Clinical Utility of Bone Marrow Culture

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Abstract

Standardized culture of bone marrow in soft agar permits the detection of a population of granulocyte-macrophage progenitor cells (CFU-c). A spectrum of qualitative abnormalities serves to distinguish myeloid leukemic CFU-c from normal and remission populations. These abnormalities in maturation and proliferation are diagnostic of a myeloid leukemic state and serve to functionally reclassify acute myeloid leukemia at diagnosis into a number of categories based on in vitro growth pattern. The virtue of this classification is that it permits detection of a substantial number of patients who are refractory to conventional remission induction protocols. The clear distinction between normal and leukemic growth in vitro permits early detection of emerging remission CFU-c during induction therapy and of early onset of relapse in patients who are otherwise in complete remission. In patients with leukemia undergoing allogeneic bone marrow engraftment, marrow culture has proved of value in documenting the reconstitution of the patient and in detecting re-emergence of the original leukemic stem line prior to its detection by cytogenetic and hematological techniques.

Serial studies on patients with chronic myeloid leukemia have allowed early diagnosis of blastic transformation and classification of blastic phase disease on the basis of in vitro growth pattern has revealed a similar spectrum of in vitro abnormalities as seen in AML.

The cloning of normal or leukemic human myeloid progenitor cells (CFU-c) in agar or methylcellulose has permitted analysis of both quantitative and qualitative changes in this cell compartment in leukemia and other myelodysplastic states (1–7). Among these changes are abnormalities in maturation of leukemic cells in vitro (4, 5, 6), defective proliferation as measured by colony size or cluster to colony ratio (5, 6), abnormalities in biophysical characteristics of leukemic CFU-c (4, 5), regulatory defects in responsiveness to positive and negative feedback control mechanisms (8, 9) and the existence of cytogenetic abnormalities in vitro (10,

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11). Detection of this spectrum of abnormalities has proved of clinical utility in diagnosis of leukemia and preleukemic states (5, 6, 12), in classification of leukemias and myeloproliferative diseases (5, 6), in predicting remission prognosis and response to therapy (5, 13), in predicting onset of remission or relapse in AML (13) and in monitoring the progession of chronic myeloid leukemia or preleukemic disease (4, 14). The present communication serves to illustrate the clinical applications of bone marrow culture in these various areas.

In vitro growth characteristics of untreated Acute Myeloid Leukemia (AML) and its morphological variants

Marrow and, in the majority of cases, peripheral blood cultures, were established from 250 cases of untreated AML and its morphological variants (acute monocytic, myelomonocytic, promyelocytic, stem cell and erythroleukemia). One hundred seventy-four cases represented a random selection of patients presenting at 8 hospitals in Melbourne, Australia, over a period of 3 years and 76 cases presenting at the Memorial Sloan-Kettering Cancer Center over a period of 12

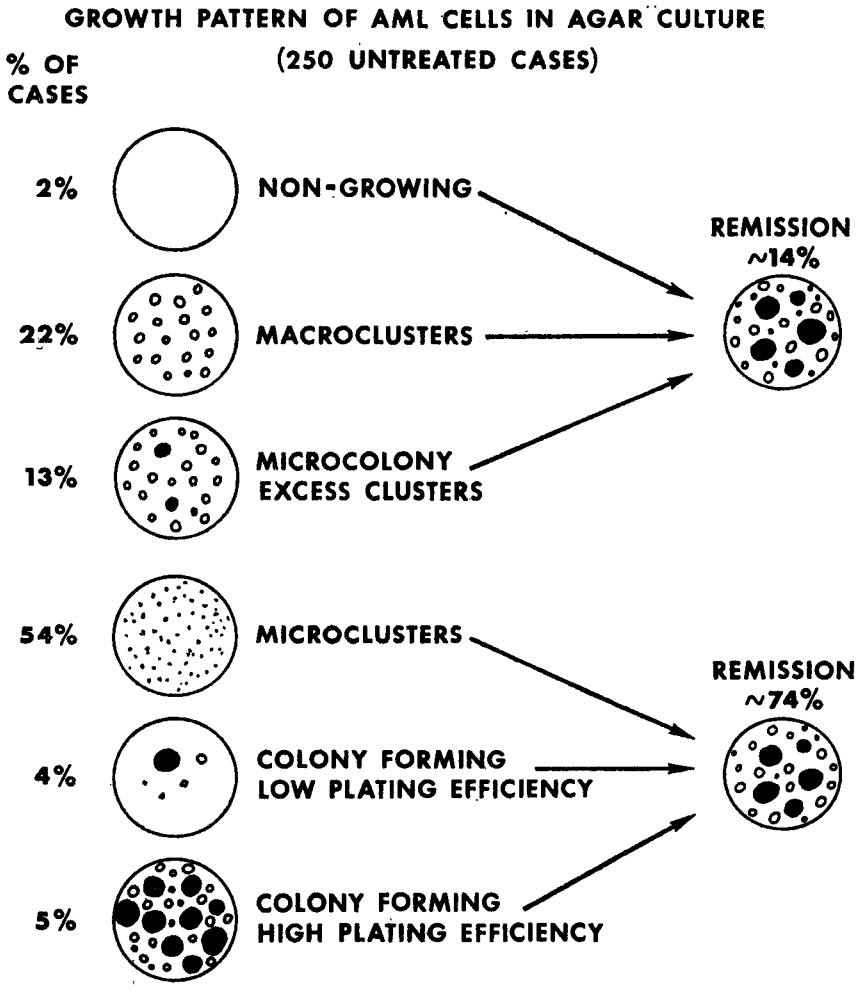

Fig. 1: Schematic representation of the in vitro growth patterns seen in 7 day cultures of marrow cells from 250 patients with untreated AML and its morphological variants. Remission incidence in the non-growing, macrocluster and microcolony category was 14 % and in the remaining categories, 74 %.

months. The clinical and hematological characteristics of these patients have been reported as have the therapeutic protocols and remission criteria (5, 13). All cultures were stimulated by a feeder layer of 1×10^6 normal WBC and were scored at 7 days for the presence of colonies of > 40 cells and clusters of 3–40 cells. For classification of leukemia according to growth in vitro we recognize the following categories of growth pattern (see Fig. 1): (a) non-growing: absence of persisting cells in CSF stimulated cultures with no colony or cluster formation detected in 4 cultures of 2×10^5 marrow cells per plate; (b) microcluster formation: absence of colonies and presence of varying numbers of clusters of 3–20 cells. The great majority of these cases exhibit a pattern of small clusters in marrow culture generally of only 3–10 cells with dispersion and degeneration. Included in this category are examples of extensive persistence of leukemic cells in CSF stimulated cultures without evidence of cluster formation at 7 days. Marrow cultures from these latter patients, when scored prior to 7 days, show cluster formation but with premature dispersion and degeneration of the clusters. The majority of microcluster forming leukemias would be considered as non-growing if scored later than 7 days; (c) macrocluster formation: absence of colonies and presence of varying numbers of clusters approaching the lower limit of colony size, i. e., up to 40 cells. If the cultures are scored later than 7 days, the majority of cases would show evidence of colony formation and merge with category (d); (d) small colonies (microcolonies) with an abnormal cluster to colony ratio: maximum colony size in this group is less than in control cultures and an abnormal excess of aggregates of less than 40 cells is seen (the normal ratio of colonies to clusters is between 2–10); (e) colony forming with a normal cluster to colony ratio at 7 days of culture: we have subdivided this category into cases showing a lower colony incidence than normal and cases with a marked elevation in marrow colony formation invariably associated with a pronounced increase in circulating CFU-c. Both groups share a similar prognosis, however, the former category is mainly comprised of cases where colony growth is non-leukemic, and thus is similar to the pattern seen in acute lymphoblastic leukemia, whereas the latter category merges with the growth patterns seen in chronic myeloid leukemia.

Classification of these patients on the basis of our previously reported correlation between growth pattern and remission rate (5, 6) indicated that 37 % of all these cases fell within a poor prognosis category (Fig. 1). The addition of 76 new cases of AML and their clinical outcome has not significantly altered the correlation we have observed between in vitro growth pattern and remission rate.

Ten patients included in this study had acute undifferentiated or "stem cell" leukemia and classification into myeloblastic or lymphoblastic type was not possible on the basis of morphology, cytochemistry or surface markers. Three of these cases showed a myeloid cluster forming growth pattern in marrow culture, whereas the remainder showed a colony forming pattern with low plating efficiency and normal granulocytic maturation. The indication that colony formation seen in these cases was due to persisting normal CFU-c coexisting with a non-myeloid acute leukemia was supported by cell separation studies. Buoyant density distribution of colony or cluster forming cells was determined by the application of a simplified density "cut" technique in which marrow or blood cells were centrifuged in bovine serum albumin of density 1.062 g/cm^3 and the distribution

of CFU-c in the supernatant and pellet fractions determined by subsequent agar culture (4, 5). 56 ± 4 % of cluster forming cells in untreated patients were of density < 1.062 g/cm³ in contrast to the normal distribution of CFU-c (1–10 % < 1.062 g/cm³). This light density distribution of CFU-c was also observed in the microcolony and high cloning efficiency colony forming acute leukemias (96 ± 5 % < 1.062 g/cm³). In contrast, the CFU-c in patients with low cloning efficiency colony forming acute leukemia had a normal density distribution (1.5 ± 1 % < 1.062 g/cm³), as did the CFU-c in untreated acute lymphoblastic leukemia.

Monitoring of relapse-remission status by marrow culture

Unequivocal complete remission was associated with return of a normal growth pattern in marrow culture (Fig. 1), a normal colony incidence and granulocytic maturation and a normal CFU-c buoyant density distribution. The correlation between return of normal colony formation and remission was investigated in a detailed analysis of 57 patients throughout the induction and consolidation phase of therapy (Table 1). patients were selected on the basis of marrow growth characteristics prior to therapy and only examples of non-colony forming AML were studied since appearance of colony formation during remission induction would provide a simple parameter for detection of non-leukemic progenitor cells. Of the 30 patients who showed return of colony formation at some point during induction, 29 achieved complete remission, on average 21 days after first detection of colonies. No examples were observed of a leukemic growth pattern persisting in clinical remission. There was no correlation between the actual number of colonies observed and the time to remission, however, preliminary analysis indicates some correlation between initial colony incidence and duration of remission.

The value of marrow culture in predicting the onset of relapse was investigated in 83 cases of cluster-forming AML where complete remission had been achieved. In this analysis, four patterns of relapse emerged.

Table I: Analysis of Colony Formation During Remission Induction in 57 Patients with Cluster Forming AML

Number of Patients Showing Colony Formation	30/57
Number of Patients Attaining Remission	29/57
Marrow Colony Incidence at Time of First Detection of Colonies (Mean + Range)	27 (1–172)/10^5
Density Distribution of Colony Forming Cells (% < 1.062 g/cm³)	8.5 ± 0.5
Time to Appearance of Normal Colonies (Days Mean + Range)	41 (5–85)
Time to Complete Clinical Remission (Days Mean + Range)	62 (20–105)

(a) Most frequently observed was a concordance of a clinical diagnosis of relapse with a complete return to a cluster forming leukemic growth pattern.
(b) Loss of colony formation and return to a cluster-forming growth pattern 1–4 weeks prior to clinical and hematological evidence of relapse.
(c) Coexistence of normal and leukemic colony and cluster forming cells for varying periods preceding overt relapse. Discrimination between normal and leukemic cells was possible on the basis of colony size, cell morphology and the dispersion or degeneration of the leukemic clusters. Density gradient separation and cytogenetic analysis of individual colonies and clusters have further confirmed the coexistence of normal and leukemic CFU-c in marrow cultures prior to clinical evidence of relapse.
(d) The fourth category comprised patients who showed evidence of early relapse based on hematological criteria including elevated marrow blast cell incidence (without detectable Auer rods), presence of immature cells in the circulation and, in the case of patients presenting with acute monocytic or myelomonocytic leukemia, abnormal monocytoid cells in marrow and blood with qualitatively normal colony and cluster formation. In this category there exists a clear discrepancy between the interpretation of marrow morphology and the in vitro culture parameters which showed no evidence of leukemic cell proliferation. This paradox was largely resolved by sequential analysis of CFU-c in the marrow of patients in prolonged remission. A striking variation in the incidence of marrow CFU-c was observed in a number of patients which could neither be attributed to technical variation nor in any direct sense, to the maintainance protocol.

Correlated with the periodicity of marrow CFU-c in many patients was a fluctuation in marrow blast cell incidence. A marked increase in CFU-c was frequently associated with or closely followed by an increase in marrow blast count to levels compatible with early relapse. The majority of such cases were treated with intensive reinduction; however, a number were continued on maintainance therapy and, in these cases, both blast cell and CFU-c incidence returned to normal levels in subsequent marrow aspirates and the patients remained in complete remission.

The detection of incipient relapse in patients who presented with leukemia characterized by the formation of microcolonies with an excess of clusters was aided by monitoring the cluster to colony ratio together with morphological analysis of colonies and buoyant density characterization of CFU-c. In the case of colony forming leukemias with a normal cluster to colony ratio, colony morphology and, specifically, CFU-c buoyant density were the main diagnostic parameters capable of distinguishing between relapse and remission status.

Marrow culture parameters in preleukemic disorders

The situation in preleukemic disorders is not unlike that observed in AML remission patients during the time immediately preceding the onset of relapse. We have observed that in such preleukemic states as refractory sideroblastic anemia associated with a variable spectrum of cytopenias or with chronic monocytosis, that a spectrum of qualitative defects can be detected in a proportion of cases. These defects are identical to those seen in overt myeloid leukemia and precede

progression to leukemia by 3–18 months. A number of such cases show coexistence of normal and leukemic cell proliferation preceding overt relapse. Clinically identical cases exhibiting no qualitative defects but generally with a depressed incidence of CFU-c in marrow culture did not progress to an acute leukemic phase without first developing qualitative defects in CFU-c maturation or proliferation.

The heterogeneity of AML in relapse is reflected in the spectrum of defects observed in progression from preleukemia to overt leukemia. Prediction of clinical progression of preleukemic disorders must therefore be based on recognition of clonal evolution with progressive derangement in CFU-c proliferation and/or differentiation similar to that observed in the progression from chronic to acute phase in CML (6). Alternatively, the demonstration by cell separation and/or marrow culture of coexisting normal and leukemic populations in "preleukemia" is similar to the situation seen in many cases of early relapse of AML and should be considered not as preleukemia but as early leukemia since the minor leukemic clone progressively expands but retains its characteristic spectrum of qualitative abnormalities.

In vitro characteristics of chronic myeloid leukemia at diagnosis and in blastic transformation

One hundred-three patients with Ph^1 positive CML were studied at various stages of their clinical course; of these 66 were investigated at the time of first diagnosis. With the exception of 8 patients, a characteristic pattern of presentation was seen. The incidence of colony and cluster forming cells in marrow was increased on average 15X normal and circulating CFU-c on average 500X normal. The ratio of clusters to colonies, an important diagnostic parameter when predicting blastic transformation, was consistently within the lower range of normal. CFU-c in CML at all stages of the disease were of an abnormally light buoyant density as determined by continuous density gradient or equilibrium density centrifugation in bovine serum albumin. The abnormal density distribution of CFU-c appears to be a characteristic of the myeloid leukemic state and is only seen in normal hematopoiesis during fetal life (15), suggesting the possibility of an oncofetal transformation associated with leukemogenesis (14).

Analysis of the in vitro characteristics of marrow and blood of 42 patients at the time of clinical diagnosis of blastic transformation revealed in every case defects in proliferation and maturation which served to distinguish this phase from chronic phase disease. A spectrum of different patterns of in vitro growth was seen and the same six categories of proliferative abnormalities were identified as seen in untreated AML (Table 2). Only one patient showed complete absence of colony and cluster formation in marrow and blood culture and the most common pattern was that of macrocluster or microcolony formation with an excess of clusters. These variants accounted for 50 % of the cases studied. The macrocluster variant tended to present with a higher WBC count, higher blast incidence, lower platelet count and after a shorter duration of chronic disease than did the microcolony variant. Both categories showed minimal response to therapy, no remissions were observed and survival in blastic phase was brief. The next most common

Table II: Characteristics of Untreated CML and Blastic Transformation

	No. of Cases	Age (Years)	% Blasts in BM	Marrow/10^5 Colonies	Marrow/10^5 Clusters	Duration of CML (Months)	Blast Phase Survival (Weeks)	Remission Rate
Untreated chronic	58	47	< 5	404	1506	–	–	–
Untreated blastic trans.								
Macrocluster	9	54	50	0	2417	20	7	0/9
Microcolony	12	51	36	22	1043	41	6	0/12
Microcluster	6	39	52	0	136	14	35	3/6
Colony Forming-Blast ProM.	8	52	8	345	1967	13	33	1/8
Colony Forming-Low Plating	6	49	49	4	17	22	27	3/6

variants were cases showing a microcluster growth pattern (14 %) and a colony forming growth pattern with a high plating efficiency and maturation arrest at the blast-promyelocyte stage. Both these categories are associated with a high remission rate in AML. The colony forming cases had higher platelet counts and considerably lower blast counts in marrow and blood than did any other category and generally followed a more subacute course reflected in their longer survival in blasts crisis. The microcluster category of patients were, on average, younger than the other groups and had the longest average survival after diagnosis of blast crisis due to the fact that 50 % achieved complete remission which in two cases was prolonged. The relatively short mean duration of chronic phase disease in the microcluster group may be attributed to the fact that 2/6 cases presented at first diagnosis in blastic crisis with no antecedent history of chronic phase disease. A final category, comprising 14 % of cases was characterized by a low WBC count, high blast count in marrow and blood and a very low incidence of colonies and clusters with normal colony maturation and cluster to colony ratio. As we have previously reported, the blast cells in these patients possessed no discernible myeloblastic features as determined by marrow culture, did not respond to or produce colony stimulating factor and had the buoyant density characteristics of leukemic lymphoblasts rather than myeloblasts (6). A similar growth pattern is seen in acute lymphoblastic leukemia (4) and in the majority of acute undifferentiated leukemias (6). In these latter leukemic states, granulocytic colony formation reflects the persistence of low numbers of normal CFU-c coexisting with a non-myeloid leukemic blast cell population which cannot totally suppress normal granulopoiesis. It appears probable that a similar situation exists in this variant of blastic transformation and that the low incidence of colonies reflect residual chronic phase CFU-c coexisting with an acute leukemic blast population which is either non-myeloid or so undifferentiated that it lacks the capacity to proliferate in response to a regulatory macromolecule (CSF) and is defective in its capacity to specifically suppress chronic phase myelopoiesis. Recognition of this variant of blastic CML may be of particular importance, since we have observed a 50 % remission rate using protocols including vincristine and prednisone.

Of 103 patients who were Ph^1 positive, 8 presented at first diagnosis with abnormalities of in vitro CFU-c proliferation and differentiation characteristic of blastic phase disease. Of these, three were unequivocally in blastic phase at presentation by clinical and hematological criteria. Of the remaining five cases, two died within a week of diagnosis and three progressed to overt blastic transformation within 8–16 weeks.

The number of CML patients who have been sequentially studied using the CFU-c assay is relatively small; however, certain generalizations may be made concerning early detection of acute leukemic clones.

(a) Progressive increase in the cluster to colony ratio in marrow and/or blood cultures may precede by weeks or months clinical or hematological evidence of blastic transformation. During this period, chronic phase CFU-c coexist with emerging acute clones characterized by a microcluster, macrocluster or microcolony growth pattern. The rate of progression may be determined by the relative proportions of the coexisting clones as determined by a changing ratio of clusters to colonies or physical separation and quantitation of CFU-c subpopula-

tions (6, 14). This progression is frequently, but not invariably, associated with cytogenetic evidence of aneuploidy involving additional Ph^1 chromosomes and/or additions or deletions of C, F and G group chromosomes (14).

(b) Progressive increase in the incidence of colony and cluster forming cells in the circulation with a normal cluster to colony ratio and in vitro maturation arrest at the blast-promyelocyte level preceeds a terminal colony forming blast crisis.

(c) A declining incidence of colony and cluster forming cells in marrow and blood with a normocellular to hypercellular marrow and normal to elevated WBC count with an increasing blast cell incidence preceeds clinical evidence of a terminal blast crisis associated with the development of a non-myeloid or undifferentiated acute leukemia. A subnormal incidence of marrow CFU-c with a normal cluster to colony ratio and normal maturation may also be seen in CML patients with myelofibrosis, however, in such cases, circulating CFU-c are increased in number.

Marrow culture studies in allogeneic bone marrow transplantation

The ability of the CFU-c assay to monitor a population of stem cells closely related to the multipotential stem cell compartment, together with its capacity to discriminate between normal and leukemic cell populations, has proved of value in allogeneic marrow transplantation of patients with acute leukemia. The selection of potential transplant recipients is assisted by detection, at first diagnosis, of patients exhibiting a poor prognosis pattern of leukemic CFU-c proliferation. The efficiency of pre-transplant cytoreduction remains a considerable problem in marrow transplantation in leukemia, possibly due to the marked heterogeneity of the disease. We have observed persisting leukemic cell proliferation in AML patients at the time of marrow transplantation and also total absence of detectable leukemic cell proliferation in patients who subsequently relapsed with their original leukemia (16). The regeneration of donor CFU-c following marrow engraftment showed considerable variation, ranging from rapid reconstitution with an overshoot and return to normal incidence, to delayed or absent marrow CFU-c repopulation despite cytogenetic evidence of marrow reconstitution. The number of transplant patient analysed by in vitro culture parameters is, as yet, too small to assign any prognostic significance to the rapidity of regeneration of CFU-c, but from a theoretical standpoint, monitoring reconstitution at the level of a stem cell compartment should provide a more significant parameter than either peripheral WBC counts or analysis of marrow cytogenetic status based on mitosis in predominantly differentiating hematopoietic cells.

The clinical utility of bone marrow culture is illustrated in the case history of marrow transplantation in a patient with acute erythroleukemia (Fig. 2). Seven months prior to transplantation the patient's marrow showed 90 % blast cells and a diagnosis of acute erythroleukemia was made. Despite six courses of cytosine arabinoside and daunomycin, only a transient partial remission was observed. Bone marrow culture 14 days and 10 days prior to transplantation showed a poor prognosis acute leukemic growth pattern of the microcolony type with an excess of poorly differentiated clusters of 3–40 cells and small colonies of 40–60 cells.

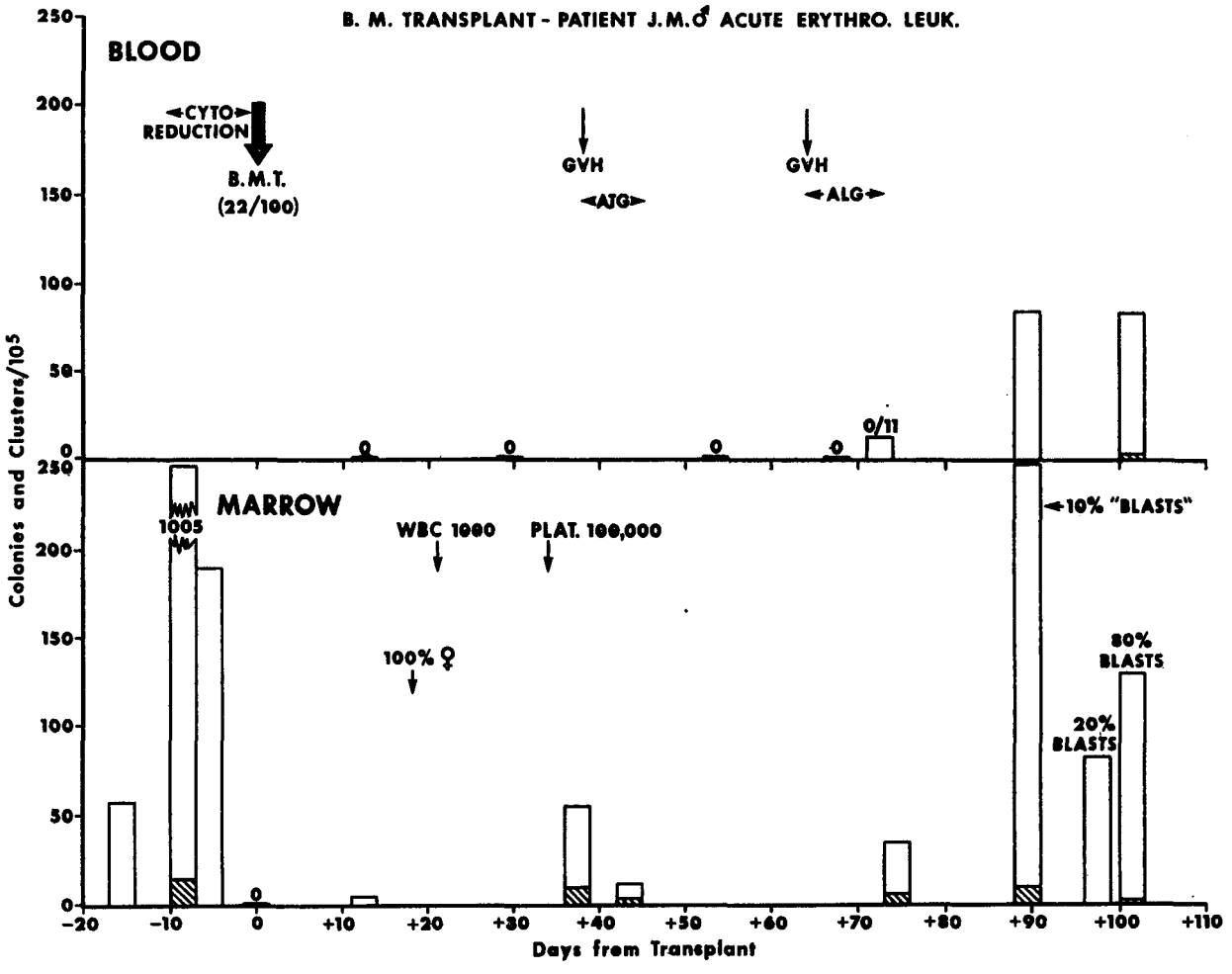

Fig. 2: In vitro culture parameters in the marrow and blood of a patient with acute erythroleukemia before and after a female sibling bone marrow transplant. Note the abnormal ratio of colonies to clusters in the pre-transplant marrow cultures and the return of this leukemic growth pattern in both marrow and blood 70–100 days post-transplantation. Methotrexate was administered at 3, 6, 11 and 18 days post-transplantation and at weekly intervals thereafter.
ATG – Horse anti-human thymocyte globulin
ALG – Goat anti-human lymphoblast gamma globulin

The clusters and small colonies could be further distinguished from normal by their compact nature rather than the dispersed morphology of normal colonies. Following cytoreduction and immunosuppression with daunomycin, cytosine arabinoside, cyclophosphamide and total body irradiation (1,000 rads), the patient received an ABO, HLA and MLC matched female sibling marrow transplant. The donor marrow contained 22 CFU-c per 10^5, indicating minimal dilution with peripheral blood. Engraftment was confirmed by cytogenetic analysis of the marrow on day 18, when 100 % female donor metaphases were observed. Although normal donor colony formation may be detected within 10–12 days following transplantation, this patient showed delayed recovery of marrow colonies which were not seen until 38 days post-transplantation. At no stage of the clinical course did the marrow CFU-c incidence approach or exceed normal levels. A diagnosis of minimal GVH disease in skin, liver and GI tract was made 38 days post-grafting

and was reversed with 7 doses of horse anti-human thymocyte globulin (HAHTg). Seventy-two days post-transplantation, peripheral blood cultures which previously had shown no detectable colony and cluster formation, showed a sharp increase in cluster incidence with no detectable colony formation. Bone marrow culture 3 days later showed a mixed population of normal colonies with leukemic clusters and colonies identifiable by their compact nature. At this stage no hematological or cytogenetic evidence of recurrence of leukemia was found. Eighty-nine days post-transplantation both marrow and peripheral blood cultures showed leukemic cell proliferation with a growth pattern and colony formation characteristic of the leukemic clone observed prior to transplantation. No evidence of persisting donor CFU-c was obtained. At this stage the marrow morphology revealed 10 % abnormal proerythroblasts and monocytoid cells; however, an unequivocal-diagnosis of early leukemic relapse was not possible on hematological or clinical grounds. Cytogenetic studies at this time revealed 24/24 normal female donor metaphases. Eight days later, marrow aspiration revealed 20 % blast cells with a persisting leukemic growth pattern and cytogenetic studies showed at least 3 different populations of cells: (1) 24/50 normal female metaphases, (2) 12/50 translocated male metaphases that resembled the original stem line defect and (3) abnormal male cells containing multiple hyperdiploid alterations. The patient expired on day 101 post-transplantation in full hematological relapse with a high circulating blast count and leukemic infiltrations in multiple organs.

It is apparent from studies of this and other patients that marrow culture can detect leukemic relapse following marrow transplantation considerably earlier than conventional diagnostic criteria and at a time when marrow cytogenetic analysis showed no evidence of emerging host leukemic stem lines. More extensive analysis of bone marrow transplantation in patients with AML, aplastic anemia and myeloproliferative disorders will be necessary before the ultimate value of monitoring in vitro culture parameters can be determined, but the preliminary observations suggest that such a venture will not be unrewarding.

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