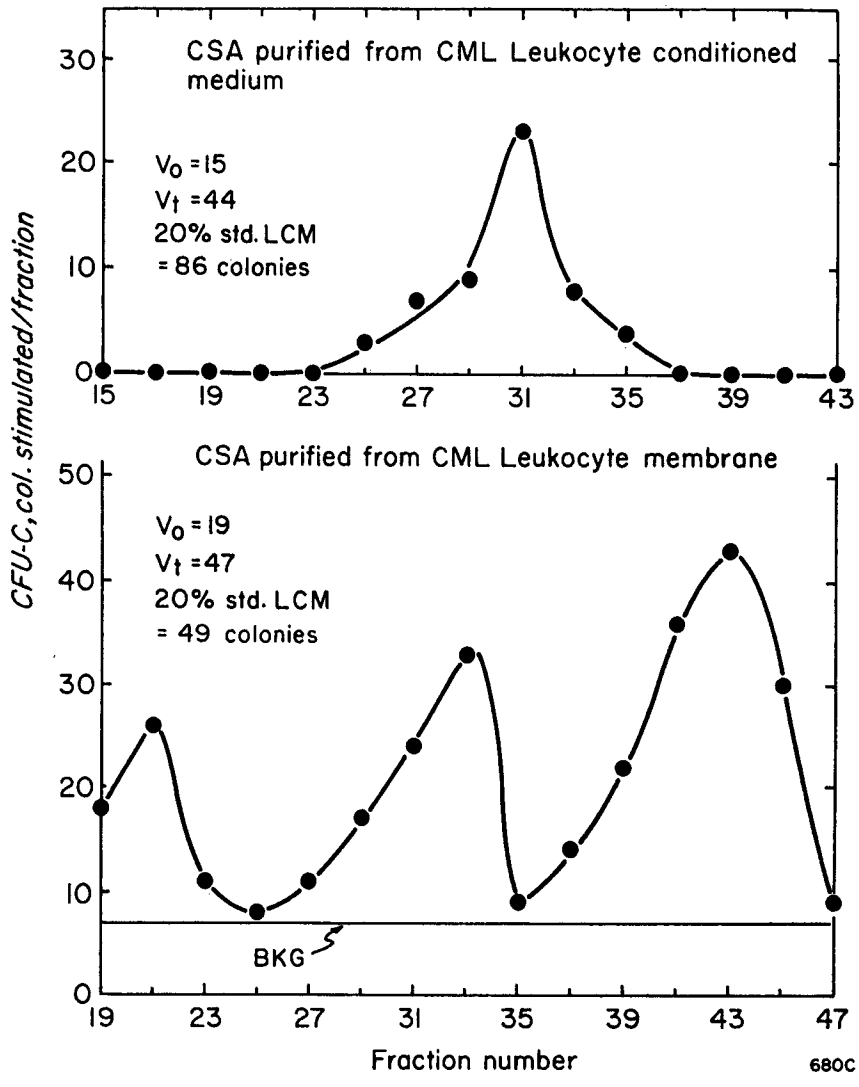

Fig. 3: Gel filtration on Sephadex G-150 of CSA assayed by stimulation of colony formation by granulopoietic progenitor cells (CFU-C). Upper panel: CSA purified from leukocyte-conditioned medium (LCM) prepared from peripheral leukocytes of a patient with chronic myelogenous leukemia (CML). Lower panel: CSA purified from surface membranes of leukocytes from the same CML patient, after solubilization of the membranes in 2 % sodium dodecyl sulfate. The methods used for isolation of membranes and purification of CSA have been described (34, 36).

detected by the mouse assay need not be the same as CSA detected using human cells (33). For this reason, much of the work on CSA in leukemia is difficult to interpret.

More recent studies using cells of human origin to assay for either CSA or CSA-producing cells have yielded a more consistent pattern. A convenient source of CSA active on human granulopoietic progenitors is medium in which normal or leukemic leukocytes have been cultured for 3–7 days. Purification of CSA from such leukocyte conditioned media has revealed four apparent molecular species; of these, three are nondialyzable and have molecular weights of approximately 93,000, 36,500 and 14,700 (34). The fourth species is dialyzable, hydrophobic and has a molecular weight of less than 1300 (35). The cellular location of the three

high molecular weight species of CSA has been investigated and they have been found in assiciation with cell surface membranes (36).

Leukemic cells also add CSA to culture media. However, when peripheral leukocytes from newly-diagnosed leukemic patients are used to prepare conditioned media, and the same purification procedure as that used for media conditioned by normal leukocytes is applied, only one of the three species of high molecular weight CSA is detected in the leukocyte conditioned media (34).

The difference in number of high molecular weight species of CSA detectable in media conditioned by normal and leukemic leukocystes does not appear to be simply the result of differences in the relative numbers of various cell classes in the two populations. Evidence supporting this view is presented in Figure 3, which shows results from a direct comparison of high molecular weight CSA in media conditioned by cells from a patient with CML and high molecular weight CSA obtained from the surface membranes of cells from the same patient. The leukocyte conditioned medium yielded only a single species of high molecular weight CSA, even though the cells in the peripheral blood of this same patient contained all three species, and the cell classes present included all those found in normal blood.

This approach has also been applied to cells from patients with idiopathic sideroblastic anemia, a condition known to be associated with a high incidence of leukemic transformation. Leukocyte conditioned media were prepared from peripheral leukocytes of six patients with idiopathic sideroblastic anemia. Although the distribution of cell classes in the blood of these patients was not abnormal, in three instances only a single species of high molecular weight CSA was detected in the leukocyte conditioned media (37). Two of these three patients subsequently developed leukemia while the third died of myocardial infarction three months after his cells were assessed. None of the patients whose leukocytes released all three high molecular weight species of CSA have developed leukemia (5–12 months of follow-up to date). The controls in these studies consisted of four patients with secondary or congenital sideroblastic anemia. Leukocyte conditioned media prepared from their leukocytes contained the usual three species of high molecular weight CSA.

These data are consistent with the view that the capacity of leukocytes to release these particular bioactive molecules into culture media is an expression of their phenotype. The findings in sideroblastic anemia support the hypothesis that a reduced capacity of leukemic leukocytes to release certain species of CSA in culture may be related to the leukemic phenotype rather than a secondary manifestation of disordered cell metabolism or altered distribution of cellular populations. Since high molecular weight CSA species are located in or near cell membranes the proposal that their abnormal release in leukemia is part of the leukemic phenotype is consistent with other evidence suggesting membrane changes in this disease and reminiscent of the model proposed for regulation of normal granulopoiesis by membrane interactions (36, 38, 2). If the molecular species that are essential for proliferation and differentiation in culture are also physiologically active *in vivo,* the decreased ability of leukemic leukocytes to release such bioactive membrane components may contribute to abnormal cell regulation in leukemia. In any event, the findings provide further support for the view that leukemic populations retain some of the regulatory features of normal hemopoiesis.

Leukemic Target Cells

The cell culture data summarized above provide clues that leukemic populations may be heterogeneous, with a hierarchy of cell classes linked by lineage relationships and responsive to regulatory mechanism in a manner analogous to the relationships found in normal hemopoiesis. These results, however, do not bear directly on the relationship between leukemic stem cells and their normal counterparts. The evidence for both granulopoietic and erythropoietic differentiation in CML and AML makes it attractive to consider that the target cell for leukemic

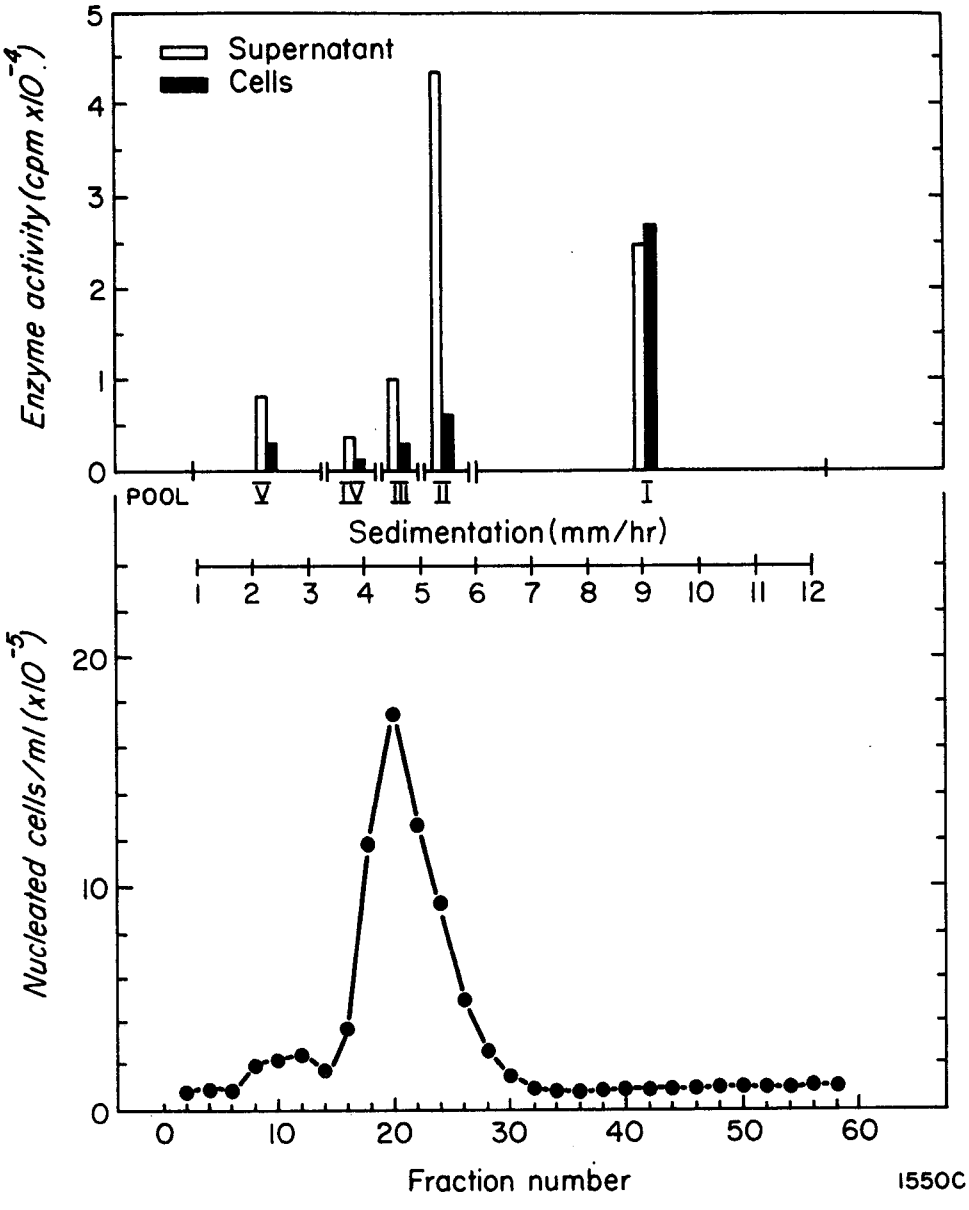

Fig. 4: Velocity sedimentation profile of marrow cells from a patient with acute lymphocytic leukemia (ALL). Lower panel: profile of nucleated cells. Upper panel: distribution of reverse transcriptase activity in pools of cells cultured for 5 days. The total enzyme activities in the supernatant medium and the cells from each pool are shown as bars. Reverse transcriptase activity as stimulated by poly $rC(dG)_{12-18}$ was assayed after the cells and supernatant medium were fractionated in sucrose gradients, as described (39, 40, 46).

transformation is the normal pluripotent stem cell. If this view is correct, then the various types of leukemia recognized morphologically must be determined by the transforming events rather than by the cell class in which they occur.

If the normal pluripotent stem cells are the target cells for leukemic transformation, then methods for studying this transformation are required in order to make a direct identification of the leukemic target cells. A start toward this goal has been taken wtih the development of culture methods which result in the production of particles with many of the physical, biochemical and morphological properties of leukoviruses (39–43). The biological significance of the production of these virus-like particles by leukemic cells is still unclear; in particular, it is not known whether or not they are able to cause a leukemic transformation of normal hemopoietic cells. However, if the cellular heterogeneity in leukemic populations is related, as in the normal, to patterns of growth, it might be anticipated that any cell properties with functional significance would be associated with specific subpopulations rather than with all cells in the leukemic populations. From this viewpoint, the capacity to produce leukovirus-like particles may be regarded simply as a functional property of cells, and one may test whether or not only a subpopulation of leukemic cells exhibit this particular function. On the basis of this reasoning, leukemic populations were separated by velocity sedimentation, and fractions were pooled to yield suspensions of approximately equal cell numbers. These were then tested for their capacity to release virus-like particles, using as the criterion of particle release the presence of RNA-dependent DNA polymerase (reverse transcriptase) whose acitivity is stimulated by poly $rC(dG)_{12-18}$ and is associated with densities from 1.17 to 1.22 gm/ml. This criterion was chosen because poly $rc(dG)_{12-18}$ is considered to be a specific template for viral-related reverse transcriptase (44, 45) and because assays for enzyme activity revealed peaks of activity in density regions characteristic of intact virus or viral cores (1.17 and 1.22 gm/ml respectively). For cells from 6 of 12 patients, including patients with AML, ALL, CML, and AMML, particle release was associated with pools containing rapidly sedimenting (large) cells. An example of data from a typical experiment is shown in Figure 4. Cell populations from 6 other patients provided no evidence of particle release in cultures from any of the cell pools (46).

As controls, marrow from 11 patients without leukemia were separated by velocity sedimentation and pools of cells of appropriate sedimentation velocities were examined for particle release using the same criterion. In 4 of these experiments small but significant amounts of enzyme activity were detected, although at lower levels than those found for the 6 cases of leukemia where particle release was detected. In contrast to the leukemic populations, however, enzyme activity was always associated with pools of slow sedimentation velocity (small cells).

As emphasized earlier, the criterion for particle release used in these experiments was limited to the detection of reverse transcriptase activity in association with appropriate densities on sucrose gradients and stimulated by the artificial template poly $rC(dG)_{12-18}$. In the absence of more detailed characterization, particularly of the material obtained from normal cells, the relationship of the enzyme-containing particles to known viruses or to preparations from different patients can be only a matter of speculation. However, the cellular specificity of particle release is consistent with the view that this property reflects functionally important aspects

of the phenotypes of specific cell populations in normal and leukemic hemopoiesis. The relationship of these particle-releasing subpopulations to the other normal or leukemic cellular subpopulations remains to be determined.

Concluding Remarks

Information about leukemic populations is much too limited to permit the construction of a detailed model of cell lineage relationships. It is reasonable only to postulate the existence of leukemic stem cells capable of extensive proliferation, including self renewal, and of giving rise to other cell classes with different properties. The latter cell classes include leukemic progenitors committed to granulopoiesis and other leukemic progenitors committed to erythropoiesis. In acute leukemia, the major morphologically-recognizable population consists of blast cells, whose relationship to the leukemic stem cells is unknown. For example, the blast cells might be at an early level of differentiation analogous to that of normal committed progenitors, but prevented by the leukemic lesion from further maturation. Alternatively, they might be cells that have progressed along another pathway unique to the leukemic population. The limited amount of information that is available does, however, provide some support for the view that leukemic populations may be organized into cell lineages and regulated by messages from the environment in a manner analogous to normal hemopoiesis. If this viewpoint is correct, it should be possible to identify a class of "managerial cells" in leukemia. At present, it is not known whether messages able to modulate leukemic growth originate from cells within the leukemic population, or from normal managerial cells, or both. For leukemic leukocytes in culture, the release of a single species of high molecular weight CSA into culture medium, in contrast to the three high molecular weight species of CSA released by normal leukocytes, could be a direct manifestation in culture of the defective regulation characteristic of leukemic populations. Alternatively, the differences may arise from surface membrane changes in leukemic cells which are part of the leukemic phenotype, but not directly related to the defective regulation characteristic of leukemia. We have proposed (36, 38, 2) that these molecular species of CSA represent a novel class of surface membrane-associated bioactive macromolecules. From this viewpoint, they represent a means to detect surface membrane differences between normal and leukemic cells. To date, the only circumstances in which a reduction in numbers of high molecular weight species has been observed were for leukemic patients and for patients proven to be pre-leukemic (37). Thus, the shift from three high molecular weight species of CSA to one species may prove to be a very useful marker of leukemic or pre-leukemic cell populations.

Another very intriguing question concerns the possible significance of the capacity of a rapidly-sedimenting subpopulation of leukemic cells to release virus-like particles. Is the capacity of this particular subpopulation to produce particles a property only of leukemic stem cells? Should this be the case, it need not necessarily imply an etiologic role of such virus-like particles in human leukemia. Instead, it might be a reflection of a more extensive capacity for gene expression in the leukemic stem cells compared with other cell types present in leukemic populations. Such a marker for leukemic stem cells, whatever its functional role, would be of

great value. However, the basis for the enzyme activity detected in pools of slowly sedimenting cells derived from non-leukemic marrow needs to be clarified; it remains to be demonstrated that the particles produced by the normal and leukemic subpopulations are different enough to be useful as specific markers. Also, it should be stressed that the culture conditions used to obtain release of particles are unlikely to be representative of events occurring *in vivo*.

Taken together, the areas of ignorance greatly outnumber those where firm information is available. Nevertheless, the elucidation of the organization and regulation of leukemic cell populations should lead to novel therapeutic modalities; one can visualize, in addition to the toxic agents used to inactivate leukemic stem cells, a second generation of non-toxic agents able to modify regulatory mechanisms and thus control, in a more selective way, the proliferation and differentiation of cells in leukemic populations.

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