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Myeloid Progenitors in Acute Non-lymphocytic Leukemia: Prognostic Value in Remission*

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Abstract—*The prognostic value of* in vitro *cloning of bone marrow and blood cells was tested in 32 patients with acute non-lymphocytic leukemia (ANLL) at various stages of disease. Using placental conditioned medium (PCM) as a stimulus and panoptical staining methods for the analysis of colony morphology, four different growth types could be distinguished at presentation. Poor response to colony-stimulating activity (GM–CSF)* in vitro *seemed to be associated with poor survival. Probably due to low patient numbers, this association could not be proven statistically. During remission a progressive decrease of morphologically normal colonies was recognized in the bone marrow and peripheral blood. Thus by* in vitro *cloning methods the quality of remission could be substantiated.*

INTRODUCTION

ACCUMULATING evidence in the literature suggests that acute non-lymphocytic leukemia is a heterogeneous disease, with entities distinguishable by chromosome analysis [1] and affecting progenitor cells at various stages of the hemopoietic stem cell hierarchy [2]. Subgroups of different prognosis have been identified by their ability to form colonies and clusters *in vitro*, but data — though numerous — are still conflicting. Some authors demonstrated that poor *in vitro* growth at presentation was associated with poor prognosis [3–6], whereas others found better response to chemotherapy in this group [7–11].

There is more agreement on the normalization of *in vitro* granulopoiesis during remission [12, 13]. However, the large fluctuations in remission observed make interpretation of the culture data difficult with respect to the quality of remission [5, 7, 14]. These discrepancies might originate from variable stimulation of colony formation and different incubation times, as well as from different patient groups and treatment protocols. Moreover, most studies determined cluster and colony counts without routinely staining the cultures for morphological analysis of the cell types grown. In fact, this might be helpful to determine if the aggregates formed *in vitro* are derived from leukemic cells or represent a population of residual normal hemopoiesis. To rely on the determination of the cluster-to-colony ratio is probably not sufficient in this respect. Thus, by staining and morphologically examining the cultures it might be possible to distinguish more meaningful subgroups of patients at presentation, and perhaps enable the detection of regrowth of leukemic colonies earlier during remission.

We report here on our experiences in 32 patients with ANLL in whom *in vitro* colony formation of bone marrow and blood cells and their morphological types were determined at various stages of disease. The results were statistically analyzed in comparison to remission rates, remission durations, survival and FAB-classification [15]. In contrast to earlier studies, we used placental conditioned medium as a source of colony-stimulating activity which can be produced in larger batches and provides a less variable stimulus for *in vitro* granulopoiesis than feeder layers [16].

MATERIALS AND METHODS

Patients and treatment protocols

Thirty-two unselected patients (14 male, 18 female), with a mean age of 44.7 (range 16–74) yr and with ANLL (no blast crisis of chronic granulocytic leukemia), were examined. Patients were treated between 1981 and 1984 at our department according to one of two protocols: (a) LAM–4/5 protocol of the EORTC Haematosarcoma Group, consisting of adriamycin (ADM) i.v. 50 mg/m^2 on day 1, vincristine (VCR) i.v. 1 mg/m^2 on day 2 and cytosine arabinoside (ara C) i.v. 80 mg/m^2 as push injection every 12 hr on days 3–9 (21 patients); or (b) LAM–6 protocol of the EORTC Haematosarcoma Group, consisting of daunorubicin (DNR)

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i.v. 45 mg/m^2 on days 1–3, VCR i.v. 1 mg/m^2 on day 2, ara C 100 mg/m^2 by continuous infusion over 8 hr plus 50 mg i.v. every 12 hr on days 1–7 (11 patients).

Two to three cycles were given for induction chemotherapy. Patients who failed to respond after three cycles received no further intensive treatment, whereas patients with CR were given one additional course as consolidation chemotherapy and thereafter were maintained: (a) by 6-thioguanine orally 70 mg/m^2 on days 1–4 and ara C subcutaneously 80 mg/m^2 on day 5 weekly until relapse; or (b) by repeated cycles of the induction regimen (DNR only day 1) at 6-week intervals, or cycles of 4′–(9 acridinylamino)methane-sulfon-*n*-aniside (AMSA) i.v. 150 mg/m^2 on day 1 plus ara C 3000 mg/m^2 i.v. every 12 hr on days 1 and 2, alternating with 5-azacytidine i.v. 150 mg/m^2 on days 1–3. Maximally a total number of nine cycles was given.

Bone marrow and blood samples were drawn 2–4 weeks following the end of the last chemotherapy.

Control bone marrow specimens were drawn from the iliac crest of patients who were biopsied for non-hematological diseases. Informed consent was given by the patients participating in the study.

Classification according to FAB criteria

Classification according to FAB criteria [15] was made on bone marrow and blood smears stained by May–Grünwald–Giemsa, peroxidase, PAS, non-specific esterase with and without sodium fluoride and acid phosphatase with and without tartrate.

Criteria for complete remission (CR) were absence of clinical signs of leukemia, bone marrow blasts below 5%, no blasts in the blood smear, granulocyte count above 1.5 × 10^9/l, platelet count of at least 100 × 10^9/l and a hemoglobin value of at least 10 mg/dl. Patients were categorized to be in partial remission (PR) when they had up to 25% blasts in the bone marrow and did improve with normal cell blood counts without fulfilling all criteria of CR. Additionally, those patients who had a bone marrow CR but relapsed within 4 weeks were retrospectively categorized as PR.

Cell preparations for culture

Bone marrow and blood cells were anticoagulated with preservative-free heparin and centrifuged at 400 ***g*** for 25 min above a Ficoll-gradient of 1077 g/l density. The resultant suspension of mononuclear cells (MNC) was washed twice in Hank's balanced salt solution and resuspended in McCoy's 5A modified medium.

Colony-forming assay

For colony formation 25,000–75,000 bone marrow MNC or 125,000–250,000 MNC of the peripheral blood were immobilized in 0.3% agar (Difco) in a volume of 0.25 ml (McCoy's 5A modified medium with amino acids, vitamins and 15% fetal calf serum) in Falcon-Multiwell plastic culture plates [17]. A liquid overlayer of 0.25 ml medium was placed over the agar when it had gelled at 37°C. Cultures were supplemented with a final concentration of 5% PCM as a source of colony-stimulating activity (GM-CSA) [18] which maximally stimulated the growth of myeloid progenitors in normal bone marrow at a concentration range of 2.5–20%. Cultures were set up in duplicate and, following 7–8 days of incubation at 37°C and 5% CO_2 in fully humidified room air, the complete agar pellicles were fixed in 2.5% glutaraldehyde, dried on slides and panoptically stained by a modification of the May–Grünwald–Giemsa stain [17]. After embedding in Eukitt®, cultures were permanently preserved and ready for microscopical evaluation. Clusters were defined as aggregates of 15–39 cells and colonies as aggregates of 40 or more cells. Although clusters and colonies were counted separately, the sum of both was statistically evaluated, because the morphological types of cells appeared to be more meaningful than colony size.

Statistical analysis

To determine the influence of single pretreatment variables on response and survival, non-parametric correlation coefficients were calculated according to Spearman. Analysis of frequency-distributions was carried out by the chi-square test.

For the comparison of independent samples in various groups one-dimensional variance analysis was applied. Correlation analysis of blood GM-CFU and time to relapse was made by linear regression. The level of significance in all tests was set up at $P = 0.05$.

RESULTS

Patients characteristics influencing response and survival

A number of conventional parameters were analyzed for their prognostic significance (Spearman rank correlation). The only variable significantly associated with better response and survival was young age ($P = 0.006$ and 0.009 respectively). All other parameters like FAB-type, duration of history, lactate dehydrogenase level, leucocyte count at presentation, percentage of blasts in the bone marrow and peripheral blood were not significantly associated with response to chemotherapy and survival.

Growth pattern at presentation

In 23 patients bone marrow cells were cultured at first diagnosis of the disease. Four different patterns were recognized: (I) non-growing cultures; (II) diffuse growth of undifferentiated cells; (III) growth of clusters and colonies with blast cell morphology; and (IV) growth of clusters and colonies of relatively differentiated granulocytic cells including banded forms.

Table 1 summarizes a number of variables analyzed statistically among these four groups of patients; age was similar in all groups. The distribution of FAB-subtypes was not significantly different in groups I–III, whereas the formation of colonies of differentiated cells occurred in patients with the M4-subtype of ANLL only (chi-square test, $P = 0.021$). Statistical analysis of 20 patients evaluable for remission rates, remission durations and survival showed no significant differences in response rates among the four groups (chi-square test). A tendency was found, however, for better survival in patients with increasing growth and differentiation *in vitro* (see means of survival in Table 1).

Colony formation of bone marrow cells during remission

During remission (partial or complete) colony morphology appeared to be of normal type, with aggregates of granulocytic cells in all maturation stages, macrophage type colonies and eosinophilic clusters. Blast clusters or colonies could not be detected; however, the possibility of a very low proportion of such aggregates cannot be entirely excluded. Figure 1 demonstrates the numbers of aggregates (sum of colonies and clusters) found at different stages of remission as compared to 27 normal control bone marrow cultures set up in the same period of time (means and standard deviations are given as well as the numbers of patients in each group). A mean 310 granulocyte/macrophage colony-forming cells (GM-CFU) were grown from 75,000 normal bone marrow MNC, a number not very different from the 321 found in patients early in first CR (1. CR, 1. value). Cultures from patients assayed later in first CR (2. value, 3. value) produced significantly less clones (177 and 130 respectively). Bone marrow cells drawn from patients in second CR again produced colony numbers within the normal range (335 as a mean). In contrast, patients with PR grew substantially less colonies and clusters with a mean of 99.5 in early PR (1. value) and 39 later in PR (2. value). The differences at various stages of remission were statistically highly significant ($P = 0.004$ in one-dimensional variance analysis).

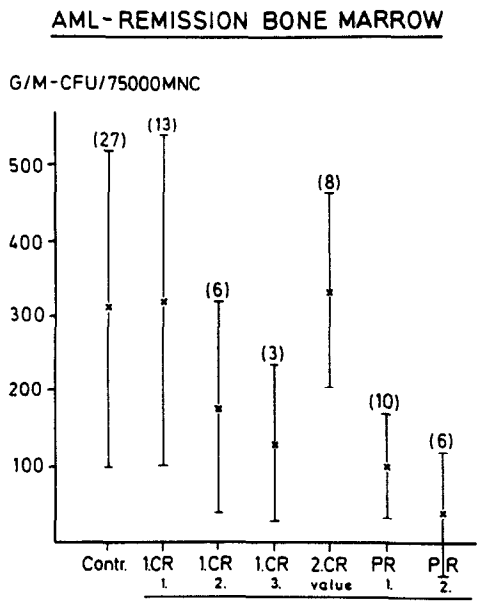

Fig. 1. Means and standard deviations of numbers of GM-clones grown from remitted bone marrow at various stages of ANLL as compared to normal bone marrow.

Table 1. Presentation growth pattern, FAB-type and prognosis in ANLL

	Growth type				
	(I) No growth	(II) Diffuse blasts	(III) Blast colonies	(IV) Diff. colonies	Significance*
No. of patients†	6 (6)	9 (7)	4 (4)	4 (3)	
Age‡	45 ± 18	52 ± 17	40 ± 25	45 ± 18	N.S.§
FAB-type	M1 = 1 M2 = 3	M2 = 6 M3 = 2	M1 = 1 M2 = 2	M4 = 4	0.021
	M3 = 1 M4 = 1	M6 = 1	M3 = 1		(group IV only)
CR-rate	2/6	4/8	3/4	2/4	N.S.
PR-rate	0/6	2/8	1/4	2/4	N.S.
Progression	4/6	2/8‖	0/4	0/4	N.S.
Dur.rem. (month)‡	—	7.6 ± 8.4	6.0 ± 3.4	16.7 ± 17.2	N.S.
Dur. CR (month)‡	7.5 ± 2.1	7.3 ± 6.1	7.6 ± 0.6	24.5 ± 14.0	N.S.
Survival (month)‡	5.8 ± 3.4	10. ±+ 7.6	17.8 ± 9.9	19.7 ± 16.5	N.S.

†In parentheses: No. of patients evaluable for survival. §N.S. = not significant. ‖One patient not evaluable because of early death. ‡Mean ± standard deviation. *Chi-square test.

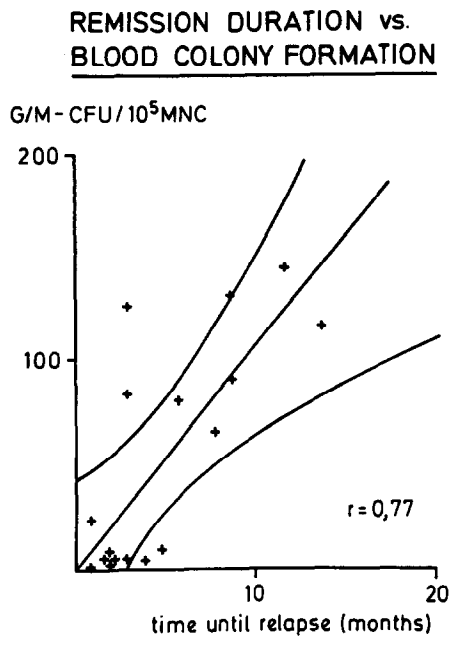

Fig. 2. Numbers of GM-clones per 10^5 peripheral blood MNC plotted against the time from sampling until relapse occurred by cytological criteria.

Colony formation of peripheral blood cells during remission

Colony formation of peripheral blood (PB) MNC was assayed in seven CR and six PR patients, in some of them sequentially. Most of the patients had GM-CFU in the circulation with only two non-growing cultures (one CR patient, and one PR patient) out of 18 samples tested. Blood-derived colony-forming cells showed a tendency to decline during the course of remission which was even more pronounced than in the bone marrow. In Fig. 2 the numbers of aggregates per 10^5 blood MNC are plotted against the time the patients remained in remission from the day of sampling onward (time from assay until evidence of relapse occurred cytologically and clinically). By linear regression analysis a highly significant correlation was found ($R = 0.77$, $P = 0.00009$).

DISCUSSION

The clinical course of patients with ANLL may vary considerably, ranging from progressive lethal disease to long-lasting remissions. Attempts to identify prospectively those patients who will respond to chemotherapy by means of cell culture criteria have rendered variable results, attributing a better prognosis to either the group of patients without *in vitro* growth at presentation [7–11] or to those with colony and cluster formation [4, 6, 19, 20]. Our data lend support to the latter group of authors, but the number of patients evaluable for remission rate and survival was too small to allow definite conclusions. Furthermore, differences in technology have to be taken into consideration when comparing the results of different studies. We used placental conditioned medium as a source of colony stimulating activity, which has been told to be a weaker stimulus than feeder layers to leukemic cells [21]. In fact, it is probably a better stimulus for normal *in vitro* granulopoiesis [22] and more easy to standardize [16]. Nevertheless, we were able to show by morphological analysis of the colonies that *in vitro* growth of blast cells occurred in a substantial proportion of presentation bone marrows (six non-growing out of 23), which is in the same order of magnitude as in most studies applying feeder layers as a source of GM-CSA. The possibility that some of the leukemic clones would have responded to other stimuli could not be ruled out, of course. The existence of leukemic clones with different responsiveness to GM-CSA and interleukin-3 has been shown by others [23].

Panoptical staining of all cultures in this study allowed the distinction of patients with *in vitro* blast colony formation from those who gave rise to colonies of relatively differentiated appearance. Remarkably, patients in the latter group all had leukemia of the M4-subtype (myelomonocytic leukemia according to FAB-criteria). For this observation two explanations may exist: those cases might have been purely monocytic leukemias (M5-subtype), possibly restricted to monocytic progenitors [24], and thus probably able to give rise to normal granulocytic colonies. On the other hand, this type of leukemia may be able to differentiate *in vitro*, giving rise to morphologically normal-appearing colonies of leukemic progeny. The latter hypothesis is supported by the fact that all four patients in group IV had cytochemical and morphological signs of both myelocytic and monocytic cells. Furthermore, cells within these showed considerable maturation defects when examined under high magnification.

In bone marrow cultures of patients in CR or PR we recognized colonies and clusters of essentially normal morphology. The cloning efficiency in early first CR was not different from normal controls. Serial cultures in first CR revealed that the number of colony-forming cells gradually declined (Fig. 1). Though large variations were observed in our study as well as by others [5, 7, 10, 14], the tendency to decline during the course of remission was obvious in our patients. Sporadic reappearance of colonies of blast-like cells was not observed. Patients in early second CR again had normal colony counts, whereas patients achieving only PR had significantly fewer colony-forming cells in the bone marrow, which decreased even more with further progression of the disease.

This tendency of colony-forming cells to decline with time elapsing in remission was demonstrable even more convincingly in the peripheral blood. A

linear correlation existed between the peripheral blood colony count and the time until relapse of ANLL could be demonstrated cytologically (Fig. 2). Thus a patient with low colony numbers in the peripheral blood is at a high risk of early relapse, an observation which has also been reported by others but has lacked statistical confirmation [25, 26].

The decline of colony-forming cells during remission may have two explanations: it might reflect the cytotoxic effect of repeated cycles of chemotherapy or it might originate from an otherwise undetected regeneration of leukemic cells which exert increasingly inhibitory effects on the normal hemopoiesis [27]. Only one of our CR-patients was in unmaintained long-term remission. This individual had 370 GM-CFU/75,000 MNC in the bone marrow, a value somewhat above the normal mean, which is in good agreement with published data on such patients [13]. Therefore a cytotoxic mechanism for the decline of colony-forming cells during remission cannot be ruled out. Some observations, however, suggest that undetected leukemic regrowth is the more important factor: colony counts normalize in second CR and are much lower in PR patients, although these received no more chemotherapy than patients with CR. These data are interpreteted as meaning that cells responding to GM-CSA in remission mainly represent the normal granulopoiesis.

Our observation that colony counts from the peripheral blood correlated better than those from bone marrow with the time of each patient to remain in remission might be somewhat surprising. When interpreting the data one should take into consideration that for GM-CFU a bone marrow–blood barrier exists which prevents earlier subpopulations of these progenitors from circulating, releasing mainly a subpopulation of smaller colony-forming cells into the blood [28]. Such a mechanism could easily explain a decline in circulating colony-forming cells at a time when those in the bone marrow are still maintained at nearly normal levels.

Our data indicate that *in vitro* differentiation of ANLL cells in response to GM-CSF at presentation seemed to be associated with better prognosis, but low numbers of patients did not allow any definite conclusions. The quality of remission, however, could be defined more precisely by cloning remitted bone marrow cells. Peripheral blood colony-forming cells proved to be an especially valuable predictive test for imminent relapse.

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