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# Acute Leukemias II

Prognostic Factors  
and Treatment Strategies

With 332 Figures and 257 Tables



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## Preface

Acute leukemia a quite homogenous disease when untreated reveals a substantial heterogeneity in its response to therapy. While cure is achieved in a certain proportion of patients other cases prove to be highly resistant. The curability is superior in acute lymphoblastic (ALL) than in acute myeloid (AML) leukemia and – within both types – higher in children as compared to adults.

The two age groups and cell types can be further subdivided into prognostic groups by special diagnostic features. Thus, in AML a longer remission duration is associated with the presence of Auer rods, myelomonocytic morphology with increased abnormal eosinophils, inversion of chromosome 16, and translocations of chromosomes 15; 17 and 8; 21 whereas AML after preleukemia, monocytic morphology and abnormalities of chromosomes 5 or 7 predict for earlier relapse. In ALL the common ALL and T-cell phenotype indicate a favourable outcome while B-cell phenotype, a high leukocyte count and translocations of chromosomes 4; 11 and 9; 22 (Philadelphia chromosome) show an unfavourable prognosis. The prognostic significance of some of the factors, however, may differ between different clinical trials and may thus be treatment dependent. Furthermore, their relevance for individual patients remains far from being understood since one patient may experience an early relapse while another patient with identical prognostic factors becomes definitely cured by the same treatment. Thus, a risk-adapted treatment concept as derived from the analysis of patient groups may fail in the individual case due to inadequate treatment intensity or overtreatment ultimately leading to treatment related death.

Risk adapted treatment strategies are well established in childhood ALL where in standard risk patients the high cure rates seem to hold up even after reducing treatment intensity. In AML, in contrast, treatment was gradually intensified in all patients, but

failed to break through the sound barrier of unsatisfactory cure rates even in special subgroups. While new protocols including more effective supportive care show some increase in the initial response rates and certain improvements in the long-term results, no beneficial effect on the relapse rate during the first 1½ years emerged from any of these regimens. Thus, high chances for cure are presently restricted to children with ALL and to lesser proportions children with AML and adults with ALL and AML.

Within these potentially curable subgroups chemotherapy – its intensity and duration – seems to have an important impact and further improvements appear possible by intensive multiple step treatment strategies including autologous bone marrow transplantation or high-dose consolidation chemotherapy. These options may be facilitated by the introduction of recombinant human hematopoietic growth factors which may diminish the limits of myelotoxicity and allow more effective antileukemic treatment.

Adoptive immunotherapy is a new antileukemic principle using immunomodulators and showing promising effects in pre-clinical studies. Similar mechanisms seem to be involved in the graft-versus-leukemia effect in allogeneic bone marrow transplantation.

Since Acute Leukemias I appeared three years ago new approaches have been coming up and relevant data of more established methods are now available. Several multicenter trials helped better understanding the role of major treatment alternatives and led to improved treatment strategies. Numerous valuable contributions from the leading groups in leukemia research provide a most up-to-date state of the art in this second volume of Acute Leukemias.

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# **Leukemia Cell Biology and Basic Science**

## Myelodysplastic Syndromes: Preleukemic or Early Leukemic Conditions?

P. Dörmer

Myelodysplastic syndromes (MDSs) comprise a heterogeneous group of homopoietic disorders sharing some probability of developing into overt leukemia [1]. According to glucose-6-phosphate dehydrogenase [9, 10], restriction fragment length polymorphism [6], and *ras* point mutation studies [7, 8], there is at least a subgroup of MDSs showing clonal growth of bone marrow cells. Up to now, however, it has been impossible to state that clonal cell growth is equivalent to malignant growth. It is conceivable that the progeny of an altered stem cell possesses some growth advantage over nonaltered cell populations but nevertheless remains subject to the regulation of steady state between growth and maturation. This regulation may be operating at an altered growth-to-maturation level. On the other hand, if preleukemia is a slowly progressing disorder, the very faint exponential character of the initial blast cell expansion may not become recognizable by standard laboratory examinations. The question whether MDSs represent early leukemic or true preleukemic hemopoietic lesions may be of significance for treatment strategies. This question has been addressed by a prospective study of cell kinetics in the MDSs [2–4]. First, a measure of the normal and disturbed proliferation-to-maturation ratio of a bone marrow cell lineage was established. Production of the earliest recognizable cells in a lineage, i.e., proerythroblasts and myeloblasts, respectively, was attributed to the proliferative activity of a lineage. Production of cells in the

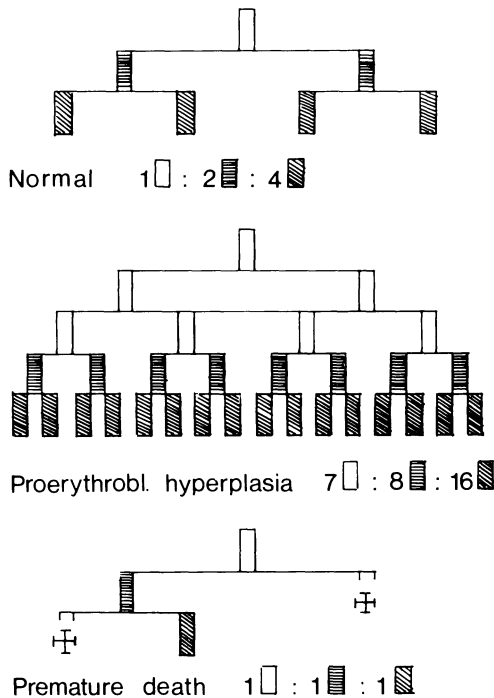
succeeding compartments, i.e., basophilic and polychromatic erythroblasts or promyelocytes and myelocytes, was attributed to maturation and balanced against the extent of proliferation.

Figure 1 schematically represents examples of normal and disturbed proliferation-to-maturation indices. The upper example of production rates in three succeeding compartments showing a ratio of 1:2:4 resembles normal conditions. If there are additional mitoses in the first compartment, as depicted in the middle graph, the ratio of production rates may be 7:8:16 or roughly 1:1:2. This indicates a decrease in maturation as opposed to proliferation. The same effect of maturation decrease is encountered in the lower graph, which exemplifies premature cell death in the bone marrow. Conditions like these were quantitatively assessed by dividing the ratio of production rates in a lineage under study by the respective normal ratio. The quotient obtained was termed the maturation index. This index by definition is 1.0 in a normal lineage and approaches zero in the myeloid lineage of acute myeloblastic leukemia (AML).

When the mean maturation indices of erythro- as well as of granulocytogenesis were calculated for the individual disease groups of MDSs at first presentation, greatly reduced figures were obtained throughout [3]. The erythroid maturation index was around 0.5 in refractory anemia (RA) and refractory anemia with excess of blasts (RAEB) and was as low as 0.36 in idiopathic sideroblastic refractory anemia (ISRA). The myeloid maturation index was 0.44 in RA, 0.39 in ISRA, and 0.17 in RAEB. Of all the

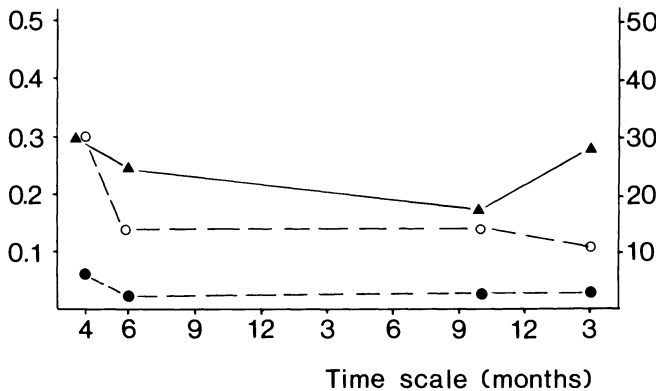
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**Fig. 1.** Schematic representation of the proliferation-to-maturation ratio in normal erythropoiesis (*upper panel*), in erythropoiesis with additional proerythroblast cell cycles (*middle panel*), and in ineffective erythropoiesis (*lower panel*). Cell production in the first compartment (proerythroblasts) was considered to reflect proliferation, while cell production in the succeeding compartments was associated with maturation of the lineage

kinetic parameters, the myeloid maturation index was the most significant concerning actuarial patient survival [3]. It was superior to the myeloblast-labeling index previously shown to be significant [5].



**Fig. 2.** Sequential observations of cell kinetics in a case with RA. ●, blast frequency/100 bone marrow cells (BMC); ○, blast S-phase frequency/1000 BMC; ▲, myeloid maturation index

In many patients the disease status remained unchanged during the whole observation period. Figure 2 illustrates an example with RA. In spite of the quite low maturation index at all instances the disease remained stable. Figure 3 shows a case of RAEB with considerable changes over the years of the values of kinetic parameters. This patient, however, did not develop leukemia during the observation period and 2 ensuing years.

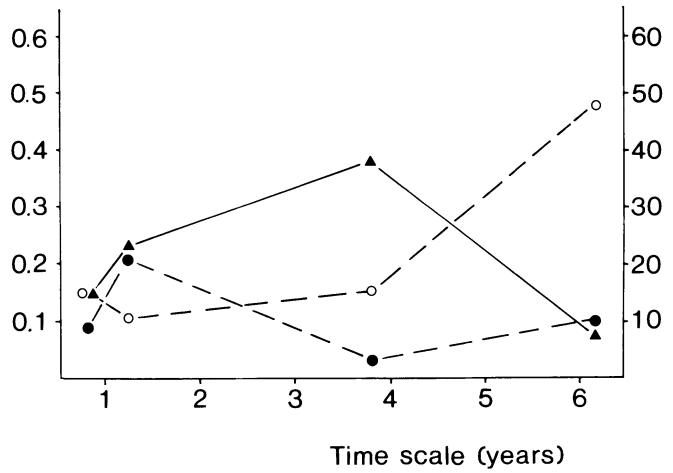
A case with continuous progression to overt leukemia from RAEB is shown in Fig. 4. Blast cell count and fraction of blasts in S-phase continuously rise, while the myeloid maturation index shows a continuous decrease. In this case the disease status at first presentation can be retrospectively classified as early leukemia. Figure 5 is another example of early leukemia in a case of RAEB. At the last instance of observation the myeloid maturation index is close to zero, although the blast frequency in the bone marrow is not yet higher than 33%.

A different type of leukemia development is illustrated in Fig. 6. This patient presented a stable phase of MDS lasting 3 months and then a steep increase in the blast count and a decrease in the myeloid maturation index to almost zero. The same pattern was observed in another patient with RAEB (Fig. 7), who was stable for the first 10 months and then developed overt leukemia within 1 month. With a single dose of vincristine the MDS status was reestablished, but the patient died shortly thereafter from an intercurrent infection.

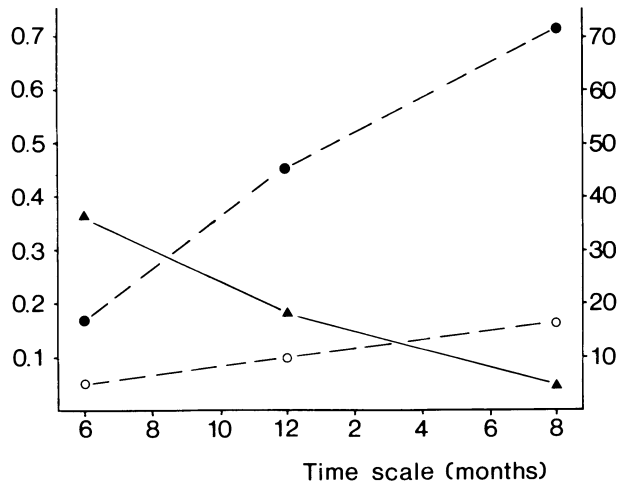
In the last two examples there was an abrupt change in the proliferation-to-maturation pattern of the myeloid cells. The ery-



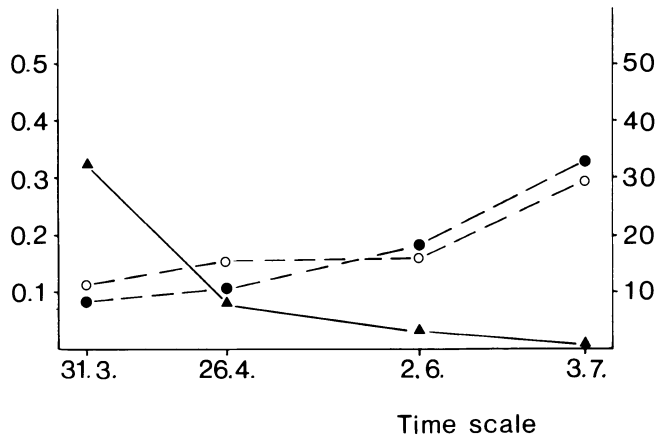
**Fig. 3.** Sequential observations of cell kinetics in a case initially presenting with RAEB. Although the FAB subgroup changed severalfold due to varying BM blast cell counts, there was no indication of leukemia development at any time. For symbols, see legend to Fig. 2

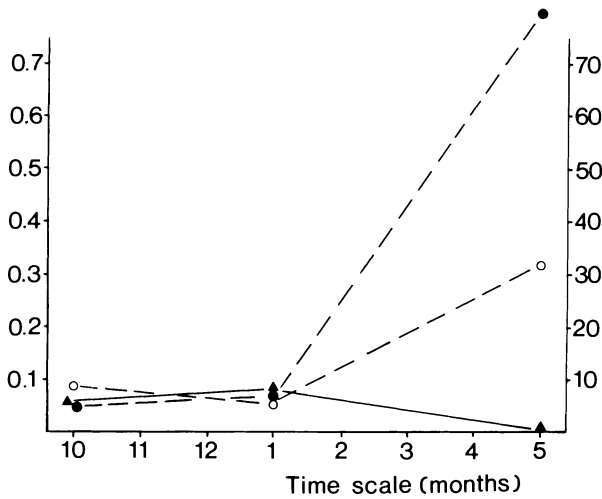


**Fig. 4.** Sequential observations of cell kinetics in a case with RAEB gradually progressing to overt AML. For symbols, see legend to Fig. 2

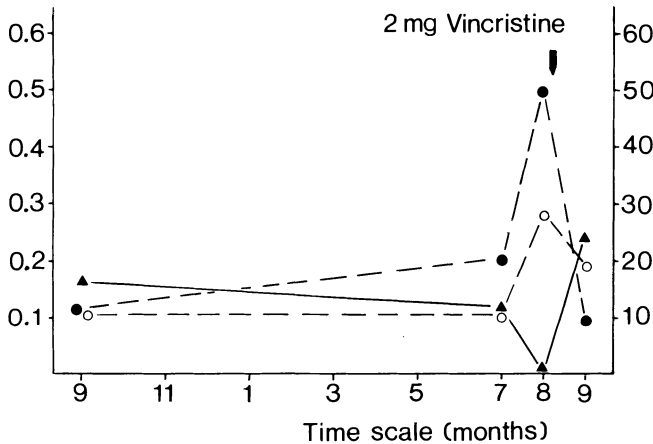


**Fig. 5.** Sequential observations of cell kinetics in a case with RAEB gradually progressing to overt AML. For symbols, see legend to Fig. 2





**Fig. 6.** Sequential observations of cell kinetics in a case with a 3-month course of stable RAEB suddenly progressing to overt AML. For symbols, see legend to Fig. 2



**Fig. 7.** Sequential observations of cell kinetics in a case with a 10-month course of stable RAEB suddenly progressing to overt AML. For symbols, see legend to Fig. 2

throid lineage in these cases did not show significant changes in the maturation index. It is therefore likely that some further transformational event had occurred in the myeloid series during our observation, shifting the myeloid growth pattern from non-leukemic to leukemic. Obviously, the last two cases initially had presented with a non-leukemic type of myelodysplastic lesion.

In summary, conditions of slowly progressing early leukemia as well as of true preleukemia may both present with the clinical picture of MDS. At the present time the only means to distinguish between these two growth conditions in the bone marrow is by performing a follow-up or a retrospective evaluation.

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## Mechanisms of Autocrine and Paracrine Growth Control in Acute Myelogenous Leukemia

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Blast cells of a high proportion of patients with acute myelogenous leukemia (AML) proliferate in response to exogenous hematopoietic growth factors, both in vitro [9, 14, 26, 36, 48, 65] and in vivo [20]. Whereas leukemic colony-forming cells (L-CFCs) from most patients share their growth factor dependence with normal committed myeloid progenitor cells (CFU-GMs), some AML samples autonomously form colonies in agar and are therefore believed to bypass growth factor requirements [17, 42, 72]. Autocrine growth factor production has been identified as one mechanism used by AML blasts to supply various growth-promoting molecules. Moreover nontransformed accessory bone marrow cells have been shown to be inducible for production of growth-promoting factors by AML-derived mediators. This paper summarizes the current knowledge of autocrine and paracrine growth-promoting mechanisms in AML whereby leukemic cells may achieve selective growth advantages and includes recent data supporting the concept that various cytokines may act in concert to induce optimal leukemic growth.

### Mediators Involved in the Regulation of Normal Hematopoiesis, Their Genetic Locations, Possible Involvement in Cytogenetic Aberrations, and Cellular Sources

Five human hematopoietic growth factor species have been identified so far, i.e., colony-stimulating factor (CSF) for granulocytes (G-CSF) [60], for macrophages (M-CSF or CSF-1) [25], for mixed granulocyte-macrophage colonies (GM-CSF) [6], for multilineage colonies (IL-3) [68], and for erythropoietin (EPO) [33]. Interleukin (IL)-6, IL-1, IL-4, tumor necrosis factor (TNF), and gamma-interferon (IFN) may affect growth promotion of hematopoiesis by exerting synergizing [23, 30, 49, 62] as well as growth factor-inducing actions [40–44, 67]. However, CSFs have also been shown to recruit each other [21, 34, 46] and to exhibit overlapping and synergistic activities [8, 10, 46].

All CSFs are encoded by single-copy genes and some are clustered on chromosome 5: The *M-CSF* gene has been assigned to 5q33.1 [50], the *GM-CSF* gene to 5q21–q32 [22], and the *IL-3* gene has been mapped to the same band of chromosome 5 as *GM-CSF* [31]. Additionally, chromosome 5 carries multiple genes coding for growth-related proteins and protein receptors, including the *M-CSF* receptor gene, which is identical to the protooncogene *c-fms* [57], the receptor for platelet-derived growth factor (5q31.3–32) [69], the beta-2 adrenergic receptor (5q31–32) [27], and genes coding for endothelial cell growth factor (5q31.3–32) [24].

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Chromosome 5 is frequently involved in cytogenetic aberrations in AML, e.g., partial loss of the long arm of the chromosome (del 5q) [56]. It has also been shown that the genes for GM-CSF, M-CSF, and IL-3 are directly affected by structural changes of chromosome 5 [22, 31, 50]. *G-CSF* has been mapped to the chromosomal region 17q11.2–21 proximal to the breakpoint involved in the translocation (15;17) [59], which is the karyotypic aberration hallmark of acute promyelocytic leukemia [56]. The *IL-6* gene has been mapped to chromosome 7 [12], which is also commonly involved in cytogenetic aberrations in AML (–7 or 7q–; q31.2q36) [56]. Physiological sources of CSFs are monocytes/macrophages, T-lymphocytes, granulocytes, endothelial cells, and fibroblasts [19, 34, 38, 42, 58]. Normal hematopoietic progenitor cells, however, are not known to have the capacity to produce CSFs. Even after stimulation by agents such as phorbol myristate acetate, known to promote expression of the *CSF* genes, mRNA for G-, GM-, M-, and multi-CSF remained undetectable in normal hematopoietic progenitor cells [17, 71].

### Effects of Exogenous CSFs on Myeloid Leukemia Cell Growth

Leukemic colony-forming cells share several features with normal hematopoietic stem cells including their requirement for exogenous growth factors for colony growth [4, 8, 51]. The ability of recombinant purified CSFs has been assessed to substitute for media conditioned by various tumor cell lines which are the most common source of growth factors. GM-CSF, G-CSF, and IL-3 have been shown to promote growth of a high proportion of L-CFCs [14, 26, 36, 48, 65]. However, responses of AML to CSF vary markedly from patient to patient.

Colony-stimulating factors have been shown to affect both renewal (“birth”) and determination (“death”) of L-CFCs. Significant differences were seen when analyzing effects of GM-CSF, which in one AML specimen only triggered “birth,” while another specimen was stimulated to mature (“death”) [36]. However, GM-CSF has also been described to be the most effective growth-inducing factor for human myeloid leukemia cells [48]. IL-3 was effective by inducing AML growth in most instances, whereas G-CSF promoted both growth and differentiation with varying intensity [36].

Our data on proliferative responses of L-CFC to hematopoietic growth factors are summarized in Table 1. In contrast to GM-CSF, G-CSF, and IL-3, M-CSF was mostly found to favor differentiation and thus to inhibit self-renewal of leukemic stem cells in culture [37]. In 17 of 25 AML samples tested for effects of M-CSF we found an increase in the number of adherent cells that became apparent after 10 days of culture with up to 20% of cells adhered (Fig. 1). Overlapping effects of CSFs may result in synergistic or additive actions on in vitro growth of some AML specimens, as shown for G-CSF and GM-CSF [26, 65]. Effects of combinations of different CSFs on blast colony formation vary in contrast to very reproducible responses seen in normal hematopoietic progenitor cells [8, 66]. Responses of AML to CSFs could not be associated with distinct immunophenotypes of whole leukemic populations or those of L-CFC, nor to morphological subtypes [2, 42, 65].

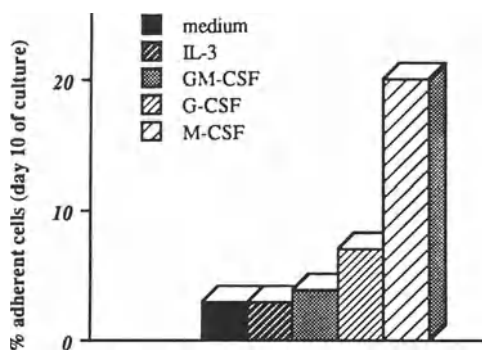
In long-term culture the combination of IL-3 with G-CSF and GM-CSF resulted in higher plating efficiency compared with the effects seen with individual CSFs. Interestingly, the tendency of AML cells to differentiate in this assay was not reported to be accelerated by exogenously added CSFs, as compared with the maturation process ap-

**Table 1.** Proliferative response of L-CFC to hematopoietic growth factors<sup>a</sup>

5637-CM (15% v/v)	GM-CSF (100 ng/ml)	G-CSF (100 ng/ml)	M-CSF (1000 U/ml)	IL-3 (100 ng/ml)	IL-1-b (20 U/ml)	TNF- $\alpha$ (50 ng/ml)
38/49 (77) <sup>b</sup>	31/49 (63)	25/49 (51)	0/19 (5)	10/19 (52)	0/19 (0)	0/19 (0)

<sup>a</sup> Colony formation of L-CFC at day 10 of culture in a double-layer agar assay

<sup>b</sup> Number positive/number investigated (percentage positive)



**Fig. 1.** Response of AML samples to various growth factors is determined by increases in adherent cells in suspension culture. Leukemic cells were cultured at  $10^6$  cells/ml in medium, or in medium with IL-3 (100 ng/ml), GM-CSF (100 ng/ml), G-CSF (100 ng/ml), or M-CSF (1000 U/ml) for a period of 10 days. Percentage of adherent cells was evaluated at the end of the incubation period for each assay. The data represent means of ten independent experiments

pearing spontaneously [66]. In rare cases of AML (FAB M7), IL-1-beta has been identified as an essential growth promoter but also as an inducing molecule for IL-2 receptor expression [55]. However, there is no evidence for a promoting effect of IL-1 on blast colony formation of AML for the FAB M1, M2, M4, and M5 subtypes (own observation, unpublished), although IL-1 may act synergistically on very primitive multipotent hematopoietic cells [62]. Recently IL-6 has been identified as a costimulator to augment CSF-induced clonogenicity of AML blasts [45]. There is also evidence for suppressive effects on growth of AML, exerted by direct action of various cytokines, including TNF-alpha, IFN-gamma [18], and transforming growth factor-beta [64].

### Colony-Stimulating Factor and Cytokine Production by AML Blasts

In contrast to normal myeloid progenitor cells, L-CFCs of some AML show colony formation in the absence of exogenous growth factors with a rate of 28% [42] (Table 2). With this information and responsiveness of AML samples to CSFs, it became conclusive to search for autocrine production of CSF in AML.

Recent studies show mRNA accumulation for various CSF species in a substantial proportion of a series of AML. G-CSF mRNA was detected in 25%–30%, GM-CSF in 23%–25%, and M-CSF in 12%–50% of the AML samples investigated [42, 53, 72]. Most frequently mRNA synthesis was found in AML specimens that exhibited autonomous in vitro growth (Fig. 1). In these cases autonomous growth was shown to be inhibited by adding neutralizing antisera for a given CSF to the experimental cultures [17, 71]. Autonomous leukemia colony growth was also not associated with a particular pattern of CSF production by AML or with distinct morphological subtypes [42].

Whereas the majority of AML samples with G-CSF and GM-CSF mRNA accumulation secreted the respective protein in a biologically active form, M-CSF was detected most often as a membrane-anchored protein [42, 53] (Table 3). The association of several eukaryotic genes to alternative promoters has been recognized. Thus alternative modes of splicing may result in generation of mRNA species coding for polypeptide products, which exhibit different secretion patterns. The presence of a hydrophobic transmembrane domain and two forms of protein encoded by two differently spliced mRNAs has been shown for M-CSF [28, 52]. Alternative use of the 5' splice

**Table 2.** Association of autonomous in vitro growth by L-CFC and mRNA accumulation pattern by AML blasts

CSF mRNA for:	G+GM+M	G+GM	G	GM	M	No. CSF mRNA
Number of cases	4 <sup>a</sup>	2	3	0	0	5

<sup>a</sup> Values are expressed as number of cases growing blast-type colonies in the absence of exogenous growth factors. Total number of AML samples tested for autonomous colony growth was 49

**Table 3.** Colony-stimulating factor and cytokine production by AML blasts

	mRNA	Protein
G-CSF	15/49 <sup>a</sup>	12/49
GM-CSF	11/49	9/49
M-CSF	6/49	0/49
IL-3	0/54	ND
Beta-IL-1	14/44	14/44
IL-2	0/49	ND
IL-4	0/25	ND
IL-6	14/54	14/54
Alpha-TFN	19/54	19/54

ND, not done

<sup>a</sup> Number positive/number investigated

donor sites in intron 2 of the *G-CSF* gene has been shown in human squamous carcinoma line (CHU-2) [39]. Other reports describe utilization of alternative promoters to transcribe the murine *GM-CSF* gene, thereby altering the hydrophobic NH<sub>2</sub>-terminal leader sequence, and generating a non-secreted form of the protein [63].

Recent studies have focused on autocrine production of various growth factor-related cytokines with growth regulatory potential on AML blasts. It was found that a high proportion of AML populations secrete IL-6, IL-1-beta, and TNF-alpha [15, 45]. Whereas IL-6 was shown to act as a cofactor to promote AML growth in vitro by enhancing numbers of CSF-driven blast colonies, IL-1-beta and TNF-alpha had little direct effect on AML growth (Table 1) [45]. Only high concentrations of TNF-alpha were shown to inhibit leukemic growth [18].

Endothelial cells which are potent sources of CSF supply in the normal marrow are known to respond to IL-1 and TNF with production of CSFs [38, 58, 73]. Consequently, AML-derived IL-1 and TNF has been shown to induce CSF synthesis by endothelial cells and has therefore been identified as a mechanism to augment growth-promoting stimuli [15, 45]. IL-4 acts in synergy with G-CSF to induce proliferation of normal CFU-GM [49]. Therefore we considered IL-4 as a candidate cytokine for synergistic growth promotion of AML blasts. In our series we found no AML sample pro-

ducing IL-4 ( $n=25$ ) (Table 3). A significant number of AML samples also express M-CSF receptors [11, 53]. No association was, however, found between growth of AML in vitro and expression of the M-CSF receptor. It has been shown that AML samples with monocytic phenotypes (FAB M4 and M5) can be induced to express surface-binding sites for IL-2 by gamma-interferon [16]. As already mentioned above, the IL-2 receptor could also be upregulated by IL-1-beta in AML M7 subtypes [55].

In series of AML samples of various subtypes ( $n=49$ ), IL-2 mRNA was not detectable (own unpublished observation) (Table 3), suggesting that IL-2 does not play a role in autocrine growth control of AML. Coordinated expression and close linkage of IL-3 and GM-CSF genes in human activated T cells [47] led us to examine AML cells for IL-3 expression; however, mRNA or protein activity for IL-3 was not detected in any of the cases studied ( $n=54$ , unpublished). Data of factor expression in our series of AML are summarized in Table 3.

Cascade induction of various cytokines in normal [19, 21, 34, 46] and malignant cells [5, 35] raises the possibility that growth factors may recruit each other. Particularly IL-1, which was never found to be expressed by AML samples as a sole factor [45], must be considered in this regard. Further studies on the inducible expression of CSFs by AML blasts are currently under way. In order to exclude that distinct differentiation states of hematopoietic cells are physiologically associated with cytokine expression, a set of experiments was performed to show that accumulation of CSF mRNA by AML blasts does not depend on induction by in vitro cell processing, as previously mentioned [1]. Moreover, ten AML populations that were negative for G-, GM-, M-, and multi-CSF mRNA were induced with phorbol myristate acetate without providing evidence for inducible mRNA accumulation after various periods of incubation. In a series of ALL ( $n=20$ ), assayed for accumulation of CSF, mRNA for G-, GM-, and M-CSF remained undetectable.

## Pathophysiological Impact of Factor Production by AML Blasts and Possible Initiation Events

It has been suggested that transformed cells may escape growth restrictions by minimizing their needs for exogenous factors [61]. Some experiments suggest that growth factor production by malignant cells may be one escape mechanism and possibly crucial to sustain transformation: Infection of chicken hematopoietic cells with MH2, an avian retrovirus containing the *v-myc* and *v-mil* oncogenes, has resulted in transformation and proliferation of leukemic cells in the absence of exogenous growth factors. Autonomous growth in this system was promoted by chicken myelomonocytic growth factor production, induced by *v-mil* in *v-myc*-transformed hematopoietic cells [13]. Moreover constitutive synthesis of IL-3 by leukemia cell line WEHI-3B has been shown to result from retroviral insertion near the gene [70]. However, in human AML no virus-induced CSF expression has been reported so far. Cytogenetic abnormalities, which involve *CSF* genes in human AML, e.g., (del 5q) or (t15;17), must also be considered to result in dysregulated expression of growth factors. However, no specific association of cytogenetic changes with factor expression has yet been unraveled.

To investigate possible genetic changes undetectable by cytogenetic analysis, Southern blot analysis from our series of AML samples ( $n=54$ ) was performed to search for reconstructed genes of *G-CSF*, *M-CSF*, *GM-CSF*, *IL-3*, and *IL-6*. So far, we have not been able to detect a case with structural abnormalities in any of these AMLs, including AML populations that proliferated autonomously in vitro and/or expressed substantial amounts of CSF activity. However, another group reported abnormal Southern patterns of *G-* and *GM-CSF* genes, each in two individuals with AML: abnormal genomic patterns of GM-CSF were associated with larger-than-normal messages, which were constitutively expressed in two patients. In one patient with an abnormal G-CSF pattern, normal-sized G-CSF and GM-CSF mRNA was detected [7].

Autocrine and paracrine mechanisms, which can be viewed as primitive mecha-

nisms to achieve growth advantages [61], may be potentially detrimental to the survival of the organism if they are not closely regulated. Thus malignant transformation may result from inappropriate delayed expression of growth factors that were required by cells in early stages of differentiation. In order to determine a possible predisposition of CSF-stimulated hematopoietic cells to malignant transformation, the *GM-CSF* gene was introduced into the germline of mice, without observing myeloid tumors in these animals [29].

This and other experiments [54] reveal that induction of growth factor expression is most likely not the sole causative principle for oncogenesis in myeloid cells. *ras* gene mutations have been considered as an initiation event for AML and *ras* protein was identified as a GTP-regulatory protein which serves as transducer element in the activation of adenylate cyclase [32]. Recent preliminary data suggest an accelerating role for a mutant *ras* gene product to augment growth factor-dependent signaling; in this experiment transfection of a mutant *ras* gene resulted in enhanced IL-3-dependent proliferation of a myeloid cell line [3]. To assay for an association with CSF expression we are currently screening a series of AML for N-*ras* point mutations.

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## Clonal Analysis of Human Leukemias by Molecular Genetic Approaches

C. R. Bartram and J. W. G. Janssen

Studies of clonality using cytogenetic markers and glucose-6-phosphate dehydrogenase (G6PD) isoenzymes have provided convincing evidence for the clonal origin of most malignancies [1, 2]. Recently, molecular genetic approaches have complemented these investigations. In this chapter we briefly discuss some clinical applications or recombinant DNA strategies as powerful tools for the identification and monitoring of clonal cell populations in hematopoietic neoplasias.

### Gene Rearrangements at Chromosomal Breakpoints

A novel set of clonal markers has been obtained through the molecular cloning of breakpoints involved in tumor-specific chromosomal aberrations. A case in point is the Philadelphia translocation (*Ph*) observed in more than 95% of chronic myelocytic leukemias (CMLs) and 20% of adult acute lymphoblastic leukemias (ALLs). The molecular hallmark of this cytogenetic marker is a recombination between the *c-abl* and the *bcr* gene [3]. Although clustered within two distinct areas of the *bcr* gene, the breakpoints on chromosome 22 differ molecularly from patient to patient. Therefore *bcr* rearrangements as demonstrated by Southern blot analysis can serve as a leukemia-specific marker in monitoring therapeutic effectiveness of regimens such as bone marrow trans-

plantation or interferon therapy [4, 5]. Application of a modified polymerase chain reaction (PCR) technique has recently revolutionized the diagnosis of Ph-positive leukemias [6, 7]. This approach allows the rapid and precise mapping of breakpoints within the two *bcr* regions involved in Ph-positive CML and ALL and, moreover, the detection of minimal residual leukemic cells at a level of 1:100 000 (Fig. 1). Using this ultrasensitive method we observed leukemic cells in a variety of transplanted CML patients during complete clinical/hematological remission, who also revealed no indication of residual leukemia by cytogenetic or conventional Southern blot analysis. An example is shown in Fig. 1 A (lane b).

Similar strategies have been successfully applied in other hematopoietic neoplasias, e.g., follicular lymphoma characterized by a t(14;18) and a corresponding rearrangement of the *bcl<sub>2</sub>* gene [8, 9]. However, the ultimate clinical significance of the PCR technique in detecting subclinical disease and predicting the clinical course of a given patient remains to be elucidated.

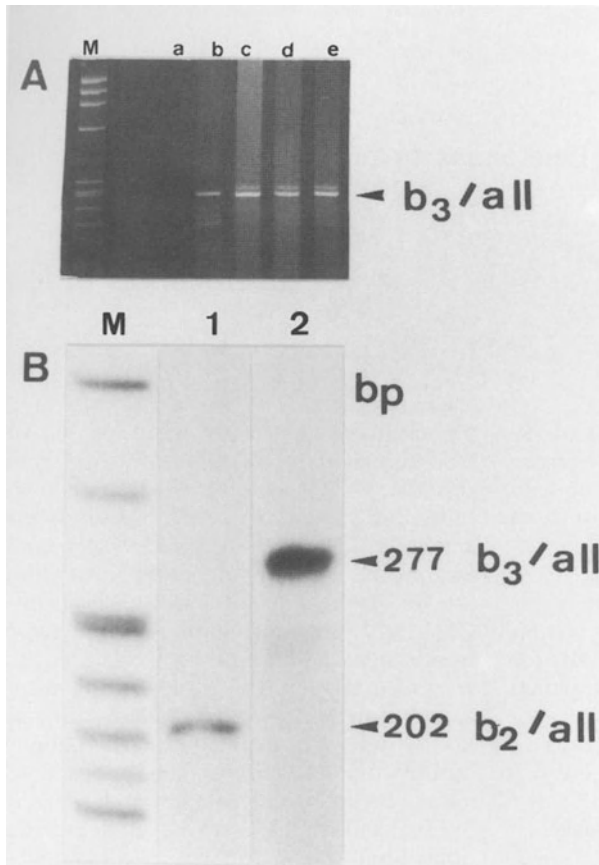
### Immunoglobulin and T-Cell Receptor Gene Rearrangements

The analysis of *Ig* and *TCR* gene structure and regulation has been of great value in

1. defining the lineage and stage of maturation of lymphoid malignancies,
2. determining the clonality of cell populations, and
3. monitoring the clinical course of hematopoietic neoplasias [10].

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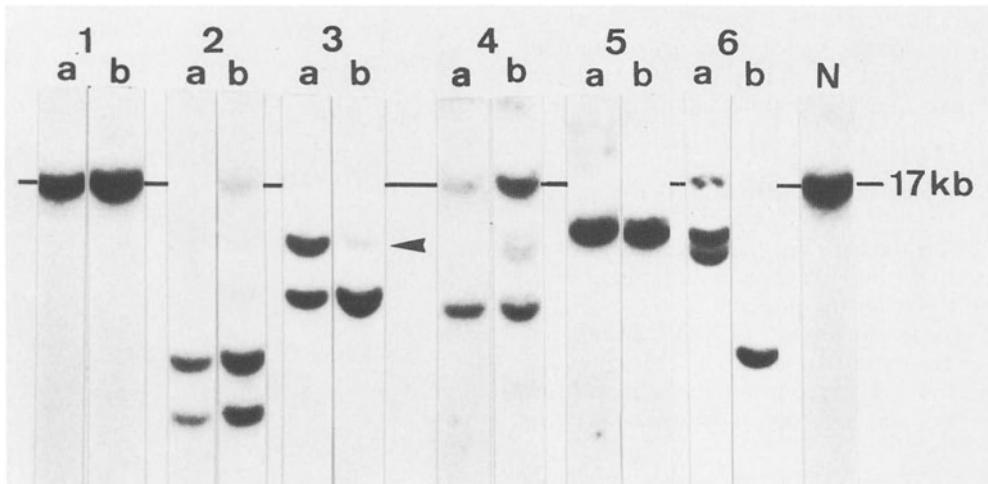
**Fig. 1 A, B.** Diagnosis of Ph-positive CML by PCR. **A** RNA samples obtained from BM cells of two CML patients 13 and 8 months after bone marrow transplantation (*a, b*) and a dilution series of a third case chronic-phase disease (*c-e*; one leukemic cell was diluted in  $10^2$ ,  $10^3$ , or  $10^4$  normal cells) was copied into cDNA, amplified, and directly run on a 2.5% agarose gel. Rearranged *bcr/abl* sequences (arrow) are observed in all lanes except *a*. **B** Amplified cDNAs obtained from peripheral blood of two Ph-positive CMLs were run on an 8% polyacrylamide gel and hybridized to a *c-abl* probe. Analysis of case 1 shows a recombination between *bcr* exon 2 and *abl* exon II, while in case 2 *bcr* exon 3 is spliced to *abl* exon II. *M*, molecular weight marker

Along this line we recently investigated 21 ALL patients combining immunological and molecular analyses to evaluate the clonal relationship of leukemic cells at first presentation and relapse [11, 12]. Using this approach we provided compelling evidence that, despite a similar phenotype at relapse, leukemic cells of the majority of cases (12/21) showed genotypic variations (Fig. 2). Extending a previous study [13], we also analyzed 31 AUL (acute undifferentiated leukemia) patients and found in 30% of cases multiple rearranged *Ig* gene fragments due to clonal evolution. Reexamination in relapse was possible in four of the latter patients and revealed reemergence of a single subclone. As a possible clue to the mechanisms leading to the predominance of one leukemic subclone in relapse, we observed concurrent amplification and/or increased expression of *mdr1* sequences. Increased expression of the *mdr* gene family, however,

has been implicated with the development of resistance against multiple chemotherapeutic agents [14].

#### Mutated *ras* Genes

Point mutations leading to the activation of the transforming potential of *ras* genes have been detected in a variety of different human malignancies with variable frequencies [15]. Preferential involvement of the Ki-*ras* gene has been demonstrated in some solid tumors such as pancreatic and colorectal carcinomas or adenocarcinomas of the lung, while activated N-*ras* genes predominate in hemopoietic neoplasias [15, 16]. Although the clinical significance of this genomic alteration remains to be elucidated, mutated *ras* genes are undoubtedly valuable markers for clonal analysis. This may be illustrated by recent investigations of AMLs demonstrat-



**Fig. 2.** Southern blot analysis of six ALLs investigated at first presentation (a) and relapse (b). *Bam*HI digests were hybridized to *Ig mu* sequences detecting a 17-kb germline fragment. *N*, placenta control DNA. Note clonal variations in both stages of cases 3, 4, and 6

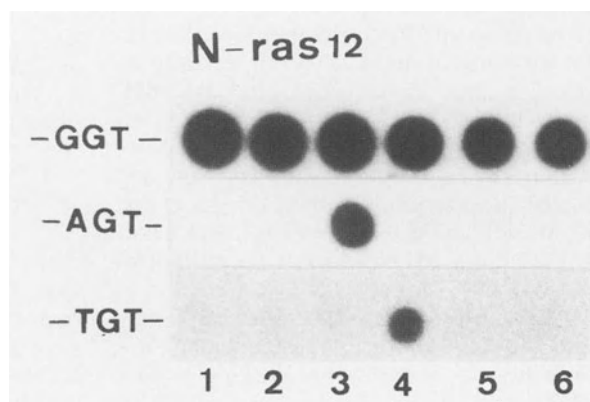
ing mutated *N-ras* alleles in approximately 20% of cases [17–19]. A remarkable heterogeneity of leukemic cells was observed by direct comparison of the *ras* gene status of individual cases at initial diagnosis and relapse [18, 19]. These studies identified AMLs with

1. identical mutations at both stages,
2. acquisition of a point mutation at relapse, or
3. absence of a mutated alleles, initially characterizing leukemic blasts (Fig. 3).

*Ras* gene mutations can obviously occur at various stages during the evolution of AML.

The detection of *ras* mutations in a proportion of patients with myelodysplastic syndromes, preleukemic entities evolving into overt AML in up to 40% of cases, supports the view that respective alterations may also represent initiating events in leukemogenesis [20, 21]. In a series of 96 myelodysplastic syndromes (MDSs) we observed *ras* gene mutations in seven patients. Cell separation analysis demonstrated the pluripotent stem cell origin of all cases by identification of mutated alleles in granulocyte, monocyte/macrophage, as well as B- and T-lymphocyte cell fractions [22, 23]. Moreover, the value of *ras* gene analyses for monitoring

**Fig. 3.** Detection of mutated *N-ras* codon 12 alleles in two AML patients investigated at initial diagnosis (1, 4), remission (2, 5), and relapse (3, 6). DNA preparations obtained from peripheral blood samples were amplified by PCR and screened using oligonucleotide probes specific for the wild-type *N-ras* 12-sequence GGT (glycine) as well as mutation AGT (serine), or TGT (cysteine). One patient (1–3) is characterized by substitution of the normal amino acid glycine by serine (AGT) in leukemic cells at relapse (3), but not at primary diagnosis (1), while the other case (4–6) exhibits a substitution of the wild-type allele by cysteine (TGT) initially (4), but not in blasts at relapse (6)



therapeutic effectiveness may be illustrated by the disappearance of *ras* gene mutations in MDS patients during the course of low-dose cytarabine treatment [24].

### X-Chromosome Inactivation Analysis

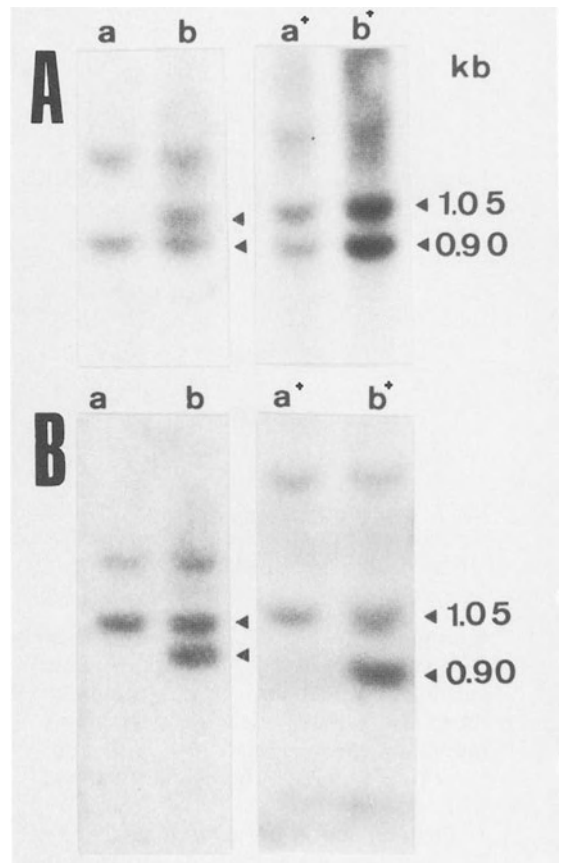
X-Chromosome inactivation analyses as introduced by Vogelstein et al. [25] are based on the following principles:

1. The random nature of X-inactivation renders females mosaic for heterozygous loci. However, in monoclonal cell populations the same X-chromosome will be active in all cells.
2. Maternal and paternal alleles of an X-linked gene can be distinguished by restriction fragment length polymorphisms (RFLPs).
3. Active and inactive copies of X-chromosomal genes such as hypoxanthine phosphoribosyl transferase (HPRT) or phosphoglycerate kinase (PGK) are identified through different methylation patterns.

This approach enables X-inactivation to be studied in almost 50% of females and thereby overcomes a major detraction of G6PD isoenzyme analysis posed by the rarity of heterozygous patients.

Using this strategy the clonal nature of acute myeloid leukemias could be unequivocally demonstrated [19, 26]. Interestingly, a single dominant hemopoietic clone can also be observed in a proportion of AML patients (Fig. 4) in otherwise complete clinical and hematological remission [19, 26, 27]. This result indicates that in some patients leukemic cells may have the capacity of differentiating into mature hematopoietic cells. From a clinical point of view it seems to be relevant that clonal remission in AML might not necessarily be associated with an unfavorable prognosis [19, 26]. This observation may be reconciled with data obtained by the same technique establishing a clonal relationship of hematopoietic cell lineages in almost every MDS patient analyzed, irrespective of disease duration [23].

The few examples discussed above may indicate the value of molecular genetic approaches in establishing diagnostic and prognostic parameters for the analysis of



**Fig. 4 A, B.** Clonal composition of bone marrow samples from two female AML patients (**A, B**) determined by X-chromosome inactivation analysis. A PGK RFLP characterized by 1.05-kb and 0.9 kb fragments in *BstXI/PstI* digests distinguishes the maternal from the paternal allele and methylation differences as shown by *HpaII* digestion distinguish between active and inactive X-chromosome. Following *BstXI* and *PstI* digestion, DNAs were divided into two equal aliquots; one was not digested further (*lanes b*) and the other was digested with *HpaII* (*lanes a*). The complete loss of one allele is demonstrated after *HpaII* digestion in both patients prior to therapy, indicating a monoclonal cell population. Analysis in remission reveals restoration of a polyclonal pattern in patient A (*lane a+*), but persistence of monoclonal hematopoiesis in patient B (*lane a+*)

human leukemias. Although we have concentrated in this chapter on some novel strategies of elucidating the clonal nature of hematopoietic neoplasias, it is obvious that the application of recombinant DNA tech-



niques is of major importance for numerous areas in clinical oncology.

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## Clinical Relevance of Cytogenetics in Acute Leukemia

A. Hagemeijer and D. C. van der Plas

### Introduction

Cytogenetic analysis of leukemic cells has shown the presence of acquired chromosome abnormalities in more than 50% of cases. Since 1970, banding techniques have been applied progressively and a preliminary analysis of the data has been performed at the First, Second, and Fourth International Workshops on Chromosomes in Leukemia (IWCL) (1978, 1979, 1982) for Acute Non-Lymphocytic Leukemia (ANLL) and at the Third IWCL (1980) for Acute Lymphoblastic Leukemia (ALL) [1–4]. These analyses revealed

1. the nonrandom occurrence of some cytogenetic changes in acute leukemias and
2. the association of specific chromosomal abnormalities with specific morphological subtypes of leukemia; and
3. they emphasized the diagnostic and prognostic value of the karyotype of the leukemic cells independent of other clinical and hematological features.

Cytogenetic abnormalities also constitute a unique marker of the leukemic cells that is used in longitudinal studies to ascertain remissions, relapses, and residual diseases [5].

More recently, development of molecular genetic techniques has stimulated investigation into molecular changes characteristic for specific leukemic chromosomal translocations. With the exception of the Philadel-

phia translocation, most progress along this line of research has been slower than originally expected. How gene rearrangement and/or activation of oncogenes cause leukemia has not yet been demonstrated but, in the meantime, the discovery of specific molecular changes has generated new tools for diagnosis and follow-up of leukemic patients [6].

### Chromosome Abnormalities in ANLL

#### *Occurrence and Type of Clonal Aberration*

A relatively large number of reports have been published describing consecutive or selected series of ANLL patients and correlating the karyotype of the leukemic cells with morphological type of leukemia and outcome [for review see 7, 9–13]. In ANLL clonal abnormalities are found in 60%–90% of cases depending on the laboratory and the selected group of patients: in de novo adult ANLL the percentage of normal bone marrow karyotype is more important than in childhood ANLL or in secondary ANLL [7]. The clonal changes can be classified into three major categories:

1. Specific translocation associated with a particular morphological subtype of leukemia according to the French-American-British (FAB) classification (Table 1). These specific changes are more frequent in children and younger patients. The overall frequency is about one-third that of the ANLL cases.
2. Nonrandom numerical or structural changes, particularly +8, -7 or 7q-, -5

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**Table 1.** Recognized specific chromosomal changes in de novo ANLL

Chromosome abnormalities <sup>a</sup>	FAB morphological subtype	Additional characteristics
t(15;17)(q22;q11 or q12) t(8;21)(q22;q22) t(6;9)(p23;q34)	M3 and M3 variant M2 M2 or M4	In more than 90% of M3 Auer rods Basophilia in M2 cases, sometimes history of MDS
t(9;22)(q34;q11) inv(16), del(16), t(16;16) (p13;q22)	M1 (M2, M4) M4-“eo”	Faint peroxidase stain Abnormal eosinophils in bone marrow
t(9;11)(p21-22;q23), t11q23 t(8;16)(p11;p13)	M5a or M4 M5b	Erythrophagocytosis

<sup>a</sup> For specific reports on these translocations see ref. 7, 16, and 17

or 5q-, but also trisomy 21 and 22, and deletion of part of 17p, 20q, 18q, 9q, and 13q. These changes are recurrent, found as isolated abnormality or in association. They are found in about one-third of de novo ANLLs, they are the most frequent changes in secondary leukemia, but they are also observed in myelodysplastic syndrome (MDS). They are not associated with a specific FAB morphological type and are the major component of the complex karyotypic abnormalities that characterize leukemia with very poor prognosis.

- The remaining cases (about 25%) show numerical (like +4) or structural changes that are random and/or specific, but every single one occurs at such low frequency ( $\leq 1\%$  of ANLL cases) that their diagnostic significance has not yet been established. A recent example of such miscellaneous structural change is the t(8;16) that is now recognized as a specific translocation associated with FAB-M5 acute leukemia with prominent phagocytosis [14, 15].

### *Clinical Significance*

For obvious reasons, determination of the clonal chromosomal aberrations in ANLL is of diagnostic value: it confirms malignancy and for the specific changes, because of their specific clinical and morphological associations, it makes possible a chromosomal subclassification of leukemia [17]. Recent large

studies correlating response to therapy and outcome of ANLL patients classified according to their leukemic karyotype indicated the prognostic value of the leukemic karyotype independent of other clinical parameters. At the Sixth IWCL [13] held in London in 1987 the follow-up data of the Fourth IWCL were analyzed and the results were in line with the study reported by Keating et al. [12] as well as other studies (Table 2). These studies demonstrated not only a difference in morphological diagnosis, therapeutic response, and survival for the various type of cytogenetic abnormalities but also that unique biological consequences follow these abnormalities that influence the (natural) course of the disease and that could be amenable or require specific therapeutic strategy. For example, the t(15;17) is found in more than 90% of cases with FAB-M3 and with the microgranular variant form of acute promyelocytic leukemia (APL): 20%–30% of these patients died early of hemorrhagic complications (DIC, disseminated intravascular coagulation); among the treated patients, the remission rate was high and the duration of first complete remission was relatively long, but relapses usually appeared to be therapy resistant. In contrast, patients with a t(8;21) showed a high remission rate, but also tendency to successive relapses followed by successive remissions, explaining the overall survival time, which is only slightly better than the cases with a normal diploid karyotype, matched for age and receiving the same type of treatment.

**Table 2.** Prognostic value of leukemic cell karyotype in ANLL median survival/CR duration in months

Karyotype	Sixth IWCL (1987) <sup>a</sup> 355 intensively treated		Yunis et al. (1988) <sup>a</sup> 185 adults		Keating et al. (1988) <sup>a</sup> 384 patients	
	<i>N</i>	Median survival	<i>N</i>	Median survival	<i>N</i> (CR)	Median CR duration
Normal	148	14	19	22+	178 (122)	14
Abn. 16	6	27	14	26+	21 (19)	22
t(15;17)	16	20	8	20	21 (11)	27
t(8;21)	25	14	9	24+	27 (25)	17
Abn.11q	15	6	16	9	5 (3)	–
–5,5q–	28	3	11	9	41 (15)	7
–7,7q–						
t(9;22)	–	–	5	3	12 (4)	–
Complex	52	12	39	3		
Very complex	21	3				

<sup>a</sup> Refs. 13, 12, and 11 respectively

The cases with *inv*(16), *del*(16), or *t*(16;16) showed a complete remission rate of 95% or more. But, after cessation of therapy, relapses as solid leptomeningeal tumors [18] or even peritoneal tumor have been reported. Later on, changes in therapeutic regimen seemed to have obviated these late complications [12].

Other specific translocations, i.e., *t*(9;22), *t*(6;9), or translocation involving 11q23 are correlated with very poor prognosis and short survival, as are also the recurrent abnormalities with losses of chromosomes 5 or 7. The very complex karyotypes are particularly refractory to treatment.

For other specific changes, the frequency is too low to enable any sensible analysis of survival data to be made. In Yunis' recent report of 185 consecutive AMLs [11], the cases with single miscellaneous defect (20% of total) did as well as the patients with a normal diploid karyotypes (10% of total in this series), with a median survival close to 2 years. In other series [e.g., 9, 10, 12, 13], where the proportion of normal diploid karyotype (NN) is much larger, the median survival time of this NN category is significantly shorter than in the cases with single recurrent effect, indicating the intrinsic heterogeneity of the "normal leukemic karyotype" group.

## Chromosome Abnormalities in ALL

### *Incidence and Classification*

In recent consecutive series of chromosome studies in ALL, including our own, 20%–40% of the cases show only normal metaphases, less than 10% are unsuccessful, and the remaining 50%–70% are abnormal. In childhood ALL, the abnormal karyotypes are distributed in various categories, based on changes in ploidy: high hyperdiploid karyotypes with more than 50 chromosomes ( $\pm 30\%$  in childhood ALL), hyperdiploid karyotypes with 47–50 chromosomes ( $\pm 10\%$ ), pseudodiploid karyotypes (25%–30% of cases), and rare hypodiploid cases (Table 3) [19, 21–24]. In adults, the percentage of normal karyotypes and of hyperdiploid karyotypes ( $\geq 50$  chromosomes) is decreased in favor of the pseudodiploid cases [20, and personal unpublished data]. The latter category is characterized by the presence of structural abnormalities and translocations that have been shown to correspond to the specific immunological phenotype with distinct prognostic significance.

**Table 3.** Distribution of the leukemic karyotypes in ALL in children and adults

	Normal	Hyperdiploid		Pseudodiploid	Hypodiploid
		> 50 chromo- somes	47–50 chromo- somes		
Children	20%–40%	±30%	±10%	25%–30%	<5%
Adults	15%–20%	10%–20%	±10%	35%–50%	±5%

### Technical Considerations

Cytogenetic analysis in ALL are more difficult than in ANLL. In ALL, despite high cellularity and a high percentage of leukemic cells in bone marrow (BM) and peripheral blood (PB), only a low yield of leukemic metaphases is obtained, with chromosomes very fuzzy and of poor morphology. Use of cultures and synchronization techniques increases the number of well-banded metaphases, but these show mostly a normal karyotype and are probably representative of the normal BM stem cells. This is particularly true in children and for the group of ALLs with hyperdiploid karyotype. Adult ALLs are more amenable to culture and in many cases a 24-h culture will be the method of choice. Despite these difficulties in childhood ALL, by using a direct method and careful handling of BM cells in an optimal setting, Williams (1985) demonstrated the presence of an abnormal karyotype in over 90% of cases [25]. To the best of our knowledge, these results have not been equaled in other institutions. DNA flow cytometry of leukemic cells can (should) be used in parallel with cytogenetics: indeed DNA cytometry will reliably identify two categories of patients: those with a high hyperdiploid karyotype (> 50 chromosomes) and those with low hypodiploidy ( $\leq 43$  chromosomes) corresponding to the groups with the best and worse prognosis, respectively [26].

### Prognostic Significance of Cytogenetic Findings in ALL

In childhood ALL, the group of patients with high hyperdiploidy shows the highest

response rate and a probability of cure of about 85% with modern chemotherapy [3, 17, 19, 21–24]. Translocations, particularly the Philadelphia translocation, the t(4;11), and translocations involving 8q24 in association with B-cell ALL have been shown to identify patients with the shortest survival. Intermediate response rate and survival time is found for the group of patients with normal karyotype and with slight aneuploidy (45–49 chromosomes) provided that the poor-risk translocations are absent. Translocation (1;19), 6q–, 9p–, and rearrangement of 12p are recurrent structural changes seen in these leukemic karyotypes (Table 4).

In adult ALL, discrimination between better- and worse-risk ALL is much less clear, as survival longer than 3 years is still exceptional. But, also in adults, the high hyperdiploid karyotype (> 50 chromosomes) is doing better than the other karyotype [3, 20, 27, personal unpublished data].

In ALL the *high-hyperdiploid karyotype* shows recurrent, nonrandom numerical abnormalities with mainly trisomy of chromosomes X, 4, 6, 10, 14, 17, 18, and often tetrasomy 21. Structural changes and translocations are unusual and are found in about 10% of cases. Classically, the leukemic karyotype correlates with FAB-L1 (80%) or FAB-L2 (20%) morphological classification and with the common ALL (sometimes pre-B) immunophenotype. Incidentally, 0-ALL and T-ALL are found, but in these cases there is usually a specific translocation corresponding to the particular phenotype, in addition to the numerical changes. Two categories of high-hyperdiploid karyotypes do not belong to this rather homogeneous group and are best diagnosed by using DNA flow cytometry in parallel with cytogenetics, i.e., (a) the near-

**Table 4.** Prognostic value of karyotype in ALL

Outcome	Karyotype	Immunophenotype
Favorable	Hyperdiploid: > 50 chromosomes	CALLA (80%)
Intermediate	Normal	
Poor	Translocations t(1;19) t(8;14) (q24;q11) t(11;14) (p13;q11) inv(14)	Pre-B T-ALL T-ALL
Very poor	t(4;11) t(9;22) t(8;14) (q24;q32) near haploid	0-ALL CALLA/Pre-B B-ALL
Unclear	6q- del/t 9p del/t 12p	No specific phenotype

CALLA, common acute lymphoblastic leukemia antigen; T-ALL, T-cell acute lymphoblastic leukemia; 0-ALL, Null-acute lymphoblastic leukemia; B-ALL, B-cell acute lymphoblastic leukemia

diploid karyotype (26–35 chromosomes), which by duplication may present with a majority of hyperdiploid metaphases, and (b) the hypotetraploid karyotype, both types with less favorable outcome.

A normal karyotype is found in various percentages in different studies and is by nature heterogeneous since it comprises ALL cells with changes undetectable by cytogenetics as well as undetected abnormalities due to technical shortcomings. All immunophenotypes are found, but more than half of adult T-ALLs and about 30% of childhood T-ALLs have a normal karyotype. Prognosis is also intermediate, with a probability of cure of 65%–70% for children, and a median disease-free survival of 12 months in adults.

In ALL, the specific translocations identified correspond to specific immunophenotypes and are indicative of unfavorable outcome, thus requiring adapted treatment strategy. Other nonrandom structural changes are deletions (6q-, 9p-, 11q-) and unbalanced translocations involving particularly 1q, 9p, 12p, as well as 2 isochromosomes i(7q) and i(9q). Most of these recurrent abnormalities are probably secondary. They are found as a single defect or in association with other aberrations and their prognostic significance is still unclear [28].

#### *ALL with t(4;11)*

We studied 12 cases of ALL with t(4;11), 6 children including 4 infants aged 2–13 months, and 6 adults aged 22–67 years [29 and unpublished data]. All presented with high-risk clinical features and an 0-ALL phenotype (Tdt+, CD19+, CD10-, HLADR+), rearrangement of IgH in the investigated cases, and paradoxical expression of some mature myeloid or monocytic membrane antigens (CD15+). Morphologically, it is the prototype of biphenotypic leukemia. Usually the majority of blasts show a FAB-L2 morphology but monoblastic features can be expressed when studied by electron microscopy, with monoclonal antibodies or after induction with phorbol esters. In our series, all adults died within 1 year of diagnosis, four of the children survived 30–51 months, and two of them are still alive 30 months and 45 months after diagnosis.

#### *Philadelphia-Chromosome-Positive ALL*

We studied ten ALL patients with Philadelphia translocation in leukemic cell karyotype, one child and nine adults (12.5% of adult ALL in our series). The standard t(9;22) was found in six cases, variant

translocations in four instances, and in four cases the karyotype showed rather complex additional abnormalities (unpublished data). In four cases, molecular studies showed a breakpoint on chromosome 22 in the 5.8-kb *BCR* region corresponding to the breakpoint cluster region in chronic myelocytic leukemia (CML) [30]. Six cases were *BCR*(-) and in four of them rearrangement of the first intron of the *bcr* gene could be demonstrated; the other two cases were not investigated [6]. The median survival time was 12 months and the longest 36 months. This was an exceptional patient who relapsed after 28 months of complete remission. This series is too small to show and to allow correlation between the site of molecular breakpoint in the *BCR* gene (either first intron or *BCR* region) and outcome.

### Cytogenetic Marker of Leukemic Cells

Cytogenetic abnormalities constitute a unique marker of leukemic cells. In longitudinal studies, they are used to assess the quality of hematological remission and of bone marrow autograft and to demonstrate impending relapse. Their usefulness in detecting minimal disease is limited by the requirement of a large number of metaphases, the majority being from normal progenitors. For this reason, combined techniques are used, aiming at selection or enrichment of the residual leukemic cell population in the sample karyotyped, for instance, immunolabeling of the metaphases [31], flow sorting of cells labeled with specific monoclonal antibodies [32], flow karyotyping [33], interphase cytogenetics using chromosome-specific alphoid probes to detect a known aneuploidy [34]. Cytogenetics and cell culture techniques can also be combined, e.g. using specific growth factors for recruitment of leukemic cells [35]. By far the most powerful tool for detecting minimal diseases is the new molecular technology that allows amplification of DNA or RNA sequences specifically altered in some leukemia (e.g.,  $\text{Ph}^1+$  leukemia), using the polymerase chain reaction (PCR) [6]. Unfortunately, so far this technique has only been applicable in the cases where molecular analyses of the specific chromosomal translocation have

demonstrated consistent molecular rearrangements.

### Molecular Studies of Specific Cytogenetic Rearrangements

In acute leukemia, the recurrent chromosomal translocations are indicative of a consistent molecular rearrangement of the genes located in the chromosomal bands involved in the translocation. Presumably, molecular analyses of these specific translocations will unravel the mechanisms causing the development of this particular morphological type of leukemia. So far the studies have been successful in demonstrating constitutive activation of the *c-myc* oncogene in Burkitt's lymphoma t(8;14) and variant translocations. Also, other leukemic T- and B-cell rearrangements of breakpoints have been characterized [36]. In the Philadelphia translocation the *c-abl* oncogene is modified and activated as a result of its translocation within the *BCR* gene on chromosome 22 [37]. These results are providing us with new tools for diagnosis of  $\text{Ph}^1+$  leukemia as well as for detection of residual disease (see above). In the past few years, many oncogenes, growth factors, and growth factor receptor genes have been mapped around chromosomal regions specifically involved in leukemia [38]. In the very near future it is to be expected that the number of specific translocations molecularly characterized will increase rapidly and that new breakpoint-specific regions will be discovered.

### Summary and Conclusions

The diagnostic and prognostic value of specific cytogenetic abnormalities has been established for most recurrent translocations. For less frequent changes, we still need to collect more cases for determination of their clinical significance. Optimal treatment of leukemia with modern therapeutic strategies requires knowledge of the prognostic factors, and leukemic karyotype should be one of the variable features systematically evaluated in all trials. The molecular analysis of the specific translocation will considerably increase our under-



standing of the mechanism of leukemogenesis and provide us with new tools for diagnosis. Systematic collection and conservation of acute leukemic cells, cytogenetically and immunologically characterized, would greatly facilitate and accelerate these fundamental studies.

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## Minimal Residual Disease in Acute Leukemia: Lessons Learned from Animal Models

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### Introduction

Minimal residual disease (MRD) in acute leukemia is defined as the relatively few ( $1-10^{10}$ ) leukemic cells which have survived successful remission-induction chemotherapy. In human acute myelocytic leukemia (AML) there is a significant lack of knowledge on the degree and biological behavior of MRD. However, this is the population of AML cells that determines the failure in the majority of patients: a relapse.

A selection of studies are presented in a rat model relevant for human AML, i.e., Brown Norway acute myelocytic leukemia (BNML). Special emphasis will be given to the heterogeneous distribution of MRD over the bone marrow compartment.

The BNML rat model shows striking similarities with human AML with respect to its slow growth rate, severe suppression of normal hemopoiesis, and response to chemotherapy [1, 6]. Detection of MRD employing monoclonal antibodies and fluorescence-activated cell sorting (FACS) has been the subject of a number of studies in BNML [2-4, 7]. In conclusion, it was shown that the minimum number of leukemic cells which can be detected in this way varies between 1 leukemic cell/10000 to 1/100000 normal bone marrow cells.

The present paper focuses on the distribution of MRD in the marrow compartment.

Little is known of the processes of (re)distribution and regrowth of leukemic cells during the phase of MRD. In the remission phase patients are examined regularly, which includes bone marrow aspiration analysis. For the interpretation of the observed leukemic cell frequency it is important to realize what might have happened in the bone marrow compartment one is actually sampling. Did any leukemic cells survive therapy and did they redistribute in the marrow cavity as a characteristic behavior of leukemia in general? Or were all original leukemic cells present at this location eradicated and did migration take place from relapse sites located elsewhere in the body? Many such questions are difficult to answer by studying human patients. In this respect studies in animal models offer an alternative.

### Materials and Methods

#### *Experimental Animals*

The experiments were performed in the barrier-derived inbred Brown Norway (BN) rat strain BNBi/Rij, produced in the Rijswijk colony. Male rats between 13 and 16 weeks of age were used (mean body weight, 260 g).

#### *Rat Leukemia Model*

Brown Norway acute myelocytic leukemia, which was induced in a female BN rat by treatment with 9,10-dimethyl 1,2-benzanthracene, shows striking similarities with human AML [1, 6]. Upon cellular transfer the

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leukemia shows a reproducible growth pattern. Some of its major characteristics are:

- a) slow growth rate;
- b) severe suppression of normal hemopoiesis due to an absolute numerical decrease in the number of hemopoietic stem cells (CFU-S: colony-forming unit spleen);
- c) presence of clonogenic leukemic cells (in vivo, LCFU-S: leukemic colony-forming units spleen; in vitro clonogenic assays); and
- d) response to chemotherapy as in human AML.

An additional advantage of this model is that normal hemopoietic stem cells and leukemic clonogenic stem cells can be distinguished by modified spleen colony assays.

#### *Treatment of Animals with Cyclophosphamide*

Leukemic animals were treated at various stages of leukemia development: day 8–14 after inoculation of  $10^7$  BNML cells i.v. Cyclophosphamide (a generous gift from Asta Werke, Bielefeld, FRG) was injected intraperitoneally in doses of 66–140 mg/kg. Two to four weeks later the animals were put to death and various bones were removed, the marrow taken out, and the number of residual BNML cells determined employing Rm124 monoclonal antibody and FACS.

Preparation of marrow cell suspensions, immunofluorescence labeling of cells, Rm124 monoclonal antibody, and the FACS procedure have all been described previously [2–4, 7].

## **Results and Discussion**

### *Variability in Leukemic Cell Frequency Before Treatment*

Four animals were put to death, two on day 7 and two on day 10 after injection of  $10^7$  BNML cells i.v. At that moment the clinical signs of leukemia became noticeable (increase in spleen and liver weights and pro-

**Table 1.** Ratios of measured maximal and minimal leukemic cell frequencies in various bones of leukemic rats before cyclophosphamide chemotherapy

Rat number	Day after $10^7$ BNML	MRV <sup>a</sup>
1	7	11.7
2	7	6.6
3	10	3.7
4	10	4.4

<sup>a</sup> Examined bones were: femur, tibia, humerus, scapula, vertebra, costa, and sternum, up to a total of 16–19 bones/rat  
MRV, maximal ratio value (i.e., highest/lowest frequency ratio)

nounced infiltration of the bone marrow by the leukemic cells).

Marrow cell suspensions were prepared from the following bones: both femora, tibiae, humeri, scapulae, and limited numbers (three to four) of the costae, vertebrae, and sternum sections. The leukemic cell frequencies in the different samples ranged from  $4 \times 10^{-3}$  to  $5 \times 10^{-2}$  on day 7 and from  $7 \times 10^{-2}$  to  $4 \times 10^{-1}$  on day 10 after leukemic cell transfer. Obviously, the increase from day 7 to day 10 is a result of leukemia growth. The ratio between the highest and lowest measured values of leukemic cell frequencies in the various bones was used as a parameter indicating the range between the two extreme values and is defined as the “maximal ratio value” (MRV).

The MRVs determined for all the animals were in a narrow range: 6.6–11.7 for day 7 and 3.7–4.4 for day 10 (Table 1).

### *Variability in Leukemic Cell Frequency During the MRD Phase*

The high variability in leukemic cell frequency between bones as reported [5] led to the assumption that the size of the marrow compartment was playing an essential role with regard to response to treatment and subsequent outgrowth of residual cells. Because the study of all individual bones from each of the animals would have been too

**Table 2.** Maximal ratio values (MRVs) for leukemic cell frequencies in femur sections and in ribs during the MRD phase after chemotherapy

Rat number	Day of treatment	Dose of cyclophosphamide (mg/kg)	Day of sampling	Ribs MRV	Femur MRV
3	8	100	29	<sup>a</sup>	<sup>a</sup>
7	8	100	32	7	7
8	8	100	36	66	> 320
9	8	140	32	2	7
1	11	66	26	38	202
4	11	100	31	> 220 (70)	> 28 300 (1900)
5	11	140	31	> 84	<sup>a</sup>
6	11	140	38	35	350
2	14	100	31	40	4
10	14	100	24	80	n.d.

n.d., not determined; 50 000–300 000 cells analyzed

<sup>a</sup> All samples less than  $1/10^5$

laborious, it was decided, guided by the data obtained so far, to make an arbitrary distinction between “large” and “small” type bones. Therefore, the second part of the study, i.e., “selected marrow site sampling,” included detailed analyses of the femoral bone and the ribs, which were considered as being representative for the “large bone type” and the “small bone type,” respectively.

Four animals were treated on day 8 after administration with  $10^7$  BNML cells i.v. with 100 mg/kg (rat 3, 7, and 8) or 140 mg/kg cyclophosphamide (rat 9). On the basis of the dose response data it could be expected that this will result in a 6 and a 9 log cell kill (LCK), respectively. At this stage the ribs contain on average  $6.7 \times 10^5$  leukemic cells, which implies that the treatment should be sufficient to eliminate all leukemic cells. The leukemic cell frequencies in ribs and femoral marrow sections were determined during the MRD phase, i.e., between days 29 and 36 after treatment with cyclophosphamide. The results are shown in Table 2. The MRV found for the animals treated with 100 mg/kg are 7 for rat 7 and 66 for rat 8. The third animal, which received 100 mg/kg (rat 3), was apparently studied too early in the MRD stage (i.e., day 29), since in all bone marrow suspensions from

the ribs as well as from the femur sections the leukemic cell frequency was below the detection limit. However, some leukemic cells must have survived in this animal because the analysis of the remaining femur, which was not cut into slices but flushed in total, yielded a leukemic cell frequency of 0.05%. If indeed these cells originated from a single leukemic focus, it must have contained  $5 \times 10^4$  cells.

The animal which was treated with 140 mg/kg (rat 9) on day 8 and analyzed on day 32 showed a pattern comparable to that in rats 7 and 8, i.e., uniformly distributed leukemia in ribs, with an MRV of 2. The majority of the ribs (15 out of 16) were, however, in a narrow range with an MRV of 10. The measured leukemic cell frequencies were extrapolated backwards on the basis of the parameter values derived from a computer simulation analysis. For the day 8 treatment group, it was found that in all investigated ribs the one leukemic cell per rib level was reached between 9 and 20 days after treatment (Table 3). In other words, the ribs of these animals were free from disease for some time, which is in agreement with the assumption that the treatment provided is more than sufficient to eradicate all leukemic cells which are present in the ribs at that time.

**Table 3.** Disease-free intervals for ribs of rats treated with cyclophosphamide and investigated during the MRD phase

Rat number	Treatment on day	Cyclophosphamide dose (mg/kg)	Sampling day	Ribs <sup>a</sup> free of leukemia per total	Disease-free interval (days)
3	8	100	29	13/13	> 16
7	8	100	32	16/16	8.8 – 11.1
8	8	100	36	14/14	15.8 – 16.7
9	8	140	32	16/16	14.3 – 19.5
1	11	66	26	16/18	0.4 – 2.0
4	11	100	31	12/12	6.0 – > 12
5	11	140	31	12/12	4.0 – > 12
6	11	140	38	12/12	16.8 – 21.9
2	14	100	31	2/13	1.0 and 1.5
10	14	100	24	10/19	0.2 – 3.4

<sup>a</sup> Immediately after treatment as derived by backwards extrapolation

A similar phenomenon was observed if rats at day 11 after leukemia transfer were treated with 66–140 mg cyclophosphamide/kg (Table 3). However, treatment at a later stage, i.e., day 14, resulted in surviving leukemic cells in the majority of ribs investigated.

With regard to the regrowth of the residual leukemic cells, the pilot study in the BNML model, i.e., the survey of extensive marrow sampling, already indicated that unexpected phenomena occurred. The study was performed on the assumption that after treatment the surviving leukemic cells in each of the bone marrow compartments would rapidly redistribute in the animal including in the bone marrow. Therefore, at any given time interval after treatment, similar leukemic cell frequencies in each marrow compartment were expected. However, when the animals were investigated during the phase prior to the imminent relapse, a totally inhomogeneous distribution of the regrowing leukemia cells was observed. Analyses of the many marrow samples obtained from a variety of different bones from the various animals investigated revealed large differences in leukemic cell frequency, up to a factor or 28 000-fold [5]. This variation was most pronouncedly found in the group of so-called smaller bones, e.g., ribs, vertebrae, and scapulae. It was obvious that the leukemic cell frequencies measured in

one of the marrow samples did not automatically reflect the situation in the total marrow. Another conclusion was that the leukemic animals which were studied had to be analyzed on an individual basis [5].

The alternative hypothesis for early spread during MRD to explain the data is that surviving leukemic cells do not immediately migrate during the MRD phase, but that residual cells show localized regrowth for some time. This is called “primary relapse growth.” However, at some later stage during the MRD phase cells migrate to other sites, which can be concluded from the fact that in all marrow sites sooner or later leukemic cells were found. This is called “secondary relapse growth.” Three types of regrowth can thus be defined:

1. “Primary relapse growth,” where many leukemic cells survive in a certain marrow compartment
2. “Secondary relapse growth,” where no leukemic cells survive and all residual cells are derived from migrated leukemic cells from a relapse site elsewhere
3. A combination of the two

If the results of the rat apply to the human situation, the following should be considered.

Provided that the methods for the detection of residual disease in leukemia patients will indeed be improved in the future, resid-

ual leukemic cells might be detected at frequencies in the order of 1/100 000–1 000 000 normal cells. The most important and largest part of the tumor load to be detected is located at the primary relapse sites. What are then the chances of detecting them by using limited marrow sampling? The number of these foci will depend on the effectiveness of the treatment. Currently used therapy for leukemia is fairly effective, indicated by the long periods of remission which are observed clinically. This may indicate that the number of surviving cells will be limited and the chances of detecting them will be small. Especially when smaller marrow compartments are sampled it is most likely that the measured leukemic cell frequency represents a secondary relapse site which is characterized by homogeneous distribution of leukemic cells. The minimal tumor load can then be calculated. Of more importance for the total tumor load, however, are the primary relapse sites. For that reason, one should focus on the detection of these. This means that other methods should be developed, e.g., radiolabeling of monoclonal antibodies or development of NMR techniques, for in situ detection of these residual leukemic foci.

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## Toward Improvement of Therapeutic Strategies in Leukemia by Amplification of the Immune Responses Against Leukemia

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### Introduction

The increase in the intensity of induction chemotherapy, either in the form of an intermittent high-dose upfront therapy or in combination with late intensification regimen, has led to improved remission induction and cure rate in malignant hematological disorders. Hence, many oncologists tend to believe that successful treatment of disseminated malignancies requires physical elimination of all tumor cells. Although it is difficult to assume on a theoretical basis that the "last tumor cell" can be eradicated, it is difficult, if at all possible, to document minimal residual disease in many instances of successfully treated patients and therefore it remains speculative whether all tumor cells were indeed eradicated or whether few residual tumor cells were being put under the host's control mechanisms that prevent clonogenic expansion of a few residual tumor cells escaping chemotherapy. Although the role of naturally occurring anticancer defense mechanisms and therapeutically induced immunotherapy remains speculative in conventional hematological and oncological practice, the role of cellular adoptive immunotherapy in conjunction with allogeneic bone marrow transplantation (BMT) is by now well established [1–6]. Evidence for effective antileukemic effects of allogeneic immune cells against residual host leukemia cells was derived by documenting

beneficial antileukemia effects in conjunction with graft-versus-host disease (GVHD) [1–5], detrimental effects of T-lymphocyte depletion [1–14], and increased relapse rates following transplantation of identical twins [6] or autologous marrow grafts [15]. An effective role of cell-mediated immune effects against the residual host's tumor cells was also documented in several animal models [3, 7–10]. It was suggested by several investigators working in murine models of leukemia that graft-versus-leukemia (GVL) effects may be accomplished without clinically overt manifestations of GVHD [3, 8, 10]. Our previously published work suggests that donor cells completely tolerant of host's alloantigens may still exert curative antileukemia effects in the murine model of B-cell leukemia (BCL1) [10, 11]. The latter experimental system suggests that GVL effects may be mediated by effector cells that are distinguished from those leading to GVHD, or else that effector cells of GVL may recognize cell surface determinants on tumor cells in distinction from alloantigens which lead to alloactivation that results in GVHD. Alternatively, other effector mechanisms may play a role in GVL effects. For example, various mediators of inflammation or products of the inflammatory process may act against proliferation of clonogenic tumor cells or may activate macrophages to react against leukemia cells. We are currently investigating several approaches that may lead to improved therapeutic strategies in leukemia therapy by activating the host's natural defense mechanisms against cancer on the one hand and donor's immune cells in conjunction with autologous and allogeneic

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BMT on the other. Our experimental work in mice suggests that potent antitumor effector mechanisms may be generated by cells syngeneic to the tumor, suggesting that, once such mechanisms are better understood, it might be possible to potentiate GVL effects that are mediated in conjunction with autologous BMT and that may lead to major progress in the treatment of malignant hematological disorders in conjunction with autologous BMT.

## Materials and Methods

### *Mice*

BALB/c (H-2<sup>d</sup>) and (BALB/c × C57BL/6)(F1) (H-2<sup>d/b</sup>) mice purchased from the Hebrew University Hadassah Animal Facility were used throughout the experiments. (C57BL/6)(H-2<sup>b</sup>) (C57) mice were used as marrow donors.

### *Bone Marrow Transplantation*

Bone marrow cells were obtained by flushing the shafts of femura and tibia. Mice received  $30 \times 10^6$  bone marrow (BM) cells in the lateral tail vein. Depletion of Thy-1-positive cells was accomplished by monoclonal anti-Thy-1.2 antibodies (Cedarlane Labs, Hornby, ONT, Canada) with 1.0 low-tox rabbit complement. Depletion of various T-lymphocyte subsets was accomplished by injecting cells treated by a monoclonal rat antimouse L3T4 or Lyt-2 (kindly provided by Drs. S. Cobbold and H. Waldmann, Cambridge University School of Medicine, UK) using no complement (lysis occurring in vivo).

### *Cytoreduction of Leukemia and Induction of Transplantation Tolerance by Total Lymphoid Irradiation (TLI) Prior to Allogeneic BMT*

F1 recipients were inoculated with a high-dose leukemia challenge of  $10^7$  BCL1 cells/mouse. After 12–14 days of incubation, mice were conditioned by TLI, consisting of eight daily doses of 200 cGy (a total of

1600 cGy) as previously described [10]. Upon completion of TLI, mice were cytoreduced by intravenous cyclophosphamide 200 mg/kg and  $30 \times 10^6$  BM cells were infused 24 h later. Confirmation of chimerism following transplantation of allogeneic (C57) BM cells was carried out as previously described by alloantisera, using complement-dependent microcytotoxicity assays [10].

### *Treatment of BCL1-Bearing Mice with Recombinant Human Interleukin-2*

Interleukin-2 (IL-2) was kindly provided by Cetus (Emerville, CA, United States) and EuroCetus (Amsterdam, The Netherlands). Mice were injected with  $10^5$  U × 3 day i.p. for five consecutive days, as previously described [13].

### *Generation of IL-2-Dependent Cytotoxic Cells*

Spleen cells of C57 mice were cultured for 4 days in IL-2 at 1000 U/ml in RPMI medium in 10% AB fetal calf serum. Cytotoxic activity was confirmed against NK-resistant (Daudi cell line cells) and NK-sensitive (K562 cell line cells) using a standard microcytotoxicity technique with radioactive chromium release assay.

### *Documentation of Minimal Residual Disease by Adoptive Transfer Experiments In Vivo*

Since none of the in vitro assay systems at hand are useful for documenting minimal residual disease by direct testing [11], spleen cells of treated mice were adoptively transferred ( $10^5$  cells/mouse) into syngeneic BALB recipients. Since leukemia develops following inoculation of as few as 10 BCL1 cells/mouse [12], development of leukemia in adoptive recipients served as indication for residual tumor cells in successfully treated hosts.

## Results

F1 recipients conditioned by TLI and cyclophosphamide showed consistent engraftment of C57 or C3H BM cells and developed donor-specific tolerance without any overt evidence of GVHD, as previously described [10]. Adoptive transfer of spleen cells obtained from F1 recipients inoculated with leukemia prior to BMT was carried out in the following experimental groups:

1. Recipients of control BALB BM cells
2. Recipients of Lyt-2-depleted C57 BM cells
3. Recipients of L3T4-depleted C57 BM cells

Adoptive transfer of spleen cells of F1 recipients (proven to be C57→F1 chimeras) was carried on within 2–4 weeks following transplantation. Fifteen out of 15 adoptive recipients of spleen cells obtained from F1 mice reconstituted with BALB BM cells developed leukemia and died and similarly all 15 recipients of Lyt-2-depleted C57 BM cells developed leukemia and died (groups A and B), whereas all 15 adoptive recipients of spleen cells obtained from F1 chimeras reconstituted with L3T4-depleted C57 BM cells (group C) remained tumor free for an observation period of >300 days. The data suggest that immunocompetent Lyt-2-positive allogeneic lymphocytes of C57 origin may play a significant curative role against residual BCL1 cells *in vivo*.

In order to test whether cytotoxic cells of syngeneic origin may also play a role in eradication of residual BCL1 cells, BALB mice inoculated with  $10^2$ – $10^4$  BCL1 cells were treated by high-dose IL-2 ( $10^5$  U  $\times$  3/day I.P. for five consecutive days) as previously described [13]. As previously shown, most recipients of high-dose IL-2 therapy showed no evidence of leukemia for a long observation period, currently extending to >1 year.

In order to investigate whether IL-2-activated allogeneic spleen cells could exert potent GVL and GVHD *in vivo*, IL-2-activated C57 spleen cells were injected into sublethally (550 cGy) irradiated BALB mice. All mice died of typical GVHD and hence it was not possible to document antitumor effects by IL-2-activated allogeneic

cells by long-term observation of original mice inoculated with leukemia. It was therefore decided to observe antitumor effects of IL-2-activated spleen cells by killing treated mice and assaying for residual leukemia cells in the spleen of treated mice by adoptive transfer experiments.

In order to test whether *in vitro* activated spleen cells are capable of inducing potent GVL effects *in vivo*, F1 recipients were inoculated with  $10^7$  BCL1 cells/mouse and subjected to lethal whole body irradiation (700 cGy) 2 weeks later. Mice were reconstituted with  $10^7$  C57 BM cells depleted of Thy-1-positive cells and subsequently received a total of  $200 \times 10^6$  IL-2-activated spleen cells on days +1, +4, and +7. None of ten mice showed any evidence of leukemia, as documented by >200-day survival of adoptive recipients of spleen cells obtained from treated mice. In contrast, all ten adoptive recipients of spleen cells obtained from BMT recipients receiving no cellular adoptive immunotherapy following BMT developed leukemia and died within 31–42 days.

## Discussion

Data recently generated in our laboratory using the animal model of human lymphoid malignancies, the murine B-cell leukemia/lymphoma BCL1, suggest that effective treatment of an extremely aggressive and invariably lethal leukemia may be accomplished by cell-mediated immune mechanisms through cells derived from syngeneic (activated by IL-2) or allogeneic sources against residual BCL1 cells. GVL effects of C57 cells, most likely mediated by Lyt-2-positive cytotoxic lymphocytes, that can be accomplished despite full tolerance to host's and hence to BCL1's alloantigens (H-2<sup>d</sup>) is of particular importance [10, 11], since it suggests that GVL effects may be mediated by reactivity against cell surface determinants other than alloantigens. Similarly, the unique efficacy of high-dose IL-2 *in vivo* with no allogeneic immune cells involved [13] suggests that potent antileukemia mechanisms may be inducible in conjunction with autologous BMT and hence with no threat of serious GVHD. Indeed, if GVL may op-

erate independently of GVHD, as suggested by several independent experimental systems in different laboratories [3, 8–11], optimal activation of effector cells of GVL in syngeneic systems may lead to new therapeutic strategies in the treatment of leukemias and other malignancies, particularly in conjunction with autologous BMT, since it may be possible to activate the reconstituting immune system, following BMT, toward faster maturation and enrichment of desirable effector cells using cytokines that may favor differentiation and activation of anti-tumor effector cells *in vivo*.

Our pilot data in the BCL1 model in mice suggest that IL2-activated allogeneic lymphocytes may convey therapeutic antileukemia effects *in vivo* in conjunction with BMT when given repeatedly and in high doses. We are currently investigating the role of syngeneic IL-2-activated cells in this setting and whether administration of IL-2 *in vivo* following adoptive transfer of activated cells may augment their antitumor effects toward more potent inactivation of residual clonogenic BCL1 cells that have escaped chemoradiotherapy given during the conditioning regimen prior to BMT.

Taken together, our preliminary results using the BCL1 tumor model system suggest that potent and even curative antileukemia effects may be accomplished *in vivo* against a nonimmunogenic tumor such as the BCL1 by immune cells capable of inducing tumor dormancy, rather than physical eradication of the “last tumor cell” in conjunction with BMT without mandatory GVHD. Activation of cells that may induce natural defense mechanisms against tumors may be accomplished in a syngeneic system by nonspecific potent cytokine-mediated activation *in vivo*, unless such immune cells are capable of self-propagation and reactivation through an alternative strong stimulation – alloactivation across major histocompatibility complex (MHC) – which would provide intrinsic IL-2 and other important factors and cells. MHC-compatible spleen cells are unlikely to propagate and be activated continuously by a nonimmunogenic tumor and hence exogenous supply of IL-2 (or a comparable cytokine) may be required for continuous activation of such antitumor effector mechanisms.

In view of the paucity of data in support of our hypothesis, it should be regarded as speculative. Nevertheless, documentation of beneficial antitumor effects by active immune cells is encouraging and warrants further study in an attempt to improve the treatment of drug-resistant hematological malignancies, especially in conjunction with autologous BMT – a procedure that may be recommended at an early stage of the disease in a large number of patients in need while avoiding the risk of insurmountable complications of allogeneic BMT. Alternatively, antitumor effector mechanisms may be inducible by allogeneic immune cells, hopefully, at least to a certain extent, independently of GVHD and hence such an approach may be clinically applicable in conjunction with methods that result in host-specific unresponsiveness, such as depletion of immunocompetent T-lymphocytes with subsequent administration of donor-type immune cells in a manner that will not result in severe GVHD (Slavin et al. unpublished data). Such an approach is currently being clinically investigated in patients at high risk of relapse in Jerusalem. Although the number of patients investigated so far is too small and the follow-up period is too short for any firm conclusions, it appears that such an approach may be feasible and hence we are optimistic that it may lead to better eradication of leukemia in conjunction with safer approaches for allogeneic BMT.

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## Double Marker Analysis for Terminal Deoxynucleotidyl Transferase and Myeloid Antigens in Acute Nonlymphocytic Leukemia Patients and Healthy Subjects \*

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### Introduction

The enzyme terminal deoxynucleotidyl transferase (TdT) is expressed on the nuclear membrane of normal precursor B and T cells as well as their malignant counterparts, i.e., acute lymphoblastic leukemias (ALLs) and some lymphoblastic lymphomas [1, 2]. TdT expression has also been found in 5%–46% of acute nonlymphocytic leukemias (ANLLs) [3–9]. In ANLL there is a large variability in the percentage of TdT<sup>+</sup> cells, and also the intensity of TdT expression varies per cell. In most studies a limit of at least 10% of TdT<sup>+</sup> cells was adopted for the diagnosis of a TdT<sup>+</sup> ANLL. However, it is likely that in some ANLLs smaller TdT<sup>+</sup> leukemic subpopulations are present.

We have analyzed 60 ANLLs for the presence of a TdT<sup>+</sup> subpopulation using double-marker analyses for TdT and differentiation markers, such as myeloid markers (CD13 and CD33), B-cell markers, and T-cell markers. In addition, we applied double-marker analyses to monitor the TdT<sup>+</sup> leukemic subpopulation in two ANLL patients during and after chemotherapy. In control studies, it was investigated whether

CD13<sup>+</sup>, TdT<sup>+</sup> cells and CD33<sup>+</sup>, TdT<sup>+</sup> cells are present in normal bone marrow (BM) and peripheral blood (PB).

### Materials and Methods

Bone marrow and/or PB samples from 60 patients having an ANLL were classified according to the revised criteria of the French-American-British (FAB) group. Immunological marker analyses using fluorescence microscopy were performed on mononuclear cells (MNCs) isolated by Ficoll (density, 1.077 g/cm<sup>3</sup>) density centrifugation [10]. We determined the expression of a series of immunological markers, including TdT, the B-cell markers CD10 and CD19, the T-cell markers CD2, CD3, and CD7, the myeloid markers CD13, CD14, CD15, and CD33, the HLA-DR antigen, and the precursor antigen CD34. Information about the monoclonal antibodies (mAbs) used is given in Table 1. The TdT-immunofluorescence (IF) assay was performed by use of a rabbit anti-TdT and a fluorescein isothiocyanate (FITC) conjugated goat antirabbit immunoglobulin (Ig) antiserum (Super-techs, Bethesda, MD, United States). Only the characteristic nuclear staining was considered positive. For double-marker analysis cells were successively labeled with one of the mAbs mentioned above and a tetramethylrhodamine isothiocyanate (TRITC) conjugated goat anti-mouse Ig antiserum. Subsequently the TdT-IF assay was performed. If possible, at least 200 TdT<sup>+</sup> cells were analyzed; when MNC samples con-

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**Table 1.** Presence of myeloid marker<sup>+</sup>, TdT<sup>+</sup> cells in 60 ANLLs

Leukemia type, according to the FAB group	Total number of ANLLs per FAB group	Number of ANLLs with myeloid marker <sup>+</sup> , TdT <sup>+</sup> cells
M1	7	6
M2	22	16
M3	3	3
M4	10	8
M5	8	3
M6	2	2
AUL	8	7

tained less than 1% of TdT<sup>+</sup> cells two cyto-centrifuge preparations (total ~50 000 MNC) were screened.

Bone marrow and/or PB samples from two patients were monitored for the presence of CD33<sup>+</sup>, TdT<sup>+</sup> cells (patient B.B.) or CD13<sup>+</sup>, TdT<sup>+</sup> cells (patient A.K.) during and after chemotherapy.

Sixty-six BM samples obtained from healthy volunteers ( $n=7$ ), from ALL patients under therapy ( $n=14$ ), from ALL patients off therapy ( $n=45$ ), as well as 25 PB samples from healthy volunteers ( $n=16$ ) and from ALL patients ( $n=9$ ) were analyzed for the presence of CD13<sup>+</sup>, TdT<sup>+</sup> cells and CD33<sup>+</sup>, TdT<sup>+</sup> cells.

## Results

### *Terminal Deoxynucleotidyl Transferase Positive Subpopulations in ANLL at Diagnosis*

The FAB classification of the 60 ANLLs is summarized in Table 1. Detailed information about hematological characteristics and results of immunological marker analysis of 12 representative ANLLs are given in Table 2. The results of all 60 leukemias will be published elsewhere [11]. A marked heterogeneity of marker expression was found between the different leukemias as well as within each leukemia. In 75% ( $n=45$ ) of the 60 ANLLs a myeloid marker<sup>+</sup>, TdT<sup>+</sup>, CD10<sup>-</sup> subpopulation was present; this subpopulation varied from 0.1% up to 90% of MNCs (Fig. 1). In Table 3 the 60 ANLLs are listed according to the size of the TdT<sup>+</sup> subpopulation. In most TdT<sup>+</sup> ANLLs the TdT<sup>+</sup> subpopulation accounted for <50%

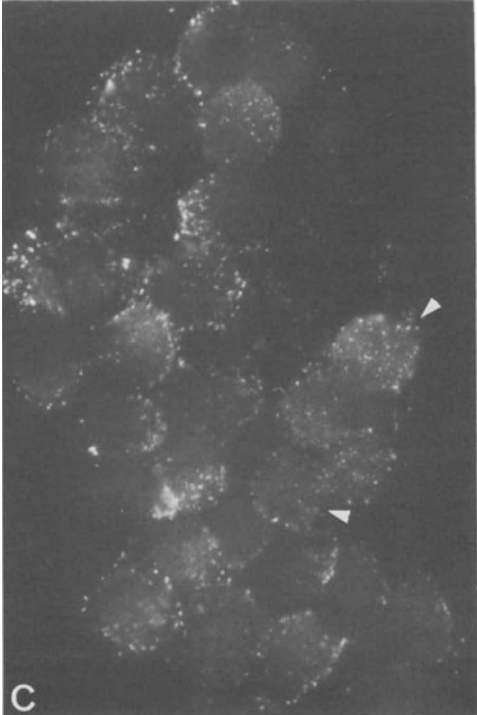
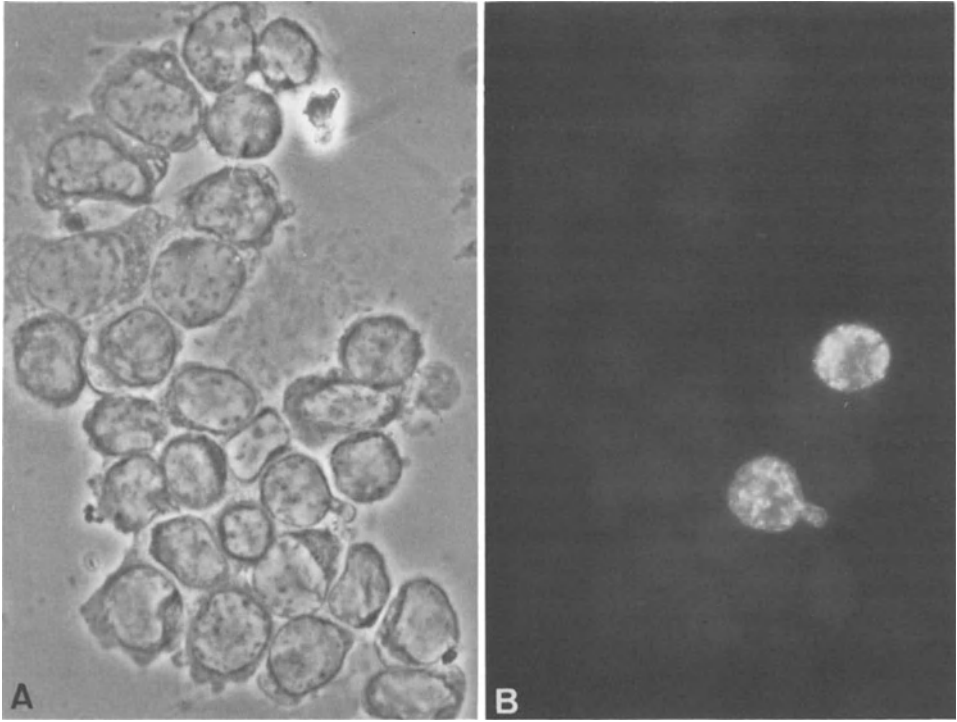
and often even <10% of MNCs. No obvious relationship between the presence or absence of myeloid marker<sup>+</sup>, TdT<sup>+</sup> cells, and the FAB subtypes could be found (Table 1). Within each leukemia the intensity of the TdT expression was variable. Generally, the TdT expression in ANLL was weaker than in ALL. In all but one of the 45 ANLLs with a TdT<sup>+</sup> subpopulation the TdT<sup>+</sup> cells were positive for CD13 and/or CD33.

### *Follow-up of ANLL Patients by Use of Double-Marker Analysis*

Follow-up studies were performed in patients B.B. and A.K. The immunological phenotype of these two ANLLs at diagnosis is given in Table 2, while the follow-up data are summarized in Fig. 2.

After stopping the maintenance therapy in patient B.B., a gradual increase in the percentage of CD33<sup>+</sup>, TdT<sup>+</sup> cells was found during the 6-month period before relapse. After reinduction treatment she obtained complete remission (CR). At this point BM was taken for autologous bone marrow transplantation (ABMT), which was performed 4 weeks later. A second BM relapse occurred 16 weeks after ABMT and the patient died. Although this patient seemed to be in second CR for a period of 20 weeks, in all BM samples tested CD33<sup>+</sup>, TdT<sup>+</sup> cells were detected (Fig. 2).

In patient A.K. both BM and PB samples were monitored. This patient achieved remission after 5 weeks of treatment and obtained CR after 12 weeks. Although at diagnosis only about 1% of the MNCs were CD13<sup>+</sup>, TdT<sup>+</sup>, during follow-up low per-



**Fig. 1A–C.** Double IF staining for CD33 and TdT on PB cells from patient P.B. at diagnosis. **A** Phase contrast morphology; **B** TdT-positive cells (FITC labeled); **C** CD33 (MY9)-positive cells (TRITC labeled). The majority of the cells are positive for CD33. *Arrowheads* indicate CD33 expression on the two TdT-positive cells

Table 2. Twelve typical examples out of the 60 ANLLs

Patient	Age/sex	WBC ( $\times 10^9$ /liter)	FAB diagnosis	Sample	Blasts (%)	Immunological markers/MNC (per TdT <sup>+</sup> cells) <sup>a</sup>												
						TdT	CD2 (T11) <sup>b</sup>	CD3 (Leu-4) <sup>c</sup>	CD7 (3A1) <sup>d</sup>	CD10 (VIL-A1) <sup>e</sup>	CD19 (B4) <sup>b</sup>	CD13 (My7) <sup>b</sup>	CD14 (My4) <sup>b</sup>	CD15 (VIM-D5) <sup>e</sup>	CD33 (My9) <sup>b</sup>	CD34 (BI-3C5) <sup>f</sup>	HLA-DR (L243) <sup>e</sup>	
K.A.	60 M	59	M2	PB	82	0	9	9	84	0	2	41	5	3	72	17	56	
J.J.	40 M	33	M5b	BM	68	0	13	8	6	0	2	2	37	18	93	3	20	
M.S.	0 F	67	M4	BM	41	2	11	8	51 (1/2)	16 (95)	11	1/2 (0)	3	14	66 (0)	1/2	45 (78)	
S.M.	16 F	3.5	M6	BM	55	0.1	8 (1)	6	10 (21)	<1 (11)	3 (0)	4 (45)	8 (0)	21 (1)	79 (87)	10 (93)	19 (73)	
A.K.	26 M	82	M4	PB	75	0.6	8 (27)	4	2 (0)	0 (0)	3 (0)	95 (62)	20 (0)	11 (1)	80 (20)	67 (97)	48 (12)	
P.B.	40 F	13	M2	PB	56	4	4 (0)	2	38 (94)	0 (0)	3 (1/2)	74 (99)	5 (0)	2 (0)	89 (99)	22 (88)	78 (99)	
J.R.	75 M	47	M1	PB	71	6	9 (2)	8	91 (95)	<1 (1/2)	2 (0)	64 (58)	1/2 (0)	1/2 (0)	13 (14)	49 (44)	67 (89)	
M.M.	17 M	1.7	M6	BM	71	11	9 (1/2)	10	10 (5)	<1 (1/2)	8 (0)	2 (39)	2 (0)	38 (0)	57 (97)	5 (26)	32 (26)	
B.B.	13 F	18	M4	BM	73	12	3	1/2	2 (0)	0 (0)	82 (89)	3 (0)	15 (0)	15 (0)	92 (95)	74 (26)	74 (26)	
A.S.	71 M	0.9	M1	BM	88	17	33 (0)	26	23 (0)	0 (0)	0 (0)	36 (99)	1/2 (0)	1 (0)	14 (54)	39 (99)	3 (0)	
P.A.	53 M	47	M3	BM	95	20	1/2 (0)	2	1/2 (0)	0	44 (2)	1	0	0	98 (97)	17 (48)	1/2 (2)	
B.M.	76 F	11	AUL	BM	95	65	21 (0)	7	13 (1/2)	0 (0)	21 (0)	48 (50)	9 (0)	39 (16)	80 (90)	87 (99)	87 (94)	

<sup>a</sup> Percentages of positivity for a surface membrane marker per TdT<sup>+</sup> cells as determined by double IF staining

<sup>b</sup> Coulter Clone, Hialeah, FL, USA

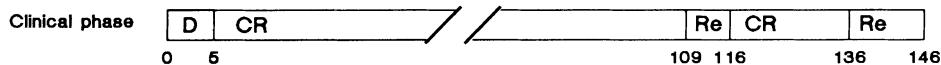
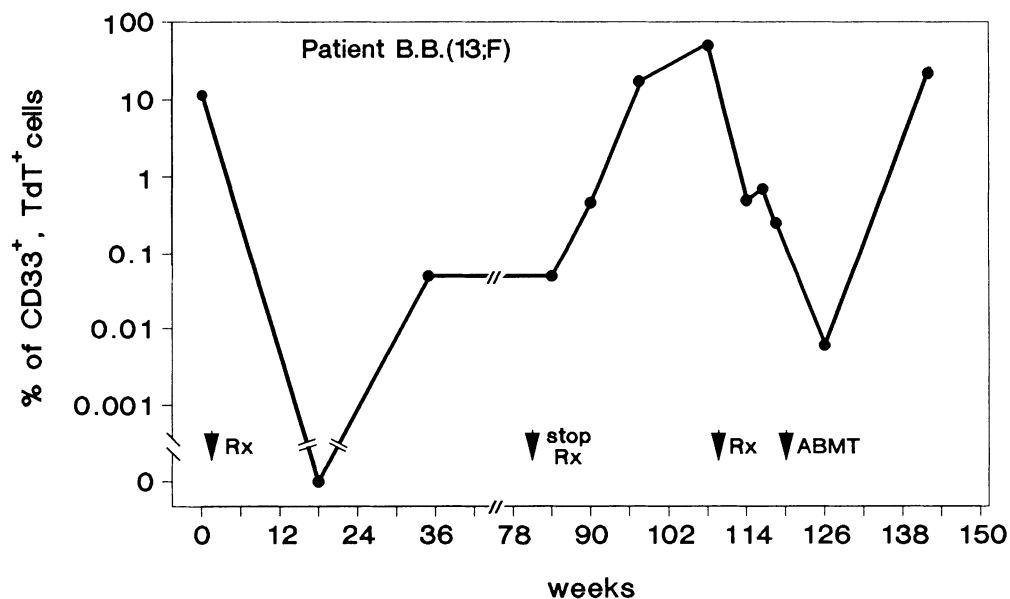
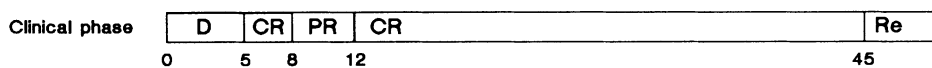
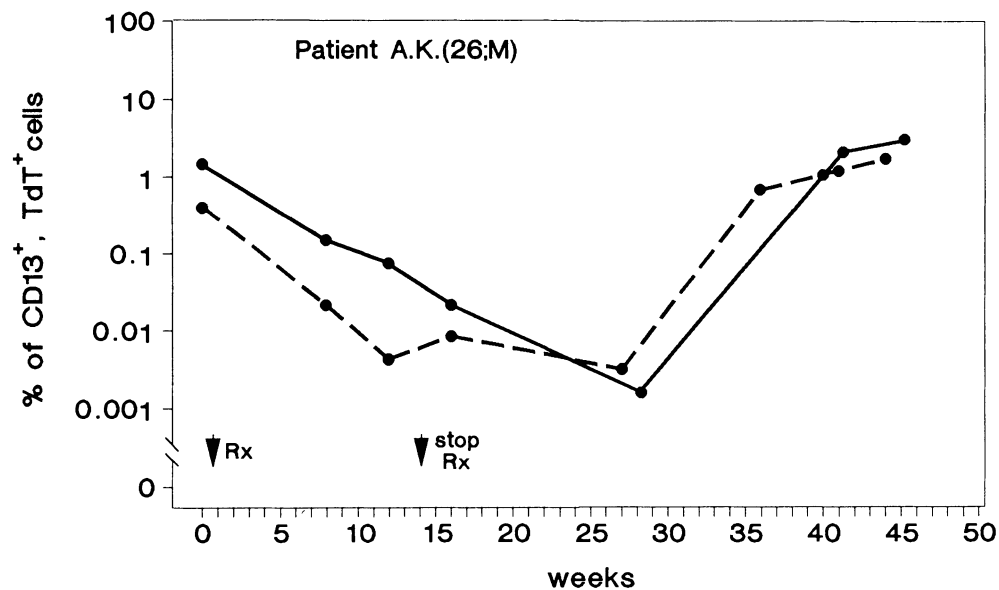
<sup>c</sup> Becton Dickinson, Sunnyvale, CA, USA

<sup>d</sup> American Type Culture Collection, Rockville, MD, USA

<sup>e</sup> Dr. W. Knapp, Vienna, Austria

<sup>f</sup> Seralab, Crawley, UK





**Fig. 2.** Follow-up of two ANLL patients by use of double IF staining for TdT and a myeloid marker [CD13(My7) or CD33(My9)]. Clinical phase is based on both clinical observation and cytomorphology of BM and PB samples; *D*, diagnostic phase; *CR*, complete remission; *PR*, partial remission; *Re*, relapse. *Arrows* indicate: *Rx*, start chemotherapy; *Stop Rx*, end of chemotherapy; *ABMT*, autologous bone marrow transplantation

percentages of CD13<sup>+</sup>, TdT<sup>+</sup> cells were detected in all BM and PB samples tested. The percentages of CD13<sup>+</sup>, TdT<sup>+</sup> cells gradually decreased, but these double-positive cells were still present at week 28 of follow-up. Subsequently the percentages of double-positive cells gradually increased to about 5% in the BM and 3% in the PB at week 45 of follow-up when a BM relapse occurred, as was proven by cytomorphology (Fig. 2).

### Control Studies

Low percentages of myeloid marker<sup>+</sup>, TdT<sup>+</sup> cells (0.001%–0.03%) were detected

in 6 out of 7 normal BM samples, in 5 out of 14 BM samples from ALL patients under therapy, and in 4 out of 45 BM samples from ALL patients off therapy (Table 4).

Taken together, in 15 out of 66 (=23%) BM samples from healthy volunteers and ALL patients in CR low percentages of CD13<sup>+</sup>, TdT<sup>+</sup> and/or CD33<sup>+</sup>, TdT<sup>+</sup> cells were detected. In 3 of these 15 BM samples both CD13<sup>+</sup>, TdT<sup>+</sup> cells and CD33<sup>+</sup>, TdT<sup>+</sup> cells were present, while in the other 12 BM samples only CD13<sup>+</sup>, TdT<sup>+</sup> cells (*n*=6) or CD33<sup>+</sup>, TdT<sup>+</sup> cells (*n*=6) were detected. In general, the nuclear TdT expression as well as the expression of the myeloid marker was weak.

**Table 3.** Percentage of myeloid marker<sup>+</sup>, TdT<sup>+</sup> cells in 60 ANLLs

Myeloid marker <sup>+</sup> , TdT <sup>+</sup> , CD10 <sup>-</sup> cells (%)	Number of ANLLs	Examples (see Table 2)
0	15 (=25%)	K. A., J. J., M. S.
0.1 – 10	24 (=40%)	S. M., A. K., P. B., J. R.
10 – 50	16 (=27%)	M. M., B. B., A. S., P. A.
> 50	5 (= 8%)	B. M.

**Table 4.** Analysis of BM and PB samples from healthy volunteers and non-ANLL patients for the presence of myeloid marker<sup>+</sup>, TdT<sup>+</sup> cells

Cell samples	CD13 <sup>+</sup> , TdT <sup>+</sup>		CD33 <sup>+</sup> , TdT <sup>+</sup>		Fraction of samples with CD13 <sup>+</sup> , TdT <sup>+</sup> cells and/or CD33 <sup>+</sup> , TdT <sup>+</sup> cells
	Fraction of positive samples	CD13 <sup>+</sup> , TdT <sup>+</sup> cells (%)	Fraction of positive samples	CD33 <sup>+</sup> , TdT <sup>+</sup> cells (%)	
Bone marrow samples from healthy volunteers aged 18–54 years ( <i>n</i> =7)	3/7	0.001–0.03	3/7	0.002–0.02	6/7
Bone marrow samples from patients (aged 5–21 years) with an ALL under maintenance therapy in CR ( <i>n</i> =14)	4/14	0.002–0.007	4/14	0.004–0.02	5/14
Bone marrow samples from patients (aged 5–18 years) with an ALL off therapy in continuous CR ( <i>n</i> =45)	2/45	0.02	2/45	0.004	4/45
Peripheral blood samples from healthy volunteers ( <i>n</i> =16) and patients with an ALL in CR ( <i>n</i> =9)	0/25	0	0/25	0	0/25

Myeloid marker<sup>+</sup>, TdT<sup>+</sup> cells were not found in the PB samples ( $n=25$ ) tested (Table 4).

## Discussion

Using double-marker analysis for TdT and several differentiation markers, we detected TdT expression in 75% ( $n=45$ ) of ANLLs. In all cases the TdT<sup>+</sup> cells only represented a subpopulation of the ANLL. Proportionally this subpopulation varied from 0.1% to 83% of MNCs. In most cases ( $n=24$ ) the percentage of TdT<sup>+</sup> cells was even <10% (Table 3). The inclusion of such low percentages explains the higher incidence of TdT<sup>+</sup> ANLL in our study as compared with the data reported in the literature [3–9]. Nevertheless, our data are comparable with those in other reports. For example, Erber et al. found TdT<sup>+</sup> cells in frequencies from 10% to 90% of MNCs in 37% of ANLL cases [8], while we detected such percentages of TdT<sup>+</sup> cells in 35% of ANLLs. If a small TdT<sup>+</sup> subpopulation (<10%) is present, it has to be demonstrated that these TdT<sup>+</sup> cells do not represent normal TdT<sup>+</sup> precursor B cells [12]. Double-marker analysis may allow discrimination between TdT<sup>+</sup> precursor B cells and TdT<sup>+</sup>, myeloid marker<sup>+</sup> ANLL cells (Table 2). Using this approach, it was proven that in the TdT<sup>+</sup> ANLL of our series the TdT<sup>+</sup> cells expressed the same pan-myeloid markers as the TdT<sup>-</sup> cells (Fig. 1).

Since a TdT<sup>+</sup> subpopulation occurs in the majority of ANLL, it is interesting to study whether normal counterparts of such ANLL cells, i.e., myeloid marker<sup>+</sup>, TdT<sup>+</sup> cells occur in normal BM and PB. Using double-marker analysis we found low percentages of CD13<sup>+</sup>, TdT<sup>+</sup> and/or CD33<sup>+</sup>, TdT<sup>+</sup> cells in 23% of the BM samples from healthy volunteers and ALL patients in CR (Table 4). As indicated in Table 4 myeloid marker<sup>+</sup>, TdT<sup>+</sup> cells were detected in the majority of the BM samples from healthy adults, while these cells were found in only 9% of the BM samples from children with an ALL off therapy in continuous CR. The latter finding may be explained by the abundance of CD10<sup>+</sup>, TdT<sup>+</sup> cells, which probably represent regenerating precursor B cells (unpublished observations). Bradstock et al.

found about 0.01% of CD13<sup>+</sup>, TdT<sup>+</sup> cells in 5 out of 11 nonleukemia BM samples [13]. Interestingly, they detected these double-positive cells in BM samples from adults, while these cells were absent in BM samples from young children. Together with our findings these data suggest an age-related occurrence of myeloid marker<sup>+</sup>, TdT<sup>+</sup> cells. In addition, Bradstock et al. reported a weak CD13 expression on these cells, which corresponds with our observations for both CD13 and CD33 expression by TdT<sup>+</sup> cells in normal BM. In PB samples we did not find myeloid marker<sup>+</sup>, TdT<sup>+</sup> cells. So far, it is unclear whether myeloid marker<sup>+</sup>, TdT<sup>+</sup> cells in normal BM represent precursor myeloid cells or whether the myeloid marker is weakly expressed by precursor lymphoid cells. In this respect, the recent finding of expression of CD13 and CD33 by ALL cells is interesting [14].

The role of TdT in ANLL cells and in normal precursor myeloid cells is unclear. In precursor lymphoid cells TdT is probably involved in the insertion of nucleotides during rearrangement of *Ig* genes or T-cell receptor (*TcR*) genes [15, 16]. In myeloid marker<sup>+</sup>, TdT<sup>+</sup> cells the TdT expression may be related to rearrangement of *Ig* or *TcR* genes or to rearrangement of a still unknown myeloid-specific gene. On the other hand, aberrant expression of TdT in immature precursor myeloid cells cannot be excluded.

If TdT is expressed by a subpopulation within an ANLL, double-marker analysis can be used to monitor this subpopulation in ANLL patients during and after chemotherapy. In analogy to our T-ALL studies, we have recently started a follow-up study using double-marker analysis for TdT and a myeloid marker in ANLL patients to detect minimal residual disease [17]. Although myeloid marker<sup>+</sup>, TdT<sup>+</sup> cells can be present in low frequencies in BM samples, our preliminary results indicate that detection of minimal residual disease is possible indeed. As illustrated in Fig. 2, myeloid marker<sup>+</sup>, TdT<sup>+</sup> cells were detected in two patients, who were in CR on clinical grounds and by cytomorphological examination of BM and PB samples. Although in both cases at diagnosis only a subpopulation of the cells were TdT<sup>+</sup>, it was possible to monitor this sub-

population during follow-up. In one patient a gradual increase of double-positive cells resulted in a hematological relapse after 6 months. In addition, it was also possible to detect myeloid marker<sup>+</sup>, TdT<sup>+</sup> cells in the autologous BM graft of this patient. The latter suggests that it might be rewarding to search for TdT<sup>+</sup> ANLL cells in autologous BM grafts of ANLL patients before transplantation.

In conclusion, TdT<sup>+</sup> cells, from 0.1% up to 83% of MNCs, can be detected in the majority of ANLLs. Using double-marker analysis it can be demonstrated that these TdT<sup>+</sup> cells belong to the ANLL cell population. This offers possibilities for the detection of minimal residual disease, and early detection of relapse.

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## Antigen Receptor Rearrangement and Expression in Acute Leukemias\*

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### Summary

Twenty-two leukemias, 11 of which were undifferentiated with respect to surface antigen markers, were investigated for their antigen receptor gene rearrangement, transcription products of these antigen receptor genes, and surface antigen pattern of the cells. Among the three less-differentiated groups rearrangement was observed in 2/10 cases for the TCR  $\beta$ -chain and in 4/11 cases for the heavy-chain gene. TCR  $\beta$ -mRNA, however, was expressed in seven out of eight cases and the  $\mu$  heavy-chain mRNA in eight out of ten cases investigated. Also mRNA of TCR  $\alpha$ , the rearrangement of which could not be detected with our probes, was expressed as frequently as TCR  $\beta$ . Although rearrangement of the appropriate gene was found regularly in the more mature leukemias, transcription of these genes was lower or even lacking. These findings indicate that expression of antigen receptor mRNA in undifferentiated leukemias can be activated by events other than maturational rearrangement.

### Introduction

Studies on antigen receptor rearrangement as earliest markers of maturation into one of

the pathways of lymphoid differentiation have revealed to be only of limited value, since lineage spillover rearrangement has been detected and rearrangement sometimes occurs also in leukemias otherwise carrying markers of myeloid differentiation. Therefore we attempted to see whether transcriptional activity is able to provide more information about differentiation into a defined lineage.

### Materials and Methods

DNA coding for the immunoglobulin  $\mu$ -constant fragment was kindly provided by Dr. M. Pech (Institute for Physiological Chemistry, University of Munich). In addition the *Jh* probe (Oncor Gaithersburg) was used. To mark the T-cell receptor (TCR) chains we used cDNA coding for the constant region of the TCR  $\alpha$  chain and the cDNA specific for the constant regions of the TCR  $\beta$  chain. Salmon sperm DNA was used as a negative control.

For blot hybridization cells were lysed and DNA was extracted with the phenol extraction method. DNA, 7.5–10  $\mu$ g, was digested with the restriction enzymes *EcoRI*, *BamHI*, *BglII*, or *HindIII* and transferred to nylon membranes after electrophoretic separation in 0.8% agarose gels [1]. DNA was radioactively labeled with <sup>32</sup>P by the random poly priming method [2]. Hybridization was carried out according to the protocol of Church and Gilbert [3] and exposure was for 1–14 days.

For cytological preparations cells from peripheral blood of patients were isolated by

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Ficoll-Isopaque separation and then cells sedimented in 10  $\mu$ l/PBS. The cells were then treated as described to make the membrane porous and fixed [4]. Then the cells were cytocentrifuged directly onto slides. As has been previously described (Pachmann et al. submitted), fluorescein isothiocyanate was conjugated to the synthetic polypeptide polyethylenimine (PEI) (Serva).

A DNA probe was denatured and then coupled to the fluoresceinated PEI and hybridization reactions were sealed under a coverslip and incubated for 24–48 h in a humidified atmosphere at 37°C. The slides were then unsealed and washed, covered with one drop of glycerine, and the fluorescence measured. Measurements were performed on a Leitz Orthoplan microscope photometer MPV 2 equipped with a Ploem optic for fluorescein and rhodamine illumination. Relative intensity values were corrected and net values plotted as a frequency distribution diagram.

#### *Surface Antigen Screening Tests*

The following antibodies were used (the clusters of differentiation according to the WHO criteria given in parentheses): Vim D2, WT1 (CD7), T28 (CD3), Vil A1 (CD10), HD 37 (CD19), B1 (CD20), BA-1 (CD24), and antipolyvalent Ig. Indirect immunofluorescence staining was performed with standard methods [5] using the monoclonal antibodies as the first reagent and affinity-purified fluoresceinated goat-anti-mouse globulin reagents (Tago Hamburg).

#### **Results**

Twenty-two leukemias which were classified by their surface marker pattern into a group lacking all markers, one with only a myeloid marker, one group carrying a myeloid and an early T-cell marker, and a group with either mature T-cell markers and or with B-cell markers, were investigated for rearrangement of the *TCR* genes and the immunoglobulin heavy-chain gene by Southern blotting and the expression of these genes by quantitative in situ hybridization. Nine out of the 11 undifferentiated

leukemias investigated carried a marker on the surface of their mononuclear cells usually assigned to the myeloid differentiation pathway but otherwise did not show morphological signs of myeloid differentiation (groups B, C in Table 1). They were subdivided according to the additional appearance of CD7 antigen (group C in Table 1), which is one of the earliest appearing markers in T-cell differentiation but has also been observed on cells of other origin. No other markers were found on these cells.

In contrast to the leukemias carrying additional maturation markers most of the leukemias of groups A, B, and C had the antigen receptor genes typical for T- or B-cell differentiation in germ-line configuration. Two of them, however, had rearranged the *TCR*  $\beta$ -chain gene and four others the immunoglobulin heavy-chain gene (Table 1; typical Southern blots, Fig. 1). In spite of germ-line configuration transcriptional activity was observed in 7 of 11 leukemias tested for the *TCR*  $\beta$  and in 9 of 10 leukemias tested for the immunoglobulin heavy chain and it was higher in the undifferentiated leukemias than in the more mature leukemias (Table 1; typical histograms, Fig. 2). *T $\beta$* -gene rearrangement was regularly found in leukemias with definite T-cell markers besides one obvious T-cell blast crisis of a CML which had the *T $\beta$*  gene in germ-line configuration. The same was true for definite B-lineage leukemias but lineage cross-over rearrangement was also observed (Fig. 1, patients 12, 22).

In addition the *TCR*  $\alpha$ -transcript was monitored, the gene rearrangement of which could not be detected with our probes and it was found to be present in seven of nine undifferentiated leukemias and, puzzlingly enough, in all B-cell leukemias tested (Table 1, Fig. 3).

#### **Discussion**

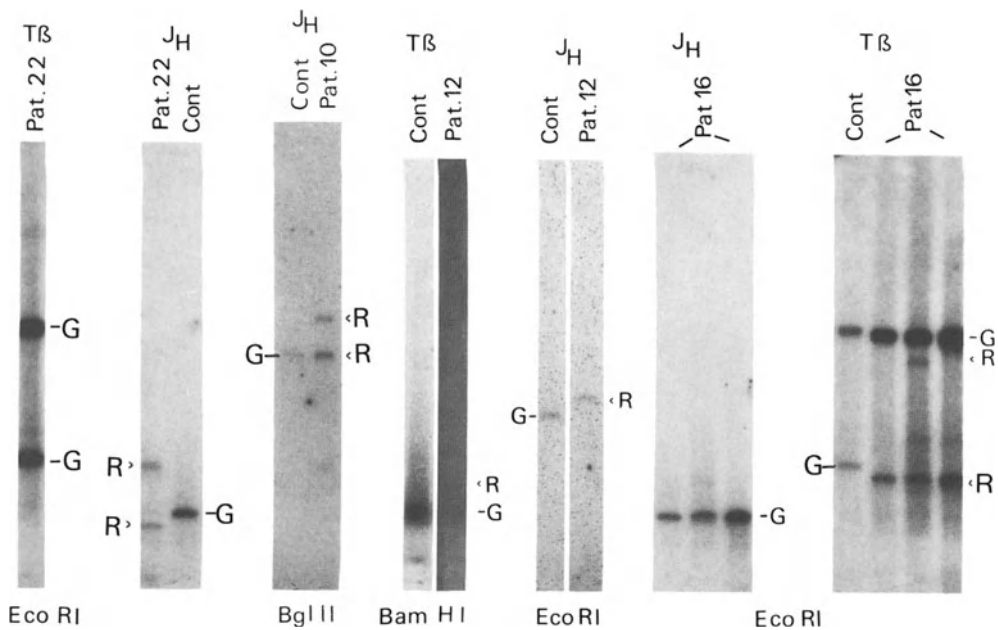
In accordance with others [6], we found rearrangement of the antigen receptor genes only in a low frequency in undifferentiated leukemias as compared with leukemias carrying more mature differentiation markers where the “appropriate” receptor genes

**Table 1.** Antigen receptor gene rearrangement and transcription and surface antigen expression in 22 lymphoid leukemias of different maturation stages. Clinical diagnosis expressed as AUL (acute undifferentiated leukemia), ALL (acute lymphatic leukemia), CLL (chronic lymphatic leukemia), and CML-BC (blast crisis of a chronic myeloid leukemia). Rearrangement of both alleles is defined as G (germ-line), R (rearranged), and D (deleted). Numbers for the detection of specific RNA are given as number of channels of specific fluorescence above that of control fluorescence in an arbitrary system comprising a maximum of 75 channels. Values for the surface markers are given as percentage positive cells

	DNA configuration				Detection of mRNA by in situ hybridization				Immune phenotyping								
	T-cell receptor		Ig-heavy chain		T-cell receptor		Ig-heavy chain		Myeloid markers		Lymphatic markers				B-line		
	Constant region	c-beta	Joining region	J-H	Constant region	c-alpha	Constant region	region	VIM13	VIM5/2	CALLA	WT1	T3	T6	T4/8	BA1	Ig
A	1 AUL		G/G					15	-	-	-	-	-	-	-	-	-
	2 AUL	G/R	G/R						-	-	-	-	-	-	-	-	-
B	3 AUL	G/G	R/R	1	7			10	92								
	4 CML-BC	G/G	G/G	9/5	17			7/3	51								3
	5 AUL	G/G	G/G	22	14			32	42								13
	6 AUL	R/?	G/G	6				4/5	59								5
	7 AUL	G/G	G/G	-	-			1	63								72
C	8 AUL	G/G	G/G	-	5			4	60								4
	9 AUL	G/G	G/G	25	17			2	41								3
	10 AUL	G/G	R/G	3	3			-	29								-
	11 AUL	G/G	R/R	4	5			-	90								-
	12 ALL	R/G	R/D						10								
D	13 CML-BC	G/G	G/G	-	-			3	18								-
	14 ALL	G/R	G/R	-	-			1	18								-
	15 ALL	R/R	R/R	2,5	-			6	-								-
	16 ALL	G/G	R/D	G/G				-	-								-
	17 CLL	G/G	G/R	5	1			-	-						57/8	7/79	-
E	18 ALL		D						-								24
	19 ALL	G/G	R/R	1	-			-	3								4
	20 IC	G/G	R/R	3	-			-	3								88
	21 CLL	G/G	R/D	5	-			-	-								91
	22 CLL	G/G	R/R	8	1			-	-								98

A, leukemias without surface markers; B, leukemias with myeloid markers; C, leukemias with lymphatic and myeloid markers; D, leukemias with T-cell markers; E, leukemias with B-cell markers





**Fig. 1.** Typical Southern blots from four patients from Table 1 as indicated. *G*, germ-line bands; *R*, rearranged bands

were detected to be rearranged almost regularly.

The *in situ* quantification of the cellular transcripts showed, however, that transcriptional activity of these genes was low or absent in the more mature leukemias, whereas there was a high frequency and accumulation of transcription products in undifferentiated leukemias with their antigen receptors in germ-line configuration.

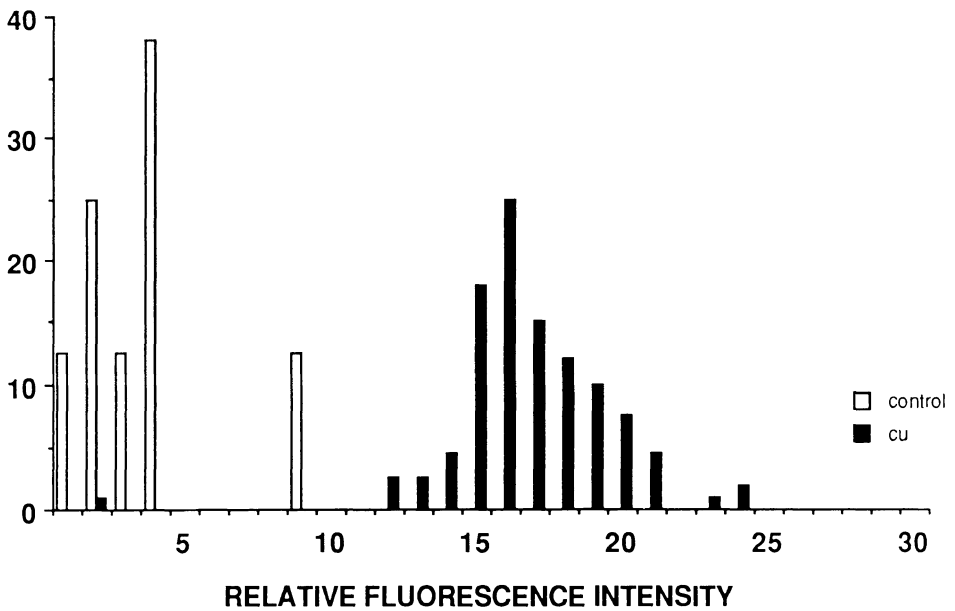
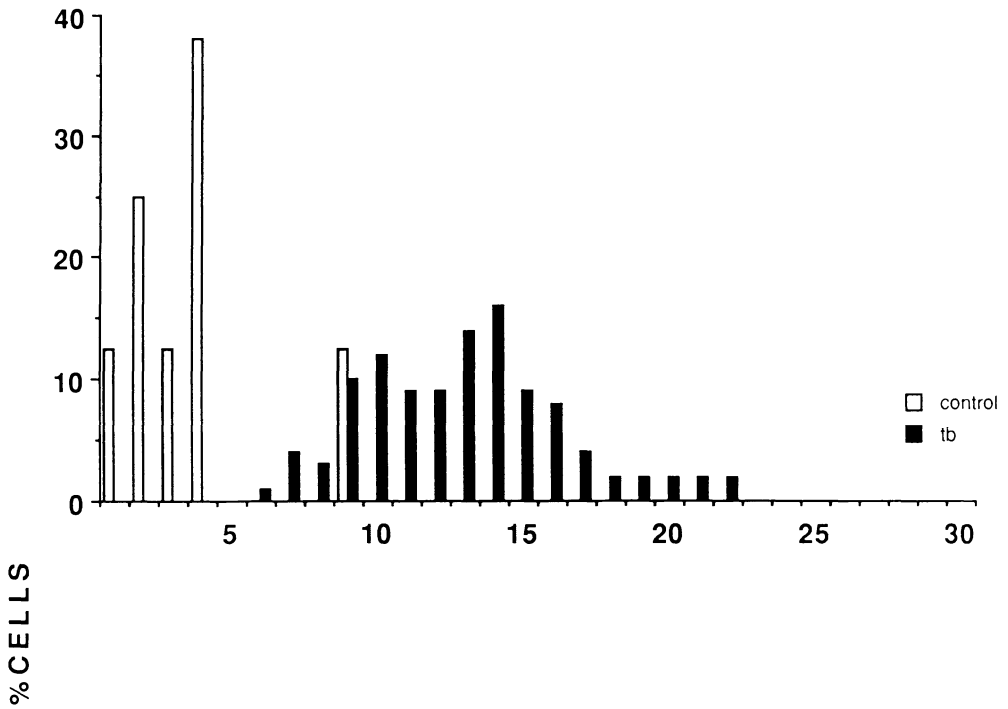
The unexpected finding of transcriptional activity without preceding antigen receptor gene rearrangement leads us to the hypothesis that in the leukemias these RNA transcripts may play another role than just ensuring rearrangement as a precursor for a functional gene product:

1. It has been reported that production of truncated transcripts may precede rearrangement and a role of these transcripts in facilitating functional rearrangement has been suggested [7]. Northern blots are now being performed to study the nature of these transcripts, and as to whether complete or truncated forms are transcribed under these conditions.

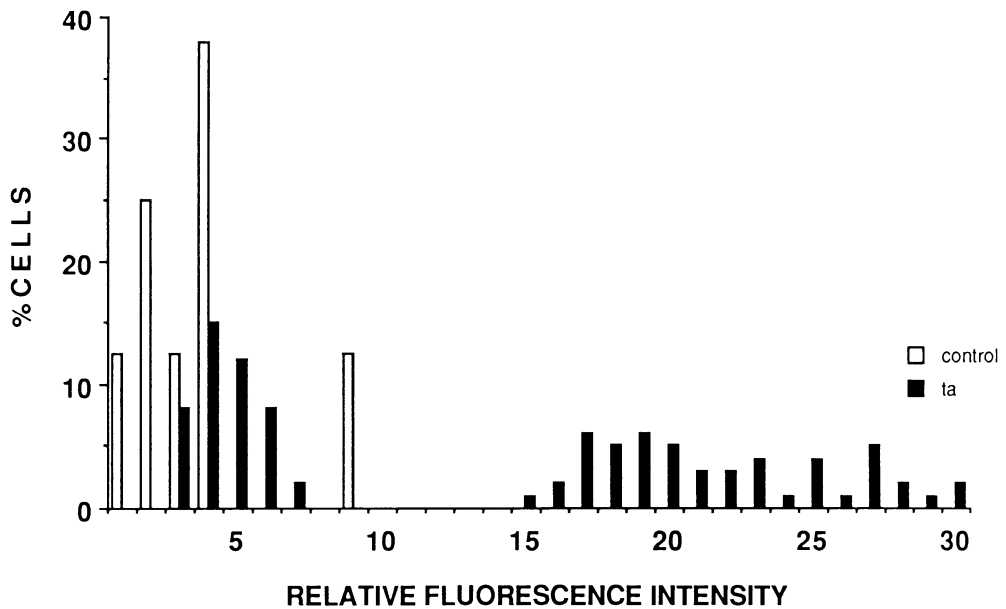
2. Transcripts of more than one antigen receptor were found simultaneously in the leukemias, the genes of which are located on different chromosomes. Therefore a far-reaching mechanism of activation must be considered in these undifferentiated leukemias. If the high transcriptional activity, in fact, indicates a maturation-enhancing process, this seems to be rather unspecific and general in character.

3. All investigated cell populations being derived from pathological clonal expansions, the unspecific transcription may reflect or be in itself a factor in malignant transformation. A connection with oncogene rearrangement into these regions with simultaneous activation of oncogenes and antigen receptor genes is currently under investigation.

Thus, expression of antigen receptor mRNA in undifferentiated leukemias may be activated by events other than maturational rearrangement.



**Fig. 2a–f.** Typical fluorescence intensity histograms from patient 3 (a), patient 5 (b), patient 10 (c), patient 4 (d), patient 9 (e) and patient 22 (f) from Table 1 for the Tβ (*top*) and the heavy chain (μ) mRNA (*bottom*) from 100 cells measured randomly from the whole population. Fluorescence intensity expressed as relative units on a 75-channel scale



**Fig. 3a-c.** Typical fluorescence intensity histograms from patient 3 (a), patient 5 (b) and patient 10 (c) from Table 1 for the T $\alpha$  mRNA from 100 cells measured randomly from the whole population. Fluorescence intensity expressed as relative units on a 75-channel scale

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## Immunoglobulin and T-Cell Receptor Gene Rearrangements in Childhood Acute Lymphoblastic Leukemia and Non-Hodgkin's Lymphoma \*

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### Introduction

Acute lymphoblastic leukemias (ALL) and non-Hodgkin's lymphomas (NHL) are clonal lymphoproliferative disorders at specific stages of differentiation. Recently, the introduction and application of molecular genetic methods have become a valuable addition to the established cytological, cytogenetic, histological, and immunological techniques in the diagnosis, classification, and monitoring of therapy of leukemias and lymphomas [1–4].

The antigen recognition molecules of B and T cells, immunoglobulins (Ig), and T-cell receptors (TCRs), respectively, are encoded by genetic loci that undergo somatic recombinations (rearrangements) during the differentiation of pluripotent bone marrow stem cells into immunoglobulin-producing plasma cells or immunocompetent T-lymphocytes [5–7]. This process generates functionally active genes and increases the diversity of Ig and TCR. Every Ig or TCR gene rearrangement produces a uniquely occurring gene in the lymphocyte, making it a specific marker of lineage and differentiation for a B or T cell and its clonal progeny [8–10].

We have analyzed DNA samples of 60 children with ALL and NHL for gene rearrangements of the Ig heavy-chain joining region (*JH*), the Ig kappa light-chain constant

region (*Ck*), and the TCR  $\beta$ -chain constant region (*CT $\beta$* ) at first presentation, in relapse, and during therapy.

### Material and Methods

#### Cells

Bone marrow, blood, lymph nodes, and other kinds of tumor tissue were obtained at the time of diagnosis and at different times of treatment. Cell morphology and cytochemistry were examined. The percentage of leukemic cells in each sample ranged from 0% to 98%. Analysis of surface markers using a panel of monoclonal antibodies, and surface and cytoplasmic immunoglobulins were carried out by Dr. W. D. Ludwig, Univ.-Klinikum Steglitz, Berlin.

#### DNA Preparation and Southern Blot Analysis

High molecular weight DNA of each patient was isolated by standard phenol-chloroform extraction. Ten micrograms of DNA was digested with the restriction endonucleases *Bam*HI, *Eco*RI, and *Hind*III. Digested DNA was sizefractionated by 0.7% agarose gel electrophoresis and transferred to nylon membranes [11]. The filters were hybridized to randomly primed P-32-labeled probes [12].

#### Gene Probes

The human Ig heavy-chain gene probe was a *Bam*HI-*Hind*III fragment that contained

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the joining region (*JH*: 5.9-kb *Bam*HI-*Hind*III fragment). The *JH* probe identified a 17-kb *Bam*HI and a 16-kb *Eco*RI fragment in germ-line DNA. A *Pst*I-*Bgl*III fragment of the T $\beta$  cDNA clone *C $\beta$ 1*, a subclone from pT10, was used as a T $\beta$  constant-region gene probe (*CT $\beta$* : 0.7-kb *Pst*I-*Bgl*III fragment). The *CT $\beta$*  probe detected a germ-line 24-kb *Bam*HI fragment containing both *CT $\beta$ 1* and *CT $\beta$ 2* and 12- and 4-kb *Eco*RI fragments containing *CT $\beta$ 1* and *CT $\beta$ 2*, respectively. The human Ig kappa light-chain gene probe was an *Eco*RI fragment that contained the constant region of the kappa chain (*Ck*: 2.5-kb *Eco*RI fragment). The *Ck* probe identified a 12-kb *Bam*HI fragment in germ-line DNA. The gene probes were kindly provided by P. Leder, Boston, United States (*JH*, *Ck*) and T. Mak, Toronto, Canada (*CT $\beta$* ).

## Results and Discussion

Of the 60 children who were studied sequentially, 43 had common ALL (10 at first presentation, 33 in relapse), 2 0-ALL, 2 pre-B-ALL, 1 B-ALL, 1 pre-T-ALL relapse, 4 T-ALL, 1 biphenotypic ALL relapse, 5 B-NHL, and 1 child with a human B-lymphotropic virus (HBLV) infection. These diagnoses were established through surface marker analysis. The results of the gene rearrangements were compared with the percentage of leukemic cells and their morphology, shown in Table 1.

Twenty-seven (90%) of 30 children with common ALL had a clonal rearrangement of the Ig *JH* gene; 6 of these patients (20%) showed an additional Ig *Ck* gene rearrangement. In 15 cases (50%) the *TCR*  $\beta$  gene was

**Table 1.** Molecular genetic analysis of children with ALL and non-Hodgkin's lymphoma in comparison with immunophenotype, percentage of leukemic cells, and their morphology

Diagnosis	<i>n</i>	<i>JH</i>	<i>Ck</i>	<i>CTB</i>	Leukemic cells	Morphology
C-ALL ( <i>n</i> =8)	5	R	G	G	75%–97%	L1/L2
	1	R	G	R	84%	L1
	2	R	R	R	91%	–
Therapeutic control	2	G	G	G	< 2%	–
C-ALL relapses ( <i>n</i> =22)	8	R	G	G	54%–98%	L1/L2
	2	R	R	G	81%–92%	L1/L2
	7	R	G	R	32%–98%	L1/L2
	3	G	G	R	5%–85%	L1/L2
	2 <sup>a</sup>	R	R	R	21%–86%	L1
Therapeutic control ( <i>n</i> =11)	2	R	G	G	< 9%	–
	9	G	G	G	< 2%	–
0-ALL ( <i>n</i> =2)	1	R	G	G	–	–
	1	G	R	G	97%	L1
Pre-B-ALL	1	R	G	G	–	–
Pre-B-ALL relapse	1	G	G	G	3%	–
B-ALL therapeutic control	1	G	G	G	0%	–
Pre-T-ALL relapse	1 <sup>a</sup>	G	G	R	92%	L2
T-ALL ( <i>n</i> =4)	2	G	R	R	83%–93%	L1/L2
	1	G	R	R	89%	L1/L2
	1	R	G	R	83%	L2
Biphenotypic ALL-relapse	1	R	G	G	76%	–
B-NHL inquiry into Bm involvement	4	G	G	G	–	–
B-NHL inquiry into lymph node involvement	1	G	G	G	–	–
HBLV infection	1	R	G	R	–	–

R, rearrangement; G, germ-line

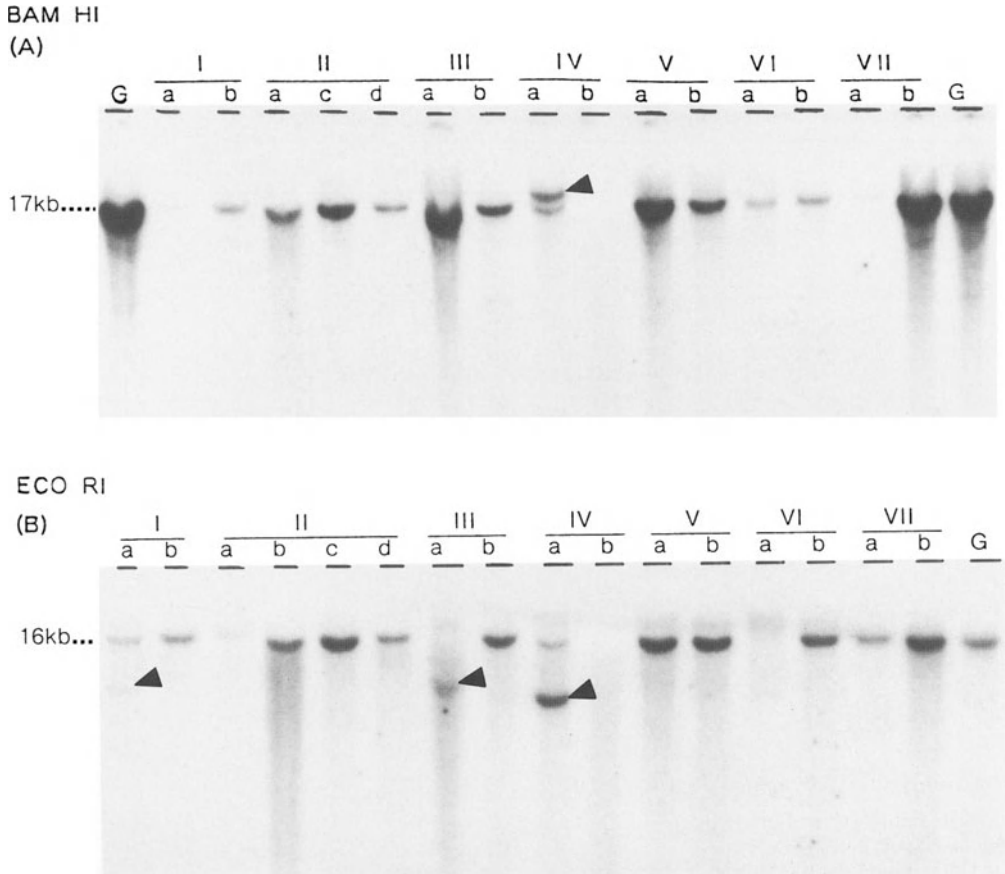
<sup>a</sup> Poor/nonresponder

rearranged: 12 (40%) were rearranged in both Ig *JH* and *TCR β* and 3 (10%) had a sole *TCR β* rearrangement. Four (13%) were rearranged in all three gene loci (Ig *JH*, Ig *Ck*, and *TCR β*). In all cases of T-ALL and precursor T-ALL a *TCR β* gene rearrangement was detected, moreover, three with Ig *Ck* and one with Ig *JH* being additionally rearranged. The examined 0-ALLs showed rearranged Ig *JH* or Ig *Ck* gene loci, indicating an initial step toward B-cell differentiation.

We would like to emphasize that in two benign cases (one child not mentioned in Table 1) a clonal rearrangement of Ig *JH* and *TCR β* was detected, one having a proven HBLV infection [13, 14]. Except for the

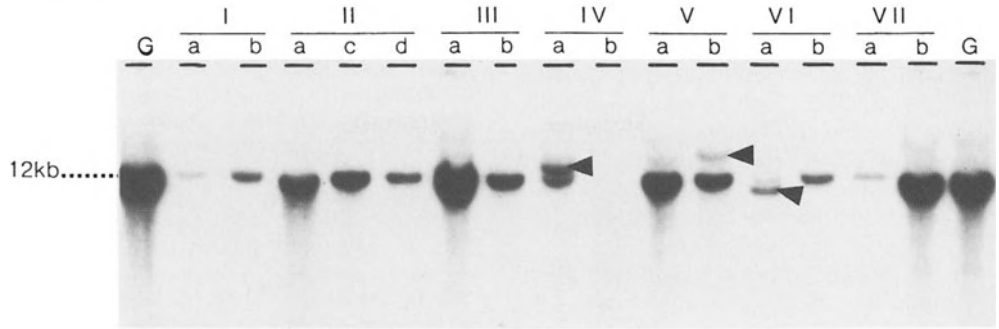
patients with remnant leukemic blast cells (<9%) the monitoring of the course of therapy through DNA analysis yielded a germline configuration. The comparison of patients with different acute leukemias at the time of diagnosis and at different stages of treatment is demonstrated in Figs. 1–3. In the five cases of B-NHL, we investigated the involvement of tissue other than the tumor site [15]. In neither case could an extranodal clone be detected.

We conclude from these results that in every analyzed case of ALL and NHL a clonal rearrangement is found at the time of diagnosis [16, 17]. These rearrangements are clonospecific for every patient and can be used as an individual genetic marker to



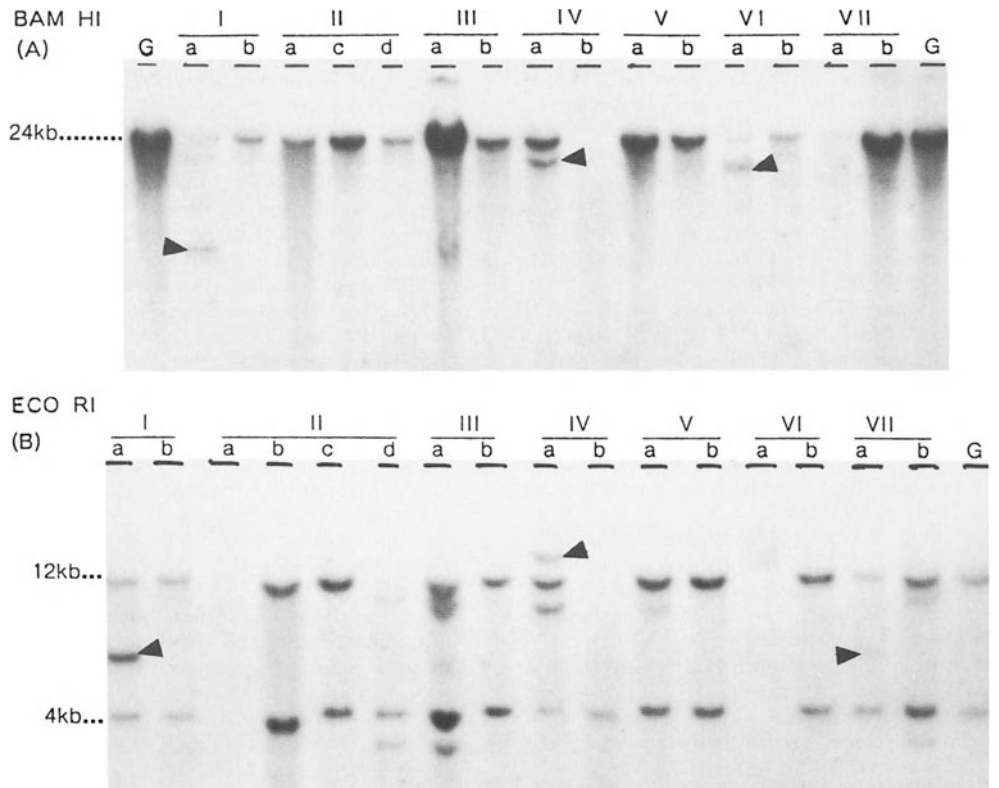
**Fig. 1 A, B.** Comparison of patients with different acute leukemias at the time of diagnosis (a), during therapy (b), before autologous BMT (c), and after autologous BMT (d) examined by Southern blot analysis, hybridized with a *JH* probe. Lanes G (human placenta DNA) show the germ-line restriction enzyme pattern after digestion with *Bam*HI (A) and *Eco*RI (B), 17 and 16 kb, respectively. The rearranged bands are indicated by arrows

BAM HI



- |                                   |                   |
|-----------------------------------|-------------------|
| I - Acute Hybrid Leukemia ( AHL ) | V - AML           |
| II - Common ALL relapse ( c-ALL ) | VI - T-ALL        |
| III - O-ALL                       | VII - AML relapse |
| IV - c-ALL                        |                   |

**Fig. 2.** Comparison of patients with different acute leukemias at the time of diagnosis (*a*), during therapy (*b*), before autologous BMT (*c*), and after autologous BMT (*d*) examined by Southern blot analysis, hybridized with a *Ck* probe. Lanes *G* (human placenta DNA) show the 12-kb germ-line restriction enzyme pattern after digestion with *Bam*HI. The rearranged bands are indicated by *arrows*



**Fig. 3A, B.** Comparison of patients with different acute leukemias at the time of diagnosis (*a*), during therapy (*b*), before autologous BMT (*c*), and after autologous BMT (*d*) examined by Southern blot analysis, hybridized with a *CTβ* probe. Lanes *G* (human placenta DNA) show the 24-kb germ-line restriction enzyme pattern after digestion with *Bam*HI (A) and the 12- and 4-kb germ-line bands after digestion with *Eco*RI (B). The rearranged bands are indicated by *arrows*

quantify remission and to monitor therapy [18]. In addition, the high sensitivity of the molecular genetic method (detection of a clonal population which comprises only about 2% of the total cell population) offers a powerful tool in detecting residual disease, especially for patients undergoing autologous bone marrow transplantation [19, 20].

A molecular genetic classification of leukemias and lymphomas is possible [4, 9, 10, 16, 17]. Comparison with the immunophenotype showed that in many cases aberrant rearrangements in the complementary cell line were detected next to rearrangements of the specific cell line [21–23].

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## Heterogeneity in Protein Patterns of CGL Blast Crisis Cells: Discrimination Between Lymphatic and Myeloic Lineages\*

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### Summary

Studies on cell-membrane-bound proteins in the human hematopoietic system revealed that the expression of certain peptides is restricted to the differentiation lineage. We applied discontinuous polyacrylamide gel electrophoresis of triton X-114 lysates to identify such proteins for a new diagnostic approach in human leukemia. A polypeptide with an apparent molecular mass range of 24 kd (p24) was found predominantly in cells of chronic granulocytic leukemia (CGL), myeloic type of blast crisis, and normal granulocytes. The data presented here suggest a role of this protein in the biology of malignant cells in chronic granulocytic leukemia throughout the course of the disease.

### Introduction

Sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a common method for analysis of biosynthetically labeled and immunoprecipitated proteins [1], as well as externally labeled cell surface polypeptides [2–6]. The problem of SDS-PAGE followed by an unspecific but high

sensitive silver stain is, in fact, its high resolution power. The multitude of visualized bands is difficult to interpret and the reproducibility may be disturbed by minor changes in technical performance. We described previously that the triton X-114 (TX-114) phase separation technique, primarily introduced by Bordier [7], yields a fraction of lipid-soluble proteins, some of them specific for different cell lineages of the hematopoietic system. Analysis of these detergent-phase-separated and so enriched membrane-bound proteins gives promising results and is reproducible [7, 8]. Using this technique, we analyzed 57 CGL blast crises considering only two proteins, p24 as a myeloic marker and p55 as a lymphatic marker, obtaining concordance with immunological typing in the vast majority of cases. However, p24 is the most specific protein observed so far. Blast crises, clearly positive for p24, were almost always immunotyped as myeloic [8].

### Material and Methods

Peripheral blood or bone marrow aspirate from CGL patients was treated as previously described [8]. Aliquots of  $2 \times 10^7$  cells were either lysed immediately in TX-114 buffer or stored until use at  $-80^\circ\text{C}$  as a dry pellet. After lysis and phase separation [7, 8], the samples were subjected to SDS-PAGE [9]. The gel was then silver stained [10] by a modified technique as previously described [8]. A protocol of the procedure will be sent out upon request.

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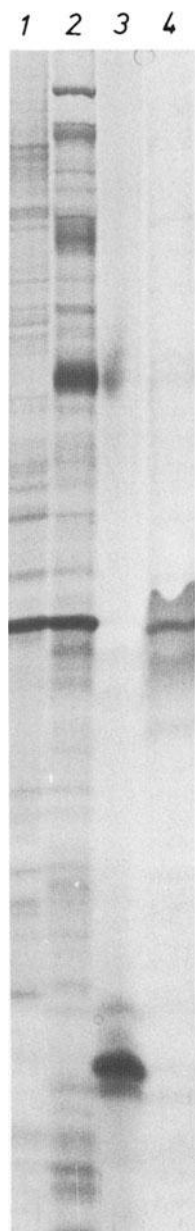
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\* Supported by the Deutsche Forschungsgemeinschaft DFG Do 313-1, and SFB102 TPC2

\*\* Present address: Biotest AG, Dreieich, FRG

## Results

Figure 1 shows the protein pattern of different peripheral blood subpopulations of a healthy individual. In conjunction to our working hypothesis the different subpopulations have different patterns. Granulocytes (lane 3) express predominantly a peptide of 24 kd apparent molecular mass. This peptide (p24) is not observed in lysates of

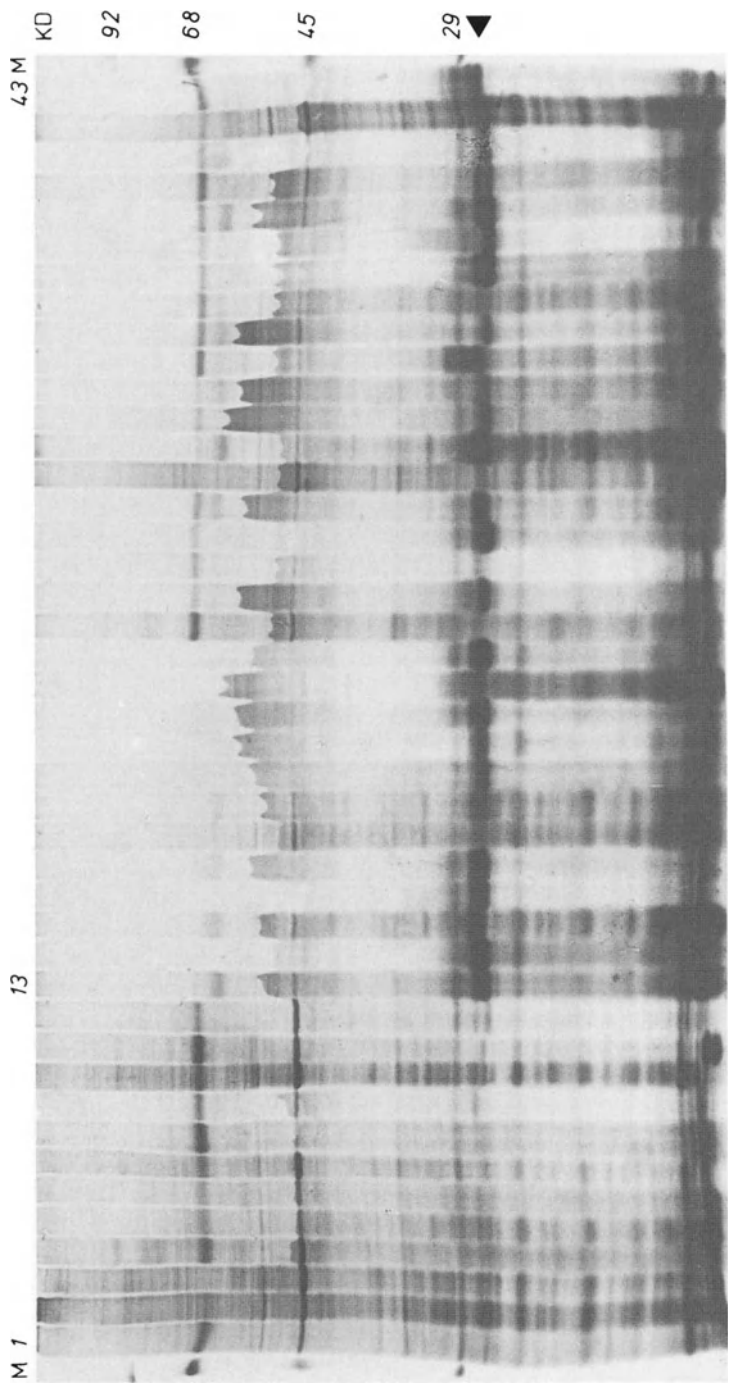


**Fig. 1.** SDS-PAGE of detergent-phase concentrated triton X-114 lysates of different peripheral blood subpopulations of a healthy individual. *Lane 1*, lymphocytes; *lane 2*, platelets; *lane 3*, granulocytes; *lane 4* monocytes

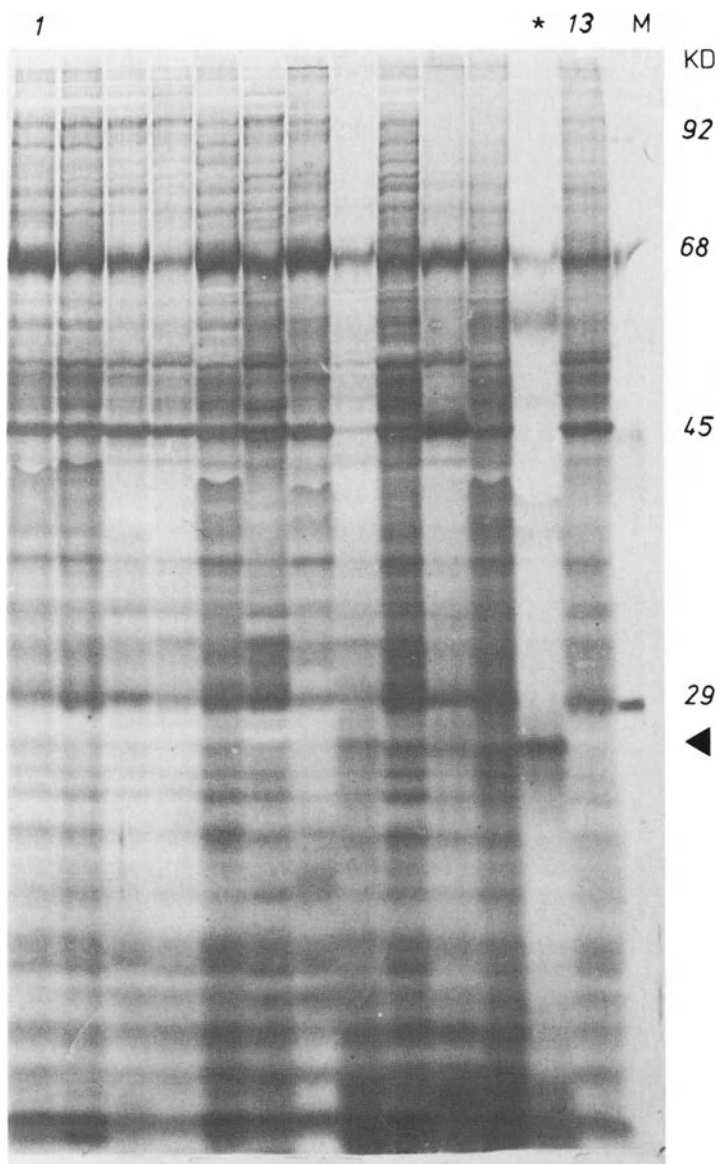
lymphocytes (lane 1), platelets (lane 2), or monocytes (lane 4). Both the granulocytes and monocytes are relatively poor in number of TX-114-phase-concentrated peptides compared with the lymphocytes and platelets. The predominance of p24 in CGL blast crisis patients is demonstrated in Fig. 2. Peripheral blood cells from 43 patients were separated according to the presented method. All patients identified immunologically by the use of several monoclonal antibodies as suffering from a myeloid blast crisis type expressed the p24 peptide (lanes 13–43). Lymphatic blast crisis cells expressed in contrast no p24 and resembled the protein pattern of normal lymphocytes (lanes 1–12). The protein pattern of acute myeloid leukemias (AMLs) is presented in Fig. 3. In 12/13 cases no predominance of p24 as shown for CGL blast crisis was observed. However, one case resembled the pattern shown in Fig. 2 and is marked by an asterisk.

## Discussion

Cells of the different lineages can be subdivided biochemically by the simple use of a detergent-phase separation and SDS-PAGE. Among several proteins we concentrated on the expression of p24. In healthy individuals p24 is found in cells of the granulocytic lineage, granulocytes, and their precursors in normal bone marrow, respectively. Among leukemias its detection is a regular finding in CGL cells in chronic phase as well as in acute blast crisis of the myeloid type [8]. Since the development of blast crisis in CGL is a prolonged process leading to clones with atypical and variable phenotypes, the finding of a constant biochemical feature in a subgroup of CGL blasts was surprising. These blasts are immunologically often defined by the absence of lymphatic markers only [3]. In addition, p24 positivity not only discriminates myeloid from lymphatic CGL blasts but myeloid CGL blasts from AML cells. These results indicate that the myeloid subgroup of blast crises in CGL can be biochemically defined by the presence of the granulocyte-related protein p24. However, it still remains unclear why CGL cells conserve this protein throughout the process of



**Fig. 2.** SDS-PAGE of detergent-phase concentrated triton X-114 lysates of CGL blast crisis cells. *M*, molecular weight markers; *lanes 1-12*, lymphathic type; *lanes 13-43*, myeloic type



**Fig. 3.** SDS-PAGE of detergent-phase concentrated triton X-114 lysates of AML cells. *M*, molecular weight markers; \*, leukemia resembling a myeloc type of CGL blast crisis

metamorphosis, involving additional chromosome mutations and variable phenotypic changes. In our opinion p24 seems not to be an essential protein for myeloc cells nor a sign of malignancy. A possible explanation is that the expression of p24 by myeloc cells is advantageous, by a not yet understood mechanism, in selection through the evolution leading to the terminal clone. In contrast AML blasts do not profit from p24 production perhaps due to another mechanism in oncogenesis.

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## Correlation Between the Expression of Myelomonocytic Surface Antigens and Ultrastructural Demonstration of Early Myeloperoxidase Expression in Null-AL(L) Cells

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### Introduction

Substantial progress in immunological and molecular analyses has now enabled most cases of acute leukemia to be classified as myeloid or lymphoid differentiated neoplasms [1]. In spite of these sophisticated methods, which have completed routine morphological and cytochemical studies, a certain number of acute leukemias remain unclassified [2]. This group of acute undifferentiated leukemias, mostly referred to as AULs or null-AL(L)s, can be characterized by the absence of morphological and cytochemical features for myelomonocytic differentiation at light microscopic level, lack of reactivity with anti-T-cell antibodies (Leu-1, OKT 11), and negativity for expression of the CD 10 antigen or cytoplasmic or surface immunoglobulin [3]. Some of these leukemias display myeloid surface antigens, alone or together with early B- or T-lymphoid surface markers, suggesting myeloid or bilineage differentiation [4]. It still remains unclear, however, whether these myeloid markers reflect true myeloid differentiation or are due to aberrant marker expression [5].

Ultrastructural studies have identified early or minimally differentiated myeloid leukemias among the AUL cases by demonstration of early, light microscopic non-

detectable myeloperoxidase expression (PO-EM), which is generally accepted as a lineage-specific marker for myelomonocytic differentiation [6]. The aim of our study was to identify early myelomonocytic leukemias among the subgroup of acute unclassified leukemias by ultrastructural analyses of PO-EM expression and to correlate POEM positivity with the expression of myeloid differentiation markers.

### Patients and Methods

#### Patients

Thirty patients aged 12–70 years with newly diagnosed acute leukemia were selected, because they remained unclassified according to stringent criteria after routine morphological, cytochemical, and immunological diagnosis [3].

#### Cells

Leukemic blast cells were isolated from peripheral blood or bone marrow by separation on a Ficoll-Hypaque gradient (1.077 g/ml). Unless the cells were not used immediately after separation they were cryopreserved in 10% demethylsulfoxide at –196°C liquid nitrogen.

#### Morphology

The morphological diagnosis was based on May-Grünwald-Giemsa and cytochemical

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staining of bone marrow and blood smears, including periodic acid Schiff (PAS), peroxidase, naphthyl-acetate esterase, and acid phosphatase. Classification was performed as previously described [7].

### Immunophenotyping

Phenotypic analyses of the blasts were performed either on bone marrow or peripheral blood cells by a broad panel of monoclonal antibodies including OKT 6 (CD 1), OKT 11 (CD 2), OKT 3 (CD 3), Leu 1 (CD 5), Leu 9 (CD 7), BA-2 (CD 9), J5 (CD 10), MY7 (CD 13), OKM 1 (CD 14), VIM-D5 (CD 15), B4 (CD 19), B1 (CD20), BA-1 (CD

24), MY 9 (CD 33), VIM 2, and OKIa. Immunofluorescence assays were performed in fresh or cryopreserved samples. Reactivity with murine monoclonal antibodies was determined as previously described [4]. For detection of the CD13 and CD33 antigen, a marker cocktail was used.

### Transmission Electron Microscopy Cytochemistry

Endogenous peroxidase activity was studied in fresh or nitrogen-stored blasts of the peripheral blood or bone marrow using the method of Roels [8]. The cells were incubated unfixed in a medium of 20 mg diamino-

**Table 1.** Three subgroups of untreated acute leukemia

No.	Myeloid antigens				POEM	Group
	CD 13/33	VIM-2	CD 14	CD 15		
1	60	40	0	56	70	I
2	50	16	0	0	90	I
3	0	46	0	72	100	I
4	0	43	nd	nd	65	I
5	0	46	0	0	20	I
6	0	44	0	28	45	I
7	20	45	0	nd	25	I
8	40	28	0	nd	16	I
9	80	0	0	nd	31	I
10	20	25	nd	nd	0	I
11	0	69	69	65	0	I
12	25	0	0	0	0	I
13	47	20	nd	0	25	II
14	96	90	0	nd	95	II
15	95	0	0	0	95	II
16	35	18	0	10	36	II
17	12	21	0	0	0	II
18	0	30	nd	nd	0	II
19	0	20	0	nd	10	III
20	95	0	0	0	40	III
21	74	0	0	0	15	III
22	0	22	0	0	95	III
23	0	73	0	25	0	III
24	60	54	0	nd	0	III
25	0	35	84	10	0	III
26	0	23	nd	nd	0	III
27	0	54	10	15	0	III
28	0	54	0	0	0	III
29	0	59	0	0	0	III
30	0	63	10	27	0	III

*n*: percentage of positive blasts; CD, cluster of differentiation; POEM, ultrastructural demonstration of myeloperoxidase; nd, not done



benzidine (DAB; Sigma, St. Louis, Mo, United States) dissolved in 10 ml 0.05 M Ringer-Tris buffer, pH 7.4, containing  $H_2O_2$  at a final concentration of 0.003% for 1 h at room temperature in the dark. After incubation cells were washed and processed for electron microscopy without block staining by uranylacetate as previously described [9].

## Results

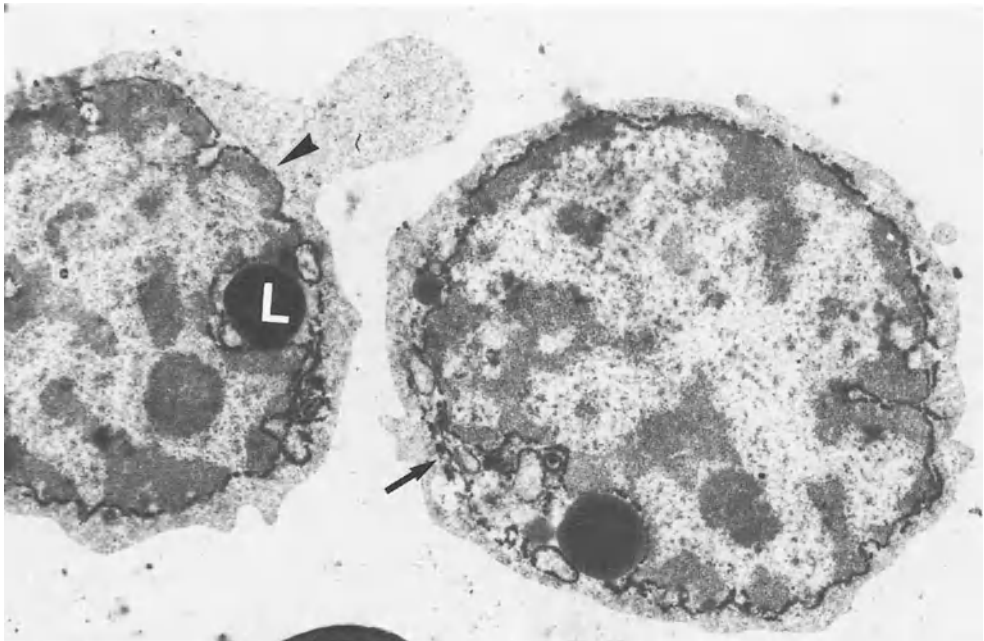
Thirty patients with newly diagnosed, untreated acute leukemia were selected for this retrospective study. Light microscopic analyses did not reveal morphological and cytochemical features of myelomonocytic differentiation. According to their immunological phenotype, which had been selected for the absence of expression of the CD 2 and CD 10 antigen and negativity for the expression of cytoplasmic or surface immunoglobulin, three subgroups among these leukemias could be identified (Table 1):

I. Presence of only myeloid surface antigens (patient 1–12)

II. Presence of both the CD 7 antigen and myeloid surface antigens (patient 13–18)

III. Presence of both B-lymphoid and myeloid surface antigens (patient 19–30)

For electron microscopic analyses of endogenous peroxidase activity the method of Roels was used, since this technique allows both detection of myeloperoxidase and platelet peroxidase and gives a stronger reaction in the membranous structures. In 17/30 patients under study a positive reaction for myeloperoxidase activity (POEM, Fig. 1) was found in at least 5% of the blasts of the peripheral blood. In the other patients less than 1% of the cells displayed positivity or were completely negative. In none of the cases studied could platelet peroxidase be detected. In 9/12 cases of group I POEM activity of the blasts could be detected. All but one of these nine patients displayed VIM 2 positivity and in five cases CD 13/33 positivity could be demonstrated. In one of the POEM-negative patients (patient 10) positivity for both the CD 13/33 antigen and



**Fig. 1.** Ultrastructural analyses of myeloperoxidase activity (POEM) reveals a positive reaction in the membranous structures as the nuclear membrane (*arrowhead*) and the endoplasmic reticulum (*arrow*). *L* denotes lipid droplet,  $\times 20\,000$

the VIM 2 antigen was found. Patient 11, also negative for POEM, displayed strong expression of the VIM 2 together with the CD 14 and 15 antigen. In four out of six patients of group II, POEM activity could be demonstrated in at least 25% of the blasts. In three POEM-positive cases and in one POEM-negative patient CD 13/33 together with VIM 2 positivity could be detected. Only in a minority of patients of group III (4/12) did ultrastructural analyses reveal POEM activity in at least 10% of blasts, and in two cases CD 13/33 positivity and in another two cases VIM 2 expression was found. All POEM-negative patients of this group displayed VIM 2 positivity and in one patient (patient 24) additionally CD 13/33 positivity could be detected.

A close correlation between the percentage of POEM-positive cells and marker positivity was found in five cases for CD 13/33 and POEM expression (patients 1, 7, 14, 15, 16) and in three cases (patients 6, 13, 14) for VIM 2 and POEM positivity. In none of the POEM-positive cases could CD 14 expression be detected. The best correlation between myeloid antigen expression and POEM positivity was found for the CD 13/33 or CD 13/33 and VIM 2 antigens (Table 2).

**Table 2.** Correlation between myeloid antigen expression and POEM positivity

		POEM positivity
CD 13/33	Positive	73%
	Negative	40%
VIM-2	Positive	52%
	Negative	80%
CD 14	Positive	0%
	Negative	71%
CD 15	Positive	44%
	Negative	64%
CD 13/33/VIM-2	Positive	70%
	Negative	50%

## Discussion

All cases under study expressed at least one myeloid surface antigen as an indication for myeloid differentiation of the blasts. Ultrastructural analyses of myeloperoxidase ac-

tivity (POEM) identified in 17/30 cases a substantial portion of POEM-positive cells, whereby the percentage of positive cells varied considerably, from 10% to over 90%, in different patients. On this basis these leukemias could be reclassified as early or "minimally" differentiated acute myeloid leukemias (AMLs) in agreement with the French-American-British (FAB) group, suggesting that ultrastructural demonstration of myeloperoxidase is sufficient to establish myeloid lineage [10, 11].

Thus POEM positivity in these cases confirms the myeloid differentiation of the blasts as suggested by the expression of myeloid markers. It remains a matter of speculation why in the majority of these cases the percentage of POEM differs from that of marker positivity. The meaning of myeloid marker expression in the 13 POEM-negative cases remains open. These cases might reflect an earlier stage of myelomonocytic differentiation prior to myeloperoxidase expression, especially in those cases of group I. It might be that application of molecular analyses of myeloperoxidase expression at messenger RNA level or in vitro studies including differentiation induction by various chemical inducers or by different growth factors could verify the myeloid differentiation of these leukemias [9, 12].

Myeloid marker expression together with T- or B-lymphoid markers (groups II, III) in POEM-negative cases might reflect, under the assumption that these markers are really lineage specific, an even earlier stage of hemopoietic differentiation, prior to definite lineage commitment to the myelomonocytic or lymphoid differentiation pathway. One has to take into consideration, however, that this phenomenon might be due to genetic misprogramming of the leukemias resulting in an aberrant marker expression [13].

Taken together we confirm data from recent reports that ultrastructural analyses of POEM activity identifies minimally differentiated myeloid leukemias among AUL patients and that most of the POEM-positive cases display myeloid surface antigens [14, 15]. In contrast to others, we found that the predictive value of myeloid surface antigens with respect to POEM activity varies considerably depending on whether myeloid markers were found alone or together with T- or

B-lymphoid surface markers. For all cases under study the best correlation between POEM positivity and different surface markers was found for the CD 13/33 cocktail used, whereas the additional analyses of the other markers studied could not improve this correlation index.

It has to be further elucidated whether the implication of other myeloid surface markers could improve the accuracy of immunological diagnosis of minimally differentiated AML, otherwise ultrastructural analyses of POEM expression seems necessary to identify this subgroup of AML [1, 5].

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## Interleukin-1 Production in Childhood Acute Lymphoblastic Leukemia During Chemo- and Radiotherapy According to BFM (Berlin-Frankfurt-Münster) Protocol

A. Chybicka and J. Bogusławska-Jaworska

### Introduction

Interleukin-1 (IL-1) is an important cytokine produced by nearly all cell types including blood monocytes and macrophages, T- and B-lymphocytes, natural killer cells, skin keratocytes, brain astrocytes, microglia, epithelial cells, mesangial cells, and vascular tissues. There are two structurally related IL-1s alpha and beta [1]. The production of IL-1 is induced by antigens, toxins, injury, and inflammatory processes. IL-1 represents a family of hormone-like polypeptides with a wide spectrum of activities, acting like an endogenous immunoregulatory adjuvant, serving as cofactor during B- and T-lymphocyte activation, proliferation, and differentiation, primarily by inducing the synthesis of other lymphokines and activation of the resting T cell. IL-1 also plays a role in antibody production. The systemic effects of IL-1 are associated with acute-phase responses. Within a few hours after the onset of infection or injury IL-1 production can be detected in the circulation. IL-1 induces fever, slow-wave sleep, synthesis of hepatic acute-phase proteins, and release of neutrophils, adrenocorticotrophic hormone (ACTH), cortisol, and insulin. It has dramatic effects on endothelial cells, leading to a procoagulant state, leukocyte adherence, prostaglandin release, and hypotension [2, 6]. IL-1 shares many of its multiple systemic properties with other molecules such as tumor necrosis factor (TNF) and colony-stim-

ulating factor (CSF) [3]. Inadequate production of IL-1 has been observed in several disease states, raising important questions about the therapeutic use of this substance. A deficiency of IL-1 production was observed in patients with cancer, who have large tumor burdens [1–3, 5, 9, 10, 12].

### Aim of Study

The purpose of this study was to investigate the effect of cytostatic treatment on the ability of mononuclear cells in ALL patients to produce IL-1.

### Material

A total of 27 children (8 girls and 19 boys) with acute lymphoblastic leukemia were investigated. The mean age at the time of the study was 6 years. Eight children of the same age without inflammatory disease served as controls. All children were treated at the Department of Children Hematology and Oncology from April to November 1988 according to the BFM 87 protocol. Heparinized blood samples were drawn from children with ALL at different stages of their disease (on diagnosis, during therapy, after therapy).

### Methods

Mononuclear cells were separated from peripheral blood cells by Ficoll-Hypaque centrifugation. The method used described by

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Zimecki and Wieczorek is based on IL-1-dependent reduction of the content of thymocytes forming autologous rosettes [12]. Mononuclear cells were counted, resuspended in Eagle's medium containing 10% fetal calf serum (FCS), and placed in an incubator at a concentration of  $2 \times 10^6$  cells/ml for 24 h. After 24 h of incubation supernatant was removed. Thymocytes,  $10^7/1.8$  ml RPMI medium, supplemented with 10% FCS and antibiotics, were incubated with 0.2 ml supernatant, at various dilutions for 24 h in a cell culture incubator.

#### Rosette Assay

The cells were resuspended at a concentration of  $3 \times 10^6$  in Eagle's medium supplemented with 10% mouse serum (preabsorbed with syngenic erythrocytes). To 0.1 ml of the cell suspension was added 0.1 ml 12% syngenic erythrocytes, mixed and centrifuged for 5 min at 200 g and 4°C. After 24-h incubation at 4°C, 0.5 ml Hank's medium and 0.1 ml 0.1% acridine orange were added, the cells gently resuspended, and kept in an ice bath. The percentage of autologous rosettes formed by thymocytes of 2-month-old CBA mice was counted and was found to vary from 28% to 33%. Con-

trol samples consisted of recombinant IL-1 dilutions. 1 U IL-1 showed 50% inhibition of rosette formation. All results were counted in units of IL-1.

## Results

### *Interleukin-1 Production in Initial Phase of ALL*

The production of IL-1 by mononuclear cells from each patient is listed in Table 1. It was found that cells deriving from untreated children with ALL exhibited 4–64 U IL-1 activity, the mean value of 33 U being similar to the control value. A significant difference was observed between IL-1 production at diagnosis of ALL, before any treatment, and that found after prednisone pretreatment. In four of five patients no detectable levels of IL-1 activity were found.

### *Interleukin-1 Production in Remission and Relapse of ALL*

After 4 weeks of continuous remission induction therapy, IL-1 activity increased to 64 units (Table 2). IL-1 production completion of the consolidation phase of treatment

**Table 1.** Interleukin-1 production by mononuclear cells of children with ALL before achieving remission

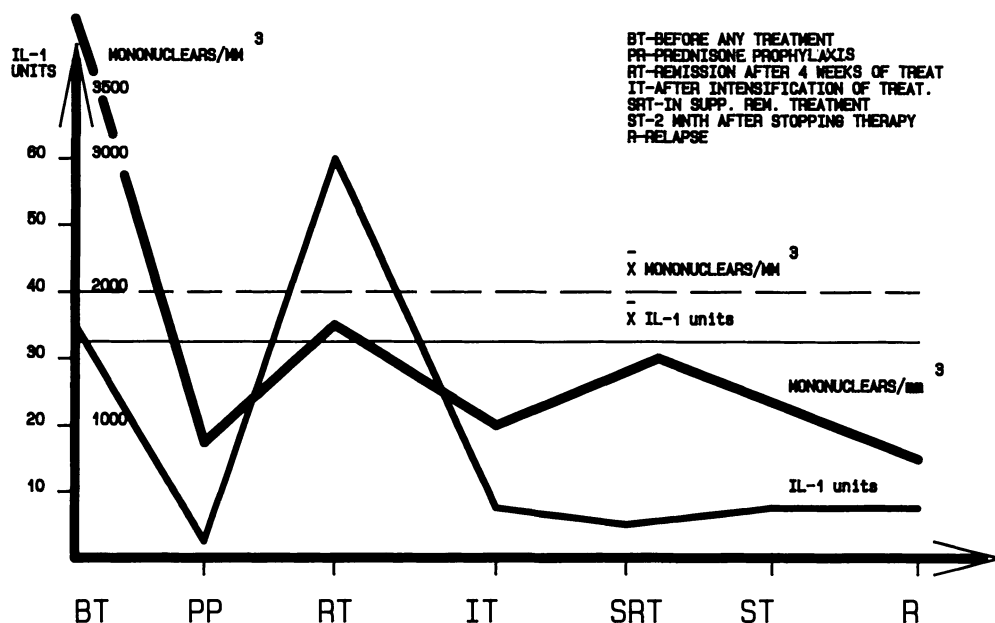
N	Name	Age (years)	Day of treatment	IL-1 units	Phase of treatment	Mononuclear cells (mm <sup>3</sup> )	B-lymphocytes (mm <sup>3</sup> )	T-lymphocytes (mm <sup>3</sup> )
1	G. A.	7	1	64	Before any treatment	10200	1224	1530
2	D. P.	4	1	31	Before any treatment	320	16	182
3	R. J.	6	1	4	Before any treatment	324	110	308
$\bar{x}$		5.3		33		4084	450	670
1	D. P.	3	3	5.4	Prednisone prophase	576	51	809
2	D. O.	4	4	0	Prednisone prophase	1854	463	238
3	B. E.	4	3	0	Prednisone prophase	992	109	386
4	U. K.	10	4	0	Prednisone prophase	702	86	86
5	I. R.	4	5	0	Prednisone prophase	128	1	42
$\bar{x}$		5		1.08		850	142	312
$\bar{x}$	Control group	8		31.5		2000	423	980

**Table 2.** Interleukin-1 production in remission and relapse of ALL

	Number of children	$\bar{X}$ IL-1 units	IL-1 units	$\bar{X}$ mononuclear cells (mm <sup>3</sup> )	$\bar{X}$ Lym-phocytes B (mm <sup>3</sup> )	$\bar{X}$ Lym-phocytes (mm <sup>3</sup> )
I. In remission	20	12.5	0-64	1260	254	1050
After remission induction protocol	2	64	64	1800	270	1060
After therapy-intensive treatment protocol	14	8.7	0-19.5	976	140	504
In supportive remission treatment	4	5.45	3- 6.5	1405	398	810
II. After stopping treatment	2	9.15	0-18.3	1051	342	460
III. Relapse	3	8.1	0-24.5	616	115	308
IV. Control group	8	31.5	11-64	2000	423	980

(which included cranial irradiation) was undetectable or very low in five of six children studied. During remission therapy the production of the factor was also suppressed when compared with that found after initial treatment and in the control group. But in this phase more variability in individual IL-1 activity was observed. A correlation between mean level of IL-1 and number of mononuclear cells was found. No direct

correlation between IL-1 activity and T- or B-lymphocyte count has been established (Fig. 1). Two children were tested 2 months after discontinuing antileukemic treatment. In one no IL-1 production was observed. The second child showed similar IL-1 activity to that found during remission therapy, where the children were studied in relapse after completion of antileukemic therapy. In neither was IL-1 activity present.



**Fig. 1.** Number of mononuclear cells and IL-1 production during treatment of ALL

## Concluding Remarks

The ability of mononuclear cells to produce IL-1 in the tested children with ALL was high at diagnosis before treatment; this could be due to monocyte activation to produce the factor stimulating lymphocyte differentiation. Throughout the period of cytostatic therapy of children with ALL the decrease in IL-1 production was observed in comparison with that of the healthy children and untreated patients. The most striking decrease in IL-1 activity was observed during the first 5 days of prednisone therapy. It is not clear whether this alteration of IL-1 activity reflected the lower production of the factor or the presence of some inhibition molecules in the period of leukemic blast destruction. Such inhibitors – if present – might mask the bioactivity of IL-1. Also the low level of IL-1 activity found during the very intensive consolidation therapy (which included the most potent cytostatics and irradiation) could be connected with an impairment of mononuclear cells to produce the factor. This hypothesis can be supported by our further observation, which demonstrated the decreased IL-1 production during the whole period of remission treatment.

The low activity of IL-1 during chemotherapy can also be related to the decrease in monocyte number.

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## Determination of Soluble Interleukin-2 Receptors After Bone Marrow Transplantation

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### Introduction

Graft-versus-host disease (GvHD) complicates allogeneic bone marrow transplantation (BMT) in up to 25%–50% of patients receiving human leukocyte antigen (HLA)-compatible grafts. The diagnosis is mainly based on the clinical findings of a skin rash, watery diarrhea, and cholestatic hepatitis, often confirmed by histopathological examination. Frequently the effects of drug toxicity or infections are difficult to discriminate from GvHD. Therefore, there is need for criteria that additionally define the diagnosis of GvHD and which possibly help to predict the occurrence of GvHD [1].

Graft-versus-host disease is caused by mature immunocompetent T-lymphocytes of donor origin, which are activated to proliferation by major histocompatibility complex (MHC) antigens of the host. T-lymphocytes stimulated by antigens or mitogens release a variety of lymphokines, one of which is interleukin-2 (IL-2), and express the IL-2 receptor (IL-2R). During the process of activation the Tac subunit of the IL-2R complex is released into the serum [2]. As a consequence of "strong" immune stimulations, like allograft rejections or infections, elevated IL-2R levels have been detected in the serum of patients [3, 4].

We addressed the question of whether increased levels of soluble IL-2R could be detected after BMT and whether they would

correlate with GvHD such that IL-2R might serve as a predictor or indicator of GvHD.

### Patients and Methods

We analyzed 26 transplant episodes, 18 after allogeneic and 8 after autologous bone marrow transplantation. Serum samples were serially drawn prior to BMT and in the early posttransplant period. The diseases for which BMT were performed were acute leukemia ( $n=11$ ), chronic myeloid leukemia ( $n=9$ ), aplastic anemia ( $n=3$ ), and solid tumor ( $n=3$ ). The conditioning regimens consisted of total body irradiation/cyclophosphamide or busulfan/cyclophosphamide for leukemias, cyclophosphamide for aplastic anemias, and new experimental regimens for solid tumors. GvHD prophylaxis was performed with the combination of methotrexate and cyclosporin A. Twelve of 18 patients with allografts developed GvHD grade II–IV and 6 patients did not develop GvHD or developed GvHD grade I. Patients were allocated to two groups depending on the presence of GvHD. Group 1 comprised patients with GvHD grade II–IV. Group 2 patients were those without GvHD or with GvHD grade I (group 2a) and with autografts (group 2b).

### Results and Discussion

There was a strong correlation between the presence of elevated IL-2R levels, fever, and GvHD. All patients (12/12) with GvHD grade II–IV had elevated IL-2R levels. This

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is in contrast to patients without GvHD or with GvHD grade I and patients with autografts in which elevated IL-2R levels were only found in two out of six (33%) and three out of eight (38%), respectively. Seventeen out of 19 (89%) patients with fever  $\geq 38^{\circ}\text{C}$  lasting for longer than 6 days had elevated IL-2R levels, whereas none of 7 patients with fever  $\geq 38^{\circ}\text{C}$  for 6 days or less had elevated levels. The comparison of the IL-2R maximum levels revealed significant differences between group 1 and 2 at a level of  $P < 0.01$ . The median of the IL-2R maximum levels in group 1 patients was 480 U/ml (range, 180–2250 U/ml) and 144 U/ml (range, 40–1178 U/ml) in group 2 patients. Patients from group 2 with fever  $\geq 38^{\circ}\text{C}$  for  $\leq 6$  days had an IL-2R maximum of 118 U/ml (range, 40–139 U/ml); group 2 patients with fever  $\geq 38^{\circ}\text{C}$  for  $> 6$  days had significantly higher IL-2R maximum levels 325 U/ml (range, 150–1178 U/ml) ( $P < 0.01$ ).

Besides differences in the IL-2R values themselves, we observed remarkable differences in the time course in patient groups 1 and 2. In patients with no or mild GvHD or in patients with autografts we constantly observed an IL-2R course which paralleled the fever curve with an initial rise of IL-2R, coinciding peaks, and a subsequent drop to background levels in the phase where the temperature normalized. In patients with GvHD grade II–IV the IL-2R levels continued to rise and reached peak values at a median of 20.5 days compared with group 2 patients in whom maximum values were observed 9 days after BMT ( $P < 0.01$ ). In group 1 patients the IL-2R peak coincided with the onset of acute GvHD at a median of 22 days after BMT.

In the search for markers that might give similar information, urinary neopterin excretion and DNA synthesis in peripheral blood lymphocytes were studied [5, 6]. Although there were good correlations with the occurrence of GvHD, neither test has become a widely accepted routine procedure. IL-2R and its determination in the serum have also attracted attention because of recent very promising attempts to suppress immune reactions in vitro and to treat autoimmune diseases and allograft rejections

in animals with monoclonal antibodies against IL-2R [7]. Hervé and coworkers have published a trial in which they successfully suppressed acute corticosteroid-resistant GvHD in man by IL-2R-targeted treatment [8].

We conclude that IL-2R determinations may be helpful in defining GvHD and possibly in predicting its occurrence, provided that they are carried out serially. Single determinations are not conclusive. Our data may contribute to a better definition of the optimum time for a start with IL-2R antibody administration in the prophylaxis of GvHD.

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## Synergism of H2 Histamine Receptor Antagonists with Alpha-Interferon to Inhibit the Growth of Leukemic and Normal Hematopoietic Progenitors

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### Introduction

A number of substances, including tumor necrosis factor and retinoic acid, have been shown to amplify in vitro the antiproliferative or differentiation-inducing activities of interferon (IFN), suggesting that their combinations may be clinically more effective than IFN alone. Histamine H2 receptor antagonist, cimetidine, given in combination with IFN to patients with malignant melanoma, had been reported to produce a clinical response when IFN alone was ineffective [1, 2]. The rationale for combining these agents was based on the immune-stimulating properties and the in vivo antitumor activity of cimetidine in experimental mouse models [3–5]. Other studies using human lymphoma and melanoma cell lines had indicated that cimetidine enhances the antiproliferative effect of  $\alpha$ -IFN in culture [6].

Human interferons can inhibit the proliferation of normal and certain malignant hematopoietic cells. Alpha-IFN inhibits the growth of leukemic colony-forming unit granulocyte-macrophage (CFU-GM) colonies from patients with chronic myeloid leukemia (CML) [7], and administration of recombinant  $\alpha$ -IFN induces hematological remissions in the chronic phase of the disease [8]. Although some investigations show mild antiproliferative effects in vitro on acute myeloid leukemia (AML) cells [9, 10],

patients with AML, in contrast to CML, do not appear to respond to  $\alpha$ -IFN therapy [11].

We investigated whether cimetidine would enhance the antiproliferative effects of recombinant  $\alpha$ -IFN on the clonal growth of the HL-60 acute promyelocytic leukemia cell line, leukemic CFU-GM colonies from patients with CML, and normal human CFU-GM burst-forming unit erythroid and (BFU-E) progenitors.

### Materials and Methods

Clonal growth of leukemic cells and normal CFU-GM was assayed by plating the cells in soft gel. Placenta-conditioned medium was added to cultures of normal bone marrow and CML cells but not to HL-60 or K562 leukemic cells. Normal bone marrow BFU-E colonies were grown in methylcellulose, with sheep erythropoietin. BFU-E colonies (>40 cells) were scored after 14 days of incubation.

Biochemical events that mediate IFN action include induction of 2'-5', oligoadenylate (2-5A) synthetase activity. To measure this activity, HL-60 cells were incubated for 18 h at 37°C with  $\alpha$ -IFN alone, cimetidine alone, or both agents. About  $2 \times 10^7$  cells were harvested, washed, and placed for 15 min at 4°C in lysis buffer. The lysates were centrifuged at 10 000 *g* for 10 min and the supernatant (S-10 fraction) was stored in small aliquots at -70°C. The 2'-5A' synthetase activity was determined in the presence of Poly(I):Poly(C). The <sup>32</sup>P-labeled 2-5A oligomers were analyzed by high-

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voltage electrophoresis and the radioactive spots identified by autoradiography.

The statistical analysis of an additive versus synergistic effect was performed according to the method described in detail by Spector et al. [12]. The probability of significance between two means was determined by Student's *t*-test.

## Results

Colony growth was not inhibited by adding cimetidine alone at concentrations of 0.1–100 µg/ml to HL-60 cells, 1–100 µg/ml to normal bone marrow CFU-GM cultures, and 0.1–10 µg/ml to normal bone marrow BFU-E cultures. For combination experiments with  $\alpha$ -INF we used cimetidine levels that had no inhibitory effect when added alone to the cultures.

Addition of  $\alpha$ -INF alone to HL-60 cells, at concentrations of 0.1–100 U/ml, did not substantially affect the growth, while, in the presence of cimetidine, a marked inhibition of colony formation was observed (Fig. 1). Within this range of concentrations, the  $\alpha$ -INF dose-response curve plateaued at 1 U/ml IFN and the mean ( $\pm$ SE) inhibition with 10 µg/ml cimetidine was 49%  $\pm$  2% compared with medium containing control cultures. Higher  $\alpha$ -INF concentrations of 1000 and 10 000 U/ml, added alone, suppressed colony growth, which was further reduced by adding cimetidine. Alpha-IFN concen-

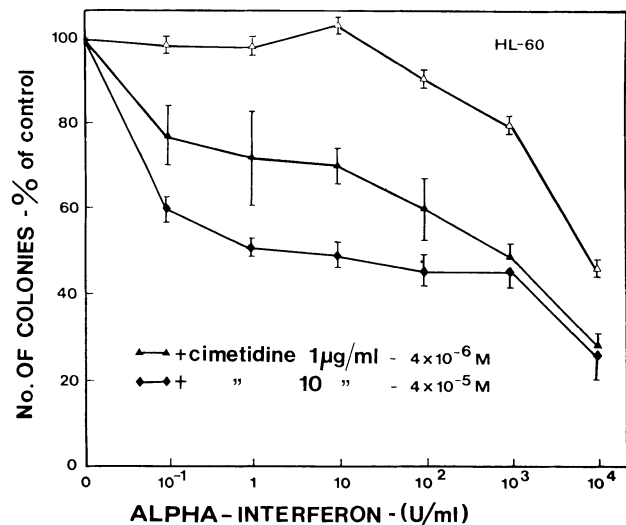
trations of 1–1000 U/ml with or without cimetidine did not affect clonal growth of K562 cells (data not shown).

In contrast to cimetidine, the combination of equimolar concentrations of chlorpheniramine, a histamine H1 receptor antagonist, cultured together with  $\alpha$ -IFN did not inhibit the growth of HL-60 cells compared with  $\alpha$ -IFN alone. The suppressive effect of the combination of  $\alpha$ -IFN and cimetidine on HL-60 clonal growth was neutralized by adding  $10^{-7}$  M histamine to the dishes. Histamine itself had no effect on cells grown with  $\alpha$ -IFN or cimetidine alone.

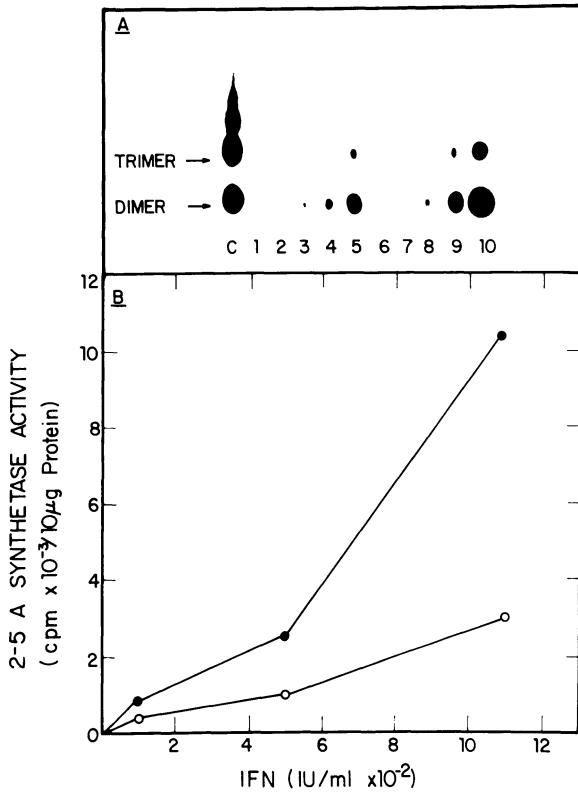
In HL-60 cells,  $\alpha$ -IFN induces a dose-dependent induction of 2-5A synthetase activity. Cimetidine alone had no effect, but it significantly increased the  $\alpha$ -IFN-induced 2-5A synthetase activity (Fig. 2).

Colony-forming unit granulocyte-macrophage growth from CML patients was not significantly affected by cimetidine alone. In three patients  $\alpha$ -IFN alone significantly inhibited CFU-GM growth. Combination of 10 µg/ml cimetidine with different concentrations of  $\alpha$ -IFN decreased CFU-GM growth to values that were significantly lower than would be expected for additive effects of both agents. In one case,  $\alpha$ -IFN alone had no effect on colony growth while combination with cimetidine led to significant inhibition (Table 1).

Alpha-interferon alone inhibited the growth of normal bone marrow CFU-GM and BFU-E colonies. Alpha-IFN in combi-



**Fig. 1.** Effect of  $\alpha$ -IFN on growth of HL-60 cells with or without cimetidine. Results are expressed as percentages of control cultures with medium only



**Fig. 2.** Effect of  $\alpha$ -IFN on 2-5A synthetase activity of HL-60 cells with (●-●) (lanes 6-10) or without (○-○) (lanes 1-5) 10  $\mu$ g/ml cimetidine. Lane C, control cells. Interferon concentrations: lanes 1, 6, none; lanes 2, 7, 1 U/ml; lanes 3, 8, 100 U/ml; lanes 4, 9, 500 U/ml; lanes 5, 10, 1000 U/ml

**Table 1.** Effect of  $\alpha$ -IFN with or without cimetidine on the growth of CFU-GM colonies from four patients with CML

IFN U/ml	Patient 1		Patient 2		Patient 3		Patient 4	
	CFU-GM <sup>a</sup> -/+	$\Delta^b$	CFU-GM -/+	$\Delta$	CFU-GM -/+	$\Delta$	CFU-GM -/+	$\Delta$
0	38/36	- 5%	166/162	- 2%	50/46	- 8%	1308/1350	+ 3%
1	29/7	-75% (S)	154/62	-60% (S)	29/21	-27% (A)	1224/1116	-10%
100	19/8	-57% (S)	100/42	-58% (S)	21/12	-43% (S)	1134/432	-62% (S)
1000	11/5	-54% (S)	ND		16/4	-75% (S)	1080/414	-62% (S)

<sup>a</sup> Mean number of colonies per  $2 \times 10^5$  low-density cells - or +10  $\mu$ g/ml cimetidine

<sup>b</sup> Percentage of difference calculated by:

$$\Delta = \frac{\text{number of CFU-GM with cimetidine} - \text{number of CFUGM without cimetidine}}{\text{number of colonies without cimetidine}}$$

(S), statistical significantly synergistic effect of combined  $\alpha$ -IFN and cimetidine (12); (A) additive effect of combined  $\alpha$ -IFN and cimetidine; ND, not done

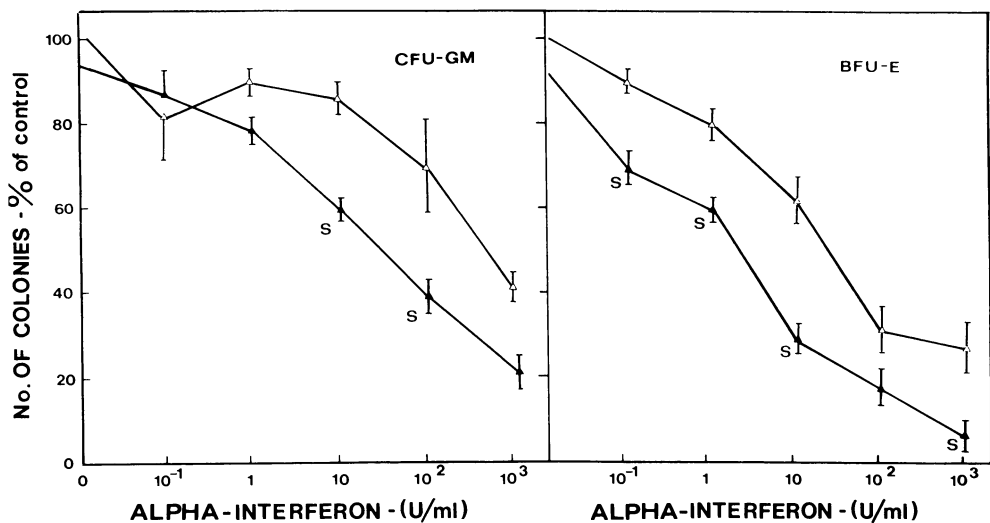


Fig. 3. Effect of  $\alpha$ -IFN on growth of normal CFU-GM and BFU-E with (▲—▲) or without (Δ—Δ) cimetidine. Results expressed as percentages of control cultures with medium only. Cimetidine concentrations: 10  $\mu$ g/ml for CFU-GM and 1  $\mu$ g/ml for BFU-E. (S), statistically significant synergistic effect of  $\alpha$ -IFN and cimetidine at each  $\alpha$ -IFN concentration

nation with cimetidine reduced synergistically the number of BFU-E and CFU-GM colonies as compared with the effect of  $\alpha$ -IFN alone (Fig. 3). CFU-GM colonies were less sensitive than BFU-E colonies to inhibition by  $\alpha$ -IFN with or without cimetidine.

## Discussion

Alpha-IFN alone has a mild inhibitory activity against AML cells, which is evident only at high concentrations. Concentrations of  $\alpha$ -IFN as low as 1 U/ml together with cimetidine inhibited HL-60 cell growth by approximately the same degree as 1000 U/ml  $\alpha$ -IFN alone. Since  $\alpha$ -IFN with cimetidine failed to inhibit the clonogenic growth of the K562 cell line, it is unclear whether this observation can be generalized to other AML cells. However, cimetidine potentiated synergistically the inhibitory activity of  $\alpha$ -IFN against leukemic CFU-GM colonies from CML patients. The effect of cimetidine on  $\alpha$ -IFN inhibition of normal BFU-E and CFU-GM indicates that synergism between the two drugs is not limited to suppression of leukemic cell growth.

The mechanism of this synergistic effect is unclear. The growth inhibition of normal

BFU-E and CFU-GM colonies by cimetidine alone, reported in the past, is probably clinically irrelevant, since the toxic drug levels were 10–100 times higher than therapeutic serum levels. However, Byron showed that lower levels (0.25  $\mu$ g/ml) of cimetidine prevented murine pluripotent hematopoietic stem cells (CFU-S) from entering the cell cycle [13]. Suppression of committed CFU-GM and BFU-E progenitors by IFN would increase the demand for cycling CFU-S cells and failure to respond to this demand in the presence of cimetidine would make hematopoietic cells more susceptible to inhibition by interferon. Cimetidine alone, at the standard therapeutic drug concentration, did not inhibit leukemic cell growth when cultured without  $\alpha$ -IFN. In mouse models cimetidine alone, at therapeutic serum levels, has no direct cytotoxicity *in vitro* though *in vivo* it can elicit an antitumor effect [4–6]. This antitumor effect of cimetidine has therefore been related to enhancement of host immune response, mediated by blocking activation of H2 histamine-bearing suppressor T cells [4, 5]. The anticancer effect of cimetidine combined with  $\alpha$ -IFN was linked to inhibition of an IFN-induced activation of these suppressor T cells [1]. In contrast, our results indicate a

direct antiproliferative effect of cimetidine with  $\alpha$ -IFN on HL-60 cellular growth, unrelated to the presence of lymphocytes. Cimetidine could act by binding to a histamine H2 receptor on the HL-60 cells since the synergism was neutralized by histamine and was not seen with histamine H1 receptor antagonist. Furthermore, the potentiating effect of cimetidine is mediated biochemically by increasing intracellular 2-5A synthetase activity induced by  $\alpha$ -IFN. Resistance to antiproliferative effects of  $\alpha$ -IFN may be related to failure to induce increased activity of intracellular 2-5A [14, 15].

In CML patients the combination of cimetidine and  $\alpha$ -IFN acting against CFU-GM in vivo might overcome the resistance to  $\alpha$ -IFN alone. In AML, in vitro studies suggest that suppression of clonogenic blasts requires toxic concentrations of  $\alpha$ -IFN. Interestingly, a complete remission in a single AML patient following treatment with low-dose  $\alpha$ -IFN together with cimetidine has been described [16]. The results with normal progenitors indicate that the drug combination might increase the hematological toxicity of  $\alpha$ -IFN. It remains to be seen if cimetidine could play a role in overcoming resistance to  $\alpha$ -IFN therapy in leukemia, and increase  $\alpha$ -IFN hematopoietic toxicity.

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## Dependence of Serum Erythropoietin Level on Erythropoiesis in Leukemia\*

W. Jelkmann, H. Johannsen, G. Wiedemann, M. Otte, and T. Wagner

### Introduction

There is some evidence that the blood level of erythropoietin (Epo) in anemia is influenced by the proliferative activity of the erythron independently of the O<sub>2</sub> supply to the tissues. Previously, very high levels of erythropoietin in relation to the degree of anemia have been found in patients with bone marrow aplasia [1–6].

In the present study, the dependence was studied of immunoreactive serum Epo on the blood hemoglobin (Hb) concentration in patients with acute or chronic leukemia. Comparative measurements were carried out in patients with anemia caused by chronic bleeding in association with ulcerative colitis. In addition, the relationship was examined between the concentration of Epo in serum and the erythrocytic activity of the bone marrow of the individual leukemic patients.

### Patients and Assay of Epo

The study encompassed 11 patients with acute myelogenous leukemia (AML) before therapy was started and 4 AML patients following their complete remission. In addition, untreated patients were included with chronic myelogenous leukemia (CML, two

cases), acute lymphoblastic leukemia (ALL, four cases) and chronic lymphatic leukemia (CLL, four cases). Comparative Epo measurements were carried out in 32 patients suffering from ulcerative colitis.

For the assay of Epo, venous blood was sampled without anticoagulant, maintained at 4°C overnight to allow for clot retraction, and then centrifuged at 2000 g for 20 min. The radioimmunoassay of Epo was carried out in duplicate using <sup>125</sup>I-labeled recombinant human Epo (Amersham Buchler, Braunschweig, FRG) and antiserum from a rabbit previously immunized with recombinant human Epo (Cilag, Alsbach-Hähnlein, FRG). Mixtures of 100 µl anti-Epo serum (1:1000, dilution buffer: phosphate-buffered saline, pH 7.4, containing bovine serum albumin 500 mg/liter and sodium azide 500 mg/liter, to which 10% Epo-free human serum was added) were incubated with 100 µl test sample or Epo standard (range 0–150 mU/ml in dilution buffer to which bovine γ-globulin 12 g/liter was added) were incubated at 4°C for 48 h. Human urinary Epo standard was calibrated against the international Epo preparation B [7]. Thereafter, 100 µl labeled Epo (about 5 × 10<sup>-15</sup> mol) was added for a further 24 h incubation at 4°C. Antibody-bound <sup>125</sup>I-Epo was separated from free <sup>125</sup>I-Epo using goat anti-rabbit IgG conjugated to *Staphylococcus aureus* cells (Tachisorb; Calbiochem, Frankfurt, FRG).

For the sake of comparison, serum Epo in the leukemic patients was also determined by bioassay in hypoxia-exposed polycythemic mice as described earlier [8].

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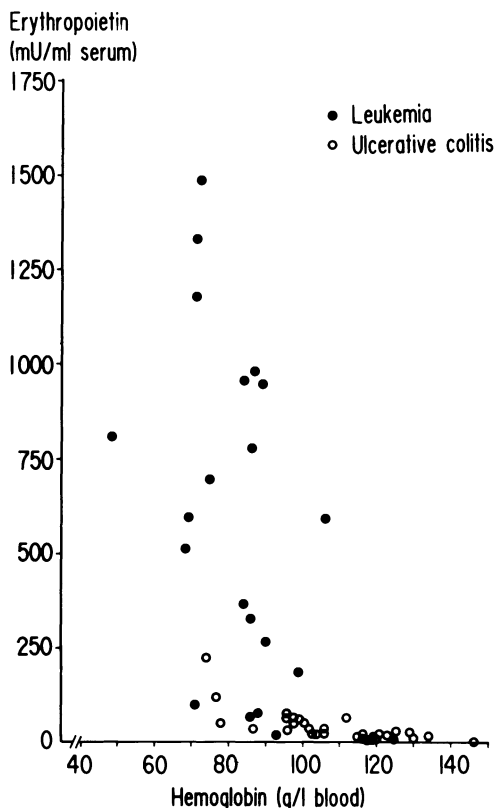


Fig. 1. Immunoreactive erythropoietin in the serum of patients suffering from untreated leukemia (21 cases) or ulcerative colitis (32 cases)

## Results

Figure 1 contrasts the serum level of immunoreactive Epo in patients suffering from either leukemia or ulcerative colitis. Several of the leukemic patients had extremely high Epo levels with regard to their degree of anemia. In Table 1, leukemic patients are identified according to the French-American-British (FAB) Classification [9]. It can be seen that the level of immunoreactive Epo was particularly high in those patients whose erythrocytic bone marrow activity was reduced. The results of additional Epo measurements by bioassay in polycythemic mice indicate that the Epo was fully bioactive.

## Discussion

Similar to the situation in other types of anemia without primary hemopoietic disorder [10, 11], a clear inverse correlation existed in ulcerative colitis between the blood Epo and hemoglobin concentrations. On the other hand, several of the leukemic patients had relatively high Epo levels for their degree of anemia. Bone marrow biopsy showed that the occurrence of erythrocytic precursors was reduced in these patients.

Extremely high Epo levels have been previously found in the blood of anemic patients with bone marrow hypoplasia [1-6]. Laboratory studies have shown that the Epo level is abnormally high in anemic mice with a congenital defect of the hemopoietic stem cells [12, 13]. In addition, a much more pronounced increase in the plasma Epo level was observed in hypoxia-exposed mice with marrow hypoplasia induced by irradiation or by the injection of 5-fluorouracil, when compared with hypoxia-exposed control mice [14]. Taken together, these findings seem to suggest that tissue hypoxia is perhaps not the only relevant factor in the control of the production of Epo in the kidney and the liver. Instead, there appears to be a feedback-inhibiting mechanism which leads to attenuated Epo production when the erythrocytic progenitors proliferate in response to the hormone. Accordingly, relatively low Epo values were seen here and in earlier studies [15] in anemic CML patients, whose erythrocytic progenitor compartment is generally enlarged [16, 17]. Furthermore, our hypothesis is supported by the finding that the Epo levels in AML patients following their complete remission fell in the range of the Epo level in patients with ulcerative colitis.

Finally, our results are of note with a view to the new possibility of treating anemic patients with recombinant human Epo. The application of Epo may prove useful in leukemic patients whose endogenous Epo formation is insufficient. Hence, we conclude from our findings that the measurement of the serum Epo level in the individual leukemic patient is a prerequisite to Epo replacement therapy.



**Table 1.** Blood hemoglobin and erythropoietin level, and bone marrow erythropoiesis in the group of leukemic patients

Case number	Disorder	Age, sex	Hemoglobin (g/liter blood)	Erythropoietin (mU/ml serum)		Erythrocytic precursors % of marrow cells	Marrow cellularity according to CALGB
				RIA	Bioassay		
Acute myelogenous leukemia (FAB M1-6), before treatment							
1	M2	33, F	99	185	167	> 15	++
2	M4	38, F	90	265	482	3	++++
3	M6	23, M	89	949	1022	d.d.	++
4	M4	49, M	87	980	742	n.a.	n.a.
5	M4	74, M	84	960	950	3-4	++ (+)
6	M1	44, M	72	1487	1526	< 3	+++ (+)
7	M4	73, M	71	1332	1470	< 3	+++
8	M5	78, F	71	1180	1163	> 15	++
9	M1	73, F	69	600	447	< 3	++
10	M4	20, F	68	560	660	n.a.	n.a.
11	M6	60, F	48	810	790	d.d.	++++
Acute myelogenous leukemia, complete remission							
1	M1	37, M	110	80	107	> 15	++
2	M1	44, M	89	64	104	> 15	++
3	M1	80, M	87	70	128	> 15	++
4	M4	74, M	84	< 2.5	73	> 15	++
Chronic myelogenous leukemia							
1		77, M	93	15	72	10-13	+++
2		52, M	88	74	151	12-17	+++
Acute lymphoblastic leukemia							
1		4, F	106	595	506	n.a.	n.a.
2		19, M	86	325	460	6-10	++
3		25, F	84	367	390	3- 5	++++
4		26, M	71	99	395	3- 5	++
Chronic lymphatic leukemia							
1		65, F	125	4	48	< 15	++
2		75, M	86	68	43	> 15	++
3		65, M	86	780	1732	1-2	++
4		71, M	75	692	1046	4-5	++

d.d., differentiation difficult; n.a., bone marrow not available; CALGB, Cancer and leukemia, Group B

## Summary

The dependence of the serum erythropoietin (Epo) level on the blood hemoglobin concentration was compared in patients suffering from leukemia and ulcerative colitis. In leukemia, the level of immunoreactive and bioactive Epo was generally much higher than in ulcerative colitis at comparable degrees of anemia. The highest Epo values were found in patients with severe bone marrow insufficiency of erythropoiesis.

These findings support the hypothesis that the plasma level of Epo depends not only on the hemoglobin concentration of the blood but is also influenced by the proliferative activity of the erythron.

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## Transferrin Derivatives with Growth Factor Activities in Acute Myeloblastic Leukemia: An Autocrine/Paracrine Pathway\*

K. H. Pflüger, A. Grüber, M. Welslau, H. Köppler, and K. Havemann

### Introduction

In contrast to conditions in patients with acute myeloblastic leukemia (AML), leukemic blast cells display only limited proliferative capacity when cultured *in vitro*. Apart from well-defined nutrients these cell lines need supplementation with serum providing a great number of specific and unspecific growth factors. In general leukemic cells are excellent models for studying malignant cell growth and differentiation arrest. For the investigation of the effects of single growth or differentiation factors adaptation of leukemic cells to minimal essential and clearly defined culture conditions is essential.

Recently, three human leukemic cell lines deriving from different patients with AML have been established [1]. These cell lines, designated EW2, LG3, and MS6, all exhibit myelomonocytic characteristics and are adapted to serum-free culture conditions. The cells grow permanently in RPMI 1640 medium supplemented with selenium, insulin, and transferrin (SIT). Conditioned media (CM) of these cell lines exhibit growth-stimulating activity when tested on the same and on other leukemic cells. Since all three cell lines synthesize and secrete cal-

citonin-related peptides such as human calcitonin, clacitonin gene-related peptide, and salmon calcitonin [1] and since these calcitonin-related peptides are frequently found in sera of patients with AML and are partly correlated to prognosis [2, 3], we examined CM of these cell lines to clear up the impact of these peptides in growth regulation. However, so far no growth- or differentiation-regulating activity of these ingredients of CM could be found. In this paper we report that growth-stimulating activity of CM of these three cell lines is predominantly represented by transferrin derivatives which are active in an autocrine or paracrine way.

### Methods

#### *Conditioned Media*

Conditioned media were harvested from cell cultures growing in log phase on day 3 or 4 and stored at  $-20^{\circ}\text{C}$ . For production of large volumes of CM the Nunc cell factory (Nunc, Roskilde, Denmark) was used. For some experiments CM were lyophilized after dialysis against distilled water.

#### *Cell Extract*

For the preparation of cell extracts, cells were washed three times with RPMI 1640 medium and sonicated for 30 s in an ice bath by use of a Branson Sonifier Cell Disruptor B 15. After centrifugation at  $20\,000\text{ g}$  for 30 min, the cell extract was stored at  $-20^{\circ}\text{C}$  until use.

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After a starvation period of 72 h in RPMI 1640 medium (Gibco) HL60 and LG3 cells were seeded in 96-well plates (Nunc) at a density of  $10^5$  cells/ml ( $2 \times 10^4$  cells/well). Growth factor activity to be tested and control substances were dissolved in RPMI medium and 20  $\mu$ l was added to each well. After a 24-h incubation period at 37°C in humidified atmosphere of 5% CO<sub>2</sub> in air, cells were pulsed for the next 24 h with 0.1  $\mu$ Ci [<sup>3</sup>H]thymidine delivered in 10  $\mu$ l RPMI medium. Cells were harvested by the use of a semiautomatic cell harvester (Flow Laboratories, Irvine, Scotland). The radio-labeled DNA was fixed to glass-fiber filters, samples were transferred to a liquid scintillation cocktail (Optifluor, Packard Instruments, Illinois, United States) and counted in a counter for liquid scintillation (LKB, Bromma, Sweden) with a counting efficiency of 60% for tritium. Colony formation was assayed in the soft agar cloning assay as described earlier [1].

#### *Gel Filtration Chromatography*

A quantity of 120 ml CM was placed in a Sephadex G 25 column (5  $\times$  50 cm, flow 280 ml/h), equilibrated with distilled water. Elution fractions were lyophilized and resuspended in phosphate-buffered saline (PBS) or RPMI medium. Proliferation activity was determined as described above and Transferrin (Tf) content was measured by the radial immunodiffusion assay of Tf on LC-Partigen-Transferrin plates.

#### *Fast Protein Liquid Chromatography (FPLC) on Superose 12*

Growth-active fractions of G25 chromatography were pooled and applied to a Superose 12 prep grade column (Pharmacia, gel volume 125 ml, flow 1 ml/min) equilibrated with 20 mM sodium phosphate buffer, pH 7.4

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the Laemmli method [4] on an LKB protein gel electrophoresis system using a 12% polyacrylamide gel under reducing conditions. Gels were fixed in 7% acetic acid and were incubated in Amplify for 15 min before drying.

#### *Western Blot*

After electrophoresis the separated proteins on the gel were blotted onto "Immobilon PVDF" Transfer Membrane (Millipore Corporation, Bedford, MA) using the Transfer Electroblothing Unit LKB 2005 (LKB, Bromma, Sweden) in a Tris-glycine buffer containing 20% methanol, pH 8.3. Following transfer, nonspecific binding sites on the membrane were blocked with bovine serum albumin (BSA) (3%). Immunostaining was carried out with an antihuman Tf antibody from rabbit as the primary antibody and a biotinylated second antibody (anti-rabbit Ig, biotinylated from donkey). Biotinylated horseradish peroxidase was linked to the biotinylated second antibody by a streptavidin bridge to obtain a stronger signal. Immunoreactive Tf was developed by incubation with the enzyme substrate 4-chloro-1-naphthol and H<sub>2</sub>O<sub>2</sub>.

#### *Radioimmunoprecipitation Assay*

Cells were washed twice with minimum essential medium (MEM) Eagle's medium (Gibco) without methionine and incubated in the same medium with a density of  $2.5 \times 10^7$  cells/ml for 2 h at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. Cells were pulsed for 4 h with 100  $\mu$ Ci/ml [<sup>35</sup>S]methionine. Tf production was determined by the reaction of cell supernatant and cell lysates with a sheep transferrin antiserum at time 0, after 15, 30, 60, and 240 min. The immune complex was precipitated after 24 h with a donkey anti-sheep antibody. The precipitates were dissolved in SDS sample buffer [4] with mercaptoethanol, and after an incubation time of 5 min at 95°C they

were separated by SDS PAGE for 6 h in a 12% gel at 150 V. After drying on a gel dryer (Pharmacia) the gels were exposed to an X-ray film for 48 h at  $-70^{\circ}\text{C}$ .

## Results

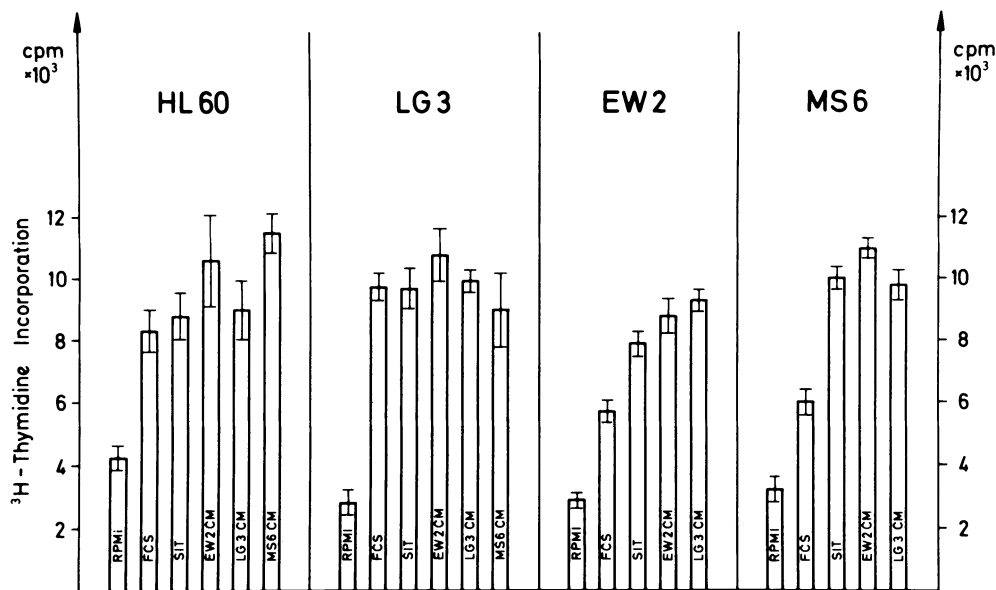
### *Growth Stimulation of Leukemic Cells by CM*

The stimulatory effects of CM of the cell lines on the growth of leukemic cells were examined. As shown in Fig. 1, CM of the cell lines LG3, EW2, and MS6 exhibit a proliferative activity on various leukemic cells comparable to the effects of fetal calf serum (FCS) and superior to those of SIT.

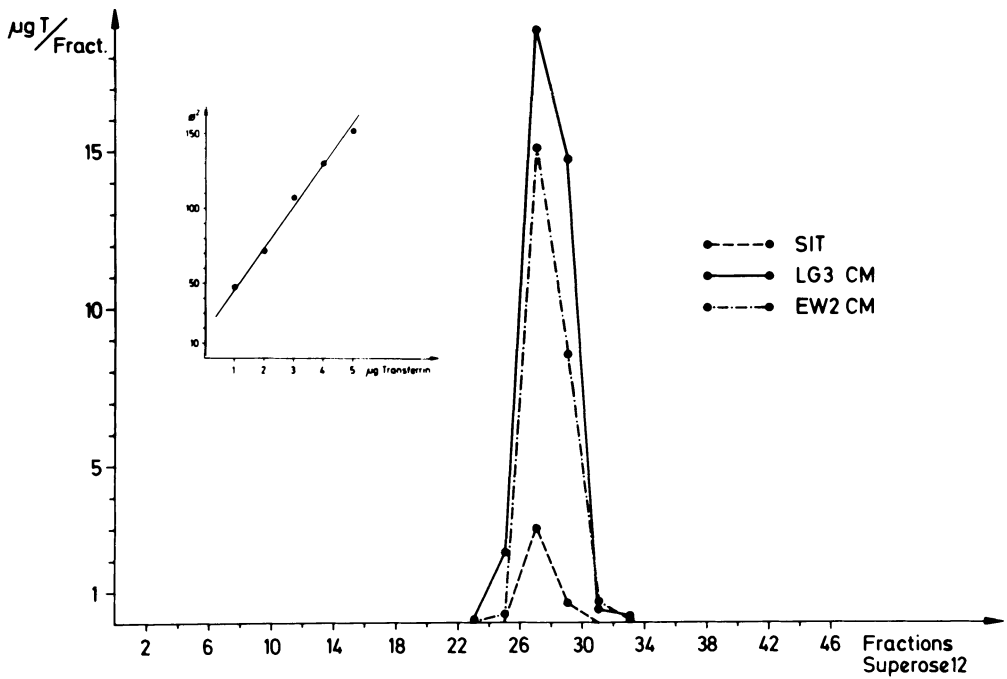
### *Isolation and Characterization of the Growth-Promoting-Activity*

Conditioned media of the cell lines LG3, EW2, and MS6 grown under serum-free conditions were desalted on a Sephadex G25 column. The eluates were tested for immunoreactive Tf and growth-stimulating ac-

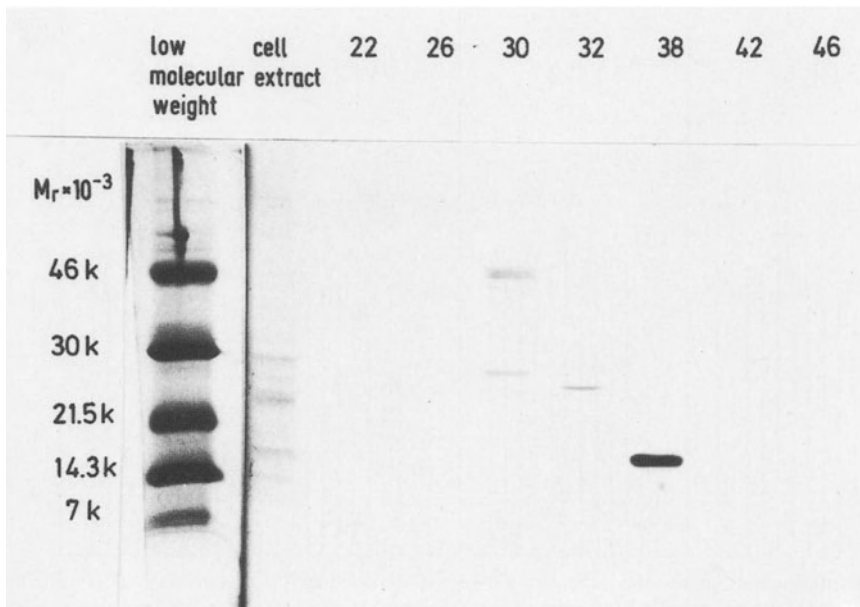
tivities. Several peaks of growth activity could be detected. The main peak coincided with the main Tf peak. After rechromatography of this fraction on Superose 12, again growth-promoting activity was present in the Tf fraction. As illustrated in Fig. 2 quantitative evaluation of Tf in CM and the original SIT medium indicated that immunoreactive Tf was accumulated in CM. After separation of an EW2 cell extract on Superose 12 four different fractions with growth-stimulating activities measured by soft agar colony assay could be identified. Further characterization of these activities is shown in Fig. 3. After SDS-PAGE of single elution fractions a western-blot analysis using a polyclonal human Tf-antibody was performed. As illustrated in Fig. 3 several immunoreactive Tfs could be detected. Apart from small amounts of normal Tf (82 kD), different low molecular weight forms were present. Growth-promoting activity was found in the fractions No. 18, 30, and 38, indicating that in addition to normal Tf two immunoreactive Tfs, Tf 45 kD and Tf 15 kD, may represent Tf-related growth factors.



**Fig. 1.**  $^3\text{H}$ Thymidine uptake into the indicated cell lines. Cells were seeded at a density of  $10^5$  cells/ml in 96-well plates ( $2 \times 10^4$  cells/well); 20- $\mu\text{l}$  control solutions or CM of the various cell lines were added to each well. After an incubation time of 24 h cells were pulsed with  $0.1 \mu\text{Ci}$   $^3\text{H}$ thymidine/well for the next 24 h. Radiolabeled DNA was measured in a  $\beta$ -scintillation counter after fixation to glass fiber filters



**Fig. 2.** Comparison of the eluted Tf content measured by the radical immunodiffusion method of CM of EW-2 ····· and LG-3 ——— with SIT medium ---- after rechromatography of the Tf peak of G25 separation on FPLC Superose 12. Insert shows the Tf standard curve measured by radial immunodiffusion



**Fig. 3.** Immunostaining with antihuman Tf-antibody from rabbit after protein blotting on transfer membrane (Western blot) of SDS PAGE in 12% polyacrylamide under reducing conditions. The *single lanes* represent indicated elution fractions of the FPLC separation of EW-2 cell extract

**Fig. 4.** Autoradiography of separated [<sup>35</sup>S]methionine-labeled Tf-antitransferrin immunocomplexes by SDS-PAGE under reducing conditions. Lanes 1, 5, and 9 represent <sup>14</sup>C-labeled molecular weight standards. The remaining lanes show LG3, HL60, and EW2 cell supernatants and cell extracts as indicated after a pulse of 4 h

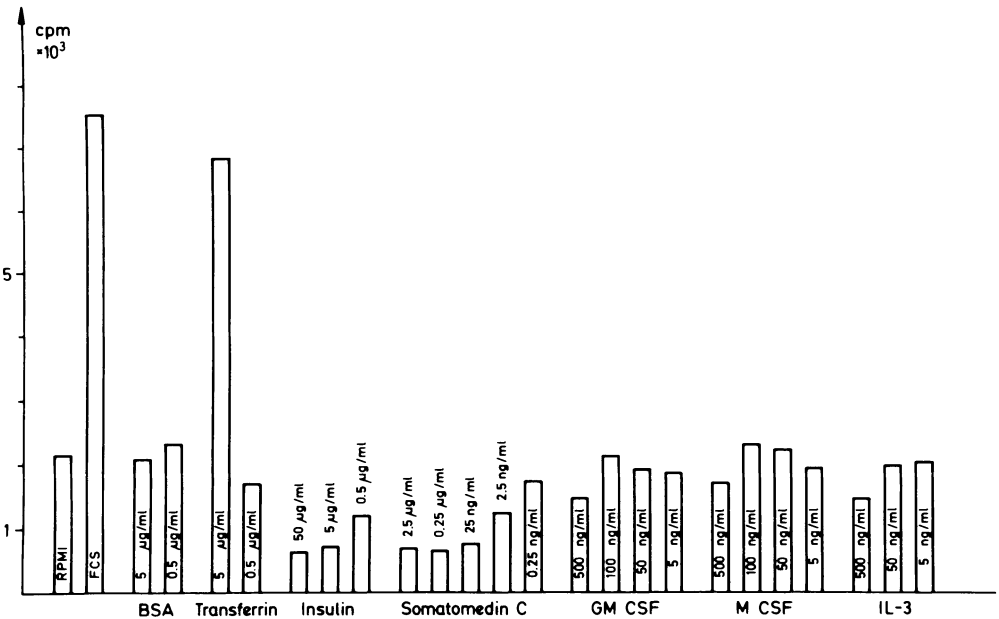


*Demonstration of Transferrin Production by the Leukemic Cells*

Radioimmunoprecipitation assay (RIP) after [<sup>35</sup>S]methionine short-term culture was performed to prove suggested synthesis of these immunoreactive Tfs by the leukemic cells. As shown in Fig. 4 after SDS-PAGE of the immunoprecipitate different fractions of newly synthesized immunoreactive Tfs are present. Again a predominant fraction is located in the range of 45 kD.

*Effects of Different Growth Factors on the Proliferation of Leukemic Cells*

In order to determine the growth-stimulating activity on the leukemic cell lines a panel of peptide hormones and growth factors were tested in different concentrations using [<sup>3</sup>H]thymidine incorporation in HL60 and LG3 cells. As shown in Fig. 5, only Tf exhibited a significant growth-stimulating activity comparable to SIT medium. Insulin and somatomedin C, as single substances, showed



**Fig. 5.** [<sup>3</sup>H]Thymidine uptake into HL-60 cells after stimulation with various growth factors. The technical details are given in Fig. 1

an inhibitory activity, whereas the other factors had no effect.

*Influence of Antibodies Raised Against Tf and Tf-Receptor on Cell Proliferation*

Several antibodies raised against pure Tf and Tf-receptor were used in growth inhibition experiments. As shown in Fig. 6, Tf-an-

tiserum specifically inhibited cell growth and its effect was dose dependently antagonized by the addition of pure Tf and CM. Antihuman-IgG antibodies had no effect, whereas antihuman antiserum showed some inhibition in higher concentrations, probably indicating the presence of Tf-specific antibodies. Even baseline growth activity of pure RPMI 1640 medium was inhibited by the addition of Tf-specific antibodies.

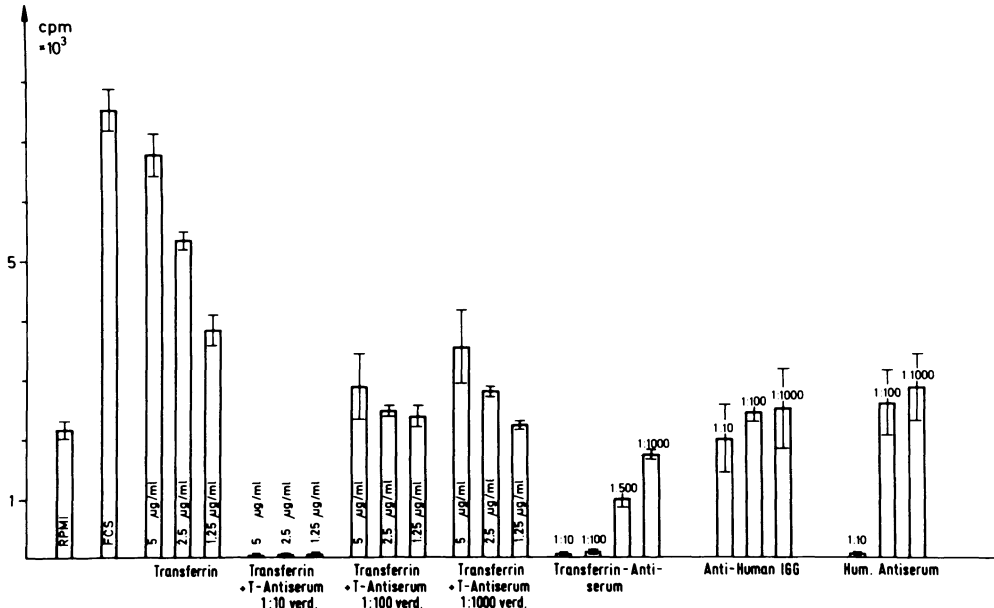


Fig. 6. Inhibition of growth-stimulating activities by Tf antiserum, anti-human IgG, and human antiserum on HL60 cells. Technical details are given in Fig. 1

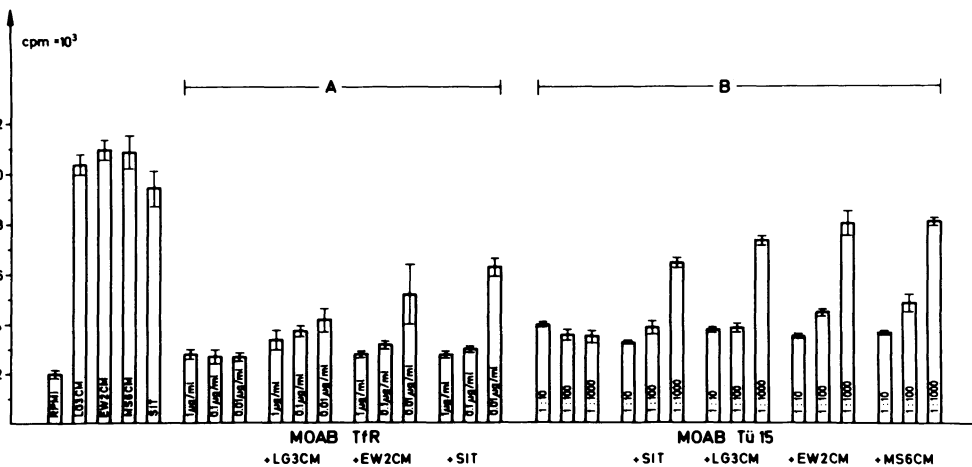


Fig. 7. Inhibitory effect of two monoclonal Tf-receptor antibodies on the growth-stimulating activities of the CM of LG3, EW2, and SIT tested on HL60 cells



The effects of two monoclonal Tf-receptor-specific antibodies are demonstrated in Fig. 7. The results are similar to those seen with the Tf-antibodies. Both antibodies inhibited the growth activity of CM of all three cell lines and were also antagonized by Tf. In contrast to the Tf-specific antibodies, a baseline inhibition was not observed.

## Discussion

A convincing growth stimulation of leukemic cell lines by a number of different factors such as colony-stimulating factor (CSF) [5, 6], insulin and IGF I [7], Tf [8], and unpurified activities [9, 10] have already been described. Serum-free CM of the three cell lines EW2, LG3, and MS6 exhibit a growth-promoting activity for different leukemic cell lines which is comparable to that of FCS and superior to that of the original SIT medium (Fig. 1).

Chromatographic separation of CM demonstrated that the produced growth-stimulating activity is coeluted with Tf. Measurement of the Tf-content of corresponding fractions, of original SIT, and of CM leads us to suggest that Tf is produced and secreted by the leukemic cells (Fig. 2). Further experiments clearly demonstrate that the immunoreactive Tfs are paraneoplastically produced by the leukemic cells. With radioimmunoprecipitation assay [<sup>35</sup>S]methionine is shown to be incorporated in immunoreactive Tf. Apart from Tf of normal molecular weight (>80 kd), several fractions in the range 15–80 kd could be demonstrated. The main amount of newly produced immunoreactive Tf exhibited an apparent molecular weight of 45 kd (Fig. 4). Gel-chromatographic separation of EW2 cell extract also demonstrates growth-stimulating activity in the low molecular weight range (15 kd). After electrophoretic separation of single fractions and western-blotting immunoreactive Tfs of low molecular weight could be seen. A distinct protein band reacting with Tf-antibody exhibited high specific growth-stimulating activity (Fig. 3). This finding demonstrates that low molecular weight immunoreactive Tfs are still biologically active. Antibodies raised against human Tf and Tf-receptor are able

to inhibit proliferation activity in a dose-dependent way (Figs. 6, 7). This effect was competitively compensated for by the addition of increasing amounts of Tf and CM. Whereas Tf-receptor antibodies only inhibit growth at the level of baseline proliferation, the Tf-antibody completely inhibits [<sup>3</sup>H]thymidine incorporation. Thus, it is apparent that the Tf-antibody on the one hand is able to recognize different Tfs and, on the other hand, is able to inhibit proliferative activity completely.

The present study indicates a production of Tfs with different molecular weights by leukemic cell lines. These Tf molecules exhibit growth-stimulating activity and thus act in an autocrine manner. Diamond et al. [11] reported a high grade of homology of the amino acid sequence between Tf-derivatives and the *B-Lym-1* oncogene product. This observation would support the suggestion that Tf might act as an oncogene product, genes which are known to be closely linked to cell proliferation. Further studies are in progress in order to purify and characterize the low molecular weight fractions of immunoreactive Tfs and to clarify the mechanisms of action in these Tfs and Tf-antibodies.

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## In Vitro Effects of G-CSF, GM-CSF, and IL-3 on Leukemic Cells of Children with Acute Nonlymphoblastic Leukemia

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### Introduction

Hematopoietic growth factors (G-CSF, GM-CSF, and IL-3) are potent stimulators of proliferation and differentiation of normal myelopoiesis in vitro and in vivo [1]. To study the effects of G-CSF, GM-CSF, and IL-3 on proliferation of leukemic cells of children with acute nonlymphoblastic leukemia (ANLL), we investigated peripheral blood (PB) and bone marrow (BM) cells of 20 children with ANLL. Furthermore, we studied production of G-CSF by leukemic cells.

### Patients and Methods

We studied 20 children (0–16.5 years old) with ANLL. As illustrated in Table 1, they were diagnosed as M1 (three cases), M1/2 (one case), M2 (four cases), M4 (three cases), M2 (three cases), M7 (two cases), and acute undifferentiated leukemia (AUL) (four cases).

Low-density mononuclear cells from peripheral blood or bone marrow obtained before initiation of therapy were isolated by Ficoll-Hypaque. T cells were depleted with AET-(2-aminoethyl)isothiuronium bromide

**Table 1.** Patient characteristics

ANLL #	FAB	Age	Sex	Blasts (%)	
				PB	BM
1	M1	16 2/12	f	39	80
2	M1	5 1/12	f	70	94
3	M1	12 11/12	f	92	90
4	M1/M2	13 9/12	m	92	97
5	M2	5 6/12	f	64	31
6	M2	2 0/12	f	56	96
7	M2	11 8/12	m	47	85
8	M2	5 7/12	m	87	95
9	M4eo	15 8/12	m	22	70
10	M4	4 4/12	f	61	78
11	M4	6 0/12	f	78	90
12	M5	10 5/12	f	06	93
13	M5	11 4/12	m	80	50
14	M5	16 6/12	f	63	78
15	M7	Congenital	m	60	–
16	M7	0 8/12	m	19	52
17	AUL	0 1/12	f	99	–
18	AUL	8 2/12	m	48	–
19	AUL	14 4/12	m	20	73
20	AUL	13 9/12	m	94	96

hydrobromide-treated sheep red blood cells and monocytes were separated by plastic adherence.

In suspension cultures nonadherent and T-cell-depleted cells were grown in RPMI medium [20% fetal calf serum (FCS)] at a concentration of  $1 \times 10^6$  cells/ml in the presence of rh G-CSF, rh GM-CSF, or rh IL-3 ( $1 \times 10^3$  units/ml). Complete medium served as control. Survival of the cultures was defined as more than 10% viable cells as measured by trypan blue exclusion.

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In clonogenic assays nonadherent and T-cell-depleted cells were cultured at a concentration of  $1 \times 10^5$  cells/plate (except for ANLL # 4 at  $2.5 \times 10^4$  cells/plate) in McCoy's medium and 15% FCS in 0.3% agar [2, 3]. Factors (rh G-CSF, rh GM-CSF, rh IL-3) were added at a concentration of  $1 \times 10^3$  units/ml. Complete medium was used as control. At day 14 colonies greater than 20 cells were counted with the inverted microscope. Autonomous growth was defined as more than 20 colonies/ $1 \times 10^5$  cells.

The murine cell line NFS-60 was used to screen for production of G-CSF. Media conditioned by the nonadherent and T-cell-depleted cells – in the absence or presence of rh GM-CSF or rh IL-3 – were prepared. These supernatants were collected after 48 h of suspension culture and assayed at a concentration of 5% or 10%. After 48 h of culture in 96-well plates, [ $^3\text{H}$ ]thymidine (0.5  $\mu\text{Ci}/\text{well}$ ) was added for 4 h and [ $^3\text{H}$ ]thymidine uptake was measured. Positive was defined as a stimulation index of greater than 5.

### Survival of ANLL Cells in Suspension Culture

Cells of 20 children with ANLL were viable in suspension culture for several weeks. While for the majority of samples survival was about 3–4 weeks, some samples grew up to 21 weeks. In most cultures survival could be improved by the addition of G-

CSF, GM-CSF, or IL-3. The median survival (and range) was in the presence of G-CSF 3.5 (2–15) weeks, GM-CSF 4.0 (2–21) weeks, IL-3 4.0 (2–10) weeks, and in the absence of any factor 3.0 (2–10) weeks.

### Proliferation of ANLL Cells in Clonogenic Assays

Table 2 indicates the number of colonies growing in agar assay for each ANLL sample. Cells from 5/17 children with ANLL demonstrated definite autonomous proliferation. Proliferation was improved by the addition of G-CSF (5/17 cases), GM-CSF (10/17 cases), or IL-3 (6/17 cases). Inhibition of clonogenic growth by additional growth factors was noted in one experiment (ANLL # 11). Cells from three children with ANLL demonstrated no growth in the presence or absence of growth factors.

### Production of G-CSF by ANLL Cells

Production of G-CSF by cells of children with ANLL as measured by proliferation of the murine cell line NFS-60 could be demonstrated in 11/18 cases in the absence of growth factors (ANLL # 4, 5, 6, 7, 8, 9, 11, 15, 16, 17, and 20). In three samples production of G-CSF was only noted after GM-CSF or IL-3 stimulation of ANLL cells (ANLL # 1, 3, and 10). These findings were confirmed by Western blot analysis of the

**Table 2.** Number of colonies in agar at day 14

ANLL # stimulus	1	2	3	4	5	6	9	11	
None	18	–	–	5	194	8	12	195	
G-CSF	87	–	19	475	220	7	10	98	
GM-CSF	141	–	20	602	593	32	26	21	
IL-3	149	–	6	353	503	252	12	36	
ANLL # stimulus	12	13	14	15	16	17	18	19	20
None	1	93	49	–	1	44	3	–	–
G-CSF	3	88	30	4	5	38	35	–	–
GM-CSF	8	144	75	1	23	95	30	–	–
IL-3	3	115	45	1	109	28	25	–	–

supernatants using an anti-G-CSF monoclonal antibody.

### Conclusion

In suspension culture cells of 20 children with ANLL survived up to 10 weeks without addition of hematopoietic growth factors. In a clonogenic assay autonomous colony formation of ANLL cells could be observed. Additionally, we demonstrated that the hematopoietic growth factors G-CSF, GM-CSF, and IL-3 could influence the proliferation of cells of children with ANLL. Studies of adult patients with ANLL showed that G-CSF, GM-CSF, and IL-3 promoted self-renewal of leukemic cells [4, 5]. These studies and our findings suggest considerable similarities in the responses to G-CSF, GM-CSF, and IL-3 of leukemic cells of children and adults with ANLL. Cells of adults with ANLL proliferated autonomously, and they expressed CSF mRNA [6]. We started to investigate whether ANLL cells of children are capable of producing their own growth factors and studied production of G-CSF by these cells. According to bioassays and Western blot analysis production of G-CSF by ANLL cells was evident for the majority of cases.

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## In Vitro Growth Kinetics of Myeloid Progenitor Cells of Myelodysplastic Patients in Response to Granulocyte-Macrophage Colony-Stimulating Factor and Interleukin-3

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### Introduction

The myelodysplastic syndrome (MDS) is a clonal disorder of the hemopoietic stem cell resulting in multilineage cytopenias *in vivo*. *In vitro* a reduced myeloid and erythroid colony formation is observed [1, 2]. The reduced colony formation may be due to functional abnormalities of the progenitor cells such as an altered response to hemopoietic growth factors. Current investigations suggest that stimulation with certain colony-stimulating factors is only effective in early G<sup>1</sup> [3]. This would imply that variations in the resting time of progenitor cells would influence the responsiveness of these cells for hemopoietic growth factors. In order to obtain more insight into this problem, we determined the colony formation kinetics of myeloid progenitor cells of MDS patients in response to rhGM-CSF, rhIL-3, and GCT-CM.

### Materials and Methods

*Normal Donors.* Normal marrow samples were obtained from six healthy volunteers.

*Patients.* Thirteen patients with MDS and five patients with leukemic transformation of MDS (LT-MDS) were studied. Relevant clinical and hematological data are given in Table 1. The MDS cases were classified

according to the French-American-British (FAB) nomenclature [4].

*Bone Marrow Cells.* Bone marrow cells of patients and normal volunteer were aspirated from the posterior iliac spine. Aspirates were collected in glass tubes containing preservative-free heparin. Light-density, T-cell-depleted, nonadherent bone marrow cells were obtained as described previously [5].

*Colony Assay.* Assays were performed in the  $\alpha$ -modification of Dulbecco's modified Eagle's minimum essential medium ( $\alpha$ -DMEM) containing 0.9% methylcellulose, and supplemented with 20% fetal calf serum (FCS), 1% dialyzed bovine serum albumin (BSA), 30  $\mu$ M egg lecithine, 0.1  $\mu$ M sodium selenite, 7.7  $\mu$ M fully iron saturated human transferrin, and 100  $\mu$ M mercaptoethanol. Cultures were performed in 24-well plates (Costar), 250  $\mu$ l/well containing  $2.5 \times 10^4$  bone marrow cells. The culture plates were incubated in a fully humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C. Colonies of more than 50 cells were counted on days 7, 10, 14, 18, and 22. In some cases colonies were plucked from the well for morphological examination.

*Hemopoietic Growth Factors.* Giant-cell-tumor-conditioned medium (GCT-CM) was prepared by culturing the confluent growing cell-line for 7 days with  $\alpha$ -DMEM containing 10% FCS and was used as a source of colony-stimulating activity in the colony assay at a concentration of 10%. RhGM-CSF was prepared and generously made available by Schering (Kenilworth, New Jersey,

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**Table 1.** Clinical, hematological, and cytogenetic data of the MDS patients studied

Risk group	Patient/ No.	Sex	Age (years)	FAB	% blasts	Cytogenetics
Low risk	1	M	17	RA	2.6	N
	2	M	73	CMML	0.4	N
	3	M	71	RARS	0.4	N/hypodiploid (17%)
	4	M	60	CMML	1.2	N/t(1;19) (12.5%)
	5	M	73	RARS	0.6	n. d.
	6	M	77	RAEB	7.6	46XY, del(II) (q21 q24)
	7	M	67	RA	1.5	N
High risk	8	M	17	RAEB	15.6	N
	9	M	83	RAEBt	20.0	N
	10	F	38	LT-MDS	40.0	N/+8 (4%)
	11	M	40	LT-MDS	34.2	N/-yt(8;21) (20%)
	12	F	75	LT-MDS	30.4	N/complex (58%)
	13	F	78	RA	4.2	N
	14	M	70	RAEB	8.6	N
	15	F	17	RAEB	7.2	N
	16	M	55	LT-MDS	77.0	n. d.
	17	M	66	LT-MDS	82.4	N
	18	F	53	RAEB	16.4	N/5q-, +21 (78%)

N; normal karyotype; n. d. not determined

United States) and was used at a concentration of 10 ng/ml, which was a saturating concentration in a CFU-GM assay. RhII-3 was obtained from RBI-TNO (Rijswijk, Holland) as a generous gift from Dr. Wage-maker and was used at a concentration of 10 ng/ml.

**Cytogenetics.** Cytogenetic analyses of patients' bone marrow cells were performed using a standard technique [6], as part of the diagnostic investigation. Chromosomes were always identified by banding (R-, Q-, G-bands). The karyotypes were reported according to the International System for Human Cytogenetic Nomenclature [7].

## Results

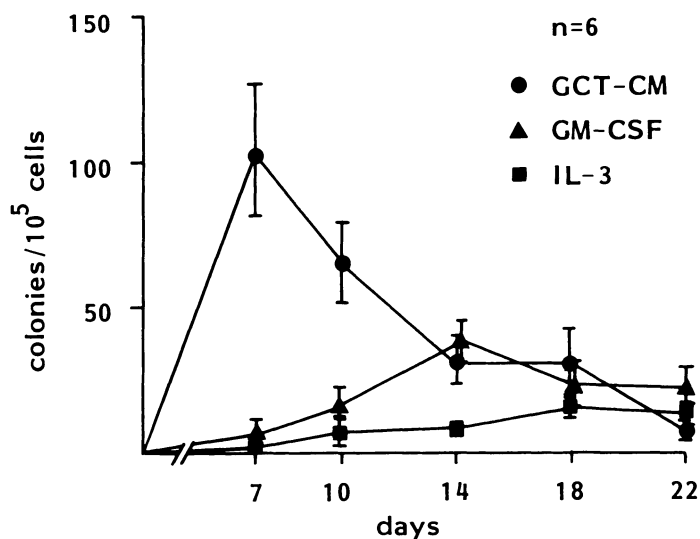
### *Classification of Patient Groups*

On the basis of clinical and hematological data patients were divided into two groups. Criteria for group 1 were: the presence of more than 10% bone marrow blast cells, complex cytogenetic abnormalities, or transformation to leukemia during the

observation time; and for group 2: bone marrow blast count below 10% and stable clinical course during the observation time. Group 1 is called the high-risk and group 2 the low-risk group. Eleven patients met the criteria for the high-risk group and seven the criteria for the low-risk group (Table 1).

### *Colony Formation Kinetics of Normal Bone Marrow Cells*

The growth of myeloid progenitors in response to GCT-CM, rhGM-CSF, and rhII-3 is shown in Fig. 1. GCT-CM supported maximally day 7 colonies (GM-CFC/10<sup>5</sup> cells: 114.9 ± 22.3 SEM) with a rapid decline in colony numbers after day 7. RhGM-CSF stimulated maximally day 14 colonies (39.5 ± 6.8). RhII-3 stimulated only a few myeloid colony-forming cells with a maximum on day 18 (16.3 ± 5.6). Colonies formed in response to GCT-CM consisted predominantly of granulocytic cells, whereas the progeny of GM-CSF and II-3-stimulated colony-forming cells consisted of granulocytes and monocytes/macrophages.



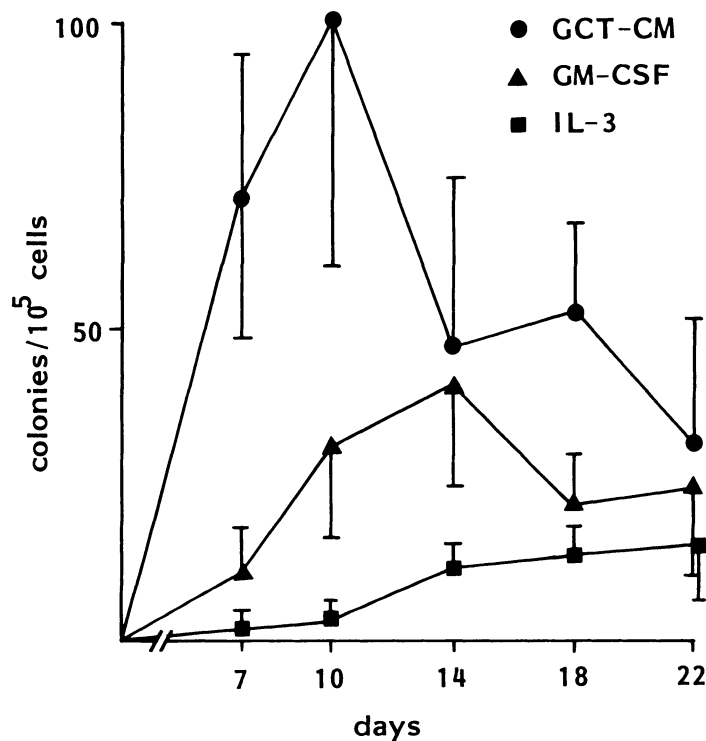
**Fig. 1.** Colony formation kinetics of normal bone marrow. Each point represents the mean colony number of six normal bone marrow cultures  $\pm$  standard error of the mean (SEM). Each culture was performed in triplicate

*Colony Formation of MDS Bone Marrow Cells*

Colony formation in the low-risk group of patients resembles that of normal bone marrow cells (Fig. 2). Maximal colony numbers in response to GCT-CM, however, were

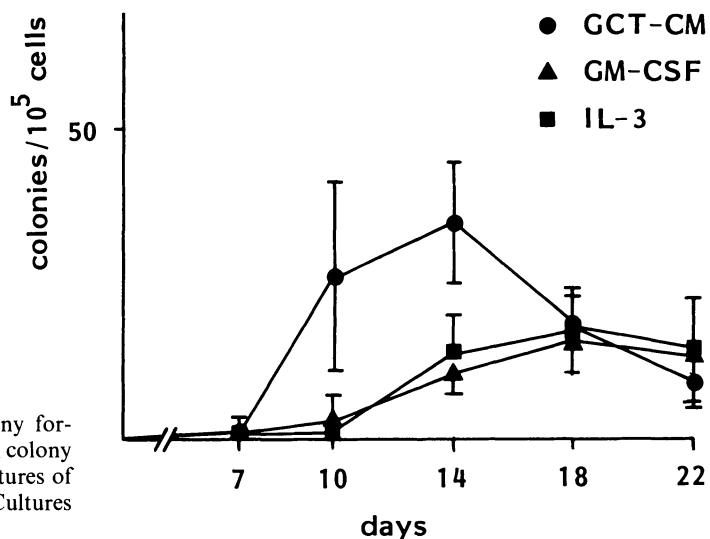
found on day 10:  $100.8 \pm 24.9$  instead of on day 7 ( $73.0 \pm 40$ ). The relative rise in colony numbers from day 7 to day 10 was found to be highly significant (Wilcoxon  $P < 0.01$ ).

The response to rhGM-CSF and rhIL-3 resembles that of NBM cells, with maximum colony numbers on day 14:  $40.5 \pm 15.5$  and



**Fig. 2.** Low-risk MDS: colony formation kinetics. Mean  $\pm$  SEM colony numbers of bone marrow cultures of seven low-risk MDS patients. Cultures were performed in triplicate



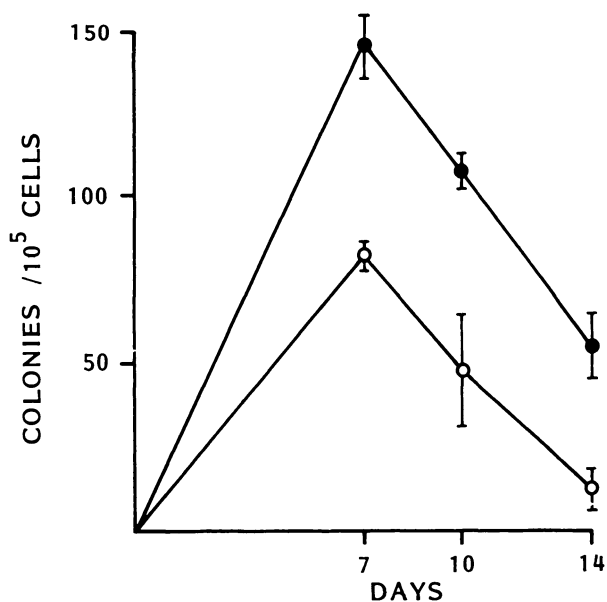


**Fig. 3.** High-risk MDS: colony formation kinetics. Mean  $\pm$  SEM colony numbers of bone marrow cultures of 11 high-risk MDS patients. Cultures were performed in triplicate

$13.3 \pm 6.6$  colonies, respectively. In the high-risk group, colony formation kinetics are clearly altered (Fig. 3). Maximum colony formation stimulated by GCT-CM is shifted from day 7 in NBM to day 14. The mean maximum colony number in response to GCT-CM is decreased to  $37.8 \pm 9.4$ . In contrast to the low-risk group the response pattern to rhGM-CSF and Il-3 is changed as well. Optimum colony numbers for rhGM-CSF as well as for Il-3 are found on day 18 (GM-CSF:  $17.3 \pm 3.9$ , Il-3:  $16.8 \pm 8.6$  CFC/ $10^5$  cells).

*Colony Formation Kinetics in Response to GCT-CM in Comparison with rhG-CSF*

Figure 4 shows the colony formation kinetics of NBM cells in response to GCT-CM and rh-G-CSF. Both stimuli result in identical colony formation kinetics with a maximum on day 7. Rh-G-CSF, in saturating concentrations, however, stimulates only 56.8% of the day 7 CFCs as compared with GCT-CM (84.0 and 148.0 CFCs/ $10^5$  cells, respectively).



**Fig. 4.** Colony formation kinetics in response to GCT-CM and rhG-CSF (100 U/ml). Each point represents the mean  $\pm$  SEM of triplicate cultures of normal bone marrow cells. Closed symbols, GCT-CM; open symbols, rhG-CSF

## Discussion

The present study has characterized the growth kinetics of myeloid progenitor cells from MDS patient bone marrow cells in response to GCT-CM, rhGM-CSF, and rhIL-3. The MDS patients were divided into a high- and low-risk group on the basis of clinical, hematological, and cytogenetic data. The low-risk MDS group is comparable to NBM in respect of the maximum numbers of colonies formed in response to the various stimuli. The time point on which this maximum is reached in response to GCT-CM, however, is shifted from day 7 to day 10. With rhGM-CSF and rhIL-3 as stimuli, the growth kinetics in this group are apparently the same as observed in NBM cultures.

The change in growth kinetics of the high-risk MDS group is more striking. Maximum colony numbers are decreased to at least 50% in cultures stimulated with GCT-CM and rhGM-CSF. Peak values of colony numbers are reached only on day 14 with GCT-CM and day 18 with rhGM-CSF. This means a delay of 7 and 4 days respectively as compared with NBM.

The data indicate that GCT-CM stimulates predominantly late progenitors, giving rise to day 7 colonies in NBM. The primarily granulocytic morphology of the colony cells suggests that the GCT-CM produced in our laboratory contains G-CSF and no effective concentrations of M-CSF or GM-CSF. Moreover, the growth kinetics of GCT-CM and rhG-CSF are identical (Fig. 4). Apart from granulocytic colony-stimulating activity, GCT-CM appears to contain additional factors distinct from M-CSF and GM-CSF since it stimulates almost twice as many CFCs to form colonies as rhG-CSF does.

Early progenitor cells are considered to be dormant in the cell cycle (i.e., in  $G^0$  phase), whereas the more mature progenitor cells are in active cycle [8, 9]. Therefore the pool of early progenitors gives rise to colonies with a greater latency than late progenitor cells. This would imply that our results may be explained by a relative left shift in the progenitor cell compartment in MDS. How-

ever, the shift to the left did not result in an enlargement of the earlier compartments. In contrast, the number of day 14 colonies is decreased in the high-risk MDS group. An increment of late progenitor cells in  $G^0$  or an increased cycling time may be alternative explanations for the observed delay in colony formation and may be caused by a decreased sensitivity for hemopoietic growth factors.

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## Lymphokine-Activated Killer (LAK) Cells Against Human Leukemia: Augmentation of LAK-Cell Cytotoxicity by Combinations of Lymphokines or Cytokines\*

J. V. Teichmann, W. D. Ludwig, H. Seibt-Jung, and E. Thiel

### Introduction

Adoptive immunotherapy with lymphokine-activated killer (LAK) cells and interleukin-2 (IL-2) or induction of cytotoxic mechanisms by IL-2 alone was shown to be a promising approach in cancer therapy [1–11]. Most of the available data come from experimental and clinical studies of solid tumors, while only little is known about the effect of LAK cells against human leukemia [12–14]. Leukemia patients have been reported to have a deficiency in natural killer (NK) cell functions which may contribute to leukemogenesis. Experimental data on correcting this deficiency by IL-2 make it possible that adoptive immunotherapy of leukemia patients may be of great value in the treatment of human leukemia [15, 16]. In the present study we investigated the ability of LAK cells to lyse fresh human leukemia cells *in vitro* and evaluated the augmentation of cytotoxic mechanisms by combined application of IL-2 and other lymphokines or cytokines and by inhibition of prostaglandin synthesis during the activation process.

### Materials and Methods

**Lymphokines.** Recombinant IL-2 and gamma-interferon ( $\text{rIFN}\gamma$ ) were generous gifts

from the Glaxo Institute of Molecular Biology, Geneva, and the Ernst-Boehringer Institute, Vienna. Recombinant tumor necrosis factor alpha ( $\text{rTNF}\alpha$ ) was purchased from Genzyme, Boston, United States.

**Induction of LAK Cells.** Allogeneic human LAK cells were generated from peripheral blood mononuclear cells (PBMCs) obtained from healthy volunteers and cultured ( $1 \times 10^6/\text{ml}$ ) for 6 days with  $\text{rIL-2}$  (1000 U/ml). In some experiments, we induced LAK cell activity by combined application of IL-2 and  $\text{IFN}\gamma$ ,  $\text{TNF}\alpha$ , or indomethacin.

**Target Cells.** Fresh leukemic cells were obtained from bone marrow or peripheral blood of untreated patients by Ficoll-Hypaque gradient centrifugation. Phenotypic analyses were performed by standard indirect immunofluorescence assays as described elsewhere [17]. K 562 and Daudi cell lines, maintained in continuous cultures, served as standard target cells. For use in the cytotoxicity assay, fresh leukemic cells and cell lines were labeled with 300  $\mu\text{Ci}$  or 50  $\mu\text{Ci}$  sodium chromate, respectively.

**Cytotoxicity Assay.** Lymphokine-activated killer cell activity was determined in a standard 4-h  $^{51}\text{Cr}$  release assay using  $5 \times 10^3$  target cells and various effector-to-target (E:T) ratios.

### Results

Fresh leukemic cells from 120 untreated patients with various types of leukemia were

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evaluated for their susceptibility to the lytic effect of allogeneic LAK cells. A significant lysis (defined as over 20% specific lysis at an E:T ratio of 50:1) was found in about half of the leukemias examined (Table 1, Fig. 1). No substantial differences could be detected between myeloid and lymphoid leukemias or with regard to the FAB subgroup or the immunological phenotype, although we observed that T-cell acute lymphoblastic leukemia (T-ALL) tended to be resistant to LAK-cell lysis. Chronic leukemias seem to be less sensitive to the cytotoxic effect of LAK cells than acute leukemias.

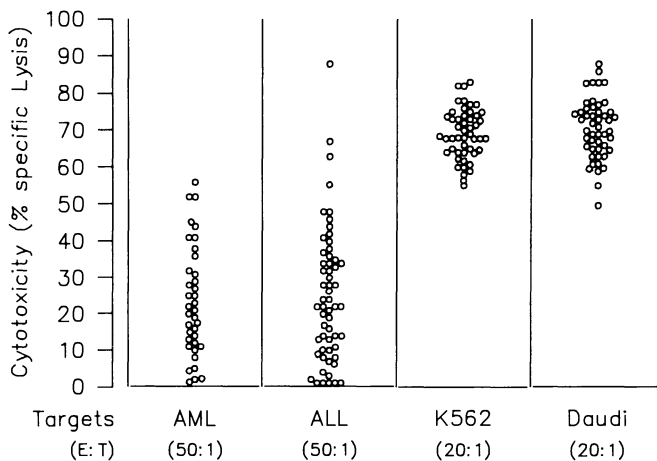
Reports that endogenous  $\gamma$ -IFN is required for IL-2 induction of LAK cells [18] led us to conjecture that it might be possible to augment LAK activity by adding rIFN $\gamma$  during the activation process. The results of our studies showed that the combined application of rIFN $\gamma$  and rIL-2 can improve the effectivity of cytotoxic mechanisms even at a low E:T ratio of 2.5:1 (Fig. 2). The sequential administration of rIFN $\gamma$  24 h before IL-2 or 48 h after IL-2 resulted in an augmentation of cytotoxicity, whereas the simultaneous application of both lymphokines or the addition of  $\gamma$ -IFN to the cultures only 24 h after IL-2 did not enhance cytotoxicity. Our observations, shown by a representative experiment (Fig. 2), revealed that the combined application of IL-2 and IFN $\gamma$  in a certain timing can generate LAK cells, even with a tenfold lower concentration of IL-2 than the standard dosage, which are able to exert a cytotoxicity level equal to that resulting from the standard IL-2 concentration.

**Table 1.** Susceptibility of fresh leukemic cells to allogeneic LAK cells. LAK cells were generated from PBMCs cultured ( $1 \times 10^6$ /ml) for 6 days with rIL-2 (1000 U/ml)

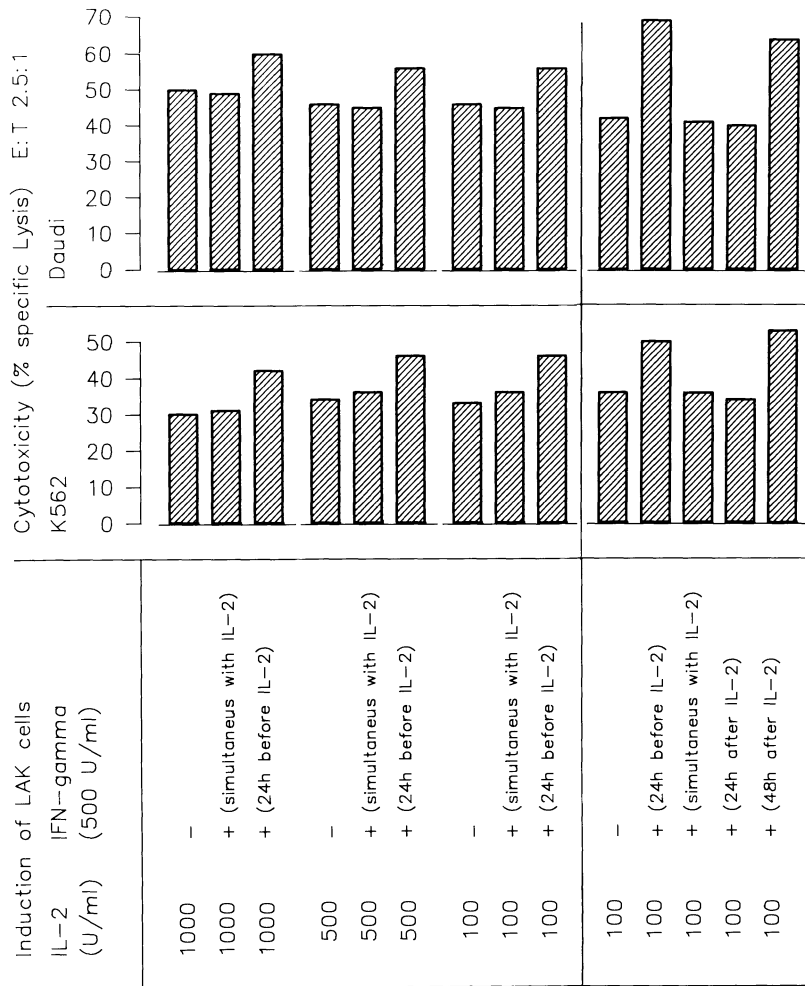
Target cells	n	Cytotoxicity (% specific lysis) E:T 50:1	
		≥ 20%	<20%
AML	37	21	16
CML-BC	8	6	2
CML chronic phase	6	2	4
ALL	58	30	28
0-ALL	5	2	3
c-ALL	39	26	13
B-ALL	2	1	1
T-ALL	12	1	11
CLL	4	1	3
PLL	3	1	2
HCL	4	0	4
Total	120	61	59

AML, acute myeloblastic leukemia; CML-BC, chronic myelocytic leukemia in blast crisis; CML, chronic myelocytic leukemia; ALL, acute lymphoblastic leukemia; 0-ALL, null-acute lymphoblastic leukemia; c-ALL, common acute lymphoblastic leukemia; B-ALL, B-cell acute lymphoblastic leukemia; T-ALL, T-cell acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; PLL, prolymphocytic leukemia; HCL, hairy cell leukemia

In another series of experiments, the improvement of LAK cell cytotoxicity by IL-2 in combination with rTNF $\alpha$  was tested. We observed a marked increase in the specific lysis of leukemia cells, especially when low



**Fig. 1.** Susceptibility of fresh leukemic cells compared with the cell lines K562 and Daudi



**Fig. 2.** Improvement of the effectivity of cytotoxic mechanisms: combined sequential application of rIL-2 and rIFN $\gamma$

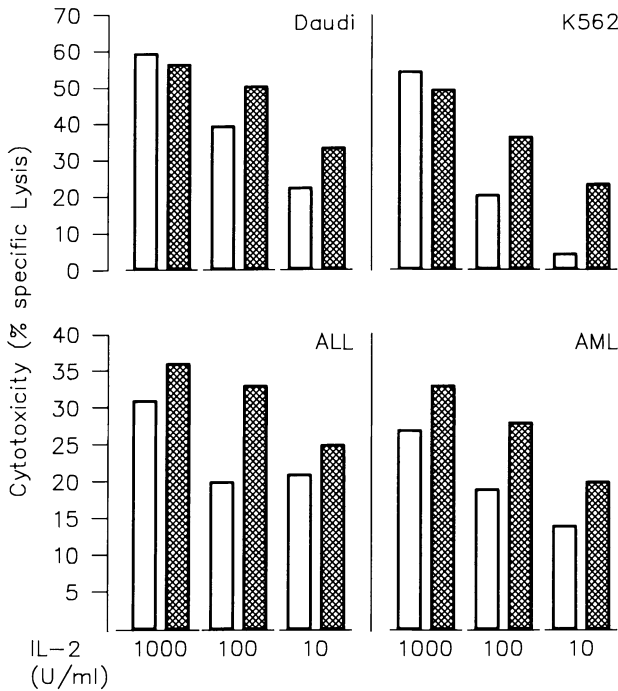
doses of IL-2 were used and at a low effector : target cell ratio (Fig. 3).

The repetitive addition of indomethacin to the cell cultures during the activation process led to a substantial augmentation of the cytotoxicity against leukemic cells as demonstrated by a representative experiment in Fig. 4.

## Discussion

Adoptive immunotherapy with ex vivo LAK cells and IL-2 or in vivo induction of cytotoxic mechanisms of the immune system

by IL-2 alone seem to be promising therapeutic approaches for certain tumor entities [1-11]. While many data are available on solid tumors, little is known about the LAK cell system in connection with human leukemia [12, 13]. This study demonstrates that about 50% of the leukemias examined are susceptible to the lytic effect of LAK cells. These findings are in contrast to those of other authors, who find most leukemias to be susceptible to LAK cell lysis [12, 14]. The discrepancies may be due to the lower numbers of leukemias examined or to the definition of "susceptibility" used. As we detected a broad spread of the specific cytolysis



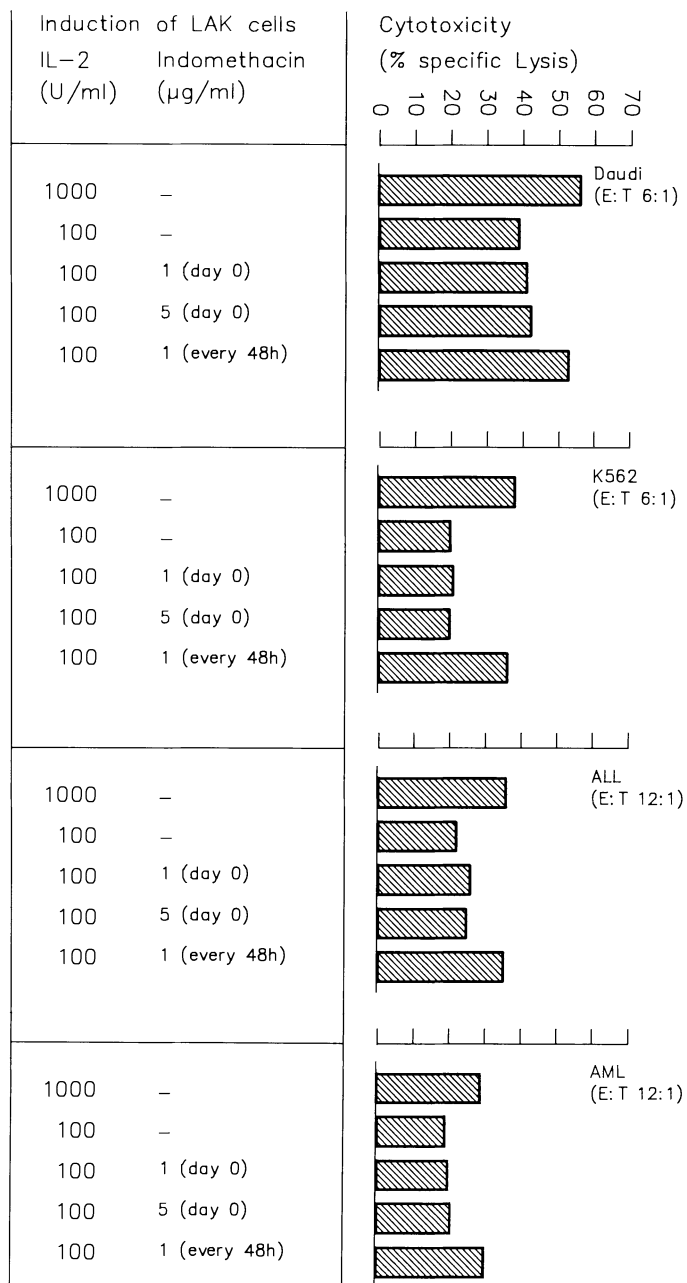
**Fig. 3.** Improvement of the effectiveness of cytotoxic mechanisms: combined application of rIL-2 and rTNF $\alpha$ : IL-2 ( $\square$ ), IL-2+TNF 500 U/ml ( $\square$ ). E:T 6:1 (Daudi, K 562). E:T 12:1 (ALL, AML)

sis of fresh leukemia cells (Fig. 1), and in view of a possible clinical application of this system, we defined a relatively high specific chromium release (over 20%) as a significant susceptibility to LAK cell cytotoxicity, while other authors give no or a much lower level as their definition of the sensitivity of a given leukemia target cell to the LAK cell cytotoxicity [12–14]. We observed a broad variability of LAK-cell-mediated cytolysis to fresh leukemic cells compared with the cell lines K 562 or Daudi. From our results depicted in Fig. 1 it is obvious that the chromium releases from fresh leukemia cells are markedly lower than those from cell lines; the specific Cr release is mostly under 50%, and this at a relatively high E:T ratio of 50:1. For a possible therapeutic utilization of the LAK cell system we tried to improve the efficacy of the cytotoxic mechanisms.

The combined sequential administration of  $\gamma$ -IFN and IL-2 led to enhanced LAK cell cytotoxicity even with a low dosage of IL-2. The augmentation of the cytotoxicity by administration of  $\gamma$ -IFN prior to IL-2 may be due to the induction of IL-2 receptors on the

effector cells before they are affected by IL-2, resulting in a more efficient activation. The enhanced LAK activity through addition of  $\gamma$ -IFN 48 h after IL-2 can be explained by a recruitment of additional cell populations which are not activated by IL-2 alone or by an induction of further lymphokine/cytokine circuits. These findings are consistent with data showing that the activation of NK cells involves collaboration between IL-2 and  $\gamma$ -IFN [18].

Another possibility of improving LAK cell cytotoxicity is the combined addition of IL-2 and TNF $\alpha$  to the LAK cell cultures. The synergy observed with TNF $\alpha$  and suboptimal IL-2 concentrations leads to cytotoxicity levels otherwise only achieved with a ten times higher concentration of IL-2 (Fig. 3). The mechanisms whereby TNF augments the killing of leukemia cells by LAK cells are not yet known. Consistent with observations of other investigators [19], we have not seen induction of LAK cytotoxicity by culturing PBMCs in the presence of TNF alone (data not shown). Therefore, and because TNF is a known participant in a number of complex lymphokine/cytokine



**Fig. 4.** Augmentation of LAK-cell cytotoxicity by addition of indomethacin during the activation process

circuits, the cytotoxicity augmentation may be indirect, possibly caused by increasing high-affinity IL-2 receptor expression [20]. In view of a possible clinical application of the system it will be interesting to examine whether combined low-dose administration of the components may result in reduced

toxicity while having a similar or even improved therapeutic efficacy.

As there are data indicating that monocytes may cause up- or downregulation of the IL-2-induced LAK activity [21], we hypothesized that LAK cell activity could possibly be augmented by eliminating mono-

cyte-suppressive influence. Our findings shown in Table 4 demonstrate that repeated addition of indomethacin to the cell cultures during LAK activation resulted in a marked augmentation of the cytotoxicity to leukemia cells. This makes it possible to apply a tenfold lower dosage of IL-2 and still obtain cytolytic effects similar to those resulting from the standard IL-2 dosage. The mechanism of this enhancement is probably the inhibition of prostaglandin-mediated suppression of LAK cell activation by monocytes.

The observations with the combined administration of lymphokines or cytokines as well as with addition of indomethacin to the cell cultures suggest that it seems possible

- a) to generate more potent LAK cells and thus improve the therapeutic efficacy and/or
- b) to reduce the toxicity by a lower dosage of biological substances while preserving the efficiency known from the standard dosage.

The results of these studies suggest that adoptive immunotherapy with IL-2-induced LAK cells may be of great value for treatment of leukemia, especially when the tumor burden is low, e.g., during maintenance therapy to eliminate minimal residual disease or in early relapse. Combined application of different lymphokines or cytokines and inhibition of prostaglandin synthesis during the activation process are possible approaches for improving the efficacy of cytotoxic mechanisms.

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## Biochemical Evidence for Synergistic Combination Treatment with Methotrexate and 6-Mercaptopurine in Acute Lymphoblastic Leukemia\*

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### Introduction

Methotrexate (MTX) and 6-mercaptopurine (6MP) are common antimetabolites in the treatment of acute lymphoblastic leukemia (ALL) since their introduction in 1947 and 1952 [1, 2]. Originally, these agents were administered separately. However, after empirical evidence for a potentiating efficacy of the combination of both in mice leukemia [3], MTX and 6MP have also been used in combination in childhood ALL. The enhanced efficacy of combination treatment in children was demonstrated in a study of the Acute Leukemia Group B in 1961 [4].

The route of administration and dose of MTX have been changed dramatically with the introduction of intrathecal and high-dose intravenous therapy. Moreover, the multidrug induction chemotherapy of ALL has been changed significantly, associated with a significant improvement of survival, especially after the introduction of enforced reinduction therapy by Riehm et al. [5]. However, with the exception of a shortening of its duration to 1 year in some protocols, the route and dosage of the oral maintenance treatment with 6MP (50–75 mg/m<sup>2</sup> once daily) and MTX (20–30 mg/m<sup>2</sup> once weekly) have remained unchanged in the past 30 years. Some authors even doubted

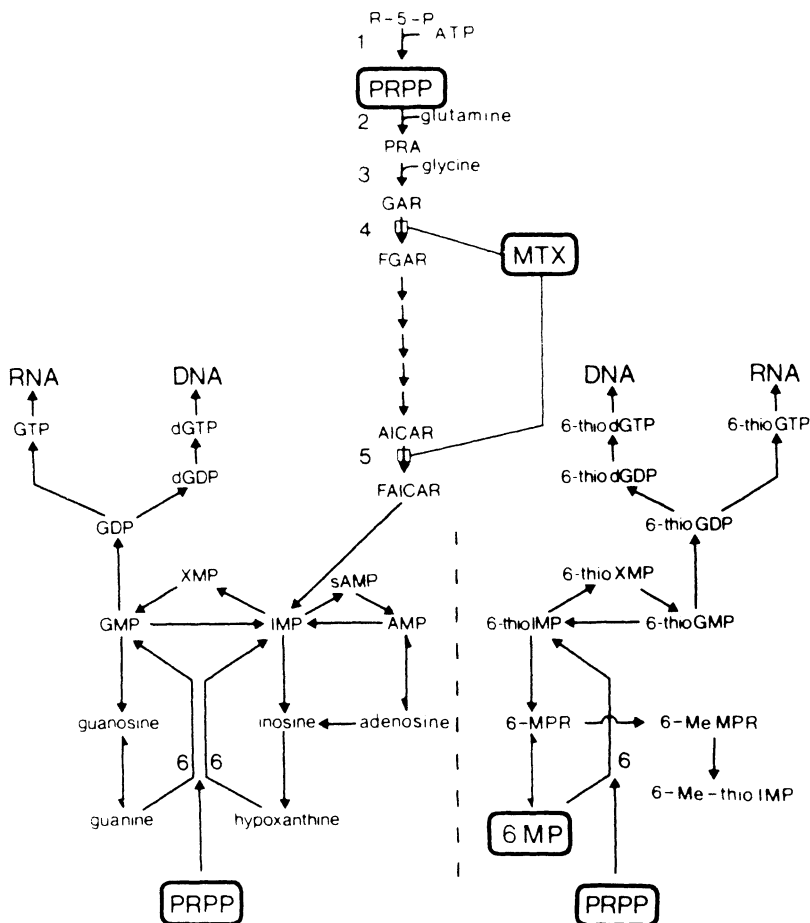
the efficacy of the maintenance therapy in ALL, based on the bioavailability of MTX and 6MP [6–8].

Because the biochemical basis for the potentiating efficacy of combination treatment with MTX and 6MP was unknown, we studied the biochemical pharmacology and cell kinetics of separate and combination therapy with both agents. Some details of these studies will be mentioned here.

Our hypothesis of the synergism concept is demonstrated in Fig. 1: MTX is an inhibitor of two folate-dependent enzymes (Nos. 4, 5) in purine de novo synthesis (PDNS), resulting in an increase of intracellular phosphoribosyl pyrophosphate (PRPP) levels. The increased availability of PRPP can be used for an enhanced conversion of 6MP – when coadministered – by hypoxanthine guanine phosphoribosyl transferase (HGPRT) (No. 6) into active thiopurine metabolites, because PRPP is a rate-limiting cosubstrate for the conversion of purine bases into purine nucleoside monophosphates. The inhibition of PDNS by MTX will also deplete intracellular purine nucleotide pools and the inhibition of thymidylate synthetase by MTX will deplete thymidylate pools, both resulting in a depletion of essential substrates for RNA and especially DNA synthesis. When MTX and 6MP are both administered, the net result will be an inhibition of DNA synthesis by MTX and an abundance of thiopurine nucleotides, which can be incorporated as false deoxythioguanine nucleotides into DNA, as far as its synthesis is still present. In this way, MTX potentiates the cytotoxic activity of 6MP.

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**Fig. 1.** Scheme of purine metabolism, metabolism of 6MP, and mode of action of MTX. 1, PRPP synthetase; 2, PRPP amidotransferase; 3, glycinamide ribonucleotide (GAR) synthetase; 4, GAR formyltransferase; 5, AICAR formyltransferase; 6, HGPRT

## Results

The biochemical pharmacology of MTX and 6MP was studied in three human malignant lymphoblastic cell-lines: T- (MOLT-4), B- (RAJI), and non-T-non-B/cALLA<sup>+</sup> (KM-3) lymphoblasts. We used two concentrations of MTX (0.02, 0.2  $\mu$ M), which can be maintained for several hours after oral administration of MTX [9], and two concentrations of 6MP (2 and 10  $\mu$ M), which can be reached after nontoxic prolonged intravenous administration of 6MP at a dosage of 2 mg/kg per hour [10, 11]. Details of materials and methods used in our studies have been published previously [12–16].

The inhibition of PDNS by MTX is demonstrated in Table 1, measuring the incorporation of [U-<sup>14</sup>C]glycine (Fig. 1) into purine metabolites as a parameter of PDNS activity. These data show an incomplete inhibition of PDNS in T cells, an increasing inhibition in B cells, an almost complete inhibition in CALLA<sup>+</sup> lymphoblasts by 0.02  $\mu$ M MTX, and a complete inhibition of PDNS in all cell lines by 0.2  $\mu$ M MTX. These data were confirmed by measuring levels of the PDNS intermediate aminoimidazole carbonamide ribonucleotide (AICAR) (Fig. 1) [14]. The result is a time- and cell-line-dependent increase of intracellular PRPP levels after incubation with

**Table 1.** Activity of purine de novo synthesis<sup>a</sup> in lymphoblasts exposed to MTX

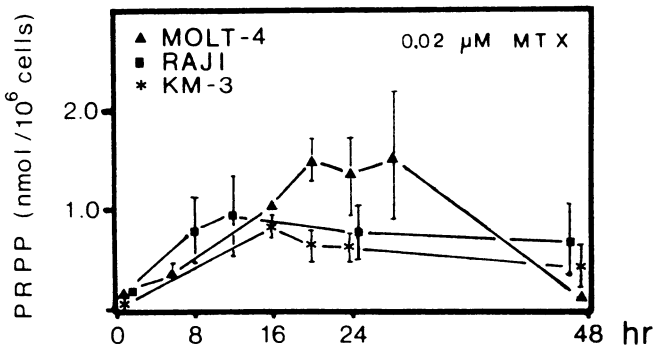
MTX ( $\mu\text{M}$ )	MOLT-4	RAJI	KM-3
At 24 h			
0	100	100	100
0.02	88	29	2
0.2	0	1	0
At 48 h			
0	100	100	—
0.02	94	10	—
0.2	0	—	—

<sup>a</sup> Measured by incorporation of [ $U\text{-}^{14}\text{C}$ ] glycine in purine metabolites and expressed as percentages of untreated cells. Mean of at least four experiments. —, not done

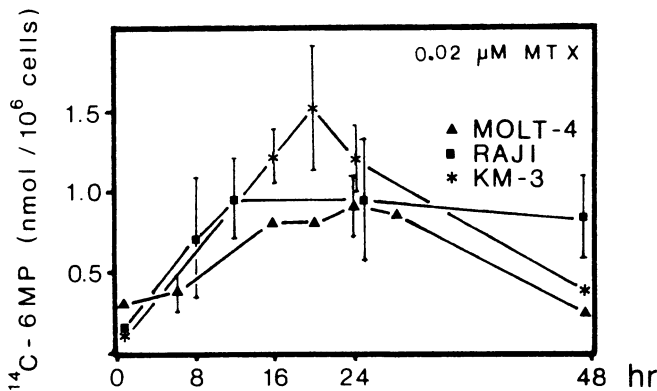
0.02  $\mu\text{M}$  MTX (Fig. 2), and also a dose-dependent earlier increase of PRPP levels with 0.2  $\mu\text{M}$  MTX [14]. In order to demonstrate that the increased availability of PRPP could be used for an enhanced intracellular

conversion of 6MP, we incubated the lymphoblasts at each time point during 20 min with 10  $\mu\text{M}$  [ $8\text{-}^{14}\text{C}$ ]-6MP and measured the intracellular uptake of 6MP in intact cells. The time-, dose-, and cell-line-dependent effects of MTX on 6MP incorporation (Fig. 3) are comparable to the effects of MTX on intracellular PRPP availability (Fig. 2). The differences in incorporation of 6MP between the cell lines can be explained by the differences in activity of the purine salvage pathway and the inhibition of the PDNS between the cell lines [14]. Because the PDNS in CALLA<sup>+</sup> cells is most severely inhibited by 0.02  $\mu\text{M}$  (Table 1), these cells will be affected most by simultaneous or sequential combination therapy with MTX and 6MP, due to a more pronounced conversion of 6MP. This will be demonstrated below in clonal growth and cell viability studies.

The inhibitory effects of MTX on PDNS and thymidylate synthesis result in a depletion of purine and thymidine nucleotides for DNA and RNA synthesis. The depletion of ribonucleotide and deoxyribonucleotide



**Fig. 2.** Effects of incubation with 0.02  $\mu\text{M}$  MTX on intracellular PRPP levels in MOLT-4, RAJI, and KM-3 cells. Mean  $\pm$  SD of three experiments in duplicate and expressed as nanomoles/ $10^6$  viable cells



**Fig. 3.** Effects of incubation with 0.02  $\mu\text{M}$  MTX on intracellular incorporation of 10  $\mu\text{M}$  [ $8\text{-}^{14}\text{C}$ ] 6-mercaptopurine in MOLT-4, RAJI, and KM-3 cells. Mean  $\pm$  SD of three experiments in duplicate and expressed as nanomoles/ $10^6$  viable cells 20 min

levels were dose, time, and cell line dependent [15]. We found a close relationship between cytotoxicity, degree of PDNS inhibition, and extent of decrease of both dGTP and dTTP pools in the three cell lines. Again, CALLA<sup>+</sup> cells were affected most and T-cells were affected less.

In order to demonstrate the potential synergistic effects of combination treatment with MTX and 6MP, we performed extensive incubations with various combinations of MTX and 6MP after simultaneous or sequential administration. In these studies MTX was added at 0 h and 6MP was added simultaneously at 0, or sequentially at those time points at which MTX caused a maximal increase of PRPP levels. Table 2 shows an example with combinations of 0.02  $\mu$ M MTX and prolonged administration of 2  $\mu$ M 6MP in the three cell lines. It should be stressed that clonal growth inhibition reflects the capacity of the cell to overcome its biochemical damage after incubation in a drug-free medium, whereas the number of nonviable cells reflects cytotoxicity, which is the aim of chemotherapy in ALL. Synergism of combination treatment [17] is present when the inhibition of clonal growth or the number of nonviable cells of combination treatment (numbers not in parentheses) exceeds the multiplication of the effects of separate treatment with MTX and 6MP (numbers in parentheses). Table 2 demonstrates that synergism (underlined numbers) with respect to cytotoxicity is most pronounced in KM-3 cells after incubation with MTX followed by sequential administration with 6MP at 16 h. In this case the difference with respect to nonviable cells between combination treatment (22 at 40 h and 50 at 64 h) and summation of separate treatment (4 and 26, respectively) is superior. As mentioned previously, synergism of sequential combination treatment with MTX and 6MP could be expected, based on the severe combined effects of MTX on dTTP levels and PDNS. On the other hand, synergism with respect to clonal growth inhibition is predominantly seen in MOLT-4 cells. The biochemical parameters (PDNS and dTTP levels) in MOLT-4 cells are hardly affected by treatment with 0.02  $\mu$ M MTX [14, 15]. Nevertheless, the disturbances are sufficient to ensure a significant disturbance in metabolism after

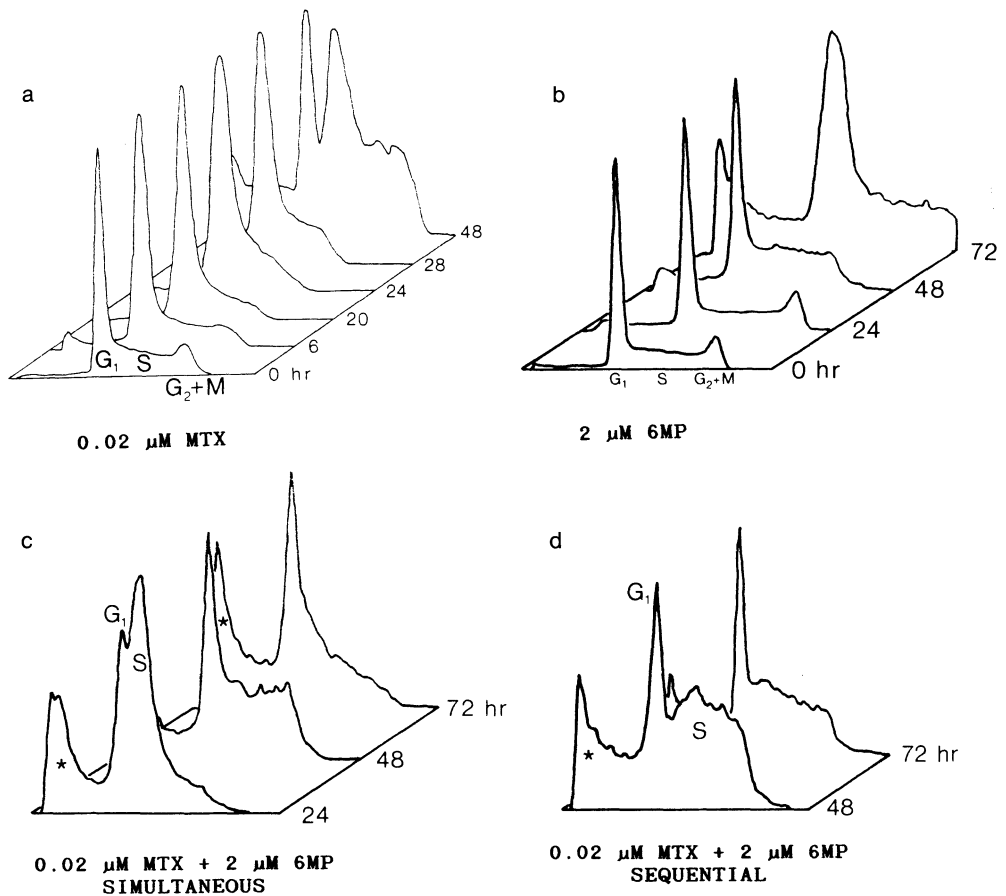
coaddition of 6MP. This phenomenon can be explained by the active purine salvage pathway in MOLT-4 cells [14] in comparison to RAJI and KM-3 cells, which favors the biochemical effects (clonal growth inhibition) of 6MP coaddition.

In order to understand the effects of MTX and 6MP on DNA synthesis, we performed DNA-flow cytometric and [<sup>32</sup>P] incorporation studies into DNA of MOLT-4 T-lymphoblasts as an example. After incubation with 0.02  $\mu$ M MTX, a decreasing number of cells in G<sub>2</sub>+M phase is demonstrated, associated with an accumulation in G<sub>1</sub> phase and subsequently in early and mid S-phase (Fig. 4a). However, cell cycle progression is not completely inhibited. These data confirm the incorporation of <sup>32</sup>P into DNA (Fig. 5, black bars): inhibition of DNA synthesis is noted in the first 24 h to 75% and in the second 24 h to 50% of controls. Incubation with 2  $\mu$ M 6MP (Fig. 4b) results in a delay of progression through the cell cycle: a decrease of cells in G<sub>1</sub> phase, associated with an accumulation of cells in S phase and G<sub>2</sub>+M phase. However, this delay has significant consequences for DNA synthesis (Fig. 5a, second bars): DNA synthesis is inhibited to 49% of controls in the first 24 h, and to 43% in the second 24 h. When MTX and 6MP are administered simultaneously at 0 h (Fig. 5a, third bars), DNA synthesis is lowered to 29% in the first 24 h and to 24% in the second 24 h. When MTX is given at 0 h followed by sequential administration of 6MP at 24 h (Fig. 5b, third bar), DNA synthesis in the second 24 h is lowered to 25% of controls. Thus, both simultaneous and sequential administration of MTX and 6MP result in augmented inhibitory effects on DNA synthesis in comparison to separate administration. Similar augmented effects are demonstrated by flow-cytometric studies after simultaneous administration (Fig. 4c): at 24 h the accumulation of cells in early S phase is more pronounced in comparison to separate treatment with MTX at 24 h; at 48 h the cells are blocked in the S phase, followed by a complete cessation of progression through the cell cycle at 72 h. After sequential administration of MTX and 6MP (Fig. 4d), the augmented effects result in accumulation and arrest of cells in the S phase. Thus, addi-

**Table 2.** Clonal growth and cell viability after exposure to simultaneous and sequential combinations of 0.02  $\mu$ M MTX and 2  $\mu$ M 6MP

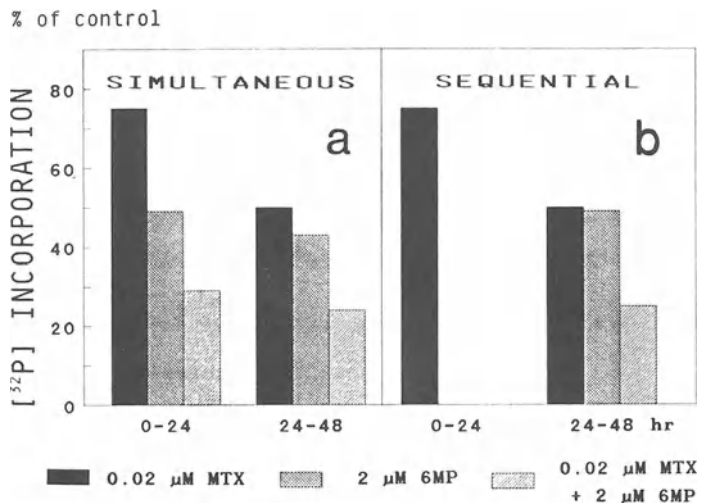
Duration of exposure	Clonal growth inhibition													
	12	16	24	36	40	48	60	64	72 h	Nonviable cells				
Cell line	MTX		6MP											
MOLT-4	0 h	0 h	0.90 (1.00)			2.22 (2.21)			2.60 (1.25)	16 (29)			66 (47)	73 (66)
	0 h	24 h				0.92 (1.11)			<u>1.02</u> (0.48)				<u>47</u> (34)	<u>60</u> (53)
RAJI	0 h	0 h			0.98 (1.13)		1.30 (1.74)					0 (5)		3 (11)
	0 h	12 h			<u>1.07</u> (0.77)		1.19 (1.47)					0 (6)		5 (9)
KM-3	0 h	0 h		0.13 (0.03)		0.82 (1.65)		3.00 (3.21)			0 (3)		15 (4)	32 (23)
	0 h	16 h				1.39 (1.70)		1.84 (2.94)					<u>22</u> (4)	<u>50</u> (26)

Mean of two experiments in duplicate. Clonal growth inhibition is defined as  $-\log$  clonal growth (corrected for untreated cells). Nonviable cells are expressed as percentages and corrected for nonviable untreated cells. Numbers in parentheses represent a summation of the results of separate exposure to MTX and 6MP. Underlined numbers indicate synergism of combination treatment [17]



**Fig. 4a–d.** Changes in DNA distribution after incubation with *a* 0.02  $\mu\text{M}$  MTX, *b* 2  $\mu\text{M}$  6MP, *c* simultaneous incubation with 0.02  $\mu\text{M}$  MTX and 2  $\mu\text{M}$  6MP, and *d* incubation with 0.02  $\mu\text{M}$  MTX, followed by sequential incubation with 2  $\mu\text{M}$  6MP at  $t=24$  h. The histograms are representative examples of at least two individual experiments

**Fig. 5.** Phosphorus-32 incorporation into DNA (i. e., activity of DNA synthesis) of MOLT-4 cells after separate or combination treatment with MTX and 6MP. Expressed as percentages of untreated cells. *Black bars*, separate incubation with 0.02  $\mu\text{M}$  MTX; *second bars*, separate incubation with 2  $\mu\text{M}$  6MP; *a*, *third bars*: simultaneous incubation with 0.02  $\mu\text{M}$  MTX and 2  $\mu\text{M}$  6MP at  $t=0$  h; *b*, *third bar*: incubation with 0.02  $\mu\text{M}$  MTX at  $t=0$  h followed by sequential incubation with 2  $\mu\text{M}$  6MP at  $t=24$  h. Mean of quadruplicate determinations in three experiments in duplicate

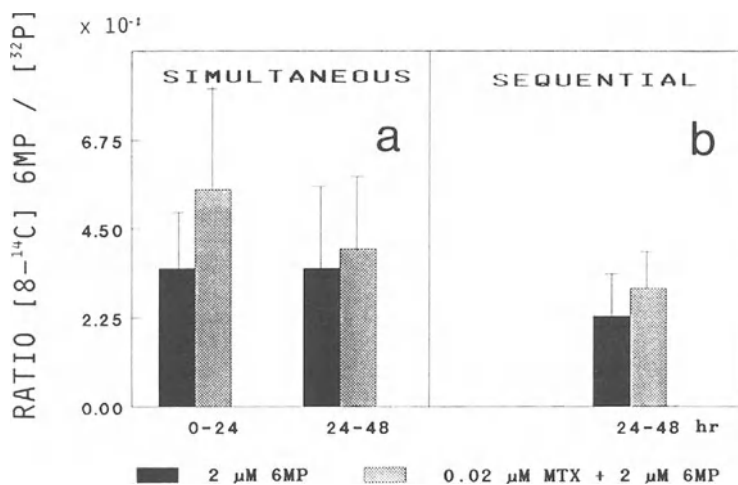


tion of 6MP augments the effects of MTX on cell cycle progression.

In Fig. 3 we demonstrated that pretreatment with MTX favors intracellular uptake and conversion of 6MP in incubations for 20 min with [ $^{14}\text{C}$ ]6MP. Figure 6 shows the results of incorporation into DNA after prolonged incorporation with  $2\ \mu\text{M}$  [ $^{14}\text{C}$ ]6MP with and without MTX. The results are expressed as the ratio [ $^{14}\text{C}$ ]6MP/ $^{32}\text{P}$  and reflect the ratio of 6MP/normal nucleotide incorporation into DNA. After simultaneous combination treatment with MTX and 6MP (Fig. 6a), the incorporation of 6MP into DNA is augmented in comparison to separate treatment with 6MP, especially in the first 24 h. In the second 24 h and after sequential treatment the enhancement of 6MP incorporation into DNA is less. The explanation for this phenomenon was mentioned in our hypothesis: MTX causes an inhibition of PDNS with enhanced intracellular PRPP levels, resulting in enhanced intracellular conversion of 6MP into thiopurine nucleotides. On the other hand, MTX causes an inhibition of DNA synthesis by depletion of normal purine and thymidine nucleotides. The net result will be an abundance of deoxythioguanine nucleotides, which can be incorporated into DNA, as far as its synthesis is still present.

## Clinical Perspectives

Our studies concerning the biochemical pharmacology of MTX and 6MP demonstrated a potential synergistic effect of combination therapy in human malignant lymphoblasts with an active PDNS and purine salvage pathway. These effects will be almost absent in normal bone marrow cells and peripheral blood lymphocytes with a low activity of PDNS. Pharmacokinetic studies in patients after oral administration of 6MP ( $50\ \text{mg}/\text{m}^2$ ) demonstrated low plasma levels of 6MP (mean,  $0.064\ \mu\text{M}$ ; range,  $0.020\text{--}0.140\ \mu\text{M}$ ) [18]. However, the presence of prolonged measurable levels of 6MP during 3–6 weeks after cessation of maintenance treatment in children indicates that the human body contains a large pool of bioavailable 6MP after prolonged oral administration. Therefore, the concept of synergism may be present during maintenance treatment with oral MTX and 6MP, as was demonstrated empirically [4]. Plasma levels of  $2\text{--}10\ \mu\text{M}$  6MP, as used in our studies, can easily be reached after prolonged intravenous administration of 6MP [10, 11]. Therefore, the synergism concept will be especially advantageous for induction therapy in children with ALL after prolonged intravenous administration of MTX and 6MP.



**Fig. 6.** Ratio [ $^{14}\text{C}$ ]6MP/ $^{32}\text{P}$  incorporation into DNA of MOLT-4 cells after separate treatment with  $2\ \mu\text{M}$  [ $^{14}\text{C}$ ] 6MP (black bars), simultaneous combination treatment with  $0.02\ \mu\text{M}$  MTX (a, second bars), and treatment with  $0.02\ \mu\text{M}$  MTX at  $t=0$  h followed by sequential combination with  $2\ \mu\text{M}$  6MP at  $t=24$  h (b, second bar).  $\times 10^{-2}$ , mean  $\pm$ SD of quadruplicate determinations in three experiments in duplicate



Because the penetration of 6MP into the CNS fluid is almost 50% [11], this synergism concept may also offer a special contribution in the (prophylactic) treatment of meningeal leukemia in patients with ALL, using intravenous administration of MTX and 6MP.

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## Pharmacokinetics of Folinic Acid in Children with Acute Lymphoblastic Leukemia\*

J. D. Borsi, E. Sagen, I. Romslo, and P. J. Moe

### Patients and Methods

We analyzed folinic acid (FA) levels in the serum samples of 20 children with acute lymphoblastic leukemia (ALL). Mean age of the patients was 7.7 years (SD, 3.96 years), median age 6.8 years, and range 2.6–17.4 years. The patients received 96 infusions of methotrexate (MTX) at a dose of 8 g/m<sup>2</sup>. A total of 320 serum samples – taken during different periods of rescue therapy – were analyzed. Determination of MTX, 7-OH-MTX, and FA levels was performed by high-performance liquid chromatography (HPLC) with electrochemical detection.

### Rescue Schedule

1. Thirty-six hours after the start of the MTX infusion: 75 mg i.v.
2. Thirty-nine to 63 h: 8 × 15 mg i.v. every 3 h
3. Sixty-three to 110 h: 8 × 15 mg per os every 6 h.

### Results

A significant difference was observed between FA levels in the periods of i.v. 75 mg,

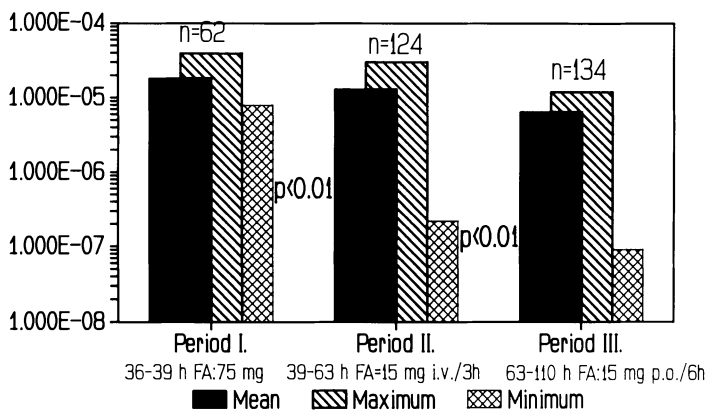
15 mg, and per os 15 mg administration of the rescue. A great difference was found between the minimum and maximum levels of FA in the latter two periods (Fig. 1). While levels of FA in the serum decreased with time, the ratio of FA to MTX increased, the variability of the FA/MTX ratio being even higher than the variability of the FA levels (Fig. 2). About a fourfold variability in FA concentration was observed in the same patient in the i.v. administration of the rescue, which increased to a 100-fold difference during the per os administration of FA (Fig. 3). The case in Fig. 2 represents an extreme: the majority of the patients showed only 2- to 30-fold inpatient variability. The variations in FA/MTX ratio also increased by the third period of rescue administration in the same patient. In other patients a three- to fivefold variability was also observed (Fig. 4). The FA dose administered was recalculated on a mg/m<sup>2</sup> and a mg/kg basis. Significant correlation was found between the dose and concentration in serum in periods I and II of rescue administration (Fig. 5). However, no significant correlation was found between the FA levels in the serum and the dose of the orally administered rescue (Fig. 6).

### Conclusions

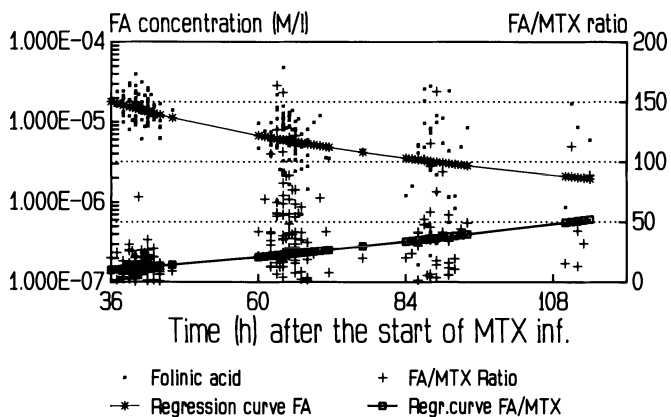
Great inpatient and interpatient variability in FA levels and FA/MTX ratios obviously represent different degrees of protection against the effect of MTX. At 36 h after the start of the MTX infusion, the 0.1- to 10-fold FA/MTX ratio may not provide a suffi-

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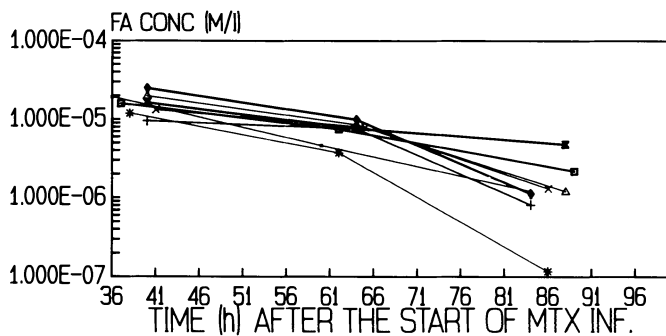


**Fig. 1.** Folic acid levels in serum at different periods of administration

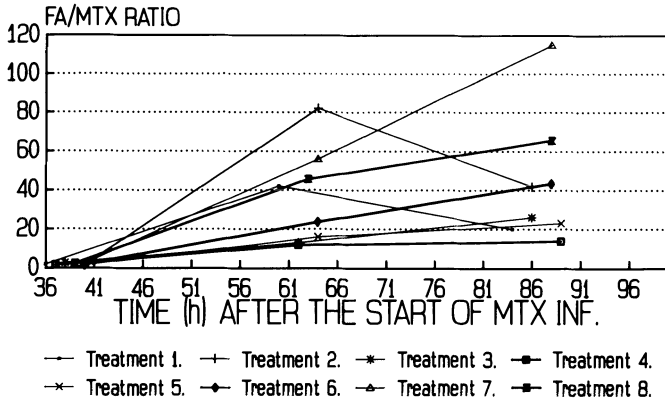


**Fig. 2.** Folic acid levels and FA/MTX ratio at different times

Dose of MTX:8g/sq.m./24h

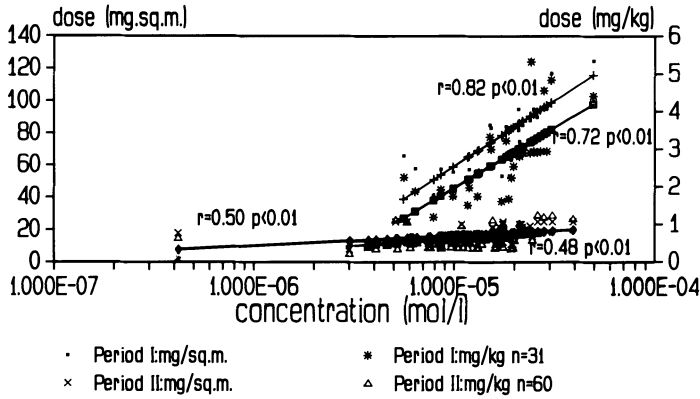


**Fig. 3.** In-patient variability of folic acid levels



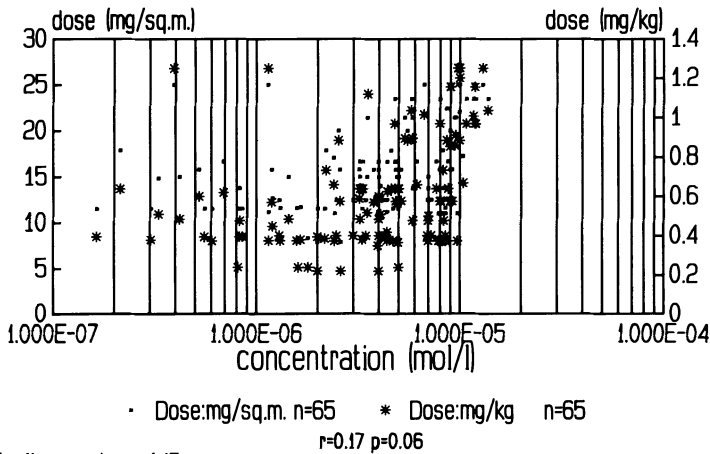
**Fig. 4.** In-patient variability of folinic acid/MTX ratios

PATIENT G.E.T.



**Fig. 5.** Dose-level relationship for folinic acid rescue

1h after iv.dose of 75 mg and 15 mg



**Fig. 6.** Dose-level relationship for folinic acid rescue

1h after p.o. dose of 15 mg

cient rescue effect judging from basic data. As no patients developed MTX-related toxicity, it may not be necessary to start the rescue administration as early as 36 h after the start of MTX infusion. However, in the later periods, patients who had around a 100-fold FA/MTX ratio were most probably “overprotected.” Determination of the dose

of the rescue on the basis of body surface area is recommended. The FA levels achieved by per os administration are rather unpredictable and must be standardized. The introduction of therapeutic drug monitoring for folinic acid may further improve the treatment results with protocols based on high-dose MTX therapy.

## Cellular Pharmacokinetics of Daunomycin in Human Leukemic Blasts In Vitro and In Vivo\*

M. E. Scheulen, B. Kramer, M. Skorzec, and W. K. Reich

### Introduction

The description of a decrease in the intracellular accumulation of a number of cytostatics, such as anthracyclines, vinca alkaloids, epipodophyllotoxins, and actinomycin D, in drug-resistant malignant cells – first demonstrated for colchicine in hamster ovary cells [4] caused by an energy-dependent outward pump in connection with the expression of the 170 000-dalton plasma membrane glycoprotein P170 [2] – has substantially contributed to the understanding of “multidrug resistance” [6]. Accordingly, the responsiveness of malignant cells to these cytotoxic drugs may more critically depend on their cellular tumor pharmacokinetics and metabolism than on their plasma pharmacokinetics, which in general only poorly correlates with their pharmacodynamics [7].

We have determined the cellular pharmacokinetics and metabolism of the anthracycline daunomycin (DNM) in leukemic blasts from peripheral blood of 30 patients with acute myelogenous leukemia treated according to the TAD protocol (6-thioguanine, cytosine arabinoside, DNM) [1] in vivo and in isolated leukemic blasts in vitro in correlation with the outcome of this treatment, to evaluate the clinical relevance of a decreased intracellular accumulation of DNM for

multidrug resistance and possibly predict the response of individual patients from in vitro parameters.

### Material and Methods

Leukemic blasts were isolated from peripheral blood by Ficoll gradient centrifugation and lysis of red blood cells up to 4 h after intravenous bolus injection of 60 mg/m<sup>2</sup> DNM, washed, and analyzed for DNM and metabolites by high-performance liquid chromatography (HPLC) with fluorescence detection after extraction (Fig. 1) [10]. DNM content was calculated by internal standardization with Adriamycin (doxorubicin, ADM) and expressed in micrograms per milliliter after determination of cellular volume by morphometry. Plasma DNM kinetics was simultaneously measured. Accumulation of DNM in leukemic blasts was defined by the quotient of the intracellular and plasma DNM concentration for each time point of the kinetics (Fig. 6). For in vitro experiments leukemic blasts were isolated in the same way before treatment and incubated with 0.02–6.0 µg/ml DNM up to 1 h before washing and HPLC analysis (Fig. 2), including efflux studies by reincubation of leukemic blasts in DNM-free medium. A typical chromatogram of an extract of mononucleated cells (MNC) after in vitro incubation is shown in Fig. 3 in comparison to the standard mix.

The outcome of TAD treatment was quantified by the relative reduction of leukemic blasts in bone marrow defined by the quotient of the percentages of blasts on

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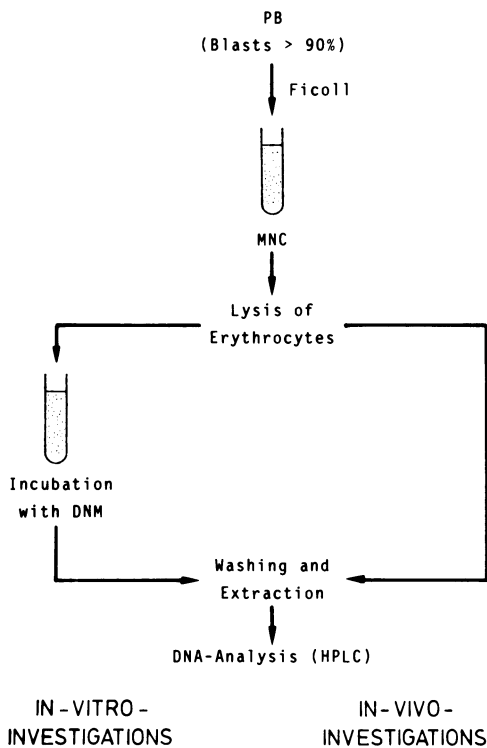
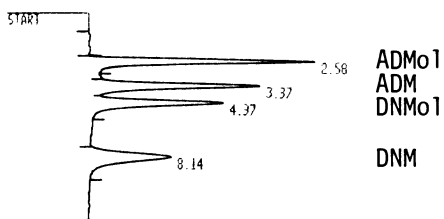


Fig. 1. Method of isolation, incubation, and analysis of the DNM content of leukemic blasts from peripheral blood

### STANDARD MIX



### EXTRACT (IN-VITRO-INCUBATION)

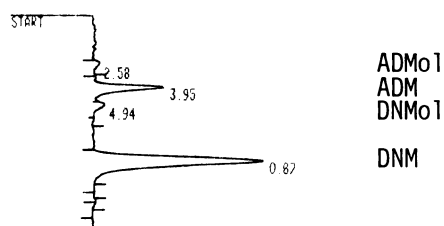
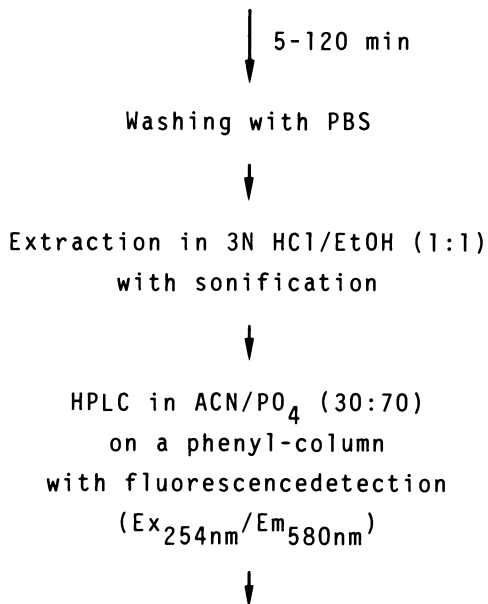


Fig. 3. Chromatograms

### DNM-UPTAKE IN VITRO

Incubation of  $10^6$  MNC/ml  
with 0.02-6  $\mu\text{g}$  DNM/ml  
in PBS + 20% FCS  $\pm$  4% HSA  
at 37°C in a shaking water bath



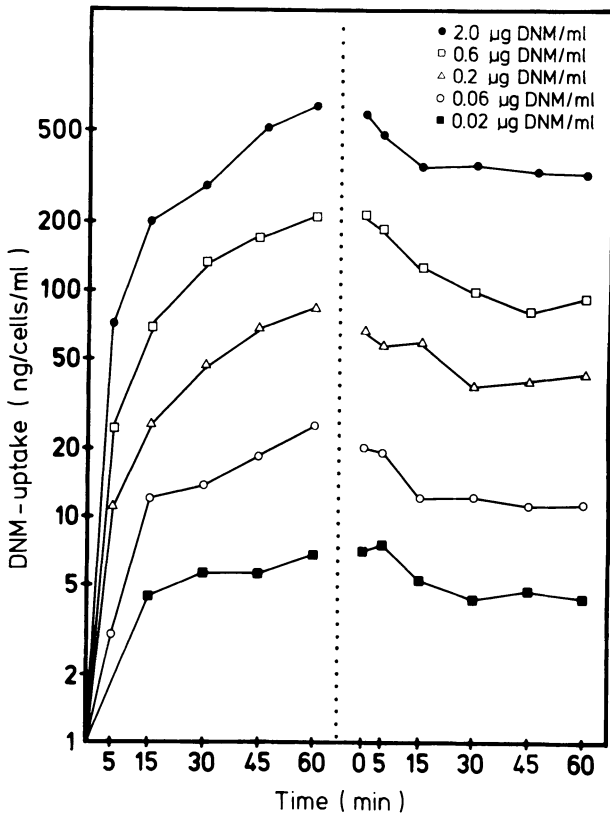
Quantification by integration  
and internal standardization

Fig. 2. Conditions for the incubation of leukemic blasts, extraction, and HPLC analysis of anthracyclines

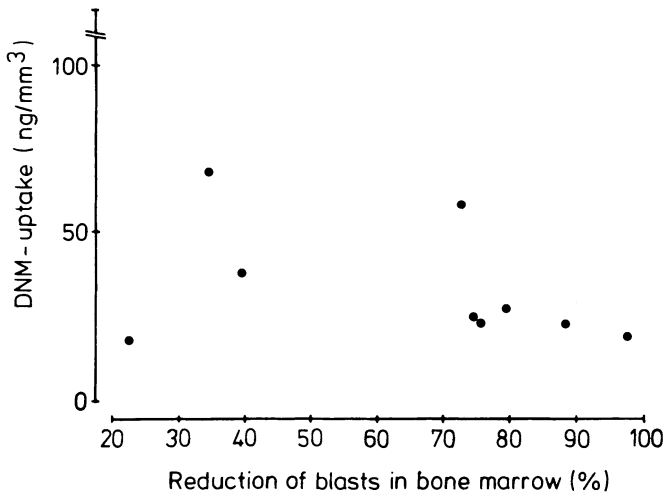
day 21 after and immediately before treatment.

### Results

Figure 4 gives an example of the concentration-dependent cellular uptake of DNM by leukemic blasts in vitro and its efflux in DNM-free medium after washing. Altogether, there was no significant correlation between the DNM uptake in vitro and response to TAD treatment (Fig. 5).



**Fig. 4.** Concentration-dependent cellular uptake of DNM by leukemic blasts in vitro and its efflux in DNM-free medium after washing



**Fig. 5.** Correlation between the DNM uptake by leukemic blasts in vitro and the response to TAD treatment

An example of the differences in the cellular pharmacokinetics of DNM during TAD induction therapy among the diverse acute myelogenous leukemias is given in Fig. 6. In spite of the nearly identical plasma kinetics of DNM in both patients, intracellular con-

centrations strongly, vary resulting in a significant change in the intracellular accumulation of the drug. In the same way, there are great *interindividual* differences in the maximal intracellular DNM concentrations between 4.0 and 23.1 µg/ml and in the areas



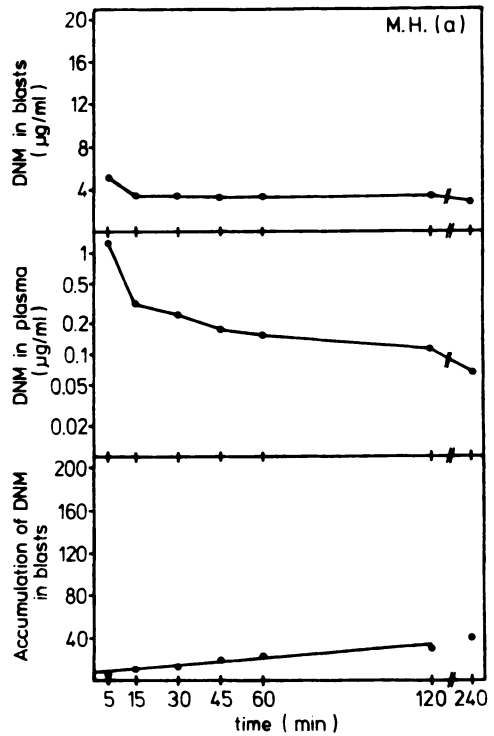
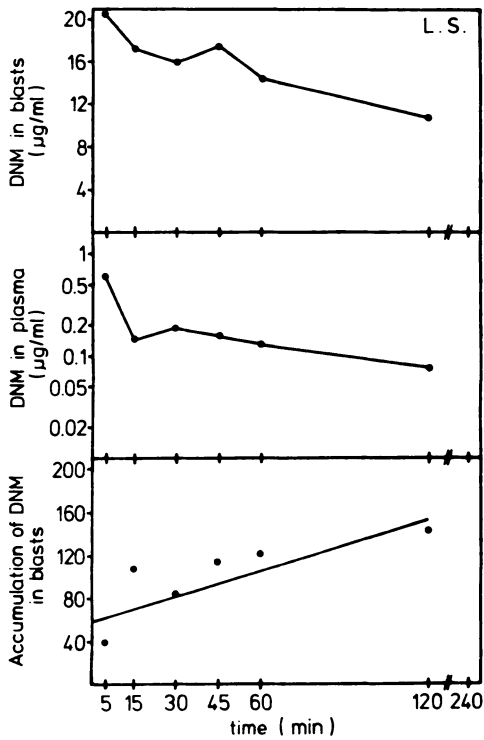


Fig. 6. Cellular and plasma pharmacokinetics and intracellular accumulation of DNM in leukemic blasts in two patients in vivo

under the concentration time curves (AUC) for DNM during the first 2 h after administration between  $6.8$  and  $30.7 \mu\text{g} \times \text{h}$  for the first TAD induction therapy in vivo (Fig. 7). A significant correlation either between the

maximal intracellular DNM concentrations or between the AUC in vivo and the outcome of TAD induction therapy did not exist in a series of six patients (Figs. 8, 9). Also, there was no relationship between the in vit-

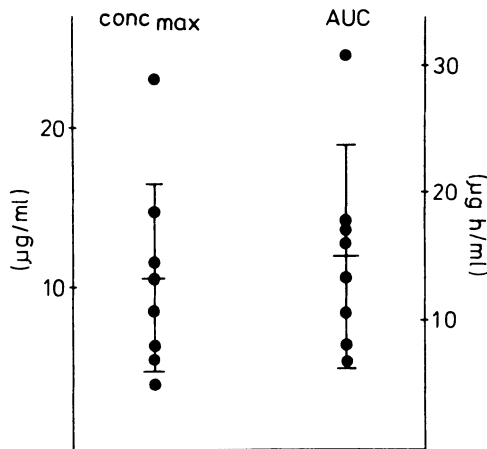
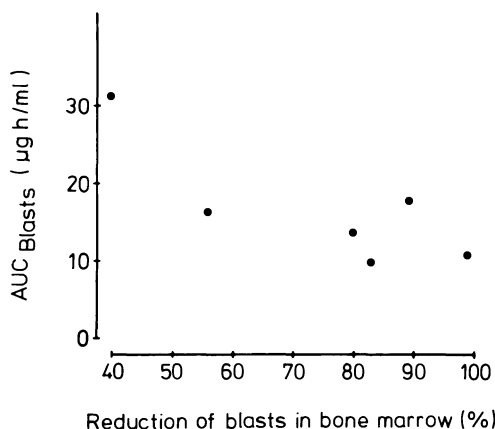


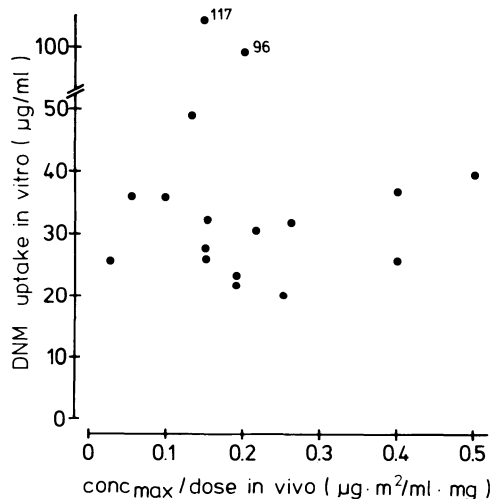
Fig. 7. Maximal concentrations ( $conc_{max}$ ) and areas under the curve (AUC) of DNM during the first 2 h after administration for the first induction TAD therapy in vivo



Fig. 8. Correlation between the maximal intracellular DNM concentrations in vivo and the outcome of induction TAD therapy



**Fig. 9.** Correlation between the areas under the curve (*AUC*) of DNM during the first 2 h after administration in vivo and the outcome of induction TAD therapy



**Fig. 10.** Correlation between the DNM uptake by leukemic blasts in vitro and the maximal intracellular DNM concentrations per dose in vivo

ro and in vivo parameters for the cellular pharmacokinetics of DNM (Fig. 10).

Daunomycin metabolites such as daunomycinol (DNMol) or aglycones were not detected in leukemic blasts.

## Discussion

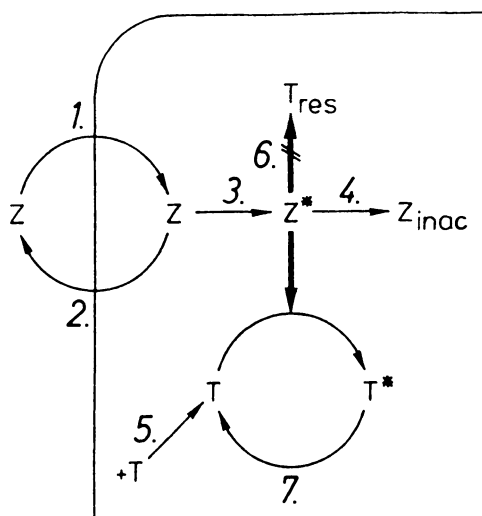
Our investigations of the cellular pharmacokinetics and metabolism of DNM in leu-

kemic blasts in the peripheral blood of patients with acute myelogenous leukemia treated according to the TAD protocol show great interindividual differences.

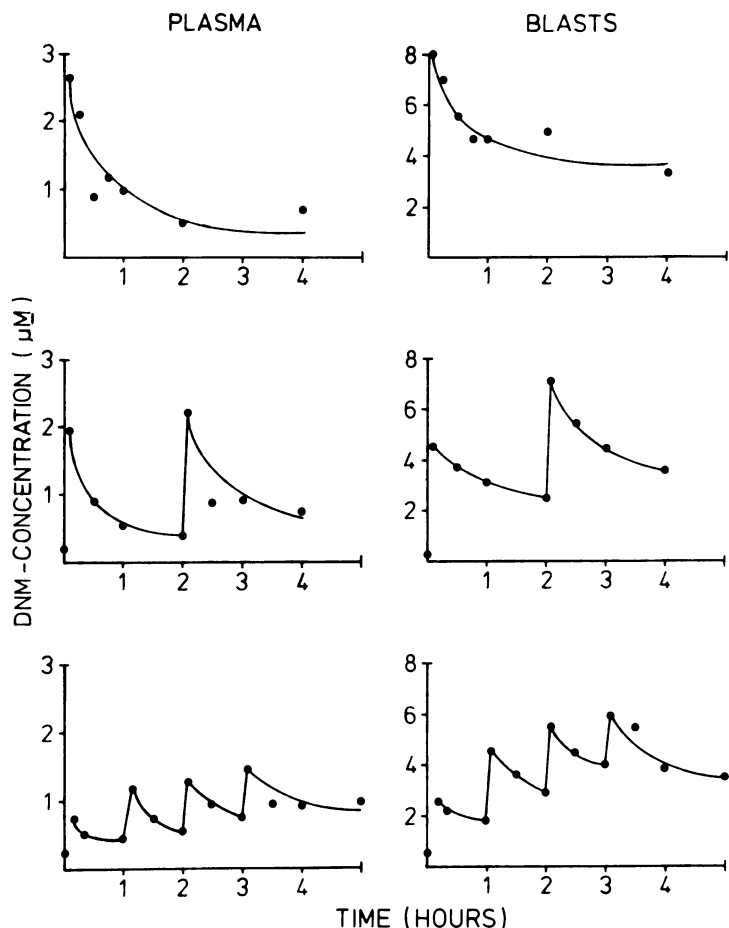
We found no significant correlation between

- in vitro pharmacokinetic parameters and the response to initial TAD therapy,
- in vivo pharmacokinetic parameters and the response to initial TAD therapy,
- in vivo and in vitro pharmacokinetic parameters.

The lack of conformity of the intracellular uptake of DNM with the outcome of treatment is in agreement with the results of Kokenberg et al. [3]. However, it has to be considered as a potential restriction of the significance of our findings that the impact of the cellular pharmacokinetics of cytosine arabinoside and 6-thioguanine combined



**Fig. 11.** Potential mechanisms of drug resistance: 1 reduction of intracellular uptake; 2 increase in extracellular efflux (e.g., multidrug resistance); 3 reduction of intracellular activation; 4 increase in intracellular inactivation; 5 increase in cellular target; 6 development of resistant cellular target; 7 increase in repair of damaged cellular target; 8 shortening of drug-sensitive phase of cell cycle; 9 reduction of number of proliferating cells. Z, drug; Z\*, activated drug; Z<sub>inac</sub>, inactivated drug; T, cellular target; T\*, damaged cellular target; T<sub>res</sub>, resistant cellular target



**Fig. 12.** Influence of the fractionation of DNM on the cellular and plasma pharmacokinetics of DNM in the same patient (*top*: 60 mg/m<sup>2</sup> on day 1; *middle*: 2 × 30 mg/m<sup>2</sup>, 0+2 h on day 2; *bottom*: 4 × 15 mg/m<sup>2</sup>, 0+1+2+3 h on day 3)

with DNM on the response to this combination chemotherapy has not been determined, in addition. Thus, only resistance to the TAD regimen is identical with DNM resistance, while response to the TAD regimen may not necessarily correspond to DNM efficacy.

Nevertheless, the feasibility of the cellular pharmacokinetics and metabolism of DNM in leukemic blasts *in vitro* and *in vivo* as a “predictive test” for the response to treatment is questionable. In the same way, though the expression of the plasma-membrane glycoprotein P170 has been demonstrated in two acute nonlymphoblastic leukemias [5], the multidrug resistant phenotype may be rare in the clinics. Thus, reduced susceptibility of acute myelogenous

leukemias may be more critically influenced by other factors (Fig. 11) [9].

However, the *intraindividual* comparison of the cellular pharmacokinetics and metabolism of DNM in leukemic blasts in the peripheral blood of patients with acute myelogenous leukemia may be a helpful tool for the assessment of

- different treatment schedules, e.g., dose fractionation (Fig. 12) or continuous infusion [11],
- concomitant cardio- or myeloprotective measures,
- concomitant chemosensibilization [8]

to ameliorate the antileukemic efficacy of anthracyclines and reduce the toxic side effects of the chemotherapy.

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## Synergistic Cytotoxicity of Cytosine Arabinoside and Mitoxantrone for K562 and CFU-GM

C. Krehmeier, M. Zühlsdorf, Th. Büchner, and W. Hiddemann

### Introduction

Clinical studies employing high-dose Ara-C combined with MX have shown this combination to be an effective treatment of refractory acute leukemia, comparing favorably with other regimens employing high-dose Ara-C [1–5]. Drugs have been used empirically with regard to their sequencing. Some investigations have focussed on optimizing drug sequencing for this kind of chemotherapy [6–9]. We sought to investigate the synergism of Ara-C and MX in an *in vitro* model using a human leukemic cell line and human CFU-GM as targets.

### Materials and Methods

The human myeloid cell line K562 was grown in RPMI 1640 medium, 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine at 37°C in a fully humidified atmosphere. Normal human bone marrow was obtained as iliac crest aspirates, separated on Ficoll-Hypaque, and washed three times with IMDM medium, 2% fetal bovine serum, penicillin, streptomycin, and L-glutamine as above. Colony assays were performed in 3% methylcellulose (Aldrich) in IMDM (GIBCO), 30% fetal bovine serum (Boehringer), 50 µM 2-mercaptoethanol (BIORAD), penicillin, streptomycin, and L-glutamine as above. For CFU-GM, 5% PHA-LCM and 1 U/ml erythropoietin (Amgen) were added.

Quadruplicate plates with 300 K562 cells or 50 000 bone marrow cells per plate were seeded. Colonies were scored on day 7 (K562) or day 14 (CFU-GM). Ara-C (Mack) and MX (Lederle) was diluted freshly in RPMI 1640 medium and 10% fetal bovine serum before each test. Cells were incubated at  $0.5 \times 10^6$  cells/ml for 24 h with Ara-C and/or 1 h with MX and washed three times before plating or applying the next drug.

### Results

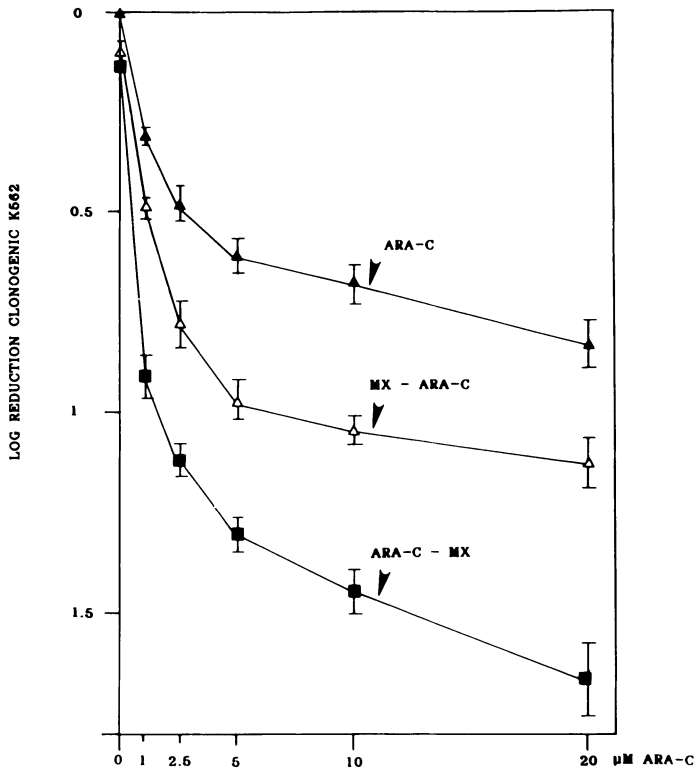
#### *Single Drugs*

K562 cells were incubated with 0–100 µM Ara-C for up to 24 h. Significant cell kill (>1 log) was observed only for incubation periods in excess of 8 h. Incubations for 24 h yielded dose-dependent cytotoxicity plateauing above 40 µM Ara-C at 1.5 logs cell reduction. K562 cells were incubated with 0–1 µM MX for 1 h. The dose response curve was nearly linear in logarithmic scale with 1.2 log cell reduction at 1 µM MX.

#### *Combinations*

K562 cells were incubated with 0–20 µM Ara-C for 24 h, washed, and exposed to 0 or 100 nM MX for 1 h after 1 h delay time. The reverse order of drugs was also tested. A cooperative index (CI) was calculated from the survival fractions (fs) with Ara-C alone, with MX alone, or with the combination:  $CI = fs(\text{Ara-C} + \text{MX}) / [fs(\text{Ara-C}) \times fs(\text{MX})]$ .

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**Fig. 1.** Combined cytotoxicity of Ara-C and MX for K562. Cells were incubated for 24 h with graded doses of Ara-C (x-axis) alone or followed or preceded by a constant 100 nM MX (1 h) after 1 h. The curves are labeled according to the sequence of the drugs. Cytotoxicity was measured in a clonogenic assay (reduction of colony numbers in logs on the y-axis)

CI < 1 showed synergism, CI = 1 summation, and CI > 1 antagonism of the cytotoxic drugs (Fig. 1). The sequence Ara-C  $\gg$  MX showed synergism with a CI of 0.23 (20  $\mu$ M Ara-C, 100 nM MX), being superior to the sequence MX  $\gg$  Ara-C, with a CI of 0.77. Results are means from three independent experiments.

A second series of three experiments tested graded doses of MX (0–2  $\mu$ M) for 1 h combined with a constant 0.5  $\mu$ M Ara-C for 24 h using a 1 h delay time. Again, the sequence Ara-C  $\gg$  MX (2  $\mu$ M MX) was superior, with a cell kill of 3.68 logs, CI 0.02, over the sequence MX  $\gg$  Ara-C with 2.64 logs kill, CI 0.31 (Fig. 2). Similar experiments on human CFU-GM also yielded higher synergism for Ara-C  $\gg$  MX (CI 0.23, 2.24 logs cell kill) with 0.5  $\mu$ M Ara-C and 10  $\mu$ M MX than with the reverse (1.44 logs kill, CI 1.11), which was even slightly antagonistic.

When graded delay times between 1 h and 8 h were tested with 0.5  $\mu$ M Ara-C (24 h) and 100 nM MX (1 h) for K562, the highest synergism was found for the sequence Ara-

C  $\gg$  MX, with 1 h delay time. This synergism could still be increased, giving MX in the last hour of an Ara-C incubation. The sequence MX  $\gg$  Ara-C showed no delay time dependence on its synergism.

In vitro testing of Ara-C and MX in a time pattern designed after the clinical high-dose Ara-C/MX (HAM) protocol gave the following results: With one complete sequence Ara-C  $\gg$  MX  $\gg$  Ara-C (3 h Ara-C (0.5  $\mu$ M), 8.5 h delay, 0.5 h MX (0–2  $\mu$ M), another 3 h Ara-C as before), 2.01 logs cell kill was achieved. For incubation with a partial sequence Ara-C  $\gg$  MX (3 h Ara-C, 8.5 h delay, 0.5 h MX), 1.73 logs kill resulted. MX for 0.5 h, followed by 3 h Ara-C, yielded 1.02 logs cell kill.

## Discussion

In vitro treatment with Ara-C and MX in the order Ara-C  $\gg$  MX was shown to be more cytotoxic for K562 and for human

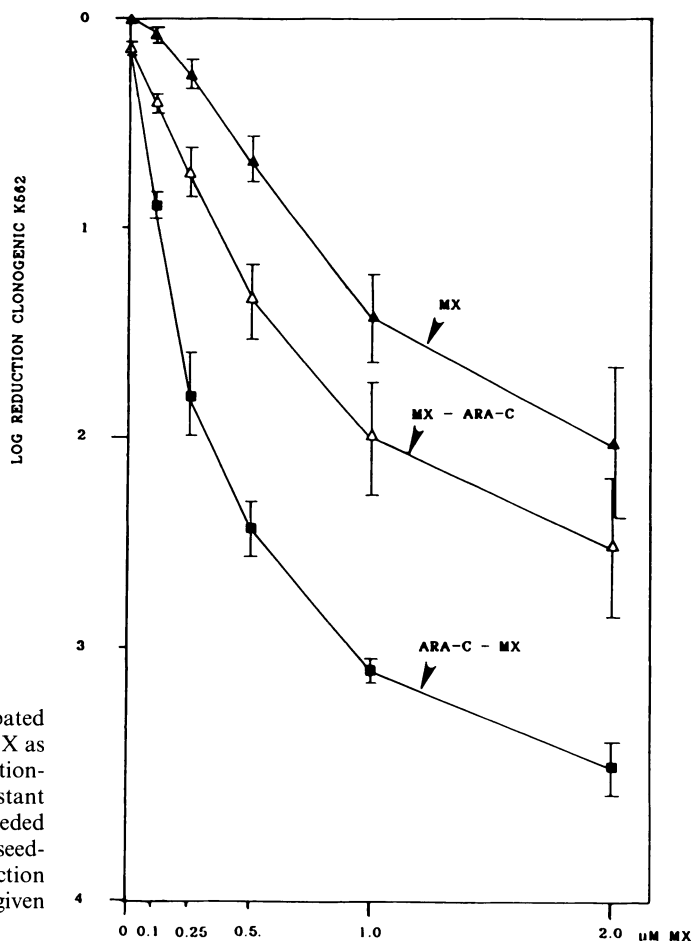


Fig. 2. K562 cells were incubated for 1 h with graded doses of MX as marked on the x-axis. An additional 24 h incubation with a constant 0.5  $\mu$ M Ara-C followed or preceded the MX exposure. Cells were seeded in a colony assay. The reduction of colony numbers in logs is given on the y-axis

CFU-GM than the reverse sequence. For the time pattern employed clinically as the HAM protocol, the partial sequence Ara-C  $\gg$  MX contributed higher synergism than MX  $\gg$  Ara-C to the whole sequence Ara-C  $\gg$  MX  $\gg$  Ara-C. Further studies will focus on the pharmacokinetic interactions of both drugs in vitro and on a biochemical basis for their synergism.

### Summary

High-dose cytosine arabinoside (Ara-C) plus mitoxantrone (MX) have proved to be effective in the treatment of refractory acute leukemia. The optimal sequence of drug administration was tested in a clonogenic assay with the leukemic myeloid cell line K562,

and with CFU-GM of normal human bone marrow. The exposure times were 24 h for Ara-C and 1 h for MX with 1 h delay between incubations. Either order of the drugs and a wide range of drug concentrations were tested. Cytotoxicity was quantified by the survival fraction (fs) of colonies scored on day 7 (K562) or day 14 (CFU-GM). Drug synergism was evaluated by a cooperative index (CI). CI < 1 indicates synergism, CI = 1 summation, and CI > 1 antagonism of the cytotoxic drugs.

In K562 the sequence Ara-C  $\gg$  MX was significantly more toxic (3.68 logs cell kill, CI = 0.02) than MX  $\gg$  Ara-C (2.64 logs kill, CI = 0.31). The highest synergism was found by adding MX during the last hour of a 24 h Ara-C exposure. For CFU-GM, Ara-C  $\gg$  MX showed higher synergistic toxicity

(2.24 log cell kill, CI=0.23) than MX  $\gg$  Ara-C (1.44 logs, CI=1.11). The clinical high dose Ara-C/MX protocol was transformed into an in vitro model and tested on K562. The highest synergism was found after the sequence of 3 h Ara-C followed by 0.5 h MX after 8.5 h delay (1.73 logs kill, whole sequence 2.01 logs kill), thus supporting the clinically applied sequence.

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## Mafosfamide Induces Less Sister Chromatid Exchange in Ph-Positive Cells Than in Normal Bone Marrow\*

R. Becher, G. Becker, and C. G. Schmidt

### Introduction

Sister chromatid exchange (SCE) is considered a highly sensitive parameter for the detection of mutagenic and/or cancerogenic effects of drugs. A close correlation between induced SCE frequency and reduction of cell proliferation and cell survival after exposure of cells to alkylating agents was described by Morris et al. [1].

This correlation provided the possibility of detecting and monitoring primary or secondary chemotherapy resistance to alkylating cytostatic drugs utilized in the treatment of malignancies. Deen et al. [2] have shown that this approach can be used to predict the clinical response in brain tumors. Furthermore, this group was able to demonstrate that significant differences in the induced SCE frequencies [3] can be used to detect clonal heterogeneity in malignant tumors.

Recently [4], we compared normal bone marrow and Ph-positive chronic myeloid leukemia (CML) cells after treatment with busulfan in vitro and observed different SCE frequencies. There was evidence of a lower susceptibility of the leukemic cells by busulfan in vitro. This observation could explain the difficulty in eradicating Ph-positive clones in CML.

Based on these results we subsequently studied mafosfamide in a similar experimental design. This drug is believed to exert a higher cytotoxic effect on leukemic cells and

therefore is currently used for the purging of autologous bone marrow grafts in acute leukemias. We wanted to answer the question whether mafosfamide would react differently than busulfan on Ph-positive CML and therefore be more effectively in the treatment of this disorder.

### Materials and Methods

#### *Patients*

Normal bone marrow was obtained voluntarily from healthy bone marrow sibling donors. Patients with Ph-positive CML were only considered for the study if they had not received any cytotoxic treatment for the previous 3 months. Normal bone marrow was obtained by iliac crest puncture. In CML patients bone marrow and also peripheral leukemic cells were used.

#### *Sister Chromatid Exchange Studies*

Cells were cultured in McCoy's medium, which was supplemented with 20% fetal calf serum (Boehringer, Mannheim, FRG) and gentamycin (Boehringer, Mannheim, FRG) in a concentration of 50 µg/ml. Cultures were set up in dark-stained glass culture flasks which were wrapped with aluminum foil in order to completely protect them from light exposure. The concentration of Bromodesoxyuridine (BrdU) (Serva, Heidelberg, FRG) used was 10 µM/ml culture medium. Total culture time was 50 h for all cases, including a final colcemide treatment

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(Gibco, Paisley, Scotland) in a concentration of 0.1 µg/ml for 2 h. Thereafter cells were treated with methanol/glacial acetic acid (3:1) and washed three times in fresh fixative. SCE staining was performed as previously reported in detail [5].

#### Sister Chromatid Exchange Frequency

Sister chromatid exchange frequency was evaluated in M2 metaphases which had incorporated BrdU through two S-phases of cell cycle. The methodology of SCE scoring included so-called peripheral and central chromatid exchanges [6]. A number of 25 M2 cells were scored for SCE frequency per case. A minimum of 15 metaphases were necessary to fulfill the criterion of evaluability (due to reduced cell growth at higher mafosfamide concentrations). Only metaphases with a modal clonal number of  $46 \pm 2$  chromosomes were scored. Leukemic cells were identified by the Ph chromosome.

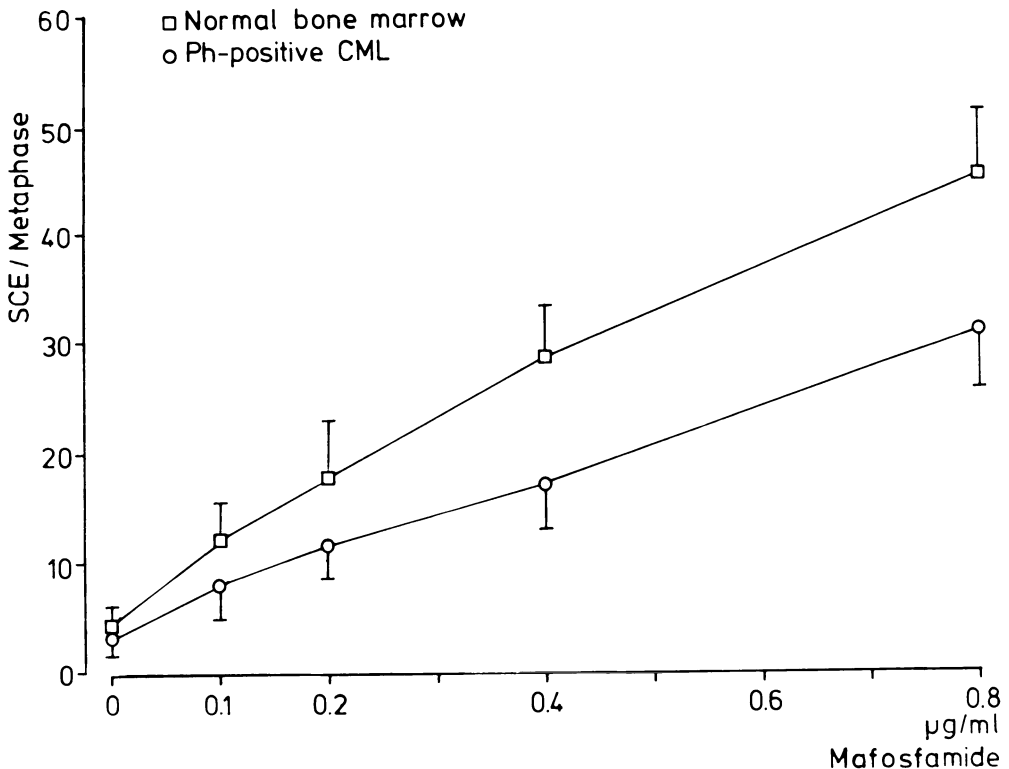
#### Mafosfamide Treatment

Mafosfamide (ASTA Z 7557) was kindly provided by ASTA-Werke Bielefeld, FRG, as a pure substance. The mafosfamide salt was dissolved in distilled water. The following mafosfamide concentrations were used in culture: 0.1, 0.2, 0.4, and 0.8 µg/ml culture medium. Mafosfamide was added at the start of cultures and remained there throughout the entire culture time.

#### Results

##### Dose Effect Curve

The SCE frequency continuously increased in a dose-dependent manner after in vitro treatment with mafosfamide in a concentration of 0.1, 0.2, 0.4, and 0.8 µg/ml in normal bone marrow (4.32–44.96 SCE/metaphase) and Ph-positive cells (3.20–30.52 SCE/metaphase) as shown in Fig. 1. The number



**Fig. 1.** Sister chromatid exchange in normal bone marrow and Ph-positive CML after treatment with increasing doses of mafosfamide in vitro

of evaluable metaphases significantly decreased with increasing doses of mafosfamide. There was only minimal cell growth above a concentration of 0.8  $\mu\text{g/ml}$ .

The correlation coefficient calculated according to Pearson confirmed a linear dose-dependent SCE increase ( $P=0.0005$ ) with a diverging course of the curves for normal bone marrow and Ph-positive metaphases. The regression coefficient as a parameter for the degree of SCE induction was 33.06 for CML and 49.49 for normal bone marrow.

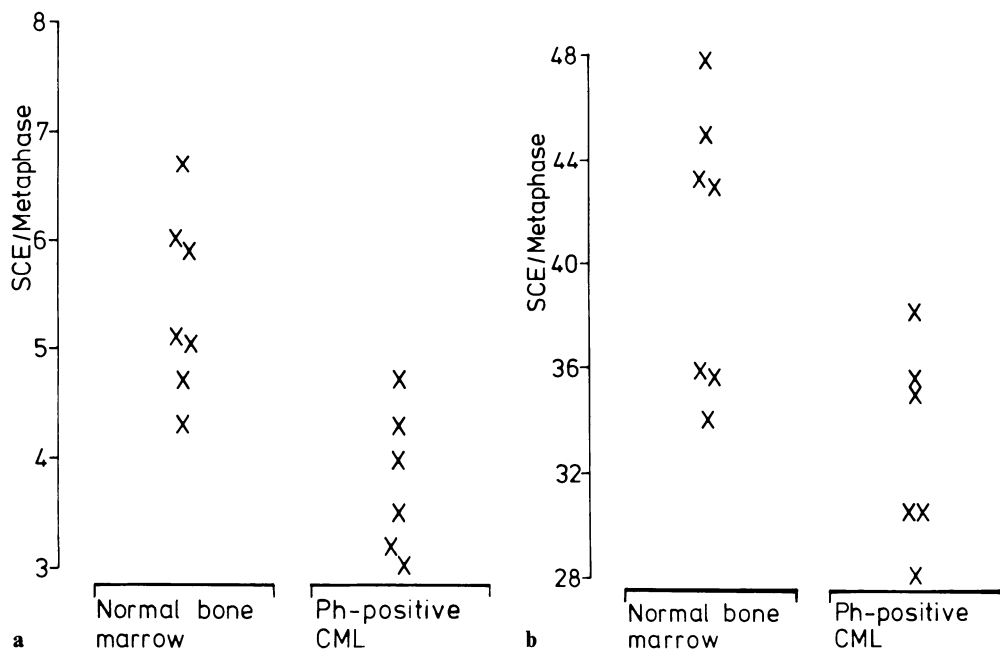
### Sister Chromatid Exchange Induction in Ph-Positive and Normal Marrow

In order to confirm the differences already detected in the dose-effect study, the induction of SCE was studied in seven additional cases of normal marrow and six cases of Ph-positive CML after treatment with 0.8  $\mu\text{g/ml}$  mafosfamide. The spontaneous SCE in Ph-positive CML ranged from 3.08 to 4.68 SCE/metaphase (median, 3.79 SCE) and in normal marrow from 4.32 to 6.72 SCE/metaphase (median, 5.41 SCE). The re-

spective data for normal bone marrow after SCE induction with mafosfamide ranged from 28.12 to 38.82 SCE/metaphase (median, 32.94 SCE) and in normal marrow from 34.00 to 47.76 SCE/metaphase (median, 40.52 SCE). The differences in spontaneous SCE ( $P<0.005$ ) and induced SCE ( $P<0.025$ ) were significant between normal and leukemic cells. The spontaneous and induced SCE frequencies are illustrated in Fig. 2 a, b.

### Discussion

Mafosfamide has been used for purging of bone marrow grafts because its cytostatic effectivity is thought to be higher on committed leukemic cells than normal stem cells. This assumption is based on in vitro data derived from murine hematopoietic stem cells, indicating a loss of aldehyde dehydrogenase during differentiation. Aldehyde dehydrogenase, present in hematopoietic stem cells, is able to inactivate mafosfamide [7], which is responsible for the marrow-sparing effect of this drug. Our study revealed that



**Fig. 2 a.** Spontaneous SCE in normal bone marrow (seven cases) and untreated Ph-positive CML (six cases). **b** Induced SCE frequency after treatment of normal bone marrow (seven cases) and untreated Ph-positive CML (six cases) with mafosfamide at a concentration of 0.8  $\mu\text{g/ml}$

the frequency of induced SCE was significantly lower in leukemic cells, suggesting less sensitivity of Ph-positive cells to mafosfamide. This result is comparable with data recently described for busulfan [4] and provides evidence that oxazaphosphorines do not seem to act differently. Therefore a karyotypic conversion of Ph-positive CML cannot be expected to result from treatment with mafosfamide.

## Summary

The frequency of induced sister chromatid exchange (SCE) is a sensitive tool for the monitoring of DNA damage and has been shown to indicate chemotherapy resistance. Mafosfamide is presently used for the purging of bone marrow in autologous bone marrow transplantation in the treatment of acute leukemia. We studied the SCE-inducing effect of mafosfamide on leukemic cells of Philadelphia (Ph)-positive chronic myeloid leukemia (CML) as a model for leukemic cells. Corresponding data from normal bone marrow were analyzed for comparison. A positive linear correlation ( $r=0.99$ ,  $P=0.0005$ ) was found between the dose of mafosfamide and induced SCE in Ph-positive CML and normal bone marrow. The concentration of mafosfamide used was 0.1, 0.2, 0.4, and 0.8  $\mu\text{g/ml}$ . Additionally, we analyzed five cases of CML and six cases of normal bone marrow. A significant difference in the frequency of induced SCE/metaphase was found between CML and normal bone marrow even after addition of 0.8  $\mu\text{g/ml}$  mafosfamide. Also, spontaneous SCE was significantly lower in CML. Our data indicate a lower sensitivity of the

leukemic cells to mafosfamide as shown by the induction of a lower frequency of SCE events.

*Acknowledgment.* This paper contains part of the data of the dissertation of G. B. We thank G. Zimmer for expert technical assistance.

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## Blood Concentration of Ascorbyl-Free Radical in Children with Acute Lymphoblastic Leukemia: Preliminary Report

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In spite of the introduction of new diagnostic tools in acute lymphoblastic leukemia (ALL), monitoring of the disease is still traditional. It is based on clinical observation and on careful evaluation of peripheral blood and bone marrow smears in search of paralympoblastic cells. Several investigators reported that measurement of terminal deoxynucleotidyl transferase activity in extracts of isolated mononuclear cells may be a useful adjunct in monitoring therapy, but this method is not applicable to all cases of ALL [2, 5]. In a series of publications, Lohmann et al. suggested that the concentration of ascorbyl-free radical (AFR) plays an indicative and informative role in patients with ALL [10, 11]. Being inspired by his observation, we measured electron spin resonance (ESR) spectra of blood in children with ALL and healthy individuals.

### Material and Method

#### *Patients*

The study comprised 13 children diagnosed as having ALL. In all cases the diagnosis of ALL was made on the basis of morphological criteria according to the French-American-British (FAB) classification [1]. All patients were treated with the same chemotherapeutic regimen – the Berlin-Frankfurt-Münster 79/81 protocol [6] modified by the Polish

Leukemia Study Group in May 1983. Patients were subdivided into three categories:

Group I: Six children at the onset of disease, before the introduction of therapy

Group II: The same six children in the phase of early remission, within 3–4 weeks from the beginning of therapy

Group III: Seven children during the phase of sustained remission, on maintenance therapy

The control group consisted of ten healthy voluntary blood donors. For at least 7 days before blood collection supplementary vitamin C was not given to any of the cases under investigation.

#### *Method*

One milliliter of venous blood was drawn into the plastic tube with heparin, shaken, and transferred to the Pathophysiology Department within 30 min. Samples of 0.5 ml were immediately desiccated in a vacuum at a temperature of 310 K. Dried samples were ground and then exposed to air. Twenty milligrams dried tissue was inserted into the quartz glass tube (5 mm in diameter) and placed in the cylindrical resonance cavity of a spectrometer. ESR spectra were obtained with the use of a Radiopan SE/X 2544 spectrometer at 9.45 GHz (3 mW) with 100-kHz modulation of 2.5 mT amplitude, a scan of 20 mT, and a time scan of 4 min. Diphenylpicrylhydrazyl (DPPH) standard ( $g = 2.0036$ ) was used as a reference for marking the resonance position ( $g$ ) and of spin concentration (spin/gram). All measurements

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were performed after 1 h from the end of desiccation. Results were statistically evaluated with Student's *t*-test.

## Results

Data presented below relate to desiccated samples. Typical ESR spectra of blood in healthy volunteers and in children with ALL are shown in Fig. 1. According to data taken from the literature, ESR spectra observed in our study were mainly caused by AFR [3]. We never observed the so-called leukemic peak, described by Lohmann et al. [12]. Blood from healthy volunteers showed the single, slightly asymmetrical signal characterized by the following parameters:  $g = 2.005$ ;  $\Delta B_{pp} \approx 1$  mT; mean spin concentration =  $0.92 + 0.14 \times 10^{16}/g$  desiccated blood. In all categories of children with ALL (groups I, II, and III) ESR spectra formed single, slightly asymmetrical signals which were characterized by the following parameters:  $g = 2.005$ ;  $\Delta B_{pp} \approx 0.8$  mT. Mean spin concentrations for the groups studied were as follows: group I:  $1.40 + 0.21 \times 10^{16}/g$  desiccated blood; group II:  $1.41 + 0.41 \times 10^{16}/g$  desiccated blood; group III:  $1.19 + 0.28 \times 10^{16}/g$  desiccated blood. Spin concentrations per gram desiccated blood in all studied cases are presented in Fig. 2. Mean values of spin concentration for studied groups were significantly higher

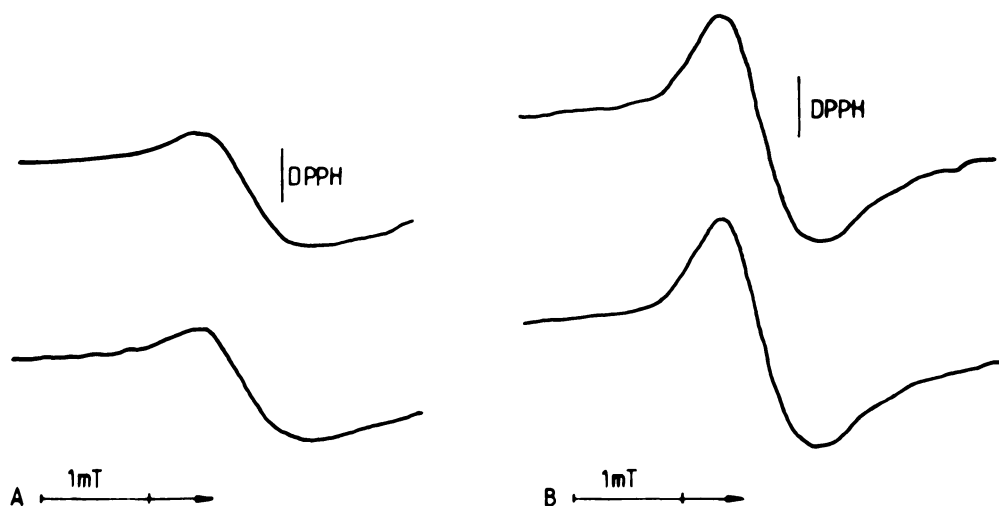
**Table 1.** Statistical evaluation of differences in spin concentration between the groups studied (Student's *t*-test)

	Group I	Group II	Group III
Control	$t = 5.598$ $P < 0.001$	$t = 3.80$ $P < 0.001$	$t = 2.647$ $P < 0.01$
Group III	$t = 1.355$ $P > 0.2$	$t = 0.048$ $P > 0.9$	

than in controls. There was no statistical difference between values in studied groups, but the tendency toward decreased values in sustained remission could be seen. Results of statistical evaluation with Student's *t*-test are presented in Table 1.

## Discussion

Increased free radical concentration in malignant disease has been observed by many investigators [4, 7, 9, 12]. It has also been seen in patients with ALL [10, 11]. The question of whether it is the cause or the result of malignancy is still open for discussion. Little is known of the nature of this phenomenon. It has been shown that the observed in vitro ESR signal is caused by adsorbed AFR [3, 8]. Lohmann et al. suggest that AFR is adsorbed by the copper protein of the erythrocyte membrane [12]. The precursor sub-



**Fig. 1.** Typical ESR spectra obtained from blood of children with ALL (B) and healthy volunteers (A)

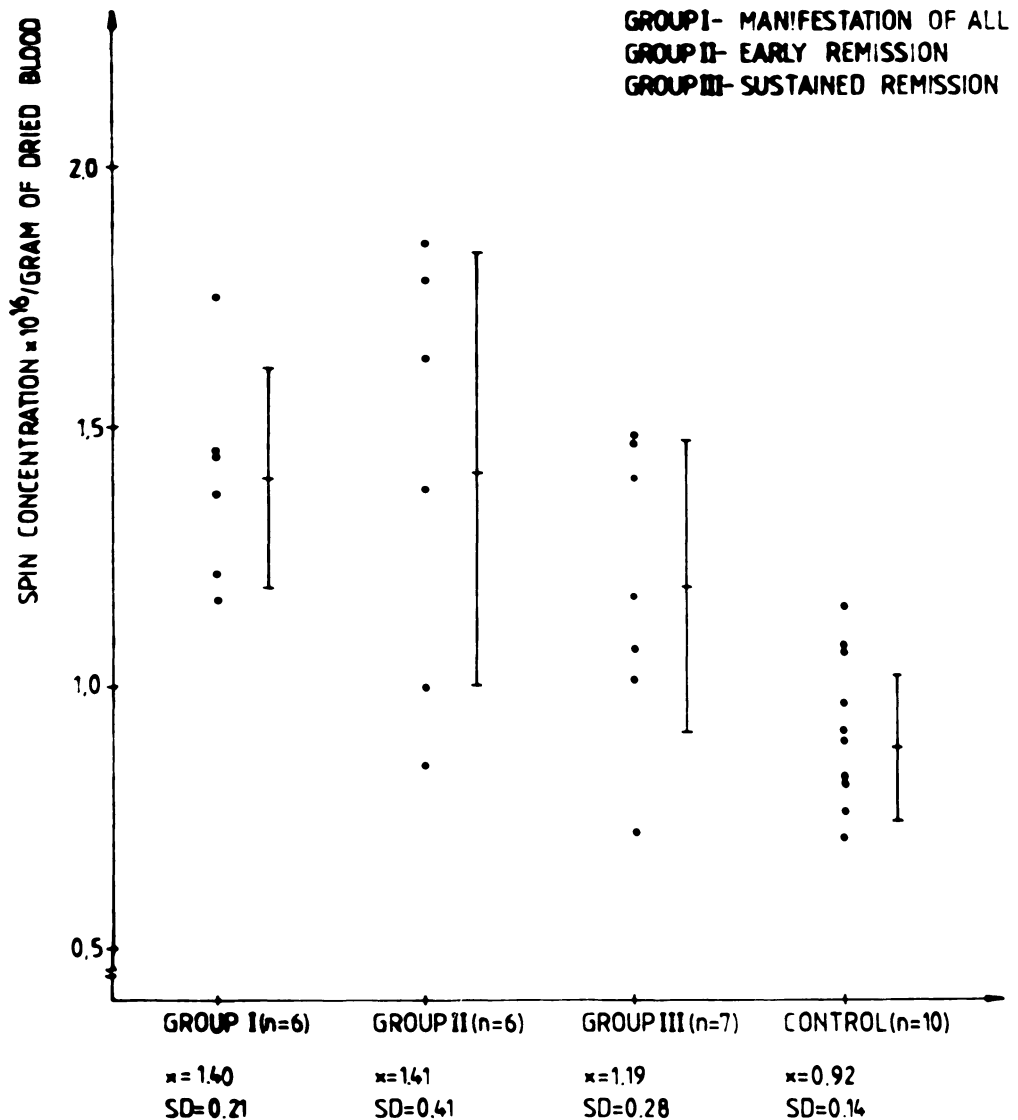


Fig. 2. Spin concentration  $\times 10^{16}/g$  dried blood in the groups studied

stance of AFR in in vitro conditions is vitamin C. Thus increased ESR signal observed in vitro in ALL should relate to increased in vivo concentration of vitamin C. This can be caused by the decreased activity of ascorbate oxidase and/or by a modification of the erythrocyte membrane receptor. The steady state of the ascorbate system can be described as follows: ascorbic acid  $\rightleftharpoons$  inert AFR  $\rightleftharpoons$  dehydroascorbic acid. Increased concentration of vitamin C should therefore

lead to increased concentration of inert AFR. It is compatible with the hypothesis of a correlation between ESR signal strength of the air-exposed lyophilisates and pathological states, where the air oxidation parallels and amplifies the situation existing in vivo [7]. This preliminary report supports the opinion that in vitro concentration of AFR in cases of ALL is significantly higher than in healthy individuals. We dare not consider it as a factor indicating the stage of disease,

but the observation that there is a statistical tendency toward decreased values in sustained remission seems to be of interest. If confirmed by further studies, the measurement of AFR concentration in the blood of children with ALL could be useful in therapy monitoring.

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## Association of GP40/CD7<sup>+</sup> Acute Myeloblastic Leukemia and Chromosome 5 Aberrations

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### Introduction

Immunological investigation of human leukemias revealed conservation of lineage specificity in the majority of cases. However, deviations of maturation-linked phenotype (i.e., asynchronous differentiation compared with normal hematopoietic progenitors) may be found in leukemic cells at diagnosis, especially in acute myeloblastic leukemia (AML) compared with acute lymphoblastic leukemia (ALL). Nonetheless, the "coexpression" of markers belonging to different but related lineages confirms the potential of interlineage infidelity of gene expression in acute leukemia [1].

Nuclear terminal deoxynucleotidyltransferase (TdT) and T-lineage-associated gp40/CD7 expression were originally regarded as lymphoid-specific markers as they are already found on very early cells of the B- and T-cell lineages. However, the existence of TdT and myeloid markers in the same cell or weak expression of the CD7 molecule on colony-forming cells (CFU-GM, GEMM, CFU-Me, BFU-E) has been unambiguously demonstrated [1, 2]. Therefore, it has been proposed that early lineage-associated markers may be transiently coexpressed on bi- or multipotential stem cells before achieving irreversible lineage commitment. Accordingly, coexpression of, e.g., TdT or

gp40/CD7, on AML cells may identify a more immature cell population.

### Results and Discussion

In a retrospective analysis of 183 patients (all tested cytogenetically, 180 for TdT and 138 for gp40/CD7), we found that leukemic cells from about 30% of all adult AML cases "coexpress" either gp40/CD7 (17%) or TdT (14%), or both (3%). Neither hematological nor clinical features of these patients differ significantly from their (TdT or gp40/CD7) negative counterparts. However, TdT positivity is more frequently observed in FAB:M1 than in the other FAB subtypes (25% vs. 11%) and in CD15-negative than in CD15-positive AML (27% vs. 12%). On the contrary, gp40/CD7 expression is more often associated with FAB:M5 than with the remaining FAB subtypes (26% vs. 16%) and with the expression of CD11b (CD11b<sup>+</sup> AML: 24% gp40/CD7<sup>+</sup> vs. CD11b<sup>-</sup> AML: 10% gp40/CD7<sup>+</sup>) (data not shown). Except for these weak correlations so far no other features have characterized AML subtypes positive for gp40/CD7 expression or for TdT.

Cytogenetic analysis obtained from 20 patients with TdT<sup>+</sup> AML and from 19 patients with gp40/CD7<sup>+</sup> AML revealed some differences between these subgroups. In only half (ten patients) of the TdT<sup>+</sup> AML (Table 1) was an abnormal karyotype demonstrated. In nine of these ten patients a single chromosomal aberration was seen, three of them being typical for AML. Complete remission is more often achieved in TdT<sup>+</sup>

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**Table 1.** Hematological and clinical data of 20 patients with TdT<sup>+</sup> AML

Sex	Age (years), median (range)	FAB classification	Karyotype	Response (CR/N)	Survival (months), median (range)
3 M/7 F	62 (26–81)		“Diploid”	5/10	10.5 (4–19)
F	51	M4	46XX	CR	12
F	51	M2	46XX	CR	11
M	26	M1	46XY	CR	18
F	69	M1	46XX	PR	7
F	71	M4	46XX	CR	5
F	56	M2	46XX	NT	5
F	41	M5	46XX	CR	19
M	72	M1	46XY	PR	10
F	77	M1	46XX	PR	4
M	81	M1	46XY	PR	13
6 M/4 F	59 (37–77)		“Aneuploid”	2/10	3.5 (1–12)
F	37	M2	45X	NT	9
F	45	M5	46XX, der3	CR	12
M	68	M2	47XY, +8	F	8
F	77	M4	47XX, +8	NT	1
M	40	M4	46XY, t(1;16)	ED	1
M	55	M2	45XY, t(6;17)	F	3
M	72	M4	47XY, +11	F	4
F	71	M2	46XX, 15q–	NT	1
M	63	M1	46XY, 21q+	F	3
M	46	M2	Hypodiploidy	CR	11

AML without abnormal karyotype but long-term survivors (> 3 years) were not observed in the whole group.

In 13 of 19 patients with gp40/CD7<sup>+</sup> AML (Table 2) an abnormal karyotype was observed, 6 of them presenting a single chromosomal aberration and 7 a complex one. Most interestingly, in seven of these cases chromosome 5 was involved in the aberration. Most frequently (five cases) we observed a monosomy 5 in addition to other aberrations (complex karyotype abnormality). None of these elderly patients (49–74 years) achieved complete remission after induction treatment (TAD or “3+7”). Even though response to therapy was poor in the remaining patients with gp40/CD7<sup>+</sup> AML, prognosis may more likely be related to age and aberrant karyotype than to the expression of gp40/CD7. Again, long-term survivors (> 3 years) were not observed in this group of patients.

Within the entire group of 183 AML patients tested, 18 displayed an aberrant karyotype involving chromosome 5 (Table 3).

Even considering the age of these patients, their response to induction therapy was usually poor (4 complete remissions out of 18). Seven of these 18 AML patients expressed gp40/CD7. Due to the small percentage of chromosome 5 anomaly or of gp40/CD7 expression in AML the correlation between both is weak, but even so was statistically significant ( $P < 0.05$ ).

We therefore assume that leukemic cells with an aberrant karyotype involving chromosome 5 or the loss of one chromosome 5 may be associated with the expression of the surface antigen gp40/CD7 on these cells. Genes coding for a number of hemopoietic growth factors and for the M-CSF receptor are located on the long arm of chromosome 5. These become responsible for growth and differentiation of various hemopoietic cells including some of their leukemic counterparts. Regarding mechanisms such as autocrine stimulation determining growth behavior or differentiation stage, we suggest that the loss of chromosome 5, upon which the genetically active genes for IL-3, GM-

**Table 2.** Hematological and clinical data of 19 patients with gp40/CD7<sup>+</sup> AML

Sex	Age (years), median (range)	FAB classifi- cation	Karyotype	Response (CR/N)	Survival duration (months), median (range)
1 M/5 F	70 (25–80)		“Diploid”	2/6	3.5 (1–35)
F	52	M5	46XX	ED	1
F	25	M2	46XX	CR	35
F	69	M1	46XX	PR	7
F	71	M5	46XX	F	1
F	71	M4	46XX	CR	5
M	80	M1	46XY	F	2
7 M/6 F	63 (23–79)		“Aneuploid”	4/13	4 (1–26)
F	63	M4	46XX, 5q–	PR	15
M	68	M2	47XY, +8	F	8
M	37	M2	46XY, t(3;5)	CR	1+
F	69	M4	46XX, inv16	CR	4
F	32	M5	46XX, 21q+	ED	1
M	29	M2	45XY, –22	CR	21
M	23	M4	47XY, inv16, +22	CR	26+
M	79	M1	46XY, 1q+, –2, +mar	ED	1
F	72	M2	49XX, –5, +15, +mar1–3	F	2
M	73	M4	46XY, –5, –16, +mar1, +mar2	F	4
F	49	M5	45XX, der3, –5, +8, t(8;22), +11, –12, –17	F	1
M	74	M1	45–49XY, –2, der3, –5, 6p–, 9q–, PR +mar1, +1–3mar2	PR	4
F	56	M2	45XX, der2, –5, t(12;19), 22q–, (+r5)	F	2

**Table 3.** Chromosome 5 anomalies in 183 cases of AML compared with TdT or gp40/CD7 positivity

	TdT		gp40/CD7		Age (years), median (range)	Sex	CR	Remission duration (months)	Median survival (months)
	+	–	+	–					
Chromosome 5 anomaly	1	17	7	11	68 (21–81)	9M/9F	4/18	1, 1+, 2, 4+	3
Mono 5	1	8	5	4	66 (21–74)	5M/4F	1/9	2	2
5q–	0	8	1	7	68 (57–81)	3M/5F	2/8	1, 4+	8
Other	0	1	1	0	37 –	1M/–	1	1+	2+

CSF, M-CSF, and M-CSF receptor are localized, may cause an immunophenotype of immature cells including coexpression of gp40/CD7 in AML [3–6]. To our knowledge, this is the first time that the association between an immunophenotypic (gp40/CD7) and cytogenetic (chromosome 5 aberration) feature has been detected in AML.

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## Acute Monocytic Leukemia with Translocation t(1;11) (p31;q23): Simultaneous Staining of Chromosomes and Cell Surface Antigens\*

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### Introduction

Leukemia is thought to be the clonal expansion of a single mutated cell. Depending on the differentiation stage of this cell, different cell lineages might be involved in tumorigenesis. Keinänen et al. [2] have shown that myeloid, erythrocytic, and megakaryocytic lineages were involved in 8 out of 12 cases of acute myeloid leukemia with monosomy 7 or trisomy 8. In order to evaluate the lineage involvement in a case of acute monocytic leukemia with trisomy 8 and a translocation t(1;11), we modified a method described by Teerenhovi et al. [1]. With this method it is possible to analyze cell surface antigens and chromosomes of the same cell, whereas by standard chromosome preparation the cell membrane and cytoplasmic structures are destroyed.

### Methods

**Case Report.** We studied metaphases of bone marrow and blood cells of a 76-year-old patient with acute monocytic leukemia French-American-British Classification (FAB) M5a. The patient showed typical symptoms of acute leukemia such as bone

pain, fever, bleeding, and weakness. No typical symptoms of acute monocytic/blastic leukemia with extramedullary involvement were seen. The patient refused to have polychemotherapy carried out. He died 2 months after diagnosis.

**Cytogenetic Studies.** Bone marrow and blood of the patient were cultured for 1–4 days without additions. Chromosome preparation was performed according to the standard air-drying technique [3]. Conventional Giemsa staining was followed by sequential R- and Q-banding [4, 5].

**Cytogenetic and Immunological Studies on the Same Cell.** Bone marrow cells were cultured as described above. After culturing cells were swollen with a mild hypotonic solution according to Teerenhovi et al. [1]. One milliliter of cell suspension and 1–2 ml hypotonic solution were gently mixed. After standing 10–30 min at room temperature, the cells were centrifuged onto glass slides with a cytocentrifuge (400 rpm for 10 min) and air dried for 1 h at least. Cells were fixed in chloroform for 30 min and acetone per analysis for 15 min at room temperature. Slides were stored at  $-80^{\circ}\text{C}$  before antigen staining. Cell antigens were investigated with monoclonal antibodies in a modification of the alkaline phosphatase (monoclonal) anti-alkaline phosphatase staining method [6]. New fuchsin was used as substrate. Monoclonal antibodies against monocytes (KiM2, KiM5, KiM6) were made by Radzun et al. [7–9]. CD3 and anti-common acute lymphoblastic leukemia antigen (CALLA) were taken to identify cells from the lymphocytic lineage, anti-glycophorin A

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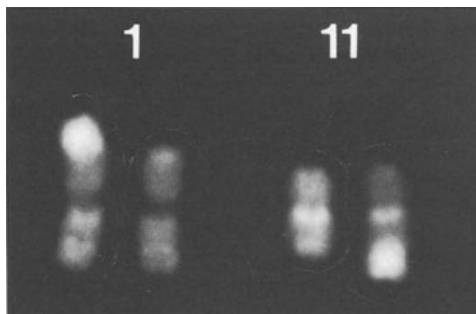
to identify cells from the erythrocytic lineage, and anti-factor VIII was used to identify cells from the megakaryocytic lineage (all from Dakopatts).

Counterstaining was carried out with hemalum. Reaction of the mitoses with the monoclonal antibodies was documented. For subsequent chromosome banding the APAAP substrate color was removed with xylene, the cells were refixed for at least 1 day with methanol: acetic acid (3:1), and the fluorescence R-banding technique (chromomycin A3/methylgreen) was performed [3].

## Results

Thirty-five mitoses from bone marrow and 20 mitoses from peripheral blood revealed the abnormal karyotype 47,XY,+8,t(1;11)(p31;q23) (Fig. 1).

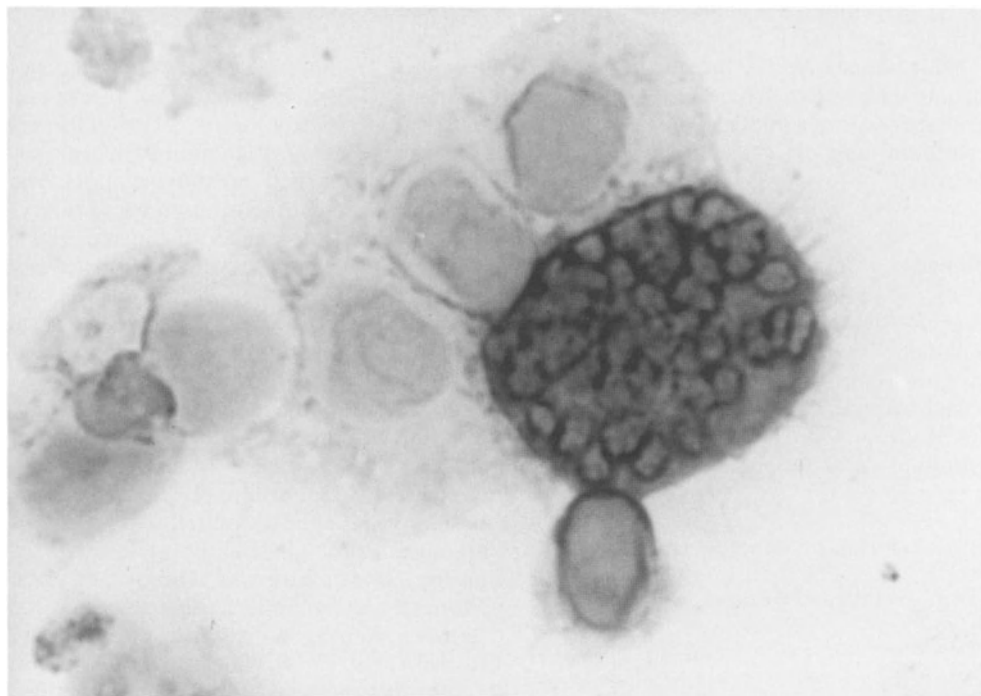
*Demonstration of Cell Surface Antigens and Chromosomes of the Same Cell.* On each



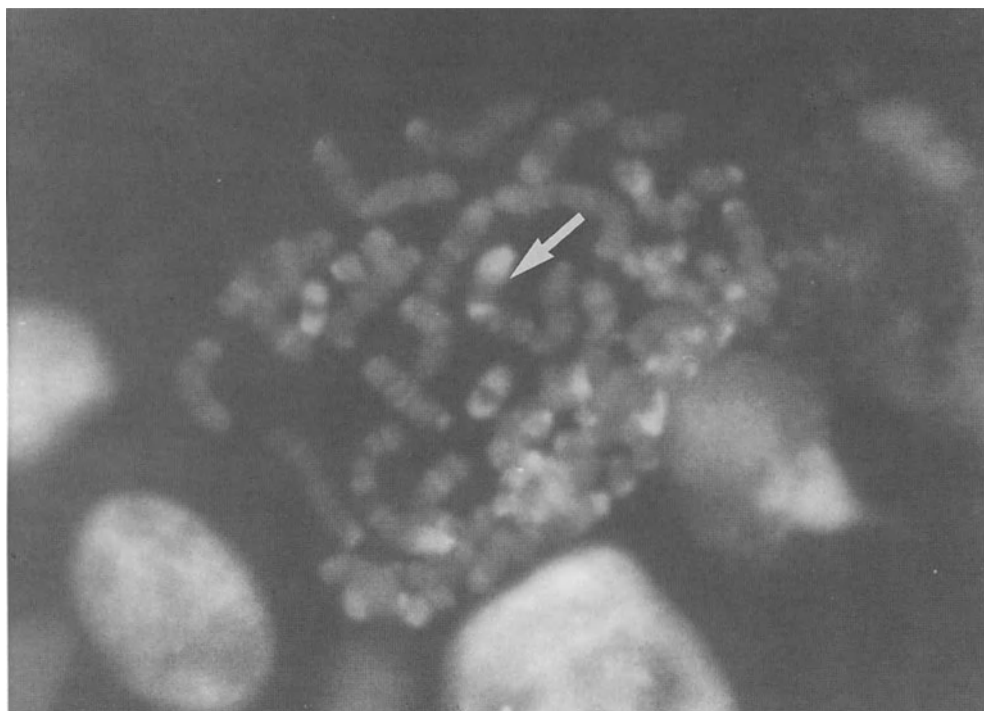
**Fig. 1.** Partial karyotype of chromosome 1 and 11 showing reciprocal translocation t(1;11)(p31;q23), R-banding technique

glass slide about seven to ten mitoses were found. It was not possible to analyze all mitoses completely, but the chromosome 11q+ was seen in nearly all mitoses as a marker of the tumor clone (Fig. 2).

*Myelomonocytic Antigens.* Nearly all mitoses (22/28) showed a positive reaction with monocytic antibodies KiM2, KiM5, and KiM6 (Fig. 3).



**Fig. 2.** 11q+ marker (arrow) is visible even in mitoses which are not optimally spread because of the mild hypotonic treatment, R-banding technique



**Fig. 3.** APAAP technique: mitosis expressing monocytic antigens detected by monoclonal antibody KiM6

*Other Cell Surface Antigens.* No mitoses showed a positive reaction with monoclonal antibodies against cell antigens of other cell lineages (glycophorin A, factor VIII, CD3, CALLA).

### Discussion

In a case of an acute monocytic leukemia (FAB M5a) we found a trisomy 8 and a translocation  $t(1;11)(p31;q23)$ . The region 11q22–25 is often involved in reciprocal translocations with different donor chromosomes [10, 11]. Abé et al. [12] have pointed out that the distal breaks in 11q22–25 are more common in the very young. The patient presented here, however, was 76 years old. So far four cases of  $t(1;11)$  with involvement of the long arm of chromosome 1 in different types of acute myeloid leukemia have been described [13–16]. To the best of our knowledge this is the first case with involvement of the short arm of chromosome 1 in a  $t(1;11)$  in acute myeloid leukemia.

On one hand, the breakpoint 11q23 is interpreted to be associated with monocytic leukemia [10]. The breakpoint was found even in a blast crisis of CML with monoblastic differentiation [17]. On the other hand, 11q23 breakpoints are also described in acute lymphoblastic leukemias [18, 19]. So far three cases of  $t(1;11)(p31-33;q23)$  in acute lymphoblastic leukemia have been published [11, 20]. Therefore translocations involving 11q23 could be related to the proliferation of a progenitor cell that may differentiate into a lymphoid or nonlymphoid cell. Kaneko et al. [11] have suggested that the differentiation may depend on the chromosome which is involved in the reciprocal translocation with 11q. Our case of a monocytic leukemia, however, cytogenetically showed the same breakpoints as the three cases of lymphoblastic leukemia with  $t(1;11)$  described above.

This case demonstrates only an involvement of the monocytic cell lineage in tumorigenesis. No other lineage involvement was detected. One can conclude that the tumor

has developed from a myelomonoblastic progenitor cell, which has lost the ability to differentiate into the lymphoid, erythrocytic, or megakaryocytic lineages.

## Summary

Cytogenetic analysis of leukemic cells from a 76-year-old patient with acute monocytic leukemia revealed the karyotype 47,XY,+8,t(1;11)(p31;q23). To the best of our knowledge this is the first case with involvement of the short arm of chromosome 1 in a t(1;11) in acute nonlymphocytic leukemia. In order to determine which hematopoietic cell lineages are involved in this case, we used a method to demonstrate chromosomes and cell surface antigens of the same cell [1]. To identify mitoses as monocytic, erythrocytic, megakaryocytic, or lymphocytic, cell surface antigens were stained with monoclonal antibodies in an APAAP detection procedure. Subsequently, an R-banding technique was performed. About 80% of the abnormal mitoses expressed monocytic markers. No erythrocytic, megakaryocytic, or lymphocytic mitoses were found. Only an involvement of the monocytic cell lineage was revealed.

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## Prognostic Significance of Chromosome Analysis in De Novo Acute Myeloid Leukemia\*

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### Introduction

Since the first report by Sakurai and Sandberg [1] in 1973, indicating a better prognosis for patients with a normal karyotype (NN) or a mixture of normal and abnormal mitoses (AN) than for patients with only abnormal mitoses (AA), several other large cytogenetic studies [2–4] have confirmed these prognostic findings in patients with de novo acute myeloid leukemia (AML). But there are also some reports denying such a prognostic value of the chromosomal status NN, AN, AA [5–7] and in more recent studies [8–10] the prognostic value of the types of chromosomal aberrations rather than the NN-AN-AA classification has been emphasized.

In 1981 we undertook a prospective cytogenetic study with the aim of evaluating the prognostic value of the karyotype in patients with de novo AML. So far, 177 patients have entered this study.

### Patients

Between 1981 and 1988, 177 patients with de novo AML were studied. Criteria for study inclusion were: no myelodysblastic syn-

drome (MDS) preceding AML and no prior chemo- and/or radiotherapy for a primary malignancy. In 31 patients no specific treatment was administered because of advancing age, poor performance status, or serious comorbidity. One hundred and forty-six patients were treated by aggressive combination chemotherapy, most of them ( $n=108$ ) by the TAD or TAD/HAM regimens. The prognostic significance of chromosome analysis was only evaluated in these patients who underwent severe treatment. The clinical data of these 146 patients were: 129 adults and 17 children, 74 males and 72 females; mean age, 43 years French-American-British (FAB) classification: m1 ( $n=29$ ), m2 ( $n=36$ ), m3 ( $n=4$ ), m4 ( $n=44$ ), m5 ( $n=23$ ), m6 ( $n=4$ ), and m7 ( $n=5$ ).

### Chromosome Analysis

Chromosome analysis was performed according to standard techniques (culture time, 24 or 48 h without cell synchronization, G-banding) prior to any specific antileukemic therapy. Chromosomes were classified according to the ISCN criteria. The karyotype was further classified as NN (only normal mitoses), AN (mixture of normal and abnormal mitoses), and AA (only abnormal mitoses).

### Results

Overall incidence chromosomal anomalies in the 146 severely treated patients was 53% (77/146). The aberrations most often found

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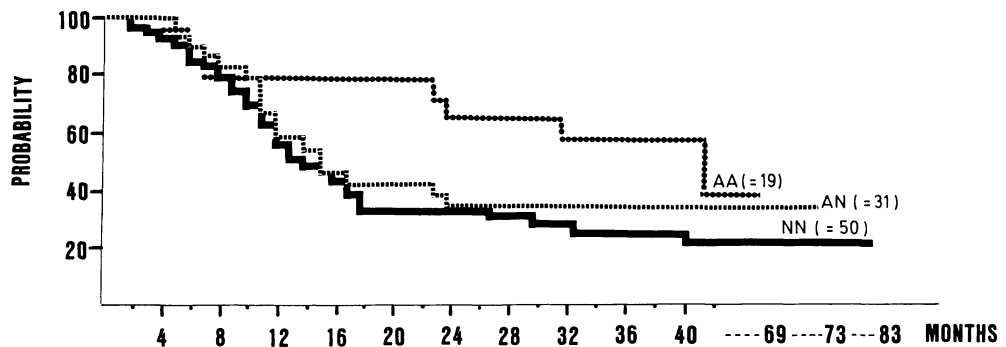
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\* This study was supported by the Hamburger Krebsgesellschaft

**Table 1.** Prognostic significance of karyotype in 146 patients treated by combination chemotherapy according to the NN, AN, AA classification and the types of chromosomal aberration

Karyotype	CR rate (%)	(Median) duration of CR (months)	(Median) survival time (months) <sup>a</sup>
NN	72 (50/69)	7	14
AN	70 (31/44)	7	15
AA	58 (19/33)	30	42
P	0,3 (NS)	0,01	0,18 (NS)
t(8;21)	100 (11/11)	40	43
inv (16)	100 (4/4)	6+, 13, 22, 62+	7+, 23, 24, 64+
Simple trisomies (+4, +6, +8, +11)	71 (12/17)	3, 3+, 4+, 5, 6, 6+, 9+, 9+, 30+, 32+, 34+, 39	4, 5+, 6, 6+, 8+, 11+, 11+, 12, 32+, 34+, 36+, 73+
t(15;17)	66 (2/3)	6, 6+	9+, 14
12p-	66 (2/3)	6, 10	11, 12+
-7/7q-	50 (4/8)	4, 5, 12, 14+	5, 11, 16+, 48+
#11q23	33 (2/6)	10, 58+	12+, 60+
Complex	23 (3/13)	1+, 4, 13	2+, 17, 36+
-5/5q-	0 (0/4)	-	-

<sup>a</sup> From the date of diagnosis, only for patients who entered CR



**Fig. 1.** Probability of survival according to the chromosomal status NN, AN, AA

were: complex chromosomal aberrations ( $n=13$ ), t (8;21) ( $n=11$ ), -7/7q ( $n=8$ ), anomalies involving 11q23 ( $n=6$ ), +8 ( $n=6$ ), -5/5q-, inv (16), +11, +6 ( $n=4$ ), 12p-, t (15;17), +4 ( $n=3$ ). The NN-AN-AA classification revealed: NN = 47% (69/146), AN = 30% (44/146), and AA = 23% (33/146).

Complete remission (CR) rate and median survival were not significantly influenced by the NN, AN, AA classification (Table 1, Fig. 1), whereas median duration of CR was significantly longer in AA patients than in NN or AN patients, but this was probably due to the high proportion of patients with t (8;21) among the AA group (7/19). On the

other hand, some types of chromosomal aberration were of prognostic value, with t (8;21) and inv (16) being associated with high and complex anomalies as well as -5/5q- with low CR rates (Table 1). The number of patients studied with these anomalies is still too limited to draw any prognostic conclusions with regard to CR duration and survival.

## Discussion

The chromosomal aberration rate of 53% and types of anomalies in this study are comparable to those in other reports [2-7,

9]. In contrast to other authors [1–4], we could not find any prognostic significance of the chromosomal NN, AN, AA classification. Neither CR rate nor survival time of our patients was influenced by it and the longer CR duration of our AA patients was certainly due to the high proportion of t (8;21) patients in this group. We have no clear explanation for this discrepancy, but technical reasons, more aggressive chemotherapy, and better supportive care during the past years may be important factors.

On the other hand, the types of chromosomal aberrations may be of major prognostic value. As in other studies [8–10], we found a relatively good prognosis in patients with t (8; 21) or inv (16) and a poor outcome in those with complex aberrations. Only large prospective cytogenetic studies can clarify the possible prognostic significance of other well-known anomalies in AML, such as t (15; 17), trisomies 4 and 11, and aberrations involving 11q23.

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## Chromosomal Aberrations in Childhood Acute Nonlymphoblastic Leukemia\*

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### Introduction

Cytogenetic analysis has become an important factor in the characterization of a leukemic cell population in addition to morphological, immunological, and cytochemical criteria [1]. Blast cells of patients with acute nonlymphoblastic leukemia (ANLL) show specific karyotypic changes, which are of diagnostic and probably prognostic value concerning the course of the disease. We report here the cytogenetic investigation of children with ANLL during a 5-year period (January 1984 – December 1988). Most of the patients were treated according to the protocol of the AML-BFM study group.

### Material and Methods

Chromosome analysis was performed on mailed specimens of heparinized bone marrow and peripheral blood using standard techniques. Bone marrow cells were washed twice in RPMI culture medium 1640 and then prepared directly and/or cultured for 24 h in RPMI 1640 + 20% fetal calf serum (FCS) including synchronization with methotrexate (MTX) for 17 h. Colcemide (10 µg/ml) was added for the last 10 min of

culture, followed by hypotonic treatment with a 0.075-M KCl solution and a final fixation in methanol/acetic acid (3:1) (six to eight times). Metaphase chromosomes were G-banded according to the method of Seabright [2]. Karyotyping followed the criteria of the ISCN [3]. An abnormal clone was defined according to the recommendations of the First International Workshop on Chromosomes in Leukemia [4].

### Results

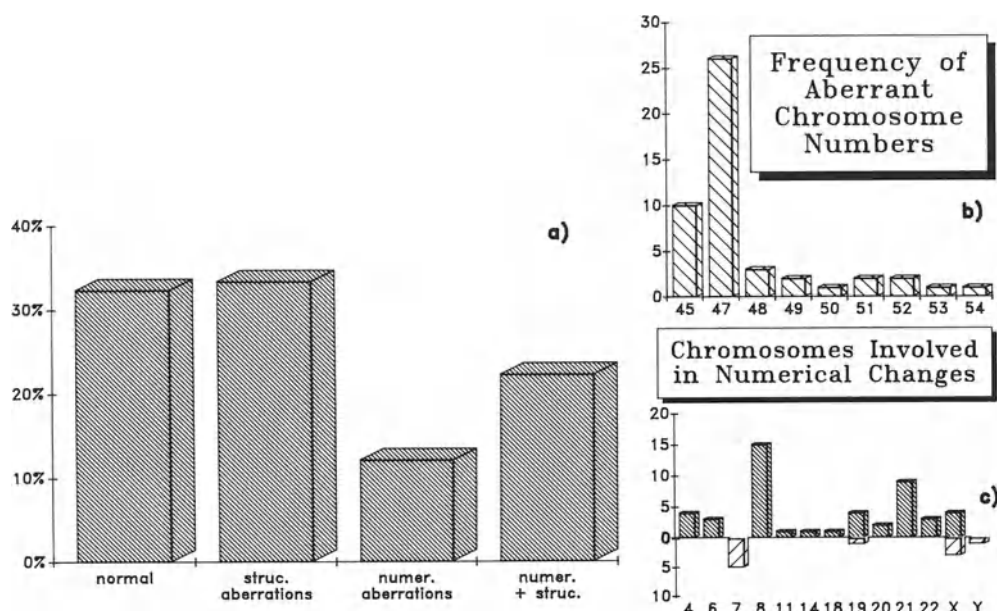
Between January 1984 and December 1988 we received 244 samples (203 at diagnosis, 37 at relapse, and 4 as secondary leukemia) of bone marrow or blood of children with ANLL for cytogenetic analysis. All in all we studied 215 patients (104 girls, 111 boys); 161 of these were treated according to the protocols of the AML83 and AML87 trials. One hundred and sixty-nine samples (69.3%) were analyzed successfully. Fifty-four patients (32%) revealed a normal diploid karyotype in their blast cells, whereas the remaining 68% showed cytogenetic changes, structural and/or numerical (Fig. 1 a).

A pseudodiploid chromosome set could be found in the leukemic cells of 56 children (33.1%), with chromosomal aberrations that could be correlated to the different FAB subtypes (Table 1, Fig. 1 a). The most frequent nonrandom chromosome abnormality was t(8;21), which could be found 16 times, mostly occurring in patients with FAB type M2 ( $n=10$ ). Karyotypes with translocation (15;17) ( $n=5$ ) were detected

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**Fig. 1.** Frequency of different chromosomal aberrations in the bone marrow of children with ANLL ( $n=169$ )

**Table 1.** Correlation and frequency (number of patients) of consistent primary chromosome abnormalities in ANLL within different FAB-subgroups

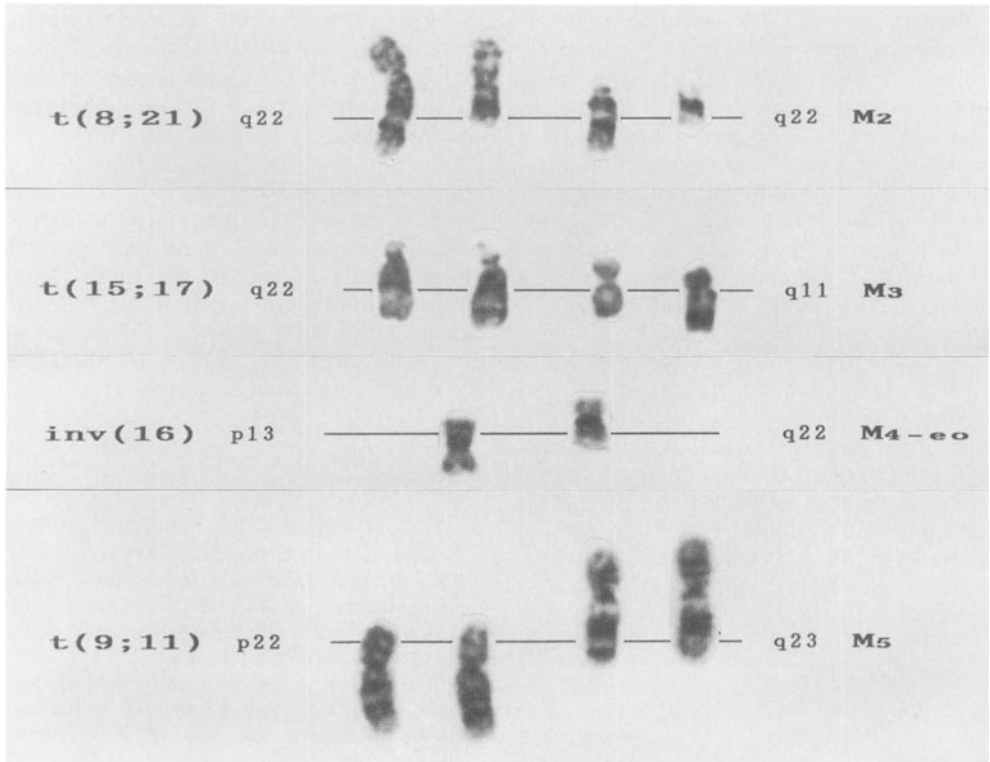
Leukemia karyotype	FAB classification							
	M1	M2	M3	M4	M5	M6	Mixed	n.d.
t(8;21)(q22;q22)	3	10	-	2	-	1	-	-
t(15;17)(q22;q11)	-	-	5	-	-	-	-	-
t(9;11)(p22;q23)	-	-	-	-	9	-	-	-
inv(16)	-	-	-	3	-	-	-	1
der(11)(q23)	1	2	-	4	4	-	1	-
+8	2	3	-	2	6	-	1	1
-7	1	1	-	-	1	1	-	1

only in children with ANLL-M3, thus showing a strong specificity to this FAB subgroup, since *inv(16)* ( $n=4$ ) appeared mainly in patients with ANLL-M4.

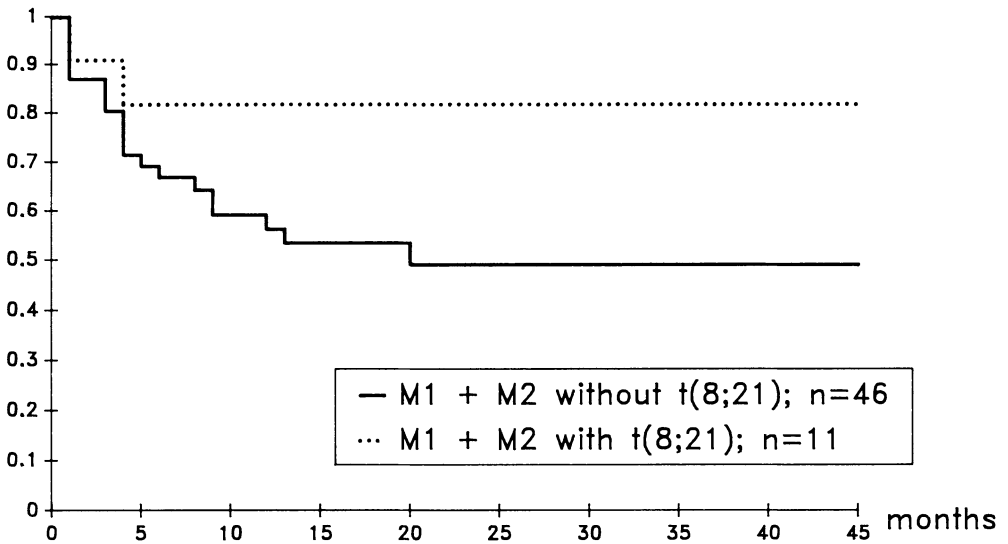
The translocation (9;11) ( $n=9$ ) exhibited a strong correlation to acute monoblastic leukemia (M5), whereas variant forms of this rearrangement involving the long arm of chromosome 11 with breakpoint q23 oc-

curred 12 times and were associated with all subgroups except M3 and M6.

Numerical abnormalities of the malignant cells could be seen in 59 patients (34.9%) (Fig. 1 a), the karyotype being either hypo- or hyperdiploid with modal chromosome numbers between 45 and 54 (Fig. 1 b). The most often encountered chromosome number was 47 followed by 45.



**Fig. 2.** Specific cytogenetic abnormalities in ANLL correlating with morphological subgroups



**Fig. 3.** Life-table analysis (Kaplan-Meier) of patients with (EFS 82%) and without (EFS 49%) t(8;21) leukemia karyotype

Twenty patients (11.8%) showed only numerical changes in their leukemic cells, whereas in 37 children (21.9%) numerical and structural aberrations of the chromosomes in combination were detected. With regard to chromosomes involved in numerical anomalies the most frequent aberration in our patients was trisomy 8 (Fig. 1c), not restricted to a particular FAB-subtype. Monosomy 7, a typical abnormality in secondary ANLL, could be found in five children.

To evaluate prognosis we performed life-table analysis (Kaplan-Meier) for children having a t(8;21) ( $n=11$ ) in their blast cells and for patients ( $n=46$ ) without this aberration. All patients had an acute myeloblastic leukemia without or with maturation (M1, M2) and were treated by either the protocol of the AML83 or AML87 therapy study. Patients with t(8;21) showed a better outcome, i.e., a higher rate of event-free survival, than those children without this cytogenetic anomaly.

## Discussion

In this study we found recurrent cytogenetic abnormalities in the malignant cells of children with ANLL; we also demonstrated the association between specific aberrations and particular FAB groups. Besides metaphases with an aberrant karyotype we detected mitoses in the bone marrow and blood without any clonal abnormalities in the same proportion of cells as described by other authors [5, 6]. The significance of these findings is not yet quite clear. In our series the most frequent structural rearrangement was t(8;21) and the results of life-table analysis for patients with and without t(8;21) are in good accordance with reports by other groups [5, 7]. Numerical aberrant cells in the

majority of cases were characterized by trisomy 8 or monosomy 7 whereas hyperdiploid chromosome numbers over 50 were relatively rare in contrast to the findings in common ALL.

*Acknowledgment.* We wish to thank all colleagues from other German pediatric centers for supplying us with bone marrow and blood samples. We also wish to thank Dr. J. Hofmann and Mrs. C. Lausch for patients' clinical data from the BFM group. For technical assistance we thank Mrs. S. Gräf, Mrs. A. Maurer, and Mrs. S. Vaupel.

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## Cytogenetic Study of 130 Childhood Acute Nonlymphocytic Leukemias

G. Schaison, G. Leverger, A. Bernheim, M. T. Daniel, G. Flandrin, and R. Berger

A systematic cytogenetic study was started in 1977 to investigate the incidence, types, and possible relations with prognosis of childhood acute nonlymphocytic leukemia (ANLL). We report on the findings of 130 de novo childhood ANLL studied at diagnosis in one department and in the same laboratories for cytology and cytogenetics between September 1977 and December 1986.

### Materials and Methods

A total of 130 children, 68 boys and 62 girls, under 16 years of age, were successfully cytogenetically studied by banding techniques. The patients were classified according to the French-American-British (FAB) nomenclature in seven classes, M1–M7. An average of 15–25 metaphases were examined in the majority of the patients, the extremes being 10 and 116 metaphases. An abnormal clone was defined according to the criteria of the Fourth International Workshop on Chromosomes in Leukemia. ANLL were classified as NN, with only normal metaphases, AN with a mixture of normal and abnormal metaphases, and AA with only abnormal metaphases.

### Results

#### *Incidence of Acquired Chromosomal Clonal Abnormalities*

A total of 89 out of 130 patients had acquired chromosomal clonal abnormalities (68.5%), of whom 59 (45.4%) were AN and 30 (23.1%) AA. Among the abnormal karyotypes, 23 (17.7% of the total) were hyperdiploid (11 with associated structural chromosome changes), 17 (13.1% of the total) hypodiploid (12 with associated structural abnormalities), and 49 (37.7% of the total) had only structural abnormalities with 46 chromosomes. Hyperdiploidy was thus less frequent than has been reported in acute lymphoblastic leukemia.

#### *Types of Chromosomal abnormality*

Trisomy 8, isolated (two cases) or associated with other changes (five cases), was numerically the commonest abnormality (5.4% of the total, 7.9% of the abnormal karyotypes), followed by monosomy 7 found in five patients (3.8% of the total, 5.6% of the abnormal). Monosomy 7 was associated with monosomy 5 in two further patients. The commonest structural abnormality was the t(8;21) translocation observed in 17 patients, i.e., in 58.6% of the M2-ANLL. A rearrangement of chromosome 11q was found in 14 patients (10.8% of the total, 15.7% of the abnormal karyotypes). Twelve were classified as M5 and two as M4. A t(15;17) was also observed in 10 of the 11 children with M3 (8 M3 and 3 M3 variants).

Nine patients had an M4 with bone marrow eosinophilia (M4EO). An abnormality of chromosome 16, inversion and/or deletion, was present in five of them. Twelve patients were studied at diagnosis and in relapse. The karyotypes were unchanged in six cases and additional changes were seen in five cases.

#### *Cytogenetics and Prognosis*

The overall complete remission (CR) rate was 81.6%. CR was significantly lower ( $P < 0.05$ ) in the AA category than in the AN and NN groups. Among the 15 patients without achievement of CR, 14 (93%) had a clonal chromosomal abnormality, of whom 7 were AA. The median survival, calculated in patients introduced in the study between 1977 and December 1985, was significantly different ( $P < 0.01$ ) in AA, AN, and NN groups and was lower in the first (respectively 6, 17, and 16 months). However, median survival since achievement of remission was not significantly different in the three categories. The CR rates were high in the patients with specific rearrangements such as

t(15;17), t(8;21), and 11q abnormalities, but the median survival was not particularly good when compared with the other ANLL. Comparison between AN and AA patients with these abnormalities, particularly with t(8;21), was not possible because of the small number of patients. The longest median survival was observed in M4EO ANLL (26 months).

#### **Discussion**

The overall incidence of chromosomal abnormalities was found to be higher in childhood ANLL (68.5%) than in adult ANLL (50.4%) studied in the same institute. The now well-established correlations between certain rearrangements and certain types of ANLL were confirmed as expected. The generally poorer prognosis of AA patients has been confirmed in the present series. The poor prognosis of ANLL with monosomy 7 and the high rate of complete remission achievement in specific translocations but without necessarily good median survival have to be underlined.

## Changes in Clonal Growth, Immunophenotype, and Morphology During a Follow-up Study of an Acute Lymphoblastic Leukemia

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### Introduction

Follow-up studies of leukemias enable interesting insights to be gained into their biological and physiological behavior. There is little opportunity to study leukemia cell clones during chemotherapy in a follow-up study because most acute leukemias treated in the actual multicenter studies show good initial response. Studying clinical drug-resistant leukemias one can elucidate changes in morphological and immunological phenotype and clonal growth during the course of disease dependent on the cytotoxic drugs administered and the different compartments involved. Here we present a helper cell T-ALL primarily resistant to chemotherapy according to the German BMFT-ALL protocol characterized by a tendency to undergo differentiation-like steps.

### Clinical Course

At diagnosis the patient (21-year-old man) showed heavy infiltration of the bone marrow, involvement of the mediastinum, lung, liver, spleen, and kidneys, pleural effusions, ascites, and a severe antibody deficiency. During the administration of vincristine, daunorubicin, prednisolone, and asparaginase according to the BMFT-ALL protocol disease was progressive. Subsequent HAM

chemotherapy [high-dose cytosine arabinoside (Ara-C), mitoxantrone] led to a cell reduction of 75% and a significant clinical improvement. The leukemia has shown no further response to the following chemotherapy: second HAM chemotherapy and additional chemotherapy in the further course with cyclophosphamide, ifosfamide, vepeside, methotrexate, teniposide, and etoposide (IMVP16, Ara-C/VM26, cyclophosphamide, prednisolone). After second HAM chemotherapy there was leukemic involvement of the knee joint, lung, and meninges. The patient died of progressive disease 5 months after diagnosis.

### Materials and Methods

We studied morphology and immunophenotype (CD 1, 2, 3, 4, 7, 8, 23, 25, OKT9) of leukemia cells in knee joint fluid, liquor, alveoli (bronchoalveolar lavage) and peripheral blood, *T $\beta$*  rearrangement in Southern blot analysis, *mdr1*-mRNA in Northern blot analysis and tumor markers (tissue polypeptide antigen, CA125) of a helper cell T-ALL in a 21-year-old man during a follow-up study. Mononuclear cells were separated by density gradient centrifugation of heparinized peripheral blood, liquor or knee joint fluid over Ficoll. For cytological analysis a smear was stained using Pappenheim staining.

### Surface Antigen Screening Tests

Indirect immunofluorescence staining was performed with standard methods [1] using

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the monoclonal antibodies as the first reagent and affinity-purified fluoresceinated goat-anti-mouse globulin reagents (Tago Hamburg) [2].

### *Northern Blot Analysis*

A quantity of  $10^8$  leukemia cells were lysated for RNA extraction with guanidiniso-cyanate in a homogenizator. The pellet was obtained after centrifugation of the lysate over a cesium-chloride gradient. The pellet was resuspended in 0.3 M sodium acetate and frozen with ethanol on dry ice. The RNA content of the precipitate was estimated by measuring the optical density at 260 nm.

After electrophoresis of total RNA in 1% agarose containing 13.4% formaldehyde RNA was blotted on nitrocellulose filters. Filters were baked and preincubated for 4 h at 42°C in 50% formamide,  $5 \times$  SSC (standard saline citrate),  $10 \times$  Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), and fish sperm DNA at 100 µg/ml. Filters were hybridized overnight in the above solution containing  $^{32}\text{P}$ -labeled probe. Filters were washed three times at room temperature in  $2 \times$  SSC, 0.1% SDS, and three times for 20 min at 50°C in  $0.1 \times$  SSC, 0.1% SDS. Hybridization was evaluated by autoradiography [3].

### *Southern Blot Analysis*

DNA coding for the immunoglobulin  $\mu$ -constant fragment was kindly provided by Dr. M. Pech (Institute for Physiological Chemistry, University of Munich). In addition the *Jh* probe (Oncor Gaithersburg) was used. To mark the T-cell receptor chains we used cDNA coding for the constant region of the TCR  $\alpha$  chain and the cDNA specific for the constant regions of the TCR  $\beta$  chain. Salmon sperm DNA was used as a negative control.

For blot hybridization cells were lysed and DNA was extracted with the phenol extraction method. DNA, 7.5–10 µg, was digested with the restriction enzymes *EcoRI*, *BamHI*, *BglII*, or *HindIII* and transferred to nylon membranes after electrophoretic sep-

aration in 0.8% agarose gels [4]. DNA was radioactively labeled with  $^{32}\text{P}$  by the random poly priming method [5]. Hybridization was carried out according to the protocol of Church and Gilbert [1]. Hybridization was evaluated by autoradiography for 1–14 days.

## **Results**

### *Morphology*

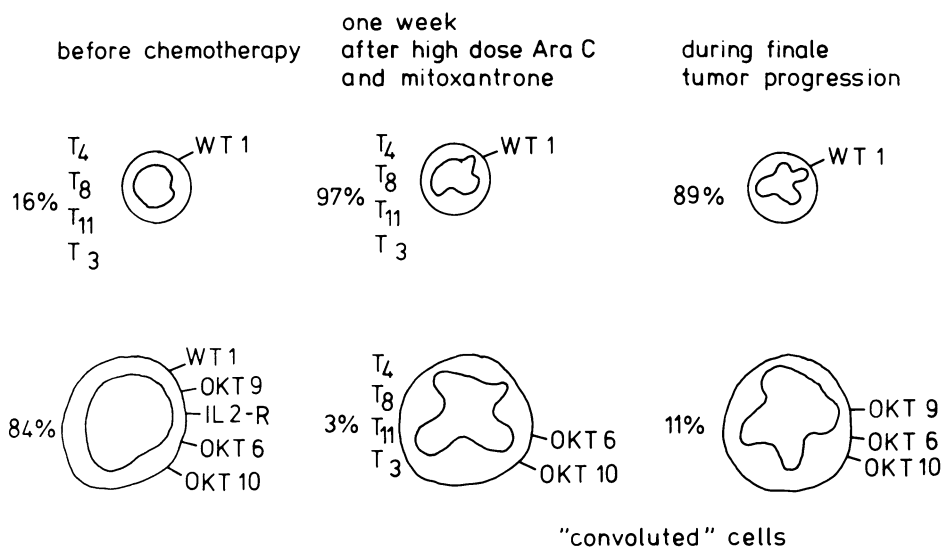
Initially there were 84% large blasts against a small number of small lymphoid cells. Subsequent HAM chemotherapy induced a drastic change in cell morphology from large blasts to mainly small lymphoid cells (98%) now showing convoluted nuclei. This cell population remained morphologically uninfluenced by further chemotherapy. Depending on the compartment studied different ratios of small lymphoid cells and large blasts were found (Fig. 1).

### *Immunophenotype*

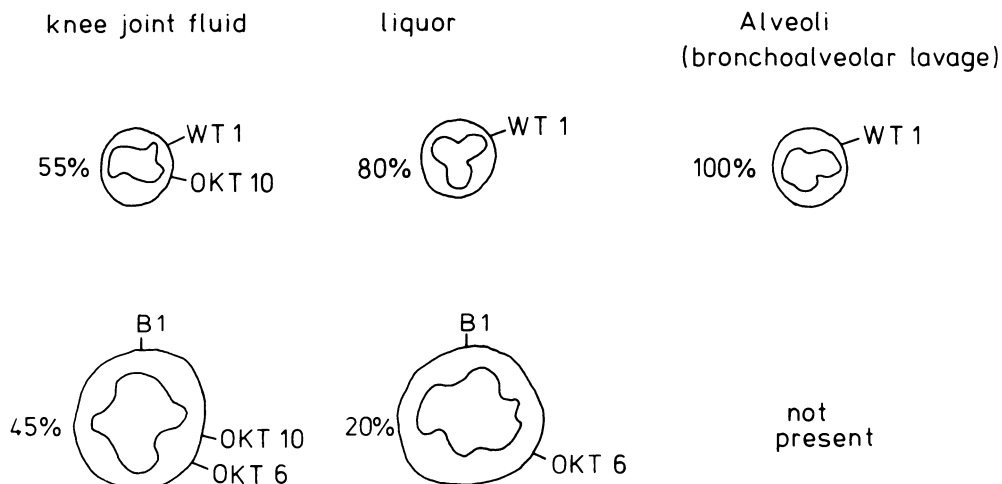
Initially 90% of the leukemia cells expressed CD7 with a prevalence of helper (60%) over suppressor cells (15%). This ratio remained constant until the start of HAM chemotherapy, which led to a sharp fall and subsequent slow increase in all T-cell markers. In contrast to pretherapeutic findings CD7 was only expressed now on the small lymphoid cells and not on blast cells (Fig. 1). In the knee joint fluid blast cells additionally expressed CD23, a typical B-cell marker (Fig. 2). Activation antigens (IL2-receptor, transferrin receptor) disappeared during initial chemotherapy (according to the BMFT-ALL protocol) but were reexpressed a short time after the start of HAM chemotherapy despite the steep decrease in all the other T-cell markers (Fig. 3).

### *Tumor Markers*

The initial blast population expressed mainly CA125 and only to a lesser extent TPA cells. After HAM chemotherapy CA125 levels decrease to nearly normal values fol-



**Fig. 1.** Morphology and immunophenotype of the predominant leukemia cell clone in the peripheral blood



**Fig. 2.** Morphology and immunophenotype of the leukemia cells in different compartments

lowed by a steep increase in TPA levels during the appearance of the small lymphoid cells (Fig. 4).

#### *Southern Blot Analysis of T-Cell Receptor Conformation*

Southern blot analysis revealed an initially monoclonal population with rearranged  $T\beta$

gene. A new band appearing during clinically ineffective therapy was indicative for the development of a second small population which did, however, not emerge in immunophenotype analysis. This second small population was eliminated by the HAM chemotherapy, leaving the initial clone responsible for the final fatal outcome (Fig. 5).

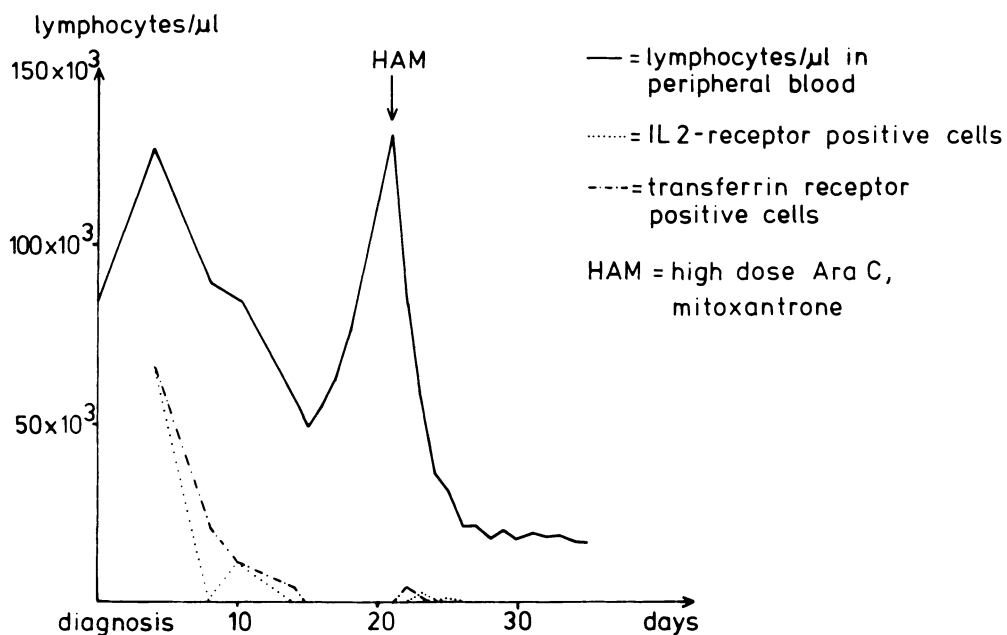


Fig. 3. Number of leukemia and activation-antigen-positive cells

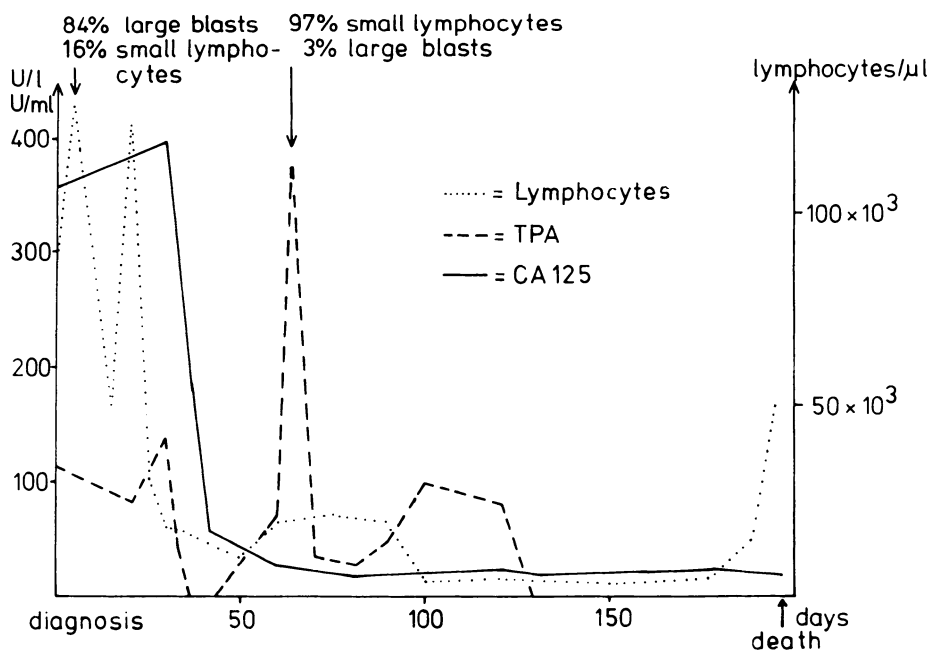
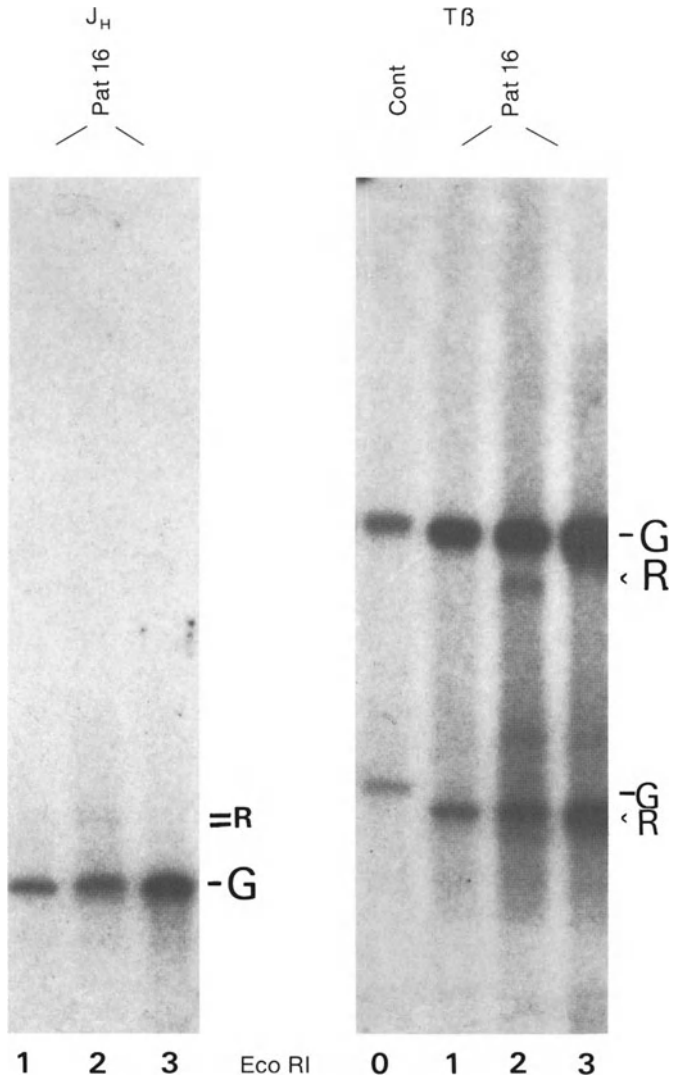


Fig. 4. CA125 and TPA serum levels dependent on the ratio of blast cells and small lymphoid cells to leukemia cell count in the peripheral blood



**Fig. 5.** *Tβ* and immunoglobulin heavy chain rearrangement. 1, before first chemotherapy; 2, during ineffective chemotherapy; 3, after HAM chemotherapy, 0, germ line

#### Northern Blot Analysis of *mdr1*-mRNA

No multidrug resistance gene activity could be detected by Northern blot analysis in the follow-up despite obviously clinical resistance to vincristine and daunorubicin.

#### Discussion

We present helper-cell T-ALL with primary resistance to chemotherapy according to the

BMFT-ALL protocol. The predominant morphological and immunological heterogeneous but clonal helper-cell T-ALL undergoes differentiation-like steps dependent to a large extent on the different compartments being involved and on the cytotoxic drugs administered, especially high-dose Ara-C. Cytotoxic drugs are able to modulate proliferation- and differentiation-associated antigens. In bone marrow of mice low-dose Ara-C activates hematopoietic stem cells to proliferation accompanied by

the secretion of humoral factors [6]. Hydroxyurea induces *in vitro* IL2-receptors on activated lymphocytes [7]. In our case administration of high-dose Ara-C led to a strong transient reexpression of IL2-receptors and transferrin receptors in a few leukemic blast cells (about 5%). This result is remarkable because levels of all T-cell receptor antigens decrease very sharply during the start of HAM administration. The initial therapy with vincristine, daunorubicin, asparaginase, and prednisolone continuously decreases the activation antigen expression in contrast to HAM chemotherapy.

A further modulating factor of immunophenotype and morphology seems to be the involvement of the compartments. Cell size, ratio of small lymphoid and blast cells, and immunophenotype vary as far as the additional expression of a typical B-cell antigen (CD23) in blast cells of the knee joint fluid and liquor. These phenotypic changes are probably induced by T-lymphocytes and macrophages in the microenvironment of the leukemia cell population. The mediators could be lymphokines and growth factors released into the extracellular space. Also an autocrine stimulation of the leukemia cells is possible. Such humoral factors modulate long-term receptor-mediated changes of cells such as differentiation and proliferation [8–10].

Non-Hodgkin's lymphomas containing both B- and T-cell clones are known [11]. In this case blast cells coexpress T- and B-cell markers. A possible mechanism to account for the patient's T-ALL is derivation from a single transformed early lymphocytic precursor. Rearrangement of immunoglobulin heavy-chain gene indicates a faint rearranged band during onset of the second small T-cell population, developing during ineffective chemotherapy (Fig. 5). This was not detected by immunophenotype and morphological analysis but by Southern blot analysis of T-cell receptor configuration. This T-ALL population shares a common feature with the predominant cell population: drug resistance to chemotherapy according to the BMFT-ALL protocol but in contrast susceptibility to HAM followed by subsequent clone elimination. Another possible action of HAM chemotherapy can be shown in the predominant T-ALL popula-

tion: cytoreduction accompanied by drastic changes in morphology, immunophenotype, and tumor marker expression. These differentiation-like steps remained uninfluenced by further chemotherapy and final leukemia progression. They were accompanied by reduced sensitivity to further chemotherapy, even to a second subsequent therapy with HAM, leading to no significant cytoreduction.

Ara-C is known to have some specific toxicity for self-renewing blast stem cells in clonogenic and suspension assays [12]. Interpreting our results against the background of these observations we have to assume that Ara-C has acted mainly on blast cells with the capacity for self-renewal, leading to a drastic cytoreduction of about 75% but favoring on the other hand the capacity of blast cells for differentiation. The action of Ara-C on proliferating cells would be consistent with the short-term promotion of proliferation during the start of HAM chemotherapy monitored by the strong transient IL2- and transferrin-receptor expression in a small blast cell population. The observation that Ara-C acts mainly on proliferating blast cells is supported by the failure of the second HAM therapy. Thus, Ara-C has shown a significant influence on the blast cell physiology.

Both cell populations show different rearranged *Tβ* genes in Southern blot analysis. Due to the quite different sensitivity to HAM chemotherapy, one can suggest that distinct *Tβ* gene conformations may correlate with differential sensitivity to cytotoxic drugs. This would be much more interesting because clinical drug resistance to vincristine and daunorubicin does not correlate with an increased expression of *mdr1* gene coding for P-glycoprotein.

We have described the different features of a primary drug-resistant T-ALL at a molecular biological level. General conclusions with regard to typical molecular biological characteristics of this group of T-ALLs and future strategies of therapy are so far premature. But the results describing the biology and physiology of the T-ALL studied are a basis for further investigation. The observations support the hypothesis that it is necessary to develop therapeutic regimens which influence the balance of self-renewal



and differentiation-like steps of blast cells. Distinct T-cell receptor conformations are possibly indicative of drug resistance.

### Summary

Cells of a 21-year-old patient with acute lymphatic leukemia were analyzed for morphology and immunophenotype and for genotype consecutively during the course of disease. Initial therapy with the BMFT-ALL protocol (Bundesministerium für Forschung und Technologie) reduced leukemic cells only marginally. The following high-dose Ara-C, mitoxantrone (HAM) chemotherapy led to a cell reduction of 75% and to a drastic change in cell morphology from initially 90% blasts to mainly small lymphoid cells. Immunophenotype, which showed 90% CD7-positive cells in the beginning with a prevalence of helper (60%) over suppressor cells (15%) remained fairly constant until the onset of HAM chemotherapy, which led to a sharp fall and a subsequent slow increase in all T-cell markers. In contrast to pretherapeutic findings, CD7 was now only expressed on the small cells and not on blast cells. Southern blot analysis of the T-cell receptor configuration revealed an initially monoclonal population with rearranged *Tβ* gene. A new band appearing during the clinically ineffective therapy was indicative for development of a second small population which did, however, not emerge in immunophenotype analysis. This second population was eliminated by the HAM chemotherapy, leaving back the initial clone responsible for the final fatal outcome. No activity of the multidrug resistance gene could be detected by Northern blotting.

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## Prognostic Significance of the Karyotype in Patients with Primary Myelodysplastic Syndrome\*

S. Suciú, H. J. Weh, and D. K. Hossfeld

### Introduction

The myelodysplastic syndrome (MDS) comprises a group of heterogeneous hematologic disorders characterized by ineffective hematopoiesis and a high risk of transformation to acute leukemia. Studies on the role of chromosomes in MDS are still controversial as contrasted with those in acute non-lymphocytic leukemia (ANLL), where specific translocations have been associated with certain morphologic types [1]. Only a few reports described cytogenetic findings in a significant number of patients with MDS [2–4]. The purpose of this study was to determine the frequency of chromosome anomalies in a large series of patients with primary MDS, their correlation with morphologic subtypes, and prognostic impact.

### Material and Methods

Between 1981 and 1988 the chromosomes of 120 patients with primary MDS were examined. Diagnosis of MDS and subtyping were performed according to the criteria proposed by the French-American-British (FAB) group [5]. Our patient population included 28 patients with refractory anemia (RA), 14 patients with RA and ring sideroblasts (RARS), 45 patients with RA with excess of blasts (RAEB), 19 patients with RAEB in transformation (RAEB-T), and 14

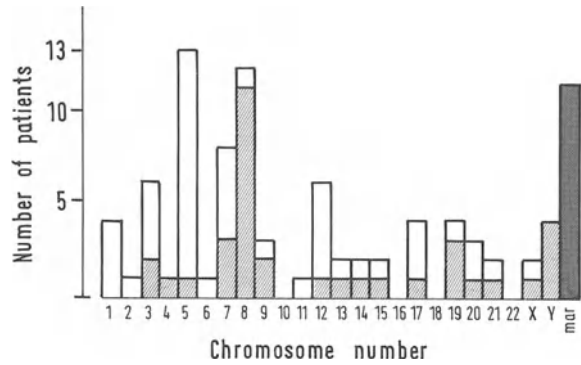
patients with chronic myelomonocytic leukemia (CMML). There are 71 males and 49 females. The median age is 62 years, the range 25–89 years. For examination of chromosomes of bone marrow cells, direct preparations, 24- and 48-h cultures, without mitogen, were performed. Chromosome banding was carried out by the trypsin-Giemsa method. In nearly all cases, at least 20 metaphases and 5 karyotypes were analyzed.

### Results

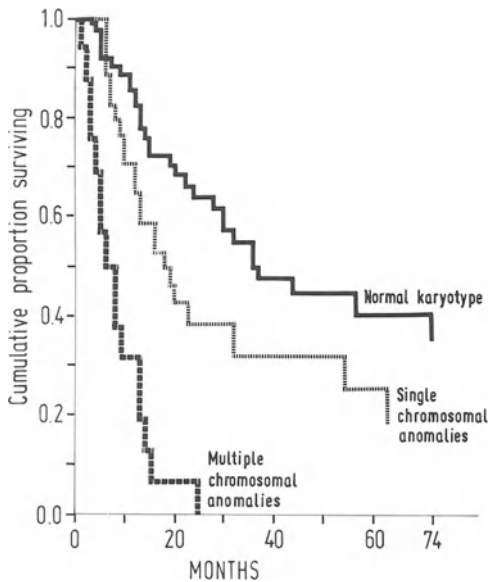
Clonal karyotypic anomalies were detected in 50 patients (42%) at initial chromosome study. Among them, 34 patients (68%) had a single karyotypic anomaly and 16 patients (32%) had multiple chromosomal anomalies. The results are summarized in Fig. 1. The most common anomalies were a 5q-chromosome, trisomy 8, the  $-7/7q-$  anomaly, and a 12p-chromosome. Nonspecific structural anomalies of chromosomes 1, 3, and 17 were also common, the majority of them in combination with other anomalies. Multiple anomalies were much more frequent in patients with RAEB and RAEB-T (20% of 64 patients) as compared with individuals with RA, RARS, and CMML (4% of 56 patients). This agrees with the view that blast excess represents a later stage in the progression of MDS to frank leukemia, and that karyotypic evolution is associated with such a progression. The median survival time for the 70 patients with a normal karyotype was 36 months, and for those with multiple anomalies 18 months (Fig. 2).

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**Fig. 1.** Frequency of involvement of individual chromosomes in numerical (▨) and structural (□) anomalies



**Fig. 2.** Survival curves of the patients with MDS according to chromosome status

All these differences are statistically significant. Leukemia transformation was observed in 26 patients (52%) with an abnormal karyotype in comparison to 16 out of 70 patients (23%) with a normal karyotype

**Table 1.** Relation of chromosome anomalies to subsequent leukemia

Patients with	Progression to ANLL
Normal karyotype	16/70 (23%)
Single chromosome anomaly	17/34 (50%)
Multiple chromosome anomaly	9/16 (56%)

( $P < 0.05$ ) (Table 1). Among the patients with RA, RARS, and CMML, 7 of 12 patients (58%) with an abnormal karyotype progressed to ANLL in contrast to 4 of 44 patients (9%) with normal karyotype ( $P < 0.01$ ).

## Discussion

In our material, chromosome anomalies were found in 50 of 120 patients (42%) with primary MDS. In the literature, the incidence of karyotypic aberrations in MDS patients has been reported to be 30%–60% [2–4, 6–12]. A higher incidence was found by Yunis et al. (79%) [13]. In 17 of 120 patients with primary MDS, a 5q- and/or -7/7q- anomaly was detected. The fact that these anomalies are more frequently observed in therapy-related MDS [14] suggests a possible exposure to unknown mutagenic agents of such so-called de novo MDS patients. Generally in our series, the 5q- and -7/7q- anomalies were detected more frequently in RAEB and RAEB-T than in other subtypes. The other cytogenetic anomalies were not correlated with certain subtypes of MDS.

Our findings extend several reports on the prognostic values of chromosome anomalies in preleukemic states [4, 12, 13, 15]. Most importantly, survival data on a large number of patients with MDS demonstrate especially the extremely grave prognosis of patients with multiple karyotypic anomalies. All these patients died within 8 months. Our survival data indicate that patients with a single karyotypic anomaly are also at significantly increased risk for early death as com-

pared with those without a demonstrable cytogenetic anomaly. There is increased frequency of leukemia in MDS patients with a single anomaly but their median survival was significantly better in comparison to that of patients with multiple chromosome anomalies.

Some studies [12, 13] indicate that certain chromosome anomalies provided a greater prognostic significance than the presence or absence of karyotypically abnormal clones. Other authors report that only MDS patients with multiple karyotypic anomalies could be expected to develop overt acute leukemia and/or have a poor prognosis [4, 15]. In our study, the patients with single anomalies as well as those with multiple cytogenetic anomalies had a shorter survival time and an increased risk of leukemic transformation.

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## Update of the Cytogenetic Study of Childhood Non-High-Risk Acute Lymphocytic Leukemia at Diagnosis in Protocol VI of the Dutch Childhood Leukemia Study Group

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### Introduction

The presence of chromosomal abnormalities at diagnosis has been demonstrated to have an independent prognostic value in childhood acute lymphoblastic leukemia (ALL) [1–5]. Individuals with a hyperdiploid (>50) clone show the most favorable treatment response whereas those with a pseudodiploid clone have the poorest responses [2]. Chromosomal translocations have been shown to have the most profound impact on treatment outcome [6–8].

Because of this correlation between cytogenetic findings and prognosis, chromosome studies were included as one of the parameters in classifying newly diagnosed cases of non-high-risk ALL of childhood entered into protocol VI of the Dutch Childhood Leukemia Study Group (DCSLG). Non-high-risk ALL was defined as an initial white blood cell count of less than  $50 \times 10^9$ /liter, no mediastinal mass or cerebromenin-

geal involvement at diagnosis, and absence of leukemic cells with B-cell morphology and immunological characteristics. This includes 70% of all children with ALL in the Netherlands. A total of six different cytogenetic laboratories throughout the Netherlands participated in this study.

### Materials and Methods

Chromosome preparations were obtained from fresh bone marrow and/or peripheral blood samples from children with non-high-risk ALL by direct techniques and/or after 24 and 48 h in tissue culture. The slides were stained with banding techniques, and the metaphases analyzed and classified according to international convention [9]. Where possible 30 metaphases were analyzed; an abnormal clone was defined as two or more metaphases with the same marker chromosome, or three or more cells missing the same chromosome. The presence of a single hyperdiploid cell (>50) was considered sufficient to assign a patient to this ploidy category.

A total of 190 children with non-high-risk ALL at diagnosis were entered into protocol VI (Veerman et al., this volume) and in 141 instances samples were obtained for cytogenetic analysis. Two individuals had Down's syndrome. There were 105 cases of morphology French-American-British (FAB) type L<sub>1</sub> and 36 of FAB type L<sub>2</sub>. The majority (100) had common ALL, 14 pre-B ALL, 4 acute undifferentiated leukemia, and 1 pre-pre-B ALL (CD19<sup>+</sup>, cALLA<sup>-</sup>); 22 cases were not classified.

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**Table 1.** Ploidy distribution in 102 cases of non-high-risk ALL of childhood studies at diagnosis

		Present study		% incidence in all types of ALL at diagnosis <sup>a</sup>
		No. cases	%	
Hyperdiploid	(>50)	34	33.3	30.0
Pseudodiploid		18	17.6	40.0
Hyperdiploid	(47–50)	16	15.7	16.0
Hypodiploid	(<46)	7	6.9	7.0
Polyploid		2	2.0	n. s.
Diploid (normal)		25	24.5	7.0
Total		102	100.0	100.0

<sup>a</sup> From Williams et al. [10]  
n. s., not specified

## Results

Chromosome studies were successful in 102 patients (72.3%), failures being due to either an inadequate sample or lack of dividing cells. An abnormal clone was identified in 77 cases (75.5%), with hyperdiploidy (>50) as the commonest abnormality (see Table 1).

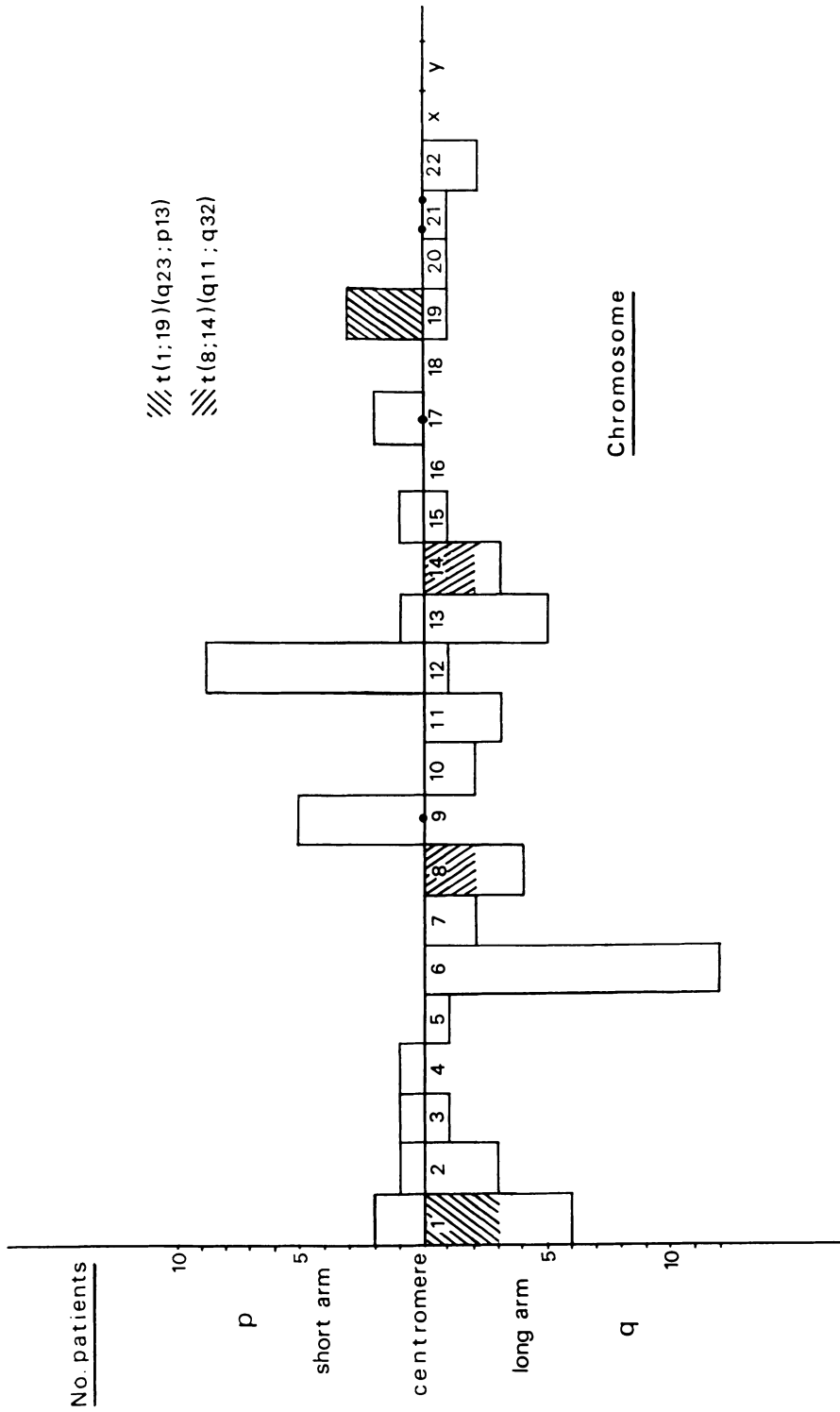
Structural abnormalities could be identified in 41 patients, which included 11 pseu-

dodiploid cases, 8 hyperdiploid (47–50), 6 hypodiploid, and 1 polyploid. Only eight of the large hyperdiploid (>50) group fell into this category. Figure 1 illustrates which chromosomes were involved in structural rearrangements. These predominantly involved chromosomes 6, 9, 12, and 13. Twelve individuals had abnormalities involving the long arm of chromosome 6 (eight FAB L<sub>1</sub>; four FAB L<sub>2</sub>), nine of whom

**Table 2.** Translocations and derivative chromosomes identified in 102 cases of non-high-risk ALL of childhood studied at diagnosis

Translocation	Immuno-phenotype	FAB type	Ploidy
t(1;19)(q23;p13)	AuL n. d. pre-B	L1 L1 L2	Pseudodiploid Pseudodiploid Pseudodiploid
t(8;14)(q11;q32)	cALL  n. d.	L2  L1	Hyperdiploid (47–50) Pseudodiploid (Down's)
t(1;12)(q22;p13)	n. d.	L1	Pseudodiploid
t(2;7)(q31;q3)	cALL	L2	Pseudodiploid
t(2;7)(q37;q22)	AuL	L1	Pseudodiploid
t(6;10)(q15;q15) <sup>+</sup>	cALL	L2	Hypodiploid
t(8;11)(q21;q23)	cALL	L1	Pseudodiploid
t(8;12)(q11;p11)	cALL	L1	Hypodiploid
t(13;22)(p11;q11)	cALL	L1	Hyperdiploid (47–50)
der(12)t(1;12)(q25;q15) <sup>+</sup>	cALL	L2	Hypodiploid
der(12)t(2;12)(q11;p13)	cALL	L1	Hypodiploid
der(17)t(13;17)(q14;p13)	cALL	L1	Pseudodiploid

n. d., not done; AuL, acute undifferentiated leukemia; +, same patient



**Fig. 1.** Chromosomes involved in structural aberrations in 102 cases of non-high-risk ALL of child hood studied at diagnosis

had deletions. The short arm of chromosome 9 was affected in five children (three FAB L<sub>1</sub>; two FAB L<sub>2</sub>), and the short arm of chromosome 12 in nine (seven FAB L<sub>1</sub>; two FAB L<sub>2</sub>). The long arm of chromosome 13 was abnormal in five patients (five FAB L<sub>1</sub>).

Fourteen of these patients had translocations or derivative chromosomes whose origin could be determined (see Table 2). These included three cases of the translocation t(1;19)(q23;p13) and two of the translocation t(8;14)(q11;q32). None were detected in the hyperdiploid (>50) group.

## Discussion

The present cytogenetic study on non-high-risk ALL of childhood detected an abnormal clone in 75.5% of patients where cytogenetic analysis was successful. As expected hyperdiploidy (>50) was the commonest abnormality with an underrepresentation of the pseudodiploid group when compared with the study by Williams et al. [10] on all types of childhood ALL. The hyperdiploid group (>50) was characterized by the apparent absence of translocations and a low incidence of other structural abnormalities; they were also cALLA positive. The commonest structural changes found in the present study, i.e., those involving chromosome 6, 9 and 12, have been reported for other cases of childhood ALL [1, 4, 6, 11]. Variation in the short arm of chromosome 9 occurs in approximately 10% cases of ALL and appears to be nonrandom for lymphoid malignancies [12, 13]. Aberrations involving the short arm of chromosome 12 have been reported by Raimondi et al. [14] as being one of the most frequent abnormalities seen in childhood ALL, predominantly of FAB type L<sub>1</sub>. Variation in the long arm of chromosome 13 was also common in the present series.

The incidence of translocations and derivative chromosomes in the present study is low, being identified in only 14 of the 102 patients. None of the "high-risk" translocations, i.e., t(9;22), t(8;14), t(4;11) were found in this group of selected patients. Three instances of the t(1;19)(q23;p13) were found. This translocation is usually associated with a pre-B immunophenotype [15, 16], but in

the present study one patient had acute undifferentiated leukemia. The incidence of translocations for cell types of childhood ALL has been reported as 45%, being found most frequently in the hypodiploid and pseudodiploid groups [8]. The low number of translocations found in the present study can be explained by the fact that only patients with non-high-risk features were included, since translocations have been shown to be associated with the presence of at least one high-risk feature [7].

At the present time the follow-up period is too short to allow any conclusions to be drawn about any relationships between the cytogenetic findings and prognosis.

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## Prognosis and DNA Aneuploidy in Children with Acute Lymphoblastic Leukemia

M. Tsurusawa, N. Katano, and T. Fujimoto

### Introduction

Childhood acute lymphoblastic leukemia (ALL) is a heterogeneous disease with respect to immunophenotype and karyotype of the blast cells, and so the biological features of the cells appear to have a strong influence on the prognosis. DNA analysis by flow cytometry (FCM) is an ideal way to detect abnormal DNA content of the leukemic cells (DNA aneuploidy), since it rapidly provides a DNA index (DI) that is independent of the mitotic index and is reliably correlated with the karyotype for DNA aneuploidy [1]. Although a favorable outcome for patients with aneuploidy has been commonly reported from our [2] and other working groups [3–5], there are some apparent differences in the prognostic value among the results of each group. In the present study we report the independent prognostic significance of the cellular DNA content in high-risk children who were stratified by WBC count and age at time of diagnosis.

### Materials and Methods

#### Patients

From January 1981 to January 1987, a total of 532 children with ALL were registered in the Children's Cancer and Leukemia Study Group (CCLSG). They were placed in a low

(or standard) – or high-risk group according to their initial WBC counts and age at the time of diagnosis (Table 1). S811A/B (1981–1983) or S841A/B (1984–1986) protocols were used for the low-risk patients, and H811A/B (1981–1984) or H851A/B (1985–1987) protocols were used for high-risk patients. No significant difference was shown among the protocols in both risk groups. The DNA contents of the leukemic cells were determined in 196 untreated and 54 relapsed children.

**Table 1.** Children with ALL, stratified to low- or high-risk group by initial leukocyte counts and age at diagnosis

WBC (/ $\mu$ l)	Age (years)				
	1	1–3	4–5	6–9	10–15
5000	LR	LR	LR	LR	HR
5001–10000	LR	LR	LR	HR	HR
10001–50000	HR	LR	LR	HR	HR
50001–200000	HR	HR	HR	HR	HR
200001	HR	HR	HR	HR	HR

LR, low-risk; HR, high-risk

#### Flow Cytometry

Leukemic marrow samples were stained with a hypotonic propidium iodide solution (0.005%) and analyzed with a FACS 440 flow cytometer, as previously described [2]. Trout red blood cells, which contain

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80% of the DNA content of human diploid values, were used as an internal reference standard. The diagnosis of “DNA aneuploidy” was made when more than two separate G1 peaks were demonstrated in a mixture of the bone marrow samples and normal human peripheral mononuclear cells. The DI was the ratio of the peak channel number of aneuploid to diploid cells in G1-phase.

### Statistical Analysis

The event-free survival (EFS) rate was calculated by the Kaplan-Meier method, and the difference between two survival curves was tested by the generalized Wilcoxon test. An “event” was defined as induction failure, first relapse, or death during the first complete remission. Patients who had no event were excluded at the time of the last follow-up. The influence of prognostic factors on EFS was analyzed by a statistical package program of the Super Application System. Factors to be analyzed were dichotomized and recorded as 1 (“better”) or 2 (“worse”). The significance of a single factor was estimated by the log-rank test of the Kaplan-Meier life-table data, and the relative magnitude of a factor’s effect on EFS was determined by the Cox life-table regression model.

## Results

### Untreated ALL

DNA aneuploidies were detected in 51 (26%) of 196 children. The incidence was high (45.4%) in low-risk patients and low (17.5%) in high-risk patients. All patients, except one hypodiploid case, showed the hyperdiploid DNA stem lines (DI > 1.0). The median value of DNA indices for the aneuploidies was 1.18 (Table 2). The initial clinical and laboratory features of the two DNA groups are shown in Table 3. Although a higher platelet count was noted in the hyperdiploid group, no other difference was shown between the diploid (DI = 1.0) and the hyperdiploid groups in the low-risk patients. In the high-risk patients, the hyperdiploid group had more favorable features (lower WBC count, high platelet count, no T-cell phenotype) than the diploid group.

When EFS curves of patients with diploid DNA stem lines were compared with patients with hyperdiploid DNA stem lines, the hyperdiploid group had significantly higher EFS rates than the diploid group (Fig. 1 A). Even after the division of the patients into the two risk groups defined by WBC and age, the patients with hyperdiploid DNA stem lines in each risk group showed apparent longer duration of EFS than the diploid group (Fig. 1 B, C).

**Table 2.** Incidence of DNA aneuploidy in childhood ALL

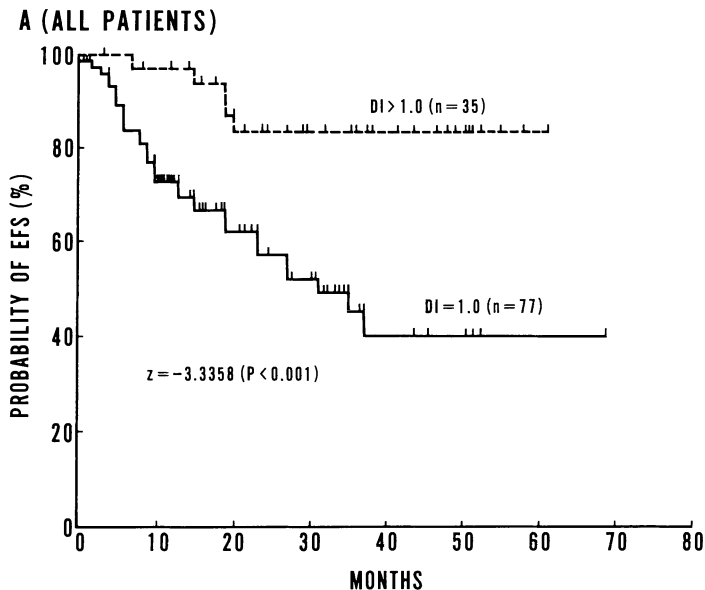
	Number	DNA aneuploidy	
		Number (%)	DNA index (median)
Untreated ALL	196	51 (26.0)	0.72–1.68 (1.18)
Common ALL	144	47 (32.6)	0.72–1.68 (1.20)
T-cell ALL	20	0	
Unclassified ALL	30	4 (13.3)	1.04–1.15 (1.13)
Others	2	0	
Relapsed ALL <sup>a</sup>	54	10 (18.5)	1.10–1.52 (1.18)
Common ALL	38	9 (23.7)	1.10–1.52 (1.19)
T-cell ALL	3	0	
Unclassified ALL	12	1 (8.3)	1.13
Others	1	0	

<sup>a</sup> DNA index was determined from the BM samples obtained at the time of relapse

**Table 3.** Distribution of features at diagnosis of childhood ALL with diploid or hyperdiploid DNA content

Feature		Risk group <sup>a</sup>			
		Low risk (55)		High risk (57)	
		Diploid (30)	Hyperdiploid (25)	Diploid (47)	Hyperdiploid (10)
Sex	Male	22	22	29	4
	Female	8	3	18	6
Phenotype	Common	24	23	26	6
	T-cell	1	0	11	0
	B-cell	0	0	0	0
	Unclassified	5	2	9	1
Age (years)	Median	3.9	3.9	7.6	9.8
	Mean $\pm$ SD	4.4 $\pm$ 2.0	3.9 $\pm$ 1.4	7.5 $\pm$ 3.7	8.2 $\pm$ 3.8
WBC ( $\times 10^3/\mu\text{l}$ )	Median	7.4	7.6	80.3	21.4
	Mean $\pm$ SD	11.3 $\pm$ 10.4	12.4 $\pm$ 10.9	176.8 $\pm$ 292.1	33.4 $\pm$ 33.3
Hemoglobin (g/dl)	Median	7.0	7.7	10.3	8.2
	Mean $\pm$ SD	7.5 $\pm$ 2.3	12.2 $\pm$ 2.1	9.7 $\pm$ 3.2	8.5 $\pm$ 2.6
Platelets ( $\times 10^3/\mu\text{l}$ )	Median	23.0	64.0	46.5	54.0
	Mean $\pm$ SD	79.9 $\pm$ 128.3	189.5 $\pm$ 409.8	90.9 $\pm$ 123.3	181.4 $\pm$ 225.1

<sup>a</sup> Defined by WBC and age (see Table 1). Numbers in parentheses indicate the number of patients. Diploid DI = 1.0; hyperdiploid DI > 1.0



**Fig. 1A–C.** Event-free survival curves of children with ALL, divided into groups based on the DI of their DNA stem-lines. Z-value was given by the generalized Wilcoxon test. **A** All patients (112 cases); **B** low-risk group (55 cases); **C** high-risk group (57 cases)

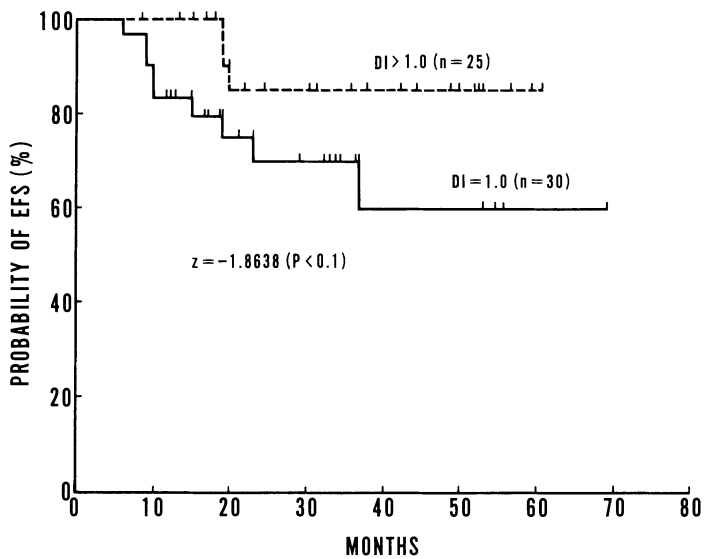
**B (LOW RISK)**

Fig. 1B

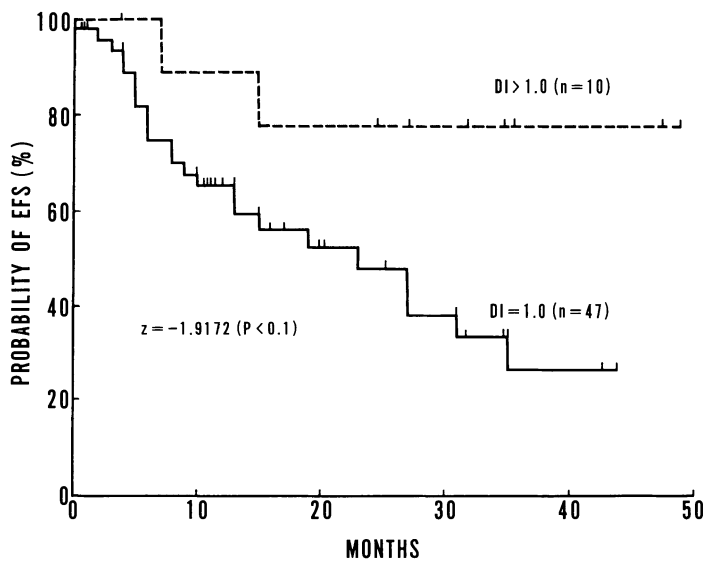
**C (HIGH RISK)**

Fig. 1C

Table 4 shows the results of univariate analysis of DNA content and other prognostic factors. In the low-risk group, the WBC count was the most significant factor, and immunophenotype [presence of common acute lymphoblastic leukemia antigen (CALLA) on the blast cell surface], platelet count, and DNA content were next in order of significance. In the high-risk group, the

DNA content was the significant prognostic factor next to the T-cell phenotype. In the second, the influence of these prognostic factors on the duration of EFS was analyzed with the Cox proportional hazards model (Table 5). After adjustment for the influence of other factors, the WBC and CALLA were still significant factors in the low-risk group, and the influence of the DNA content was

**Table 4.** Univariate analysis of prognostic factors of duration of EFS of patients in low- and high-risk groups

Factor	Category		$\chi^2$ value from log-rank test
	Better	Worse	
<b>Low risk</b>			
WBC ( $\times 10^3/\mu\text{l}$ )	<8 (27)	$\geq 8$ (28)	9.8137***
Phenotype	CALLA+ (47)	CALLA- (8)	6.8845***
Platelets ( $\times 10^3/\mu\text{l}$ )	$\geq 30$ (32)	<30 (20)	4.6461*
DI	>1.0 (25)	1.0 (30)	3.3688*
Sex	Male (43)	Female (12)	1.0350
Hemoglobin (g/dl)	$\geq 8$ (22)	<8 (29)	0.3973
Age (years)	$1 \leq, <4$ (27)	$1 >, \geq 4$ (28)	0.0517
<b>High risk</b>			
DI	>1.0 (10)	1.0 (47)	4.3182**
Phenotype	CALLA+ (35)	CALLA- (21)	3.3933*
	T-cell- (45)	T-cell+ (11)	6.7237***
Sex	Female (24)	Male (33)	3.9987**
WBC ( $\times 10^3/\mu\text{l}$ )	<80 (32)	$\geq 80$ (25)	3.2581
Hemoglobin (g/dl)	< 8 (18)	$\geq 8$ (30)	2.1032
Platelets ( $\times 10^3/\mu\text{l}$ )	$\geq 50$ (23)	<50 (25)	0.4774
Age (years)	$1 \leq, <9$ (28)	$1 >, \geq 9$ (29)	0.4511

Numbers in parentheses indicate the number of patients

\*  $P < 0.1$ ; \*\*  $P < 0.05$ ; \*\*\*  $P < 0.01$

**Table 5.** Relative importance of factors predicting EFS duration considered together by Cox life-table regression model

Factor	Category	$\beta^a$	$P$ value	Risk <sup>b</sup>
<b>Low risk</b>				
WBC ( $\times 10^3/\mu\text{l}$ )	<8 vs. $\geq 8$	2.94	0.0116	18.89
Phenotype	CALLA+ vs. CALLA-	1.68	0.0116	5.40
Age	$1 \leq, <4$ vs. $1 >, \geq 4$	1.08	0.0979	2.95
DI	>1.0 vs. 1.0	1.11	0.1621	2.86
<b>High risk</b>				
DI	>1.0 vs. 1.0	1.65	0.0413	5.23
Sex	Female vs. Male	1.14	0.0559	3.11
Age	$1 \leq, <9$ vs. $1 >, \geq 9$	0.81	0.0958	2.25
WBC ( $\times 10^3/\mu\text{l}$ )	<80 vs. $\geq 80$	0.75	0.0958	2.13

<sup>a</sup>  $\beta$  was estimated by maximum-likelihood methods

<sup>b</sup> Relative risk of induction failure, relapse, or death at any given time for a patient having the worse category of a factor to one having the better category

less significant; whereas, in the high-risk group, the DNA content showed a strong influence on EFS: the relative risk of failure to treatment for hyperdiploid cases was one-fifth that of diploid cases. The T-cell pheno-

type, which had the most significant relationship to EFS as a single factor, lost its significance after adjustment for other prognostic factors.

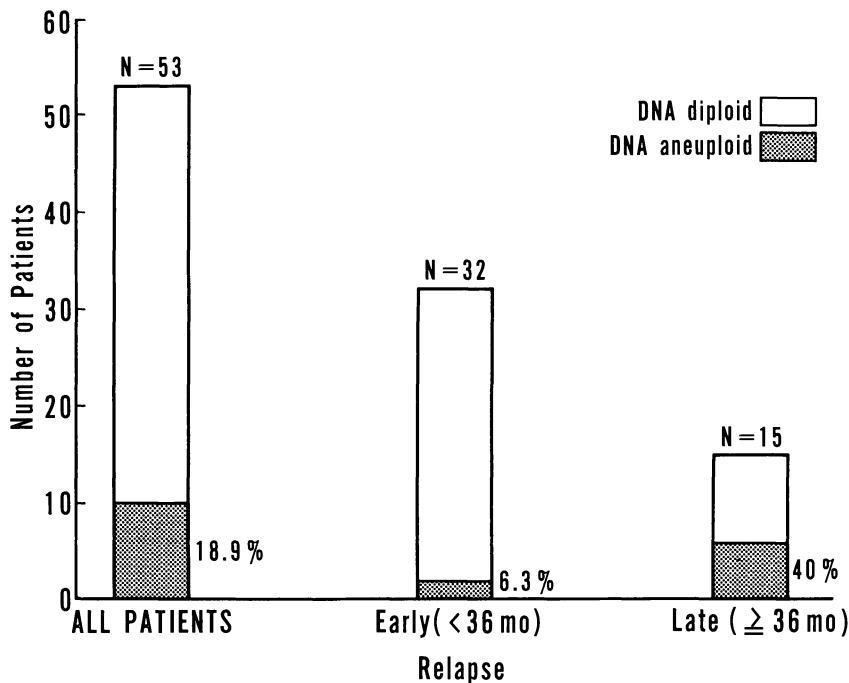


Fig. 2. Incidence of DNA aneuploidy in early and late relapsed ALL

### Relapsed ALL

DNA aneuploidies were detected in 10 patients (18.5%) from 54 relapsed children. All of the aneuploidies showed hyperdiploid DNA stem lines and the median value of this DNA index was 1.18 (Table 2).

There was no significant correlation of the aneuploidy to the clinical and biological features (sex, age, WBC count, platelet count, Hb concentration, immunophenotype, site of relapse) at the time of relapse. When the patients were divided into early (within 36 months after the initial therapy) or late (after this deadline) recurring cases based on the time of first relapse, the latter group showed a significantly higher incidence of aneuploidy than the former group (Fig. 2). The clinical outcome of the relapsed children was compared between the two DNA groups. The duration of the complete remission after the reinduction therapy was not significantly different, and a high but not significant survival rate was shown for the hyperdiploid group (Fig. 3).

### Discussion

Present results in our study confirmed the favorable outcome of patients with hyperdiploid DNA stem lines in childhood ALL which was previously reported from our working group [2]. After adjustment for the influence of other factors, the prognostic influence of aneuploidy was not evident in the low-risk group, which is inconsistent with results reported by the Memphis group [3, 6]. It may be due to the small size of the patient population analyzed in this study. Although a high frequency of long-term remission of hyperdiploid ALL was reported from the BFM group [7], a statistically defined prognostic value of aneuploidy has not been reported for the high-risk patients. In the high-risk group, the children with hyperdiploid DNA stem lines showed a very low mean WBC count as compared with the children with diploid DNA stem lines, whereas the influence of aneuploidy on EFS was significantly greater than WBC count. These results suggest that DNA aneuploidy

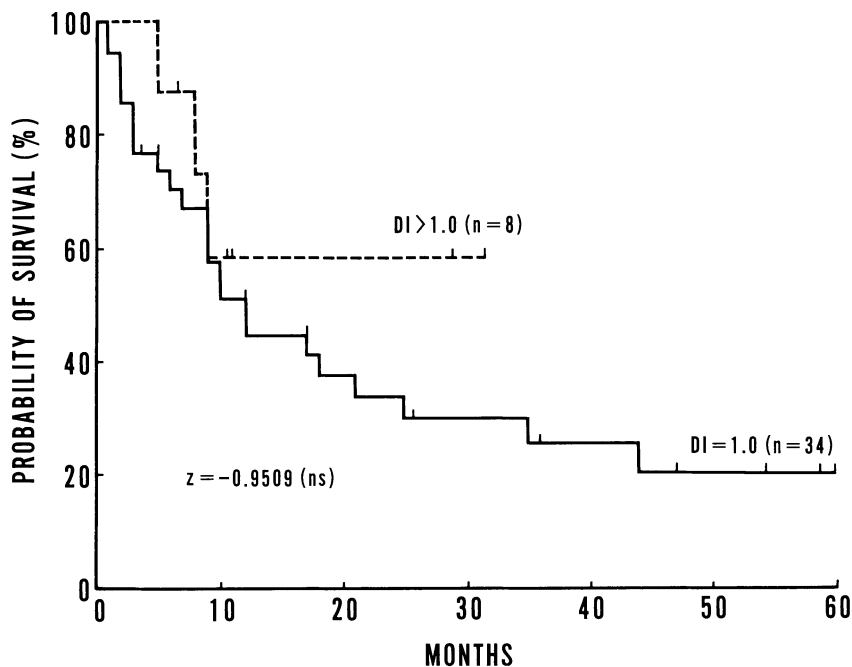


Fig. 3. Survival curves of relapsed ALL, divided into groups based on the DI of their DNA stem-lines. Z-value was given by the generalized Wilcoxon test

had a strong impact on the outcome of patients with a relatively low WBC count among the patients registered in the high-risk group.

The frequency of DNA aneuploidy in relapsed ALL in our study was relatively lower than the results reported by others [8], but a similar tendency toward high frequency of aneuploidy in late recurring children was observed. Since the present results are preliminary, further studies on a large population of recurring children are needed to clarify the clinical implications of aneuploidy in relapsed ALL.

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# **Acute Myelogenous Leukemia in Children**

## Improved Treatment Results in the Myelocytic Subtypes FAB M1–M4 but not in FAB M5 After Intensification of Induction Therapy: Results of the German Childhood AML Studies BFM-78 and BFM-83\*

J. Ritter, U. Creutzig, and G. Schellong

Acute myelogenous leukemia (AML) represents a heterogeneous group of leukemias. It is a rare disease in children, accounting for approximately 20% of acute leukemia in children and adolescents [10]. In contrast to the major advances in childhood lymphoblastic leukemia (ALL), the progress achieved in childhood AML has been less satisfactory. Approximately 20%–25% of children with AML fail to achieve complete remission and a significant number of children in remission still relapse in spite of postremission treatment with polychemotherapy. Since 1978 the German BFM Group conducted three consecutive studies for the treatment of childhood AML, in which 33 hospitals participated [3, 10, 12]. This report updates the latest results of the first two studies BFM-78 and BFM-83 with special emphasis on the main differences between them.

### Patients and Methods

Three hundred and thirty-three children under the age of 17 years entered the two consecutive multicenter studies AML-BFM-78 ( $n=151$ ) between December 1978 and October 1982 and AML-BFM-83 ( $n=182$ ) between December 1982 and October 1986.

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\* Supported by the Federal Minister for Research and Technology, Federal Republic of Germany

Children with secondary leukemia (AML) after myelodysplastic syndromes and patients with prior treatment for more than 14 days according to different protocols were excluded.

The diagnosis of AML and its subtypes was established according to the French-American-British (FAB) classification [1] on Pappenheim-stained bone marrow and blood smears. Special stains including myeloperoxidase, PAS, alpha-naphthylacetate-esterase, and acid-phosphatase were performed at the reference laboratory for the trial in Münster. All diagnoses were confirmed by an independent panel of morphologists. Diagnostic criteria were identical in both studies except for inclusion of two patients with the newly defined FAB subtype M7 [2] in study AML-BFM-83.

Patient characteristics and the FAB subtypes of all patients of both studies are summarized in Table 1.

As shown in Fig. 1, the therapy outline was similar in the two studies. The main difference was an 8-day induction phase consisting of cytosine arabinoside (Ara-C) ( $100 \text{ mg/m}^2$  per day, 24-h infusion, for 2 days, followed by 12-hourly 30-min infusion of the same dose for 6 days), daunorubicin ( $60 \text{ mg/m}^2$  per day, days 3–5), and VP-16 ( $150 \text{ mg/m}^2$  per day, 60-min infusion on days 6–8) which preceded the extended and intensive 8-week induction/consolidation of study AML-BFM-78 [3].

After completion of the extended induction/consolidation, maintenance was started with daily 6-thioguanine (6-TG) ( $40 \text{ mg/m}^2$

**Table 1.** Patient characteristics – studies AML-BFM-78 and -83

	BFM-78	BFM-83
All patients	151	182
Boys:girls (%)	54:46	54:46
Age (median)	9 11/12	8 2/12
WBC (median/ × 10 <sup>3</sup> /μl)	24	25
CNS involvement (%)	9	6
FAB type (%)		
M1	36 (24)	37 (20)
M2	34 (23)	37 (20)
M3	6 (4)	5 (3)
M4	40 (26)	45 (25)
M5	32 (21)	48 (26)
M6	3 (2)	7 (4)
M7	– (–)	3 (2)

orally), and monthly Ara-C (40 mg/m<sup>2</sup> s.c. × 4) and Adriamycin (25 mg/m<sup>2</sup> i.v.) every 8 weeks during the 1st year (up to a maximum cumulative dose of 300 mg/m<sup>2</sup> in study BFM-78 and 400 mg/m<sup>2</sup> in study BFM-83). Maintenance therapy was discontinued after 2 years for children in continuous complete remission (CCR) in study BFM-78 and after a total treatment duration of 2 years in study BFM-83.

All patients received CNS prophylaxis consisting of cranial irradiation with 18 Gy (reduced dosage in patients under the age of 2 years) in combination with intrathecal Ara-C (40 mg/m<sup>2</sup>; reduced dosage in children under the age of 3 years) on days 31, 38, 45, and 52 of the consolidation treatment.

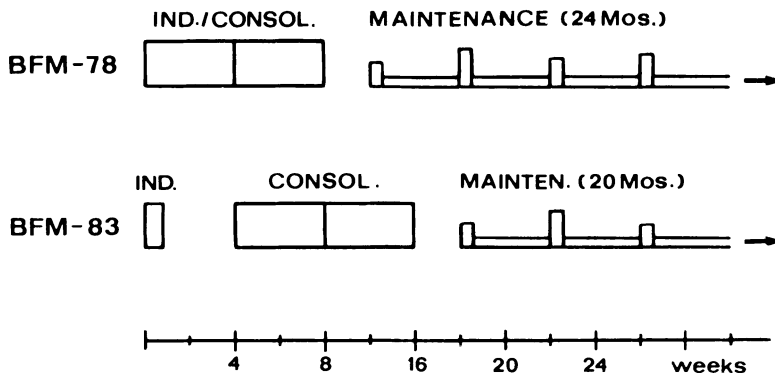
Kaplan-Meier life table analyses [7] were based on the following definitions:

1. Event-free survival (EFS): The analysis was based on the respective total group of patients. All events leading to remission failures or to termination of survival in remission (first relapse, death in remission) were evaluated.
2. Event-free interval (EFI): The analysis was based on patients achieving complete remission. The time of the first relapse was evaluated. Patients who died in first remission were also counted as failures.
3. Withdrawals from the study were always censored at the time of withdrawal.

The standard deviations for life table estimations were determined according to the Greenwood formula. Statistical comparisons between life table curves were performed by the log-rank test [9]. All results were updated as of 15 January 1989.

### Treatment Results

The overall results of study BFM-78 after a median follow-up of 8 ½ years and of study BFM-83 after a median follow-up of more than 4 years are presented in Table 2. Eleven children died of hemorrhage and leukostasis before the onset of therapy, leaving 149 protocol patients in study BFM-78 and 173 protocol patients in study BFM-83. In both studies, 80% of the protocol patients achieved complete remission. Ten children died in complete remission because of infectious complications and 13 patients were withdrawn, mostly due to bone marrow transplantation in first remission. Fifty-five



**Fig. 1.** Outline of therapy for AML studies BFM-78 and BFM-83

**Table 2.** Results of the AML studies BFM-78 and BFM-83, 15 January 1989

	BFM-78	BFM-83
Patients	151	182
Death prior to therapy	2	9
Protocol patients	149	173
Death during induction		
Hemorrhage/leukostasis	12	9
Other complications	5	3
Nonresponder	13	22
Complete remission	119 (80%)	139 (80%)
Death in CCR	6	4
Withdrawals (BMT)	6 (2)	7 (7)
Relapses, total	55	46
With CNS involvement	(9)	(7)
In CCR	52	82
Alive	63	97
Follow-up (months)	74–119	27–72

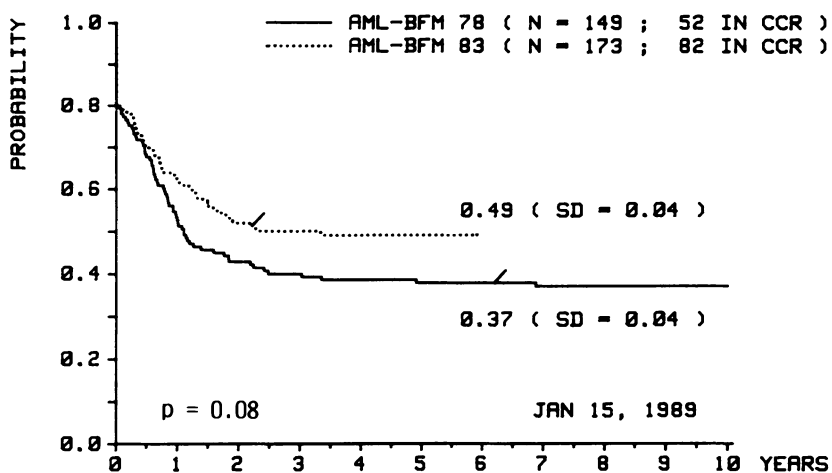
relapses occurred in study BFM-78 and 46 relapses in study BFM-83. The relapse sites are shown in Table 3. Most relapses occurred in the bone marrow, whereas in nine patients in study BFM-78 and in seven patients of BFM-83 the CNS was involved. Life table estimations for EFS (Fig. 2) are 37% (SD 4%) after 10 years in study BFM-78 and 49% (SD 4%) after 6 years in study BFM-83 ( $P=0.08$ , log-rank test). In both studies most relapses occurred during the first 30 months after diagnosis. Treatment

**Table 3.** Sites of relapse in studies AML-BFM-78 and -83, 15 January 1989

	BFM-78	BFM-83
Total relapses	55	46
Isolated BM	40	37
Isolated CNS	1	4
Combined (with CNS involvement)	8	3
Others (without CNS involvement)	6	2

results according to the morphological FAB subtypes for study BFM-83 are given in Table 4. The number of deaths prior to therapy and of early deaths from hemorrhage and/or leukostasis during induction treatment was relatively high in the M5 subtype, whereas nonresponders were evenly distributed among the four subtypes M1, M2, M4, and M5. The relatively high number of relapses in patients with M2 and M4 observed in study BFM-78 [3] was not seen in study BFM-83. However, the proportion of relapses in patients with the FAB M5 subtype was higher in study BFM-83 than in study BFM-78 [3].

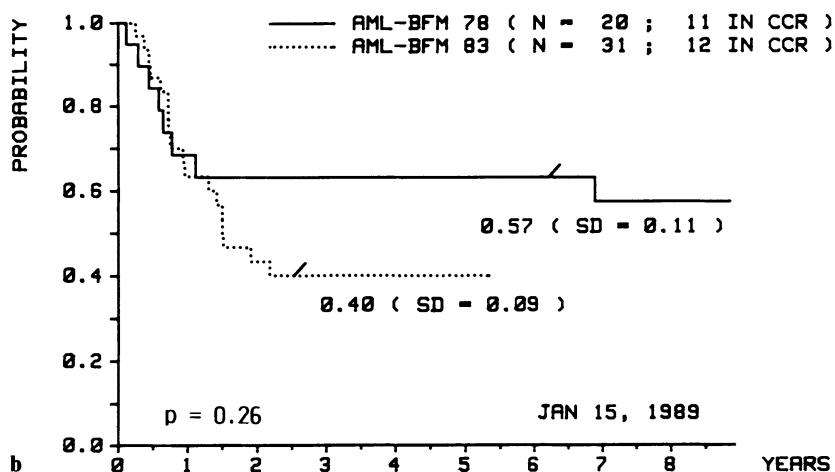
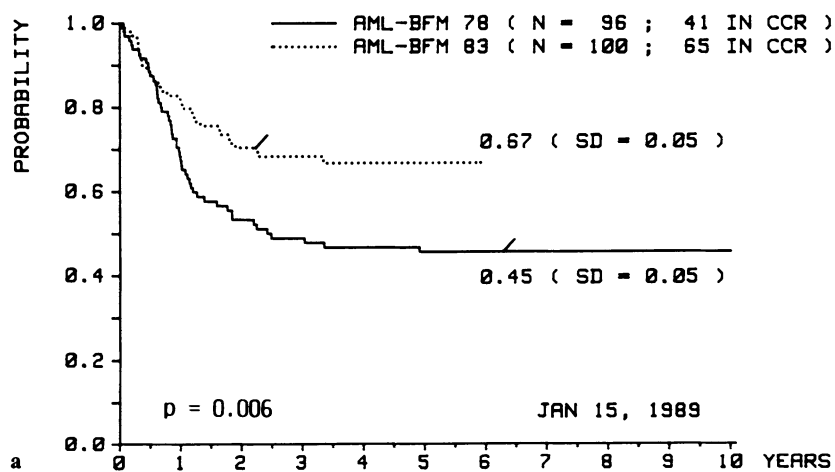
Life table analysis for EFI demonstrates a significantly ( $P=0.006$ ) better outcome for patients with M1–M4 in study BFM-83 (EFI, 5 years: 67%, SD 5%) as compared with study BFM-78 (EFI of 5 years: 45%,



**Fig. 2.** Probability of event-free survival in AML studies BFM-78 and BFM-83. /, last patient entering the study

**Table 4.** AML-BFM-83: results by morphological subtype

	M1	M2	M3	M4	M5	M6	M7
Total (n)	37	37	5	45	48	7	3
Death prior to therapy	-	-	-	2	7	-	-
Death during induction	1	3	1	2	5	-	-
Nonresponder	5	4	-	6	5	1	1
Complete remission	31	30	4	35	31	6	2
Death in remission	-	1	1	1	1	-	-
Withdrawals (BMT in first CR)	-	-	-	4	2	1	-
Relapses	8	9	1	10	16	1	1



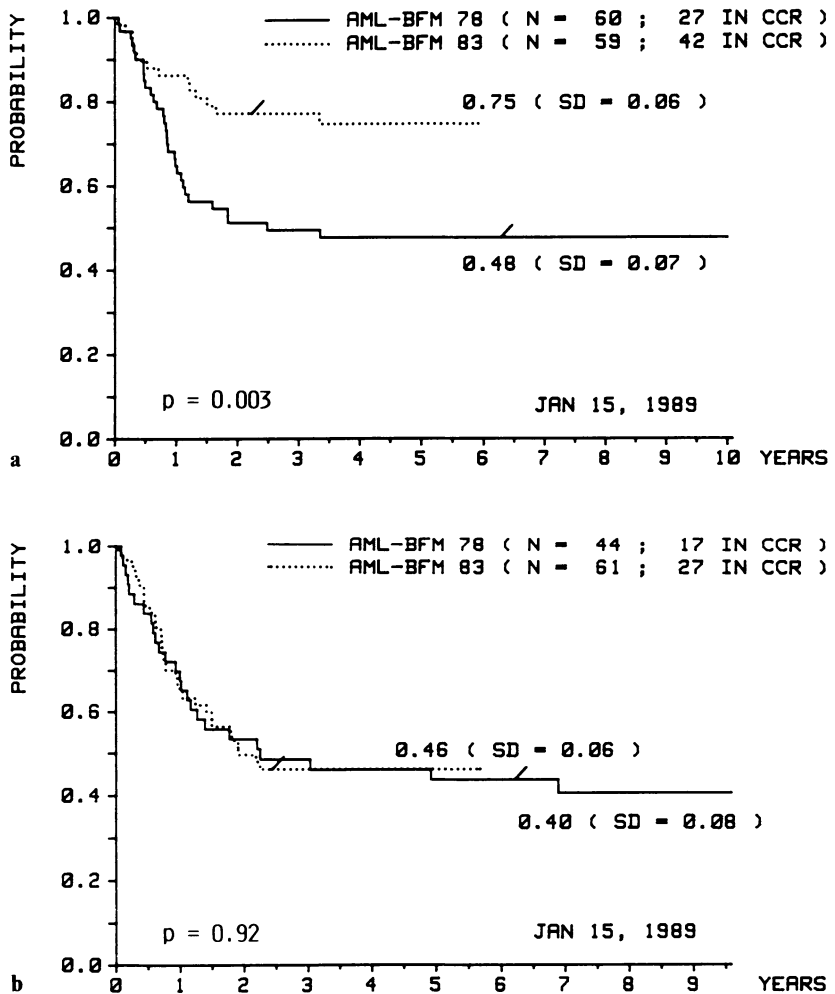
**Fig. 3 a, b.** **a** Probability of event-free interval for patients with the myelocytic subgroups FAB M1–M4 for AML studies BFM-78 and BFM-83. /, last patient entering the study. **b** Probability of event-free interval for patients with acute monoblastic leukemia FAB M5 for AML studies BFM-78 and BFM-83. /, last patient entering the study

SD 5%) as shown in Fig. 3a. However, the EFI for children with the M5 subtype was not improved in study BFM-83 (EFI of 5 years: 40%, SD 9%) over study BFM-78 (EFI of 5 years: 57%, SD 11%; Fig. 3b).

In addition to the morphological FAB subtypes another two morphological features of prognostic significance were found in study BFM-83, namely presence or absence of Auer rods [11] and of eosinophils (Creutzig et al., this volume) in the bone marrow. Figure 4a shows the significantly ( $P=0.003$ ) improved prognosis for patients with Auer-rod-positive blasts in study

BFM-83 as compared with study BFM-78, whereas the EFI in patients with Auer-rod negative blasts in study BFM-83 (probability of EFI of 5 years: 46%; SD 6%) was not significantly different from the EFI in study BFM-78 (probability of EFI of 5 years: 40%; SD 8%, Fig. 4b).

Children with  $\geq 3\%$  of eosinophils in the bone marrow had a significantly ( $P=0.006$ ) improved long-term prognosis in study BFM-83 as compared with study BFM-78, which is shown in Fig. 5a. However, children with less than 3% eosinophils in the bone marrow had no improvement in long-



**Fig. 4a, b.** **a** Probability of event-free interval for patients with Auer-rod-positive blasts in AML studies BFM-78 and BFM-83. /, last patient entering the study. **b** Probability of event-free interval for patients with Auer-rod-negative blasts in AML studies BFM-78 and BFM-83. /, last patient entering the study

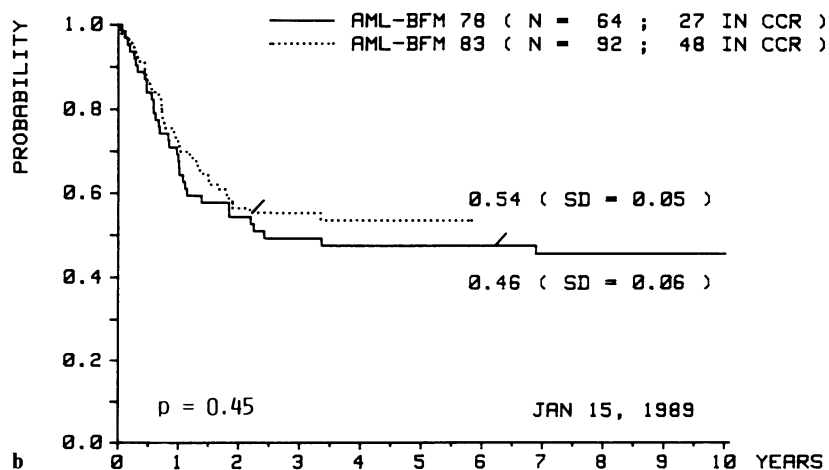
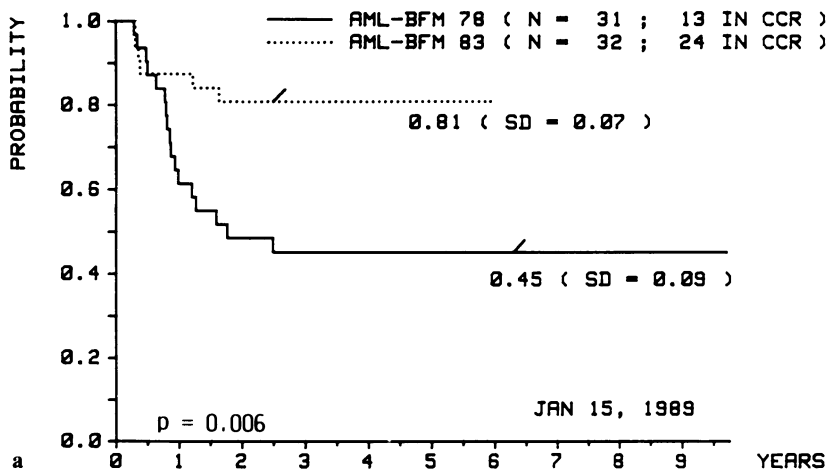


Fig. 5a, b. **a** Probability of event-free interval for patients with  $\geq 3\%$  eosinophils in AML studies BFM-78 and BFM-83. /, last patient entering the study. **b** Probability of event-free interval for patients with  $< 3\%$  eosinophils in AML studies BFM-78 and BFM-83. /, last patient entering the study

term prognosis (probability of EFI of 5 years: 54%; SD 5%) over study BFM-78 (probability of EFI of 5 years: 46%; SD 6%, Fig. 5b).

## Discussion

The updated results of the first two consecutive German multicenter studies for childhood AML demonstrate that intensification of remission induction treatment did not further improve the remission rate in childhood AML. However, there was a trend to-

ward improvement of the remission duration for the whole group of children with AML. Furthermore, specific subtypes of childhood AML had a significantly improved long-term prognosis, such as the myelocytic FAB types M1, M2, M4, and children with Auer-rod-positive blasts or bone marrow eosinophilia. The prognostic significance of Auer rods [8] and of bone marrow eosinophilia [6] has also been reported in adult AML.

Children with acute monoblastic leukemia (FAB M5) had no improved long-term prognosis after intensification of remis-



sion induction treatment in our studies. These results are confirmed by the Boston group, who found that only the M5 subtype independently predicted for poor long-term survival [5].

Multivariate risk factor analysis of study BFM-83 [4] led to the identification of two risk groups with an expected EFI of 5 years of more than 80% (risk group I) and less than 50% (risk group II) as shown in Table 5. In our present study AML-BFM-87, bone marrow transplantation in first remission for patients with an HLA-identical sibling is recommended only for children of the poor risk group but not for those with favorable prognostic factors.

**Table 5.** Risk groups according to AML-BFM-83 (multivariate analysis [5])

Expected EFI of 5 years	I >80%	II <50%
M1	Auer +	Auer -
M2	WBC <20000/mm <sup>3</sup>	WBC >20000/mm <sup>3</sup>
M3	All	-
M4	Eo ≥3%	Eo <3%
M5	-	All
M6 + 7	?	?
Time to CR		≥ 10 weeks

In conclusion, intensification of remission induction therapy improved the long-term results in specific subtypes of childhood AML. For further improvement a modification or intensification of therapy including allogeneic or autologous bone marrow transplantation in first remission is warranted, especially in children with acute monocytic leukemia.

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## Intensive Sequential Chemotherapy for Children with Acute Myelogenous Leukemia

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Acute myelogenous leukemia (AML) is a heterogeneous group of leukemias derived from cells of the myeloid lineage. Progress in therapy has increased the complete remission rate and the percentage of children achieving prolonged disease-free survival (>5 years). From 1976 to 1988 we have treated 228 consecutive patients with AML at our institutions on three consecutive protocols: VAPA, 80-035, and Hi-C DAZE. All three protocols featured intensive consolidation chemotherapy. Results of the first two protocols have been published previously [1, 2], and will be updated here. In addition a preliminary analysis of the third study, Hi-C DAZE, will be presented.

### Material and Methods

#### *Patients*

Sixty-one consecutive untreated children < 18 years of age with AML were treated on the VAPA protocol from 1976 to 1980. From 1980 to 1984, 64 patients were treated on the 80-035 protocol. The third protocol, Hi-C

DAZE, enrolled 103 patients from 1984 to 1988. Hi-C DAZE was modified after entry of 33 patients (group 1) because of CNS toxicity, and the subsequent 70 patients were designated as group 2. The diagnosis of AML was based on morphological examination of bone marrow and histochemical stains. Morphological subtypes of AML were determined according to the French-American-British (FAB) method of classification [3].

#### *Treatment*

The details of the VAPA and 80-035 protocols have been published [1, 2]. Table 1 summarizes the induction regimens for VAPA, 80-035, as well as both groups of Hi-C DAZE. For all the protocols, patients not entering remission after two induction regimens were considered induction failures and taken off study. In the VAPA study, remission was induced with two courses of vincristine, Adriamycin, prednisolone, and standard-dose cytosine arabinoside (Ara-c) (Table 1). Patients entering remission were treated with 14 months of intensive sequential chemotherapy (Table 2). 80-035 differed from VAPA in that:

- a) daunorubicin was substituted for doxorubicin in an attempt to reduce the incidence of enterocolitis,
- b) the Ara-c dose during induction was doubled, and
- c) intrathecal Ara-c was added during induction and maintenance to decrease primary CNS relapse.

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Division of Pediatric Oncology and Biostatistics, Dana-Farber Cancer Institute; Division of Hematology/Oncology, The Children's Hospital, Boston; University of Puerto Rico, San Juan; Midwest Children's Cancer Center, Division of Hematology/Oncology, Children's Hospital of Wisconsin, Milwaukee; Division of Hematology/Oncology, Children's Hospital at Stanford.

**Table 1.** Induction therapy

Protocol	Drug	Dose	Route	Course 1	Course 2
VAPA	Vcr	1.5 mg/m <sup>2</sup>	i.v.	d1,5	d1
	Adr	30 mg/m <sup>2</sup>	i.v.	d1,2,3	d1,2
	Pred	40 mg/m <sup>2</sup>	i.v. q12 h	d1-5	d1-5
	Ara-c	100 mg/m <sup>2</sup>	i.v. c.i.	d1-7	d1-5
80-035	Dauno	45 mg/m <sup>2</sup>	i.v.	d1,2,3	d1
	Ara-c	200 mg/m <sup>2</sup>	i.v.	d1-7	d1-5
	IT Ara-c		i.t.	d1	d1
Hi-C DAZE (gp1)	Dauno	30 mg/m <sup>2</sup>	i.v.	d1,2,3	d1,2
	HD Ara-c	3 g/m <sup>2</sup>	i.v. q12 h	d1-4	d1-4
Hi-C DAZE (gp 2)	Dauno	30 mg/m <sup>2</sup>	i.v.	d1,2,3	None
	HD Ara-c	3 g/m <sup>2</sup>	i.v. q12 h	d1-4	None
	VP-16	200 mg/m <sup>2</sup>	i.v.	None	d1-3,6-8
	5-aza	150 mg/m <sup>2</sup>	i.v. c.i.	None	d3-5,8-10

*Drugs:* Vcr, vincristine; Adr, doxorubicin; Pred, prednisolone; Ara-c, cytosine arabinoside; HD Ara-c, high-dose cytosine arabinoside; VP-16, etoposide; 5-aza, 5-azacytidine; d, day; c.i., continuous infusion

**Table 2.** Consolidation therapy

VAPA	80-035	Hi-C DAZE group 1 (alternating for six cycles)	Hi-C DAZE group 2 (alternating for six cycles)
Sequence I (courses 1-4) Adr 45 i.v., d1 Ara-c 200 c.i., d1-5	Sequence I (courses 1-4) Dauno 45 i.v., d1 Ara-c 200 c.i., d1-5 6 TG 200 p.o., d1-5	VP-16 200 i.v., d1-3 5-aza 150 c.l., d3-5,	Dauno 30 i.v., d1,2 HD Ara-c 3 g i.v., q12 h d1-3
Sequence II (courses 5-8) Adr 30 i.v., d1 5-aza 150 c.i., d1-5	Sequence II (courses 5-7) Dauno 30 i.v., d1 5-aza 150 c.i., d1-5	Dauno 30 i.v., d1,2 HD Ara-c 3 g i.v., q12h d1-3	VP-16 200 i.v., d1-3 5-aza 150 c.i., d3-5
Sequence III (courses 9-12) Vcr 1.5 i.v., d1 Pred 800 i.v., d1-5 6-MP 500 i.v., d1-5 MTX 7.5 i.v., d1-5	Sequence III (courses 8-11) Ara-c 200 c.i., d1-5 6-TG 200 p.o., d1-5		
Sequence IV (courses 13-16) Ara-c 200 c.i., d1-5			
CNS treatment: none	CNS treatment: intermittent i.t., Ara-c	CNS treatment: intermittent i.t., Ara-c	CNX treatment: intermittent i.t., Ara-c

*Drugs:* Adr, Adriamycin; Ara-c, cytosine arabinoside; 5-aza, azacytidine; Vcr, vincristine; Pred, methyl-prednisolone; 6-mp, mercaptopurine; MTX, methotrexate; Dauno, daunorubicin; 6-TG, thioguanine; VP-16, etoposide; HD, high dose; c.i., continuous infusion; d, day; i.t., intrathecal; p.o., oral; i.v., intravenous; g, grams

Hi-C DAZE utilized high-dose Ara-c during induction and consolidation and paired VP-16 with azacytidine. At the time we designed this protocol, high-dose Ara-c was shown to be active in patients with AML whose leukemia was refractory to standard doses, and schedules of Ara-c [4, 5] and VP-16 was most effective in patients with the M4 and M5 subtypes of AML [6-8]. The Hi-C DAZE induction regimens are outlined in Table 2. The two courses of high-dose Ara-c during induction resulted in a high incidence of cerebellar toxicity. The protocol was therefore modified to include VP-16 and 5-azacytidine for the second induction course (group 2). Consolidation therapy differed conceptually from the previous protocols in that pairs of drugs were given on an alternating basis rather than in sequential blocks.

### Statistical Analysis

Disease-free survival (Kaplan-Meier method) was measured from the time of complete

remission [9]. Leukemic relapses and deaths in remission were both counted as failures in this analysis. Patients removed from study for bone marrow transplantation in first remission are censored at the time of transplantation. Withdrawals for other reasons were handled similarly. Statistical tests of significance were made with the log rank test [10] or the Cox model [11] when appropriate. All probability measurements were two sided.

### Results

#### VAPA and 80-035

The results for VAPA and 80-035 are shown in Table 3. The complete remission rate was 74% on VAPA and 70% on 80-035. There were no remission deaths on VAPA, but 20% of the failures on 80-035 were due to death during remission. There were fewer primary CNS relapses on 80-035 (where intrathecal drug was used) compared with VAPA, but this did not achieve statistical signif-

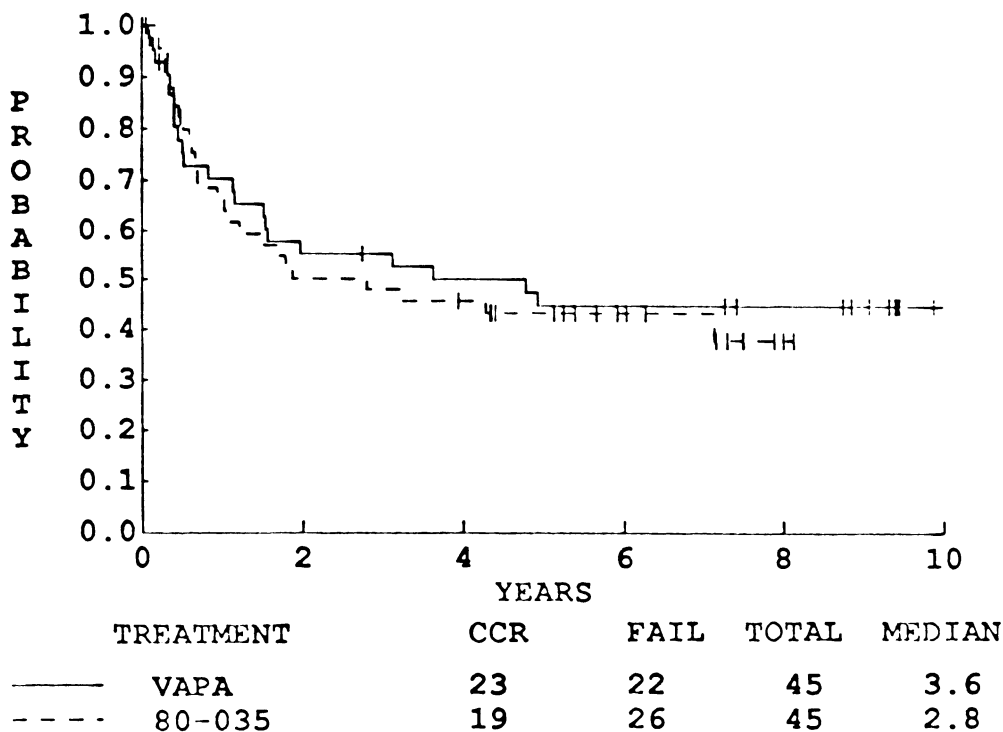


Fig. 1. Kaplan-Meier estimate for probability of disease-free survival for patients entering complete remission on VAPA (solid line) or 80-035 (dashed line). Tick marks indicate patients continuing in remission

**Table 3.** VAPA and 80-035 results

	VAPA	80-035
No. patients	61	64
No. complete remission	45 (74%)	45 (70%)
No. rem. deaths	0	5
No. relapses (CNS)	22 (8)	20 (3)

icance. Figure 1 shows the disease-free survival curves for VAPA and 80-035. As illustrated, both VAPA and 80-035 resulted in a 45% probability of continuous complete remission at 5 years.

There were no clinical or laboratory findings that had a statistically significant effect upon the remission induction rate in VAPA and 80-035. However, by univariate analysis, the following were adverse factors for duration of remission: white blood cell count greater than 100000/mm<sup>3</sup>, age less than 2 years, and FAB subtypes M4 (myelomonocytic) and M5 (monocytic). By multivariate analysis white blood cell count and FAB type appeared to have independent prognostic importance.

### Hi-C DAZE

The remission induction results for both groups of Hi-C DAZE are shown in Table 4. The complete remission rate for group 1 was 85% and 78% for group 2. One patient was withdrawn after the first induction course because of patient preference. The original induction regimen (group 1) was modified because of CNS toxicity. Five patients developed severe cerebellar signs at the end of the second induction course. Because of the toxicity, these five patients received no Ara-c during consolidation.

**Table 4.** Hi-C daze induction results

	Group 1	Group 2	Overall
Total patients	33	70	103
Induction withdrawals	0	1	1
Entered CR (%)	28 (85%)	54 (78%)	82 (80%)
CNS toxicity	5	0	5

### Discussion

The VAPA, 80-035, and Hi-C DAZE protocols were designed to improve long-term disease-free survival for children with AML. Disease-free survival on VAPA and 80-035 was 45% at 5 years. The median follow-up for both studies is greater than 5 years and relapse after 3 years in continuous complete remission has been unusual. Growth and development appeared normal in the long-term survivors. The modifications of therapy in 80-035 (intrathecal Ara-c, daunorubicin for Adriamycin and addition of thioguanine) did not result in an improvement in overall disease-free survival compared with VAPA. The results of VAPA and 80-035 were comparable or better than most other chemotherapy trials in childhood AML [12–15]. High white count and M4 and M5 FAB subtypes predicted for short duration of remission on both VAPA and 80-035. The biological basis for poorer outcome in patients with monocytic subtypes was unclear. Other investigators have not uniformly corroborated this finding [12, 13]. Monocytic leukemia tends to present and relapse in extramedullary sites [2, 16]. Perhaps better therapy to these areas could improve survival in this subgroup. However, the decreased primary CNS relapse rate on 80-035 did not improve outcome for the patients with M4 and M5 leukemic subtypes.

Hi-C DAZE was designed to incorporate a new chemotherapeutic agent (VP-16) and to use Ara-c in a new and potentially more powerful way [4–8]. In addition to excellent activity against relapsed medullary disease, high-dose Ara-c has excellent CSF penetration, and might decrease leukemia in this sanctuary spot [17]. The CNS toxicity of high-dose Ara-c forced us to modify the regimen and use VP-16 and azacytidine as the second induction drugs. Remission duration results are still too early to report. By increasing the Ara-c dose we, of necessity, shortened the duration of therapy. The efficacy of Ara-c is clearly dose and time dependent [4, 18]. Hopefully, the possible gain in efficacy by the logarithmic increase in dose will be greater than any possible decreased efficacy of shortened duration. Further progress awaits other careful analysis of factors that affect duration of remission

and better application of chemotherapeutic modalities.

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## Therapy of Childhood Acute Nonlymphocytic Leukemia: The Pediatric Oncology Group Experience (1977–1988)\*

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### Introduction

In 1977, the members of the Pediatric Oncology Group (POG, formerly the Pediatric Division of the Southwest Oncology Group) initiated a series of protocols designed for the investigation and therapy of childhood acute nonlymphocytic leukemia (ANLL). In general, studies of childhood ANLL prior to the early 1970s were concerned primarily with induction response rates. Those studies usually employed single agents or drug combinations which had proven effective for the more frequently occurring acute lymphocytic leukemia of childhood. Due to thera-

peutic limitations, little distinction was made with regard to morphologic subclasses of acute leukemia and therapeutic choices [1]. These early reports were particularly unrevealing with regard to prognostic factors and therapeutic directions in childhood ANLL [2, 3]. After the introduction of combination therapy with cytosine arabinoside and the anthracyclines, significant improvements in induction treatment responses were observed but in the next decade disease-free survival improved little [4]. While advances in supportive care modalities were important contributions to better patient survivals, it was recognized that improvements in specific therapies for ANLL would be dependent upon controlled, randomized trials designed to investigate not only induction therapies, but also postremission management. Many of the early studies of childhood ANLL were based on trials in adults with ANLL because the disease incidence and patient numbers were greater in that population. This report details the results of two completed randomized phase III studies and outlines the preliminary results of a third pilot study performed from 1977 to 1988 by the POG in newly diagnosed childhood ANLL. These studies were analyzed for selected possible prognostic factors and their relative importance.

### Methods and Materials

#### *Patients*

On all three studies, patients under 21 years of age with newly diagnosed, untreated ANLL were eligible for registration. In-

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**Table 1.** Pediatric Oncology Group**Remission induction**

V	Vincristine, 2.0 mg/m <sup>2</sup> /week × 3 (max. 2.0 mg)
Adr	Doxorubicin, 30 mg/m <sup>2</sup> /day × 3
P	Prednisone, 60 mg/m <sup>2</sup> /day in 3 doses × 21 (max. 60 mg/day)

VAdrP was repeated if the marrow was responding on day 21; if not, Ara-C/6-TG was given as described below and repeated in 15 days if marrow response was then documented

**Continuation and CNS therapy****ARM I**

6-TG	6-Thioguanine, 100 mg/m <sup>2</sup> /day × 5 followed in 12 h by
Ara-C	Cytosine arabinoside, 100 mg/m <sup>2</sup> /day × 5 alternating with
C	Cyclophosphamide, 35 mg/m <sup>2</sup> i.v. every 8 h × 12
O	Vincristine, 2.0 mg/m <sup>2</sup> (max. 2.0 mg) on day 1
A	Ara-C, 35 mg/m <sup>2</sup> i.v. every 8 h × 12
P	Prednisone, 35 mg/m <sup>2</sup> every 8 h × 21

**Arm II**

This therapy was identical to arm I with the addition of VAdrP pulses as in induction every fifth cycle

**Central nervous system therapy (given with first COAP only)**

Triple intrathecal therapy (given twice weekly for a total of five doses)

Methotrexate, 12 mg/m <sup>2</sup> (max. 15 mg)
Hydrocortisone, 15 mg/m <sup>2</sup>
Cytosine arabinoside, 30 mg/m <sup>2</sup> (max. 30 mg)

Cranial irradiation (12 fractions)

2400 cGy if aged over 2 years
2000 cGy if aged 1–2 years
1500 cGy if aged <1 year

formed consent was obtained according to both institutional and Food and Drug Administration (FDA) guidelines. Acute measures for the management of extreme leukocytosis, such as leukopheresis, low-dose cranial radiation, and/or brief treatment with hydroxyurea were permitted. Stabilization of individuals with significant hematologic or metabolic derangements or with severe infection was required prior to registration. The ANLL subtype was classified corresponding to the six groups originally described by the French-American-British (FAB) consortium in 1976 [5]. When the criteria for the diagnosis of acute megakaryocytic leukemia were established that subtype also was eligible for study entry [6]. Early death was defined as death before evaluation of response to induction therapy

could be performed. Responses were classified according to previously published guidelines [7].

*Treatments*

Pediatric Oncology Group 7721 (Table 1)

Vincristine, doxorubicin, and prednisone (VAdrP) were given initially for remission induction. Individuals failing to respond to that combination received alternate induction therapy with cytosine arabinoside (Ara-C) and 6-thioguanine (6-TG). All induction responders subsequently underwent cranial radiation and triple intrathecal chemotherapy. Systemic therapy during the period of CNS treatment consisted of a single course

of cyclophosphamide, vincristine, Ara-C, and prednisone (COAP). Upon completion of the CNS phase, responders were then randomized to receive continuation therapy with one of two treatments. Patients who had achieved remission after the initial VAdRP combination were randomized to continuation therapy either with the two chemotherapy cycles described above (Ara-C/6-TG and COAP) alone (arm I) or with the addition of VAdRP therapy pulses every fifth cycle (arm II). There were two courses each of Ara-C/6-TG and COAP between the VAdRP treatments on arm II. Those 20 patients who had failed VAdRP induction but entered remission after Ara-C/6-TG were not randomized and continued therapy alternating the Ara-C/6-TG and COAP pulses alone (arm I) without additional anthracycline. The duration of therapy was 3 years of continuous remission. Patients were re-

moved from the study if they failed to enter remission or if disease recurred at any site.

The objectives of the study were to evaluate:

1. the remission induction response rates;
2. the duration of remission and the impact of the periodic reinforcement with VAdRP pulses on that duration,
3. the incidence of early CNS leukemic involvement in childhood ANLL, and
4. the outcome in relationship to various clinical and laboratory features assessed at diagnosis.

#### Pediatric Oncology Group 8101 (Table 2)

All patients, except those with acute promyelogenous leukemia (APL), were randomized to induction therapy with either vincristine, Ara-C, and dexamethasone (VADx) or daunorubicin, Ara-C, and 6-TG

**Table 2.** Pediatric Oncology Group

#### Induction

V	Vincristine, 2.0 mg/m <sup>2</sup> weekly × 3 (max. 2.0 mg)
A	Ara-C, 100 mg/m <sup>2</sup> /day × 7 as continuous infusion (c. i.)
Dx	Dexamethasone, 6.0 mg/m <sup>2</sup> /day p.o. in three doses
	versus
D	Daunorubicin, 45 mg/m <sup>2</sup> /day × 3
A	Ara-C, 100 mg/m <sup>2</sup> /day × 7 as continuous infusion
T	6-TG, 100 mg/m <sup>2</sup> /day p.o. single dose × 7
	Courses repeated to CR as long as patient responding

#### Consolidation and CNS therapy

6-TG	100 mg/m <sup>2</sup> /day on days 1–4; Ara-C, 100 mg/m <sup>2</sup> /day c. i. on days 5 and 6
5-Az	100 mg/m <sup>2</sup> /day c. i. on days 7 and 8
	Intrathecal therapy was routinely given on day 1 of each induction course and during the period of cranial radiation for a total of six doses. Dosimetry was as in POG 7721

#### Continuation therapy

Arm I: two-cycle therapy (as in arm I of POG 7721)

Cycle 1: Ara-C/6-TG

Cycle 2: COAP

Arm II: four-cycle therapy

Cycle 1: daunorubicin, 90/mg/m<sup>2</sup> on day 1

Ara-C, 100 mg/m<sup>2</sup>/day c. i. on day 1–5

Cycle 2: Ara-C/6TG

Cycle 3: 6-TG/Ara-C/5-Az as in the consolidation therapy scheme

Cycle 4: COAP

The two cycles in arm I are identical to the second and fourth cycles in this arm

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6-TG, 6-thioguanine; Ara-C, cytosine arabinoside; 5-Az, 5-azacytidine

(DAT). Patients with APL were not randomized and received the anthracycline-containing DAT induction therapy. Individual and cumulative anthracycline doses were reduced 30% for patients under the age of 2 years. Intrathecal therapy was begun at diagnosis using the three agents given on the previous study (POG 7721). Trimethoprim-sulfamethoxazole prophylaxis was given daily throughout the induction period. All responders received cranial irradiation and additional triple intrathecal chemoprophylaxis as well as a single course of consolidation therapy with 6-TG, Ara-C, and 5-azacytidine (5-AZ). At the end of this second phase of treatment, all patients remaining in remission were rerandomized to continuation therapy with either two-cycle (arm I) or four-cycle (arm II) therapy as outlined in Table 2. Note that the two-cycle arm is identical to arm I in the preceding study, POG 7721. Therapy was continued for 2 years if the patient remained in complete continuous remission (CCR). Failure at any site completed the study.

The objectives of the 8101 study were:

1. to compare the responses and toxicities of remission induction with VADx versus DAT;
2. to measure the effect, if any, of the particular induction regimen on selected leukemic subgroups and on remission duration;
3. to compare the remission durations observed in patients receiving continuation therapy with the two-cycle regimen from POG 7721 with a more intense four-cycle regimen; and
4. to accumulate clinical and laboratory data regarding features present at diagnosis and to relate that data to outcome.

#### Pediatric Oncology Group 8498: A Pilot Study (Table 3)

This limited participation pilot study was developed in preparation for the third groupwide POG phase III study of childhood ANLL. It was designed to define the tolerable toxicities associated with intensification of both induction and postremission therapy for ANLL and was conducted in two phases. During the first phase, patients received induction therapy with two courses

**Table 3.** Pediatric oncology group 8498 – a pilot study

#### Phase 1 Induction

DAT was given as in POG 8101

#### Postremission therapy

##### Pulse 1<sup>a</sup>

HD ara-C High-dose Ara-C, 3.0 g/m<sup>2</sup>  
every 12 h × 4 doses

L-Asp L-Asparaginase, 10000 U/m<sup>2</sup> i. m.  
3 h later

##### Pulse 2<sup>a</sup>

VP-16 Etoposide, 250 mg/m<sup>2</sup> on days 1, 2, 3  
5-Az 5-Azacytidine, 300 mg/m<sup>2</sup>  
on days 4 + 5

##### Pulse 3<sup>a</sup>

P 6-Mercaptopurine, 500 mg/m<sup>2</sup> i. v.  
on days 1–5

O Vincristine, 1.5 mg/m<sup>2</sup> on day 1  
(max. 2.0 mg)

M Methotrexate, 7.5 mg/m<sup>2</sup> i. v.  
on days 1–5

P Methylprednisolone, 800 mg/m<sup>2</sup> i. v.  
on days 1–5

##### Pulse 4<sup>a</sup>

Ara-C Ara-C, 200 mg/m<sup>2</sup>/day c. i.  
on days 1–5

#### Phase 2

The induction regimen was changed to one course of DAT followed by six doses of high-dose Ara-C (3.0 g/m<sup>2</sup>/dose). If the patient was in remission after those two treatments, a second course of high-dose Ara-C was given and thereafter the regimen was identical to the phase 1 pulses 2, 3, and 4

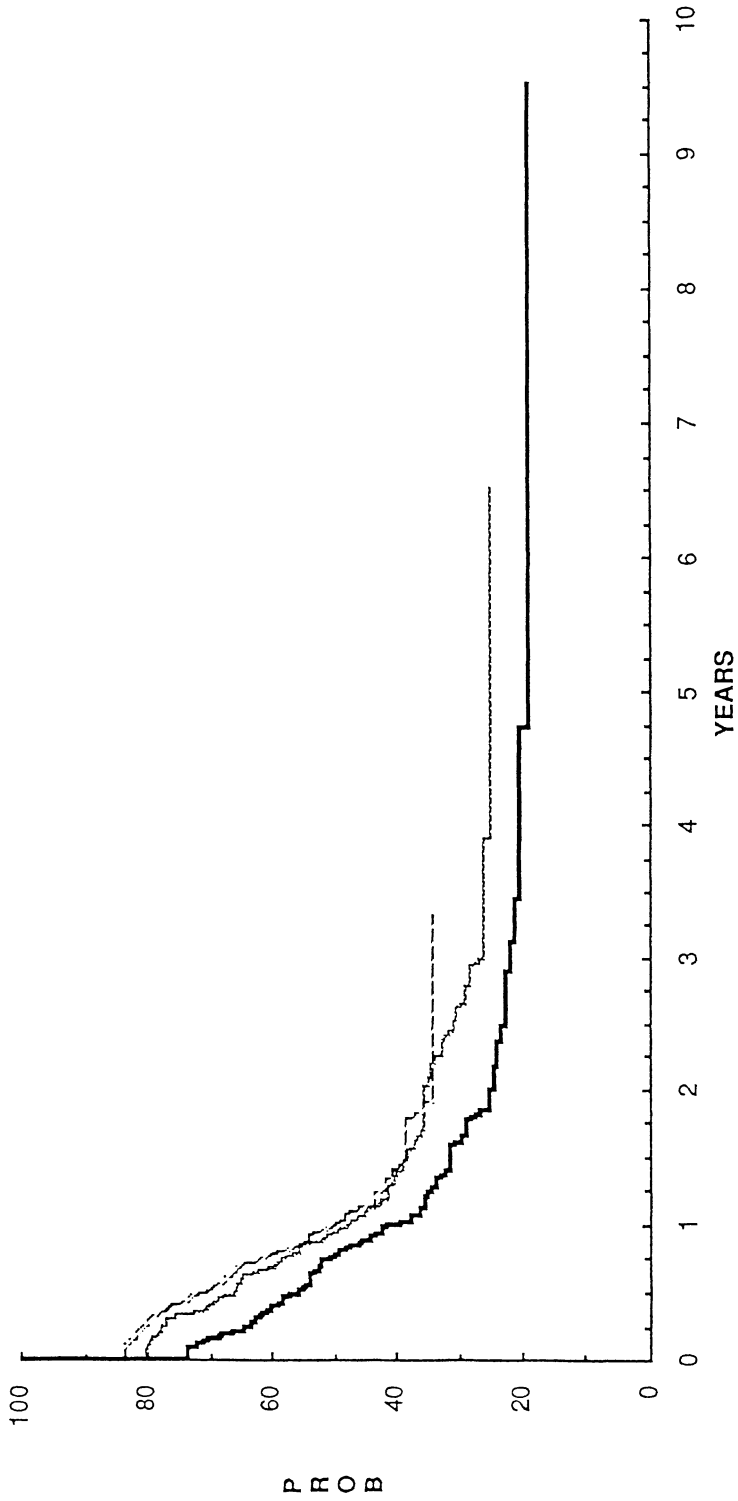
#### Central nervous system therapy

All patients received a total of six intrathecal dosages of cytosine arabinoside, 40 mg/m<sup>2</sup>/dose, during the initial 3 months of therapy

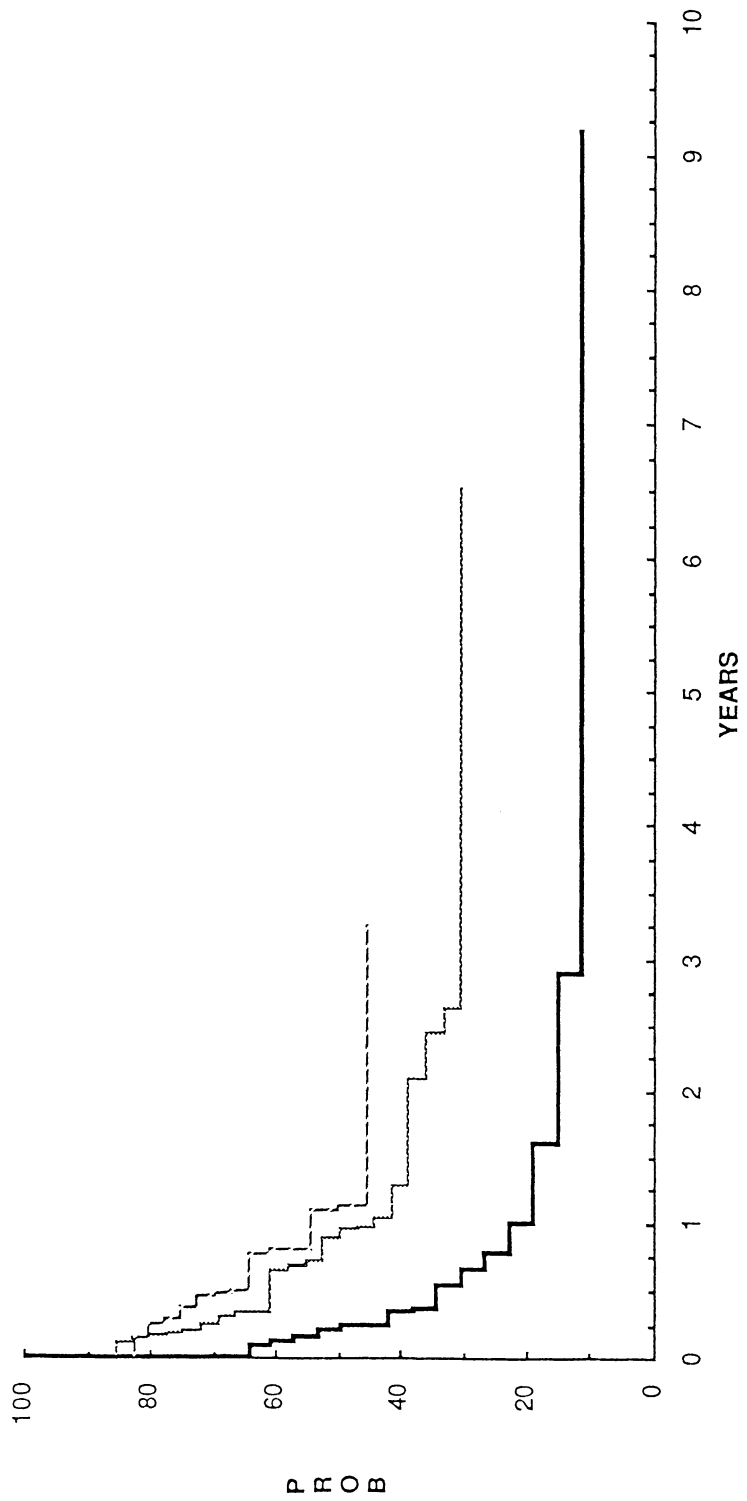
<sup>a</sup> Each pulse is repeated four times in succession

of DAT as was administered in the 8101 regimen, although the interval between courses was shortened and the doses in the second course were reduced by 30%. Responders received four sequential courses each of the following four combinations: high-dose Ara-C (four doses)/L-asparaginase (L-Asp); etoposide/5-Az; POMP (purinethol, vincristine, methotrexate, methylprednisolone); and a 5-day continuous infusion of Ara-C.

— POG 7721    - - - POG 8101    ···· POG 8498



— POG 7721    - - - POG 8101    ···· POG 8498



Four successive cycles of each combination were given to maximize the antileukemic effect of each. The duration of therapy was approximately 18 months.

During the second phase of the pilot study, the induction therapy was intensified further by eliminating the second course of DAT and substituting a more intense course of high-dose Ara-C (six doses). Induction therapy was followed by a second identical high-dose Ara-C course if the patient was in complete remission. The four courses of high-dose Ara-C (four doses)/L-Asp were dropped and the remainder of the treatment was unchanged. CNS therapy in both regimens was identical and consisted of a total of six intrathecal doses of Ara-C alone beginning at the time of diagnosis. Cranial irradiation was not employed routinely. All patients received trimethoprim/sulfamethoxazole prophylaxis. Infants less than 6 months of age had drug dosages calculated by the formula:

$$\text{Weight (kg)} \times (\text{dose/m}^2)/30 = \text{infant dose}$$

The total duration of therapy was 15 months in this second phase of the study. Nonresponse or relapse in any site completed the study.

The primary objectives of this pilot study were twofold: (1) To explore the feasibility and toxicity, particularly myelosuppression, of administering the five intensive drug combinations in sequence at close intervals and to determine minimal peripheral blood count criteria for instituting each cycle of therapy. [Initially, criteria for administering each drug combination consisted of an absolute granulocyte count (AGC) of  $\geq 1000/\mu\text{l}$  and a platelet count of  $\geq 150\,000/\mu\text{l}$ .] (2) To establish preliminary efficacy data for the new regimen before committing the membership of the POG to a lengthy therapeutic investigation.

The original regimen as outlined above was satisfactorily tolerated. It became apparent that further intensification would be feasible and even desirable and the protocol was modified to include not only additional doses of the high-dose Ara-C (an increase from four to six doses) but also to allow for the earlier introduction of the next, presumably non-cross-resistant, drug regimen, etoposide/5-Az. The minimum blood count

criteria were reduced to an AGC of  $\geq 750/\mu\text{l}$  and a platelet count of  $\geq 100\,000/\mu\text{l}$ . Although not the primary thrust of the study, there was an analysis of selected clinical and laboratory features with response correlation. Such early response correlations will be discussed herein and the toxicity data presented elsewhere.

## Results

### *Pediatric Oncology Group 7721*

Distribution of the FAB subtypes by institutional diagnosis is as shown in Table 4. From September, 1977 until July, 1981, 183 patients with newly diagnosed ANLL were treated in this study [8]. Morphologic subclassification was not subject to review. The incidence of CNS disease at diagnosis in 179 patients was 15.6%. Complete responses (CR) to VAdRP were seen in 115 (62.8%) patients and an additional 20/39 patients failing VAdRP achieved CR after receiving Ara-C/6-TG. The overall induction response rate was 73.8%. There were 19 deaths during induction. Other features and response correlations are listed in Tables 4 and 5. Patients with WBC  $\geq 100\,000/\mu\text{l}$  at diagnosis had a lesser chance of achieving CR. Acute monocytic leukemia (FAB M5) was associated with a more favorable induction response rate than the other morphologic subtypes. No other factors tested (age, sex, race, or presence of CNS disease at diagnosis) were significantly predictive of induction response. Individuals achieving remission after VAdRP induction had 30% disease-free survival (DFS) at 5 years while those who were anthracycline resistant and required the addition of Ara-C/6-TG to enter remission fared less well, with a DFS of 12% at 5 years. The median duration of remission for all responders was approximately 12 months and the median survival was 16 months. The 3-year event-free survival (EFS) for all patients entered in the study was  $22\% \pm 0.03\%$ . Children less than 2 years of age at diagnosis had a 3-year EFS of 11% vs. 24% for those over 2 years ( $P=0.04$ ). Those patients with FAB subtypes M4 and M5 had a 3-year EFS of 30% vs. 15% for the M1/M2 group regardless of the continuation arm

**Table 4.** Induction responses by morphology<sup>a</sup>, age, and WBC

	POG study number		
	7721 (%)	8101 (%)	8498 (%)
<b>FAB subtype</b>			
M1/2	84 (73)	108 (84)	117 (82)
M3	5 (20)	16 (81)	8 (67)
M4	60 (73)	83 (78)	65 (91)
M5	27 (93)	31 (77)	34 (82)
M6	6 (50)	2 (100)	0
<b>Age</b>			
<2 years	28 (64)	42 (86)	49 (88)
≥2 years	154 (75)	214 (79)	205 (84)
<b>WBC</b>			
<100 000/ $\mu$ l	155 (77)	217 (83)	204 (89)
>100 000/ $\mu$ l	27 (52)	37 (65)	48 (73)

( ), percentage patients entering complete remission

<sup>a</sup> Forty-six additional patients have been treated on these three studies but FAB types are either unknown or not otherwise specified

**Table 5.** Three-year event-free survivals by morphology, age, and WBC

	POG study number		
	7721	8101	8498
<b>FAB subtype</b>			
M1/M2	0.15 (0.04)	0.20 (0.05)	0.25 (0.15)
M4/M5	0.30 (0.05)	0.29 (0.05)	0.43 (0.19)
<b>Age</b>			
<2 years	0.11 (0.05)	0.30 (0.07)	0.46 (0.24)
>2 years	0.24 (0.04)	0.25 (0.04)	0.32 (0.13)
<b>WBC</b>			
WBC < 100 000	0.24 (0.04)	0.26 (0.04)	0.35 (0.14)
WBC > 100 000	0.13 (0.06)	0.36 (0.09)	0.27 (0.10)
All patients	0.22 (0.03)	0.26 (0.04)	0.34 (0.12)

( ), standard error

( $P=0.02$ ). In terms of remission duration, there were no differences detected between the continuation arms for the entire group or when analyzed according to sex, race, platelet count, or the presence of CNS disease at diagnosis. No failures have been noted after 5 years of continuous remission.

#### *Pediatric Oncology Group 8108*

From June 1981 until January 1986, 256 patients were treated according to this protocol and were evaluable [9]. Their FAB subtypes, age, and WBC at diagnosis are represented in Table 4. The incidence of CNS in-

volvement at diagnosis was 9.3%. The DAT regimen was more effective than VADx for induction (82% vs. 61%). After that observation was established (July 1983), the subsequent 151 patients received DAT induction alone. The CR rate for the 207 patients (56 randomized and 151 nonrandomized) who received the DAT induction regimen was 85%. Overall, there were 13 early deaths. From univariate analysis, significant differences favoring DAT induction were found between the induction arms for Caucasian race, FAB M4 morphology, WBC between 10000/ $\mu$ l and 100000/ $\mu$ l, normal hepatic function studies, and platelet count <100000/ $\mu$ l. There were no differences in outcome for induction responders with respect to the specific induction regimen. There did not appear to be a difference between the continuation therapies (two-cycle vs. four-cycle) with respect to duration of remission or survival. The overall 3-year EFS regardless of treatment was 26%  $\pm$  0.04%. No differences in EFS were detected by age, WBC, or morphology (Table 5). However, significant differences for CCRs were found between the two continuation arms for selected subgroups of DAT-induced patients. Those analyses demonstrated a remission advantage at 2 years on arm II (four-cycle) for the FAB M1/M2 subgroup (48% vs. 20%,  $P=0.005$ ) and for patients older than 10 years (62% vs. 32%,  $P=0.002$ ). When the number of courses of DAT (one versus two) required to achieve remission were examined with respect to remission duration, the greater number was an adverse indicator for patients treated on the two-cycle continuation arm (arm I) but was not significant on the more intense four-cycle arm (arm II). For all DAT responders, the 2-year CCR rate was 0.45 ( $\pm$ 0.04) with an overall survival of 0.50 ( $\pm$ 0.06). The results for DAT responders who received four-cycle continuation therapy were 0.50 ( $\pm$ 0.06) and 0.57 ( $\pm$ 0.05) respectively. Univariate analysis of remission duration for selected patient characteristics indicated an advantage for patients with CNS disease at diagnosis ( $P=0.02$ ), and for those of non-Hispanic origin ( $P=0.003$ ). Differences in remission duration for other characteristics (age, sex, morphology, initial WBC, platelet count, hepatic function, and presence of ab-

normal coagulation studies) were not statistically significant between or within the continuation arms.

#### *Pediatric Oncology Group 8498*

From June 1984 until July 1988, 295 children with ANLL were registered on this study. Preliminary data have been evaluated on 254 of these patients and were presented recently [10]. With respect to response and prognostic factor analyses, that presentation may be summarized as follows: 138 patients received the DAT induction alone (phase 1) and 117 entered remission (85%). The incidence of CNS disease was 12.3% in that group. There were nine early deaths. The second phase of the study using DAT plus high-dose ara-C induction therapy began in December 1986 and 99 of 116 children achieved remission (85%). The incidence of CNS disease at the time of the diagnostic lumbar puncture was 17.4%. There were nine early deaths in this group. The known FAB subtypes for 225 of the patients are listed in Table 4 as are response correlations with morphology, age, and WBC. There are no statistical differences between the two induction regimens and only a WBC  $\geq$  100000/ $\mu$ l was a significant prognostic factor for poor induction response to either regimen ( $P=0.005$ ). Age, morphology, and the presence of extramedullary disease at diagnosis were not predictive for induction response. Features analyzed in the previous study (8101) such as hepatic dysfunction, presence of a coagulopathy, and level of the platelet count were not studied on this pilot regimen. The median duration of remission for patients on phase 1 of the study (DAT only induction) was approximately 13 months and the EFS for the entire group was 34%  $\pm$  0.12% at 3 years (Table 5). It is too early to perform extensive analyses or projections on the later phase 2 patients.

#### **Discussion**

This series of three POG studies demonstrates a substantial improvement in remission induction rates (62% to approximately 85%) using daunorubicin, cytosine ara-



binoside, and 6-thioguanine. Over 500 children with ANLL have received this type of induction regimen during the last 7 years, attesting to its validity and reliability. Advances in supportive care modalities have reduced the early death rate from as high as 33% during ANLL induction therapy in children [11] to 5%–12% in these three studies as well as in other large series [12, 13]. It would appear that, with therapies similar to these, induction drug-resistant

disease accounts for less than 10% of patients with newly diagnosed ANLL. On the basis of induction response alone, it would be difficult to demonstrate improved response rates using comparative trials unless new induction strategies are developed.

The persistent high early relapse rate suggests that, although ANLL may respond well initially, drug resistance either develops very rapidly or exists in a subclinical population of leukemic cells present at diagnosis.

#### Appendix A. Principal investigators of POG participating in this study

Institution	Investigator	Grant #
Alberta Pediatric Oncology Consortium, Edmonton, Alberta, Canada	J. Akabutu	
Baylor College of Medicine, Houston, TX, USA	D. Fernbach	CA-03161
Bowman Gray School of Medicine Winston-Salem, NC, USA	R. Patterson	
Children's Hospital of Michigan, Detroit, MI, USA	Y. Ravindranath	CA-29691
City of Hope, Duarte, CA, USA	P. Konrad	
Duke University Medical Center, Durham, NC, USA	J. Falletta	CA-15525
Emory University School of Medicine, Atlanta, GA, USA	A. Ragab	CA-20549
Johns Hopkins University, Baltimore, MD, USA	B. Leventhal	CA-28476
McGill University, Montreal, Quebec, Canada	V. M. Whitehead	CA-33587
Medical College of Virginia, Richmond, VA, USA	H. Maurer	CA-28530
Medical University of South Carolina, Charleston, SC, USA	H. B. Othersen	
Mt. Sinai Hospital, New York, NY, USA	J. Lipton	CA-38859
New England Pediatric Oncology Consortium, Providence, RI, USA	E. Forman	CA-29293
Oklahoma University, Oklahoma City, OK, USA	R. Nitschke	CA-11233
St. Christopher's Hospital, Philadelphia, PA, USA	R. Wimmer	CA-41573
University of Texas Southwestern Medical Center at Dallas, TX, USA	G. Buchanan	CA-33625
State University of New York, Syracuse, NY, USA	R. Dubowy	CA-41721
Swiss Pediatric Oncology Group Bern, Switzerland	H. Wagner	
Uniformed Services Oncology Washington, DC, USA	D. Maybee	CA-28572
University of Alabama, Birmingham, AL, USA	R. Castleberry	CA-25408
University of Arkansas, Little Rock, AR, USA	D. H. Berry	CA-41188
University of California, San Diego, CA, USA	F. Kung	CA-28439
University of Florida, Gainesville, FL, USA	S. Gross	CA-29281
University of Kansas, Kansas City, KS, USA	T. Vats	CA-28841
University of Miami, Miami, FL, USA	S. Toledano	CA-41083
University of Mississippi, Jackson, MS, USA	J. Pullen	CA-15989
University of South Florida, Tampa, FL, USA	E. Hvizdala	
University of Texas, M. D. Anderson Cancer Center, Houston, TX, USA	D. Pinkel	CA-03713
University of Virginia, Charlottesville, VA, USA	R. B. Raney	
Washington University Medical Center, St. Louis, MO, USA	V. Land	VA-05587

Comparing current “best therapies,” long-term EFS in children with ANLL has remained relatively unchanged in this decade, with about 30%–35% of all newly diagnosed patients and 40%–50% of induction responders remaining in remission for prolonged periods [12]. As with the studies reported here, the great majority of relapses occur within the first 6–12 months after remission usually while the patient is still receiving therapy. The duration of therapy in these POG studies varies from 3 years in the first ANLL study (POG 7721) to 15 months in the most recent without adverse impact on overall disease-free intervals. This reflects the findings of others [13], that shortened intensive therapy is equivalent to more prolonged but less intense regimens and further emphasizes the importance of initial or early therapy. There is a small but significant improvement in the EFS ( $P=0.04$ ) (see Fig. 1) and this appears to be primarily attributable to improved overall induction response rates and to better disease-free survivals in patients less than 2 years of age at diagnosis. Seeking further to increase leukemic cell destruction, therapeutic trials designed to approach problems of early relapse have (as these studies here) progressively employed more intensive induction regimens and/or postremission therapies [14–16]. This approach equates or attempts to correlate response improvement with dosage increase over time. It is difficult to define optimal duration of therapy when there is continual modification of the initial therapy. It is during the early remission “window” that allogeneic bone marrow transplantation is most efficacious as a means of therapy amplification [17].

Simultaneously, efforts to improve response rates also have sought to identify, within the therapeutic framework, favorable or unfavorable clinical and/or laboratory subgroups with the intent of eventually developing subgroup-specific therapies. To date, those efforts have been largely unrewarding. In our studies, the only consistent prognostic indicator for at least induction response, if not remission duration, has been the elevation of the diagnostic WBC over 100 000  $\mu\text{l}$  – perhaps a crude measure of tumor burden. Multivariate analysis does not identify that degree of leukocytosis with

any of the other features measured. While one study will demonstrate favorable or adverse significance for a specific clinical or laboratory characteristic, another will fail not only to confirm those data but may even demonstrate contradictory findings. In these three POG studies, for instance, of note is the absence of consistent data suggesting that age less than two years at diagnosis or the acute monocytic leukemia subgroup are predictive of poor outcome. Although the younger age group fared less well on the first study ( $P = 0.006$ ) (see Table 5 and Figure 2), there is no difference in their current EFS when compared with those patients two years or older. No differences were detected when the three-year EFS for patients on all three studies were compared with respect to morphology (M1/M2 and M4/M5) and WBC at diagnosis ( $\geq$  or  $< 100\,000\ \mu\text{l}$ ) (see Table 5).

However, studies at Dana-Farber Cancer Institute between 1976 and 1984 have consistently demonstrated that monocytic leukemia (FAB M5) is a major negative prognostic indicator. Others have reported similar observations [18, 19]. There does not appear to be an adverse effect on long-term DFS for the FAB M5 subgroup in any of the studies reported here – again emphasizing the significance of therapy as a major determinant of prognostic factors. It has been demonstrated that the monocytic subtypes of ANLL appear to respond better to regimens containing an epipodophyllotoxin [14, 20], and more recent studies emphasize use of those agents.

Since there are no established or consistent prognostic factors other than the presence of an elevated WBC at diagnosis and no defined therapeutic alternatives among the various subgroups, therapeutic trials in childhood ANLL continue to employ the strategies discussed earlier. The POG has recently begun a randomized study (POG 8821) using the induction regimen tested in the second phase of study 8498 (DAT/high-dose Ara-C) and compares the duration of postremission therapy observed using intense chemotherapy combinations with that obtained by giving irreversible myeloablative therapy and purged autograft rescue. Investigation of alternative induction regimens using etoposide, amsacrine, and azacy-

tidine is also ongoing and may address problems of early resistance.

Improving induction responses and EFS, such as demonstrated in the studies reported in this paper, provides an expanded observational time frame which allows more discriminatory investigations into the nature of the nonlymphocytic leukemias. However, while awaiting maturation of these new data, it would appear that intensive chemotherapy trials, utilizing, where appropriate, effective new agents either during induction and/or immediate postremission therapy and with or without marrow grafting offer the best management approach at this time.

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## Preliminary Results of Intensive Therapy of Children and Adolescents with Acute Nonlymphocytic Leukemia – A Childrens Cancer Study Group Report

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### Introduction

The treatment of acute nonlymphocytic leukemia (ANLL) has improved significantly in the past decade. Today approximately 75% of children will enter a complete remission using cytosine arabinoside (Ara-C) and daunorubicin with or without other drugs [6]. Recently, several groups of investigators including Childrens Cancer Study Group (CCSG) investigators have reported the superiority of the disease-free survival of children and adolescents with ANLL transplanted in their first remission with a compatible donor [7, 9]. On the other hand, equally good results have been reported by Grier et al., Creutzig et al., and Amadori et al. [5, 3, 1] with the use of intensive chemotherapy. The most recently completed CCSG study, CCG-213, attempted to determine whether bone marrow transplantation (BMT) done in first remission is better than very aggressive postinduction chemotherapy and whether or not maintenance chemotherapy is needed after postinduction intensification.

A multidrug arm for induction was also compared with standard induction therapy using Ara-C and daunorubicin. Preliminary

results of this pilot protocol have been presented and more mature data will be submitted for peer review in the future [10].

### Methods

The schema for CCG-213 is shown in Fig. 1. The experimental arm for induction (Denver) was based on results of a pilot single-arm study done at Children's Hospital in Denver and consisted of giving 24-h infusions of Ara-C for 5 days; daunorubicin on days 0, 1, 2; VP16-213 on days 0 and 3; and thioguanine and dexamethasone daily [8].

The standard arm (7 and 3) consisted of giving 24-h infusions of Ara-C for 7 days and 3 days of daunorubicin as had been given in the previous frontline ANLL study (CCG-251). The dose of daunorubicin, however, was increased from 30 mg/m<sup>2</sup> to 45 mg/m<sup>2</sup>. Patients were randomized between these two arms and, irrespective of whether or not a remission was achieved with either regimen, the opposite regimen was given for course II of induction. If there was a compatible donor, a BMT was done as soon as remission was achieved. If scheduling for BMT was a problem, thioguanine 60 mg/m<sup>2</sup> was given on days 1–4, 8–11, 15–18, and Ara-C 50 mg/m<sup>2</sup> was given subcutaneously on days 5, 12, and 19.

For those patients not transplanted, the postinduction intensification consisted of three courses of therapy. The method of giving high-dose Ara-C as described by Capizzi et al. [2] was used for the first course and consisted of 3 g/m<sup>2</sup> Ara-C over 3 h every 12 h for four doses starting on day 0. Three

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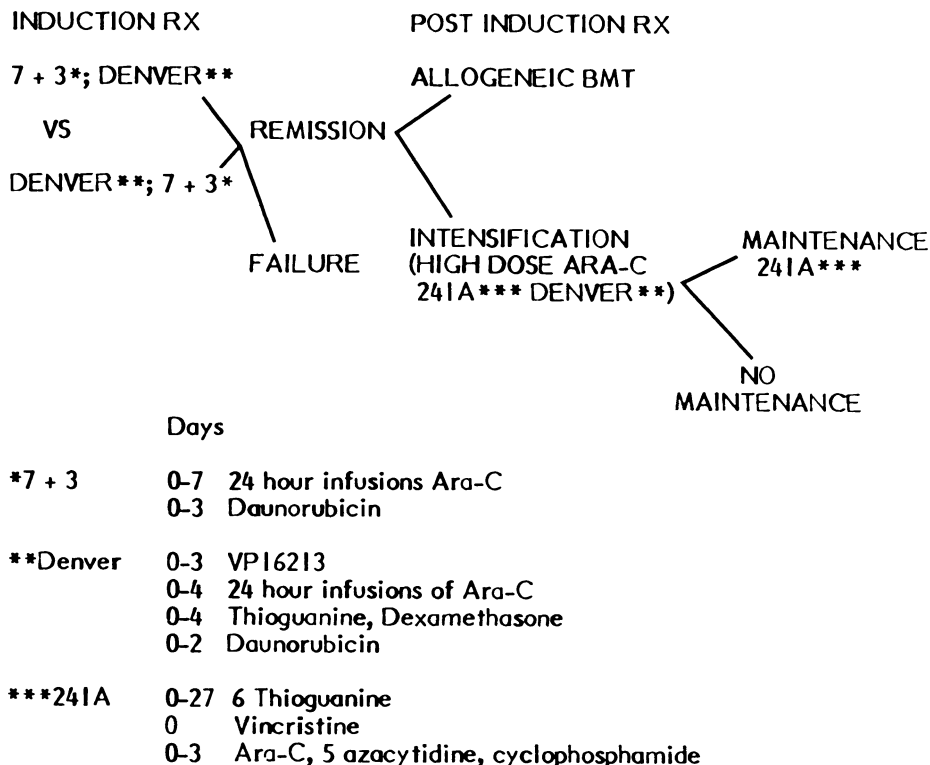


Fig. 1. Schema of CCG protocols 213P and 213. See text for details

hours after the last dose, L-asparaginase was given. This therapy was repeated on day 6. Course II was two cycles of therapy consisting of 6-thioguanine, vincristine, Ara-C, 5-azacytidine, and cyclophosphamide as was given for maintenance therapy in the CCSG study, CCG-241A. Course III was one cycle of therapy as was given for the experimental arm of induction; that is Denver therapy.

After completion of postinduction intensification the patients were randomized between no further therapy and maintenance therapy. Maintenance therapy consisted of repeating course II (241A therapy) of postinduction intensification therapy for 24 months.

A pilot study was necessary to determine the toxicity of the experimental induction arm and the postinduction intensification, which included high-dose Ara-C. High-dose Ara-C had not been reported in children at the time of writing the CCG-213 protocol.

There were 194 patients entered on the pilot protocol (Fig. 2).

## Results

### Induction Phase

#### Outcome

Approximately 70% entered a complete remission, with either arm of induction therapy. Interestingly, patients who had 0%–5% marrow blasts on day 13 were more likely to enter a complete remission irrespective of the regimen used for induction (Table 1) and these patients had a significantly better event-free survival rate.

#### Toxicity

Three percent of patients initially started on Denver induction died during induction and

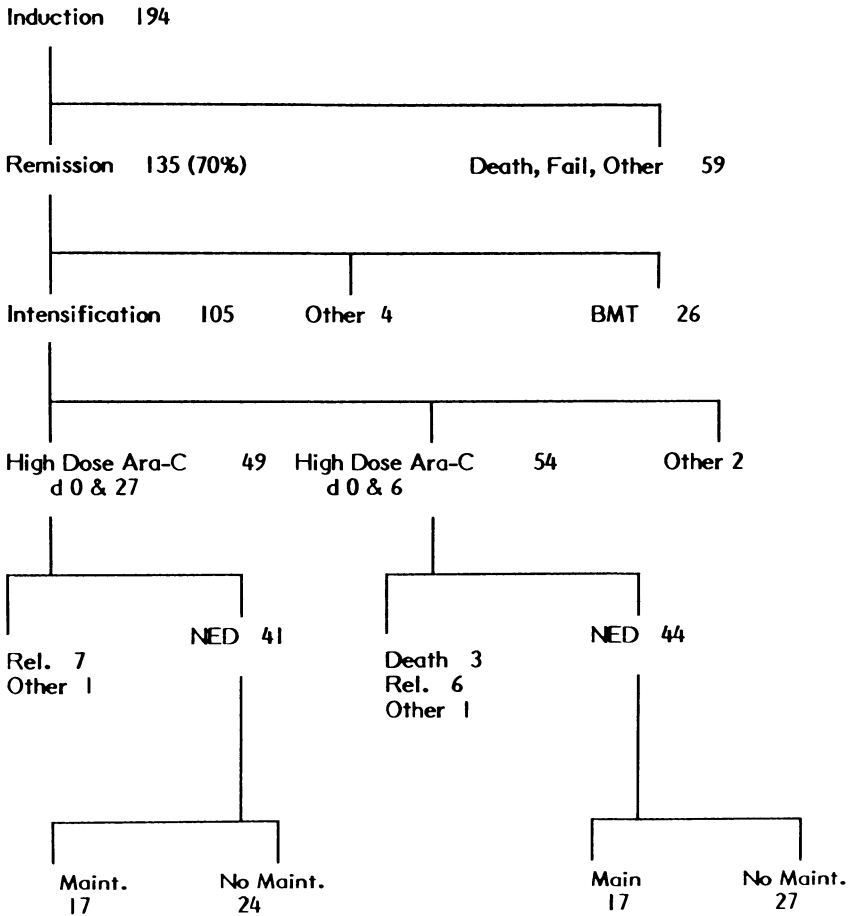


Fig. 2. Diagram of flow of patients for 213P study

8% died who were initially started on the 7 and 3 arm. After the second course of induction, 7% died if initially randomized to Denver and 9% died if initially randomized to 7 and 3. Bone marrow suppression was produced in almost all patients but was more

pronounced in the patients who initially received 7 and 3.

The next most common toxicity after either arm of induction was gastrointestinal toxicity. Nausea and vomiting were present in almost all patients. Approximately 8% of the patients with either arm of induction had grade 3 or 4 liver or other gastrointestinal toxicity when nausea and vomiting were excluded.

**Table 1.** Correlation of percentage of marrow blasts on D13 and complete remission rate

% blasts	0-5	6-15	16-40	>40
% complete remission	86.5	74.1	55.2	45.0
% death	1.4	1.7	7.5	4.8
% withdraw	1.8	0.0	6.0	6.3
% fail	7.8	17.2	25.4	38.1
% no data	2.5	6.9	6.0	4.8

#### Postinduction Intensification Therapy

#### Outcome

Patients who received high-dose Ara-C repeated on day 6 and had therapy discontin-

**Table 2.** Sequelae of bone marrow toxicities of course I of intensification

Drug	# patients	Deaths	Fever and neutropenia	Documented infections	Average days to neutrophil recovery	Average days to platelet recovery
High-dose Ara-C day 0 and 27	49	0	11	4	25	19
High-dose Ara-C day 0 and 6	54	3	51	32	31	31

ued after postinduction intensification did better than those in whom the drug was repeated on day 27 and therapy subsequently discontinued. There was no difference in the survival of patients receiving high-dose Ara-C on days 0 and 6 and 0 and 27 if maintenance therapy was given after the postinduction intensification phase. The 2-year disease-free actuarial survival from end of induction of the patients receiving intensification was  $54\% \pm 10\%$  and for 3 years was  $49\% \pm 10\%$ . The results of the bone marrow transplantation are too immature to evaluate.

### Toxicity

The most prominent toxicity during postinduction intensification was also bone marrow depression. This depression was particularly profound when high-dose Ara-C was repeated on day 6 (Table 2). Almost all of the patients developed fever and neutropenia when the drug was repeated on day 6 and over half had documented infection. Three patients died. The causes of death were as follows: pneumococcal sepsis and CNS hemorrhage in one, alpha streptococcus sepsis and *E. coli* sepsis in another, and bronchopneumonia in a patient with Down's syndrome in the third patient. As expected, the time to recovery was much longer when Ara-C administration was repeated on day 6. Sequelae of BM depression in courses II and III of the postinduction intensification phase and the maintenance phase were much less severe and there were no deaths. Gastrointestinal toxicity, particularly hepatotoxicity, which in most cases was reversible, was the next most common finding.

### Discussion

The results of this pilot study indicate that approximately 70% of children can be induced into a complete remission by the multidrug chemotherapy arm originally reported from Denver by Odom et al. [8]. This result is no better than therapy with the two drugs, Ara-C and daunorubicin, and no better than the results of previous investigators [6]. The toxicity of the postinduction phase after giving high-dose Ara-C on days 0 and 6 was marked but less than seen after preparative therapy for a bone marrow transplantation where toxic deaths represent 20% of patients treated [4]. The mortality seen in this nonablative intensification regimen was considered acceptable if an improvement in overall survival occurred. The preliminary results of the postintensification phase indicate that a greater leukemic cell kill occurs if the postintensification phase is very aggressive.

Although the numbers of patients are small, the results of the pilot study indicate that maintenance therapy may not be necessary if very aggressive postintensification therapy is given. To date, over 500 children have been entered in the definitive study for this pilot study. The results of this large study will be important to confirm the role of postinduction intensification, the value of maintenance therapy, and the role of bone marrow transplantation in first remission.

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## High-Dose Ara-C as a Single-Agent Consolidation Therapy in Childhood Acute Myelogenous Leukemia

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Acute myelogenous leukemia (AML) represents a difficult and heterogeneous group of leukemias from cells of myeloid origin [6, 8]. In the Nordic countries, all cases of acute childhood leukemias have been registered since 1 July 1981 [17]. By 31 December 1987, 1297 cases of leukemias had been registered, among which 184 (14%) were classified as AML. Traditionally the results of therapy in this group of leukemias are markedly inferior to those with acute lymphocytic leukemias, where progress is well known and well documented [8]. In recent years, however, the treatment of AML has slowly improved due to more aggressive and intensive chemotherapy. Many protocols now report a 70%–80% induction response rate with about 25%–50% long-term survivors after intensive consolidation therapy [1, 9, 11, 12, 16, 25, 28, 31, 34]. This paper is a preliminary report of the first Nordic trial on AML in children. Its main contribution is that it is a population-based study, and that it investigates the role of cytosine arabinoside (Ara-C) given at a high dose as the only drug in consolidation therapy.

### Materials and Methods

One hundred and thirteen children with AML less than 15 years of age were entered into the trial from 1 July 1984 through 31 December 1987. To the best of our knowledge this represents every child with AML in our countries during this period. The diagnosis of AML was based on morphological examinations of bone marrow and histochemical stains. In most cases an extensive investigation with monoclonal antibodies was included. The study had no central review panel. Chromosome analysis of the malignant clones was performed in some centers but is not included in the present report.

### Therapy

An outline of the protocol is shown in Fig. 1. Induction therapy consisted of three series including bolus Ara-C (100 mg/m<sup>2</sup> i.v. q12 h days 1, 2, 3, 4), 6-thioguanine (100 mg/m<sup>2</sup> p.o. q12 h days 1, 2, 3, 4), and doxorubicin (75 mg/m<sup>2</sup>, given either as the DNA complex [21] on day 5 or as free drug divided in equal doses on days 5, 6). Consolidation therapy consisted of high-dose Ara-C (2 g/m<sup>2</sup> q12 h days 1, 2, 3) repeated four times with a 3- to 4-week interval (total length of therapy, 7–9 months). Some children received high-dose retinol as maintenance [22, 23], but its role will not be analyzed further in this report.

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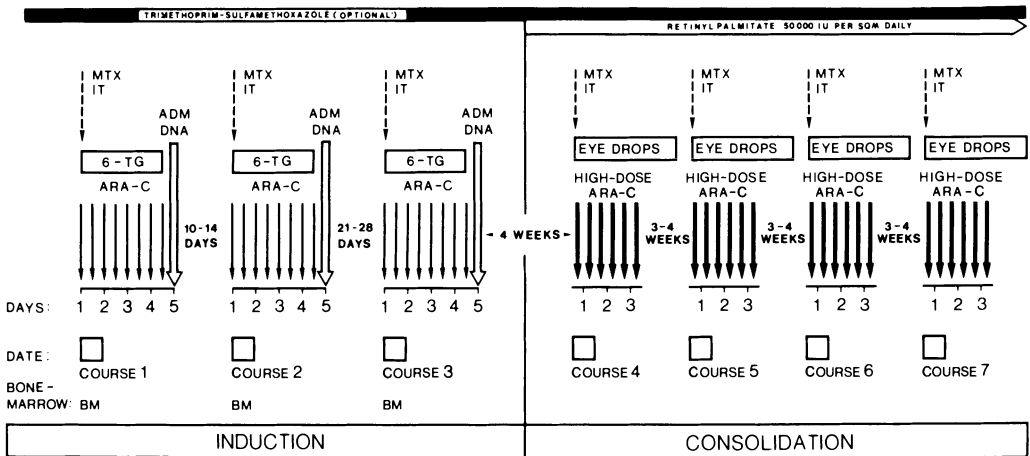


Fig. 1. Induction and consolidation regimen in AML study NOPHO-84

### Prognostic Factors

Age, sex, presenting white blood cell count, platelet count, morphological subtypes, and the number and interval between induction courses needed to achieve a remission were evaluated for influence on remission induction rate and disease-free survival. The statistical methods used were life table analysis according to Kaplan Meyer and significance tests according to the chi square distribution test [18].

### Results

#### Epidemiological Data

Table 1 presents the total number of cases reported to the study from the participating countries each year. Table 2 compares the

Table 1. No. of AML cases/year

	1984*	1985	1986	1987	Total
Denmark	6	5	7	8	26
Finland	2	11	2	4	19
Iceland	1	0	0	1	2
Norway	4	7	8	8	27
Sweden	3	6	15	14	39
Total	16	29	32	34	113

\* (6 months)

Table 2. FAB subtypes in NOPHO 84 and BFM 78

FAB	NOPHO n (%)	BFM 78 n (%)
1	22 (21)	36 (24)
2	33 (31)	34 (23)
3	7 (7)	6 (4)
4	18 (17)	40 (26)
5	12 (12)	32 (21)
6	8 (8)	3 (2)
7	2 (2)	—
	102 (100)	151 (100)

French-American-British (FAB) subtypes in the 102 patients where this information was available, with the distribution presented by the BFM-78 study [11]. The relative frequencies are rather similar, but with a somewhat higher incidence of monocytic leukemia in the German series.

We were surprised to find a high frequency of Down's syndrome in our study. Eighteen out of 109 de novo AML cases had trisomy 21 (17%). Table 3 shows the distribution according to country and sex. It is surprising that in each country there is a preponderance of trisomy 21 girls developing AML. Table 4 shows that the age distribution in children with Down's syndrome is different from that in the total material.

**Table 3.** NOPHO 1984: Down's syndrome and AML

	Male	Female
Denmark	1	3
Finland	0	1
Iceland	0	0
Norway	1	4
Sweden	1	7
Total	3	15

Eighteen out of 109 de novo AMLs=17%

**Table 4.** Age distribution

Years	n	Down's+ AML
<1	14	0
1-2	20	10
2-5	28	8
5-10	20	0
>10	27	0
Total	109	

Most cases of Down's syndrome were close to 24 months of age at diagnosis, and only one was more than 3 years old.

There was also an unsuspected high frequency of preleukemic syndrome in our patients. Twenty-two out of the 109 children had been evaluated for a hematological disorder for more than 2 months because of a cytopenia in at least two of the three cell lines. Ten of these were Down's syndromes.

#### *Induction of Remission*

The patient material is summarized in Table 5. Seventeen cases were excluded from further analysis, four because of a secondary malignancy, seven children were electively not treated (six with Down's syndrome, one with a probably transient leukemoid reaction), five were treated on a different protocol, while one died of a massive disease before therapy could be initiated. Remission was obtained in 70 of the 96 remaining evaluable cases (73%). Seven children died in aplasia, while 19 had resistant disease. Three of the seven that died in aplasia had

**Table 5.** Acute myelogenous leukemia: NOPHO 1984 (January 1988)

Total No. entered	113
Exclusions: secondary malignancy	4
No therapy	7
Other protocol	5
Death before therapy	<u>1</u>
	17
On study	96
Death in aplasia	7
Resistant disease	<u>19</u>
	26
Complete remission	70 (73%)
Bone marrow transplant	14
Chemotherapy group	56

M5 and one had Down's syndrome. There was no identifiable prognostic variable that indicated resistant disease. However, of the 19 resistant cases, 14 received a variety of other protocols after the failure of the initial therapy. Twelve of these patients were resistant also to these other very intensive therapies. Only two achieved a remission; both of these were bone marrow transplanted and remain in remission at 4+ and 22+ months.

Therapy of Down's syndrome cases is usually considered to be difficult. Only 9 of the 18 children received the NOPHO (Nordic Society for Pediatric Hematology and Oncology) protocol. Eight of the nine achieved a remission. Two received other protocols and both died. One received a therapy unknown to us and six received no therapy. All six untreated children died from progressive disease.

Preleukemia may be a prognostic factor in children with AML. Twenty-two were diagnosed as having a definite preleukemic phase in their disease. Twelve of the children with preleukemia received the NOPHO protocol and only seven of these achieved a complete remission.

#### *Consolidation Therapy*

Fourteen children were bone marrow transplanted in first remission at various time points after remission was obtained. They

will not be analyzed further here – but are censored at 5 months from diagnosis.

Fifty-six children received a total of 224 courses of high-dose Ara-C. One girl, 13 years of age, with an M2 leukemia, died unexpectedly and from unexplained reasons after the first course of high-dose Ara-C. It remains unclear whether or not the fatal outcome was related to the high-dose therapy. No other death in complete remission has been reported.

### *Duration of Remission*

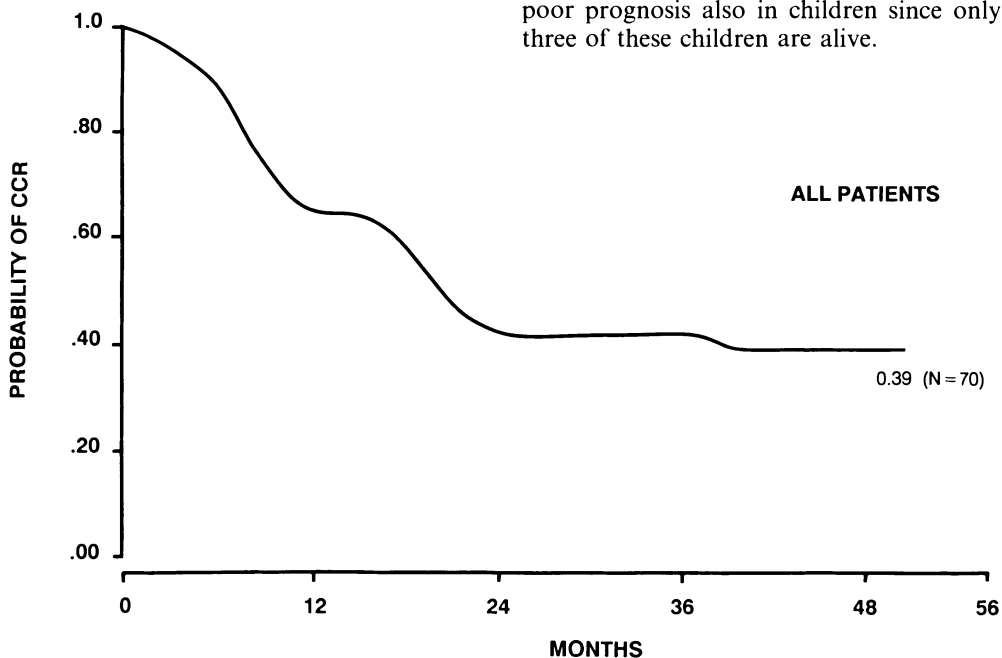
Figure 2 shows the probability of remaining in complete remission for the 70 patients achieving a complete response on the NOPHO induction protocol. The 5-year actuarial disease-free survival is about 40%. Of the prognostic factors analyzed, only white blood cells at diagnosis turned out to be a statistically significant prognostic factor (Fig. 3). The promising aspect of the survival curve is that there seems to be a definite plateau, with most of the relapses taking place during the first 2 years and with only one relapse observed after 24 months from

diagnosis. Seven of the eight patients with Down's syndrome responding to induction therapy remain in remission.

### **Discussion**

This study on acute myelogenous leukemia in the Nordic countries is a population-based study. Two interesting epidemiological findings deserve emphasis. Seventeen percent of the cases had Down's syndrome. The increased incidence of leukemia in Down's syndrome is well known [24, 29] and affects both the myeloid and lymphoid lineages. However, in most of the multicenter studies on AML reported so far they contribute less than 5% of the cases [29], which probably must mean that patients with Down's syndrome are not included in these studies.

The high frequency of preleukemia (20%) is also a new finding. In adults this is certainly well known and carries a grave prognosis [3]. In children it is reported to be less frequent [4, 10, 19, 20, 30, 32, 33]. In our series half the patients with preleukemia also had Down's syndrome, 12 children not having this diagnosis with a preleukemic phase. It seems that this factor carries a significant poor prognosis also in children since only three of these children are alive.



**Fig. 2.** Probability of disease-free survival in the complete responders

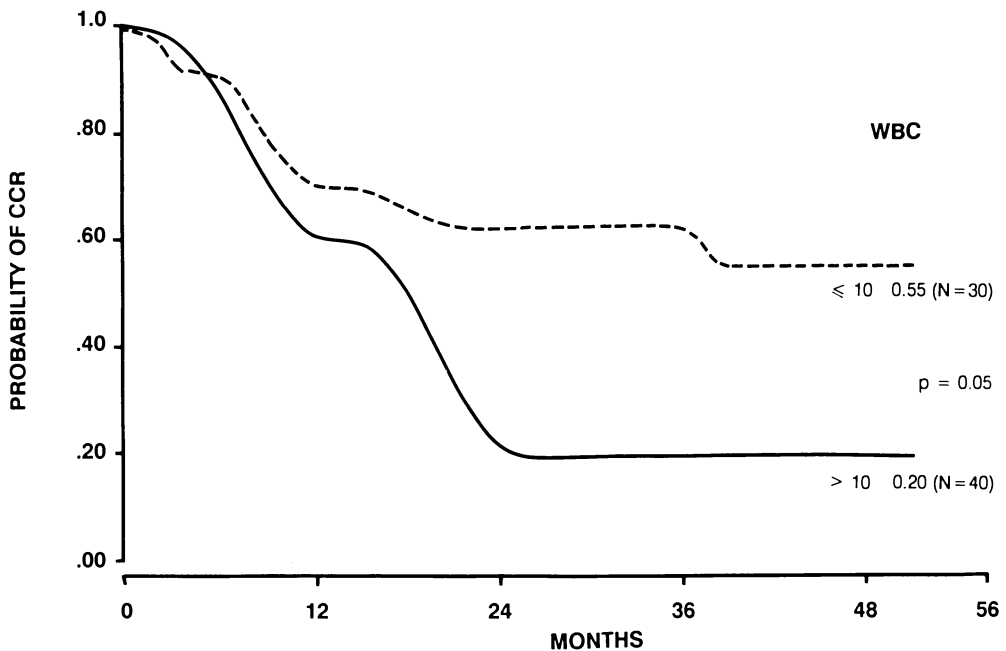


Fig. 3. Probability of disease-free survival in the complete responders according to WBC at diagnosis

The results of the induction part of the protocol are comparable to most intensive studies reported so far. Perhaps the frequency of resistant disease is a little higher while death in aplasia is lower. However, even though 14 of the 19 resistant cases were tested on other very high intensity protocols, only 2 of these children achieved a new remission, indicating that resistance to first-line therapy as used in this study carries a very grave prognosis.

The consolidation phase of the protocol is very simple, consisting of only four courses of high-dose Ara-C (2 g q12 h for six doses). This drug is certainly the mainstay of antileukemic therapy [13, 15] and its use in high doses is part of many protocols today [2, 5, 7, 14, 26, 27, 34]. In our multicenter study it was shown to be a safe therapy with acceptable side effects.

The shape of the survival curve certainly indicates that 40% of the responders may be cured of their disease since there is a definite plateau in the survival curve. This compares favorably with many other reported studies but leaves much room for improvement.

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## Therapy of Childhood Acute Myelogenous Leukemia: An Update of the AIEOP/LAM 8204 Study\*

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Over the past decade there have been significant advances in the treatment of acute myelogenous leukemia (AML). The introduction of intensive induction and post-remission chemotherapy programs, together with the improvement in supportive care, have resulted in a probability of long-term survival in 30%–50% of children with AML [1–4]. In 1982 the AIEOP (Italian Pediatric Hematology-Oncology Association) cooperative group began a multicenter prospective trial for pediatric AML in order to establish the value of a polychemotherapeutic regimen consisting of intensive induction followed by intensive consolidation and continuation chemotherapy.

The trial included 171 previously untreated children (aged under 17 years) with AML, treated at 22 AIEOP institutions between August 1982 and March 1987. Patients' characteristics at diagnosis are shown in Table 1. Patients with FAB M3 morphology were not included in this trial. Induction therapy consisted of two cycles of daunorubicin and cytosine arabinoside given according to the "3+7" and "2+5" schedules. Patients in complete remission (CR) received consolidation therapy with four courses of DAT (daunorubicin, 6-thioguanine and escalated doses of cytosine arabinoside) followed by six courses of monthly pairs of Etoposide (VP-16)/cytosine arabinoside, cy-

**Table 1.** Patient characteristics

Number of patients entered	171
Male/female ratio	93/78
Median age in years	6.1
Range	0.2–16.4
Morphology (FAB)	
M1	44
M2	49
M3v	2
M4	27
M5	44
M6	2
M7	3
WBCs × 10 <sup>9</sup> /l	
Median	32.0
Range	1.2–710.0
Platelets × 10 <sup>9</sup> /l	
Median	48.0
Range	3.0–724.0
CNS disease	6

tosine arabinoside/6-thioguanine and daunorubicin/cytosine arabinoside (Table 2). Central nervous system (CNS) prophylaxis consisted of intrathecal cytosine arabinoside, administered on day 1 or 2 of each course of therapy.

The overall results are outlined in Table 3. CR was achieved in 141 patients (84%) after a median of 27 days (range, 13–62). Three patients died before treatment due to intracranial bleeding and one due to multiple organ failure. Eleven patients (6%) died during induction therapy: seven due to infection, three to hemorrhage, and one to renal failure. Fifteen patients (9%) failed to

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**Table 2.** Protocol AIEOP/LAM 8204*Induction* (two courses)*Course 1*DNM 45 mg/m<sup>2</sup>

i.v. days 1–3

Ara-C 200 mg/m<sup>2</sup>

c.i. days 1–7

*Course 2*DNM 45 mg/m<sup>2</sup>

i.v. days 1–2

Ara-C 200 mg/m<sup>2</sup>

c.i. days 1–5

*Consolidation* (four courses)DNM 60 mg/m<sup>2</sup> i.v. day 16-TG 70 mg/m<sup>2</sup> p.o. q8h days 1–5Ara-C 60 mg/m<sup>2</sup> s.c. q8h days 1–5*Dose of Ara-C escalated to:*80 mg/m<sup>2</sup> q8h (course no. 2)110 mg/m<sup>2</sup> q8h (course no. 3)150 mg/m<sup>2</sup> q8h (course no. 4)*Continuation* (six courses)*Sequence 1*VP-16 100 mg/m<sup>2</sup>

i.v. days 1–3

Ara-C 150 mg/m<sup>2</sup>

s.c. q8h days 1–3

(months 1, 2)

*Sequence 2*Ara-C 150 mg/m<sup>2</sup>

s.c. q8h days 1–5

6-TG 70 mg/m<sup>2</sup>

p.o. q8h days 1–5

(months 3, 4)

*Sequence 3*DNM 40 mg/m<sup>2</sup>

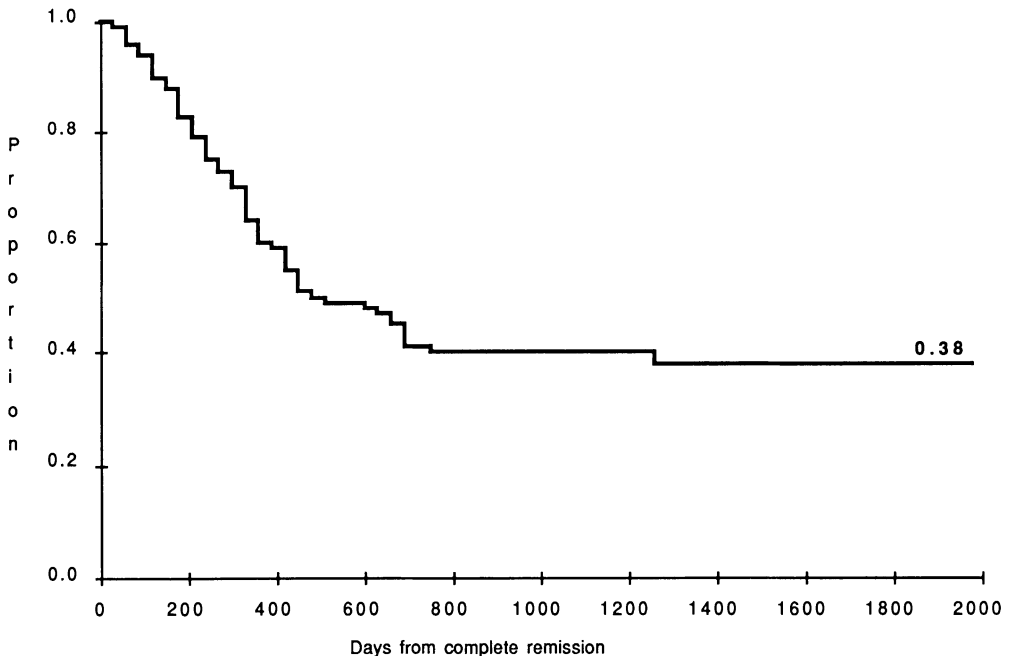
i.v. day 1

Ara-C 300 mg/m<sup>2</sup>

c.i. days 1–3

(months 5, 6)

DNM, daunorubicin; ara-C, cytosine arabinoside; 6-TG, 6-thioguanine; VP-16, etoposide

**Fig. 1.** Disease-free survival (AIEOP/LAM 8204)

**Table 3.** Induction results

Entered	171
Died prior therapy	4
Evaluable	167
Achieved CR	141 (84%)
Course no. 1	80
Course no. 2	61
Died during induction	11 (7%)
Resistant leukemia	15 (9%)
Days to CR	
Median	27
Range	13–62

**Table 4.** Follow-up

Entered	171
Complete remissions	141
Withdrawals <sup>a</sup>	30
Deaths in CR	6
Total relapses	61
BM	56
BM + CNS	2
BM + Skin	1
CNS	2
Time to relapse (weeks)	
Median	43
Range	5–178
Remain in CCR	44
On therapy	–
Off therapy	44

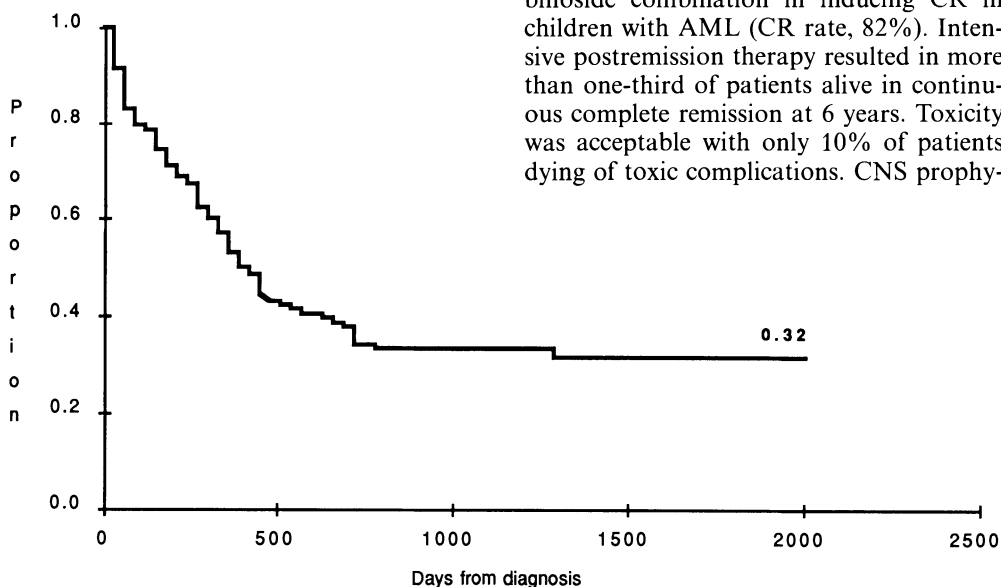
respond to therapy. There were 30 withdrawals from the study for reasons including allogeneic bone marrow transplant (BMT) in 14 patients and autologous BMT in 16 patients. Of the remaining 111 responding patients, six children died in CR of treatment-induced complications (five infections and one congestive heart failure), and 61 relapsed. Relapses occurred predominantly in the bone marrow (56 patients), with a median time to relapse of 43 weeks (range, 5–178). Forty-four patients are alive in first continuous complete remission and are off therapy (Table 4). At 6 years, 38% of complete responders (Fig. 1) and 32% of the entire population of patients (Fig. 2) are pre-

dicted to remain alive in continuous complete remission.

<sup>a</sup> Allogeneic BMT 14; autologous BMT 16

dicted to remain alive in continuous complete remission. In a multivariate analysis, FAB M5 morphology and a leukocyte count equal to or greater than 100 000/mm<sup>3</sup> correlated adversely with the probability of response and event-free survival. None of the variables examined was significantly predictive of the duration of CR.

The results of this large multicenter trial, as well as those recently reported in the literature [1–4], confirm the efficacy of the conventional daunorubicin plus cytosine arabinoside combination in inducing CR in children with AML (CR rate, 82%). Intensive postremission therapy resulted in more than one-third of patients alive in continuous complete remission at 6 years. Toxicity was acceptable with only 10% of patients dying of toxic complications. CNS prophylaxis

**Fig. 2.** Event-free survival (AIEOP/LAM 8204)

laxis with periodic intrathecal cytosine arabinoside was effective in preventing CNS leukemia, with only two isolated CNS relapses.

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## Prognostic Significance of Eosinophilia in Acute Myelomonocytic Leukemia in Relation to Induction Treatment\*

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### Introduction

The association of initial bone marrow eosinophilia, structural rearrangements of chromosome 16, and acute myelomonocytic leukemia (FAB M4) was described in adults by Arthur and Bloomfield [1] in 1983. Holmes et al. [2] suggested a favorable prognosis for this group of patients. The German AML studies BFM-78 and -83 have demonstrated increasing rates of long-lasting remissions [3]. Special analyses indicate that this improvement was not seen in all children with AML, but only in certain subgroups which can be described predominantly by morphological features including eosinophilia. The objective of our investigation was to evaluate the prognostic significance of the parameter eosinophilia in childhood AML.

### Patients and Methods

The initial Pappenheim-stained bone marrow smears of 112/151 (74%) patients of study AML-BFM-78 and of 157/182 (86%) children of study AML-BFM-83 were reviewed for eosinophils. All patients were under the age of 17 years. In 39 patients from the AML-BFM-78 study and in 25 children

from the AML-BFM-83 study the number of eosinophils could not be evaluated retrospectively. Diagnostic criteria and treatment of children in the AML-BFM-78 and -83 studies have been described elsewhere [4, 5]. The main therapy difference between the AML-BFM-78 and -83 studies was the addition of the 8-day intensive ADE (cytosine-arabioside, daunorubicin, etoposide) induction in study AML-BFM-83. Chromosome analyses using G-banding techniques were performed in Giessen (for methods see [6]). Life-table analyses for event-free survival (EFS) and event-free interval (EFI) were calculated according to the Kaplan-Meier method [7] and the corresponding statistical comparisons were performed with the log-rank test [8]. The value of prognostic factors has been examined by means of the chi-square test and by multivariate analysis using the Cox proportional hazard regression [9]. The cut-off date for the analysis was 1 March 1988.

*Definition.* Eosinophilia is defined as  $\geq 3\%$  eosinophils in the bone marrow.

### Results

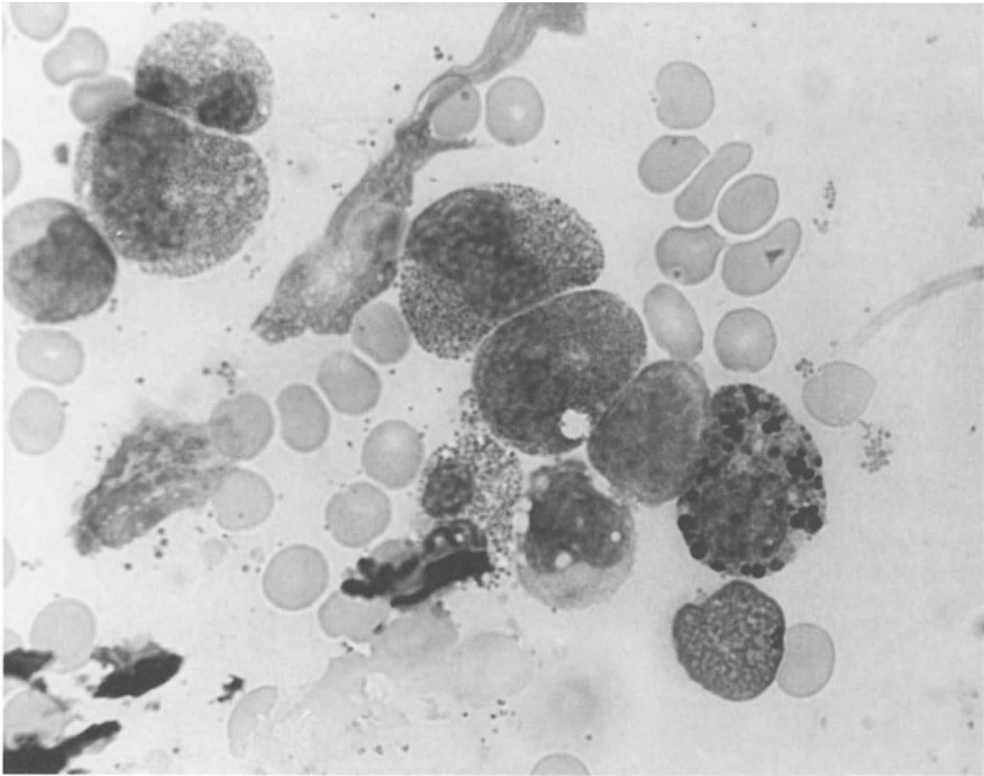
Seventy-three out of 269 (27%) patients from both studies showed bone marrow eosinophilia. Mostly children with FAB types M2 (28/64=44%) and M4 (39/70=41%) had  $\geq 3\%$  eosinophils. Atypical eosinophils were only found if  $\geq 3\%$  of these cells were present, and predominantly in FAB type M4 (20/28=71%). The dysplastic eosinophils showed a wide range of different stages of maturation (see Fig. 1). They often contained a mixture of eosino-

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**Fig. 1.** Abnormal, dysplastic eosinophils with eosinophilic and basophilic granules in a patient with FAB M4. (Pappenheim staining,  $\times 1000$ )

philic and basophilic granules in their cytoplasm. In FAB type M4 there was large variation in the percentage of eosinophils in the bone marrow (Fig. 2), and predominantly patients with  $\geq 5\%$  eosinophils had this abnormal morphology. In the other FAB types the percentage of children with  $\geq 5\%$  eosinophils was low, and abnormal eosinophilic cells were rarely seen (Fig. 3). The initial patient data of children with FAB M4 with and without eosinophilia in the bone marrow were not very different (NS  $\chi^2$ -test, Table 1). Cytogenetic studies in eight children with FAB M4 and eosinophilia showed normal karyotypes in four children, inversion 16 in one, 11q23 aberration in two, and trisomy 22 in one.

#### *Therapy Results*

Treatment results from studies AML-BFM-78 and -83 were compared for the patients

with FAB M4 with and without eosinophils in the bone marrow. In addition, the results for children with FAB M4 and atypical eosinophils are shown (Table 2). Outcome in study AML-BFM-78 did not indicate any differences in prognosis for patients with or without eosinophilia, whereas in study AML-BFM-83 an increase in EFS and EFI was seen in children with FAB M4 and eosinophilia (Fig. 4). None of the 12 children in study AML-BFM-83 with FAB M4 and atypical eosinophils relapsed, but 2 patients died initially from cerebral bleeding and sepsis. In study AML-BFM-78 there were three relapses in eight patients with FAB M4 and atypical eosinophils. One relapse occurred simultaneously in the CNS and bone marrow. Abnormal eosinophils were rare in other FAB types (three children in study AML-BFM-78 and five in study AML-BFM-83). This group also had a favorable outcome, with only one relapse in a patient with the specific morphology of

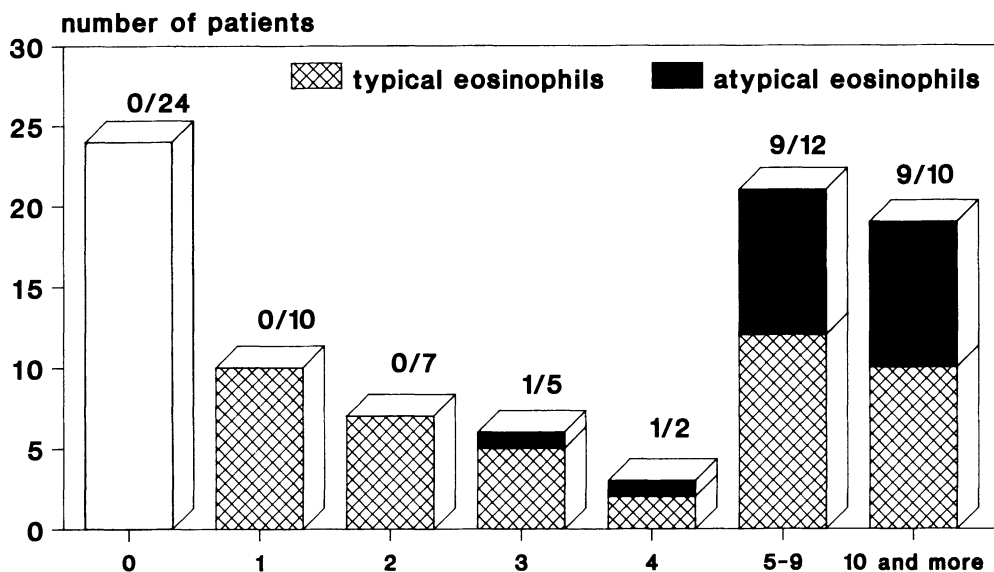


Fig. 2. Distribution of the portion of eosinophils in the bone marrow in patients with FAB M4 in the studies AML-BFM-78 and -83. *Black* indicates portion of atypical eosinophils

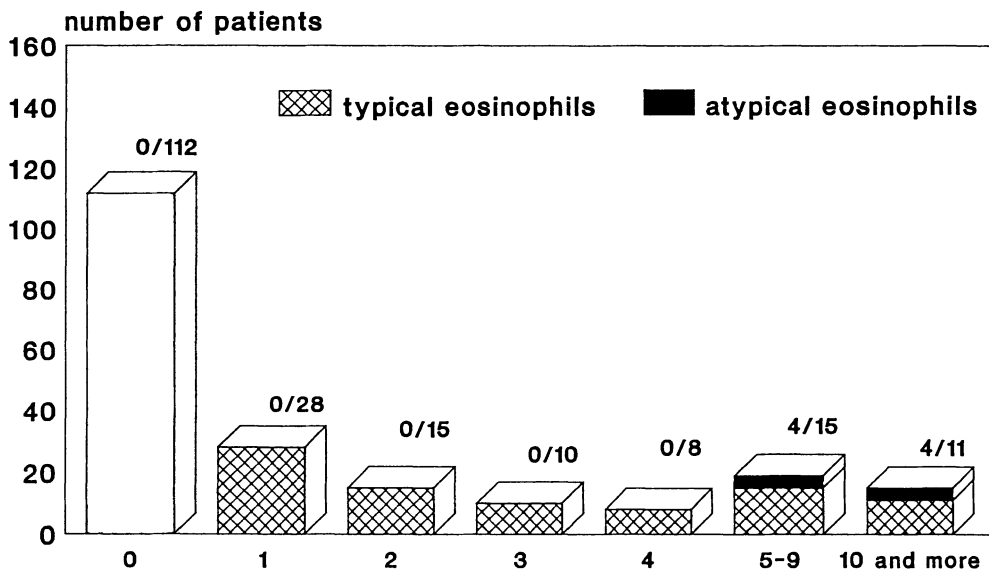


Fig. 3. Distribution of the portion of eosinophils in the bone marrow in patients without FAB M4 in the studies AML-BFM-78 and -83. *Black* indicates portion of atypical eosinophils

bubbly granules in the eosinophils. Risk factor analysis was performed for the parameters: age, sex, initial peripheral white blood and thrombocyte count, hepato- and splenomegaly, CNS and extramedullary organ involvement, eosinophilia in the bone

marrow, and evidence of Auer rods. In study AML-BFM-83 only eosinophilia in FAB M4 remained as an independent favorable prognostic variable (for details see [10]).

**Table 1.** Initial patient data of children with FAB M4 with and without eosinophilia and with atypical eosinophils in the bone marrow: studies AML-BFM-78 and -83

Eosinophils in the bone marrow	<3%	≥3%	
		all patients	with atypical eosinophils
Number of patients	41	29	20
Age, median (years; months)	9; 3	9; 3	10; 1
Boys (%)	56	55	55
WBC ( $\times 10^3/\text{mm}^3$ ) median	37	44	39
Range	1–465	5–285	5–285
CNS involvement (%)	18	8	11
Extramedullary organ involvement (%)	35	32	32
Auer rods (%)	41	55	65
POX-positive blasts in bone marrow $\geq 80\%$ <sup>a</sup> (%)	38	64	60

<sup>a</sup> Only study AML-BFM-83

**Table 2.** Comparison of treatment results of patients with FAB M4 with and without eosinophilia and with atypical eosinophils in the bone marrow: studies AML-BFM-78 and -83

Eosinophils in the bone marrow	AML-BFM-78			AML-BFM-83		
	<3%	≥3%		<3%	≥3%	
		all patients	with atyp. eo.		all patients	with atyp. eo.
Number of patients	20	12	8	21	17	12
Death prior to therapy	–	–	–	–	1	1
Death during induction therapy	1	2	1	–	1	1
Nonresponders	5	–	–	5	1	–
Complete remission	14	10	7	16	14	10
Death in remission	2	–	–	1	–	–
Withdrawals (BMT)	1	1	1	(3)	(1)	1
Relapses	7	5	3	7	1	–
(with CNS involvement)	(2)	(1)	(1)	(–)	(–)	(–)
In CCR	4	4	3	5	12	9
Event-free survival <sup>a</sup>	22%	42%		23%	81% <sup>b</sup>	
Event-free interval <sup>a</sup>	31%	50%		30%	93% <sup>b</sup>	

<sup>a</sup> Kaplan-Meier estimation for 5 years in both studies, standard deviations according to patient numbers 7%–16%

<sup>b</sup> Eosinophils <3% vs.  $\geq 3\%$ ,  $P < 0.01$  log-rank test; atyp. eo., atypical eosinophils; BMT, bone marrow transplantation

## Discussion

The particular subtype of FAB M4 with eosinophils, which has previously been described in adults [1, 2], was also found in childhood AML. As described by Le Beau et al. [11], especially in FAB type M4 eosinophils with a distinctly abnormal

morphology were seen, obviously a mixture of eosinophilic and basophilic stained granules in the cytoplasm (Fig. 1). Cytogenetic findings revealed inversion 16 in only one patient, whereas other authors reported this structural abnormality in every patient with FAB M4 and abnormal eosinophils [11].

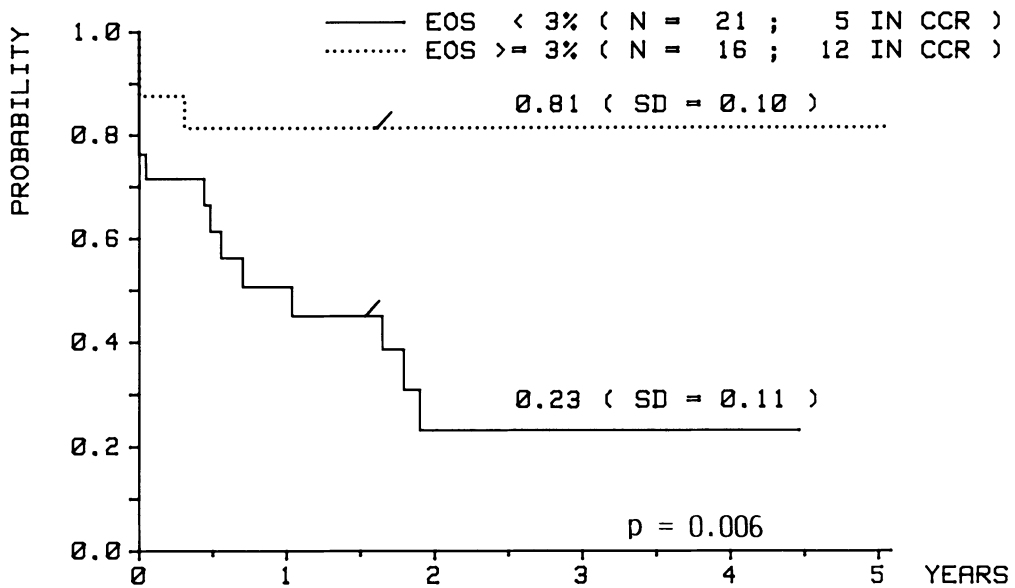


Fig. 4. Probability of event-free survival for 5 years in patients with FAB M4 with and without eosinophilia in the bone marrow in the study AML-BFM-83. /, last patient entering the group

The presence of abnormal eosinophils seems to be related to the percentage of  $\geq 5\%$  of marrow eosinophils. The dysplastic features, however, were also seen in two patients with 3% and 4% of eosinophils in the bone marrow, which was also reported by Le Beau et al. [11], who included these patients in the M4 group with eosinophilia. The presence of marrow eosinophilia at diagnosis is associated with a better prognosis in adults [11, 12]. In our studies, children with FAB M4 and eosinophilia have a better prognosis compared with the group without eosinophilia (Table 2). The differences for EFS and EFI, however, are only significant in study AML-BFM-83 (Table 2, Fig. 4). The prolonged duration of remission in children with FAB M4 with eosinophils in study AML-BFM-83 as compared with the first study is remarkable (event-free interval of 93%, SD 7% vs. 50%, SD 16%,  $P=0.03$ ), and indicates the relationship between prognostic factors and treatment. Due to the intensification of induction therapy in study AML-BFM-83, the event-free interval was significantly prolonged in children of the FAB types with predominantly myeloid differentiation, such as FAB M4 with eosinophilia, or FAB M1 with Auer rods [13].

None of the 12 patients with abnormal eosinophils in study AML-BFM-83 relapsed, but there were two early deaths, whereas three relapses and one early death occurred in eight of these patients in study AML-BFM-78. The small group of patients with different FAB types and atypical eosinophils (three children in study AML-BFM-78 and five in study AML-BFM-83) also had a favorable outcome; there was only one relapse in a patient with bubbly granule morphology.

The fact that inversion 16 was only rarely identified in our patients may be for methodological reasons. On the other hand, we can conclude that the morphological finding of FAB M4 with eosinophilia, especially with atypical forms, is sufficient to indicate a good prognosis. The percentage of eosinophilia varies considerably. It seems to us that the cutoff point may be 3% or 5% of eosinophils in the bone marrow, but the abnormal morphology – even if present in only a small number of these cells – is also characteristic for this specific subtype of FAB M4 with eosinophilia. Holmes et al. [2] reported the difficulty of assessing the percentage of eosinophilia in hyperleukocytosis and nearly 100% of blasts in the bone marrow. In



these cases the eosinophilia only became evident after blast cell reduction during induction chemotherapy, the eosinophil population possibly having been masked by the high blood count before treatment.

Our results indicate that the specific entity of FAB M4 with eosinophils is also seen in childhood AML and has an excellent prognosis with an intensive therapy regimen. It is suggested that the eosinophils are neoplastic cells. Löffler [15] has described the reactivity of eosinophilic granules to chloroacetate esterase which was not seen in normal eosinophils. This fact and the finding that in cell cultures Auer-rod-positive myelocytic leukemia cells differentiated to the eosinophilic pathway [16] confirm this presumption. Recently, clonal chromosomal abnormalities suggested multiple-lineage involvement in a high proportion of patients with AML [17]. In our study, most patients with eosinophilia also showed maturation of the neutrophilic and sometimes of the basophilic lineage. Le Beau et al. [18] reported that the metallothionein gene cluster was split by chromosome 16 rearrangements and due to this involvement the differentiation of granulocytic and monocytic cells may be disturbed.

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## Treatment of Childhood Acute Nonlymphocytic Leukemia: Cooperative Austrian-Hungarian Study AML-IGCI-84

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### Introduction

Improved remission rates of 70%–80% in childhood acute nonlymphocytic leukemia (ANLL) have been achieved by several groups. Anthracyclines (daunorubicin, doxorubicin) are of great value in the treatment of ANLL. Protocols effective for ANLL commonly include combinations with cytosine arabinoside [1–3]. Aclarubicin, a new alternative anthracycline antibiotic, has proven efficacy in relapsed or advanced as well as in untreated ANLL in adults [4–6]. In 1984 a cooperative multicenter study (AML-IGCI-84) was initiated which incorporated aclarubicin in the first-line therapy of previously untreated childhood ANLL.

### Patients and Methods

Children with newly diagnosed untreated ANLL were eligible for the study. Children with Down's syndrome were excluded. Patients were treated at several pediatric clinics in Austria and Hungary. Leukemias were classified according to the cytologic and cytochemical criteria of the French-American-British (FAB) classification [7, 8]. In addition, in most of the cases immunologic classification of cell surface markers, determination of terminal deoxynucleotidyltransferase (TdT), and cytogenetic analysis were per-

formed. Standard definitions for event-free survival, event-free interval, and relapse-free interval were applied for life-table analysis (Kaplan-Meier). The estimations were computed with BMDP-85 statistical software.

### Treatment

The study design (Fig. 1) is based on the AML-BFM-83 protocol with the exception of the different induction regimen I1 (aclarubicin, cytosine arabinoside, etoposide, Fig. 2) [1]. If complete remission (<5% blast cells in the bone marrow aspirate) was achieved between days 21 and 28, intensive consolidation therapy (doxorubicin, vincristine, cytosine arabinoside, prednisone, 6-thioguanine, cyclophosphamide, cytosine arabinoside intrathecally, cranial irradiation – BFM-83), and low-dose maintenance until 2 years from diagnosis (6-thioguanine, cytosine arabinoside, doxorubicin – BFM-83) was followed. If only partial or no response was found after I1, the original BFM-83 induction course (daunorubicin, cytosine arabinoside, etoposide) followed immediately before consolidation and maintenance [1]. Patients with initial hyperleukocytosis and/or extensive organomegaly received a cytoreductive pretreatment (6-thioguanine, cytosine arabinoside).

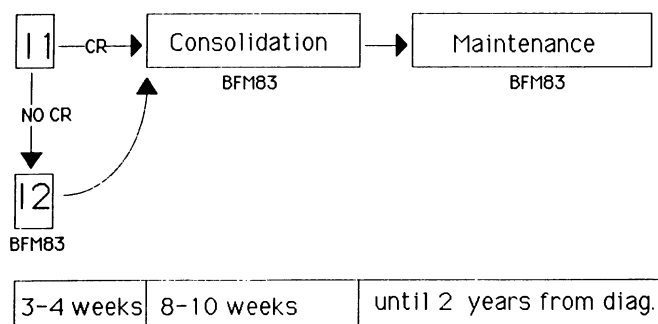
### Results

From January 1984 to August 1988, 80 children entered the study; 79 of them were evaluable (one child had just begun induc-

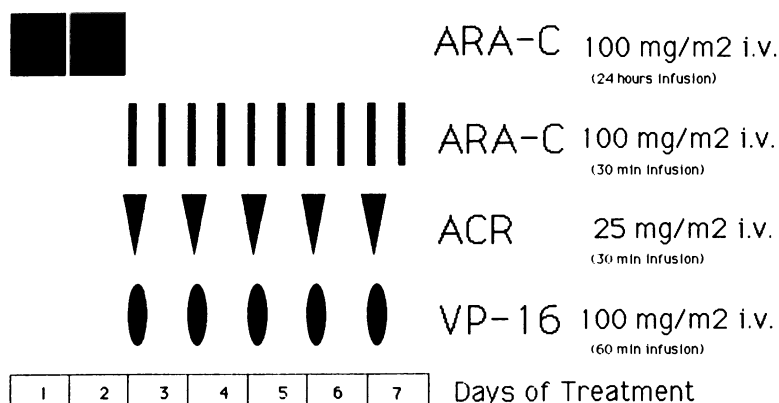
For the IGCI Pediatric Study Group.

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**Fig. 1.** Study design: *I1*, induction 1; *I2*, induction 2; *CR*, complete remission; *NO CR*, no CR achieved



**Fig. 2.** Induction I1: *ACR*, aclarubicin; *ARA-C*, cytosine arabinoside; *VP-16*, etoposide

tion therapy and was too early for evaluation – but was included in survival analysis). The study patients consisted of 37 boys and 42 girls with a median age of 7;06 years (0;02–17;10 years) and a median initial WBC of 18.0 G/liter (1.6–1350.0 G/liter). An initial CNS involvement was found in three children, extramedullary disease (in addition to hepatosplenomegaly and lymph node involvement) in 17 patients. Myeloblastic and myelomonoblastic leukemias were predominantly found, but the rare types of ANLL were represented as well (Table 1).

Complete remission (CR) was achieved in 55 patients (69.6%), in 44 patients (55.6%) after one induction course. The 24 induction failures were mainly due to early deaths before bone marrow reevaluation (10 fatal septicemias and 3 cerebral bleedings following induction I1, deaths occurring between days 8 and 27). Four children without CR after I1

**Table 1.** Distribution of FAB subtypes

FAB subtype	<i>n</i>	%
M1	26	32.9
M2	12	15.1
M3	1	1.2
M4	21	26.5
M5	13	16.4
M6	3	3.7
M7	3	3.7
Total	79	100.0

died during aplasia following induction I2 (two septic complications, two cerebral bleedings). One patient not in remission after I1 died before continuation of chemotherapy due to postoperative shock following laparotomy for obstructive ileus on day 36. In six patients induction failure was

caused exclusively by nonresponse: One child died on day 28 due to paralytic ileus caused by diffuse leukemic infiltration of the bowel. Five patients did recover from the second induction course and failed to achieve CR thereafter.

Of the 55 children who achieved CR, 4 patients proceeded to bone marrow transplantation in first CR and were removed from further evaluation at this date (2 of them died – 1 relapse, 1 septicemia; 2 of them remain in first CR). Of the remaining 51 children, 30 remain in first CR at the date of evaluation, 15 relapsed and 6 died in CR (1 bleeding, 5 septicemias).

Life-table analysis (Kaplan-Meier plots) predicts an event-free survival of 36.6% at 4;06 years (SE 6.1%,  $n=80$ ) and an event-free interval of 52.3% at 4;05 years (SE 7.8%,  $n=55$ ).

## Discussion

This prospective cooperative multicenter study, AML-IGCI-84, basically is a variant of the study AML-83 of the BFM group with a different first-line induction. With 69.6% CR it yielded a rate of complete remissions comparable to other effective regimens applied for childhood ANLL today [1, 2]. Remarkably, 80% of these remissions have already been achieved after a single induction chemotherapy course including aclarubicin, but no other anthracycline. This again compares well with the experience of other groups, who rely on daunorubicin or doxorubicin for first-line chemotherapy [1, 2]. Children treated with high doses of daunorubicin are at risk from lethal cardiotoxicity [9]. The reduction of total doses of the two established anthracycline antibiotics together with their substitution by the less cardiotoxic aclarubicin [10, 11] apparently does not reduce treatment efficacy.

The main cause of induction failure was fatal septicemia during bone marrow aplasia, surprisingly at a higher rate than reported by the BFM group, who utilized a very similar treatment approach [9]. Many of the septic deaths eventually were due to systemic mycoses. Improvement of supportive care, particularly early institution of effective systemic antimycotic treatment in

febrile agranulocytic patients, may increase remission rates.

## Conclusion

When combined with cytosine arabinoside and etoposide, aclarubicin seems to offer a similar efficacy in first-line induction chemotherapy for childhood ANLL compared with daunorubicin or doxorubicin. Without impairment of treatment efficacy, total doses of these two established anthracyclines may be reduced when substituted by the less cardiotoxic aclarubicin.

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# **Acute Myelogenous Leukemia in Adults**

## Morphology, Immunology, Cytochemistry, and Cytogenetics and the Classification of Subtypes in AML

H. Löffler<sup>1</sup>

According to the revised version of the FAB classification of 1985 [1] and the 1986 working group classification on morphology, immunology and cytogenetics of acute myeloid leukaemias (MIC) [6], there are 11 subtypes of AML. In addition to panoptic staining (Pappenheim, Giemsa) the employment of cytochemical techniques and the use of monoclonal myeloid antibodies can identify almost all cases. The defining criteria for the different subtypes are listed in Table 1. In

comparison to the first version, the 1985 version of the FAB classification includes a better distinction between M1 and M2, M2 and M4, M5a and M5b subtypes as well as a modification in the diagnosis of M6 and its separation from myelodysplastic syndromes. Also, three new subtypes are added: M4Eo, characterized by the presence of abnormal eosinophils in bone marrow, and M7 (megakaryoblastic leukaemia) and M2Baso, characterized by basophilic matu-

**Table 1.** Defining criteria for AML subtypes according to the FAB classification

FAB type	Granulocytes (%)	Monocytes (%)	Erythroblasts (%)
M1	<10	<20	<50
M2	>10	<20	<50
M2Baso	>10 Evidence of basophil maturation	<20	<50
M3	Hypergranular, Auer rods	<20	<50
M3V	Microgranular, monocytoid nuclei	<20	<50
M4	>20	>20	<50
M4Eo	>20 Abnormal eosinophils	>20	<50
M5a	<20	>80 Immature	<50
M5b	<20	>80 Mature	<50
M6	Variable >30% of NEC are blasts	Variable	>50
M7	Variable >30% megakaryoblasts	Variable	<50

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ration. This revision and these additions were necessary since cytogenetic findings have pointed to the existence of specific karyotypic anomalies corresponding in part to morphology.

The specific chromosomal anomalies which have attracted attention are considered below.

The karyotype  $t(15;17)(q22;q12)$  and its association with hypergranular promyelocytic leukaemia (M3) has proved to be the most specific since it has never been seen in any of the other acute leukaemias or in any other tumour. According to recent studies, every patient with M3 morphology may have a 15;17 translocation [4]. Therefore morphology is the defining feature for clinical purposes. Problems arise in the later defined variant form of M3. The discovery of the 15;17 translocation in atypical forms with monocytoid (reniform, bi- or multi-lobed) nuclei and with faint or invisible cytoplasmic granulations by light microscopy prompted the inclusion of the variant form into the M3 category. On careful search one finds rare cells with heavy granulation and/or cells containing multiple Auer rods. In these variants (M3V) myeloperoxidase and/or chloroacetate esterase cytochemistry are essential for the first step in definition of this subtype.

The karyotype  $t(8;21)(q22;q22)$ , occurring almost exclusively in the M2 subtype, may be identified if markedly heterogeneous blast cells – among them large blasts with abundant cytoplasm – occur containing a thin large Auer rod or very large granules [2, 7]. A marked marrow eosinophilia ( $\geq 5\%$ ) in approximately one-third of cases has been described as well as signs of dysgranulopoiesis in the course of granulocytic maturation, sometimes with Auer rods in mature granulocytes. Abnormally localized positivity can be seen using Sudan black B or myeloperoxidase stain. An unusually strong localized acid esterase reaction ( $\alpha$ -naphthylacetate) in blasts and early maturing granulocytes in some cases can lead to confusion with monocytic leukaemias, especially of the M4 type. In contrast to the abnormal eosinophils in subtype M4Eo, eosinophils in M2/ $t(8;21)$  are CE negative. The unexpected finding of peroxidase-positive Auer rods in eosinophils in two cases (not seen after pan-

optic staining) [7] and the accumulation of tissue mast cells in two others add to the peculiarities in M2/ $t(8;21)$  [2]. The  $t(8;21)$  is not strictly confined to M2 morphology since Swirsky et al. [7] found 6 cases out of 30 with M1 morphology. The anomaly appears to be specific for the neutrophil (and eosinophil?) cell line, combined with total or almost total disappearance of the monocytes.

A rare variant of M2 is associated with basophilic maturation (M2Baso) which has been described in cases of  $t(6;9)$  or  $t(\text{del}12)$ . But these chromosomal aberrations have been seen without basophilia, and about 20% of patients have a history of myelodysplasia [6]. The morphological recognition of the basophils may be difficult, and it is necessary to use toluidine blue metachromatic staining to confirm this finding.

Abnormalities of chromosome 16 form the third now well-established karyotypic anomaly associated with morphological alterations. This comprises inversions, deletions and rarely translocations of chromosome 16, all with the involvement of band q22. Most patients with these anomalies have the morphological subtype M4 (myelomonocytic), with abnormal eosinophils containing large irregular basophilic or black-violet staining, sometimes dysplastic-appearing granules. There are reports of  $\text{inv}(16)$  in connection with the subtypes M2 or M5; we have seen one case with M1 morphology. They all have abnormal eosinophils in common, which might reach 82% (one case of our series). Thus the abnormalities of chromosome 16 are uniquely associated with abnormal eosinophils. Together with chloroacetate esterase positivity [3] the morphological features are so specific that one can predict which patient will have an abnormality of chromosome 16 (q22).

In 1987 a new specific anomaly associated with involvement of the monocytic cell line (mostly subtype M5 b) together with significant erythrophagocytosis was described in patients with  $t(8;16)(p11;p13)$ . A less well defined group comprises a variety of translocations and deletions, all with involvement of 11q23. Morphologically most of these cases belong to the monocytic group; about 50% have M5a morphology. Some of these translocations can also be found in acute

**Table 2.** Morphological, cytochemical, immunological and karyotypical interrelationship in AML subtypes

FAB type	Morphology	Cytochemistry	Immunology	Karyotypic change	Frequency (%) <sup>a</sup>
M1	+	++	+	t(9;22)	3.0
M2	++	+	+	t(8;21)	12.0
M2Baso	++	++	?	t(6;9)	1.0
				t/del(12)	<0.1
M3	++	+	+	t(15;17)	} 10.0
M3V	+	++	+	t(15;17)	
M4	++	++	+	t/del(11)(q23)	6.0 <sup>b</sup>
				+4	<0.1
M4Eo	++	++	?	inv/del(16)	5.0
M5a	++	++	+	t/del(11)(q23)	6.0 <sup>b</sup>
M5b	++	++	+	t(8;16)	<0.1
M6	++	+	+	?	-
M7	+	(+)	++	?	-

+, helpful criteria; ++, defining criteria.

<sup>a</sup> According to second MIC Cooperative Study Group [6].

<sup>b</sup> The frequency of t/del(11)(q23) is 6.0% in total.

lymphoblastic leukaemias or in hybrid acute leukaemias.

In the Second MIC Workshop [6] cytogenetic anomalies not associated with FAB subtypes were summarized. These include the most common cytogenetic anomaly in AML trisomy 8 (+8), as well as monosomy 7 (-7) and deletion of 7q(7q-), deletion of 5q(5q-), and loss of the Y chromosome. The anomalies +21,9q-, i(17q), and +22 are rare. Each of these anomalies may occur as the sole karyotypic change or as an additional anomaly.

With regard to the frequency of the various karyotypic changes there exist only gross estimates from the chromosome workshops or from single institutions which have collected data over several years (Table 2). The most common specific translocation is t(8;21) in subtype M2; the most common anomaly not presently associated with a FAB type is trisomy 8 (+8).

The value of immunological techniques utilizing anti-myeloid monoclonal antibodies is in confirming the diagnosis of AML in situations in which morphology and cytochemistry do not clearly identify the lineage of leukaemic cells; they are particularly useful where there may be a hybrid acute leukaemia. Without immunology or the

more complicated and time-consuming electron microscopic platelet peroxidase technique megakaryoblastic leukaemia (subtype M7) cannot be identified with sufficient certainty.

With regard to prognosis associated with the different subtypes one must keep in mind that at the moment there exist no data of randomized prospective studies with sufficient numbers of karyotyped patients from the beginning, and there is no uniformity in the therapy of the patients collected for the chromosome workshops. However, there is a tendency for better prognosis in patients with M4Eo/inv16 and for long duration of remissions in patients with M3/t(15;17). However, this may be a reflection of age since most of the patients with specific anomalies are younger. In contrast, patients with monosomy 7 (-7) or 7q-, with -5/5q- or trisomy 8 have a higher median age and a poor prognosis [5].

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## Remission Induction and Postremission Therapy in Acute Myelogenous Leukemia: British MRC Study

J. K. H. Rees<sup>1</sup> and R. G. Gray<sup>2</sup>

At the first International Symposium on Acute Leukaemias – Prognostic Factors and Treatment Strategies in 1986, we outlined the background and preliminary results of the Ninth MRC AML trial. The protocol is summarised in Fig. 1. The trial closed for patients under 55 years of age in July 1988 but remains open for older patients.

In the 5 years up to the end of January 1989, 890 patients have been entered into the study with a median age of 55 years. Very few (14) children have been admitted because of a concomitant multicentre paediatric trial in the United Kingdom. The overall remission rate among the first 846 patients, which includes 763 cases of de novo AML and 83 cases of secondary disease, is

63%. The remission rate according to age and induction therapy is shown in Table 1. In each age group among the “de novo” group the more intensive 3 + 10 regimen has proved superior to the 1 + 5 combination. The fact that this is not reflected in the older patients with secondary AML is probably due to the small numbers of patients in the groups.

The number of days patients spend in hospital is substantially lower for the more aggressive 3 + 10 combination than for the 1 + 5 treatment. This is significant even when one considers only those patients who achieve complete remission (CR) (Table 2). The 3 + 10 combination is therefore more effective and more economical. The advantage conferred by the 3 + 10 regimen is continued after remission has been achieved. Figure 2 shows the duration of survival after the end of the 1st month when the majority of patients who do not achieve remission have died. There is no significant difference in survival between those patients who re-

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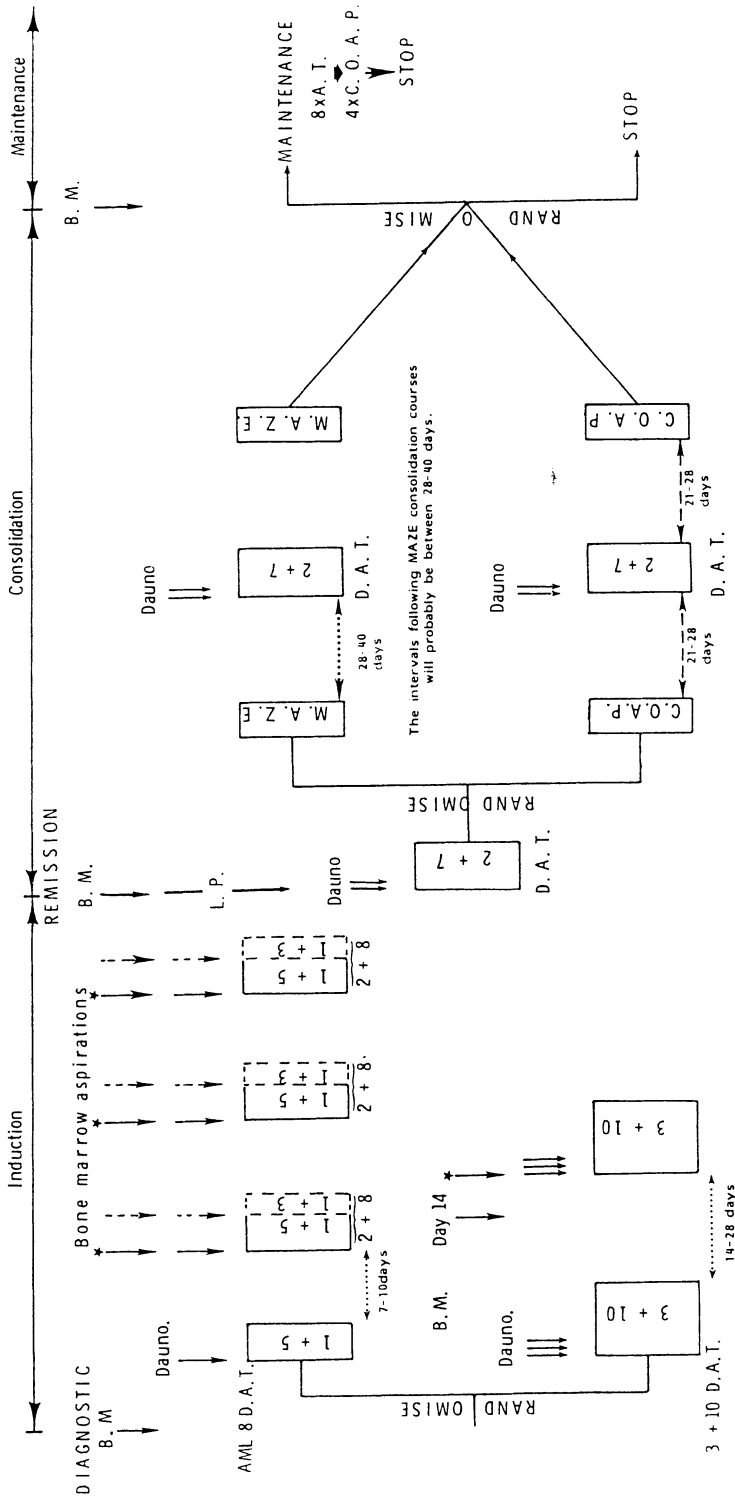
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**Table 1.** AML 9: remission rate

Age (years)	De novo		Secondary	
	1 + 5	3 + 10	1 + 5	3 + 10
0–49	77% (134/174)	84% (152/181)	50% (7/14)	67% (4/6)
50–59	64% (54/84)	63% (52/82)	20% (2/10)	67% (8/12)
60–69	44% (39/88)	49% (45/92)	42% (8/19)	23% (3/13)
70 +	34% (11/32)	47% (14/30)	50% (2/4)	20% (1/5)
All ages	63% (238/378)	68% (263/385)	40% (19/47)	44% (16/36)

$\chi^2_1$  for treatment effect = 2.73  $P = 0.099$   
 $\chi^2_1$  for trend of decreasing benefit with age = 0.67  $P = 0.4$

AML 9



\* If remission picture proceed to consolidation DAT (2+7)  
 If no remission continue induction phase.

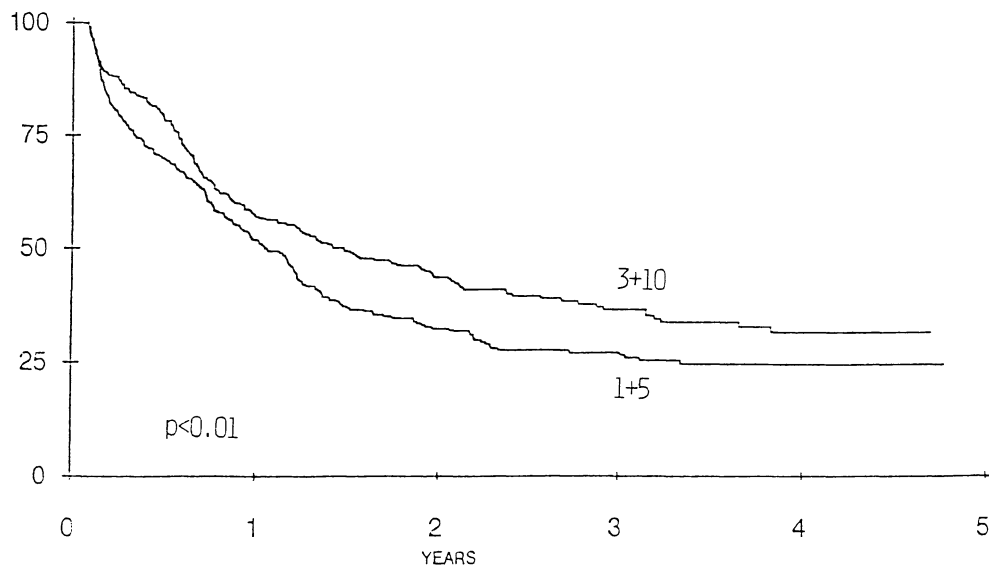
Fig. 1. Summary of protocol

Intervals between COAP consolidation courses should be kept as short as possible 21-28 days will be feasible in the majority of cases

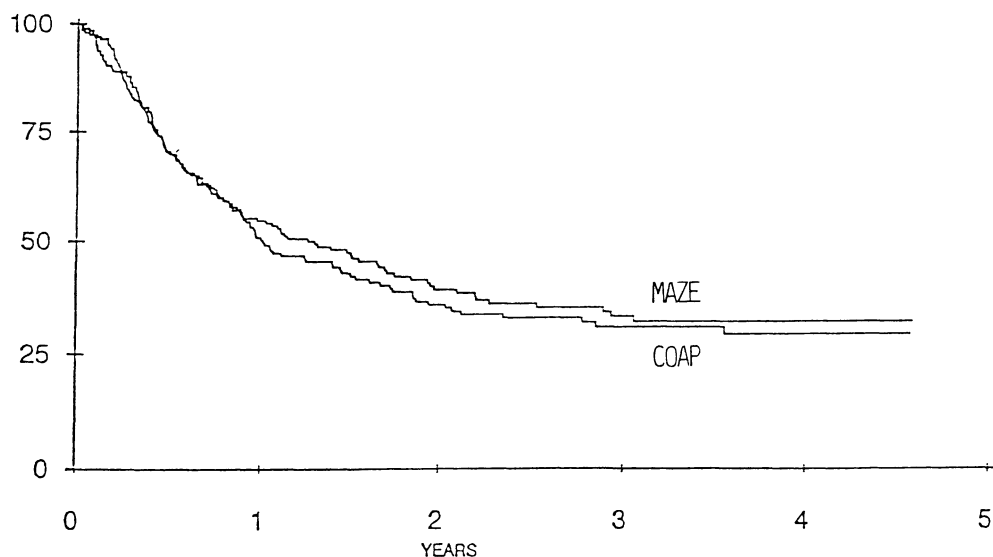
ceived COAP consolidation therapy and those who received MAZE (Fig. 3) although the MAZE combination was far more toxic and required a great deal more supportive care the median interval for the completion of the four courses of consolidation therapy was 120 days (range 70–266 days) for the COAP arm and 153 days (range 84–302

days) for the MAZE arm. The rate of death in remission due to the toxicity of postremission therapy is currently 8.5%. The majority of patients in this group were > 60 years of age.

Comparisons of the disease-free survival between patients receiving maintenance with monthly cytosine arabinoside (Ara-C)



**Fig. 2.** MRC AML 9 trial: survival month 2 onwards, February 1989



**Fig. 3.** MRC Ninth AML trial: disease-free survival from MAZE/COAP randomisation, February 1989

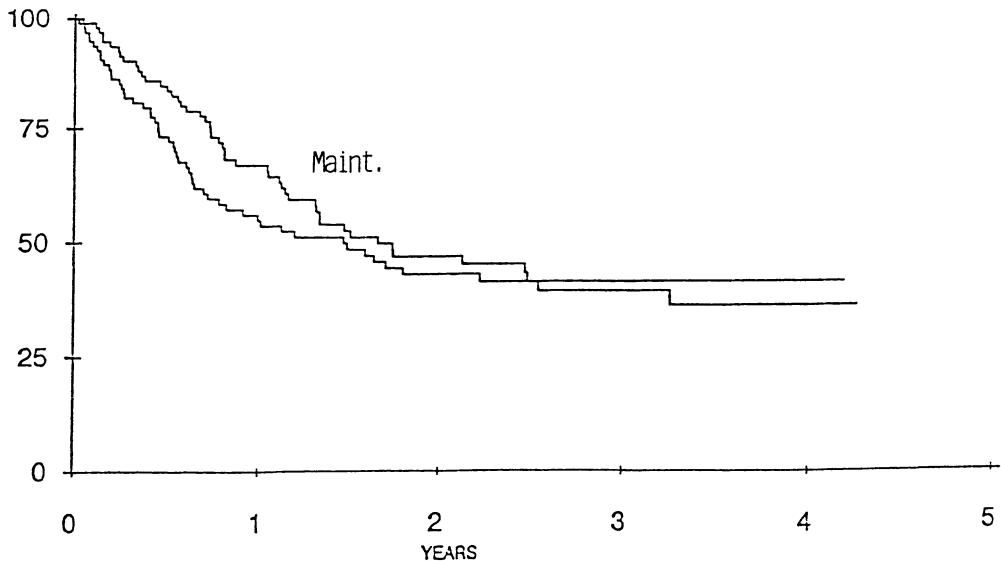
**Table 2.** AML 9: in-hospital days during induction

Age (years)	n	Hospital days		P
		1+5	3+10	
<b>All patients</b>				
0-49	350	46	38	
50-59	168	43	38	
60-69	184	44	33	
70+	64	35	32	
All ages	766	44	36	0.0001
<b>CR</b>				
0-49	269	44	38	
50-59	97	41	39	
60-69	82	50	36	
70+	24	38	46	
All ages	472	44	38	0.0001

and 6-thioguanine and those who received no treatment after the consolidation phase showed no long-term difference although there was a slight excess of relapses in the STOP group in the 1st year (Fig. 4). There was a high second remission rate in this group so that the overall survival was not affected. Maintenance therapy has therefore

been excluded from the next MRC trial - AML 10.

This trial opened in July 1988 and is designed for the treatment of children and adults under 55 years of age with de novo or secondary AML. The protocol with the doses of drugs is shown in Fig. 5. The central question is the role of bone marrow transplantation in postremission treatment of AML. Following remission, patients who do not have an HLA-compatible sibling receive one course of MACE consolidation therapy (mAMSA, Ara-C, etoposide) before proceeding to bone marrow harvest when the peripheral blood count has recovered. After a successful harvest has been carried out patients are randomised to an early autograft following cyclophosphamide and TBI conditioning or to STOP treatment after a second course of consolidation with MidAC (mitozantrone and intermediate dose Ara-C.) Patients randomised to STOP treatment who relapse >6 months after remission will receive reinduction therapy and an autograft, using the marrow which was stored earlier, following conditioning with busulphan and cyclophosphamide. In order to recruit a sufficiently large number of patients into the second phase of the trial the most successful induction therapy in AML 9 - 3+10 DAT is compared with a combina-



**Fig. 4.** MRC Ninth AML trial: disease-free survival from STOP/continue randomisation, February 1989

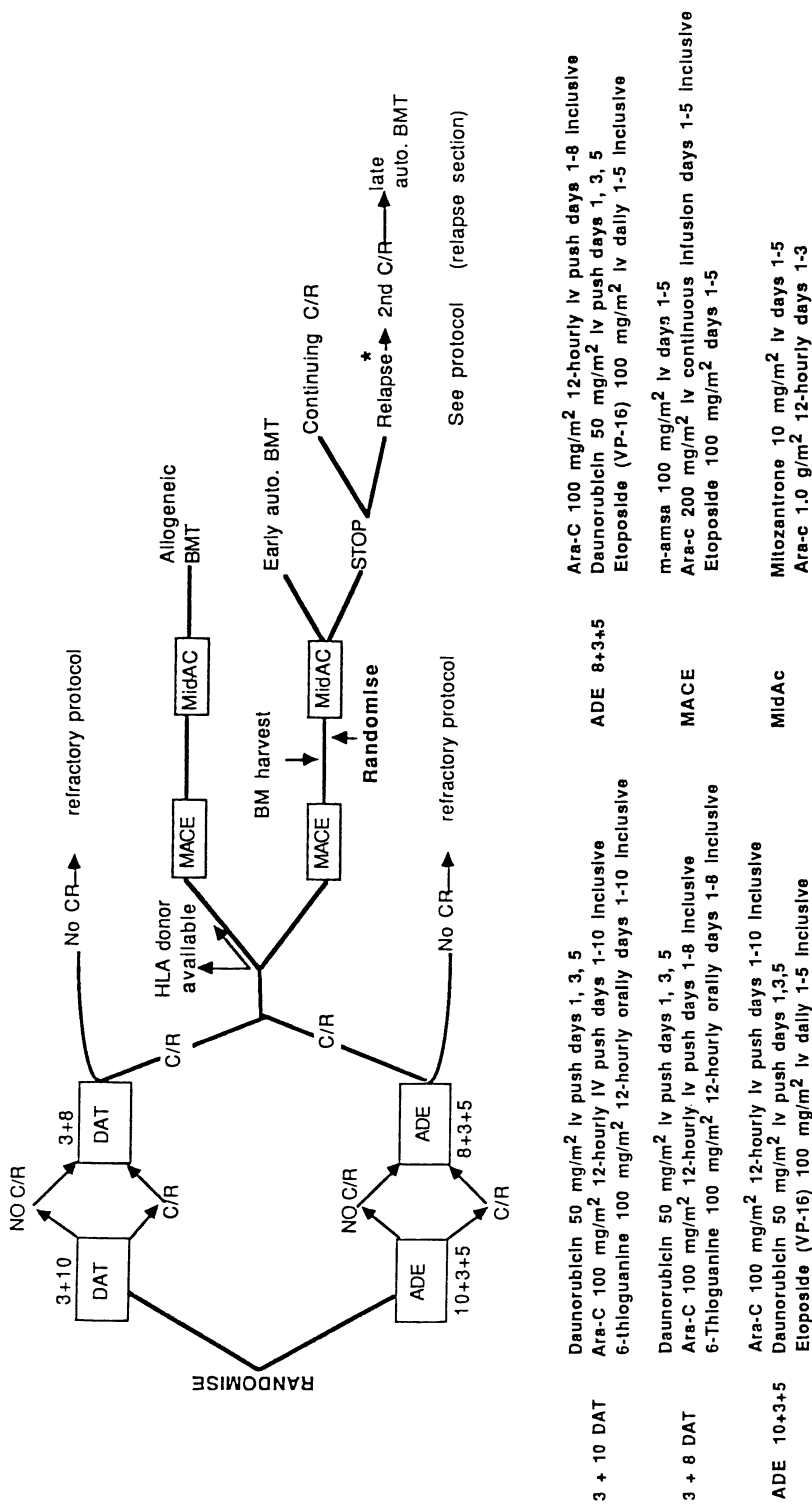
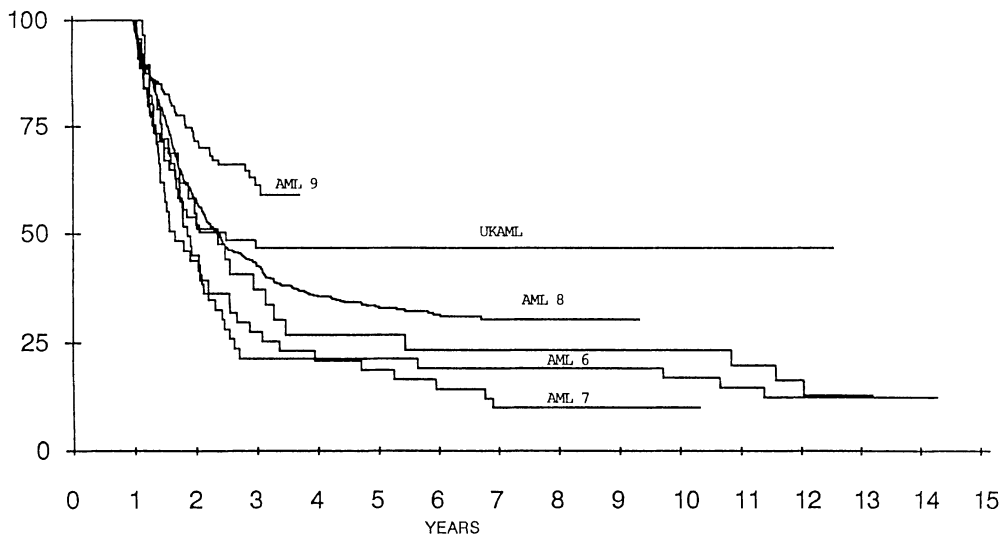


Fig. 5. MRC AML10 trial





**Fig. 6.** Disease-free survival by trial: year 2 onwards

tion in which 6-thioguanine is replaced by etoposide (ADE: Ara-C, daunorubicin, etoposide).

Up to the end of January 1989, 120 patients had been entered into the trial. At

an early stage in the study the remission rate is >85%. A review of previous trials has shown that the percentage of long-term survivors in the successive MRC trials is steadily improving (Fig. 6).

## Therapy of Acute Myelogenous Leukemia in Adults\*

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At present major issues regarding optimal therapy of acute nonlymphocytic leukemia (ANLL) remain unresolved. Controversial areas include the importance of consolidation and the role of postconsolidation therapy (either conventional or intensive). To investigate these problems the Italian Cooperative Group GIMEMA designed a prospective treatment program based on standard induction therapy with daunorubicin (DNR) plus cytosine arabinoside (Ara-C) followed by intensive consolidation. Thereafter, patients were randomized in three arms to receive an aggressive postconsolidation treatment with sequential combinations of active agents given in short courses at myelosuppressive doses, conventional maintenance, or no treatment at all. This multicenter trial began in 1982 and was closed to patient entry in May 1987.

### Materials and Methods

#### Patients

Between March 1982 and May 1987, 452 consecutive previously untreated adults with ANLL (aged 15–55 years, median 38 years) were entered into this study from 26 Institutions (Table 1). Diagnosis was established by bone marrow aspirate or biopsy examina-

**Table 1.** Member institutions of the GIMEMA cooperative group

	No. patients
Cattedra di Ematologia-I Università – Roma	66
Divisione Ematologia – Palermo	39
Ematologia-Università – Bari	32
Ematologia – Pescara	25
Divisione Ematologia – Catanzaro	27
Cattedra di Ematologia – Catania	26
Ematologia S. Camillo – Roma	24
Università Cattolica – Roma	24
Ematologia-Molinetto – Torino	19
Cattedra di Ematologia – Perugia	20
Clinica Medica – Palermo	17
Ematologia-Businco – Cagliari	16
Divisione Ematologia – S. G. Rotondo	16
Sezione Ematologia-Cardarelli – Napoli	15
Ematologia-Osp. Riuniti – Reggio Calabria	12
Divisione Ematologia – Latina	12
Ematologia – Avellino	11
Ematologia-Nuovo Pellegrini – Napoli	11
Clinica Medica – Ancona	11
Ematologia – Messina	9
Ematologia – Nuoro	5
Cattedra di Ematologia – Torino	5
Cattedra di Ematologia-10 Facoltà – Napoli	4
Ematologia – Potenza	3
C.R.O. – Aviano	2
Cattedra di Ematologia – Sassari	1
	<b>452</b>

GIMEMA Cooperative Group, Italy

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tion at the parent institutions, using accepted French-American-British (FAB) morphological classification criteria [1]. All pa-

tients with ANLL but hypergranular promyelocytic leukemia (M3) were eligible. Patients with prior hematological disorders, but blastic crises of chronic myelogenous leukemia or myeloproliferative disorders, were also included. Five patients had antecedent hematological disorders with a documented cytopenia for more than 6 months before the diagnosis of ANLL. All slides were submitted to the GIMEMA Central Office for review by members of the morphology committee. Eligibility criteria included no significant cardiac disease and adequate hepatic and renal function.

### *Induction and Consolidation Therapy*

Induction chemotherapy consisted of DNR, 45 mg/m<sup>2</sup>, i.v. days 1–3, and Ara-C, 200 mg/m<sup>2</sup> by continuous infusion i.v. days 1–7. An additional course of DNR and Ara-C (2 + 5) was administered either upon hematological recovery or on day 14 from the first course if hypoplasia was not induced. Patients were considered as failures and removed from the study if a complete remission (CR) was not achieved after the two courses of therapy. Patients achieving CR received consolidation therapy, which was started when granulocytes were  $\geq 1000/\text{mm}^3$  and platelets  $\geq 100\,000/\text{mm}^3$ , and consisted of four courses of DNR 60 mg/m<sup>2</sup> i.v. on day 1, 6-thioguanine (6-TG) 70 mg/m<sup>2</sup>, p.o. every 8 h, days 1–5, and escalated doses of Ara-C (60 mg/m<sup>2</sup> every 8 h, s.c., days 1–5 course I, 80 mg/m<sup>2</sup> every 8 h, s.c., days 1–5 course II, 110 mg/m<sup>2</sup> every 8 h, s.c., days 1–5 course III, and 150 mg/m<sup>2</sup> every 8 h, s.c., days 1–5 course IV). Consolidation courses were given at 3- to 4-week intervals as soon as hematological recovery was observed (granulocytes  $> 500/\text{mm}^3$  and platelets  $> 80\,000/\text{mm}^3$ ).

### *Postconsolidation Therapy*

Patients still in CR after consolidation were allocated to one of three arms of the postconsolidation phase using randomly permuted blocks without any stratification. The postconsolidation phase consisted of arm A, no further treatment; arm B, conven-

tional maintenance with 6-TG 200 mg/m<sup>2</sup> orally days 1–4 followed by Ara-C 200 mg/m<sup>2</sup> s.c. on day 5 (the courses were repeated after 5 days of rest for a total of 18 courses); arm C, intensive treatment with sequential administration of: VP 16 100 mg/m<sup>2</sup> i.v. days 1–3 and Ara-C 150 mg/m<sup>2</sup> every 8 h s.c. days 1–3 (two courses), Ara-C 150 mg/m<sup>2</sup> every 8 h s.c. days 1–5 and 6-TG 70 mg/m<sup>2</sup> every 8 h orally days 1–5 (two courses), and DNR 40 mg/m<sup>2</sup> i.v. day 1 and Ara-C 300 mg/m<sup>2</sup> by continuous infusion day 1–3 (two courses). Courses were given at hematological recovery. Preventive CNS therapy was also employed consisting of Ara-C (40 mg/m<sup>2</sup>) given intrathecally at remission and on day 1 of each course of consolidation therapy (total five instillations). In the case of CNS leukemia at diagnosis intrathecal Ara-C was injected weekly until the spinal fluid was free from leukemic cells plus two additional doses; thereafter patients in CR resumed the CNS therapy program dictated by the protocol. Toxicity was scored according to the criteria of the World Health Organization (WHO).

### **Results**

Of the 452 registered patients, 450 are evaluable for response to induction because 2 patients died before starting therapy. The overall CR rate was 68% (307/450) (Table 2). Complete remission was achieved by 159 patients after the first course of therapy, while the remaining 148 required two courses; the median time to CR was 33 days (range 14–94 days). Of the 143 failures, 68 patients had resistant leukemia and 75 died because of infection, hemorrhage, or renal or cardiac failure before response to treatment could

**Table 2.** LANL 8201: response to induction therapy

Registered patients	452
Pretherapy deaths	2
Evaluable patients	450
Complete remissions	307 (68%)
Resistants	68 <sup>a</sup> (15%)
Induction deaths	75 (17%)

<sup>a</sup> One patient did not complete induction therapy

be assessed. One patient interrupted induction treatment because of intolerable toxicity. The response rate was significantly affected by splenomegaly and presence of infection at diagnosis; both these features were inversely related as independent factors to the probability of achieving CR ( $P=0.01$  and  $P=0.001$ , respectively).

The 307 patients achieving CR were followed for a median of 1100 days (range, 360–2550 days). There were 48 withdrawals for allogeneic bone marrow transplantation (22) or autologous bone marrow transplantation (26). Forty-three patients interrupted the treatment program: 23 because of side effects and 20 because of their refusal to continue treatment.

Thirty-five patients (11.4%) died while in CR; causes of death were infections (24), bleeding (3), and heart failure (2), while they were not evaluable in 6. Fourteen patients died before starting consolidation from late toxicities, 20 died during consolidation, and 1 died during the postconsolidation phase (arm C). A total of 145 patients have relapsed: median time to relapse was 309 days (range 34–1410 days).

Median duration of disease-free survival (DFS) was 12 months and 23% of the patients are projected to be alive and free from

disease 5 years from remission (Fig. 1). Of the 302 patients achieving CR, 156 were randomized, with 59 patients assigned to arm A, 48 to arm B, and 49 to arm C. The three groups of patients did not differ significantly by age, FAB subclassification, WBC count, and other features. No significant difference in DFS was observed in the three groups of randomized patients (Fig. 2).

No substantial toxicities (more than WHO grade 2) were observed in postconsolidation arms A and B. On the contrary, during consolidation and in intensive post-consolidation treatment (arm C) all patients experienced profound myelosuppression, with nadirs of less than 200 polys/mm<sup>3</sup> and less than 30000 platelets/mm<sup>3</sup> after each course of therapy. More than 60% of the courses were associated with febrile episodes and antibiotic treatment was administered for presumed or proven infections. Fatal infections occurred in 18 patients (1 in intensive post-consolidation). Most patients required platelet support. Fatal hemorrhage was observed in two patients during consolidation.

Among a series of clinical and hematological presenting features investigated for their prognostic influence on DFS and overall survival, FAB M2, M5, and infection at diagnosis were found to be independent prognostic factors. The DFS was significantly better in patients with FAB class M2 ( $P=0.02$ ) while the presence of infection and FAB class M5 were significantly related to a shorter overall survival ( $P=0.009$  and  $P=0.02$  respectively).

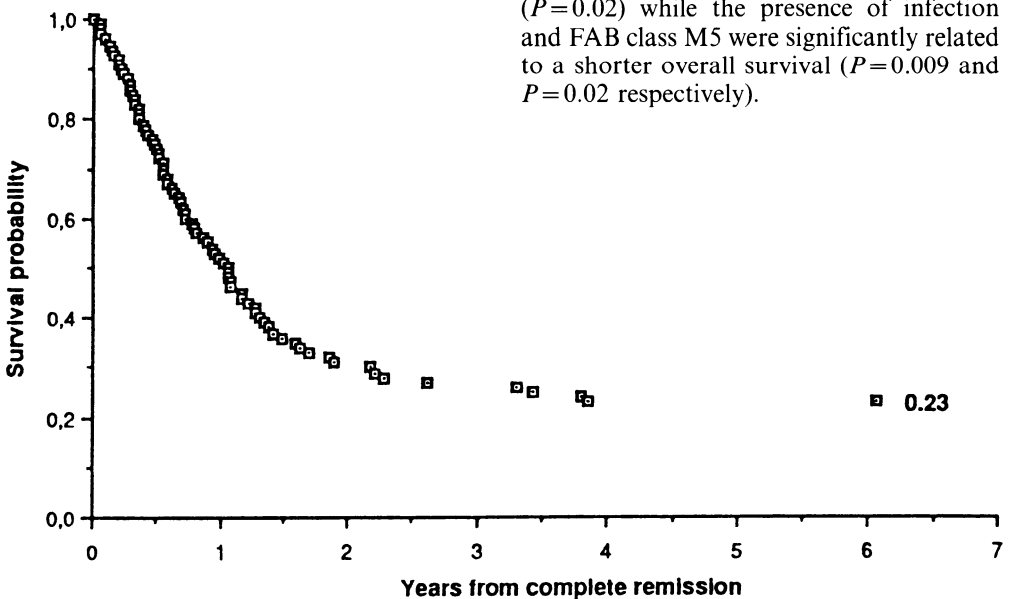


Fig. 1. LANL 8201: Disease-free survival

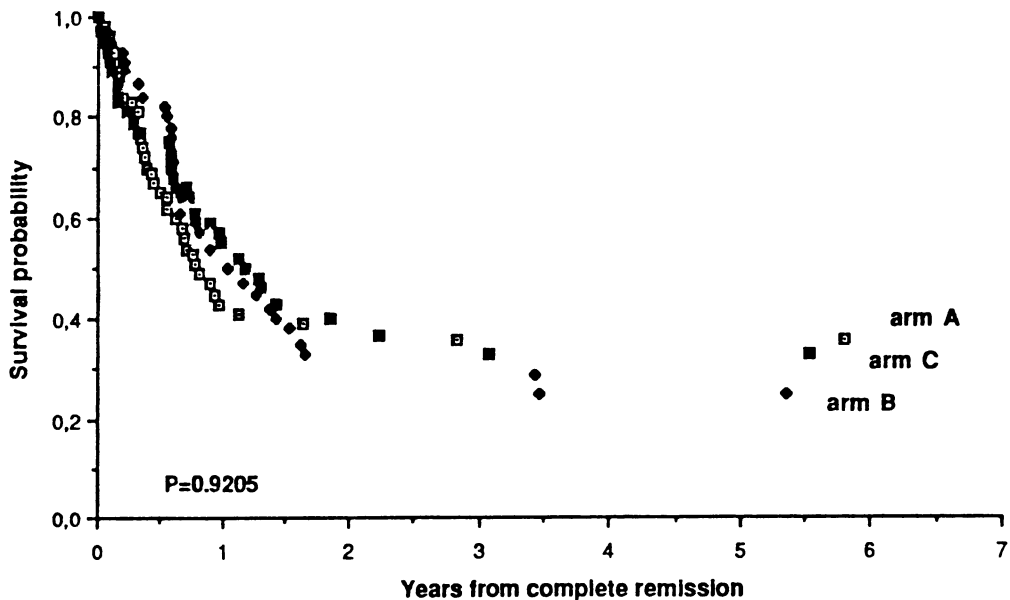


Fig. 2. LANL 8201: Disease-free survival by random

## Discussion

Four hundred and fifty adult ANLL patients were treated on GIMEMA protocol LANL 8201: 68% achieved CR. Twenty-six institutions contributed patients, and results did not differ between large and small hospitals, indicating that on average the quality of medical and supportive care among the participating institutions was comparable. Our data confirm that with the standard 7 days of Ara-C and 3 days of DNR ("3+7") remission induction regimen almost two-thirds of an unselected group of patients with ANLL aged >15 and <55 years will obtain CR. These results compare favorably to those reported so far by other similar cooperative studies [5–7]. In fact, a few non-randomized studies, either in single institutions or in cooperative groups, reported that improvement in CR rate might be achieved by using a multidrug induction regimen (including 6-TG and/or vincristine) [8], by prolonging treatment period (to 9–10 days), or by increasing DNR dosage, however, a recently published randomized trial from CALGB failed to demonstrate an improvement in CR rate by adding 6-TG to DNR-Ara-C or by prolonging Ara-C infusion to

10 days [9]. Despite the encouraging results in the frequency of CR, response duration is long lasting only in a minority of patients (usually less than 20%). In fact the best postremission policies to prolong remission are not as yet well established.

The median duration of DFS in our study is 12 months and 23% of the patients are projected to be alive and free from disease at 5 years. A total of 35 patients died in CR, 14 from late toxicity from induction therapy, whereas 20 deaths are related to complications which occurred during the consolidation phase; our consolidation therapy produces significant toxicity; however, only a small proportion of patients discontinued the planned treatment for the above reason.

The projected long-term remission rate in patients who did not receive maintenance (random A), in the upper range of published data, might reflect the therapeutic efficacy of the consolidation therapy. Indeed these results may support the idea that a short intensive consolidation chemotherapy is associated with a high proportion of long-term survivors and are consistent with the experience of other trials [9, 10]. The data presented here failed to demonstrate any statistically significant difference of overall and DFS

between patients randomized to receive either conventional or aggressive postconsolidation treatment or no treatment at all. These results are consistent with the experience of other groups and suggest that, if the induction/consolidation is sufficiently intensive, maintenance (or more aggressive postconsolidation therapy) might offer only a slight or no DFS advantage.

Recently, Büchner et al. [11] reported longer remission duration in patients receiving maintenance treatment compared with unmaintained patients. However, in the randomized study unmaintained patients received only one course of TAD-9 as consolidation while the maintenance treatment consisted of an intensive chemotherapy with a rotating multiple-drug regimen. It seems therefore that the group of unmaintained patients was given a very weak postremission therapy, not comparable to the consolidation treatment used in the present or other trials.

The frequency of CNS relapse in this trial was very low compared with that in other published series [12, 13]. However, no conclusions can be drawn because all patients received a intrathecal prophylactic regimen; moreover, no pediatric patients were included in this study.

Spleen size and the presence of infection have emerged as independent factors directly associated with a less favorable response to induction therapy. The adverse effect of infection at diagnosis may reflect an impaired capability to sustain chemotherapy. The role of spleen size as negative prognostic factor is difficult to assess. In the present series, risk factor analysis indicates a negative influence of the M5 subtype on overall survival; this finding may be due to the combined effect of lower response rate and shorter DSF, confirming the reported poorer outcome of the patients with monocytic subtype.

In conclusion, the results of the GIMEMA 8201 trial confirm the effectiveness of the standard DNR + Ara-C combination for remission induction in adult AN-LL patients and suggest that, if a program of induction/consolidation therapy is sufficiently intensive, additional therapy, either standard or intensive, might offer no significant DFS advantage to the patients.

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## Consolidation Therapy with High-Dose Cytosine Arabinoside: Experiences of a Prospective Study in Acute Myeloid Leukemia\*

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### Introduction

At the present time 60%–80% of adult patients suffering from acute myelogenous leukemia (AML) will achieve complete remission with the available induction chemotherapy including anthracycline antibiotics and cytosine arabinoside [1–5]. With standard chemotherapy, however, the majority of the patients will suffer leukemia relapse within the first 2 years after complete remission and only about 20% of the complete responders will have long-term disease-free survival. Substantially better results can be achieved with allogeneic bone marrow transplantation, which provides long-term remission in 50% or more of the patients who are transplanted in first remission with bone marrow grafts from histocompatible siblings [6, 7]. Unfortunately only a minority

of the patients with AML can be treated with allogeneic bone marrow transplantation because this approach is limited to patients of less than 45–50 years of age and because histocompatible donors are often not available. Therefore other modalities for postremission therapy are being investigated such as intensified maintenance or consolidation therapy and autologous bone marrow transplantation. As far as intensified consolidation therapy is concerned the administration of high-dose cytosine arabinoside (HD Ara-C) seems to be a promising approach. A series of studies have documented that HD Ara-C alone or in combination with other cytotoxic drugs provides complete remissions in a relatively high percentage of patients with relapsed acute leukemia or with acute leukemia refractory to conventional doses of chemotherapy [8–10]. These results suggest that HD Ara-C might be able to eliminate residual leukemic cells which survived induction therapy with conventional doses. Another advantage of HD Ara-C is that it achieves therapeutic levels in the CNS and may decrease the need for prophylactic CNS treatment. On account of these considerations HD Ara-C was introduced into consolidation therapy of AML. The preliminary results of the studies which have been published so far seem to be promising and indicate a probability of complete continuous remission (CCR) after 6 years of up to 50% [11–17].

In 1985 a study group of the Süddeutsche Hämoblastosegruppe started a prospective study on consolidation therapy with HD Ara-C and daunorubicin (HD ARA-C/

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DNR) in a series of consecutive patients with AML who were in complete remission after induction and early consolidation with DNR, Ara-C, and etoposide (DAV) and who were not candidates for allogeneic bone marrow transplantation [18]. The aims of this study were to evaluate the efficacy and side effects of the induction regimen with the DAV protocol and whether intensive post-remission therapy with HD Ara-C/DNR could yield long-term, unmaintained remissions. Since substantial toxicity of HD Ara-C had been reported in elderly patients [19], the upper age limit of this study was 50 years. This report will summarize the preliminary data of this trial which were obtained during the first 3 years of the study.

## Materials and Methods

### *Selection of Patients*

Patients with the diagnosis of AML (FAB M1–M6) who were admitted to one of the participating hospitals between 1 April 1985 and 1 November 1988 were eligible for this study. Patients were not considered for this study if one of the following conditions were fulfilled:

1. age above 50 years;
2. secondary leukemia or history of myelodysplastic syndrome, aplastic anemia, or smouldering leukemia;
3. severe diseases of heart, lung, liver, kidneys, or CNS;
4. severe complications of leukemia such as shock, pneumonia with hypoxia, or uncontrolled bleeding. If the latter complications were treated successfully the patient became eligible for the study.

### *Induction Therapy and Early Consolidation*

For remission induction therapy patients were given a combination of daunorubicin (DNR), cytosine arabinoside (Ara-C), and etoposide (VP 16-213) as described previously [18]. In the first course (DAV I) the following doses were given: DNR (60 mg/m<sup>2</sup> per day i.v.) days 3–5, Ara-C (100 mg/m<sup>2</sup> per day continuous i.v. infusion) days 1–8, and VP 16-213 (100 mg/m<sup>2</sup> per day i.v.

infusion over 2 h) days 4–8. A second course of induction therapy (DAV II) was given after day 21: DNR (45 mg/m<sup>2</sup> per day i.v.) days 3–4, Ara-C (100 mg/m<sup>2</sup> continuous i.v. infusion) days 1–7, and VP 16-213 (100 mg/m<sup>2</sup> per day i.v. infusion over 2 h) days 3–7. During the first 2 years of the study VP 16-213 was given in the same daily dose over 3 days. If after two courses of induction therapy a complete remission was achieved, a further course of the DAV regimen was applied for early consolidation. Failure to achieve complete remission after two courses of induction constituted treatment failure and such patients were removed from protocol therapy.

### *Late Consolidation*

Patients in complete remission after induction and early consolidation therapy who were not candidates for allogeneic bone marrow transplantation based on standard criteria were given one to two courses of HD Ara-C and DNR for late consolidation. The first course (HD Ara-C/DNR I) was given 4 weeks after recovery from early consolidation: Ara-C (3 g/m<sup>2</sup> i.v. infusion over 2 h every 12 h) days 1–6 and DNR (30 mg/m<sup>2</sup> per day i.v.) days 7–9. Eight weeks after recovery from the first course patients were given HD Ara-C/DNR II: Ara-C (3 g/m<sup>2</sup> i.v. infusion over 2 h every 12 h) days 1–4 and DNR (30 mg/m<sup>2</sup> per day i.v.) days 5–7. Patients with severe complications during the HD Ara-C/DNR I course such as life-threatening infections or pulmonary complications and patients with prolonged aplasia were not given the second course of late consolidation. All patients who were given HD Ara-C were treated prophylactically with corticosteroid eye drops.

### *Supportive Care*

The patients were treated under conventional ward conditions in single or two-bed rooms and received a normal hospital diet. Prophylactic platelet transfusion was given when the platelet count dropped below  $20 \times 10^9$ /liter. For antimicrobial prophylaxis, methods of selective decontamination



were applied [20]. In the case of proven or suspected infections empiric antibiotic therapy using combinations of beta-lactam antibiotics and aminoglycosides was administered. Patients who did not respond to the antibiotic therapy within 8 days received empirical antifungal therapy with amphotericin B and 5-fluorocytosine.

### Evaluation

Life tables were calculated according to the method of Kaplan and Meier. Patients who underwent bone marrow transplantations were censored at the time of transplantation. Toxicity was scored according to the criteria of the World Health Organization [21].

## Results

### Induction Therapy

A total of 132 patients entered the study. The clinical data of these patients are shown in Table 1. The mean age was 40 years (range 15–50 years). Complete remission was achieved in 89 patients (67%) and a partial remission in six patients (5%). Fourteen patients (11%) died within the first 6 weeks during remission induction and 23 patients (17%) were treatment failures (Table 2). All patients achieving complete remission were given one course of early consolidation therapy.

**Table 1.** Patient characteristics

No. of patients	132
Age (years)	
Mean	40
Range	15–50
Sex (no. of patients)	
Male	65
Female	67
FAB classification (No. of patients)	
M1	22
M2	52
M3	8
M4	27
M5	20
M6	3

**Table 2.** Result of remission induction therapy

Complete remission	89/132 (67%)
Partial remission	6/132 (5%)
Failure	23/132 (17%)
Death	14/132 (11%)

### Late Consolidation Therapy

Out of the 89 patients achieving complete remission, 52 were given late consolidation with one or two courses of HD Ara-C/DNR. Of the remaining 38 patients, 1 refused further therapy, 11 had severe medical complications during previous treatment, 1 died during early consolidation therapy in first remission, 6 relapsed before late consolidation, and in 12 bone marrow transplantation was performed. Six further patients are still waiting for late consolidation after they have finished induction therapy.

The side effects of late consolidation therapy with HD Ara-C/DNR were substantial and mainly related to myelosuppression. The mean duration of granulocytopenia ( $<0.5 \times 10^9/\text{liter}$ ) and thrombocytopenia ( $<25 \times 10^9/\text{liter}$ ) after the HD Ara-C/DNR I course was 24 and 22 days, respectively. The maximum duration of neutropenia was 46 days and that of thrombocytopenia was 84 days. As a consequence of the long duration of myelosuppression, infections were the most frequent and most severe complications. One patient died during late consolidation from infectious complications. Nonmyeloid toxicity consisted mainly of nausea, vomiting, liver dysfunction, diarrhea, and dermatitis. Central nervous toxicity was not observed.

### Survival and Remission Duration

A life table analysis for the probability of survival of all 132 patients who entered the study revealed a median survival time of 22.5 months and an actuarial probability of being alive after 42 months of 37%. The median duration of remission for all 89 complete responders was 20 months and the probability of relapse-free survival after 42 months was 39% (Fig. 1). For those patients who received one or two courses of late consolidation with

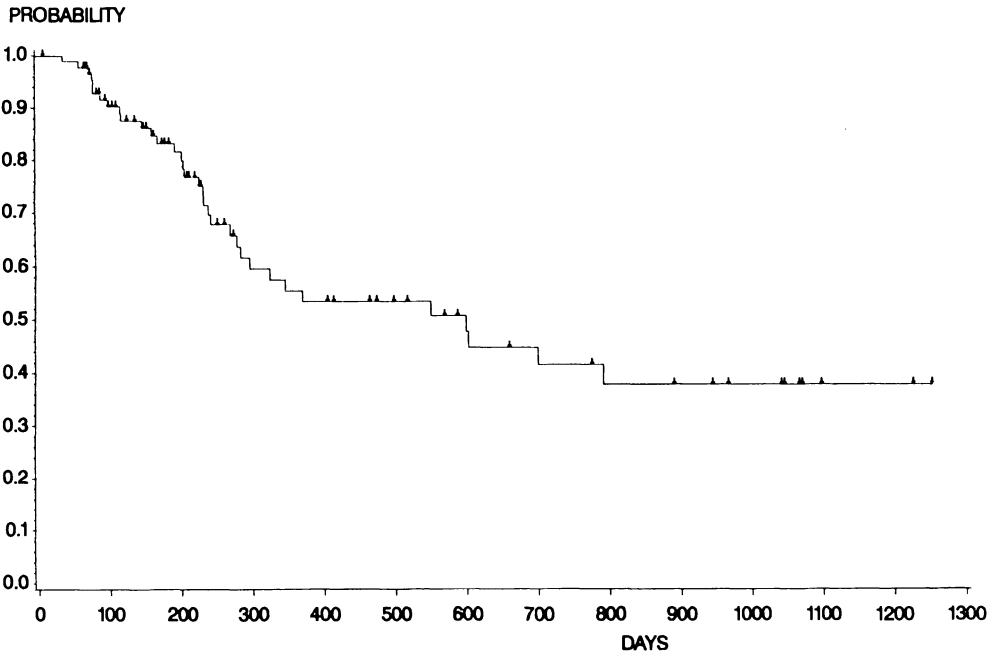


Fig. 1. Relapse-free survival of all 89 patients who achieved complete remission

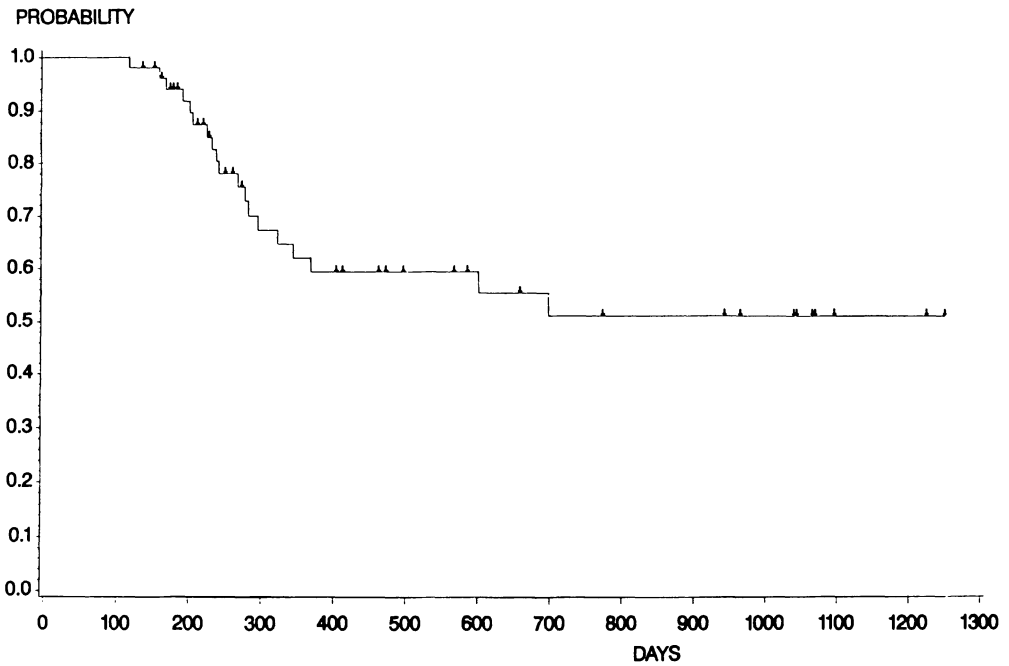


Fig. 2. Relapse-free survival of the 52 patients who received consolidation therapy with HD Ara-C/ DNR in first complete remission

HD Ara-C/DNR the actuarial probability of relapse-free survival was 51% at 42 months after complete remission (Fig. 2).

## Discussion

In this prospective multicenter study, a remission induction therapy including DNR, Ara-C, and etoposide was used which resulted in a complete remission rate of 67% and an early death rate of 11%. These data are well comparable with the results of other studies in which patients of the same age group were treated with combinations of anthracycline antibiotics and Ara-C in conventional doses [1–5]. There are no indications as yet that the addition of etoposide to DNR and Ara-C might have a positive influence on the outcome of remission induction in this study. Etoposide has been reported to be particularly effective in monocytic or myelomonocytic acute leukemias [22]. However, the number of cases with these subtypes of acute leukemia is too small in this trial to evaluate these cases separately.

The major aim of this study was to investigate whether intensive consolidation therapy with HD Ara-C and DNR yields long-term, unmaintained remissions in patients with AMLs. A pilot study by Wolff [12] demonstrated that this kind of therapy might be feasible and gave a relatively high percentage of long-term remissions although this study has been criticized for patient selection since patients were included in this trial after being in remission for various periods. Therefore we decided to perform a prospective study with a similar drug regimen. Because of the known substantial toxicity of HD Ara-C in elderly patients – especially neurotoxicity – an upper age limit of 50 years was used. Patients with secondary leukemias or a previous history of myelodysplasia or aplastic anemia were also excluded from this study because these patients might have a reduced capacity for bone marrow regeneration.

Because of the relatively short follow-up and median observation time it is not yet possible to answer the main question addressed in this study definitively. If all 89 patients who achieved a complete remission are considered, the median duration of complete remission was 20 months and the prob-

ability of being in complete remission after 42 months was estimated to be 39%. In those patients who received postremission therapy with one or two courses of HD Ara-C/DNR the probability of being in remission after 42 months was calculated to be 51%. These results seem to be superior to those which can be obtained with maintenance or consolidation therapy in standard doses. However, in the interpretation of these data it has to be considered that because of the reasons mentioned above the actuarial rates for CCR can still undergo substantial changes during further observation. Meanwhile a series of other trials have been published which also indicate that HD Ara-C may be superior to conventional postremission therapy although different therapeutic protocols and varying patient selection make the interpretation and comparison of these studies rather difficult [11–17]. In these studies rates of CCR varying from 30% at 3 years to 51% at 6 years have been reported.

The data of this prospective study indicate that patient selection can be hardly avoided in trials which include only patients being in complete remission at the time of consolidation therapy and that this selection might have a substantial influence on the therapy outcome. If only those patients were considered who received postremission therapy with HD Ara-C/DNR, the CCR rate after 42 months was 51% whereas in all patients who achieved complete remission this figure was 39%. The reason for this difference is that not all patients in complete remission could be given late consolidation therapy with HD Ara-C mainly because of medical problems (11 patients) or because of early relapses before late consolidation (6 patients). For a critical assessment of this therapeutic procedure it therefore seems necessary to include all patients in the evaluation who achieved a complete remission after induction therapy, otherwise the clinical efficacy of the postremission therapy with HD Ara-C may be overestimated.

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## Adult AML: The Role of Chemotherapy Intensity and Duration. Two Studies of the AML Cooperative Group\*

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### Introduction

Further improvement of chemotherapy for acute myeloid leukemia (AML) requires an evaluation of alternative treatment strategies as investigated in adequate prospective multicenter trials. Since no superior new drugs are available, treatment can be improved only by finding superior treatment strategies and combining them successfully, if possible. The major treatment strategies to be discussed here are

- long-term cyclic myelosuppressive chemotherapy concepts (maintenance) and
- intensified postremission or preremission chemotherapy concepts (intensification).

The AML Cooperative Group (AMLCG) in its 1981 [1] and 1985 [2] studies investigated the role of these two strategies. In addition, an interstudy evaluation of strategies used in other multicenter studies published between 1981 and 1988 gives answers about the role of maintenance.

### Patients and Methods

In the 1981 AMLCG study [1] adult patients at all ages received for induction one or two courses of TAD9 (Fig. 1) and after achieving complete remission (CR) were randomized to an additional course of TAD9 for consolidation with or without subsequent monthly maintenance by rotating courses MI–MIV (Fig. 1) for a total of 3 years. In the 1985 AMLCG study [2] all patients up to the age of 60 years received two induction courses before CR criteria in blood and bone marrow were achieved (double induction). Before treatment started, patients were randomized to double induction either by a TAD9/TAD9 (9-day course of 6-thioguanine, cytosine arabinoside, and daunorubicin) or by a TAD9/HAM (high-dose cytosine arabinoside/mitoxantrone) sequence [3] (Fig. 1). Uniformly, the second course started on day 21 even if bone marrow was aplastic with no blasts. After achieving CR patients received TAD9 consolidation and MI–MIV maintenance (Fig. 1) as in the 1981 study.

### Results

In the 1981 AMLCG study 501 patients with a median age of 47 years (range 16–78) were treated for induction. CR was achieved

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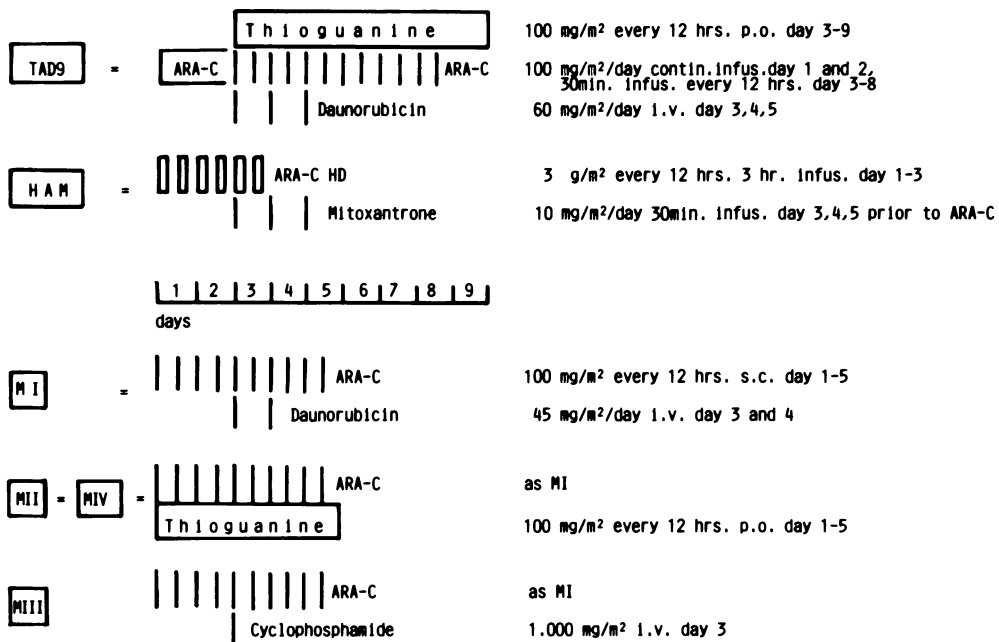


Fig. 1. Chemotherapeutic regimens applied in the 1981 and 1985 AMLCG studies

in 59% of patients; 70% of those achieving CR did so after one course only. Of the 125 patients 60 years of age and older, 41% achieved CR. During the period until randomization in this trial was stopped 161 patients were randomized to maintenance or

nonmaintenance. Remission duration for the two groups is shown in Fig. 2. The median observation time of patients still in remission is 5 years (range, 3-6.5). Median survival in the maintenance arm is 27 months versus 19 months in the nonmaintenance arm ( $p=0.02$ ). Median survival after the first relapse in the two groups has been 7 and 8 months.

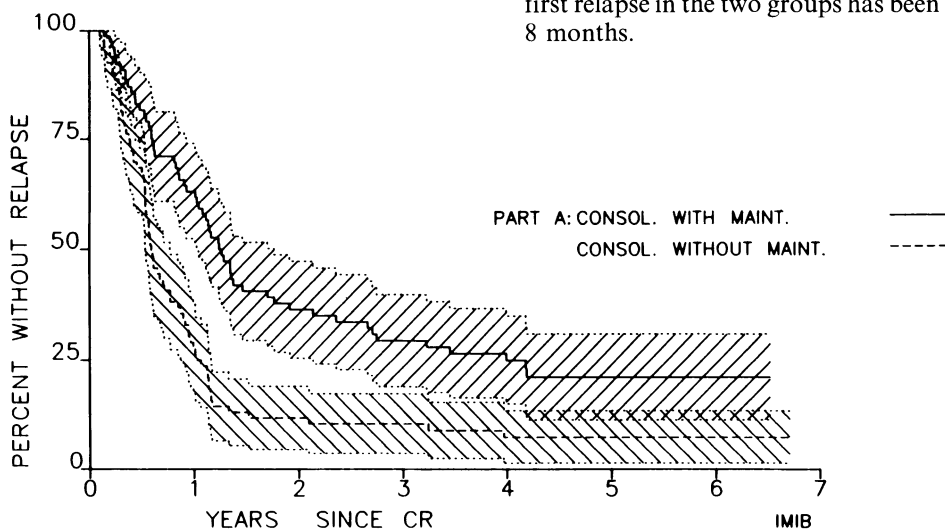
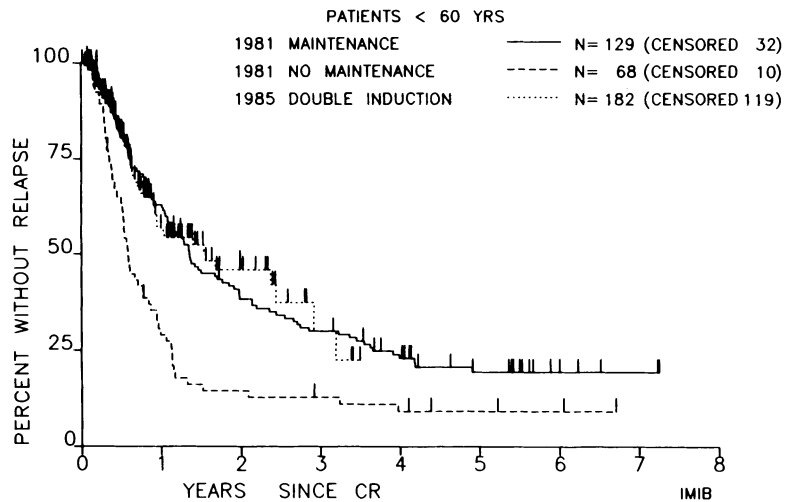


Fig. 2. AMLCG 1981 study: Kaplan-Meier plots of remission duration with 95% confidence intervals for patients randomized to one course of TAD9 consolidation with (79 patients) or without (82 patients) subsequent monthly maintenance for 3 years ( $p=0.0001$ )



**Fig. 3.** AMLCG 1985 study on double induction followed by TAD9 consolidation and monthly maintenance. Comparison of remission duration to that in two control groups, one in the 1981 study with no maintenance and one comprising all patients receiving maintenance in two different 1981 studies. *Tick marks*, censored patients in ongoing CR at last update. Only patients, aged up to 60 years are included

**Table 1.**

	1981 Standard Induction	1985 Double Induction
Patients 16–60 yrs	506	272
CR	64.6%	73.2%
ED	15.8%	9.9%
	2/89	

In the 1985 AMLCG study 272 patients with a median age of 41 years (range 16–60) were randomized and treated by one of the two double induction sequences. Table 1 shows the CR rate and the rate of early death occurring during the first 6 weeks. In addition, the response data are compared to those of the same age group in the 1981 study. Comparing the two randomized groups in the 1985 study the CR rates after TAD9/TAD9 and TAD9/HAM double induction are 69% and 77%, respectively. The median remission duration for the same two groups is 28 and 18 months ( $p=0.4$ ). Figure 3 shows remission duration of the entire group of patients receiving double induction compared to two historical control groups in our 1981 study receiving conventional

induction by mostly one course and in CR monthly maintenance or nonmaintenance. The 1985 study is still ongoing.

### Discussion

The results of the 1981 AMLCG study presented here show an important advantage of the maintenance arm which from the present observation time is significant up to at least 4 years. This is not the effect of an uncommonly poor result in the control arm. Remission duration in the nonmaintenance arm is in the range of several sets of similar or even inferior results in other multicenter studies (Fig. 4). In the maintenance arm remission duration is among the most favorable results, showing a long-term effect far beyond the 3 years of maintenance treatment (Fig. 2). In the maintenance arm total survival also shows a significant advantage. This is clearly an effect of the longer remission duration in this group. On the other hand, identical survival after the first relapse in the maintenance group as in the nonmaintenance group strongly suggests that treatment of relapse after maintenance is as effective as after nonmaintenance. Thus, there is little evidence for an induction of



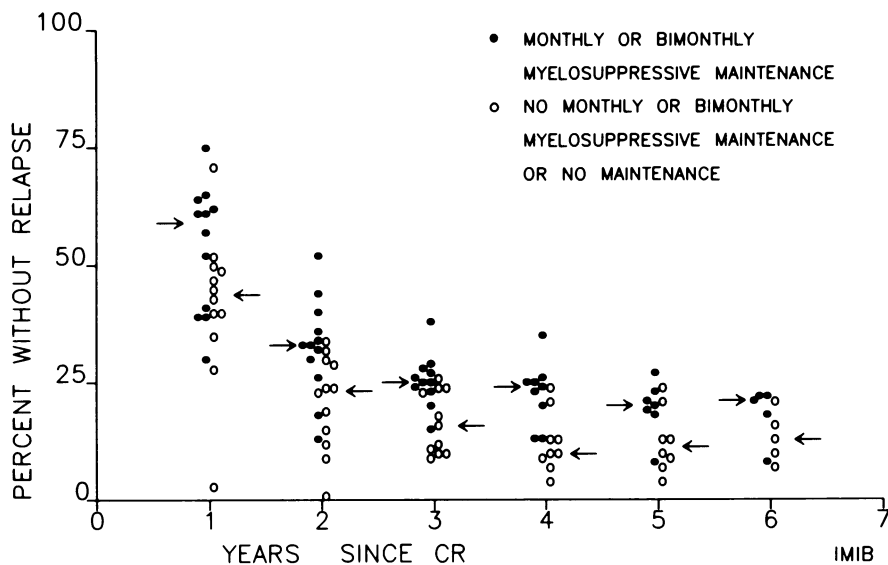


Fig. 4. Probabilities of ongoing remissions in ten multicenter studies, comparing study arms with myelosuppressive maintenance (*closed circles*) and those without comparable maintenance (*open circles*). *Arrows*, medians of probabilities after each year

drug resistance by maintenance chemotherapy.

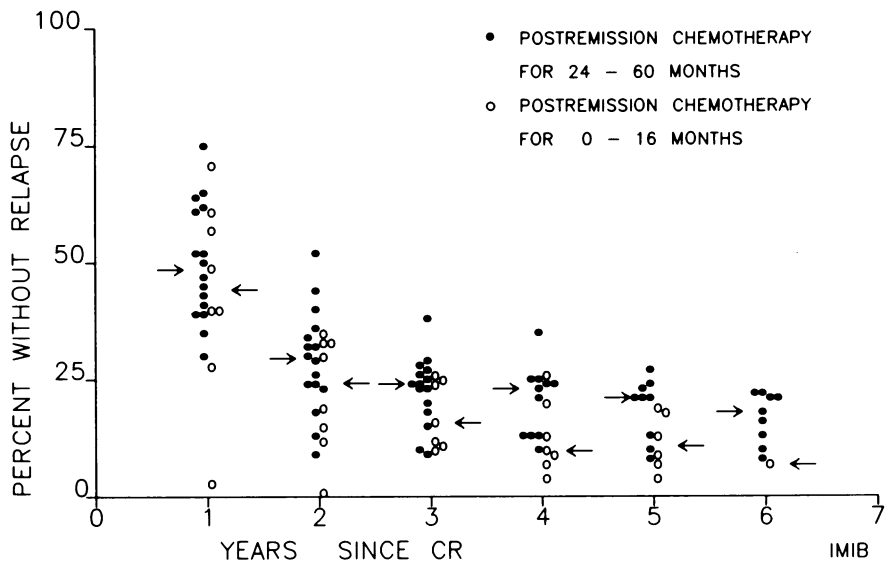
In an analysis of ten multicenter trials [1, 4–12] comprising 24 groups of patients we compared remission duration from maintenance strategies similar to that in our 1981 study to remission duration from strategies without comparable or any maintenance. Figure 4 shows a marked advantage of maintenance strategies, with median long-term remission rates after 4–6 years twice that of those from nonmaintenance strategies. A similar comparison has been made according to strategies with longer or shorter duration of any type of postremission chemotherapy including consolidation courses. Figure 5 shows a marked advantage of strategies using longer postremission chemotherapy, with median long-term remission rates after 4–6 years twice that of those from shorter chemotherapy.

A difference in long-term remission rates of only 10%–15% resulting from a single study may easily be overlooked and is hardly statistically significant due to the small number of patients at risk who are left after 4–5 years. However, as in our 1981 study this difference may account for a doubling of cures within the fraction of patients being

curable by chemotherapy. This interstudy comparison gives additional evidence of a gain in cure rate by long-term and maintenance postremission therapy strategies.

In recent years strategies of an intensification of chemotherapy early in remission have been investigated in several multicenter studies [13–16], most of the regimens including high-dose cytosine arabinoside. The common observation that most relapses occur in the first 1.5 years of CR may have led to the design of early intensification.

The different regimens concentrate all postremission chemotherapy on three or four induction-type consolidation courses of which two to four regimens contain high-dose cytosine arabinoside. The three or four courses are not followed by further chemotherapy except in one study [15] subsequently giving four additional courses of maintenance-type therapy. The four studies show a probability of ongoing remission of 51% after 5 years [13], 40% after 4 years [14], up to 50% after 2 years [15], and 39% after 3 years [16]. A positive selection of patients obviously contributes in part to the results when patients up to 14 months in CR entered intensification [13], and almost no relapse occurred in the first 6 months [14]. Thus, it has



**Fig. 5.** Corresponding comparison of study arms with a longer (*closed circles*) and those with a shorter (*open circles*) postremission chemotherapy of any type (see also Fig. 4)

not been shown so far that postremission intensification improves long-term remission rate over that achieved by maintenance strategies. In addition, it is remarkable that this intensive treatment, producing a treatment mortality of about 6% (1%–11%) failed to prevent the high relapse rate during the first 1.5 years since about 50% (40%–70%) of patients went on to relapse during this period.

An alternative to postremission intensification is represented by a preremission intensification or double induction strategy investigated in the 1985 AMLCG study [2]. All patients up to the age of 60 years received two induction courses before CR criteria were achieved. By randomization a recycling TAD9/TAD9 sequence was compared to an alternating TAD9/HAM version introducing the HAM combination which proved highly effective in relapsed and refractory AML in a phase II study of our group [3]. Besides double induction, treatment in the 1985 study was identical to that in the 1981 study. Importantly, as shown in Table 1, the very early intensification of chemotherapy by double induction is not found to increase the risk of treatment since the early death rate could even be reduced and the CR rate increased by double induc-

tion. Thus, it seems that a standardized double induction regimen facilitates induction treatment for AML.

Remission duration after double induction is compared in Fig. 3 to related historical control groups from our 1981 studies. Another 2–3 years of observation are needed to see whether double induction improves the long-term remission rate. It is obvious, however, that double induction also fails to reduce the high relapse rate in the first 1.5 years.

The question remains to be answered as to whether this phenomenon can be influenced by double induction combined with postremission intensification. Furthermore, the curative potentials of the two approaches could add to that of maintenance therapy. Such a strategy, however, appears limited by toxicity. Most recently recombinant human hematopoietic growth factors open a way to overcoming myelotoxicity as the most limiting toxicity of antileukemic chemotherapy [17].

## Summary

The curative potential of chemotherapy for AML may not be limited to a few months

around remission induction. Thus, in a randomized study of the AMLCG long-term monthly maintenance clearly improved the long-term remission rate. This effect of maintenance strategies is confirmed in an interstudy comparison of 10 multicenter trials. More recent post remission intensification strategies – as from preliminary trends – may produce cure rates similar to those from maintenance regimens. Our group introduced a preremission intensification by the double induction strategy. A successful combined double induction – post remission intensification – maintenance strategy mainly limited by myelotoxicity may become possible by the use of recombinant human hematopoietic growth factors.

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## Comparison of Postremission Therapies in Adult Acute Myeloid Leukemia: Preliminary Analysis of an ECOG Study\*

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### Introduction

Induction therapy regimens for adult acute myeloid leukemia (AML) consisting of daunorubicin and cytarabine with or without thioguanine produce complete remission in approximately two-thirds of patients [1–3]. The potential for cure and/or long-term survival correlates directly with the duration of initial remission. A variety of approaches have been employed to maintain initial remission, including long-term outpatient chemotherapy (maintenance therapy) [4], courses of intensive chemotherapy (consolidation therapy) [5], combined consolidation and maintenance therapies [1–3, 6], and allogeneic bone marrow transplantation [7]. Because it is unclear which one of these approaches is best, the Eastern Coop-

erative Oncology Group (ECOG) in 1984 began a randomized trial (EST 3483) of postremission therapies in AML. The initial study design involved randomization of patients in complete remission to receive no postremission therapy, prolonged maintenance therapy, or short-term high-dose consolidation therapy. Patients in complete remission eligible for allogeneic bone marrow transplantation were offered the option of allogeneic bone marrow transplantation instead of being randomized. Interim monitoring of the trial revealed substantially inferior results in the group of patients receiving no postremission therapy. The initial 26 patients randomized to this treatment arm all relapsed with a median duration of remission of 4.1 months. This led to early closure of the nontreatment arm. The study's accrual goals were reached in January 1988 and the study was terminated. The data presented here are an interim analysis of the outcome for patients receiving maintenance therapy vs. consolidation therapy vs. allogeneic bone marrow transplantation based on information available as of November 1988.

### Patients and Methods

All ECOG member institutions participated in this study. Adult patients, 15–65 years old with de novo AML, were eligible for this study providing they met the criteria for the diagnosis of AML by French-American-British (FAB) typing and had no prior hematologic disorders, no significant cardiac

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disease, adequate hepatic and renal function, and documentation of informed consent.

Induction therapy consisted of one or two courses of daunorubicin, 60 mg/m<sup>2</sup> per day by i.v. push on days 1, 2, and 3; cytarabine, 25 mg/m<sup>2</sup> i.v. push followed by continuous i.v. infusion of 200 mg/m<sup>2</sup> per day on days 1 through 5; and 6-thioguanine, 100 mg/m<sup>2</sup> orally every 12 h on days 1 through 5.

At the time of complete remission patients <41 years old with a histocompatible sibling were referred for allogeneic bone marrow transplantation; all remaining patients were randomized to maintenance therapy or consolidation therapy. Maintenance therapy consisted of 6-thioguanine, 40 mg/m<sup>2</sup> orally every 12 h for 4 days of the week, followed by cytarabine, 60 mg/m<sup>2</sup> s.c. on the 5th day of each week. Maintenance therapy was continued for 2 years. Consolidation therapy consisted of a single course of high-dose cytarabine, 3 g/m<sup>2</sup> i.v. every 12 h for 12 doses (6 days) followed by amsacrine, 100 mg/m<sup>2</sup> i.v., per day on days 7, 8, and 9. After completing this course of consolidation therapy, no further therapy was administered.

Postremission therapy with either maintenance therapy or consolidation therapy began 4 weeks after complete remission if patients had a performance status of 0 or 1, nor persisting infection, and adequate renal and hepatic function. The data described below are based on the 439 patients who were eligible for the study and who have adequate and analyzed data. The median duration of fol-

low-up for this study as of November 1988 was 1.5 years. The median age of patients entered on study was 44 years, with a range of 15–65 years.

## Results

### Induction Therapy

The overall complete remission rate was 67% (295/439). Of the patients obtaining complete remission, 61% required only one induction therapy course and 39% required two induction courses. The complete remission rate was age related; the remission rate for patients ≥60 years old was 54% (37/69) and for patients less than 60 years old it was 70% (258/370).

### Postremission Therapy

Overall, 10% of patients in complete remission refused to be randomized. Of patients randomized to maintenance therapy, 6% declined this treatment, leaving 85 analyzable patients; and of those randomized to consolidation therapy, 12% refused, leaving 82 analyzable patients. Of 53 patients in complete remission who were <41 years old and who had histocompatible siblings, 10% declined assignment to allogeneic bone marrow transplantation, leaving 48 analyzable patients. There were no significant differences among the three groups with regard to a variety of demographic factors present at

**Table 1.** Event-free survival

	Median (months)	2 year EFS	% alive in CCR
All patients			
maintenance (n = 85)	9	22% (±10%)	40%
consolidation (n = 82)	9	33% (±11%)	47%
Patients < 41 years old			
maintenance (n = 22)	7	14% (±16%)	18%
consolidation (n = 28)	13	38% (±18%)	43%
allogeneic BMT (n = 48)	17	40% (±15%)	48%

n, number of patients; (), ±2 SE; EFS, event-free survival; BMT, bone marrow transplantation; % alive in CCR, percent age of patients alive and in continuous complete remission at the time of this analysis

the initiation of induction therapy. The outcome of postremission therapy in terms of event-free survival (EFS, where either relapse or death is counted as an event) is shown in Table 1. Considering all patients randomized to maintenance therapy or consolidation therapy, there is no difference in the median duration of EFS. The 2-year EFS (33% vs. 22%) and percent age of patients currently alive and in continuous complete remission (47% vs. 40%) favors the consolidation therapy arm, but is not statistically significant. The results for patients of comparable age (<41 years old) who were randomized to maintenance therapy or consolidation therapy or who were assigned to allogeneic bone marrow transplantation is shown in the bottom half of Table 1. The outcomes for consolidation therapy ( $P=0.09$ ) and allogeneic bone marrow transplantation ( $P=0.02$ ) appeared to be superior to the results obtained from maintenance therapy. Thus far, there are no significant differences in outcome between allogeneic bone marrow transplantation and consolidation therapy ( $P=0.5$ ). The difference in the results for patients <41 years old compared with those for the entire group of patients receiving consolidation therapy is related to age-related differences in mortality from the toxicity of consolidation therapy.

### *Toxicity*

There was no fatality from maintenance therapy. Toxicity from consolidation therapy included the known side effects of high-dose cytarabine administration, including: conjunctivitis in 19%; skin rash in 35%; diarrhea in 59%; and cerebellar dysfunction in 17% of patients. The combination of amsacrine and high-dose cytarabine resulted in fungal infections in 30% of patients. Fungal infection was the principal cause of fatality occurring in 17% of patients receiving consolidation therapy. The risk of a fatal outcome from consolidation therapy was directly related to the age of the patients as reflected in a mortality rate of 60% (11/17) in patients  $\geq 60$  years vs. 7% in patients <41 years old (2/28).

### **Discussion**

The interim analysis of accruing data from this ECOG study suggests that one course of consolidation therapy with high-dose cytarabine plus amsacrine provides long-term, disease-free survival that is at least as good as, and may be better than, lengthy maintenance therapy. The lethal toxicity of high-dose cytarabine plus amsacrine consolidation therapy probably reduced its efficacy, especially in older patients. In patients of comparable age, that is <41 years old, both allogeneic bone marrow transplantation and consolidation therapy appeared to be superior to maintenance therapy. Thus far, the results of allogeneic bone marrow transplantation do not differ from the outcome from a single course of consolidation therapy. The validity of these statements needs to be confirmed by longer follow-up of the data from this study. Nevertheless, some tentative conclusions can be drawn. The results of this study support the view that escalating the intensity of postremission therapy is of value for patients with AML in initial complete remission. The use of further escalation of postremission dose-intensity by means of autologous bone marrow transplantation [8] is currently under study by ECOG and other cooperative groups and institutions. Whether autologous bone marrow transplantation will prove to be superior to less-intensive consolidation therapy such as was employed in the current study and whether the results will rival those of allogeneic bone marrow transplantation remains to be determined. For older patients, >60 years old, alternative, less toxic, consolidation therapy requires exploration.

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### **Appendix A**

Other participating institutions include: Albany Medical College, NY (CA 06594); Albert Einstein College of Medicine, Bronx, NY (CA 14958); American Oncologic Hospital, Philadelphia (CA 18281); Brown Uni-

versity and Roger Williams General Hospital Providence, RI (CA 15947); University of California, California College of Medicine, Orange; Charleston Area Medical Center, WV; Colorado Cancer Research Program, Denver; Hahnemann Medical College, Philadelphia (CA 13611); Medical College of Ohio, Toledo; New York University Medical Center, New York (CA 16395); Newark Beth Israel Medical Center, Newark NJ; Northwestern University Medical Center, Chicago (CA 17145); University of Pittsburgh (CA 18653); Roswell Park Memorial Institute, Buffalo (CA 12296); Rush-Presbyterian-St. Luke's Medical Center, Chicago (CA 25988); SUNY-Downstate Medical Center, Brooklyn, NY; Tufts University, Walpole, MA (CA 07190); Natalie Warren Bryant Cancer Center, Tulsa, OK; Vermont Regional Cancer Center, Burlington; West Virginia University, Morgantown.

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## EORTC Leukemia Group Trials on Acute Myeloid Leukemias: An Overview

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Originally designated as the Leukemia and Hematosarcoma Group, the EORTC Leukemia Group is one of the oldest of co-operative groups. It was created by Prof. G. Mathe in 1964 and been chaired since then by: G. Marchal (France), J. Bousser (France), W. F. Stenfert-Kroese (Netherlands), J. S. Malpas (United Kingdom), J. Bernard (France), H. O. Klein (Federal Republic of Germany), J. Revol (France), C. Hannen (Netherlands), P. Strychmans (Belgium), and R. Zittoun (France). This group has focused its efforts on the treatment of acute myeloid leukemia (AML). The objective of the present chapter is to present an overview of the trials hitherto conducted and the policy adopted for the ongoing trials on AML.

### AML Trials Conducted and Analyses of These Trials

Trial 1 lasted 3 years [5]. For the induction there was a three-arm comparison by randomization. The first schedule included 55 patients treated by with cytosine arabinoside (ara-C) 100 mg/m<sup>2</sup> per day for 4 days and by intravenous push. In the second schedule 53 patients were treated with ara-C plus thioguanine 100 mg/m<sup>2</sup> per day for 5 days. In the third arm were 55 patients treated with ara-C plus daunorubicin on day 1 at

a dose of 60 mg/m<sup>2</sup>. The incidence of complete remission (CR) was 16%, 15%, and 21%, respectively (no statistical difference was observed).

In trial 2 the same three-arm schedule was used but with two modifications:

- ara-C was given every 12 h for 8 days at a dose of 100 mg/m<sup>2</sup> and
- thioguanine was given every 12 h for 8 days at a dose of 80 mg/m<sup>2</sup>. The incidence of CR was 25%, 30%, and 31%, respectively [6]. It was also useful in this trial to evaluate the influence of supportive care in AML. CR was significantly higher in the centers with hematological intensive care units (45.5%; *n* = 33) than in centers without these facilities (25.5%, *n* = 146).

In these two trials the maintenance treatment was compared in two arms by randomization: mitoguanone (methyl-GAG): 200 mg/m<sup>2</sup> weekly for 2 years and ara-C 120 mg/m<sup>2</sup> subcutaneously weekly for 2 years. Again, no statistical differences were observed between the two arms. In trial 1 no patient had a long CR, and none of the patients survived, while in trial 2 ten patients survived with a CR of more than 3 years [1].

In trial 5 (Fig. 1) the induction treatment was the same for all patients; this consisted of doxorubicin 50 mg/m<sup>2</sup> on day 1, vincristine 1 mg/m<sup>2</sup> on day 2, and ara-C 80 mg/m<sup>2</sup> every 12 h by push injection on days 3–9 [3–4].

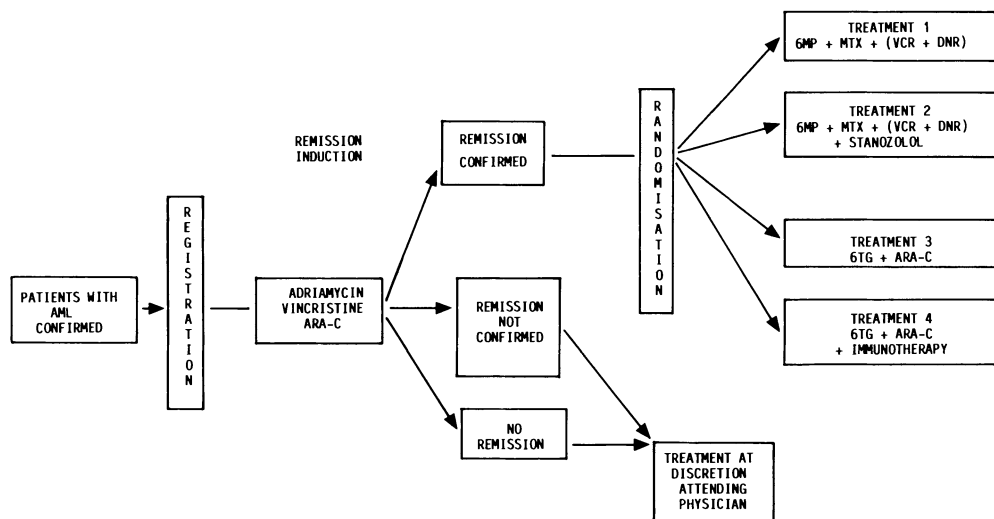
Patients in CR were randomized to four groups:

- 6-mercaptopurine 70 mg/m<sup>2</sup> on days 1–14, methotrexate 15 mg/m<sup>2</sup> twice weekly

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\* On behalf of the EORTC leukemia group





**Fig. 1.** Design of trial 5. *6MP*, Mercaptopurine; *MTX*, methotrexate; *VCR*, vincristine; *DNR*, daunorubicin; *6TG*, 6-thioguanine; *ARA-C*, cytosine arabinoside

- on days 15–28, and reinduction with daunorubicin 35 mg/m<sup>2</sup> and vincristine 1 mg/m<sup>2</sup> on day 29;
- chemotherapy as in the former group plus stanzolol 0.15 mg/kg per day continuously;
  - 6-thioguanine 70 mg/m<sup>2</sup> orally for 4 consecutive days and ara-C 80 mg/m<sup>2</sup> subcutaneously on day 5 every week; and
  - chemotherapy as in the former group plus irradiated blast cells treated with neuraminidase.

A total of 295 patients were evaluable; CR was achieved in 64%. From these, 158 patients were randomized to the four maintenance arms. No difference in disease-free survival or survival was observed among the four arms of maintenance treatment (Fig. 2). The disease-free survival for patients reaching CR was 40 weeks. The overall survival was 15% at 4 years, but it appears that the group receiving bone marrow transplantation (BMT;  $n=19$ ), although not randomized (but included in the trial for induction of CR) fared best of all, particularly as regards patients 40 years of age or under with 45% long-term survival. In contrast, if all 133 evaluable patients aged 40 years or under are considered, the 4-year survival was about 20%; CR among these patients was achieved by 30%. In terms of relapse,

factors influencing the rate of induction of another CR was age under 40 years ( $p=0.024$ ), presence of Auer rods ( $\geq 2.5\%$ ;  $p=0.002$ ), and treatment by immunotherapy during maintenance ( $p=0.09$ ) [7, 8].

Trial 6 (515 patients) will be described later in this book. The conclusions are:

- the incidence of CR (67.4%) is the highest observed of all our trials, and
- there was no difference in survival or disease-free survival between the two arms with maintenance chemotherapy using the same induction drug or an alternating schedule of non-cross-resistant drugs (Fig. 3).

The event-free survival curve for BMT patients is relatively close to that for the same age groups treated by chemotherapy. This is a major rationale for the design of trial 8, which is ongoing. In the trial 6 the two significant prognostic factors for disease-free survival are age (above or below 50 years;  $p<0.005$ ) and whether CR was achieved after one or two induction courses ( $p<0.04$ ) [9].

The objective of trial 7 in elderly patients (65 years of age or older) was to compare immediate intensive induction chemotherapy [2] (arm A) versus “wait and see” and supportive care (arm B). This study shows that the latter approach yielded extremely

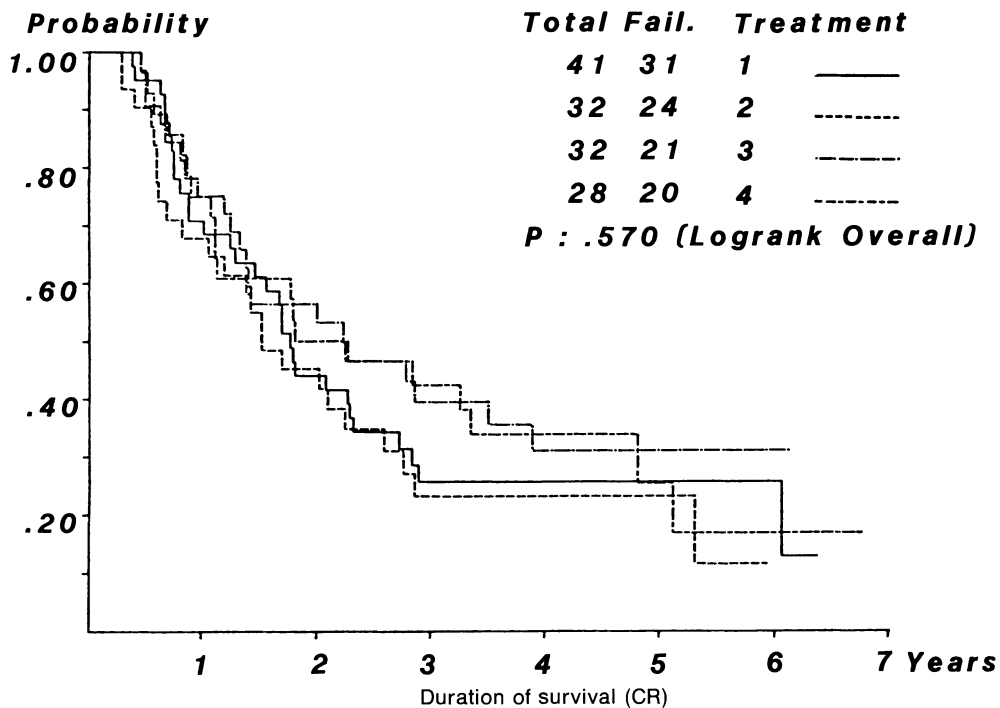


Fig. 2. Survival in the four randomized groups of trial 5. For explanation of treatment schedules see Fig. 1

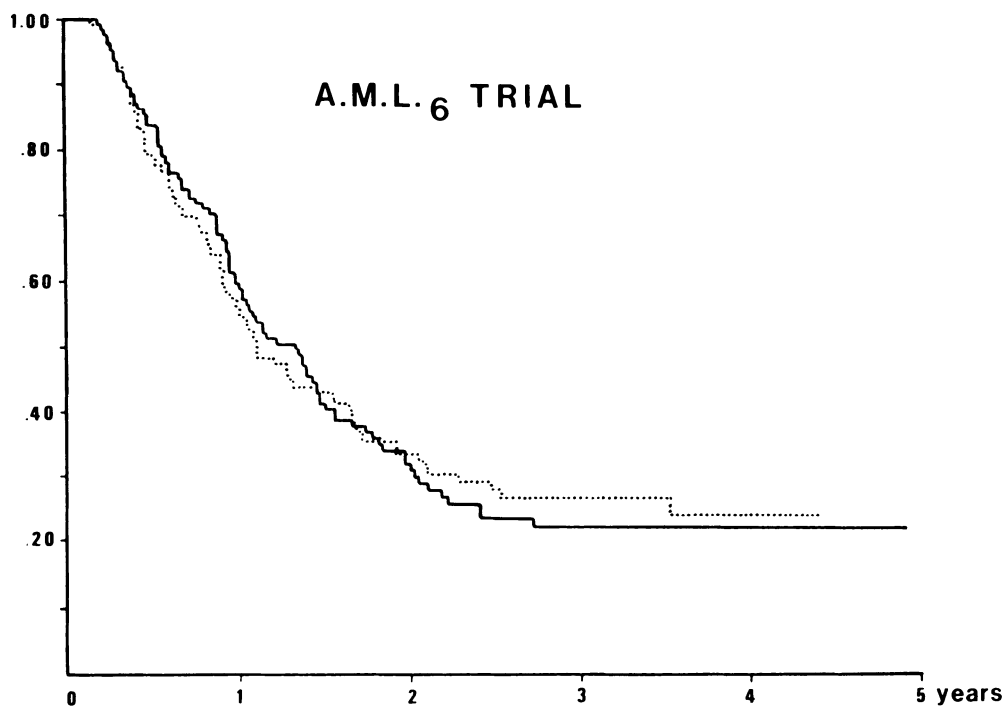
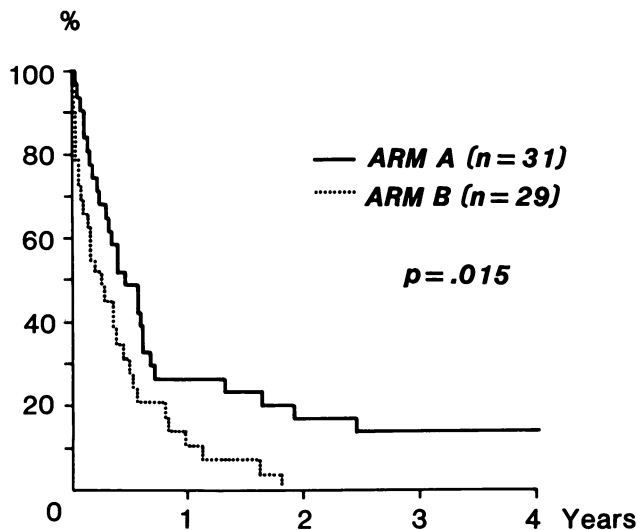


Fig. 3. Survival in the two randomized groups of trial 6. *Dotted line*, patients treated with same drug as used for induction; *solid line*, alternating regimen



**Fig. 4.** Survival in the two randomized groups of trial 7. *Arm A*, immediate intensive induction chemotherapy (cytosine arabinoside, vincristine, daunorubicin); *arm B*, “wait and see” and supportive care

poor results; it failed to avoid hospitalization and also failed to prolong survival (Fig. 4).

A general consensus now exists regarding the results of these trials (Tables 1, 2):

- a) For induction of CR, the two most active drugs as first-line therapy are antracycline and ara-C;
- b) During the past 20 years, it is a better use of the drugs which has been responsible for an improvement in CR. The incidence has increased from 17.5% in trial 1 to 70% in the last trials. In the latter, daunorubicin was given daily for 3 days at 50 mg/m<sup>2</sup> plus ara-C given daily for 7 days at 200 mg/m<sup>2</sup> by continuous infusion.
- c) The main prognostic indicators remain age, number of leukocytes, and obtaining

**Table 2.** Leukemia group EORTC: overview of the results in AML trials

	Median survival	Proportion surviving (4 years)
Trial 1 (1969–1971)	5 months	0%
Trial 2 (1972–1974)	5 months	< 5%
Trial 5 (1976–1982)	12 months	15%
Trial 6 (1983–1986)	12 months	23%
Trial 7 in elderly patients	3–5 months	0%–13.5% (2 years)

CR after the first course of chemotherapy.

- d) So far, BMT has not been evaluated in a controlled trial in our group. This is the aim of the present trial (trial 8).

**Table 1.** Leukemia group EORTC: overview of the results in AML trials

	Number of evaluable patients	Rate of CR
Trial 1 (1969–1971)	163	17.5%
Trial 2 (1972–1974)	179	29.0%
Trial 5 (1976–1982)	348	63.7%
Trial 6 (1983–1986)	515	67.4%
Trial 7 in elderly patients	71	58.0%
Total	1276	

### Ongoing Trials

Trial 8A addresses the question concerning patients aged under 45 years. Three groups of patients in CR are compared; the first group of patients undergo allogeneic BMT, and those in the other two groups have been randomized to receive autologous BMT or intensive consolidation. While it is still far too early for results, it is nonetheless important to note that only 41% of patients in CR initially enrolled were able to be random-

ized. As presented in Figs. 5 and 6, initially 228 patients were registered. Of these, 137 reached CR, and the final number of patients in the three arms are 30 for allogeneic BMT, 17 for the autologous BMT, and 20 for intensive consolidation. The reasons for substantial number of exclusions and refusals are listed in Table 3.

Trial 8 B included patients between 45 and 60 years of age. Patients in CR are randomized to receive an intensive consolidation or a standard consolidation maintenance.

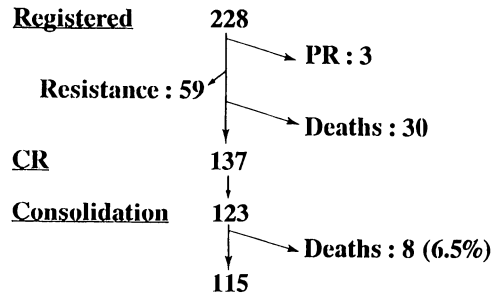


Fig. 5. Patients randomized in trial 8

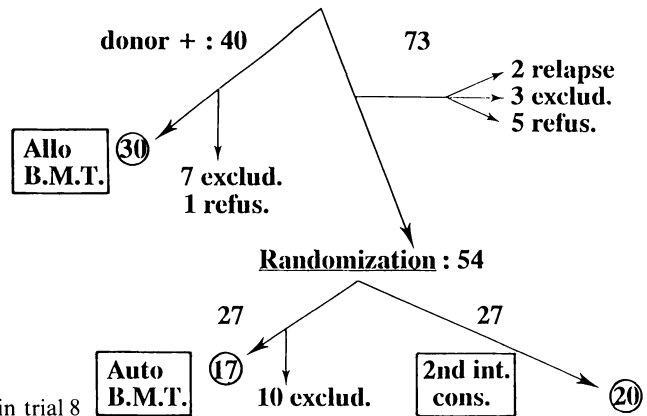


Fig. 6. Patients registered and CR in trial 8

Table 3. AML trial 8A: Reasons for exclusions and refusals (September 1988)

	<i>n</i>	Reasons
<b>Exclusions</b>		
First consolidation	2	Aspergillosis, hepatitis
Randomization	6	Candidosis, pneumonitis, slow recovery, CR with abnormal cytogen, cerebellar toxicity, abnormal bone marrow growth
Allogeneic BMT	5	Cerebellar abscess, poor condition, meningitis, pregnant donor, hepatitis (2)
Autologous BMT	6	Lung problem, hepatitis, poor harvest (2) and hepatitis, delay for hepatitis → relapse, thrombopenia, abnormal in vitro growth
Second consolidation	3	Hepatitis (3)
<b>Refusals</b>		
First consolidation	2	Foreigner, unclear
Randomization	10	Sterility (2), foreigner, unclear (4), professional, fear of second consolidation, fear of ABMT
Allogeneic BMT	2	Brother died following BMT, not yet fully convinced
Autologous BMT	0	
Second consolidation	2	Chose Autologous BMT (2)

For elderly patients (over 60 years of age), trial 9 is a controlled phase III study on the value of intensive remission induction chemotherapy followed either by maintenance chemotherapy with low-dose ara-C or by no maintenance treatment.

## Phase II Trials

The phase II trials in relapsing or refractory AML include the following:

- a) high-dose ara-C plus amsacrine in relapsing or refractory AML, a trial now completed with 57% CR in 79 patients; and
- b) tumor necrosis factors in refractory AML, an ongoing trial.

## Conclusions

The most important points in this presentation are the following:

- a) the incidence of CR in AML treated by a cooperative group such as the EORTC Leukemia group, which is somewhat heterogeneous, is at its maximum, i.e., approximately 70%;
- b) the challenge is to transform a CR into a cure; and
- c) the present trial (trial 8) will answer the question; intensive chemotherapy or BMT?

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## A Randomized Comparison of Intensive Maintenance Treatment for Adult Acute Myelogenous Leukemia Using Either Cyclic Alternating Drugs or Repeated Courses of the Induction-Type Chemotherapy: AML-6 Trial of the EORTC Leukemia Cooperative Group\*

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### Introduction

Standard induction treatment of patients with de novo acute myelogenous leukemia (AML) results in a complete remission (CR) rate of 60%–80%. However, remission duration is usually short with a relapse rate of about 70% within the first 2 years independent of various consolidation and maintenance modalities. The rate of long-term remitters at 4–5 years is no higher than 20%–30%. The role of maintenance treatment is controversial. Generally, repeated short courses of intravenous or subcutaneous cytarabine with thioguanine or daunorubicin are given monthly for 2–3 years. Numerous controlled and uncontrolled trials with few exceptions have failed to demonstrate a substantial benefit in patients receiving maintenance therapy compared with those receiving induction and consolidation alone [1–11]. Two other studies [12, 13] reported superior results with maintenance therapy, but this was probably due to unexpectedly poor results among controls [14].

Factors potentially responsible for relapse are inadequate reduction of leukemic cells during induction treatment or the development of a resistant leukemic subpopulation surviving the first drug combination. One study [15] covering the first aspect of both

possibilities claimed that intensification of maintenance treatment improves survival.

The objectives of our trial therefore were:

1. Intensive short-term (10 months) maintenance treatment in regard to DFS and survival
2. Induction type (continued) versus non-cross-resistant drugs (alternate)
3. Value of bone marrow transplantation (BMT)
4. Toxicity and quality of life in both intensive maintenance arms
5. Whether patients who relapse after CR respond to the same or alternate drugs combined in reinduction regimens

### Patients and Methods

Between January 1983 and October 1986, 578 patients from 25 institutions were registered. All patients with newly diagnosed untreated AML from 11 to 65 years of age were eligible. Patients already treated or patients with blast crisis of chronic myelocytic leukemia (CML), acute leukemia following myelodysplastic syndromes or myeloproliferative diseases, secondary leukemias, and severe concomitant diseases including cancer were excluded.

Induction chemotherapy consisted of daunorubicin (DNR) 45 mg/m<sup>2</sup> i.v. on day 1, 2, and 3; vincristine (VCR), 1 mg/m<sup>2</sup> i.v. on day 2; and Ara-C 200 mg/m<sup>2</sup> on days 1–7, half by continuous intravenous infusion and half by i.v. push injection every

EORTC Leukemia Cooperative Group, Brussels/Paris

\* Dedicated to Prof. Dr. W. Wilmanns on the occasion of this 60th birthday

12 h on days 1–7. Blood and bone marrow (BM) were checked 3 weeks following the end of the first course, and patients who did not achieve a CR received a second identical induction course.

Patients entering CR received one consolidation course identical to induction, except that DNR was administered only on day 1. They were randomized 3 weeks following the end of consolidation, providing that CR was confirmed in blood and BM. Most patients with a human leukocyte antigen (HLA)-identical sibling were taken out of the study and treated with an allogeneic BM transplantation (BMT). During the terminal phase of the trial, 17 patients were also taken out of the study in several centers for pilot studies with autologous BMT.

The two randomized arms were as follows:

1. Arm 1 (“continued” treatment: DNR, 45 mg/m<sup>2</sup>, and VCR, 1 mg/m<sup>2</sup>, i.v. day 1, cytosine arabinoside (Ara-C), 100 mg/m<sup>2</sup> s.c. every 12 h days 1–5.
2. Arm 2 (“alternating” treatment): acridinylamine methansulfon-m-anisidide (AMSA), 150 mg/m<sup>2</sup> i.v. day 1, combined with either high-dose Ara-C 3 g/m<sup>2</sup> in i.v. infusion over 1 h every 12 h days 1, 2 (four infusions), or 5-azacytidine 150 mg/m<sup>2</sup> i.v. days 1–3. 5-Azacytidine was kindly supplied by the National Cancer Institute through an NCI-EORTC agreement. AMSA was associated with high-dose (HD) Ara-C in courses 1, 3, and 5, and with 5-azacytidine in courses 2, 4, and 6.

In the two randomized arms, the maintenance treatment consisted of six courses administered at 6-week intervals. There was no further treatment, except in the case of relapse. Blood counts were performed at least every week during treatment. Bone marrow aspirate was performed before each course during treatment, then every 3 months during the two consecutive years.

## Results

Of 578 registered patients, 29 were ineligible and 22 inevaluable. Furthermore, institutions with a inevaluability rate of >30% were excluded. Five hundred and fifteen pa-

**Table 1.** Patient characteristics

		Patients	% patients
Age (years)	≤20	37	7.2
	21–30	75	14.6
	31–40	91	17.7
	41–59	234	45.5
	60–66	77	15.0
Performance status	0	83	16.4
	1	217	42.8
	2	150	29.6
	3	43	8.5
	4	14	2.8
Fever (>38.0°C)	Absent	293	57.5
	Present	217	42.5
WBC	≤ 5000	153	29.8
	≤ 25000	160	31.1
	≤ 100000	132	25.7
	> 100000	69	13.4
FAB subtype	M1	128	19.2
	M2	165	33.7
	M3	42	8.6
	M4	70	14.3
	M5	74	15.1
	M6	11	2.2
Auer rods	Absent	307	64.2
	Present	171	35.8
Cytogenetic abnormality	Absent	135	26.2
	Present	103	20.0
	Not done	277	53.8
Total		515	100.0

Age: median, 47 years; range, 11–66 years

tients were evaluable for final analysis. Patient characteristics are shown in Table 1. Complete remission was achieved by 67.4% of patients, 74% of them after the first, and 26% after the second cycle of induction; 3.7% achieved only a partial remission, 15% proved to be resistant, 2.7% died during the first induction cycle, and 11.3% died during hypoplasia after one or two cycles of induction or consolidation.

Highly significant prognostic factors for achieving CR or death rate were age, performance status, fever before start of treatment, duration of hematological symptoms, WBC and platelet counts, absence or presence of cytogenetic abnormalities and Auer

rods, and blood urea nitrogen (BUN) level before treatment. The French-American-British (FAB) subtypes were of no importance (Table 2).

Two hundred and thirty-three patients were randomized for either maintenance arm and no BMT had been planned or performed. Sixty patients were transplanted in first remission, 17 received an autologous and 43 an allogeneic graft. The median age

of the BMT group was 30 years, and median time from CR to BMT 15 weeks (4 months). An additional 12 patients were prepared for BMT but either relapsed or died shortly before BMT. Forty-two patients were not randomized after consolidation because of toxicity (18), relapse or death (10), treatment refusal (6), protocol violation (5), loss to follow-up (2), or death (unknown cause) (1). There was no difference in DFS between the

**Table 2.** Prognostic factor analysis for CR and death rate

		Patients	% CR	% Resistance	% Death	<i>P</i> value
Age (years)	≤20	37	78.4	18.9	2.7	<0.0001
	21–30	75	77.3	14.7	8.0	
	31–40	91	72.5	16.5	11.0	
	41–59	234	65.0	20.5	14.5	
	60–65	77	53.2	19.5	27.3	
Performance status	0	83	83.1	13.3	3.6	<0.0001
	1	217	71.9	18.9	9.2	
	2	150	58.0	25.3	16.7	
	3	43	55.8	9.3	34.9	
	4	14	28.6	14.3	57.1	
Fever (>38.0°C)	Absent	293	72.0	20.5	7.5	0.0005
	Present	217	61.3	16.1	22.6	
Duration of hematological symptoms	≤1 month	223	71.7	17.0	11.2	0.01
	≤2 months	133	69.9	17.3	12.8	
	>2 months	104	57.7	22.1	20.2	
WBC (×10 <sup>9</sup> /liter)	≤5	153	66.7	14.4	19.0	0.27 (0.0075)
	≤25	160	70.6	20.6	8.8	
	≤100	132	69.7	21.2	9.1	
	>100	69	56.5	18.8	24.6	
Platelets (×10 <sup>9</sup> /liter)	≤100	376	65.7	17.3	17.0	0.03
	>100	137	72.3	21.9	5.8	
FAB subtype	M1	128	66.4	21.1	12.5	(0.16)
	M2	165	65.5	21.8	12.7	
	M3	42	64.3	14.3	21.4	
	M4	70	77.1	11.4	11.4	
	M5	74	66.2	17.6	16.2	
	M6	11	45.5	27.3	27.3	
Cytogenetic abnormality	Absent	135	73.3	15.5	11.1	0.0005
	Present	103	51.5	31.1	17.5	
Auer rods	Absent	307	63.5	19.5	16.9	0.004
	Present	171	72.5	17.5	10.0	
BUN	≤1.25 <i>N</i>	460	67.8	19.6	12.6	0.07 (0.009)
	>1.25 <i>N</i>	50	60.0	12.0	28.0	
Total		515	67.4	18.7	14.0	

*P* value given by Kendall's tau test (chi-square test)

*N*, Upper limit of normal value



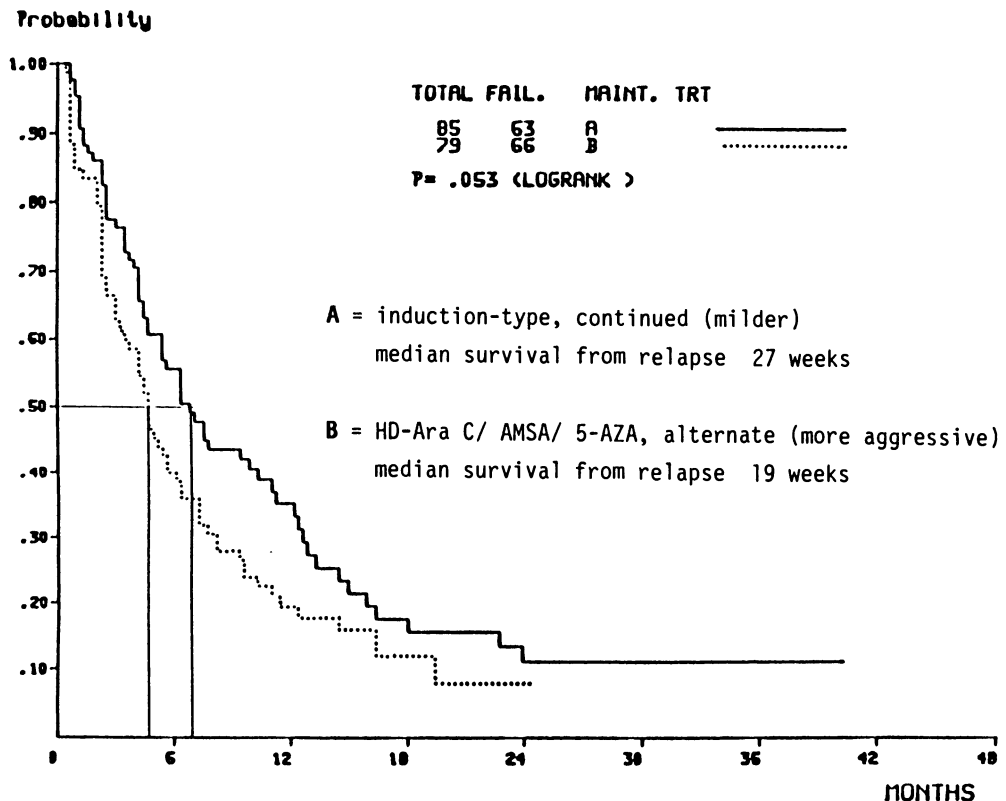
two intensive maintenance strategies. The median was 13 months, with 23% in CCR at 5 years. Also, there was no difference in survival in those patients reaching CR. The median was 26 months, with 32% alive at 5 years. The only prognostic factor of significance for survival was age. Nevertheless hematological toxicity of the HD Ara-C containing "alternating" maintenance arm was much more pronounced and, accordingly, the incidence of septicemia twice as high as compared with the milder induction-type arm (19.1% vs. 9.1%); also, the incidence of hemorrhage: 23.5% vs. 18.2% and, most importantly – as parameter of quality of life and hospital costs – the median time spent in the hospital: 2.5 weeks vs. 1 week. Figure 1 shows that the duration of survival from relapse was shorter with borderline significance ( $P=0.053$ ) in the more aggressive "alternating" arm with a median of 19 weeks as compared with the milder "induction-type" arm with a median of 27 weeks. Interestingly (Table 3), the incidence of achieving a sec-

**Table 3.** Results (% CR) of treatment reinduction of the first relapse according to the maintenance arm and occurrence of relapse

	Arm A	Arm B
DNR + Ara-C	6/ 8 (75%)	9/15 (60%)
HD Ara-C + AMSA	17/27 (63%)	12/17 (70%)
Other treatment	9/23 (39%)	6/15 (40%)
Relapse during maintenance	9/21 (43%)	7/19 (37%)
Relapse after completion of maintenance	23/37 (62%)	20/28 (71%)
Total	32/58 (55%)	27/47 (57%)

ond remission after relapse is independent of the type of pretreatment during maintenance and independent of the type of reinduction program used.

Figure 2 shows in the upper curve the survival probability of the 60 transplanted patients, and in the middle curve their DFS.



**Fig. 1.** Duration of survival from relapse according to pretreatment during maintenance

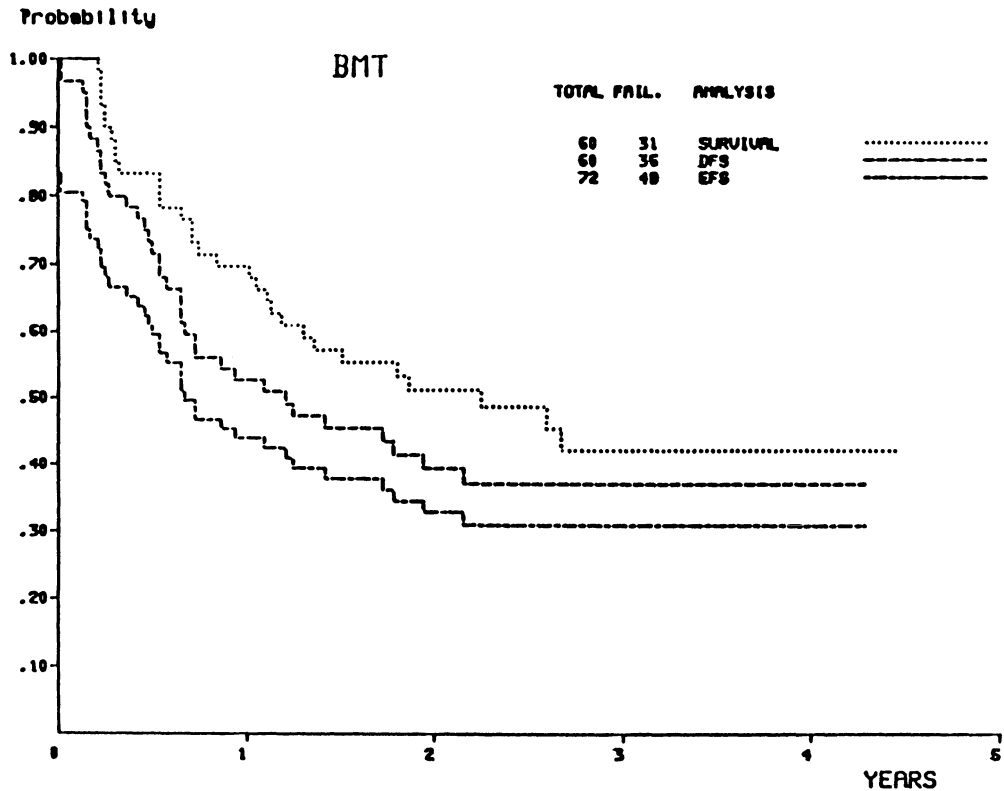


Fig. 2. Survival, disease-free survival, and event-free survival of patients who underwent allogeneic BMT

Taking the 12 patients into account, who were prepared for BMT, but relapsed or died shortly before performing BMT, and calculating the event-free survival of these 72 patients, the lower curve is close to those observed in the two maintenance arms.

Comparing DFS of all patients treated with chemotherapy – but age adjusted – up to the age of 49 years with those receiving BMT, a slight trend in favor of the BMT group is observed but with no significant difference (Fig. 3). Comparing the survival of all patients treated with chemotherapy under the age of 50 years with those receiving BMT, no difference is seen at 5 years (Fig. 4).

### Discussion

In contrast to others [15], the present trial failed to prolong remission duration or sur-

vival despite intensification of maintenance treatment. It also failed to show any improvement by introducing new drugs, non-cross-resistant to those utilized during induction. There was no improvement in DFS despite the fact that the “alternating” arm was more aggressive than the induction-type maintenance, the only consequence being 3.4% excess mortality and shorter survival after relapse.

The alternating maintenance arm was certainly more expensive: 60.3% of patients received platelet transfusions vs. 38.7% of patients in the induction-type maintenance arm ( $P \leq 0.0001$ ). The quality of life was probably also worsened, as shown by the longer total duration of hospitalization.

The present results of intensive maintenance chemotherapy in regard to DFS tend to be somewhat inferior to those obtained with BMT. However, the overall survival is probably not significantly different, espe-

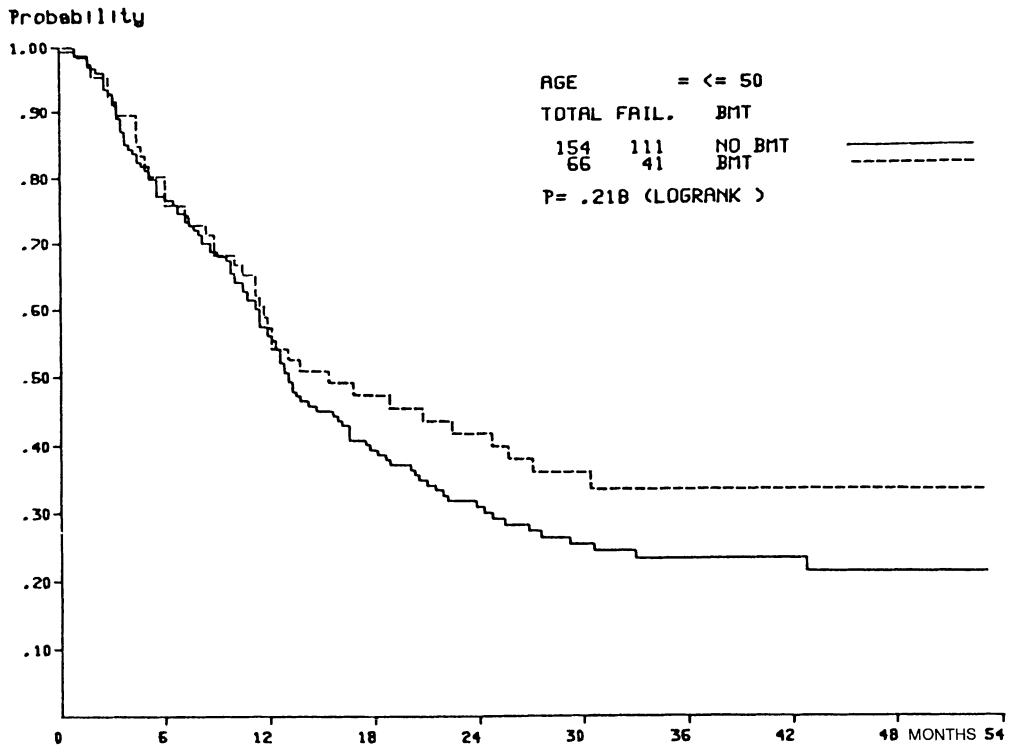


Fig. 3. Disease-free survival of patients undergoing BMT < 45 years of age and those receiving chemotherapy during maintenance < 50 years of age

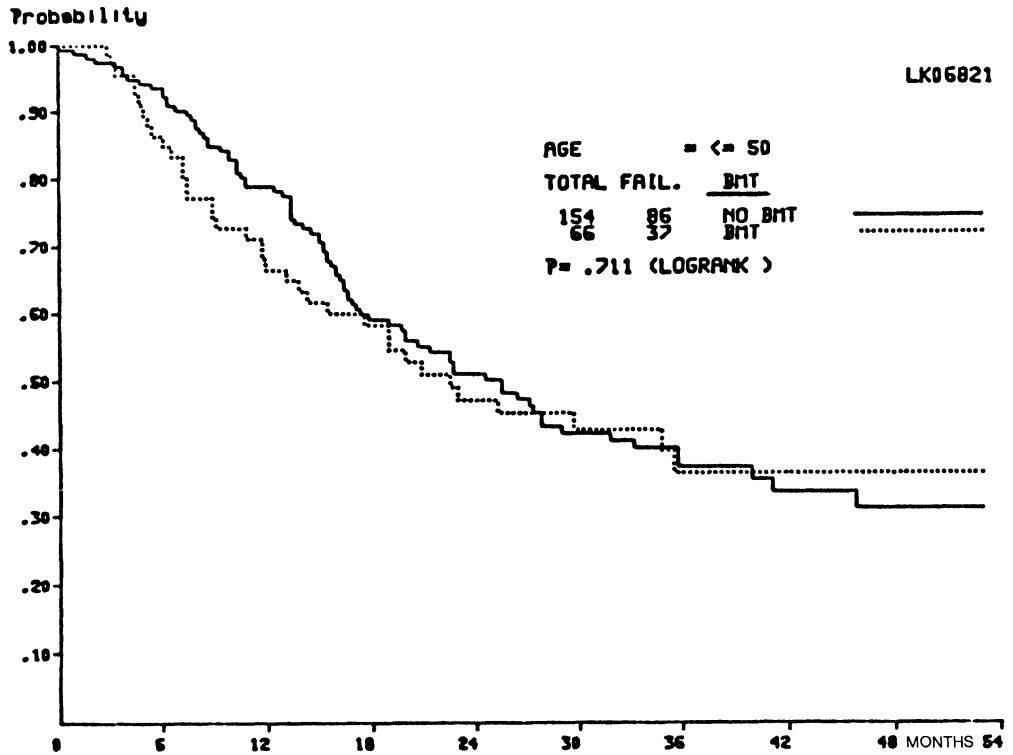


Fig. 4. Survival of patients undergoing BMT < 45 years of age and remitters receiving chemotherapy during maintenance < 50 years of age

cially in patients of the same age groups, and the antileukemic effect of allogeneic BMT seems to be counteracted by the excess toxicity of this treatment procedure. The small number of patients treated by BMT could explain the failure to find a major difference. A prospective study by Appelbaum [7] has shown superiority for long-term DFS of allogeneic BMT when compared with more conventional chemotherapy. While allogeneic BMT seems to decrease relapse rate, the actuarial long-term survival is not significantly better than following consolidation treatment [14, 16].

### Summary

Out of 515 evaluable patients (median age, 47 years) who entered the study from 1983 to 1986, 67.4% achieved complete remission (CR) after one cycle (256) or two cycles (91) of daunorubicin (DNR) (45 mg/m<sup>2</sup> days 1–3), cytosine arabinoside (Ara-C) (200 mg/m<sup>2</sup> i.v. days 1–7), and vincristine (VCR) (1 mg/m<sup>2</sup> day 2). A partial remission was achieved by 3.7% of patients, 15% were resistant, 11.3% died during hypoplasia, and 2.7% died during induction. Patients achieving CR received one consolidation course in which administration of DNR was limited to 1 day. Two hundred and forty-eight patients were randomized for six courses of maintenance every 6 weeks: either DNR + VCR day 1 + Ara-C s.c. days 1–5, or AMSA 150 mg/m<sup>2</sup> day 1 alternating with high-dose (HD)-Ara-C 3 g/m<sup>2</sup> q12 h day 1 + 2 or 5-azacytidine 150 mg/m<sup>2</sup> days 1–3. Two hundred and thirty-three patients were randomized when bone marrow transplantation (BMT) had not been planned or performed and 15 patients were randomized before the BMT. Sixty patients received BMT, 17 autografts, and 43 allografts. Median time from CR to BMT was 15 weeks. Forty-two patients were not randomized mainly because of toxicity or treatment refusal. Median DFS for both chemotherapy groups was 12 months and 23% were alive at 4 years. Median survival from CR was 22 months, and 34% were alive at 4 years. There was no difference in disease-free interval (DFI) and disease-free survival (DFS) between the two chemotherapy arms. Of 60

transplanted patients, 42% were alive at 4 years. Patients over 50 years of age responding to chemotherapy had an identical survival rate to those transplanted in first complete remission. Median duration of survival from first relapse is 22 weeks and only 9% have the chance of survival. The impact of the initial maintenance treatment on subsequent survival is that patients who received the more aggressive program (HD Ara-C/AMSA) had a shorter survival than those with the milder treatment (19 vs. 27 weeks;  $P=0.053$ ).

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## Predictive Models for Achievement of Complete Remission and Duration of First Remission in Adult Acute Myeloid Leukemia\*

A. Heinecke<sup>1</sup>, M. C. Sauerland<sup>1</sup>, and T. Büchner<sup>2</sup>

### Introduction

We describe some of our efforts to identify prognostic factors for achievement of complete remission (CR) and duration of relapse-free survival. So far the results have not been very encouraging.

### Mathematical Models and Methods

#### *Achievement of Complete Remission*

The probability  $P$  of achieving CR is mathematically modeled as:

$$P = \frac{1}{1 + e^{-X}}$$

where

$$X = a_0 + a_1 \cdot (x_1 - \bar{x}_1) + \dots + a_k \cdot (x_k - \bar{x}_k).$$

The unknown coefficients  $a_i$  are to be estimated from the data ( $i = 0, 1, \dots, k$ ), the  $x_j$  coefficients are certain patient characteristics such as age or WBC at start of therapy ( $j = 1, 2, \dots, k$ ). The appropriate choice of these characteristics is based on certain statistical criteria. This is the well-known

regression model described in detail by Cox [2].

#### *Duration of Relapse-Free Survival*

Calculation is based on the proportional hazards model for survival times introduced by Cox [3]. The probability  $S(t)$  that duration of relapse-free survival exceeds time  $t$  is mathematically modeled as:

$$S(t) = H(t) \exp(b_1 x_1 + b_2 x_2 + \dots + b_m x_m).$$

Again the fixed but unknown coefficient  $b_j$  are to be estimated from the data, the  $x_j$  coefficients are certain patient characteristics ( $j = 1, 2, \dots, m$ ), and  $H(t)$  is any continuous survivor function.

### Results

#### *Achievement of CR*

We refer to the data of the 1981 study of the German AML Cooperative Group reported in detail by Büchner et al. [1] and at this meeting:

Participants	Twenty-two clinical centers in the Federal Republic of Germany
Recruitment	11/81–4/85
Patients	Included are 501 patients at least 16 years old with confirmed untreated acute myeloid leukemia (AML), fulfilling certain require-

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\* The studies of the AML Cooperative Group are supported by grant 01 ZP 0123 from the German BMFT

**Table 1.** Six-factor model of Keating et al. [4]. AHD, antecedent hematological disorder; BUN, blood urea nitrogen; RCM, right costal margin

$j$	Factor $x_j$	Code	$\bar{x}_j$	$a_j$
0	Constant	–	–	0.495
1	Age	1 = '< 20' 2 = '20– < 50' 3 = '50– < 65'	2.54	–0.744
2	AHD	0 = 'no'      1 = 'yes'	0.19	–1.680
3	Temperature	1 = '< 38.3'      2 = '≥ 38.3' (°C)	1.34	–1.109
4	BUN	1 = '< 23'      2 = '≥ 23' (mg/dl)	1.12	–1.031
5	Hemoglobin	1 = '< 12'      2 = '≥ 12' (g/dl)	1.11	1.039
6	Liver	0 = < 5 cm below RCM 1 = ≥ 5 cm below RCM	0.05	–1.297

ments on heart, liver, and kidney function  
 Median age 49 years  
 Overall CR rate 59%

All patients received one or two courses of TAD9. At first we tested the predictive value of Keating's six-factor model for our data (Table 1). Using the same prognostic classes as in [5], expected and observed CR rates were compared, showing only poor correspondence (Table 2). To construct a new model we considered the following characteristics:

Age	Temperature	BM blasts
Sex	Hemoglobin	Cellularity
State of health	WBC	FAB-M classification
Infections	Platelets	

Using 5% as the significance level for stay or entry in the model, a best fit to our data was accomplished by the three-factor model shown in Table 3. As this is a retrospective model the good correspondence of expected

**Table 2.** Expected and observed CR rates of the 1981 study using Keating's model

Prognostic class	Patients		% CR rate	
	Num-ber	%	Ex-pected	Ob-served
[0.0, 0.2]	8	1.6	15	62
[0.2, 0.4]	21	4.2	29	43
[0.4, 0.6]	102	20.4	47	41
[0.6, 0.8]	179	35.7	67	57
[0.8, 1.0]	191	38.1	85	70

**Table 3.** Three-factor model based on the 1981 study

$j$	Factor $x_j$	Code	$\bar{x}_j$	$a_j$
0	Constant	–	–	0.601
1	Age	1 = '< 20' 2 = '20– < 50' 3 = '50– < 65'	2.54	–0.647
2	FAB-M	4 = '≥ 65' (years) 1 = 'M3'      2 = 'M5' 3 = 'M2'      4 = 'M1' 5 = 'M4'      6 = 'M6'	3.65	–0.301
3	State of health	1 = 'capable of working' 2 = 'not capable of working' 3 = 'seriously ill'	2.18	–0.366

**Table 4.** Expected and observed CR rates in our three-factor model

Prognostic class	Patients		% CR rate	
	Number	%	Expected	Observed
[0.0, 0.2]	9	1.8	16	44
[0.2, 0.4]	64	12.8	31	30
[0.4, 0.6]	128	25.5	51	50
[0.6, 0.8]	258	51.5	68	67
[0.8, 1.0]	42	8.4	82	83

and observed CR rates shown in Table 4 is not surprising. To test whether our model is of any prospective value we used the pooled data of both of the 1985 studies of the German AML Cooperative Group.

**Participants** Twenty-nine clinical centers  
**Patients** Included are 419 patients at least 16 years old with confirmed untreated AML, fulfilling certain requirements on heart, liver, and kidney function  
**Median age** 52 years  
**Overall CR rate** 63%

Patients under 60 years old received double induction with TAD9/TAD9 or TAD9/HAM, whereas patients 60 years and older received

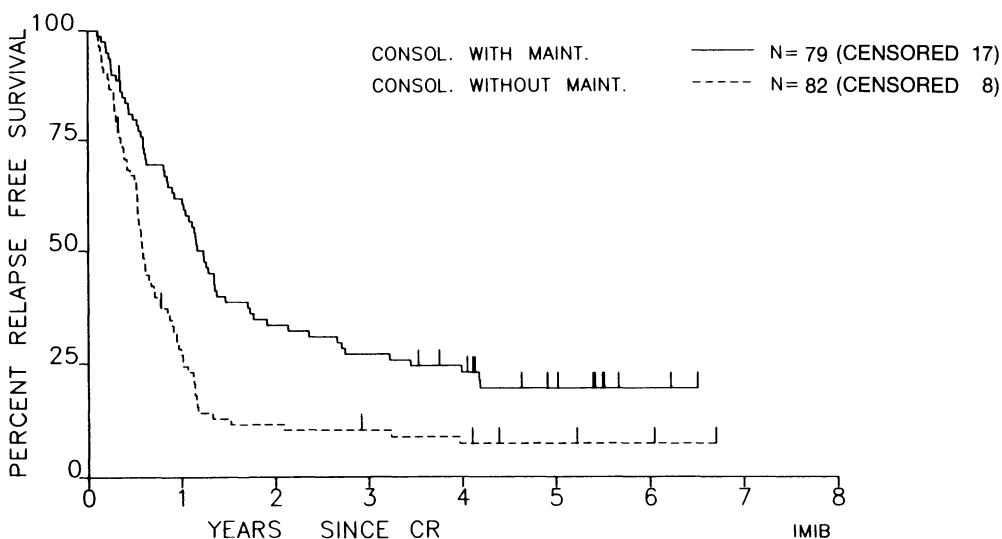
induction with TAD9 or TAD9 (DNR30). Table 5 exhibits the comparison of expected and observed CR rates. Though induction in the 1981 study was different from that in the 1985 study, the correspondence of the expected and observed CR rate is not as bad.

**Table 5.** Expected and observed CR rates of the 1985 study in the three-factor model

Prognostic class	Patients		% Cr rate	
	Number	%	Expected	Observed
[0.0, 0.2]	19	4.5	16	21
[0.2, 0.4]	70	16.7	29	37
[0.4, 0.6]	88	21.0	52	66
[0.6, 0.8]	186	44.4	69	70
[0.8, 1.0]	56	13.4	84	79

*Duration of Relapse-Free Survival*

In the 1981 study responders were randomly assigned to one of two maintenance therapy arms: therapy I, consolidation by one additional course of TAD9 followed by monthly maintenance; therapy II, consolidation by one additional course of TAD9 and no further maintenance. The result of this study was a clear advantage for patients in therapy I (see [1]) as shown in Fig. 1. The medians



**Fig. 1.** Kaplan-Meier plot of the 1981 study, update 1/89



of relapse-free survival were:  $\mu_1$ , 15 months (therapy I) and  $\mu_2$ , 7 months (therapy II).

The purpose of the following analysis was to identify possible subgroups of patients who do not benefit from monthly maintenance. Both arms were analyzed separately. We included the following characteristics:

Age	At start of therapy
WBC (log scale)	At start of therapy
Platelets (log scale)	At start of therapy
NCourses	Number of induction courses (one or two)
TCR	Time to CR
TR	Time to recovery (i.e., neutrophils $\geq 500$ , platelets $\geq 20000$ )

Table 6 represents the result of this analysis. Figure 2 shows:

**Table 6.** Multivariate analysis of relapse-free survival

Maintenance	Factors related to length of relapse-free survival
Consolidation followed by monthly maintenance	–
Consolidation without further maintenance	WBC
Level of significance:	10%

a) though significant at the 10% level there is only a slight effect of WBC on duration of relapse-free survival and

b) the probability that duration of relapse-free survival exceeds time  $t$  for patients with favorable WBC is still significantly below the respective probability for patients with monthly maintenance.

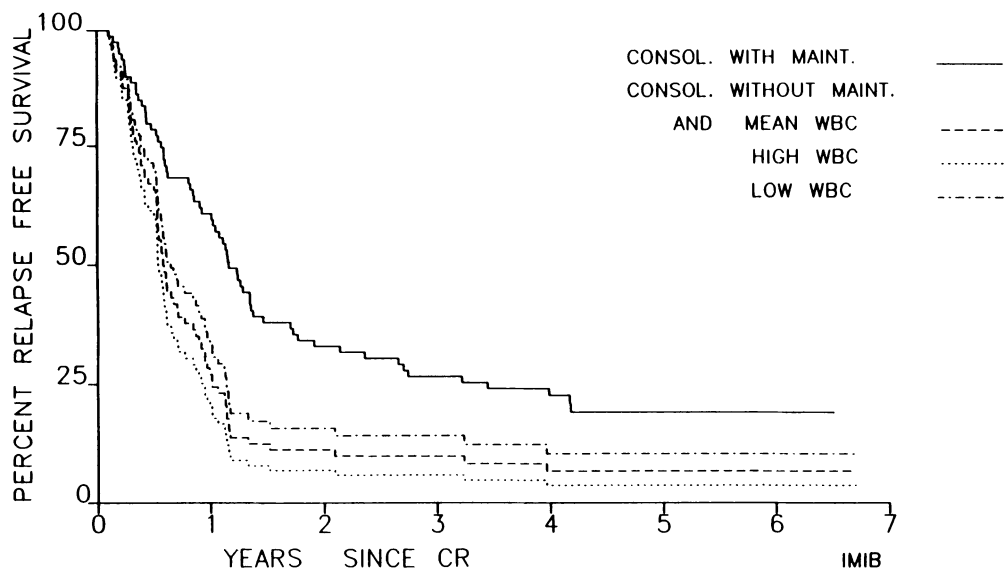
### Summary

*Achievement of CR.* Using the data of 501 patients treated identically with the TAD9 regimen we could not find any factor of predictive value besides age and state of health. The effect of FAB-M seems to be spurious as it disappeared using prospective data.

*Duration of Relapse-Free Survival.* In patients with monthly maintenance, the maintenance overrides the possible effects of the considered factors. In patients without monthly maintenance we only found a slight effect of WBC.

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**Fig. 2.** Cox PH model for relapse free survival, 1981 study, update 1/89

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## Sequential Decision Strategy of the AML Cooperative Group Studies\*

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### Introduction

The purpose of this poster is to show that sequential procedures are appropriate for clinical studies with prolonged observations. We present the sequential log-rank test used by the German AML Cooperative Group in its randomized studies.

### Mathematical Model and Method

Every statistical analysis is based on a mathematical model. Here the proportional hazards model for survival times is chosen. It is formulated as:

$$S_I(t) = S(t)^{1-1/2\theta}, \\ S_{II}(t) = S(t)^{1+1/2\theta},$$

where  $S_i(t)$  is the probability that the considered survival time under therapy  $i$  exceeds time  $t$  ( $i=I, II$ ).  $S(t)$  is any continuous survivor function. In the settings of this model the hypotheses:

$H_0$ : both therapies are equivalent with respect to the considered survival time  
 $H_1^+$ : therapy I offers some advantage with respect to the considered survival time

$H_1^-$ : therapy II offers some advantage with respect to the considered survival time  
 $H_1$ : the considered therapies are not equivalent with respect to the considered survival time

are formulated respectively as:

$$H_0: \theta = 0, \quad H_1^+: \theta > 0, \\ H_1^-: \theta < 0, \quad H_1: \theta \neq 0.$$

$H_1^+$  and  $H_1^-$  are both one-sided;  $H_1$  is two-sided. The study committee decides which alternative to consider. In this model the appropriate statistical test is the one- or two-sided sequential log-rank test described in detail by Whitehead [2].

Every time a terminal event occurs  $z$  and  $v$  are calculated and plotted in the  $(v, z)$  plane. If the sequence  $(v_i, z_i)$  reaches the boundary, randomization is stopped and the appropriate hypothesis is accepted ( $i=1, 2, \dots$ ) (Figs. 1 and 2). At a prescribed significance level  $\alpha$  and prescribed power of test  $(1-\beta)$  – for a specified  $\theta$  – this procedure guarantees that the expected number of randomizations is kept to a minimum. The calculation of the boundaries is based on the prescribed values of  $\alpha$  and  $\beta$ .

### Results

The medical rationale of the randomized studies of the AML Cooperative Group is reported in detail by Büchner et al. [1] and at this meeting. Here only the results and updates of the sequential procedures are presented.

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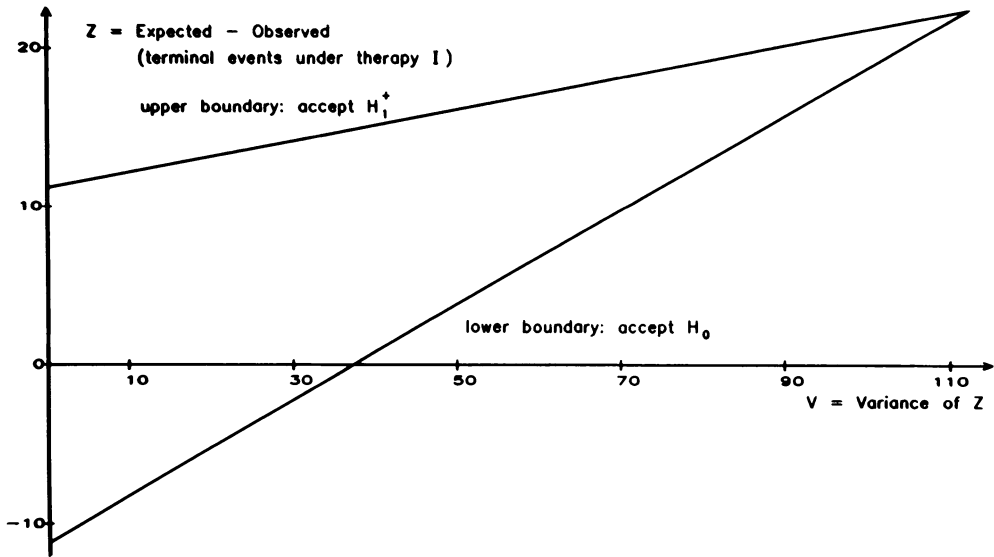


Fig. 1. One-sided sequential log-rank test

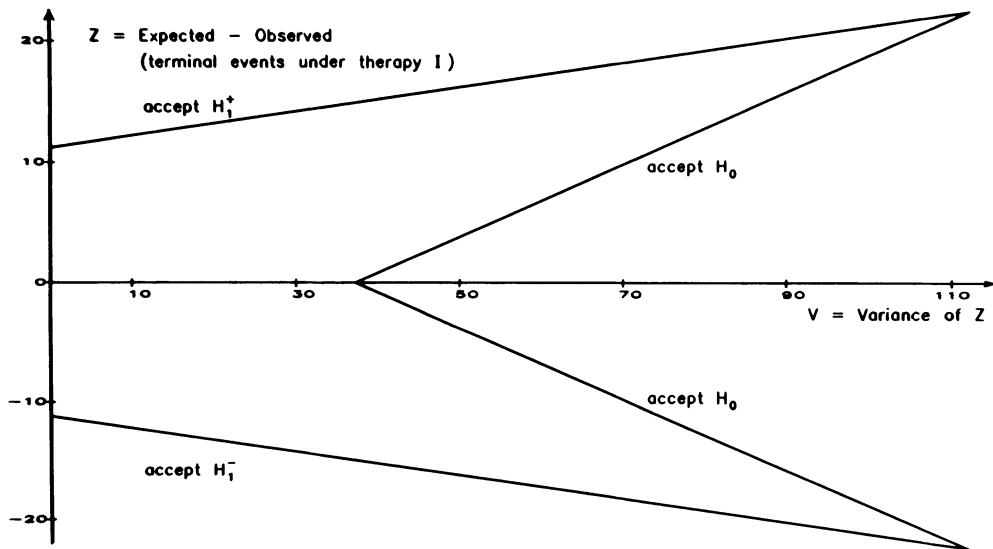


Fig. 2. Two-sided sequential log-rank test

### 1981 Study

**Participants.** Twenty-two clinical centers in the Federal Republic of Germany.

**Patients.** Included are all patients at least 16 years old with confirmed untreated AML fulfilling certain requirements on heart, liver, and kidney function.

**Main Objective.** Comparison of the effect of consolidation by one additional induction course and monthly maintenance (therapy I) versus consolidation and no further maintenance (therapy II) on the duration of relapse-free survival.

The study-committee decided to consider the one-sided alternative

$H_0: \theta = 0,$   
 $H_1^+: \theta > 0.$

The values of  $\alpha$  and  $\beta$  were chosen to be:  
 $\alpha = 0.05, \beta = 0.05$  (at  $\theta = 0.4$ ).

Figure 3 shows the sequence  $(v_i, z_i)$  in the course of the study. At the 85th terminal event (i.e., relapse or death in remission) the upper boundary was reached, randomization was terminated, and  $H_1^+$  was accepted, that is to say:

At the given level of significance consolidation followed by monthly maintenance has a

positive effect on the duration of relapse-free survival.

Until termination: 161 patients in complete remission had to be randomized, 79 to therapy I (consolidation followed by monthly maintenance) and 82 to therapy II (consolidation and no further maintenance).

Out of the 85 terminal events observed until termination, there occurred 38 under therapy I and 47 under therapy II. Figure 4 displays the Kaplan-Meier plot of the present update of this study.

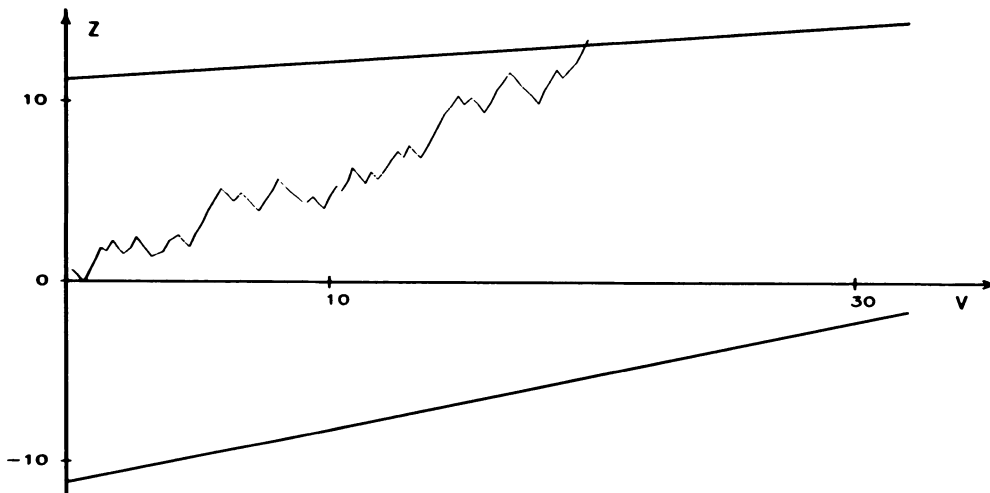


Fig. 3. One-sided sequential log-rank test used in the 1981 study

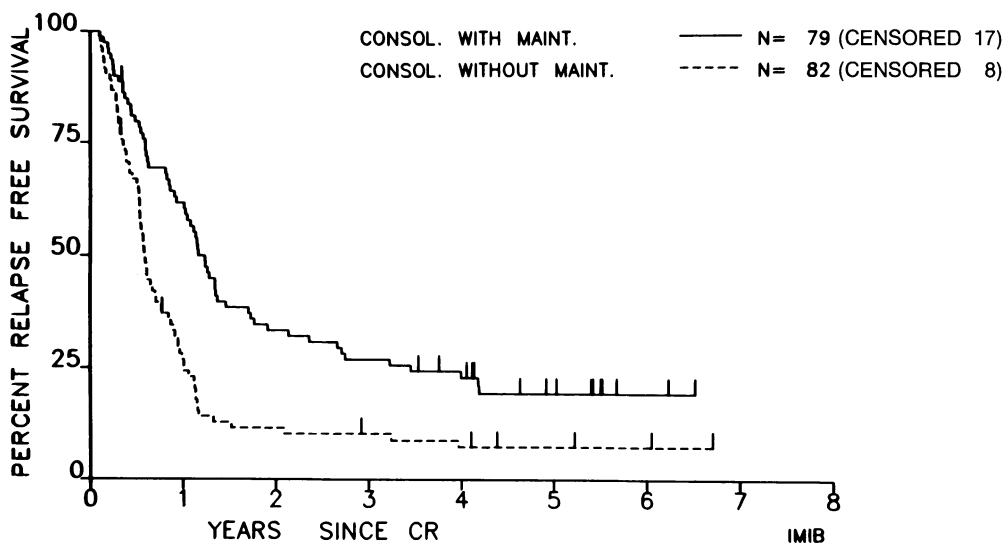


Fig. 4. Kaplan-Meier plot of the 1981 study, update 1/89

*Participants.* Twenty-six clinical centers in the Federal Republic of Germany.

*Patients.* Included are all patients at least 16 and up to 59 years old with confirmed untreated AML fulfilling certain requirements on heart, liver, and kidney function.

*Main Objective.* Comparison of the effect of double induction by two courses of TAD9 (therapy I) versus double induction by one course of TAD9 followed by one course of HAM (therapy II) on the duration of relapse-free survival. The study committee decided to consider the two-sided alternative:

$$H_0: \theta = 0,$$

$$H_1: \theta \neq 0$$

with  $\alpha = 0.1$  (two-sided) and  $\beta$  as above. There are now (update 1/89) 272 patients randomized and 182 patients in complete remission, 89 in therapy I (TAD9/TAD9), and 93 in therapy II (TAD9/HAM). Seventy-five terminal events have been observed, 33 in therapy I and 42 in therapy II. So far the two-sided sequential log-rank test has not shown a clear advantage for either therapy. Figure 5 displays the Kaplan-Meier plot of the present update of this study.

*Participants.* Twenty-three clinical centers in the Federal Republic of Germany.

*Patients.* Included are all patients older than 60 years with confirmed untreated AML, fulfilling certain requirements on heart, liver, and kidney function.

*Main Objective.* Comparison of the effect of induction by one or two courses of original TAD9 (therapy I) versus induction by one or two courses of TAD9 with DNR reduced to 30 mg/m<sup>2</sup> (therapy II) on the duration of survival. The study committee decided to consider the two-sided alternative:

$$H_0: \theta = 0,$$

$$H_1: \theta \neq 0$$

with the same specifications as  $\alpha$  and  $\beta$  as above. There are now (update 1/89) 144 patients randomized, 68 to therapy I (TAD9) and 76 to therapy II (TAD9/DNR30). Ninety-one terminal events have been observed, 38 in therapy I and 53 in therapy II. As above the two-sided sequential log-rank test has not yet shown a clear advantage for either therapy. Figure 6 shows the Kaplan-Meier plot of the present update of this study.

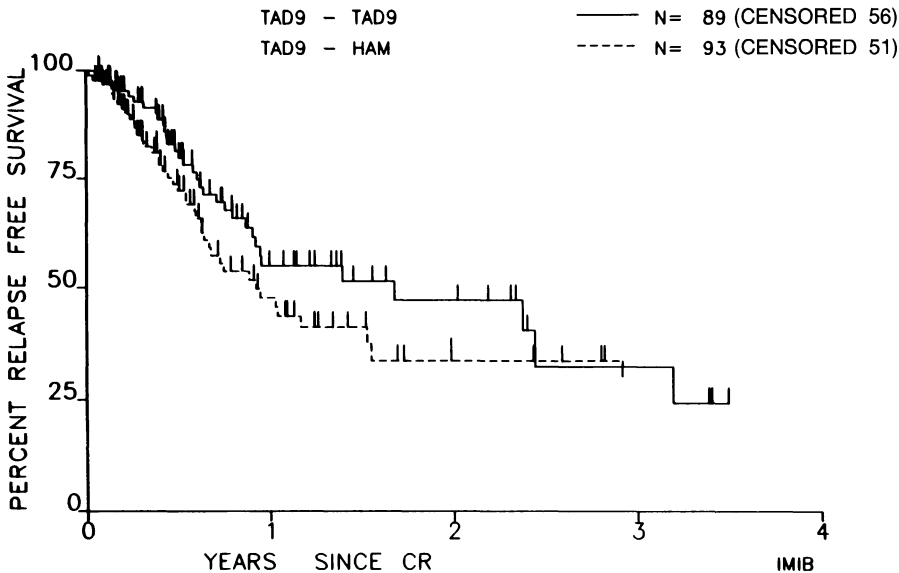


Fig. 5. Kaplan-Meier plot of the 1985 study (<60 years), update 1/89

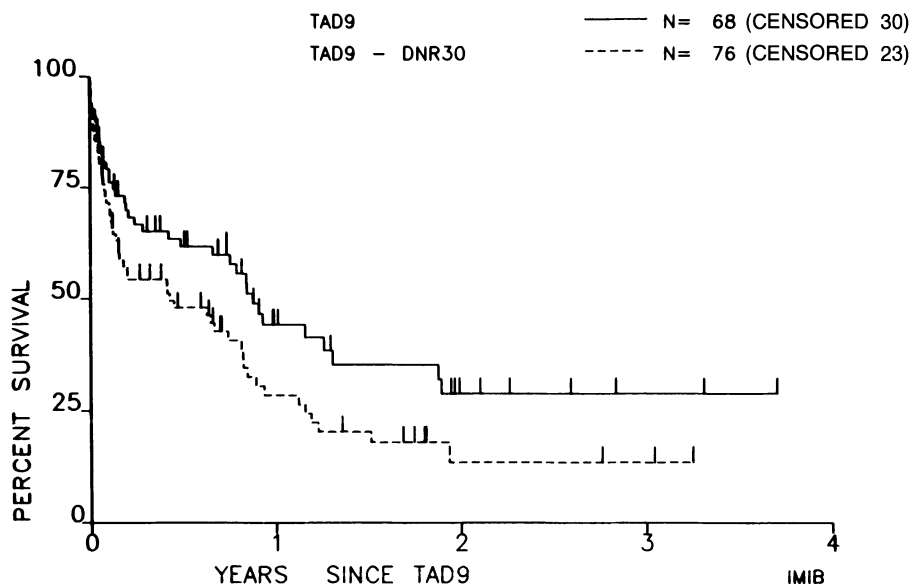


Fig. 6. Kaplan-Meier plot of the 1985 study ( $\geq 60$  years), update 1/89

### Summary

The ongoing 1985 studies are showing at present no clear advantage for either therapy. In the 1981 study, randomization was terminated by the sequential procedure after 161 randomizations. A test with the same  $\alpha$  and  $\beta$  but fixed sample size would have required at least 200 patients, 100 for each therapy. So in this case the use of the sequential procedure saved about 40 randomizations.

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## Pretherapeutic Drug Testing in Acute Leukemias for Prediction of Individual Prognosis

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### Introduction

Treatment of patients with acute leukemia is usually based upon utilization of multidrug regimens. Although the response rates for AML and ALL are quite high, the therapeutic efficacy of a drug against an individual leukemic cell population is not known. In contrast, toxicity of the drugs is unavoidable. It would therefore be desirable to know in advance which patient will or will not respond to a specific therapy.

Methods for prediction of sensitivity or resistance of leukemic cells have been proposed, but are limited by a variety of serious problems. The theoretically attractive cloning or stem cell assays have various technical problems, i.e., poor culture results, need for large amounts of cells, as well as long duration [1–3]. Short-term assays, based either upon dye exclusion [4] or [<sup>3</sup>H]thymidine incorporation [5], have limited value, since selective evaluation of malignant cells (blasts) has not been possible.

To overcome these limitations, we have modified a differential staining cytotoxicity (DiSC) assay, initially developed by Bosanquet et al. [6] for chronic lymphocytic leukemia. The DiSC assay offers the advantages of short-term in vitro tests, but, in contrast to those, evaluates exclusively the malignant cells. We have previously shown that the assay is suitable for testing acute leukemia, because selective evaluation of leu-

kemic blasts is easily performed and the assay is completed within 4 days [7].

The object of the present study was to evaluate how the results obtained in vitro correlate with the clinical outcome of the patients, in order to define prognostic criteria based upon drug sensitivity testing.

### Material and Methods

One milliliter of heparinized bone marrow or 5–15 ml blood were obtained through routine diagnostic workups from adult patients with acute myelogenous leukemia (AML) or acute lymphoblastic leukemia (ALL) at first diagnosis or in relapse. The leukocyte population was separated by Ficoll-Hypaque, washed, and adjusted to  $5 \times 10^5$  cells/ml in RPMI 1640 medium with 10% fetal bovine serum (all Gibco). A quantity of 180  $\mu$ l of this sterile suspension was pipetted into Micronic tubes placed in a 96-well Micronic plate (Flow Laboratories) and 20  $\mu$ l drug solution at tenfold concentration, or 20  $\mu$ l phosphate-buffered saline (PBS) was added. The tubes were placed for 10 s on a Vortex and then incubated at 37°C in humidified atmosphere with 5% CO<sub>2</sub>.

After 4 days incubation 200  $\mu$ l of a mixture of 1% Fast Green and 0.5% Nigrosin (Sigma) containing  $1 \times 10^7$  fixed duck red blood cells was added to each tube, mixed, and incubated for 10 min. This mixture selectively stained the dead cells, since viable cells are capable of excluding the dye. Duck red blood cells (DRBCs) served as an internal standard. The cells were cytocentrifuged



onto collagen-coated slides, air dried, and then counterstained with May-Grünwald/Giemsa (Pappenheim).

Under the microscope, four cell populations were evaluated:

1. dead cells, stained dark blue,
2. viable blasts
3. viable regular hemopoietic cells,
4. DRBCs. Due to the counterstain, blasts could easily be distinguished from normal cells by morphology.

All experiments were set up in triplicate and the mean was calculated. For quantification of results, the ratio of live blasts over simultaneously counted DRBCs was determined for each slide. For drug-treated groups, this ratio was expressed as a percentage of that in the control and termed tumor cell survival (TCS):

$$q = (\text{no of viable blasts})/(\text{no. of DRBCs})$$

$$\text{TCS} = (q \text{ drug}/q \text{ PBS}) \times 100$$

The addition of easily distinguishable DRBCs prevents interpretation problems of the assay caused by cell autolysis or in vitro proliferation. On day 4 the viability of leukemic blasts in the control group had to be at least 20% for an experiment to be evaluable for drug sensitivity information. A TCS of  $\leq 30\%$  was used as the definition of in vitro sensitivity.

## Results

### Technical Success of the Assay

A total of 50 subsequently received acute leukemia samples were set up in the DiSC assay (Table 1). The majority originated from patients with acute myelogenous leu-

kemia (AML). Overall, in 42 of the 50 specimens the viability of untreated control cells on day 4 was greater or equal to 20%, qualifying these specimens for drug sensitivity information. This represents a technical success rate of 84%. The viability of control cells on day 4 averaged 60%, with a range of 0%–98% (Table 1). With 65% the mean viability was higher for the 42 AML specimens than for the 8 ALL specimens. An average of eight different cytotoxic drugs at four to five different concentrations were tested.

### Cytotoxicity Results

The pattern of drug sensitivity resembled the clinical experience: cytosine arabinoside, daunorubicin, and mitoxantrone were usually active, while bleomycin or *cis*-platin showed lack of antileukemia activity. No differences were noted for these drugs in AML and ALL specimens. In contrast, vincristine was more often active against ALL than against AML samples, and, interestingly, prednisolone was solely active in ALL specimens (data not shown). Since appropriate in vitro test concentrations have not yet been defined, each drug was investigated at several concentrations, and for all active compounds a clear dose–response effect was noticed.

### Clinical Correlations

A total of 46 patients, 38 of these with AML, were tested for chemosensitivity with the DiSC assay (Table 2). In 9 of the 46 specimens the viability of the control group was insufficient on day 4. Of the remaining 37 patients, 8 were not treated or they received mild therapy without curative intention, and another 8 patients were not evaluable for clinical response due to early death. Therefore, only 21 of the initial 46 patients were evaluable for clinical correlation studies. Table 2 details the results for patients with AML and ALL.

Patients were treated with multidrug therapy as part of standard or experimental treatment protocols, and the in vitro tests were performed with the same drugs. Table

**Table 1.** Culture results

	Total No. of assays	No. of successful assays <sup>a</sup>	Mean viability of control cells on day 4 (range)
AML	42	37 (88%)	65% (0%–98%)
ALL	8	5 (63%)	30% (0%–85%)
Overall	50	42 (84%)	60% (0%–98%)

<sup>a</sup> Viability of control cells  $\geq 20\%$  on day 4

**Table 2.** Clinical correlations

	AML	ALL	Total
Overall No. of patients	38	8	46
No. of specimens without sufficient viability on day 4	6	3	9
No. of patients that did not receive aggressive chemotherapy	6	2	8
No. of patients not evaluable for clinical response (early death)	8	–	8
No. of patients evaluable for in vitro/in vivo correlation	18	3	21

**Table 3.** Summary of clinical correlations

No. of patients	Results		Correlation
	In vitro	In vivo	
14 (11 AML/3 ALL)	Sensitivity <sup>a</sup>	Complete response	True positive
1 (AML)	Sensitivity	No response	False positive
5 (4 AML/1 ALL)	Resistance <sup>b</sup>	No response	True negative
1 (AML)	Resistance	Complete response	False negative

<sup>a</sup> Less than or equal to 30% tumor cell survival for at least one of the clinically used drugs

<sup>b</sup> Greater than 30% tumor cell survival for all clinically used drugs

3 is a summary of the clinical correlations for the 21 evaluable patients. The in vitro concentrations of the tested drugs are summarized in Table 4. In vitro sensitivity was defined as  $\leq 30\%$  tumor cell survival obtained for *at least one* of the clinically used drugs, while in vitro resistance was defined as  $> 30\%$  tumor cell survival for all of the tested, clinically used drugs. The retrospec-

tively analyzed data, as detailed in Table 3, indicate a significant correlation between the in vitro chemosensitivity results by the DiSC assay and the clinical outcome of the patients. In 14 of 15 patients (93%) the DiSC assay predicted correctly for clinical response, while in five of six patients (83%) it predicted correctly for lack of response. Among the 21 patients only 1 false-positive and 1 false-negative prediction was obtained through the assay; in 19 of 21 cases the assay was truly predictive.

**Table 4.** In vitro test concentrations of clinically used drugs

Aclacinomycin	0.05 $\mu\text{g/ml}$
Asparaginase	0.1 U/ml
Cytosine arabinoside	1.0 $\mu\text{g/ml}$
D Daunorubicin	0.05 $\mu\text{g/ml}$
Doxorubicin	0.05 $\mu\text{g/ml}$
Mitoxantrone	0.05 $\mu\text{g/ml}$
Prednisolone	0.5 $\mu\text{g/ml}$
Rubidazone	0.1 $\mu\text{g/ml}$
Vincristine	0.1 $\mu\text{g/ml}$
VP-16	3.0 $\mu\text{g/ml}$

## Discussion

Treatment of patients with acute leukemia is performed with empirically developed multidrug regimens. At first diagnosis and even more in relapse it is uncertain which patient will respond to a specific therapeutic protocol. While for ALL some prognostic factors like the immunological subtype and initial

cell count have been established, for AML no reliable prognostic criteria besides age are known that would influence the therapeutic approach.

Although our data are based on a limited number of experiments, the strong correlation between the *in vitro* cytotoxic activity of individual drugs and the clinical response of the patients to the same drugs offers the opportunity to exploit the DiSC assay results as prognostic factors. Patients whose cells are sensitive to specific drugs in the assay are most likely to respond to these drugs. Patients whose cells are resistant *in vitro* are at high risk not to respond.

In the present study 84% of the tests were evaluable for drug sensitivity information and results were available within 4 days. Therefore, in contrast to sensitivity tests based upon colony formation, the DiSC assay is well suited for the *pretherapeutic* evaluation of a rapidly progressing disease like acute leukemia. Compared with other short-term tests, its major advantage is the selective evaluation of the leukemic blasts. The present study was a retrospective analysis. The retrospectively established *in vitro* criteria need to be evaluated in a prospective study, in order to prove that drug sensitivity results are major prognostic factors for the outcome of patients with acute leukemia.

Since the spectrum of anti-tumor activity varied for the different drugs, the assay could also be a tool for future individualization of acute leukemia therapy.

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## Double Intensive Consolidation Chemotherapy (ICC) for Acute Myeloid Leukemia

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### Introduction

The role of maintenance chemotherapy in prolonging survival in AML was questioned by Powles et al. [1] in the 1970s. Later, Champlin et al. [2] and Sauter et al. [3] confirmed the limited benefit of maintenance after a CR was achieved with intensive chemotherapy. Given the high CR rates achieved in relapsing AML with high-dose cytarabine and amsacrine reported by Hines et al. [4], it could be hypothesized that residual leukemic blasts remaining after CR would be sensitive to cytarabine at high concentration. Meanwhile, the first encouraging results of autologous bone marrow transplantation (BMT) were reported [5]. This information prompted us to design a pilot protocol consisting of two courses of post remission intensive consolidation chemotherapy (ICC). The first course contained high-dose cytarabine. The second course contained drugs used in the conditioning regimen for autologous BMT but at lower doses.

### Patients and Methods

#### *Eligibility Criteria*

Previously untreated patients younger than 65 years of age with a diagnosis of ANLL were eligible for this study after they gave

their informed consent. The diagnosis of ANLL was established on morphological and cytochemical criteria. An absolute infiltration of bone marrow with at least 30% blasts was required. Patients with a history of myelodysplasia, or with blastic transformation of CML, or with severe organ failure at presentation were excluded.

#### *Treatment Regimen*

The induction phase consisted of cytarabine 200 mg/m<sup>2</sup> per day (continuous infusion) on seven consecutive days and of zorubicin (Rubidazone) 200 mg/m<sup>2</sup> per day bolus days 1–4. For patients achieving CR, less than 45 years of age with no HLA identical donor, or between 45 and 65 years of age, postremission treatment consisted of two courses of ICC. The first course was cytarabine 3 g/m<sup>2</sup> (2 h i.v. infusion) days 1–4 and amsacrine 100 mg/m<sup>2</sup> per day (30 min i.v. infusion) day 5 to day 7. The second course of ICC was 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) 200 mg/m<sup>2</sup> i.v. bolus day 1, cytarabine 300 mg/m<sup>2</sup> per day (continuous intravenous infusion) day 1 to day 4, cyclophosphamide 1.5 g/m<sup>2</sup> per day (1 h i.v. infusion) day 2 and day 3, and etoposide 300 mg/m<sup>2</sup> per day (i.v. bolus) days 2 and 3. No further treatment was administered. Patients less than 45 years of age and with a suitable donor were allografted according to methods used in each center. Patients excluded from intensive chemotherapy received maintenance therapy with monthly courses of subcutaneous cytarabine 200 mg/

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m<sup>2</sup> per day for 5 days in association with amsacrine 100 mg/m<sup>2</sup> 1 day, etoposide 100 mg/m<sup>2</sup> 1 day, or cyclophosphamide 750 mg/m<sup>2</sup> 1 day.

### *Supportive Care*

For induction and intensive consolidation, patients were housed in laminar air flow rooms, single rooms with reverse barrier isolation, or standard rooms according to their availability in each participating center.

### *Statistical Method*

Estimation of the probability of survival and disease-free survival was made according to the Kaplan-Meier method. Estimates of the median times were also obtained by this method. Comparison between patient groups was assessed with the log-rank test. The Cox regression model was used to assess differences in a multivariate setting.

## **Results**

### *Patient Characteristics*

The 115 eligible patients ranged in age from 13 to 65 years (median age, 44 years); 53 patients (46%) were men and 62 (54%) were women. The initial WBC count ranged from 0.8 to 314 × 10<sup>9</sup>/liter (median, 9.8). The platelet count ranged from 6 to 305 × 10<sup>9</sup>/liter (median, 49). The median hemoglobin level was 8.4 g/dl (range, 4–15 g/dl). The French-American-British (FAB) type of blasts was M1 in 18 patients, M2 in 44, M3 in 9, M4 in 16, M5 in 20, M6 in 3, M7 in 1, and undifferentiated in 4. The characteristics of patients according to the treatment they received after the CR was achieved were not different except that none of the patients who received only the first course of ICC had more than 30 × 10<sup>9</sup> WBC at presentation and that the M4 and M5 FAB types were more frequent in transplanted patients than in any other group.

### *Treatment Results*

*Response.* Eighty-seven (75.5%) patients achieved CR after induction chemotherapy. Achievement of a CR was not influenced by age, sex, fever at presentation, initial WBC or platelet count, hemoglobin, FAB type of ANLL.

### *Outcome of Patients not Treated with ICC*

Out of 13 patients achieving CR who were excluded because of refusal or medical opposition, 4 are alive without disease at 15–31 months. For these patients the 4-year projected DFS is 30% (±12%). Seventeen patients received a supralethal chemoradiotherapeutic treatment followed by BMT; seven are alive free of disease after 30–53 months (median, 33 months). The projected 4-year DFS is 44% (±12%).

### **Outcome of Patients Treated with ICC**

*Disease-Free Survival.* The median DFS for the 57 patients who received at least the first ICC was 18 months and the projected 4-year DFS is 44% (±6%) for this group of patients (Fig. 1). Comparing the 4-year DFS for the 42 patients who received two ICC courses and for the 15 patients who received the first course only shows no difference (42% ± 7% and 47% ± 12%, respectively; *P* = 0.7). Patients with a low initial WBC count (30 × 10<sup>9</sup>/liter) had significantly longer DFSs than those with higher counts, as shown in Fig. 2 (*P* = 0.02). A short interval between start of induction and the first ICC (< 70 days) was also statistically associated with longer DFS (*P* = 0.02) (Fig. 3). Using a Cox regression model to examine for both factors simultaneously we found that both factors remained significantly associated with DFS duration. The DFS duration was not found to be associated with age, sex, platelet count, hemoglobin level, or FAB type.

### *Pattern of Relapse*

Of the 57 patients treated with ICC, 27 relapsed, 3 between the first ICC and the sec-

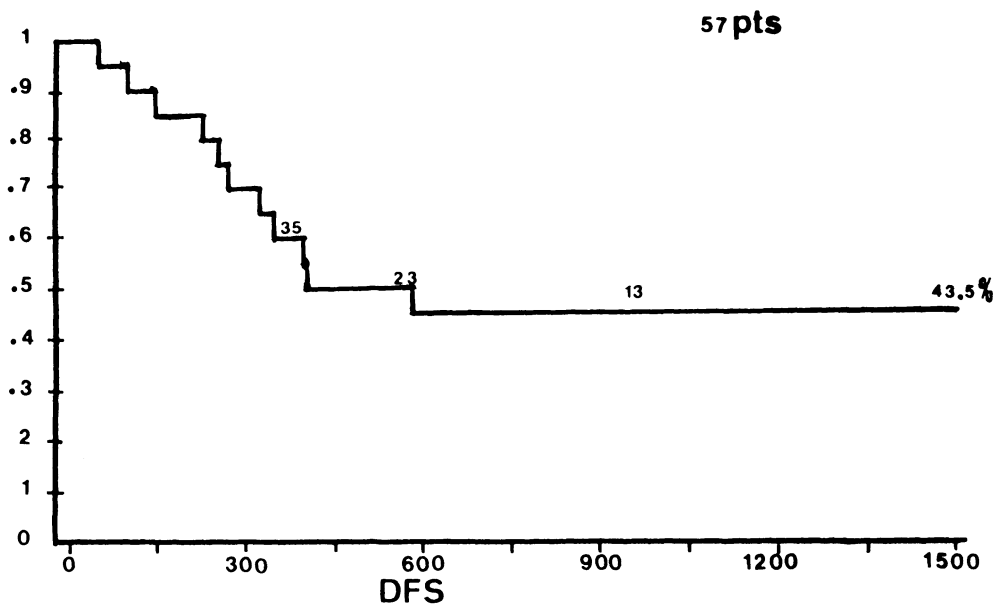


Fig. 1. Probability of disease-free survival for patients treated with ICC. *Number on the curve* represents patients at risk

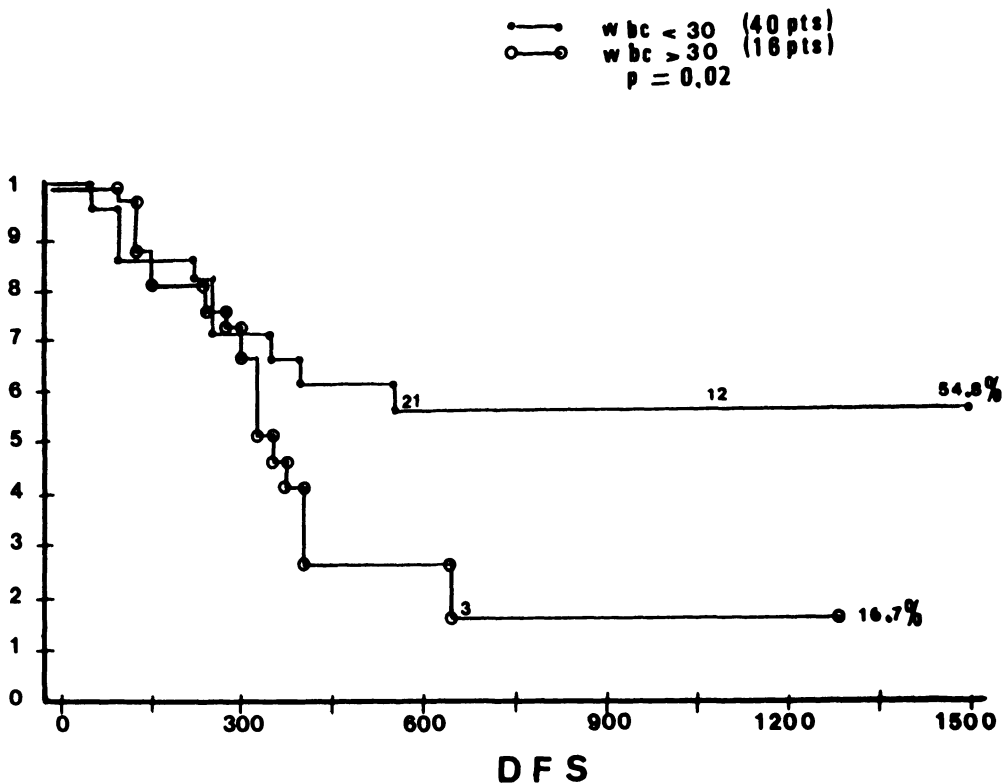


Fig. 2. Probability of disease-free survival for patients treated with ICC according to the initial WBC count

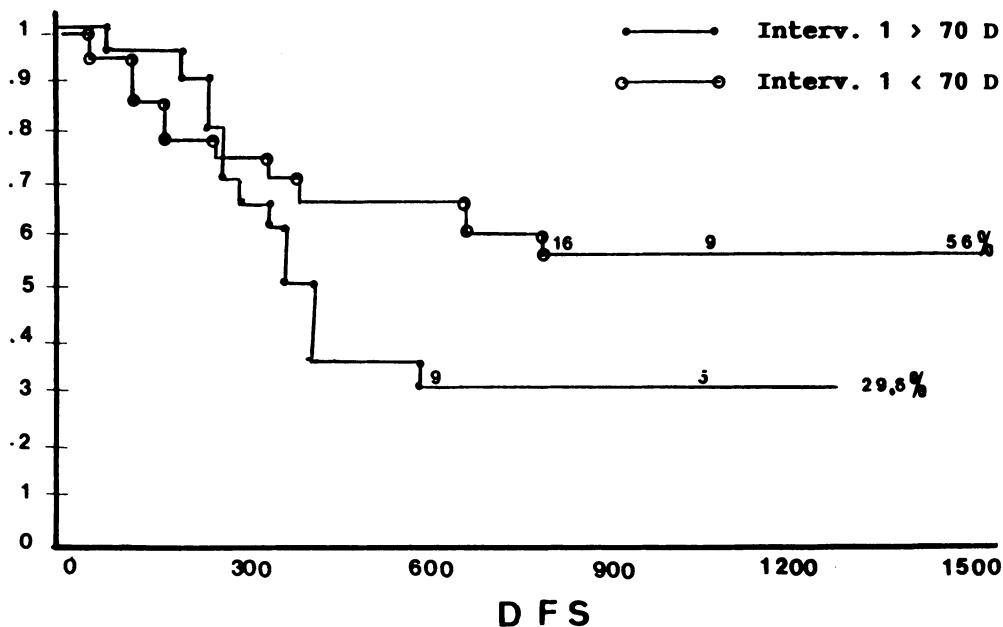


Fig. 3. Probability of disease-free survival for patients treated with ICC according to the interval between start of induction and the first ICC

ond ICC, one after the first only, and 23 after the second ICC. The last relapse occurred at 26 months after CR. The actuarial risk of relapse at 2 years is 52%. Owing to the small number of patients at risk for relapse in the group which received only the first course of ICC, there is no statistical difference for the actuarial risk of relapse between the group of patients which received one and two ICC courses (57% and 34% at 2 years, respectively).

#### Tolerance of ICC

The median duration of neutropenia ( $<0.5 \cdot 10^9/\text{liter}$ ) was 18 days (7–36 days) after the first course and 14 days (5–42 days) after the second. As a consequence of this durable neutropenia, septicemias occurred in 57% and 28% of the first and second ICCs, respectively. Extra hematological toxicity was limited during both first and second ICC courses; overall, three patients experienced mild cerebellar toxicity and WHO grade  $>3$  toxicity was encountered in less than 25% of the courses. The

mortality rate was 4% (4 deaths out of 99 ICCs performed).

#### Discussion

In the study described here, the administration of ICC was associated with a median DFS of 18 months with 44% of patients alive in CR at 4 years and an apparent plateau in the survival curve. Using an early consolidation with one to three courses of high-dose Ara-C alone or with daunorubicin, Wolff et al. report similar results [6]. These results compare favorably with conventional maintenance therapy administered monthly since with the latter therapy the median duration of remission is approximately 12 months with only 20% of patients alive at 5 years [7]. Whether or not the two planned courses were necessary is questionable since there is no difference in DFS duration according to the number of courses performed in our study. However, these results are to be examined cautiously since few patients had only one course and they had lower WBC counts at presentation. More-

over with only one consolidation using high-dose Ara-C and amsacrine (m-AMSA), Cassileth et al. reported a median duration of CR of 12 months and a probability of CCR at 3 years of 30% [8]. Champlin et al. report similar results with nearly the same therapy [9].

The median duration of cytopenia was not very different from that encountered after standard dose chemotherapy. The overall therapy-related mortality was 4%. However, the associated infections complications or the occurrence of nonmyeloid toxicity precluded the administration of the second planned course in nine cases.

Two factors were found to be statistically associated with a longer DFS; an initial WBC count lower than  $30 \times 10^9$ /liter and an interval between start of induction and of first ICC shorter than 70 days. Given these results and in order to gauge precisely the exact value of intensive postremission consolidation chemotherapy, a multicenter protocol is underway, comparing double ICC to unpurged autologous BMT performed after the first course of ICC. A maximum time of 70 days between start of induction and consolidation is now mandatory.

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## **Intensive Induction Therapy with Behenoyl, Cytosine Arabinoside, Daunorubicin, and 6-Mercaptopurine Followed by Intensive Consolidation with Mitoxantrone, Etoposide, Vincristine, and Intermediate-Dose Continuous Cytarabine (M-85 Protocol) for Adult Acute Myelogenous Leukemia**

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### **Introduction**

Over 70% of adults with acute myelogenous leukemia (AML) are induced into complete remission (CR) [1–4]. Our current target is not only to increase the remission rate, but to increase the cure rate of this disease, hopefully to over 50%. Two previous protocols for adult AML in our hospitals, BHAC-DMP [2] and BHAC-DMP (II) [4], from 1979 to 1985 showed that the percentage of blasts in the bone marrow at 2 weeks after the start of therapy is the most significant prognostic factor for predicting longer continuing CR by a multivariate analysis. However, in BHAC-DMP (II), where we gave very intensive induction therapy to reduce blasts in the marrows as quickly as possible, we were forced to stop this protocol because of a high incidence of severe infections, especially aspergillosis and other fungal infections, owing to prolonged myelosuppression during the induction period. Therefore, in the following M-85 protocol, the intensity of the induction therapy was reduced to intermediate strength between the previous two protocols, and the consolidation therapy,

instead, was more intensified, employing non-cross-resistant drugs.

### **Patients and Methods**

#### *Remission Induction Therapy*

During the 2-year period starting from 1985, adult AML patients who were admitted to Nagoya University Hospital, Aichi Shokuin Hospital, and Nagoya First Red Cross Hospital were consecutively treated with induction therapy consisting of 10- to 14-day treatment of daily 2-h infusion of behenoyl cytosine arabinoside (Ara-C) (BHAC, 200 mg/m<sup>2</sup>), daily 6-MP 6-mercaptopurine (70 mg/m<sup>2</sup>, p.o., with 300 mg/m<sup>2</sup> per day allopurinol), and intermittent daunorubicin (DNR) (25 mg/m<sup>2</sup>, i.v., days 1–3), and if necessary on days 8, 9, 11, 12, and 14). A response-oriented strategy was employed as in the previous two protocols [2, 4], and the treatment period and the administration frequency of DNR were adjusted depending on individual patient responses so as to attain a certain level of myelosuppression. At the end of therapy, peripheral WBC should be less than 1200/mm<sup>3</sup> and nucleated cell counts in bone marrows less than 15 000/mm<sup>3</sup> with less than 20% of blasts. These targets must be reached between 10 and 14 days after the start of therapy. BHAC and 6-MP were given during the entire treatment period, while DNR was given on the first 3 days, and on days 8, 9, 11, 12, and 14 if the

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bone marrow examination on days 8, 11, and 14 did not reach the above target. The treatment was stopped when the targets were reached. For acute promyelocytic leukemia (FAB, M3), 40 mg/m<sup>2</sup> DNR was given daily.

### Consolidation Therapy

Three courses of consolidation were given. The first consisted of BHAC (150 mg/m<sup>2</sup>, 2-h infusion, twice a day, days 1–6), DNR (40 mg/m<sup>2</sup>, i.v., days 1–3), and 6-MP (70 mg/m<sup>2</sup>, p.o., days 1–6). The second consisted of continuous Ara-C (400 mg/m<sup>2</sup>, days 1–5), mitoxantrone (MIT) (8 mg/m<sup>2</sup>, 30-min infusion, days 1–4), and vincristine (VCR) (1.5 mg/m<sup>2</sup>, i.v., day 1). The third consisted of BHAC (200 mg/m<sup>2</sup>, 2-h infusion, days 1–7), etoposide (ETP) (100 mg/m<sup>2</sup>, 1-h infusion, days 1–5), and vindesine (VDS) (2 mg/m<sup>2</sup>, i.v., day 10). Intrathecal methotrexate (MTX) (10 mg/m<sup>2</sup>) and Ara-C (25 mg/m<sup>2</sup>) were given after each course of consolidation therapy for CNS leukemia prophylaxis.

### Maintenance Therapy

A total of six courses of three different regimens were given every 2 months for a period of 1 year. The first consisted of DNR (25 mg/m<sup>2</sup> × 3), BHAC (170 mg/m<sup>2</sup> × 6), and 6-MP (70 mg/m<sup>2</sup> × 6), the second of MIT (6 mg/m<sup>2</sup> × 2 or 3), BHAC (170 mg/m<sup>2</sup> × 6), and 6-MP (70 mg/m<sup>2</sup> × 6), and 6-MP (70 mg/m<sup>2</sup> × 6).

### Supportive Therapy

Intensive supportive care including high-dose antibiotics and platelet transfusion from blood cell separators was given if indicated, and patients were placed under a laminar air flow if their WBC counts dropped below 1000/mm<sup>3</sup>.

### Results

Out of 41 consecutive patients, 29 (70.7%) achieved CR. Response rates related to

**Table 1.** M-85 protocol for adult AML; complete remission rate related to FAB classification and age

Type/age	No. of cases	CR	Rate
M1	8	6	75%
M2	18	9	64.3%
M3	6	4	66.7%
M4	7	5	71.4%
M5	6	5	83.3%
13 ~ 19 years	3	1	33.3%
20 ~ 29 years	7	5	71.4%
30 ~ 39 years	8	7	87.5%
40 ~ 49 years	9	8	88.9%
50 ~ 59 years	7	5	71.4%
60 ~ 74 years	7	3	42.8%
Total	41	29	70.7%

French-American-British (FAB) classification and age are shown in Table 1. Patients aged 60 years or more had a low response rate. Twenty-seven patients achieved CR by one course, and one each by two and three courses. CR was reached between 21 and 106 days with a median of 30 days. The treatment period for the first course ranged from 6 to 17 days with a median of 10 days, although the median for M3 was 8 days, and for the other subtypes 11 days. The DNR dose in the first course ranged from 60 to 340 mg/m<sup>2</sup> with a median of 133 mg/m<sup>2</sup>. The median DNR dose for M3 was 300 mg/m<sup>2</sup>, and for the other subtypes 134 mg/m<sup>2</sup>. The dose of BHAC in the first course ranged from 1200 to 3400 mg/m<sup>2</sup> with a median of 1700 mg/m<sup>2</sup>. The lowest WBC count after the first course of induction ranged from 200 to 1700/mm<sup>3</sup> with a median of 400/mm<sup>3</sup>. Circulating blasts disappeared within 1–34 days with a median of 7 days. The days when WBC was less than 1000/mm<sup>3</sup> during and after the first course of induction ranged from 0 to 38 days, with a median of 12 days. Blasts in the bone marrow at 1 week after the start of therapy ranged from 1% to 96% with a median of 22%. Blasts in the marrow at 2 weeks ranged from 1% to 76% with a median of 7%. The lowest WBC count after the first consolidation ranged from 200 to 2700/mm<sup>3</sup> with a median of 700/mm<sup>3</sup>, after

**Table 2.** Intensity of induction and consolidation therapies in BHAC-DMP (1979–1982), BHAC-DMP (II) (1983–1985), and M-85 protocols at Nagoya University hospitals

Therapy and items		Protocol		
		BHAC-DMP	BHAC-DMP (II)	M-85
<b>Induction period</b>				
Total DNA given	(mg/m <sup>2</sup> )	120	187	133
Total BHAC given	(mg/m <sup>2</sup> )	2040	3120	1700
Lowest WBC	(mm <sup>3</sup> )	700	350	400
Period WBC < 1000/mm <sup>3</sup>	(days)	5 (0–16)	11 (1–24)	12 (0–38)
<b>After first consolidation</b>				
Lowest WBC	(mm <sup>3</sup> )	2350	600	600
Period WBC < 1000/mm <sup>3</sup>	(days)	0 (0–3)	2.5 (0–13)	2 (0–13)
<b>After second consolidation</b>				
Lowest WBC	(mm <sup>3</sup> )	2400	1300	400
Period WBC < 1000/mm <sup>3</sup>	(days)	0 (0–2)	0 (0–9)	8 (0–15)
<b>After third consolidation</b>				
Lowest WBC	(mm <sup>3</sup> )	2650	–	700
Period WBC < 1000/mm <sup>3</sup>	(days)	0 (0–0)	–	3 (0–14)

Number indicates the median, and parentheses the range

the second ranged from 200 to 1500/mm<sup>3</sup> with a median of 400/mm<sup>3</sup>, and after the third ranged from 300 to 1800/mm<sup>3</sup> with a median of 700/mm<sup>3</sup>. The days when WBC counts were lower than 1000/mm<sup>3</sup> ranged from 0 to 13 with a median of 2 days after the first consolidation, from 0 to 15 with a median of 8 days after the second, and from 0 to 14 with a median of 3 days after the third (Table 2).

Serious complications during the induction period included three cases of sepsis, two of pneumonia, three of interstitial pneumonia (IP), three of perianal abscess, two of tonsillitis and one of cellulitis. Complications after the first consolidation were one case of sepsis and one of IP, after the second four cases of sepsis, four of pneumonia, four of perianal abscess, two of liver abscess, and one of pulmonary tuberculosis, and after the third one of sepsis and one of pneumonia. One patient died of cytomegalovirus IP after the first consolidation, two of bacterial sep-

sis after the second, and one 68-year-old female of myocardial infarction after the third, all in CR.

Four patients underwent allogeneic bone marrow transplantation at 2, 4, 4, and 5 months after CR, and were excluded at the respective time. The Kaplan-Meier curves of survival time and CR duration are shown in Figs. 1 and 2. At a median follow-up period of 30 months, seven patients relapsed, of whom three died and four achieved second CR with various regimens and are alive. Fourteen are still in their first CR. The initial CR duration ranged from 5 to 41+ months, with a predicted 3-year continuing CR rate of 56.4% (95% confidence limits, 26.4%–86.4%). The predicted 3-year survival and disease-free survival rate (DFS) of CR cases are 73.7% and 48.6%, respectively. The predicted 3-year survival rate of all patients is 51.0% (95% confidence limits, 21.7%–75.3%).

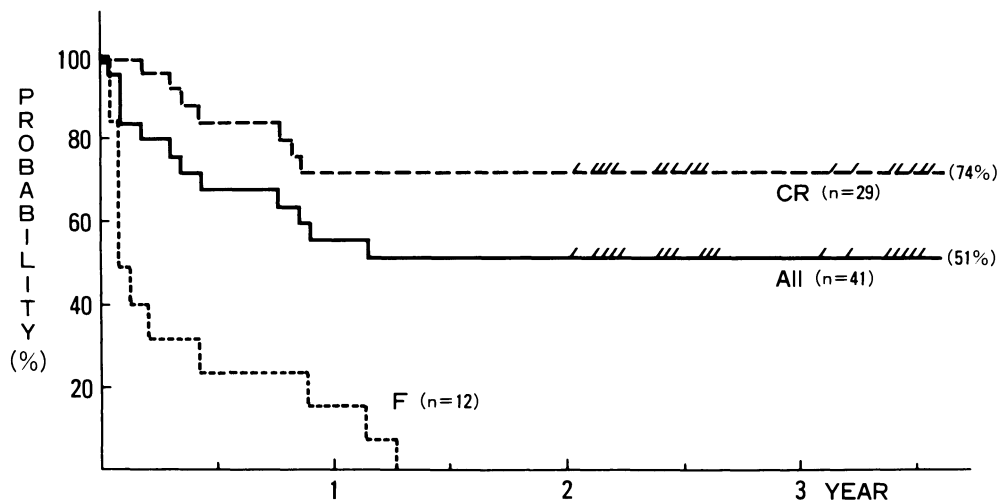


Fig. 1. Survival curves of all CR and failure cases treated with the M-85 protocol for adult AML

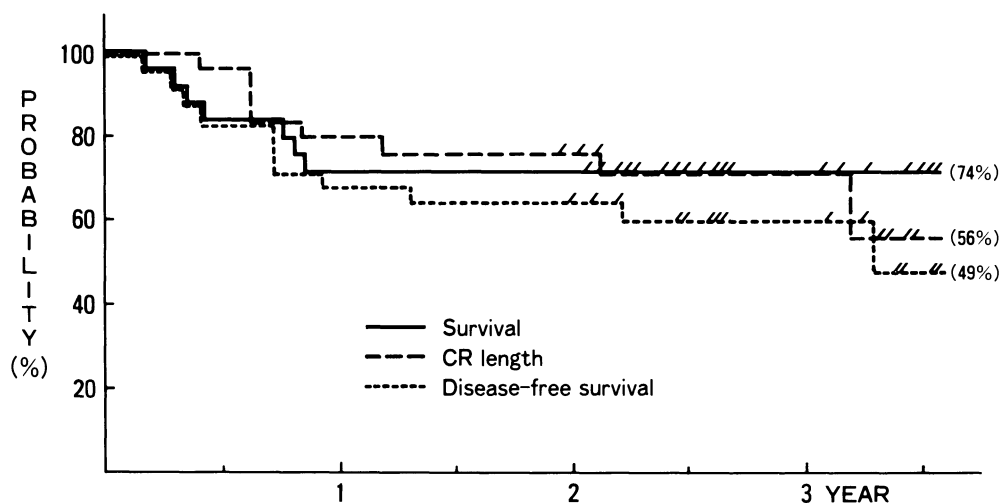


Fig. 2. Continuing CR, survival, and disease-free survival (DFS) curves of CR patients treated with the M-85 protocol for adult AML

## Discussion

The previous two protocols, BHAC-DMP and BHAC-DMP (II), for adult AML at our hospitals from 1979 to 1985 produced 84% and 76% CR, and showed 5-year continuing CR and survival rates of CR cases of 14% and 34%, respectively. Multivariate analysis in these two studies disclosed that the percentage of blasts in the bone marrow at 2 weeks after the start of therapy is the most significant prognostic factor to predict

longer continuing CR [2, 4]. However, in BHAC-DMP (II), where we gave very intensive induction therapy to reduce blasts in the marrow as quickly as possible, there was a high incidence of severe infections due to prolonged myelosuppression during the induction period. Therefore, in the subsequent M-85 protocol, we reduced the intensity of the induction slightly, and tried, instead, to intensify the consolidation therapy, employing non-cross-resistant new antileukemia drugs such as MIT and ETP. Intermediate-

dose (400 mg/m<sup>2</sup>) continuous Ara-C was also given in the consolidation.

As shown in Table 2, the intensity of the consolidation was strongest in M-85, whereas the intensity of the induction of M-85 is almost the same as that of BHAC-DMP(II), being judged from the period of WBC counts less than 1000/mm<sup>3</sup>. And the 3-year predicted continuing CR rate is highest in M-85. The intensity of the consolidation in M-85 was strong, and unfortunately produced so-called chemotherapeutic death in three patients in CR.

Thus from our three studies on adult AML, it seems to be most important to reduce blasts in the bone marrow to less than 20% at 2 weeks after the start of therapy and give intensive consolidation therapy including non-cross-resistant drugs. To accomplish this kind of intensive chemotherapy, not only intensive supportive care with high-dose antibiotics and platelet transfusions is indispensable, but an environment to protect the severely myelosuppressed patients from infectious pathogen is necessary.

This trial has shown that intensive induction therapy followed by intensive consolidations and by fairly intensive maintenance therapy produces high DFS among adult AML.

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## Acute Nonlymphocytic Leukemia in Adults: Results Obtained with TAD Remission Induction Therapy

E. O. Witteveen, L. F. Verdonck, H. K. Nieuwenhuis, and A. W. Dekker

### Introduction

The purpose of our study was to evaluate the outcome of 78 consecutive patients with acute nonlymphocytic leukemia (ANLL) referred to our hospital from 1981 to 1987. They were treated with conventional remission-induction therapy consisting of 6-thioguanine, cytarabine, and daunorubicin (TAD) [1], and the characteristics of the patients which could be associated with remission failure or relapse were analyzed [2–5].

### Patients and Methods

The records of 78 adult patients with ANLL referred to our hospital between September 1981 and June 1987 were analyzed retrospectively. Four patients with a preceding myelodysplastic syndrome (MDS) and two with secondary leukemia after previous alkylating therapy were also included. All patients received the same remission-induction treatment, which consisted of 6-thioguanine 100 mg/m<sup>2</sup> orally twice a day for 7 days, cytarabine 100 mg/m<sup>2</sup> intravenously twice a day for 7 days, and daunorubicin 60 mg/m<sup>2</sup> intravenously on days 5, 6, and 7, the TAD regimen [1]. In patients older than 60 years the daily dose of daunorubicin was adjusted to 40 mg/m<sup>2</sup>. One diagnostic lumbar

puncture with the administration of 80 mg cytarabine intrathecally was performed after remission was achieved. Patients with leukocytes > 100 000  $\mu$ l or extramedullary disease at presentation were regarded as high risk for CNS involvement [6, 7] and received a total of five injections with cytarabine, 80 mg, intrathecally. All complete responders received one TAD course for consolidation. No maintenance therapy was given. Since bone marrow transplantation was introduced in 1985 in our hospital, 7 patients underwent allogeneic and 13 patients autologous bone marrow transplantation. For prevention of infections all patients received infection prophylaxis with cotrimoxazole plus colistin or ciprofloxacin [8].

The data were calculated as of 1 January 1989. The median follow-up time for survivors was 31 months (range 10–72 months). The probability of staying alive was calculated according to the method of Kaplan and Meier. Groups of patients were compared using Student's *t*-test and the chi-square test.

### Results

#### *Remission Induction Treatment (Table 1)*

Seventy-three from a group of 78 admitted patients were treated according to the TAD regimen. Five patients died before therapy could be started, the median age in this group being 70 years, range 65–78 years. The median age for the patients treated with TAD was 50 years, range 15–80 years. The

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**Table 1.** Initial characteristics and response to TAD remission induction therapy in ANLL

	Number (%)	Achieving complete remission
Total	73 (100%)	49 (67%)
Age, 15–45 years	31 (42%)	22 (71%)
45–60 years	22 (30%)	20 (90%)
> 60 years	20 (28%)	7 (35%) $P < 0.01$
Sex, male	32 (43%)	19 (43%)
female	41 (56%)	30 (54%)
After one TAD course		25 (51%)
After two TAD courses		24 (49%)
De novo ANLL	67 (92%)	49 (73%)
Previous MDS	4 (5%)	0 (0%)
Secondary ANLL	2 (3%)	0 (0%)
Blood counts		
Hb < 5 mmol/liter	34 (47%)	24 (70%)
Leukocytes > 30000/ $\mu$ l	20 (27%)	13 (65%)
Platelets < 20000/ $\mu$ l	19 (26%)	12 (63%)
FAB classification		
M1–2	42 (58%)	24 (57%)
M3	10 (14%)	7 (70%)
M4–5	17 (24%)	14 (82%)
M6–7	4 (5%)	3 (75%)

median age for patients with previous MDS or secondary leukemia was 55 years, range 30–63 years. Forty-nine patients achieved a complete remission (67%). Of the pretreatment characteristics such as sex, infection, or bleeding at admission, extramedullary disease, FAB classification, or white blood cell counts, only age above 60 years and secondary leukemia or previous MDS had a significant influence on achievement of complete remission ( $P < 0.01$ ).

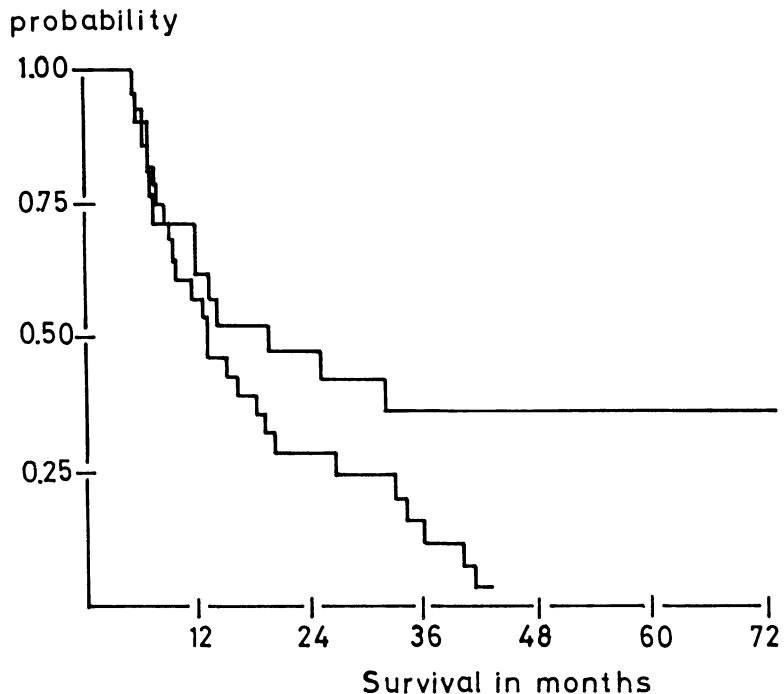
### Toxicity

Five patients who died during remission induction therapy were regarded as treatment failures. Three died from intracerebral bleeding, one from septicemia, and one in partial remission died from acute respiratory distress, possibly due to pulmonary embolism. One patient died in the 3rd month of complete remission of venoocclusive disease, probably caused by one of the cytotoxic agents [9]. No evidence of cardiac failure by the TAD regimen was observed.

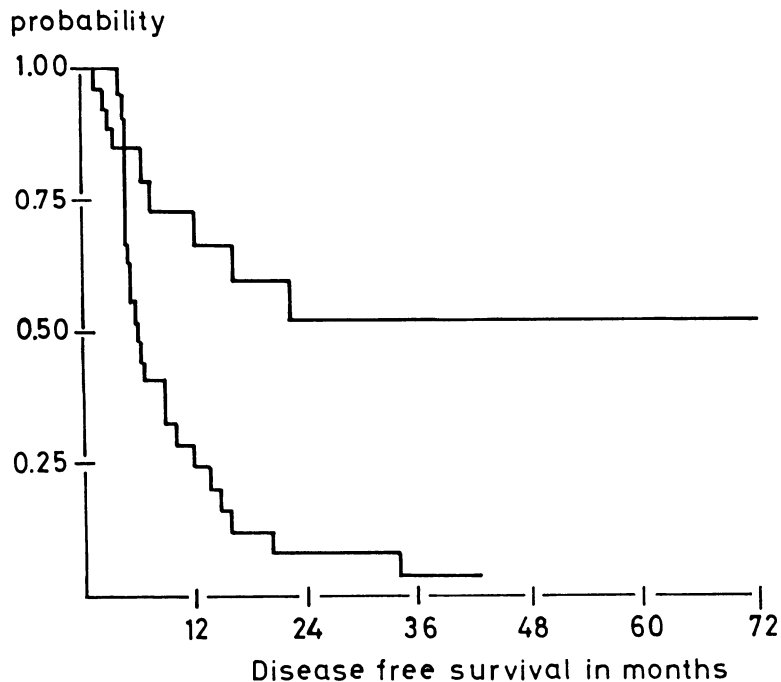
### Overall Survival and Remission Duration

The median survival for all treated patients was 9 months (range 0.1–73 months) and for the patients who achieved complete remission 14 months (range 6–73 months). The median survival of patients who did not enter complete remission was 2.4 months (range 0.2–16 months). The median duration of remission was 8 months (range 2–72 months).

Ten patients remained alive and disease free for more than 3 years (20%), 32% for the transplanted patients and 14% for the group receiving chemotherapy alone (Fig. 1). The long remitters had a lower number of leukocytes at presentation (mean  $7 \times 10.9$ /liter) compared with patients who relapsed within 36 months (mean  $34 \times 10.9$ /liter); this difference was significant ( $P < 0.05$ ). Thirty-two patients had a medullary relapse and one patient had a simultaneous bone marrow and CNS relapse; no isolated CNS relapse occurred. Twenty patients received a bone marrow transplantation: 7 allogeneic and 13 autologous. Proce-



**Fig. 1.** Kaplan-Meier plot of probability of survival from time of complete remission for 49 patients. Twenty received bone marrow transplantation (*upper curve*) and 29 chemotherapy alone (*lower curve*)



**Fig. 2.** Kaplan-Meier plot of probability of disease-free survival for 49 patients. Six patients who died in first complete remission were excluded, five in the transplantation group (*upper curve*) and one in the chemotherapy group (*lower curve*)



dure-related mortality occurred in five patients and seven patients relapsed (six after autologous bone marrow transplantation). Disease-free survival for the transplanted group looked favorable compared with the chemotherapy group (Fig. 2).

## Discussion

The complete remission rate for the whole group was 67% and for patients with de novo ANLL 73%. No remission occurred in six patients with a preceding myelodysplastic syndrome or secondary leukemia. Although Gale [1] found no influence of age on the remission rate, most studies are in agreement with our low remission rate in patients above 60 years, only 28% [11–18]. The dose adjustment we have made for older patients might have been a contributory factor. Other pretreatment characteristics such as FAB subtype, presence of infection or bleeding at admission, or blood counts had no influence on the remission and consolidation rate. The toxicity of the TAD induction and consolidation regimen was low; only five patients died during the induction phase, three from bleeding, one from infection, and one from respiratory distress. Despite this encouraging remission rate the duration of remission was disappointingly low, with a median duration of 8 months, only ten patients (20%) having a remission duration of more than 3 years. There is evidence that prolonged intensive postremission treatment can improve the results [15–19]. More intensive consolidation therapy including high-dose cytarabine is another approach, especially in suitable younger patients. However, consolidation therapy with high-dose cytarabine has a therapy-related mortality of 10%–20% [20–22]. In contrast to our previous experience [6], the incidence of CNS relapse in this study was very low, only one patient having a CNS relapse simultaneous with a bone marrow relapse. This can be attributed to the use of more intensive remission induction therapy combined with prophylactic administration of cytarabine intrathecally, for patients at risk. Allogeneic bone marrow transplantation for younger patients in first remission is the most effective approach for preventing re-

lapse of leukemia [23–27]. Bone marrow transplantation was introduced during the past 3 years of this study. Only 20 patients underwent bone marrow transplantation, 13 autologous and 7 allogeneic, so no good comparison between these data and other studies is allowed. Four out of 7 patients, who received an allogeneic graft died from therapy-related toxicity versus 1 out of 13 patients who received an autologous graft. Six of ten patients with a disease-free survival of more than 3 years were recipients of marrow transplantation. Although the groups are small and transplantation was introduced later, the outlook for patients receiving a transplant seemed much better.

In conclusion, the efficacy of the TAD regimen for adult ANLL was confirmed; however, despite a high remission rate of 73% for de novo ANLL, relapse-free survival was disappointingly low for patients receiving conventional chemotherapy. Long-term disease-free survival in ANLL remains exceptional unless the patient can be treated by an intensive consolidation program, including autologous or allogeneic bone marrow transplantation.

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## Combination Therapy with Mitoxantrone and Etoposide in Adult Acute Myelogenous Leukemia

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### Introduction

Complete remission (CR) rate in adult acute myelogenous leukemia (AML) was improved by the use of aggressive chemotherapy. Today, more than 70% of patients can achieve a CR [1, 2]. But the relapse rate remains high as well as the rate of failure to therapy in myelodysplasia transformed into overt AML. Moreover, so far the value of aggressive chemotherapy is not clarified in the case of a relapse after intensive treatment like autologous blood stem cell transplantation (ABSCT). Mitoxantrone and etoposide have both been shown to be effective as single agents in the treatment of AML [3–6] and the ability to induce a CR in refractory AML was proven for a combination of the two [7]. We therefore studied the effectiveness of a combination therapy of mitoxantrone and etoposide as first-line therapy in AML, myelodysplastic syndromes (MDSs) transformed into AML, and salvage therapy in AML relapsed after ABSCT.

### Patients and Treatment

Overall, 38 patients entered the study. The inclusion criteria are as follows:

1. Acute myelogenous leukemia in elderly patients (>45 years)
2. Acute myelogenous leukemia refractory to standard treatment with TAD

3. Myelodysplastic syndromes transformed into overt AML (>30% blasts in the bone marrow)
4. Acute myelogenous leukemia in relapse after ABSCT

Twenty-two patients suffered from de novo AML; seven of them had received previous chemotherapy according to the TAD protocol [1], but were refractory. In 11 patients MDS was transformed into overt AML with more than 30% blasts in the bone marrow. Five patients relapsed after having received ABSCT, which was followed by a CR for 3–12 months. The chemotherapy regimen is as follows:

1. Regimen
2. Mitoxantrone 10 mg/m<sup>2</sup> per day; single 30-min infusion days 1–5
3. Etoposide 100 mg/m<sup>2</sup> per day; single 240-min infusion days 1–5

The patients received one course of therapy.

### Results

The results are shown in Table 1. The overall CR rate was 52.6% after one course of therapy. In addition, five patients reached a partial remission (PR) which was completed to a CR in one patient with transformed MDS after a second course of therapy. In the elderly patients, 11 out of 15 responded to the combination therapy and 8 reached a CR. Median time to relapse (TTR) was 7.5 months. Also three out of seven patients refractory to the TAD regimen reached a CR. All these three patients could be transplant-

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**Table 1.** Results of combination therapy with mitoxantrone and etoposide in adult AML

	Number of patients	Median age (years)	Response rate after one course of treatment			TTR in months
			CR	PR	NR	
Elderly patients	15	56 (45–69)	8	3	4	7.5 (2–14)
Refractory to TAD pretreatment	7	33 (27–42)	3	0	4	<sup>a</sup>
Transformed MDS	11	64 (20–70)	6	2	3	3.5 (2–6)
In relapse after ABSCT	5	44 (24–48)	3	0	2	<sup>b</sup>

<sup>a</sup> The three patients in CR received ABMT after one consolidation course with mitoxantrone/etoposide

<sup>b</sup> Two patients died in CR (one cardiac failure, one liver failure); one is still alive after 5+ months

ed with autologous bone marrow after one consolidation course with mitoxantrone/etoposide. Eight out of the 11 patients with transformed MDS responded to the therapy and six of them achieved a CR. But the duration of CR was short and median TTR was 3.5 months. Even in the heavily pretreated patients in relapse after ABSCT, complete remissions could be achieved. Three of the five patients of this group were in CR after one course of therapy. One of them died in CR caused by cardiac failure, which was interpreted as a toxic effect of the therapy. Another was transplanted for a second time, but died due to liver failure. The third is still alive in CR with an observation time of 5 months.

### Toxicities

Following treatment, severe aplasia was observed in all patients with the need to substitute red blood cells and platelets. The majority of patients developed stomatitis and mild nausea. One young woman, aged 24 years, died in CR by sudden cardiac failure. This patient has been treated because of a relapse after ABSCT. It was assumed that toxic side effects caused the death in this heavily pretreated patient.

### Conclusions

In this study we demonstrated that the combination therapy with mitoxantrone and etoposide is effective in adult AML. An

overall CR rate of 52.6% was documented after one course of therapy. In de novo AML and MDS transformed into overt AML complete remissions were achieved. Moreover, even in AML relapsed after ABSCT, this regimen is able to induce a CR again.

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## Combination of Mitoxantrone and Etoposide in Patients Aged over 60 Years with Untreated Acute Myelogenous Leukemia

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### Introduction

The antileukemic efficacy of mitoxantrone as a single drug or in combination has been reported by several investigators [1–8]. A previous phase II study demonstrated the efficacy of mitoxantrone in combination with etoposide (MV) in refractory acute myelogenous leukemia (AML) [9]. Our study was undertaken to assess the antileukemic activity and toxicity of MV in patients aged over 60 years with prior untreated AML.

### Patients and Methods

Thirty-one hospitalized adults with previously untreated AML classified according to the French-American-British (FAB) criteria participated in this multicenter study. Patient characteristics are given in Table 1. The regimen consisted of mitoxantrone 10 mg/m<sup>2</sup> per day and etoposide 100 mg/m<sup>2</sup> per day both on days 1–5 given as a short infusion. Toxicity was assessed by the WHO criteria and response by Cancer and Leukemia Cooperative Group B (CALGB) criteria.

### Results and Discussion

Thirty-one patients with AML (age range, 61–76 years) entered the study; 28 are evalu-

**Table 1.** Patient characteristics

N	31
Age (years)	
Mean	68.5
Range	61–76
Sex	
Male	14
Female	17
FAB (N)	
M1	4 (13%)
M2	16 (52%)
M4	5 (16%)
M5	2 (6%)
M6	1 (3%)

able for efficacy. Two patients were too early to evaluate. One patient received an inadequate dosage. Fourteen patients attained a complete remission (50%). Twelve out of these 14 patients achieving complete remission were treated with 2 induction cycles. Five cases of early death (18%) within the first 6 weeks of treatment were recorded. Thirty patients are evaluable for toxicity. Severe myelosuppression was observed in all patients. The maximum duration of neutropenia was 36 days and of thrombocytopenia 39 days. The major nonhematological side effects are summarized in Table 2. In this group of elderly patients the efficacy of MV as induction protocol is comparable with that of daunorubin- and cytarabine-containing regimens. The early death rate was relatively low in this group of elderly patients. However, patient selection might have influenced this parameter.

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**Table 2.** Toxicity (MV I)

	WHO-grade				
	0	1	2	3	4
Nausea	10	10	8	3	0
Stomatitis	11	4	9	7	0
Hepatic	24	5	1	1	0
Cardiac rhythm	22	5	0	3	1
Cardiac function	24	2	0	4	1
FUO	17	0	9	2	3
Pneumonia	28	1	1	1	0
Sepsis	21	0	2	5	3
Granulocytopenia	0	0	0	0	30 <sup>a</sup>
Thrombocytopenia	0	0	0	0	30 <sup>b</sup>

<sup>a</sup> Maximum duration 38 days (mean 19.3)

<sup>b</sup> Maximum duration 45 days (mean 19.5)

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## Mitoxantrone in the Treatment of Acute Leukemia \*

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### Introduction

Mitoxantrone is an anthracenedione with structural similarities to doxorubicin [1]. Preclinical studies showed activity against a variety of experimental mouse leukemias both in vitro and in vivo, including partial activity against a subline of P388 leukemia resistant to doxorubicin [2–4]. Clinical trials with mitoxantrone in patients with relapsed and/or refractory acute leukemia have shown activity close to that seen with the anthracycline antibiotics with a lower incidence of side effects [5–11]. In previously untreated patients with acute lymphatic leukemia (ALL) and acute nonlymphatic leukemia (ANLL), mitoxantrone has shown promise as a frontline treatment [11–14] or in combination with other chemotherapy agents in refractory leukemia [15–17].

### Materials and Methods

From September 1984 to August 1988, 33 patients with ANLL or ALL in relapse or refractory to standard treatment were initially entered on study. From August 1985 to September 1986, 17 patients with previously untreated ANLL were subsequently entered on study. Patients of any age and performance status were allowed on study. Two of the untreated patients had previous

exposure to chemotherapy for metastatic ovary and breast cancer respectively and one was an ANLL patient with myelodysplastic syndrome. All patients had normal renal functions and adequate liver functions and gave written informed consent. The left ventricle ejection fraction (LVEF) was measured at resting nuclear ventriculography at the start of treatment and before each cycle of mitoxantrone. The Eastern Cooperative Oncology Group (ECOG) toxicity criteria were used [18]. Recovery from the side effects of prior chemotherapy was mandatory in previously treated patients.

Mitoxantrone was administered i.v. at a dose of 12 mg/m<sup>2</sup> on five consecutive days (days 1–5). The same dose was used in the induction, second induction, consolidation, and late intensification. Patients whose bone marrow, on day 12, was rendered aplastic or hypocellular with <5% leukemic cells, had a weekly bone marrow aspiration to evaluate bone marrow recovery. Patients not achieving at least M2 marrow (normocellular marrow consisting of 5.1%–25.0% blast cells or 10.1%–30% blast cells plus promyelocytes) after two courses of treatment were removed from study and considered nonresponders.

If significant recovery of marrow elements was present, and an M1 (normocellular marrow consisting of 0%–5% blast cells or 0%–10% blast cells with promyelocytes) or M2 marrow was achieved after one or two courses of therapy, further consolidation therapy was given as soon as the peripheral blood count had recovered to granulocytes  $\geq 2000$  and platelets  $\geq 100\,000$  and the general condition of the patient was acceptable for therapy. Then the refractory/re-

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lapsed group was followed with no further treatment.

In our group of patients with previously untreated ANLL, the induction, second induction, and consolidation were given as the dose described above. Postconsolidation, if patients remained in complete remission the patients were then randomized to the second phase of therapy between observation or late intensification, given twice every 12 weeks. Bone marrow examinations were performed monthly to exclude the possibility of an early relapse. In the second phase of the study, if a patient relapsed on observation, a reinduction was given and, if remission was not achieved, the patient was removed from study. If a patient relapsed on late intensification the patient was removed from study.

The supportive care included blood transfusion – not allowing the hemoglobin to drop below 9 g/liter. Patients received platelet transfusion when the platelet count fell below 20 000/mm<sup>3</sup> or when a tendency to bleed was noted clinically. Surveillance cultures (nose, throat, rectum) and urine and blood cultures were performed on admission and as indicated. Febrile agranulocytic patients were treated with broad-spectrum parenteral antibiotics (amikacin and piperacillin). In patients not responding to the broad-spectrum antibiotics, amphotericin B and/or vancomycin were empirically added to the combination even if no culture was positive for the specific organisms.

## Results

### *Ten Previously Treated Patients with ALL*

There were five males and five females. The median age was 22.5 years (3–47 years), and three patients were <15 years. One patient had had one prior treatment, eight had had two prior treatments, and one had received three prior chemotherapy regimens. Complete remission was achieved in one patient and a partial remission in another patient. Six patients were nonresponders and two died too early to be evaluated for response or toxicity (<6 weeks on study). The response rate was 10%. The median duration of response was 24 weeks. The median sur-

vival from diagnosis was 78 weeks (7–480 weeks) and the median survival on study was 16.5 weeks (3–66 weeks).

### *Twenty-three Refractory/Relapsed Patients with ANLL*

There were 12 males and 11 females. The median age was 41 years (3–68 years) with two patients <15 years and two patients ≥60 years. All the patients had exposure to one standard chemotherapeutic regimen. Complete remission was achieved in 11 patients. Partial remission was achieved in three patients, and seven patients were nonresponders.

Two patients died too early for evaluation. The response rate in this group of patients was 47.8%. The median duration of response was 10.5 weeks (1–35 weeks). The median survival from diagnosis was 54 weeks (6–468 weeks) and the median survival on study was 18 weeks (13–80 weeks).

### *Seventeen Previously Untreated Patients with ANLL*

There were seven males and ten females. The median age was 53 years (2–70 years), with one patient <15 years and six patients ≥60 years. Complete remission was achieved in 11 patients (9 after first induction); 4 of the responders were ≥60 years of age. One of the patients, previously treated for ovarian cancer, also achieved a complete remission. Partial remission was achieved in one patient. Three patients were nonresponders and two patients died too early for evaluation of response. Three of the 11 patients who responded died with aplasia during consolidation treatment. Two patients should have been randomized to late intensification: one refused randomization and in another relapse occurred after consolidation. The three patients randomized to observation all relapsed and died despite the fact that second complete remissions were achieved in two of the patients when reinduced with mitoxantrone. Three patients were randomized to late intensification. One patient (<15 years) refused further treatment, relapsed, and died later. The other



**Table 1.** Toxicity in all the patients treated with mitoxantrone

	ECOG grade				
	1	2	3	4	5
Nausea and vomiting	13 (26%)	7 (14%)	2 (4%)	0	0
Infection	10 (20%)	16 (32%)	8 (16%)	1 (2%)	2 (4%)
Mucosa	3 (6%)	7 (14%)	3 (6%)	0	0
Alopecia	0 (0%)	11 (22%)	0	0	0
Hematological	0	0	0	42 (84%)	0
Diarrhea	3 (6%)	2 (4%)	0	0	0

two patients received late intensification. One of the patients received three lower doses of late intensification, due to a decrease of 49% in the LVEF (total dose of 400 mg). The other patient received two full doses of late intensification (total dose of 400 mg). These two patients are still in complete remission at 184 weeks and 159 weeks, respectively, without any further treatment. The response rate was 65%. Median duration of response was 19 weeks (3–180 weeks). Median survival was 28 weeks (1–184 weeks).

### Toxicity

Toxicities encountered were hematological, infection, nausea and vomiting, mucositis, alopecia, and diarrhea. The incidence and severity of side effects are shown in Table 1. Eight patients developed cardiac toxicity, with a median decrease of LVEF of 20% (range 10%–49%). Six of the patients had prior exposure to anthracyclines.

### Conclusion

In this study mitoxantrone was not an active drug in patients with acute lymphatic leukemia, probably because the majority of patients were in second relapse. The study further confirms the activity of mitoxantrone administered as a single agent in the treatment of acute nonlymphatic leukemia. The most common nonhematological side effects experienced by the patients were infection, nausea and vomiting, alopecia, and mucositis. The cardiac toxicity was acceptable. The two living patients (3 years + 3

years and 5 months postdiagnosis) suggest that mitoxantrone, as a single agent, with late intensification, could totally eradicate nonlymphatic leukemic cells. We suggest that prospectively controlled randomized trials to assess the efficacy of mitoxantrone combinations compared with standard treatment for acute nonlymphatic leukemia are justified.

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## Mitoxantrone, Cytosine Arabinoside, and VP-16 in 36 Patients with Relapsed and Refractory Acute Myeloid Leukemia

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### Introduction

With intensive induction therapy, complete remission can be achieved in 60%–80% of previously untreated adult patients with acute myeloid leukemia (AML) when a combination of cytarabine arabinoside (Ara-C) and daunorubicin is used [1]. However, half of these patients relapse within 12–16 months. Effective cytostatic drugs and combination therapy schemes need to be developed for those patients whose leukemia cells are primary refractory to Ara-C and daunorubicin or become resistant in relapse.

Recently, phase II studies have shown the effectiveness of mitoxantrone in refractory and relapsed acute leukemia [3]. Further studies showed that the combination of mitoxantrone and VP-16 is efficacious with these patients [2]. Ara-C is very effective against AML and has already been used with success in combination with mitoxantrone.

### Aims of This Study

The purpose of this multicenter phase II study was to test the efficacy and toxicity of the combination mitoxantrone, Ara-C, and

VP-16 (MAV) in the treatment of relapsed and refractory acute myeloid leukemia.

### Patients and Methods

#### Patients

Thirty-six patients with a median age of 51 (20–73) years were treated. Diagnosis and classification of AML were carried out following the French-American-British (FAB) classification system.

#### Therapy Schedules

MAV protocol, first dosage level (A):

Mitoxantrone (M)	10 mg/m <sup>2</sup> per day i.v.	Days 1–5
Ara-C (A)	100 mg/m <sup>2</sup> per day, continuous infusion over 24 h	Days 1–5
VP-16 (V)	100 mg/m <sup>2</sup> per day i.v.	Days 1–5

MAV protocol, second dosage level (B):

Mitoxantrone (M)	10 mg/m <sup>2</sup> per day i.v.	Days 4–8
Ara-C (A)	100 mg/m <sup>2</sup> per day, continuous infusion over 24 h	Days 1–8
VP-16 (V)	120 mg/m <sup>2</sup> per day i.v.	Days 4–8

Whenever possible, two consolidation cycles were carried out after reaching complete remission.

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## Results

The results are shown in Tables 1–4 and Fig. 1.

## Conclusions

Mitoxantrone combined with Ara-C and VP-16 is an effective therapy scheme for patients with relapsed or therapy-resistant AML. The complete remission rate of 58.3% is remarkable for high-risk patients or patients who had already been subjected to extensive therapy. This remission rate lies in the upper range of or above the results of comparable studies. Remission lasted a median of 4.5 months and a maximum of 12

months. Although there was marked hematological toxicity, it lasted no longer than after other therapy protocols. However, after a longer therapy with Ara-C and higher doses of VP-16, hematological toxicity lasted substantially longer without any recognizable therapeutic benefit. Nonhematological toxicity and infection rates were not low, but on the other hand not excessive for the treatment of a life-threatening disease and in patients who had been previously subjected to aggressive therapies.

We conclude from these results that MAV therapy represents a very effective antileukemic combination with acceptable toxicity and also that the 5-day MAV treatment is a suitable induction therapy for primary untreated acute myeloid leukemia.

**Table 1.** Therapy results of MAV induction therapy

	MAV A + B	MAVA	MAV B
Complete remission	21 patients (58.3%)	9	12
Partial remission	1 patient (2.7%)	–	1
Treatment failures	9 patients (25.0%)	6	3
Early death cases	5 patients (13.8%)	1	4
Total	36 patients (100%)	16	20

**Table 2.** Response in relation to pretreatment, number of patients

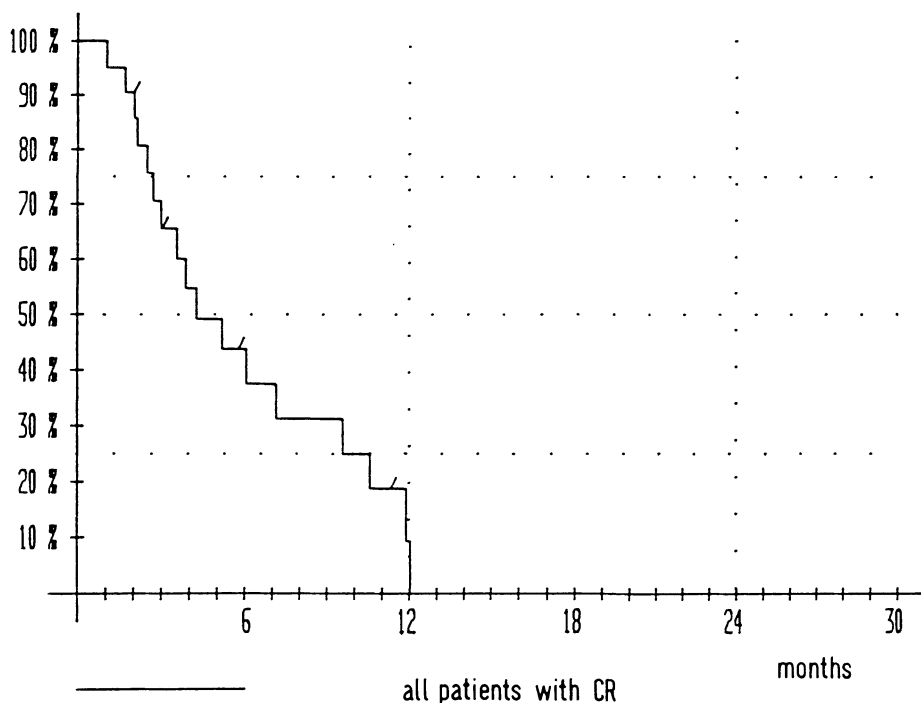
FAB-type	CR	PR	Failures	Early death	Total
High-dose Ara-C	9	–	6	1	16
Other	12	1	3	4	20

**Table 3.** Hematological toxicity of evaluable MAV cycles in surviving patients without previous granulocytopenia

	Evaluable MAV cycles <i>n</i>	Granulocytopenia < 500/μl, median days (range)	Thrombocytopenia < 25000/μl, median days (range)
MAV total	33; 35	23 (7–46)	23 (6–44)
MAVA	15; 18	21 (7–45)	18.5 (6–44)
MAV B	18; 17	23.5 (11–46)	27 (11–43)

**Table 4.** Nonhematological toxicity in 54 MAV cycles; 10 patients died from therapy-related causes: aspiration 1; infection 6; sudden cardiac death 1; hemorrhage 1; unknown 1

	WHO grade (%)			
	1	2	3	4
Hemorrhage	29.6	12.0	5.6	3.7
Bilirubin	1.9	5.6		5.6
Alkaline phosphatase	1.9	1.9		
Glutamic pyruvic transaminase	7.4	9.3	7.4	5.6
Nausea	20.4	37.0	25.9	
Diarrhea	24.1	16.7	11.1	
Mucositis	18.5	29.6	14.8	7.4
Creatinine	5.6	1.9		1.9
Proteinuria	18.5			
Hematuria	25.9	1.9		1.9
Skin reaction	11.1	22.2		
Local infection	5.6	7.4	3.7	1.9
Sepsis		3.7	11.1	9.3
Fever of unknown origin	5.6	38.9	13.0	
Cardiac arrhythmia	7.4			1.9
Congestive heart failure	1.9	1.9	5.6	1.9
Neurological symptoms	11.1	1.9		



**Fig. 1.** Event-free survival of patients in complete remission ( $n = 21$ ), median 4.5 (1–12) months from time complete remission was achieved

## Summary

Mitoxantrone in combination with VP-16 proved to be effective in refractory and relapsed acute myeloid leukemia (AML), with 42% of patients achieving complete remission (CR) [2]. The aim of this study was to assess whether the addition of cytosine arabinoside increased the response rate at a tolerable toxicity. The regimen consisted of mitoxantrone (M) 10 mg/m<sup>2</sup> i.v. days 4–8, cytosine arabinoside (A) 100 mg/m<sup>2</sup> continuous infusion days 1–8, and etoposide (VP-16) (V) 100–120 mg/m<sup>2</sup> i.v. days 4–8 (MAV protocol) for relapsed and refractory AML. Thirty-six patients were treated, with a median age of 51 (20–73) years. For induction therapy one to two MAV cycles and for consolidation therapy two courses were scheduled. Twenty-one (58.3%) patients attained a complete remission (CR), with a median duration of 4.5 (1–12+) months. The median survival of all patients was 5.5 (0.5–15.5+) months. Four patients died in CR from chronic infections or after consolidation therapy with MAV. In evaluable patients, times to > 500 granulocytes/ $\mu$ l and > 25 000 platelets/ $\mu$ l were 23 (7–46) and 23 (6–44) days, respectively. In 54 evaluable MAV

courses the following toxicity was observed (WHO grades 3/4): 26%, nausea and vomiting; 9%, hemorrhage; 6%, bilirubinemia; 11%, diarrhea; 22%, mucositis; 6%, local infection; 20%, septicemia; 13%, fever of unknown origin; 2%, cardiac arrhythmia; 7%, congestive heart failure. We conclude that MAV therapy is a highly active antileukemic combination with acceptable toxicity, which is recommended for further clinical trials in untreated AML.

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## Mitoxantrone and Etoposide in Patients with Relapsed and Refractory Acute Nonlymphocytic Leukemia\*

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### Introduction

The treatment of relapsed or refractory ANLL remains mostly unsatisfactory. New drug combinations are being studied to determine the most efficacious therapy for these patients. Mitoxantrone is a synthetic aminoanthraquinone that intercalates into DNA [1, 2] and has previously been shown to have activity in ANLL [3–5]. Similarly, etoposide, or VP-16-213, has been shown to have activity in ANLL when used as a single agent [6, 7]. Because of the significant antileukemic activity of these two drugs, a clinical phase II study was initiated by the Eastern Cooperative Oncology Group (ECOG) to evaluate the drug combination mitoxantrone and VP-16-213, in relapsed and refractory patients with ANLL.

### Materials and Methods

In May 1987, the ECOG activated a study for relapsed and refractory ANLL. The number of patients treated, morphological subtypes, and other characteristics are shown in Table 1.

Patients treated in this study were categorized into three groups:

- first relapse, including those who relapsed within 6 months of attaining a first remission;
- second relapse; and
- patients refractory to either initial induction therapy or to reinduction therapy given for treatment of relapse.

Criteria for ineligibility were:

- age > 65 years;

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**Table 1.** Study population

Patients treated	70
Data available on	50
evaluable patients	44
M:F	1:1.5
Median age	46 years (18–63) 8 patients ≥ 60 years
Morphological subtype	
FAB classification	Number of patients
M1	18
M2	10
M3	1
M4	7
M5	6
M6	4
M7	1
Unclassified	3

**Table 2.** Nonhematological toxicity

Toxicity	N (%)	Grade ½ (%)	Grade 3 (%)	Grade 4 (%)
Vomiting	29 (69)	27 (64)	2 (5)	–
Diarrhea	17 (40)	15 (36)	2 (5)	–
Mucositis	28 (67)	13 (31)	15 (36)	–
Hepatic	12 (29)	11 (26)	–	1 (2)
Cardiac	1 (2)	–	1 (2)	–

Numbers and percentages (in parentheses) refer to toxicity in 42 patients with available data. There was no grade 5 (lethal) toxicity from any of the above causes

- b) prior therapy with either VP-16-213 or mitoxantrone;
- c) prior treatment with >550 mg/m<sup>2</sup> Adriamycin or >800 mg/m<sup>2</sup> daunorubicin;
- d) patients with performance status (ECOG) 3 or 4; and
- e) major cardiac, renal, or hepatic dysfunction.

Therapy consisted of mitoxantrone 12 mg/m<sup>2</sup> and VP-16-213 100 mg/m<sup>2</sup>, both drugs given daily on days 1–5. Because of early toxicity monitoring, the first five patients received the mitoxantrone at a dose of only 10 mg/m<sup>2</sup>. The dose of VP-16-213 remained identical for all patients. The mitoxantrone was administered i.v. over 15 min and the VP-16-213 was given i.v. over 30–60 min. The study design included a repeat cycle of mitoxantrone/VP-16-213, in identical doses, on days 12–14 if the bone marrow showed residual leukemia.

Evaluation of antileukemic response was based on ECOG criteria as previously described [8]. Toxicity was quantitated using the ECOG grading system [9], where the grades 1, 2, 3, 4, and 5 refer to mild, moderate, severe, life-threatening, and lethal toxicity, respectively (Table 2).

## Results

Table 3 outlines the overall response seen in this patient population. Included are the results for all 50 patients as well as the responses for just the 44 evaluable patients. Six patients were considered non-evaluable mainly because of major protocol violation

**Table 3.** Response to treatment

	Response	# of patients	Percentage
Of 50 treated patients	Complete remission	20	40%
	Partial remission	2	4%
	No response <sup>a</sup>	28	56%
Of 44 evaluable patients	Complete remission	20	45%
	Partial remission	2	5%
	No response <sup>a</sup>	22	50%

<sup>a</sup> Includes six early deaths

affecting the total dose of mitoxantrone and VP-16-213 that was given. Of the 44 evaluable patients, 50% responded; 45% achieved a CR and 50% a PR.

Table 4 outlines the response rate among the 44 evaluable patients in the three different patient groups. The best results were seen in patients who entered the study in first relapse. The CR rate was 63% and it should be noted that more than half the patients in this group relapsed within 6 months of achieving their first CR. For second-relapse patients the CR rate was 50%, but, to date, only six patients have been treated in this group. Refractory patients did not do well, with only 2 out of 14 achieving a CR. Six patients died (12% of all treated patients) while receiving their first or second induction course, all from bacterial or fungal sepsis. These patients are considered in this study as “non-responders.”



**Table 4.** Response by disease state

Patients who entered study as	Total number of patients	Complete remission	Partial remission	No response <sup>a</sup>
First relapse	24	15 (63%)	1	8
Second relapse	6	3 (50%)	1	2
Refractory	14	2 (14%)		12

<sup>a</sup> Includes six patients who died while receiving their first or second induction course

The median duration of CR has not yet been achieved, but is at least 104 days (range, 14–426 days). The median survival of all patients (50) was 90 days (range, 18–470 days). There was no correlation between CR rate and morphological subtype (by FAB classification). The nonhematological toxicity is summarized in Table 2. The regimen was well tolerated and there was only one patient with grade 4 (hepatic) toxicity and there was no grade 5 toxicity from any nonhematological cause.

### Conclusions

The combination of mitoxantrone and VP-16-213 appears to be an effective and well-tolerated chemotherapeutic regimen for patients with ANLL, thus confirming other reports [10, 11]. It is difficult to compare results with other reports or treatment modalities because of the often different patient populations studied.

The greatest efficacy of this combination in this study appeared to be for treatment of patients in first relapse, even after a short (<6 months) first remission. The combination is also active in the second relapse and in refractory patients, although the results in this latter group were poor (14% CR). It must be emphasized that this is a very difficult group of patients to treat and many published reports on “refractory” patients also include patients in first relapse (<6 months).

This is an ongoing ECOG study and more patients are being accrued. The overall results presented must therefore be considered tentative and, as more patients are being

treated, a more precise determination will be made of the true efficacy of this drug combination.

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## Continuous Infusion of Mitoxantrone Combined with High-Dose Cytarabine in Refractory/Relapsed Acute Myeloblastic Leukemia and Blast Crisis of Chronic Myelogenous Leukemia

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### Introduction

Cell kinetic studies revealed a conditioning effect of cytosine arabinoside (Ara-C) for the subsequent administration of daunorubicin or mitoxantrone [1–3]. These data are further supported by experimental results indicating a time-dependent synergistic effect of high-dose cytosine arabinoside (HD Ara-C) and mitoxantrone [4]. The significant antileukemic activity of a combination chemotherapy consisting of HD Ara-C and mitoxantrone (HAM) could be demonstrated in patients with refractory acute myeloblastic leukemia (AML) by achieving complete remissions in 30%–53% of the patients [5, 6]. Using rigid eligibility criteria for patients with refractory AML, the original HAM protocol revealed a remarkably low rate of 12% HAM-resistant leukemias, but an unacceptably high early death rate of 33% [5]. In an attempt to reduce early death rates and nonhematological toxicity we developed a modified protocol (c-HAM) including continuous administration of mitoxantrone combined with HD-Ara-C in patients with refractory/relapsed AML and chronic myeloblastic leukemia (CML) blast crisis.

### Patients and Methods

The prospective phase II study comprised 20 patients (median age, 49 years; range, 18–78 years) from Vienna and Innsbruck. Eleven patients suffered from AML, two having an antecedent myelodysplastic syndrome which turned into an overt AML. Two patients were classified as having ALL, one as having double leukemia, and one showed a relapse after allogeneic bone marrow transplantation (HBMT). One patient had refractory anemia with an excess of blasts in transformation (RAEB-t) and four patients presented with blast crisis of chronic myelogenous leukemia (CML-BC).

Patients with AML were refractory or relapsed after induction courses with daunorubicin, cytarabine, thioguanine (DAT) or amsacrine, cytarabine, thioguanine (AAT) according to the protocol we published recently [7]. The c-HAM treatment regimen consisted of HD-Ara-C 3 g/m<sup>2</sup> every 12 h by a 3-h infusion on days 1+2 combined with mitoxantrone 10 mg/m<sup>2</sup> given by 21-h infusion on days 1+2. In eight patients the pharmacokinetics of mitoxantrone were evaluated using a specific HPLC assay.

Toxicity was assessed according to the WHO criteria [8] while the antileukemic efficacy was judged according to the cooperative acute leukemia group-B (CALGB) criteria [9].

### Results

Ten of the 20 patients were treated with one course of c-HAM while a second cycle was

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**Table 1.** Nonhematological toxicity

WHO grade	0	1	2	3	4
GI tract	3	11	3	3	–
Kidney	15	4	1	–	–
Liver	16	4	–	–	–
Cerebral	19	1	–	–	–
Heart	20	–	–	–	–

given in 7 cases and a third cycle in 3 patients, accounting for a total of 33 treatment courses. A summary of the nonhematological side effects is given in Table 1. Severe side effects with toxicity grades 3 and 4 according to the WHO criteria were seen only in 3 patients (7%) suffering mainly from diarrhea, nausea, vomiting, and mucositis. We observed no significant toxicity (grades 3+4) concerning kidney, liver, CNS, or heart.

Results of hematological toxicity after 33 cycles of a c-HAM regimen are illustrated in Table 2. A decrease in leukocytes  $< 500/\mu\text{l}$  was achieved after 7 (2–24) days, and a nadir of leukocytes occurred after 9 (2–18) days. The median time for the recovery of granulocyte counts to values above  $500/\mu\text{l}$  was 24 (30–40) days, for thrombocyte counts above  $20\,000/\mu\text{l}$  16 (10–25) days from the onset of therapy. In eight patients plasma mitoxantrone concentration was measured using a specific HPLC assay. Plasma was obtained before treatment and then ½, 1, 3, 6, 24, 27, 33, and 48 h later and then daily up to day +15. The exact pharmacokinetic studies including plasma concentration versus time data have been described previously [10].

**Table 2.** Myelosuppression during therapy with c-HAM ( $n=33$ )

		Days median (range)
Leukocytes ( $10^6/\text{liter}$ )	$< 500$	7 (2–14)
Nadir of leukocytes		9 (2–18)
Granulocytes	$> 500$	24 (13–40)
Leukocytes	$> 1000$	21 (14–42)
Leukocytes	$> 2000$	26 (9–48)
Thrombocytes	$> 20\,000$	16 (10–25)

Table 3 shows response rates and survival of all patients after treatment with c-HAM. Eight patients achieved a complete remission (CR) which was obtained in 7 of the 11 cases (64%) with AML and in 1 patient with RAEB-t. Five patients (three AML, two ALL) achieved a partial remission (PR), accounting for an overall response rate (CR + PR) of 81%. Four patients underwent successful HBMT. Four patients with CML-BC achieved remission to the chronic phase with survivals of 8, 8, 10+, and 13+ months.

## Discussion

The preliminary results clearly demonstrate a high antileukemic effect of the c-HAM regimen. Although we did not use such rigid eligibility criteria defining refractory disease as described by Hiddemann et al. [11], the response rate of 64% CR compares favorably with other HD Ara-C combinations. It has been demonstrated for bolus administration of mitoxantrone that the mean plas-

**Table 3.** c-HAM regimen in refractory/relapsed ANLL and CML-BC

Patient	Diagnosis	CR	PR	NR	Survival (months)
11	ANLL	7	3	1	4, 4, 5, 5, 6, 7, 6+, 10+, 12+, 14+, 21+
1	Double leukemia			1	3
1	ANLL-relapse after HBMT			1	1
1	RAEB-T	1			16+
2	ALL		2		5+, 14
4	CML-BC		4		8, 8, 10+, 13+

ma mitoxantrone level 60 min after a 1 h infusion decreased to below the mean lethal concentration reported for human cells exposed for 1 h in vitro [12]. In addition, patients who achieved a CR had higher plasma levels of mitoxantrone at their daily nadir than did those patients who manifested resistant disease [13]. In our study the mean plasma concentration of mitoxantrone remained constant and ranged about 200 ng/ml 15 days after onset of therapy. Thus administering mitoxantrone as continuous infusion we succeeded not only in significantly increasing the daily nadir but also in achieving a constant level of the drug above a concentration necessary to kill human cells in vitro [12].

In no case of that study did we observe an early death. Nonhematological toxicity in our series compared favorably with similar treatment protocols [5, 14]. Considering the limits of interstudy comparison our data suggest that the c-HAM regimen is at least equivalent to the original HAM protocol [5] or to the S-HAM protocol [14]. The combination of HD Ara-C and the continuous infusion of mitoxantrone (c-HAM) achieves a constant, high, long-lasting plasma level of mitoxantrone. The regimen seems to offer comparable response rates, with less toxicity and better tolerance, resulting in a high therapeutic index for the patient.

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## Intermediate-Dose Ara-C/m-AMSA for Remission Induction and High-Dose Ara-C/m-AMSA for Intensive Consolidation in Relapsed and Refractory Adult Acute Myelogenous Leukemia

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### Introduction

High-dose Ara-C regimens (HD-Ara-C) designed as intermittent infusions of  $3 \text{ g/m}^2$  over 1–3 h at 12-h intervals have shown good effectivity either as single-drug treatment or in combination with anthracyclines, m-AMSA, or L-asparaginase [1–5]. However, remission induction has been achieved at the expense of a considerable treatment-associated death rate. In our present approach we examine intermediate-dose Ara-C (ID-Ara-C) for treatment of refractory and relapsed acute AML. The dose reduction to  $1 \text{ g/m}^2$  performed in ID Ara-C was anticipated to decrease treatment-related toxicity, while treatment efficacy should not be impaired [6–8].

### Patient Characteristics and Methods

Patients of all age groups and French-American-British (FAB) subtypes with relapsed or primary refractory AML were included in this phase II study. The majority of relapsed patients experienced their relapse during or after completion of intensive maintenance treatment according to the EORTC-AML 6 study [9, 10]: they had been randomized to either repeated courses of the induction-type (DNR  $45 \text{ mg/m}^2$  i.v. day 1 plus Ara-C  $100 \text{ mg/m}^2$  s.c. days 1–5) or to non-cross

resistant drugs alternating m-AMSA ( $150 \text{ mg/m}^2$  i.v. day 1) plus Ara-C ( $3 \text{ g/m}^2$  i.v. q 12 h days 1 and 2) with m-AMSA plus 5-AZA ( $150 \text{ mg/m}^2$  i.v. days 1–3). A total of six intensive maintenance courses were given every 6 weeks. Refractory patients were defined as being resistant to two courses of an anthracycline-containing induction regimen totaling six doses of DNR ( $6 \times 45 \text{ mg/m}^2$ ) combined with 14 doses of Ara-C ( $14 \times 200 \text{ mg/m}^2$ ). Patients with a history of myelodysplastic syndrome (MDS) or a second malignancy were included.

The remission induction regimen consisted of ID Ara-C,  $1 \text{ g/m}^2$  i.v. every 12 h by a 2-h infusion for 6 days and m-AMSA  $120 \text{ mg/m}^2$  i.v. on days 5, 6, and 7. One or two cycles for induction were given. When CR was reached, one consolidation course was administered consisting of HD Ara-C,  $3 \text{ g/m}^2$  i.v. every 12 h by a 2-h infusion for 4 days and m-AMSA  $120 \text{ mg/m}^2$  i.v. day 5. The treatment-free interval between two induction cycles was 3 weeks. The interval between the end of induction and the beginning of consolidation was 4 weeks. No further therapy was given thereafter.

### Results

A total of 34 patients entered the study: 6 were refractory and failed previous standard remission induction treatment, 28 were treated for relapse (26 in first and 2 in second relapse). One relapsed and one refractory patient had a previous history of MDS, another patient suffered in addition to re-

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**Table 1.** Patient characteristics

Total number	34
Age (years), median (range)	44 (18–66)
FAB M1	1
M2	13
M3	4
M4	10
M5	5
Refractory	6
Relapsed	28
First	26
Second	2
Duration of preceding remission (months, median)	8
Time from last chemotherapy to relapse (months, median)	3.1
Type of preceding maintenance	
Intensive (EORTC AML 6):	
DNR, Ara-C (induction type)	13
HD Ara-C, mAMSA/5-AZA, mAMSA	8
Conventional (EORTC AML 5)	1
No maintenance	6

fractory AML from a cervical cancer stage II–III. The median age was 44 years. The patient characteristics are shown in Table 1.

One out of six refractory patients achieved CR after 1 cycle of ID Ara-C/m-AMSA. Two patients remained refractory to two reinduction cycles and three died in hypoplasia, one with concomitant cervical cancer and one with a history of MDS. Twenty-two out of 28 relapsed patients (79%) reached CR, 17 after 1 cycle of ID Ara-C. Three patients were refractory to two courses of this regimen; three died during hypoplasia without evidence of leukemic regrowth. Three patients died in CR after intensive consolidation with HD Ara-C (Table 2). Three patients were transplanted in second remission, two of them received an allograft and are in continued CR 14 and 21.5 months after bone marrow transplantation (BMT), and one patient received an autograft but died shortly thereafter.

Table 3 shows that responding patients had a longer duration of preceding remission (9.5 vs. 4.5 months) and a longer interval from last chemotherapy to relapse (3.5

**Table 2.** Response to treatment

Total number	34
Refractory	6
Complete remission (one cycle)	1
Failure (refractory)	2
Hypoplastic death	3
Relapsed	28
Complete remission	22/28 (79%)
After one cycle	21
After two cycles	1
Failure (refractory)	3
Hypoplastic death	3
Hypoplastic death in CR (after consolidation)	3

**Table 3.** Remission incidence according to pre-treatment characteristics

Total relapsed	28
Complete remission	22
Duration of preceding remission (months, median)	9.5
Time from last chemotherapy to relapse (months, median)	3.5
Type of preceding maintenance	
Intensive (EORTC AML 6):	
DNR/Ara-C (induction type)	11
HD Ara-C, m-AMSA/5-AZA, m-AMSA	6
Conventional	1
No maintenance	4
Failure	6
Duration of preceding remission (months, median)	4.5
Time from last chemotherapy to relapse (months, median)	2
Type of preceding maintenance	
Intensive	
DNR/Ara-C (induction type)	2
HD Ara-C, m-AMSA/5-AZA, m-AMSA	2
No maintenance	2

vs. 2 months) than nonresponders. The type of preceding maintenance (induction-type vs. HD Ara-C-containing regimen) had, most remarkably, no impact on achieving another CR or not. It is noteworthy that two patients in first relapse, who reached a sec-

ond remission of 10 months and 24 months duration respectively with this program, achieved a third remission with the identical regimen and are still in CR at +6 and +7 months. The median DFS of relapsed and refractory patients was 3.3 months, the median survival of responders 4.6 (Fig. 1), and the overall survival 4.7 months (Table 4). Patients receiving BMT were excluded from this analysis at the time of BMT.

The major toxicity seen in these patients was a non-cardiogenic pulmonary edema due to ID/HD Ara-C as substantiated in detail elsewhere [12]. So far 7 out of 34 patients (20%) (3 patients have not yet received consolidation therapy using HD Ara-C) experienced this type of lung toxicity either in combination with or without infection. Three patients recovered, and four died. From our data, the incidence of pulmonary edema was significantly related to the type of preceding intensive maintenance: 2/12 = 17% arriving from the induction type and 3/8 = 38% ( $P \leq 0.05$ ) pretreat-

**Table 4.** Response to treatment

Disease-free survival (months, median)	
Relapsed ( $n=20$ )	3.3
Relapsed + refractory ( $n=21$ )	3.3
Survival CR	
Relapsed	4.5
Relapsed + refractory	4.5
Survival all	
Relapsed ( $n=25$ )	4.7
Relapsed + refractory ( $n=31$ )	4.5

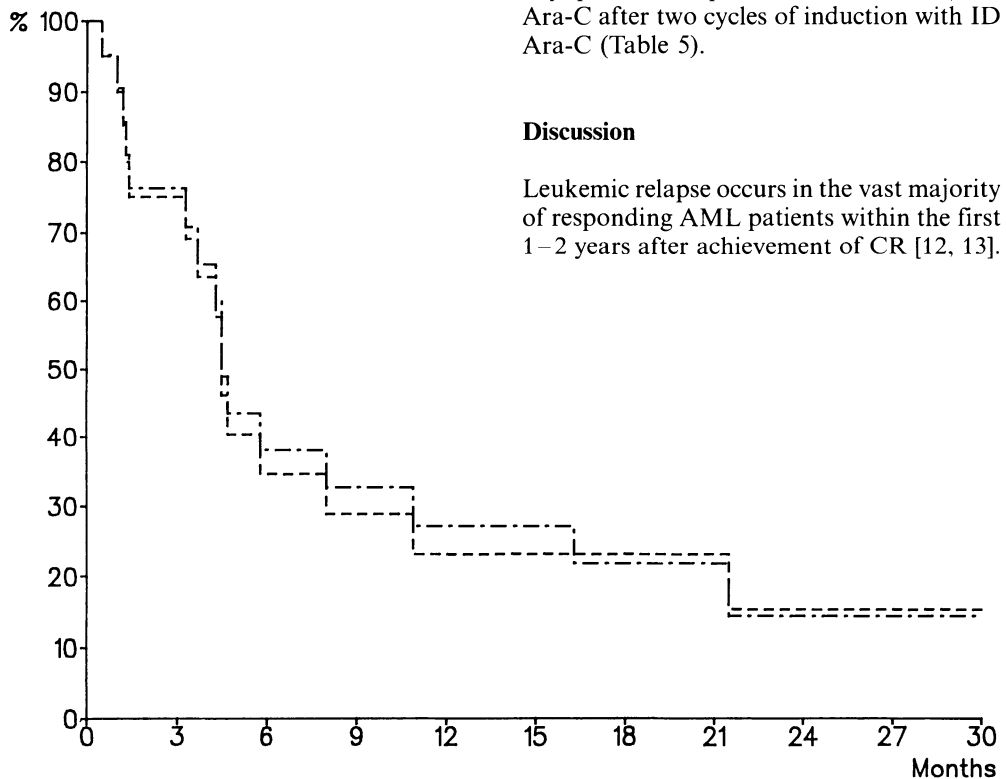
<sup>a</sup> Patients receiving BMT ( $n=3$ ) were excluded at the time of BMT

<sup>b</sup> Three patients are too early for this evaluation

ed with the HD Ara-C containing maintenance. One patient with this type of toxicity underwent a previous "low-dose" conventional maintenance program 3 years in duration and developed a lethal non-cardiogenic pulmonary edema after the HD Ara-C consolidation course. Another relapsed patient died of this complication without having any previous exposure to ID Ara-C/HD Ara-C after two cycles of induction with ID Ara-C (Table 5).

## Discussion

Leukemic relapse occurs in the vast majority of responding AML patients within the first 1–2 years after achievement of CR [12, 13].



**Fig. 1.** Survival: - - - - relapsed patients ( $n=20$ ); - - - - - relapsed + refractory patients ( $n=21$ )



**Table 5.** Lung toxicity

Total	7/34	
Recovery	3	
Death	4	
Type of preceding maintenance		
Intensive (EORTC AML 6)		
DNR, Ara-C, (induction type)	2/13 (15%)	<i>P</i> =0.05
HD Ara-C, m-AMSA/5-AZA,	3/8 (38%)	
m-AMSA		
Conventional	1/1	(after consolidation)
No maintenance	1/6 (16%)	(after second cycle of induction)

Reinduction with HD Ara-C as single drug or in combination with anthracyclines, m-AMSA, or L-asparaginase achieves CR rates between 60% and 70% [1, 3–5]. However, HD Ara-C-containing regimens are associated with severe toxicity, contributing to a substantial treatment-induced mortality. In fact, Ara-C-induced toxicity correlates directly with the cumulative amount of drug administered [14, 15].

Sufficient pharmacological data have been accumulated to advocate that reduction of Ara-C to intermediate-dose regimens (1 g/m<sup>2</sup> per 2 h) will not impair maximal accumulation of the active Ara cytidine triphosphate (CTP) in leukemia cells [6, 8, 16] and thereby the treatment outcome. Following this approach we investigated a regimen consisting of ID Ara-C and m-AMSA for its efficacy in relapsed and primary refractory leukemia (*n* = 29) (Table 2). Complete remission was achieved in nearly 80% (18/23) of patients with relapsed AML. This observation is in accordance with recent reports showing CR rates of 71%–83% induced by ID Ara-C [17–19] which are equivalent to those reached with HD Ara-C-containing regimens.

A treatment-related mortality of 11% in our study compares favorably with mortality rates between 27% and 33% observed in HD Ara-C regimens [4, 5, 20]. Interestingly, despite a marked reduction of treatment-related mortality using ID Ara-C, the remission incidence is at least equivalent to or superior to HD Ara-C in relapsed AML. Duration of preceding remission or time from last chemotherapy to relapse appear to

be prognostic factors for remission induction. In patients achieving CR, the duration of preceding remission was 9.5 months vs. 4.5 months in failing patients (Table 3). Comparably, the time from last chemotherapy to relapse amounted to 3.5 months in responding patients vs. 2 months in failing patients. The type of maintenance therapy preceding relapse had no influence on the outcome of the reinduction therapy (Table 3). Thus treatment failures were equally distributed between patients who had undergone either no maintenance or induction-type maintenance (DNR/Ara-C) or HD Ara-C containing maintenance. The significance of this observation will have to be evaluated with greater numbers of patients.

Despite a good CR rate using ID Ara-C/m-AMSA and despite intensive consolidation with HD Ara-C, disease-free survival was short and the overall survival time 4.7 months. It should be stressed, however, that three patients receiving BMT were censored at the time of transplantation. In view of considerable toxicity seen after the consolidation treatment (3/18 toxic deaths), the value of an intensive consolidation regimen for prolongation of DFS and survival appears questionable in patients with relapsed leukemia.

In primary refractory patients only one out of six achieved CR after treatment with ID Ara-C/m-AMSA (Table 2). It should be noted that the incidence of hypoplastic death in this group was considerable at 50% (3/6). Previous studies using HD Ara-C in refractory AML, either as single-drug regimen or in combination, induced CR rates of

14%–56% [1, 3, 18, 20]. Although the number of patients in this analysis is too small to allow final conclusions, it appears that ID Ara-C/m-AMSA is insufficient as treatment of refractory AML. Life-threatening toxicity occurred as non-cardiogenic pulmonary edema [11, 21, 22] in 20% (7/34) of the patients (Table 4). The incidence of pulmonary edema was significantly related to the type of preceding maintenance treatment.

In conclusion, guided by pharmacokinetic studies, we have reduced the Ara-C dosage in relapsed and refractory AML patients and thereby reduced the treatment-related death rate markedly without loss of treatment efficacy in respect to CR rate. The impact of intensive consolidation on DFS and survival using HD Ara-C is questionable.

### Summary

Thirty-four consecutive patients with either relapsed ( $n=28$ ) or primary refractory AML ( $n=6$ ) were treated with one or two cycles of intermediate-dose (ID) cytosine arabinoside (Ara-C) ( $1 \text{ g/m}^2$  i.v. q 12 h days 1–6) and amsacrine (m-AMSA) ( $120 \text{ mg/m}^2$  i.v. days 5–7). Patients reaching complete remission (CR) were consolidated with one cycle of Ara-C  $3 \text{ g/m}^2$  i.v. q 12 h days 1–4 and m-AMSA  $120 \text{ mg/m}^2$  i.v. day 5. The median duration of the preceding remission was 8 months and median time from last chemotherapy until relapse 3.1 months. Of the relapsed patients, 22/28 (79%) achieved CR regardless of the type of prior intensive maintenance (HD Ara-C/m-AMSA/5-azacytidine) (AZA) or daunorubicin (DNR/CD-Ara-C). Three of the 28 (11%) patients died during hypoplasia; 3/28 (11%) were refractory to  $2 \times$  ID-Ara-C/m-AMSA. Three of the 28 patients died in CR during hypoplasia after intensive consolidation with HD-Ara-C. Predictive factors for remission were duration of preceding remission and the time from last chemotherapy to relapse. Three patients were transplanted in second CR. One of the six refractory patients reached CR, two remained refractory, and three died during hypoplasia. The median duration of disease-free survival (DFS) of relapsed patients was 3.3 months without further treatment; median survival of re-

sponding patients (20 relapsed patients, 1 refractory patient) was 4.5 months, overall survival ( $n=29$ ) was 4.8 months. Patients receiving BMT were censored at the time of BMT. Seven patients experienced lung toxicity due to Ara-C, four of whom died. The incidence of lung toxicity was clearly related to the extent of Ara-C pretreatment during intensive maintenance. In conclusion, ID Ara-C/m-AMSA is a very effective reinduction treatment in these patients with acceptable toxicity, and the impact of HD-Ara-C during consolidation for DFS and survival is questionable.

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## Treatment of Recurrent Acute Myelogenous Leukaemia at a Single Centre Over a 10-Year Period

C. L. Davis, A. Z. S. Rohatiner, J. Amess, J. Lim, and T. A. Lister

### Introduction

Complete remission (CR) may be achieved in the majority of younger adults with acute myelogenous leukaemia (AML). However, with conventional treatment, only 25% are cured since most will subsequently relapse and die. The results of treatment in 232 newly diagnosed patients referred to St. Bartholomew's Hospital over the past 10 years illustrate these points and highlight the difficulties inherent in the treatment of AML at relapse. All patients were initially treated with curative intent with "short-term therapy" according to one of four protocols as described previously [1]. Complete remission was achieved in 144/232 (62%) patients; 86/144 (60%) subsequently relapsed. The management and outcome of the latter patients form the basis of this report.

### Patients and Methods

#### Patients

The study population comprised 86 consecutive adults (age range 15–59, median 44 years) who had relapsed following treatment with short-term therapy [1]. The median time from first remission to relapse was 8 months (range 1 month to 3¼ years).

#### Treatment (Table 1)

Further therapy was considered appropriate in 75 patients; combination chemotherapy was administered to 58 patients according to phase II protocols in use at that time, and 17 received single-agent therapy amsacrine (m-AMSA) or alpha-interferon ( $\alpha$ -IFN) in a phase I setting [2–6]. Eleven patients received supportive care only.

Table 1. Response

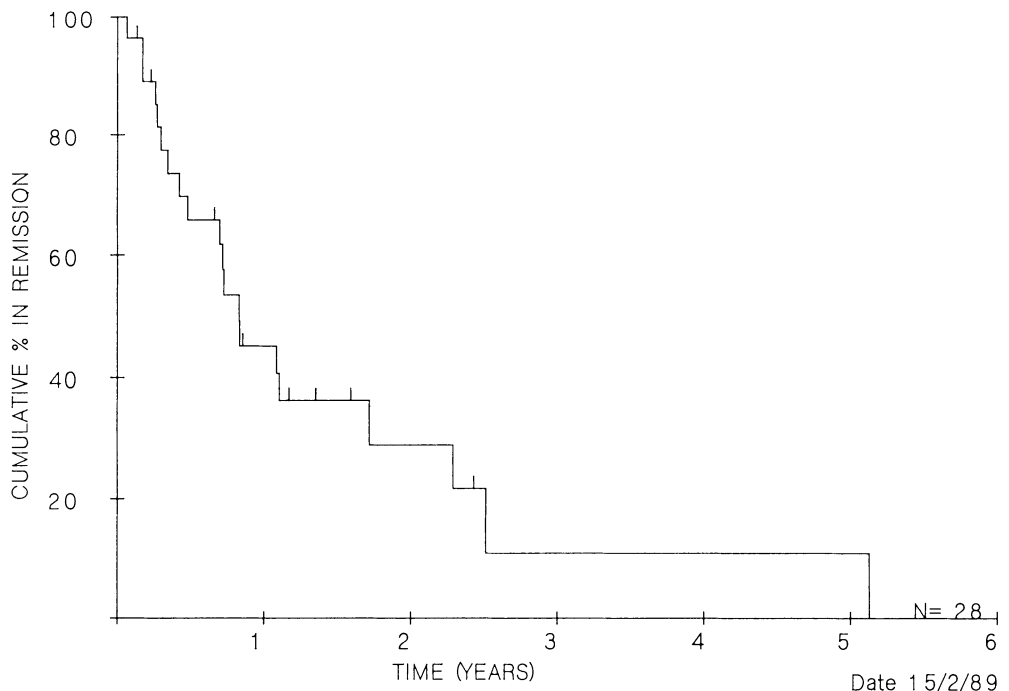
Treatment	Second CR rate
m-AMSA/Ara-C (2)	9/19
High-dose Ara-C-containing regimes (3, 4)	8/25
Adr/Ara-C/6-TG (1)	5/6
MTN/Ara-C (current study)	5/8
m-AMSA (phase I study) (5)	1/10
IFN (phase I study) (6)	0/7
	28/75

m-AMSA, amsacrine; Ara-C, cytosine arabinoside; HD Ara-C, high-dose cytosine arabinoside; ADR, Adriamycin; 6-TG, 6-thioguanine; MTN, mitoxantrone; IFN, interferon

### Results

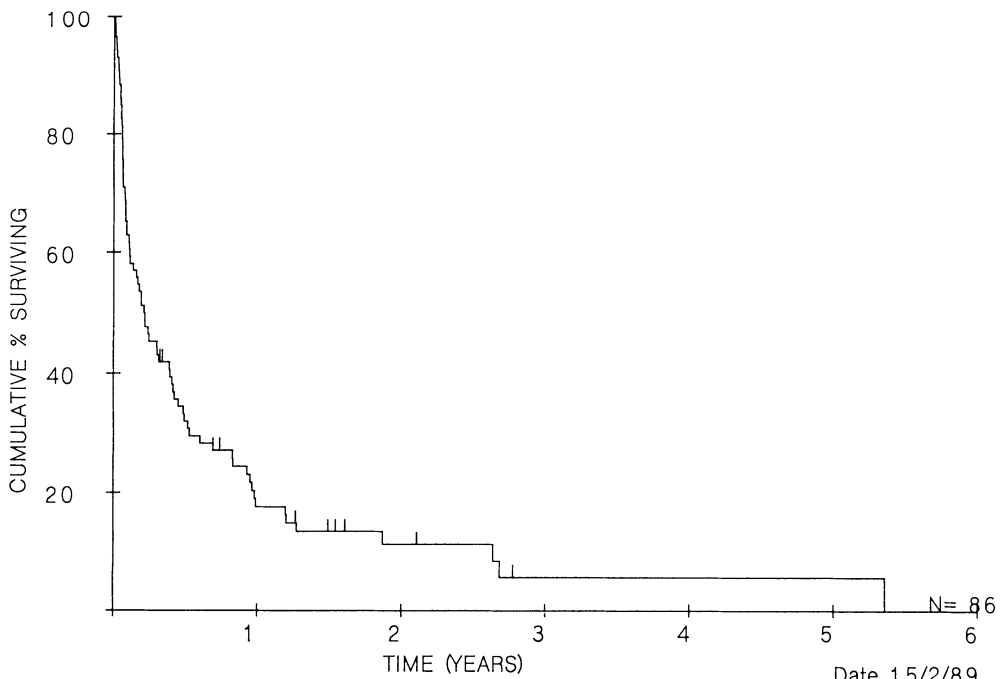
Overall, second CR was achieved in 28/75 (37%) patients (1/17 and 27/58 treated in the phase I studies and combination chemotherapy respectively). There was no correlation

Imperial Cancer Research Fund (ICRF), Department of Medical Oncology, St. Bartholomew's Hospital, London, UK



**Fig. 1.** Duration of second remission

Date 15/2/89



**Fig. 2.** Survival from first relapse

Date 15/2/89

between duration of first remission and the frequency of achievement of second remission. The median duration of second CR was 9½ months (range 1 month to 2¾ years) (Fig. 1). Only four patients continue in second CR between 14 months and 2½ years, three having received consolidation with ablative therapy (cyclophosphamide and total body irradiation) supported by autologous bone marrow transplantation, the bone marrow having been treated in vitro with 4-hydroperoxycyclophosphamide (4-HC) [7, 8]. The durations of remission in the latter three patients currently range between 14 and 30 months. Ten patients remain alive, the median survival from first relapse being 6 months (Fig. 2).

## Discussion

These results illustrate the appalling prognosis of recurrent acute myelogenous leukaemia, even though second complete remissions may be achieved in approximately half of those retreated with combination chemotherapy, while adding circumstantial support for the use of very intensive consolidation treatment with bone marrow transplantation. They emphasize the absolute necessity for addressing the issue of the quality of life following chemotherapy, particularly when the anticipation of long-term survival and cure is very low. The fact that good quality of life can, however, be attained, even for only short periods, makes it essential to continue to investigate both palliative and curative treatment within a closely monitored research setting, with the full understanding of the patient.

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## Oral Idarubicin in Elderly Acute Leukemia and Refractory Anemia with Excess of Blasts

A. Berrebi<sup>1</sup> and A. Polliack<sup>2</sup>

Two anthracyclines, daunorubicin (DNR) and doxorubicin (DXR), are widely used in the treatment of leukemias, lymphomas, and solid tumors. However, their utilization is limited due to the cumulative cardiac toxicity. 4-Demethoxydaunorubicin (Idarubicin, IDR) is a new anthracycline synthesized by Arcamone et al. in the Farmitalia Carlo Erba Research laboratory [1] which differs from DNR by the replacement of the methoxy group in position 4 with a proton [2]. IDR is more potent than DNR in inhibiting RNA synthesis in *Escherichia coli* and murine fibroblasts [3]. When given i.v. IDR is five times more effective than DNR against experimental leukemia models in mice (L 1210 or P 388) [4]. IDR is more active against Gross leukemia than either DNR or DXR. Unlike these two drugs, IDR has a clear biological activity when administered per os [5]. Finally, IDR has less cardiotoxicity than either DNR or DXR in mice, dogs, and rabbits [6]. Phase I trials [7] of IDR in patients with solid tumors have established the recommended dose of 15 mg/m<sup>2</sup> intravenously, and 40 mg/m<sup>2</sup> per os for the phase II trials. We present our preliminary results of a phase II open study with oral IDR in elderly acute nonlymphoid leukemia (ANLL) and refractory anemia with excess of blasts (RAEB).

### Patients and Treatment Regimens

Tables 1 and 2 summarize the patient characteristics, treatment regimens, and outcome. The study began in January 1988 and 15 patients have been included: three with acute megakaryoblastic leukemia (M7), three with ANLL complicating a dysmyelopoietic syndrome (DMS), and two with de novo ANLL. All but two (Nos. 1, 5) had been previously treated and unresponsive to further therapies. Five patients were in leukemic transformation of RAEB and needed frequent blood transfusion. One (No. 10) received several courses of chemotherapy with partial response. Finally the two last patients, Nos. 14 and 15, had an RAEB. The treatment regimens were 30 mg/day for three consecutive days in acute leukemia and a single 50-mg dose for the RAEB patients. These dosages were repeated every 3 weeks for a total of six courses.

### Results

In the acute leukemia group no complete remission was achieved. Three patients died after the first course because of sepsis and/or bleeding not related to IDR administration, and these cases were not evaluable. One M7 patient did not respond after two IDR courses but another had stable disease for 6 months following three courses. Four patients had a partial response, resulting in a decrease of peripheral blastosis; one of them who received six courses of IDR is living with stable disease 14 months after the initiation of treatment. In patient 8 the re-

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**Table 1.** Acute nonlymphoid leukemia patient characteristics, treatment regimens, and outcome in eight patients

Case sex, age	Diagnosis	Previous treatments	Status before Idarubicin	Idarubicin treatment	Outcome
1 F, 74	M4, 1988	No	Hb 6.8 L 10 300 Bl 78% Thr 25000	30 mg × 3 1 course	Sepsis, exitus
2 M, 64	RAEB-T M4 1988	Ara-C-DNR 2 courses	Hb 8 L 250 000 Bl 99% Thr 10000	50 mg 1 course	NR, sepsis, exitus
3 F, 70	M7, 1987	Low-dose Ara-C	Hb 8 L 15000 Bl 70% Thr 20000	50 mg 1 course	NR, sepsis bleeding, exitus
4 F, 74	M7, 1987	Low-dose Ara-C × 6	Hb 9 L 2700 Bl 14% Thr 34000	50 mg 2 courses	NR, sepsis, exitus
5 F, 86	M7, 1987	No	Hb 8 L 15000 Bl 80% Thr 30000	30 mg × 3 3 courses	PR, 6 months Hb 11 L 2400 Bl 0, sepsis, exitus
6 F, 67	RAEB-T M2 1987	Low-dose Ara-C DNR	HB 9 L 67 000 Bl 90% Thr 17000	30 mg × 3 3 courses	PR, 4 months Hb 10 L 15000 Bl 12% Pl 50000 sepsis, exitus
7 M, 66	RAEB-T M4 1987	Ara-C DNR 2 courses	HB 9 L 2900 BL 70% Thr 16000	30 mg × 3 6 courses	PR 14 months Hb 10.5 L 6700 Bl 0 Pl 70000 Alive
8 M, 74	M5 1986	VAPA CR 12 months Low-dose Ara-C	Hb 9 L 2500 Bl 50% Thr 14000 BM full relapse	30 mg × 3 5 courses	PR 6 months Hb 11.5 L 3000 Bl 3% Pl 70000 Relapse, exitus

DNR, daunorubicin; Ara-C, cytosine arabinoside; CR, complete remission; BL blasts; VAPA, vincristine, Adriamycin, prednisone, Ara-C; PR, partial remission; NR, no response; Hb, hemoglobin g/dl; L, leucocyte/ $\mu$ l; Thr, thrombocyte/ $\mu$ l

sponse to IDR was documented by a frank diminution of the bone marrow blasts and appearance of erythroid foci and megakaryocytes.

In the RAEB-T group, two patients had a partial response, reducing the peripheral blasts and the need for transfusion from 3 to 6 weeks. Two patients remained in a stable disease state after four and five courses. Finally, one patient responded dramatically after one course and changed his bone marrow from RAEB to RA, and one patient with sideroblastic anemia had a stable hemoglobin level and was not transfused for 3 months after one single course. In all the patients the toxicity was low and the major complaints were nausea, vomiting, or headache. No clinical cardiac toxicity was noted.

## Discussion

Idarubicin given orally was first evaluated in phase II studies in breast cancer [8, 9] and is currently being tried in chronic myelodysplastic syndromes. These disorders comprise a group of serious hematological diseases (RA, RAEB, RAEB-T, chronic myelomonocytic leukemia [10]) affecting elderly patients or previously treated cancer patients with a high mortality due to progression to acute leukemia. The major causes of death are hemorrhage and/or infections related to the cytopenia and bone marrow failure [11]. The poorest prognostic group is RAEB in transformation (RAEB-T), in which the median survival is 6–9 months. Chemotherapy in these conditions is associated with severe toxicity, requiring intensive



**Table 2.** Refractory anemia with excess of blasts (RAEB) and RAEB-T patient characteristics, treatment regimen, and outcome in seven patients

Case sex, age	Diagnosis	Previous treatments	Status before Idarubicin	Idarubicin treatment	Outcome
9 M, 73	RAEB-T 1988	No 1 BT	Hb 8 L 12000 Bl 15% Thr 50000	50 mg × 1 6 courses	PR Hb 9 L 9900 Bl 0 Pl 20000 BT 6 weeks
10 M, 75	SA 1976 SAEB 86 SAEB-T 88	Low-dose Ara-C Thioguanine COAP BT 3 weeks	Hb 8 L 36000 Bl 30% Thr 480000	50 mg × 1 5 courses	PR 3 months HB 9 L 9000 Bl 20% No further response
11 M, 75	RAEB-T 87	No BT 3 weeks	Hb 7 L 1000 Bl 10% Thr 4000 BM eryth. aplasia	50 mg × 1 4 courses	PR Hb 8 L 5000 PMNs 70% Bl 5% Pl 4000 BT 3 w bleeding, exitus
12 F, 74	RAEB-T 88	No BT 3 weeks	Hb 8 L 1900 Bl 30% Thr 34000	50 mg × 1 6 courses	PR Hb 8 L 2400 Bl 0 Pl 26000 on therapy. BT 6 weeks
13 M, 80	RAEB-T 87	No BT 3 weeks	Hb 7 L 1100 Bl 6% Thr 92000	50 mg × 1 2 courses	Hb 6 L 1300 Bl 0 Pl 97000 on therapy. BT 5 weeks
14 M, 71	RAEB 88	No	Hb 9.7 L 3900 Bl 0 Thr 70000	50 mg × 1 1 course	Hb 10.5 L 2600 Pl 140000 BM: DMS
15 F, 70	SAEB 85	BT every 3 weeks	Hb 9 L 5000 Bl 0 Thr 346000	50 mg × 1 1 course	No need for BT 3 months

SAEB, sideroblastic anemia with excess of blasts; COAP, cyclophosphamide, Oncovin, Ara-C, prednisone; BT, blood transfusion; PMNs, polymorphonuclear neutrophils; PR, partial remission; Hb, hemoglobin g/dl; L, leucocyte/ $\mu$ l; Thr, thrombocyte/ $\mu$ l; Bl, blasts

hematological support and long periods of hospitalization. In addition, the management of these patients must take into consideration the maintenance of quality of life and its prolongation. Hematinic treatment as well as differentiating drugs give poor results [12–14]. Low-dose Ara-C given in these patients and elderly ANLL result in a low response rate and a significant myelosuppression [15, 16]. Therefore the choice of an oral agent which is effective and can be given on an outpatient basis holds great attraction.

A phase II open study performed by De Bok et al. [17] included 19 patients with RAEB, RAEB-T, and acute leukemia post-DMS. The treatment scheme was different than ours and included 50 mg/m<sup>2</sup> IDR p.o. in four divided doses on days 1 and 14 with

five further doses at 14- and 28-day intervals. Five patients (three RAEB and two acute leukemia) achieved a CR and two had a partial remission. The toxicity was low and only one patient died from aplasia.

In conclusion, we treated 15 cases of ANLL and RAEB with oral IDR and obtained 4 partial responses of ANLL and a favorable response (partial response) or stable disease in 6 out of 7 RAEB patients. This easy single therapy with few toxic effects seems to be an encouraging approach for treating leukemia in the elderly.

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## Acute Promyelocytic Leukemia: Clinical Findings and Therapeutic Results in 30 Patients

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### Introduction

Acute promyelocytic leukemia (APL) is a rare variant of acute myeloid leukemia (AML) characterized by the presence of atypical promyelocytes in the bone marrow and peripheral blood [1]. In contrast to other forms of leukemia, APL is frequently associated with severe hemorrhagic complications which may lead to premature death of patients [2, 3]. As suggested by the French-American-British (FAB) Cooperative Group, two subtypes can be distinguished on morphological grounds:

1. the typical hypergranular promyelocytic leukemia (FAB-M3) and
2. a microgranular variant in which the azurophilic granules of the promyelocytes are often too small to be recognized by light microscopy [4].

Recently, a unique cytogenetic abnormality characterized by a balanced translocation between the long arms of chromosomes 15 and 17 has been reported in most patients with APL [5].

The use of aggressive induction regimens has led to improved remission rates in APL [6], but initiation of chemotherapy is often associated with an exacerbation of clinical symptoms and the laboratory features of disseminated intravascular coagulation (DIC). Although the precise nature of this coagulopathy remains unclear, treatment

with heparin is usually recommended for prevention of hemorrhagic diathesis [7–9]. So far, however, the efficacy of heparin has not been demonstrated in a prospective randomized trial [10].

Up to now, larger series of patients have rarely been reported. Thus, even at referral centers, experience in the management of APL is limited. In this paper, we report on 30 adult patients who were admitted to our hospitals between 1974 and 1988 and treated with various forms of antileukemic therapy. In a retrospective analysis, we reviewed the clinical and laboratory data at the time of diagnosis in order to examine their prognostic influence on response to remission induction therapy. Furthermore the different treatment strategies were analyzed with regard to short-term and long-term outcome of patients.

### Patients and Treatment

#### Patients

From 1974 through 1988, 30 patients (15 men, 15 women) were diagnosed as having APL at the Universities of Düsseldorf ( $n=21$ ) and Liège ( $n=9$ ). The median age of patients was 44 years, with a range of 20–83 years. Diagnosis was based on the morphology of leukemic cells in bone marrow and peripheral blood smears stained with May-Grünwald-Giemsa. In accordance with the FAB criteria, abnormal promyelocytes were defined as cells with large azurophilic granules and/or bundles of Auer rods (“fag-gots”) staining positively for myeloperoxi-

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dase. In one patient presenting with DIC, the promyelocytic nature of blast cells could only be confirmed by ultrastructural investigations (microgranular variant of APL).

### Induction Chemotherapy

Twenty-four out of 30 patients were treated with aggressive chemotherapy. For remission induction, various antileukemic protocols were used which, in most cases, included an anthracycline and cytosine arabinoside (Ara-C). Details of induction protocols are given in Table 1. Two patients were treated with low-dose Ara-C. Complete remission (CR) was defined according to CALGB criteria as less than 5% blasts in the bone marrow aspirate and restoration of peripheral blood counts [11]. Early death (ED) was defined as death during the first 6 weeks of treatment, and nonresponse (NR) as failure to achieve CR in patients surviving more than 6 weeks of therapy.

### Supportive Care and Therapy of Coagulopathy

Supportive care was provided by oral antibiotic (trimethoprim-sulfamethoxazole plus

colistine, norfloxacin, ciprofloxacin, or ofloxacin), and antimycotic (amphotericin B, nystatin, or pimaruficin) prophylaxis. If patients became febrile intravenous antibiotic therapy was started with an empirical regimen including a combination of extended-spectrum penicillins, third-generation cephalosporins, and/or aminoglycosides. This regimen was thereafter modified according to the results of appropriate cultures. Platelet transfusions were employed in patients with hemorrhagic diathesis or if the platelet count was lower than  $20 \times 10^9$ /liter. Transfusions of packed erythrocytes were given to maintain the hemoglobin level above 9 g/dl. To reduce the risk of DIC, 14 patients were treated with low-dose heparin. In addition, 20 patients received infusions of fresh frozen plasma (2–4 U/day), 4 were substituted with fibrinogen concentrates, 6 received antithrombin III concentrates, and 1 was treated with high doses of aprotinin.

### Postremission Treatment

After achieving CR, nine patients received additional consolidation therapy, which usually consisted of the primary induction protocol. Thereafter five patients were treated with maintenance chemotherapy, accord-

**Table 1.** Remission induction protocol

Protocol	Chemotherapeutic agents	Day	Pa- tients (n)	Period
TAD-9	Ara-C 100 mg/m <sup>2</sup> c. i. v.	1+2	10	1981– 1988
	Ara-C 100 mg/m <sup>2</sup> i. v. 2 × /day	3–8		
	Thioguanine 100 mg/m <sup>2</sup> p. o.	3–9		
	Daunorubicin 60 mg/m <sup>2</sup> i. v.	3–5		
AML-6	Ara-C 100 mg/m <sup>2</sup> i. v.	1–7	3	1978– 1988
	Vincristine 1 mg/m <sup>2</sup> i. v.	1		
	Daunorubicin 45 mg/m <sup>2</sup> i. v.	1–3		
COAP	Ara-C 60 mg/m <sup>2</sup> i. m 2 × /day	1–5	3	1974– 1980
	Vincristine 1.5 mg/m <sup>2</sup> i. v.	1		
	Cyclophosphamide 400 mg/m <sup>2</sup> i. v.	1		
	Prednisone 40 mg/day p. o.	1		
AML-85	Ara-C 100 mg/m <sup>2</sup> c. i. v.	1–7	4	1986– 1988
	<sup>a</sup> Ara-C 100 mg/m <sup>2</sup> c. i. v.	8–10		
	or <sup>a</sup> Ara-C 3000 mg/m <sup>2</sup> 2 × /day i. v.	8–10		
	Daunorubicin 45 mg/m <sup>2</sup> i. v.	1–3		

<sup>a</sup> Dosage depending on the bone marrow blast count on day 7

ing to the recommendations of the German AML Study Group. In five patients aged 21, 24, 31, 40, and 55 years, bone marrow transplantation (allogeneic = 1, autologous = 3, syngeneic = 1) was carried out at a median of 9 weeks after induction of CR.

## Results

### *Initial Clinical and Laboratory Findings*

Detailed information of our patients is given in Table 2. The patients' complaints at presentation were largely attributable to hemorrhagic diathesis. Hemorrhage was present in 22 (73%) out of 30 patients at the time of diagnosis and commonly involved the skin, gums, and oral cavity as well as the gastrointestinal and genitourinary tract. On the contrary, there were only eight patients presenting with fever or other clinical signs of infection. Spleen and liver enlargement was observed in five and three patients, respectively. In our series, enlargement of lymph nodes or gingival hypertrophy was not observed.

Anemia and thrombocytopenia were common findings at presentation. In 23 patients (77%) a pronounced decrease in platelet count (less than  $50 \times 10^9$ /liter) was noted. The median white blood count was  $3500 \times 10^9$ /liter (range,  $0.8-220 \times 10^9$ /liter). Among 26 patients in whom more detailed coagulation studies were performed, 15 cases (58%) had laboratory evidence of disseminated intravascular coagulopathy (fibrinogen level less than 150 mg/dl, fibrin degradation products more than 40  $\mu$ g/ml).

### *Remission Duration and Survival*

Regarding the entire group of patients, 19 (63%) achieved CR whereas 8 (27%) died during the first 6 weeks of treatment (ED). In five cases ED was due to cerebral bleeding. With regard to initial clinical and laboratory features, patients succumbing to early death showed markedly higher leukocyte counts than patients entering CR (median,  $31.4 \times 10^9$ /liter versus  $2.8 \times 10^9$ /liter). After a median follow-up time of 36 months, 10

(33%) of our patients, representing 53% of the complete responders, were still alive without relapse of their disease. Two patients have remained in continuous CR now for more than 36 months. No superiority of any remission induction regimen could be demonstrated. Both patients treated with low-dose Ara-C died within a period of 1–8 weeks after the start of chemotherapy before a repeat bone marrow examination could be performed. Up to now all patients receiving a bone marrow transplant survived without evidence of relapse (median follow-up period after achieving CR, 24 months).

## Discussion

Acute promyelocytic leukemia is a rare variant of AML with an incidence of about two to three cases per year in our department. According to other studies, this subtype represents 5%–15% of all cases with AML [12]. Therefore, more precise knowledge of this distinct entity of AML can only be obtained by study of larger groups of patients. In a retrospective analysis we reviewed the clinical and laboratory data as well as the therapeutic results in 30 consecutive patients with APL. As shown in this study, patients with APL may have a good chance of entering a complete bone marrow remission if initial hemorrhagic complications can be managed sufficiently. APL responds to the same chemotherapeutic agents that are effective in treating other subtypes of AML. Our results of induction therapy are comparable with those published by Stone et al. [13], who treated 21 patients with a combination of Ara-C and daunorubicin and observed a complete remission rate of 71%.

There is considerable controversy regarding the optimal supportive care in this group of patients. In order to prevent lethal bleeding, most authors currently recommend the transfusion of large quantities of platelets, whereas the benefit of other measures such as low-dose heparin or antifibrinolytic drugs has not yet been proven. On theoretical grounds, one might assume that macromolecules such as heparin which cannot penetrate into the extravascular tissue are unlikely to prevent the initiation of DIC. To clarify the relative values of the various

**Table 2.** Response rate by pretreatment characteristics

		All patients ( <i>n</i> )	CR ( <i>n</i> )	ED ( <i>n</i> )	Non-ED ( <i>n</i> )
<b>Clinical features</b>					
Age (years)	≤ 20	2	0	2	0
	21 – 40	11	10	0	11
	> 40	17	9	6	11
Sex	male	15	12	3	12
	female	15	10	5	9
Symptoms	Anemic symptoms	18	11	5	11
	Hemorrhagic diathesis	22	14	6	11
	Hematuria	14	8	6	8
	Fever	8	4	3	5
	Liver enlargement	3	4	0	5
	Spleen enlargement	5	4	0	3
Gingival hypertrophy	0				
<b>Laboratory data</b>					
Hemoglobin (g/dl)	median	9.4	10	9.3	9.5
	range	5.8–14.5	5.8–14.5	7.8–12.2	5.8–14.4
Leukocytes ( $\times 10^{12}$ /liter)	median	3.5	2.8	31.4	2.8
	range	0.8–220	0.8–40	2.1–220	0.8–400
Platelets ( $\times 10^9$ /liter)	median	29	35	23	35
	range	7–221	9–221	10–179	7–221
Fibrinogen (mg/dl)	median	170	180	150	170
	range	<10–400	<10–400	100–240	<10–400
Antitrombin III (%)	median	84	73	84	100
	range	65–150	68–100	67–125	65–150
Prothrombin time (%)	median	48	57	45	57
	range	14–100	36–100	14–60	20–100
Partial thromboplastin time	median	34	34	35	34
	range	38–145	27–55	26–50	27–120
Lactate dehydrogenase ( $\mu$ /liter)	median	323	323	430	257
	range	134–2320	134–2320	160–1312	134–2320
Creatinine (mg/dl)	median	1	0.9	1.3	0.9
	range	0.7–2.4	0.7–1.3	1–2.4	0.7–1.3
<b>Cytological features</b>					
Bone marrow blasts and promyelocytes (%)	median	78	71	90	70
	range	45–100	45–100	58–98	45–90
Peripheral blood blasts and promyelocytes (%)	median	16	14	64	14
	range	0–93	0–90	0–93	0–90
FAB-M3 variant ( <i>n</i> )		1	0	1	0
Presence of Auer rods ( <i>n</i> )		23	16	7	16
Presence of faggot cells ( <i>n</i> )		13	8	4	9

ED, death during the first 6 weeks of treatment; CR, complete remission; non-ED: surviving the first 6 weeks of treatment

hemostatic approaches, a prospective randomized study would be required.

Once CR is achieved, bone marrow transplantation appears to provide the best chance for long-term remission and even cure of patients.

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## Magnetic Resonance Imaging Follow-up in Patients with Acute Leukemia During Induction Chemotherapy

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### Introduction

With increasing age red bone marrow (BM) is substituted by yellow marrow in a centripetal direction. Moreover, a higher proportion of fatty tissue is observed within hematopoietic BM. In acute leukemia normal bone marrow elements are displaced by blasts. In this study the potential of magnetic resonance imaging (MRI) for visualization of leukemic BM infiltration and for control of chemotherapy in acute adult leukemia was investigated. MRI results were correlated with cytology of bone marrow aspiration and compared with healthy volunteers of various age groups.

### Materials and Methods

Eleven patients with acute leukemia and seven healthy volunteers were examined. Seven out of eight patients with AML were treated according to the TAD9 protocol [1] [thioguanine, cytosine arabinoside (Ara-C), daunorubicin for 9 days] in first-line therapy and one patient with sequential high-dose Ara-C and mitoxantrone (seq. HAM) [2] in second-line therapy. They were examined prior to (day 0) and one to two times during induction chemotherapy. Two out of three patients with acute lymphoblastic leukemia (ALL) were treated according to the ALL protocol [3] with "eight drugs in 8 weeks"

**Table 1.** Characteristics of the examined groups

Name	Sex	Age (years)	Diagnosis
K. M.	f	17	AML M1
T. E.	f	46	AML M4
E. G.	f	49	AML M1
I. J.	f	56	AML M4
L. S.	m	28	AML M4
W. K.	m	58	AML M6
B. F.	m	77	AML M1
B. H.	f	43	AML relapse
K. B.	m	20	Null-ALL
E. K.	f	19	c-ALL
H. S.	f	61	AUL
M. S.	f	22	Healthy
D. M.	f	30	Healthy
B. L.	m	22	Healthy
P. K.	m	67	Healthy
M. S.	f	70	Healthy
H. O.	m	31	Healthy
N. R.	m	42	Healthy

**Table 2.** Semiquantitative estimation of cellularity

Score	Meaning
4	Highly increased
3	Increased
2	Normal
1	Decreased
0	Greatly decreased

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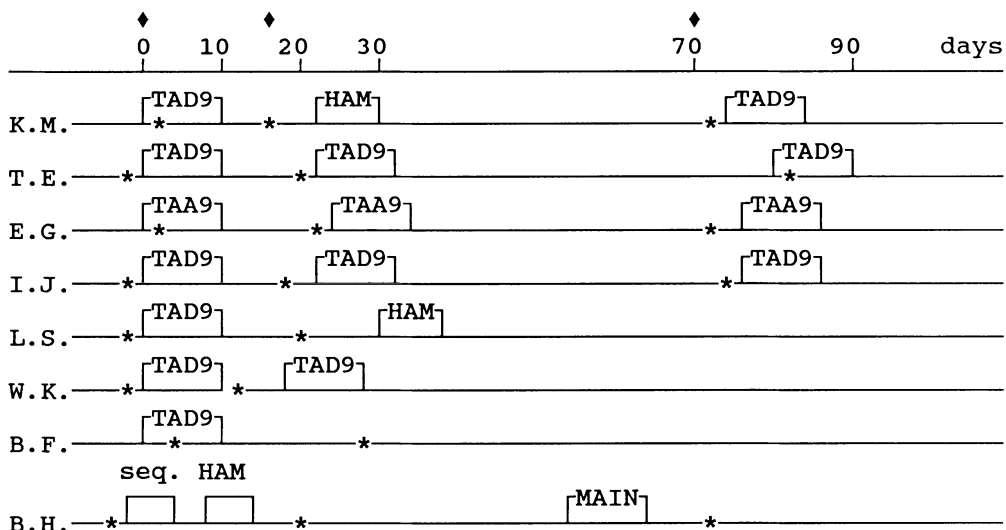


Fig. 1. Protocol of chemotherapy, MRI (\*), and BM aspiration (♦) in AML. *TAD9*, thioguanine, Ara-C, daunorubicin for 9 days; *HAM*, high-dose Ara-C plus mitoxantrone; *TAA9*, thioguanine, Ara-C, amsacrine for 9 days; *MAIN*, maintenance therapy; *seq. HAM*, sequential high-dose Ara-C plus mitoxantrone

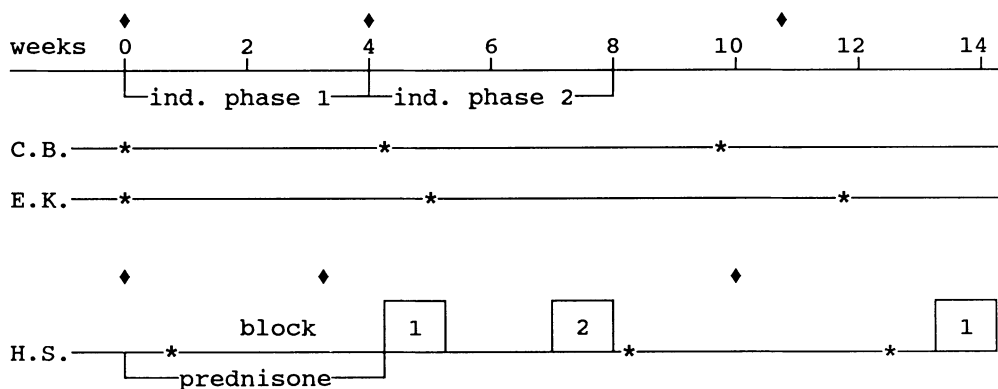


Fig. 2. Protocol of chemotherapy, MRI (\*) and BM aspiration (♦) in ALL

and one patient with secondary acute undifferentiated leukemia (AUL) was treated according to a B-cell ALL (B-ALL) protocol. Cytology examinations were performed in acute myeloblastic leukemia (AML) prior to therapy, in aplasia, and in complete remission; in ALL prior to, after phase 1, and after phase 2 of induction therapy; in AUL prior to therapy, after block 2, and in complete remission (CR), respectively. For grading of cellularity the following criteria were employed.

Magnetic resonance imaging examinations were conducted within short intervals from BM aspiration. Relatively T1-weighted spin-echo-sequences with TE = 22 ms and TR = 0.4, 0.6, 0.8, and 1.2 s were used. Signal intensities and calculated T1 relaxation times were recorded in lumbar vertebrae (LV), pelvis (P), and femoral diaphysis (FD). In addition, the degree and extension of MRI changes were evaluated using a new scoring system (modification of the Olson [4] classification).

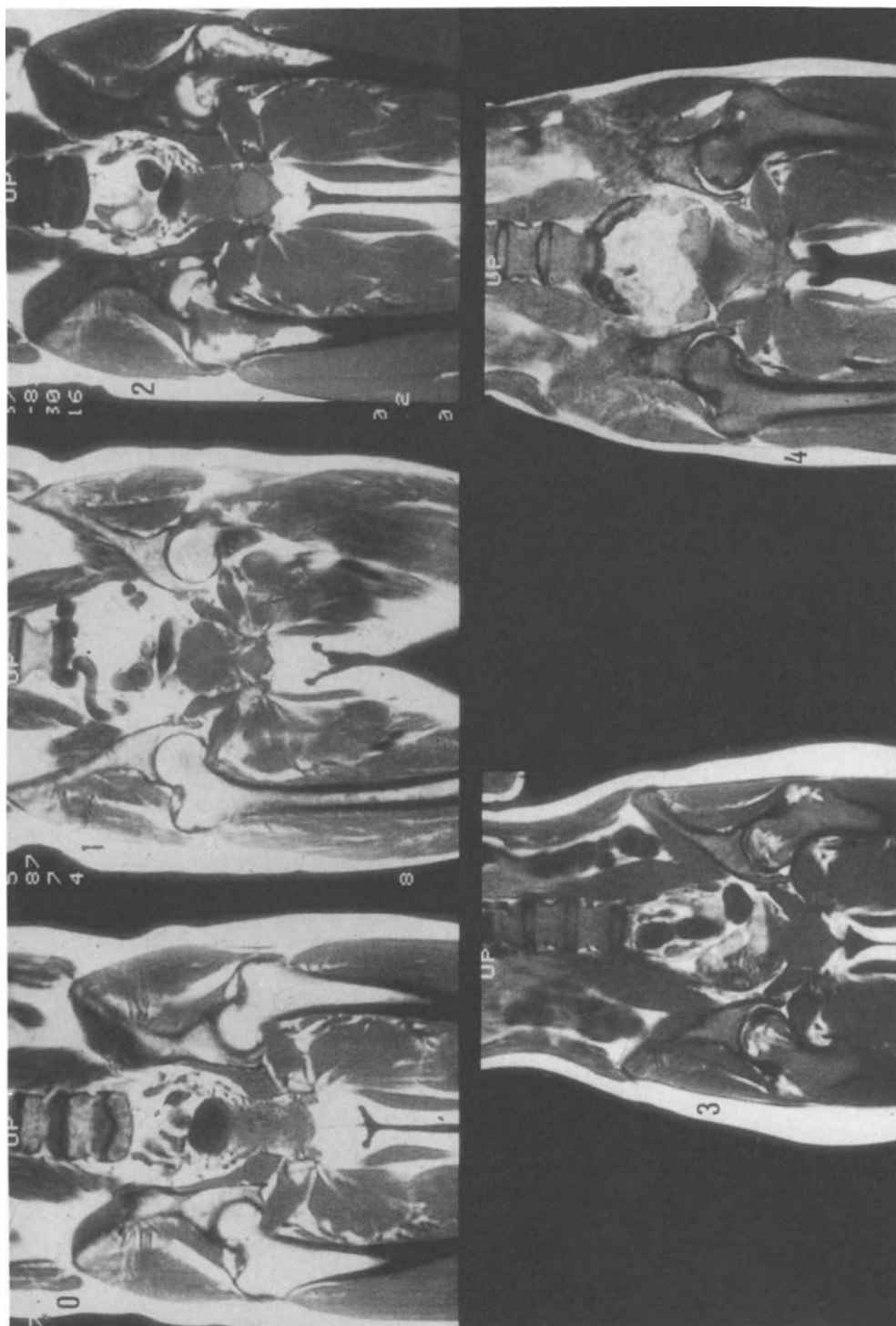


Fig. 3. Illustration of the MRI score (see table 3)

**Statistics**

The median value as well as the lower and upper quartile of signal intensities and T1 times were calculated using Friedman's test

for intragroup comparison and the two-sided Wilcoxon test for intergroup comparison. The MRI scores and hematological scores were correlated using the Mantel-Haenszel test.

**Table 3.** Semiquantitative estimation of the MR images

Score	Meaning
0	Homogeneous, high signal intensity
1	Inhomogeneous, high signal intensity
2	Inhomogeneous, mild decreased signal intensity
3	Inhomogeneous, marked decreased signal intensity
4	Homogeneous, low signal intensity

0–3, sparing of the greater trochanter and the femoral head; 4, without sparing of the trochanter and the femoral head

**Table 4.** Score of cellularity and MRI prior to therapy

	N	Score value					Mean value
		0	1	2	3	4	
<b>Cellularity</b>							
AML	7	0 0.0%	0 0.0%	0 0.0%	2 28.6%	5 71.4%	3.7
Volunteers	7	0 0.0%	0 0.0%	7 100%	0 0.0%	0 0.0%	2.0
<b>Magnetic resonance imaging</b>							
AML	7	0 0.0%	1 14.3%	3 42.9%	2 28.6%	1 14.3%	2.4
Volunteers	7	7 100%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0.0

$P < 0.0005$

**Table 5.** Intragroup comparison of SES 0.8 and T1 times in pelvis of patients with AML prior to and during induction therapy

Name	SES 0.8			T1 time		
	First	Second	Third	First	Second	Third
AML K. M.	340	357	757	2326	747	360
T. E.	–	–	529	970	1182	538
E. G.	285	363	875	1487	979	380
I. J.	459	601	955	910	651	229
L. S.	409	294	–	1668	1216	–
W. K.	495	544	–	493	493	–
B. F.	294	339	–	1471	1232	–

$P < 0.05$  for the first versus third examination

## Results

Untreated leukemic patients exhibited a decrease of signal intensity in the T1-weighted spin-echo-sequences (SEs) and an increase in T1 relaxation time in all investigated areas along with an elevation of higher values of cellularity and MRI scores which disclosed statistically significant differences from healthy volunteers.

Following treatment, normalization of cellularity scores and MRI scores were observed in ALL on day 28 and 56, and in AML in CR. While the cellularity score in AML in CR showed no statistical significant difference ( $P=1.0$ ) against healthy volunteers, the MRI score showed a smaller but significant difference ( $P<0.005$ ) compared with the examinations prior to therapy. The intragroup comparison in patients with AML revealed significant differences for

SES 0.4, 0.8 P (Table 5), and T1 times of FD and P (Table 5) on day 70 vs. day 0 ( $P<0.05$ ). The intergroup comparison in patients with AML and healthy volunteers exhibited significant differences in cellularity score, MRI score, MRI signal intensities (LV,P (Table 6), FD), and T1 times in all investigated areas (Table 6) on day 0 ( $P<0.005$ ) and day 15 ( $P<0.01$ ). In ALL a clear-cut difference from volunteers was also found. However, the small number of patients precluded statistical evaluation.

## Discussion

It can be concluded from our results that MRI allows noninvasive macroscopic evaluation of blast infiltration within large areas of bone marrow. The displacement of the normal bone marrow results in an

**Table 6.** Intergroup comparison of four SEs in pelvis and T1 times in LV, P, and FD of patients (day 0) and healthy volunteers

	Name	SES pelvis				T1 time		
		0.4	0.6	0.8	1.2	LV	P	FD
AML	K. M.	204	267	340	491	2424	2326	1997
	T. E.	284	—	—	652	1149	970	1064
	E. G.	159	244	285	405	—	1487	915
	I. J.	250	342	459	546	1519	910	942
	L. S.	203	354	409	551	1829	1668	1087
	W. K.	356	419	495	594	1593	493	834
	B. F.	159	252	294	403	3946	1471	1967
Median patients		204	267	374	546	1711	1471	1064
H	M. S.	523	657	766	886	681	542	505
E	D. M.	423	525	598	677	483	470	258
A	B. L.	470	559	637	770	667	471	385
L	P. K.	684	823	902	965	517	333	508
T	M. S.	582	704	821	898	320	432	358
H	H. O.	762	928	1035	1165	594	402	322
Y	N. R.	639	795	897	820	332	352	261
Median volunteers		582	704	821	886	517	432	358
$P<$ patients v.s. volunteers		0.005	0.005	0.005	0.005	0.005	0.005	0.005
Calculation for the second and third examinations (patients versus volunteers)								
Second examination								
$P<$		0.005	0.005	0.005	0.005	0.005	0.005	0.01
Third examination								
$P<$		NS	NS	NS	NS	NS	NS	NS

evident difference of MRI parameters between leukemic patients and healthy volunteers. In AML and ALL normalization of MRI was not observed before complete remission.

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## Thrombin Generation in Acute Myeloblastic Leukemia\*

R. E. Scharf, U. Stoffels, and W. Schneider

### Introduction

Bleeding complications are responsible for 10%–30% of early deaths in acute leukemia [1]. Despite appropriate supportive care, including prophylactic platelet transfusion therapy, this rate has remained almost unchanged [2]. In addition to disease-related or chemotherapy-induced severe thrombocytopenia and platelet dysfunction, disorders of coagulation may be involved in the pathogenesis of hemorrhages in acute leukemia [3]. Thus, overt disseminated intravascular coagulation (DIC) occurs commonly in patients with promyelocytic leukemia [4], but may also be seen in acute monocytic leukemia and other subtypes of acute myeloblastic leukemia (AML) [5]. DIC has been attributed to procoagulants released from granular fractions of leukemic blast cells [4, 6].

Plasma fibrinopeptide A (FPA, A $\alpha$  1–16) results from cleaving the A $\alpha$ -chain of fibrinogen by thrombin. The increase in FPA plasma levels is therefore considered to be an indirect but highly sensitive marker of thrombin generation [7]. Here, we report the results of a study designed [1] to evaluate thrombin activity in 27 patients with AML by measuring plasma FPA levels prior to

chemotherapy and [2] to examine whether a relationship between FPA levels and the number of peripheral blast cells exists.

### Patients and Methods

#### *Patient Selection*

The study was conducted on 27 patients with AML. Patients with impaired renal function, fever, or other evidence of an inflammatory or infectious process (e.g., pulmonary infiltrates on chest X-ray examination) on admission were not included in the study, since these conditions may lead to an elevation of plasma FPA levels in a non-specific manner [7]. The diagnosis of AML was made by standard morphological and histochemical criteria. The AML subtypes, as classified according to the French-American-British (FAB) Cooperative Group [8], and pretherapeutic blast cell counts are shown in Table 1.

#### *Collection and Processing of Blood Samples*

Specimens for determination of FPA were collected prior to initiation of antileukemic treatment and any anticoagulant or substitution therapy with fresh frozen plasma, antithrombin III, and platelet transfusions. Clean venipunctures were performed after slight tourniquet with a 21-gauge butterfly infusion set (Venisystems, Abbott Ireland Ltd., Sligo, Ireland). After discarding the first 2 ml, 4.5 ml blood was drawn into a

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**Table 1.** Acute myeloblastic leukemia subtypes, peripheral blast cell counts, and plasma fibrinopeptide A (FPA) levels in 27 patients at initial diagnosis

Patient	FAB M	Peripheral blast (cell count/ $\mu$ l)	FPA (ng/ml)
1	1	13200	7.1
2	1	8300	8.7
3	1	11700	2.0
4	1	1900	5.4
5	1	15200	11.1
6	2	19000	13.5
7	2	4700	9.1
8	2	117000	11.0
9	2	39200	6.2
10	2	48300	3.9
11	2	151000	13.1
12	2	64700	5.9
13	2	11200	12.1
14	2	3600	8.2
15	3	2100	29.5
16	3	9800	13.4
17	3	56000	62.1
18	3	12500	37.2
19	3	37600	48.7
20	4	12300	2.4
21	4	62100	6.5
22	4	84200	12.7
23	4	49500	8.0
24	4	21000	4.9
25	4	9400	7.1
26	5	71500	12.5
27	5	43000	6.4
Normal range	-		0.1-2.5

precooled 5-ml syringe preloaded with 0.5 ml of an anticoagulant-thrombin inhibitor cocktail, as described previously [9]. After incubation of blood samples on melting ice for 30 min, plasma was obtained by centrifugation at 4°C for 30 min at 2500 *g*. Plasma samples were stored in polypropylene tubes at -20°C prior to determination of FPA within 2 weeks after blood processing.

*Determination of Fibrinopeptide A*

Plasma levels of fibrinopeptide A (FPA) were determined using a commercially available radioimmunoassay kit (Mallinckrodt, Inc., St. Louis, Missouri, United States). To remove fibrinogen and the majority of elas-

tase-induced fibrinopeptides (A $\alpha$  1-21), known to cross-react with the FPA antiserum used in this assay [10], plasma samples were treated with bentonite [11] prior to further processing. Plasma FPA levels in 120 healthy volunteers, evaluated prior to this study, ranged from 0.1 to 2.5 ng/ml, with a mean of 1.5 ng/ml [9].

*Statistical Analysis*

Statistical evaluations were performed using the CLINFO programs of the Department of Health and Human Services, United States. All data are given as mean  $\pm$  SEM. Student's *t*-test (two-tailed) for unpaired sample groups was used when the data were normally distributed (Wilk-Shapiro test). Otherwise, the Wilcoxon sign rank test was chosen. Differences having a *P* value of <0.05 were considered significant.

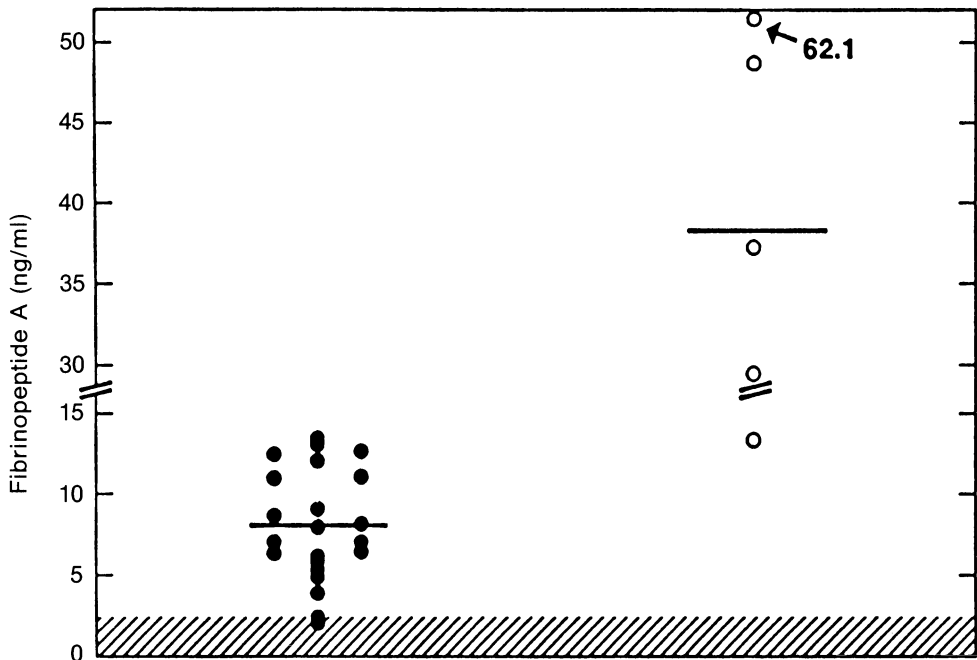
**Results**

Peripheral blast cell counts and pretreatment plasma levels of FPA obtained in the 27 patients on admission are presented in Table 1. All patients, except two (one with AML FAB m1, one with FAB m4), showed elevated FPA values (>mean + 2 SD normals) when compared with those of 120 healthy volunteers. The mean pretreatment plasma level of FPA (38.2  $\pm$  8.3 ng/ml) was significantly higher (*P*=0.021) in the five patients with acute promyelocytic leukemia (FAB m3) than in patients with any of the other AML subtypes (Table 2). In the 22 patients with AML FAB m1, m2, m4, and

**Table 2.** Mean pretreatment plasma levels of FPA in different AML subtypes

FAB M	Number of patients	FPA (ng/ml) (mean $\pm$ SEM)
1	5	6.9 $\pm$ 1.5
2	9	9.2 $\pm$ 1.2
3	5	38.2 $\pm$ 8.3
4	6	6.9 $\pm$ 1.4
5	2	<sup>a</sup>

<sup>a</sup> No mean given due to the small number of patients in this subgroup



**Fig. 1.** Plasma fibrinopeptide A levels in acute leukemia. *Closed circles* represent FPA values of patients with AML (FAB M1, M2, M4, M5), *open circles* those of patients with AML FAB M3. *Shaded area* represents the range of FPA levels in 124 healthy volunteers (1.5 + 1.0 ng/ml, mean + 2 SD). *Horizontal bars* indicate the mean of each group

m5, the mean pretreatment plasma level of FPA was  $8.1 \pm 0.7$  ng/ml (Fig. 1).

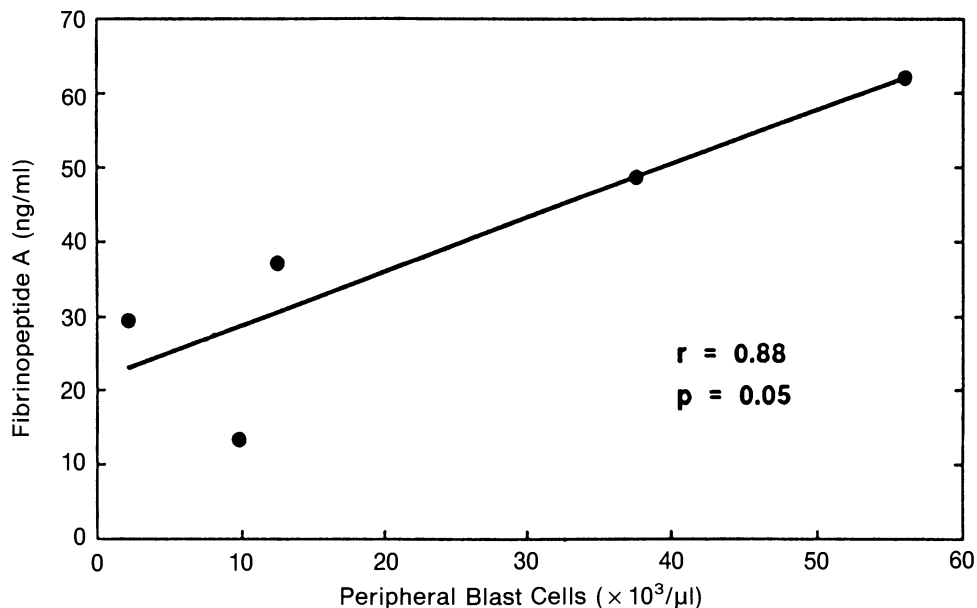
Peripheral blast cell counts ranged from 2100 to 56 000/ $\mu$ l (mean  $\pm$  1 SEM, 23 600  $\pm$  10 100/ $\mu$ l; median, 12 500/ $\mu$ l) in patients with AML FAB m3 and from 1900 to 151 000/ $\mu$ l (mean  $\pm$  1 SEM, 39 200  $\pm$  8.400/ $\mu$ l, median, 20 000/ $\mu$ l) in patients with other subtypes of AML. No relationship was found between the peripheral blast cell counts and the corresponding FPA levels in the total group of 27 patients. However, when the five patients with AML FAB m3 were considered separately, a significant correlation ( $r=0.88$ ,  $P=0.050$ ) was observed between the peripheral blast cell counts and the FPA levels (Fig. 2).

## Discussion

The present study demonstrates that fibrinopeptide A (FPA) levels are elevated in patients with AML at the time of initial di-

agnosis. This observation confirms previous findings by Myers et al. [12], Bauer and Rosenberg [13], and Gugliotta et al. [14], who reported that plasma FPA is high in patients with AML at clinical presentation and during relapse. Also in agreement with these studies [12–14], we found strikingly elevated FPA levels in the five patients with acute promyelocytic leukemia (FAB m3) in comparison to those with other subtypes of AML ( $P=0.021$ ). Furthermore, analysis of the relationship between FPA and peripheral blast cell count revealed a significant correlation ( $r=0.88$ ,  $P=0.050$ ) in patients with AML FAB m3. In contrast, no relation between peripheral blast cell count and formation of FPA was observed in the 22 patients with other subtypes of AML. Based on these findings, acute promyelocytic leukemia can be considered a separate entity by virtue of a different degree of coagulation abnormality and a different pattern in relation to the number of blast cells. Similar observations have been reported by Bauer and Rosenberg





**Fig. 2.** Relationship between peripheral blast cell counts and plasma FPA levels in patients with acute promyelocytic leukemia (FAB M3). The regression conforms to  $y = 21.1 + 7.23 \cdot 10^{-4} x$

[13] and Gugliotta et al. [14]. Thus, both the number and type of circulating blast cells and their related capacity to express procoagulant activities [6] appear to be major determinants of excessive fibrinogen degradation in AML.

Fibrinopeptide A (FPA,  $A\alpha$  1–16) is a highly sensitive marker of fibrin I formation by thrombin [7]. Thus, elevated plasma levels of FPA in patients with AML are considered to reflect the *in vivo* enzymatic activity of thrombin upon fibrinogen [12, 13]. Furthermore, it has been concluded that the thrombin generation *in vivo* is related to procoagulant activity induced by leukemia blast cells [6, 12–14]. However, for a number of reasons this conclusion may be inappropriate.

First, there are several major problems that limit the usefulness of FPA measurements as an indicator of thrombin generation *in vivo*. These limitations include artifactually elevated FPA levels due to inappropriate blood collection and processing; nonspecifically increased FPA levels due to inflammatory and infectious processes; and nonspecifically elevated FPA levels due to impaired clearance in renal failure. In this

study, these limitations were taken into account by including only patients in whom a clean venipuncture could be performed and no infectious processes or renal impairment were present. Further, the study was restricted to the pretreatment phase only since antileukemic therapy, anticoagulation with heparin, transfusions of erythrocytes or platelets, or substitution therapy with fresh frozen plasma may lead to alterations of plasma FPA levels [12, 14].

Secondly, elastase-like proteases released by leukemic blast cells may induce fibrinogenolysis [15]. Elastase-mediated degradation of the N-terminal  $A\alpha$ -chain of fibrinogen results in fibrinopeptides ( $A\alpha$  1–21) that may cross-react with the fibrinopeptide A ( $A\alpha$  1–16) antiserum, and thereby lead to non-thrombin-specific elevations of plasma FPA levels [15].

Thus, it has been shown that 8%–18% of the elastase-induced fibrinopeptide  $A\alpha$  1–21 bind to the FPA ( $A\alpha$  1–16) antiserum used in this study [10]. To overcome this limitation, plasma samples were treated with bentonite to remove cross-reactive fibrinogen [11] and elastase-mediated fibrinopeptides [15] prior to radioimmunological determina-

tion of FPA. Therefore, it is concluded that the fibrinopeptide A levels reported in this study truly reflect the thrombin generation in vivo.

This conclusion is also supported by two independent observations. Gugliotta et al. [14] reported that elevated plasma FPA levels in AML are reduced by heparin administration, indicating that the pretreatment FPA levels resulted from increased intravascular thrombin activity. Further support for the thrombin specificity of elevated plasma FPA levels in patients with acute promyelocytic leukemia was provided by Bauer and Rosenberg [13], who demonstrated a significant correlation between elevated levels of FPA and the thrombin-antithrombin (TAT) complex. Based on their findings, direct evidence was provided that the fibrinopeptide A generated in vivo is a consequence of the excessive conversion of prothrombin to thrombin [13].

In summary, our study demonstrates that thrombin generation is considerably greater in patients with acute promyelocytic leukemia than in those with other subtypes of AML. We also confirm that the measurement of FPA prior to chemotherapy is a sensitive indicator of coagulation hyperactivity that may help to identify alterations of the hemostatic system in patients prone to develop disseminated intravascular coagulation. However, it remains to be established to what extent elevated plasma FPA levels may be an indicator of the disease activity in patients with AML.

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## Acute Megakaryoblastic Leukemia: A Case Report

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### Introduction

Acute megakaryoblastic leukemia (M7) recently included in the French-American-British (FAB) classification for acute leukemias [1] is a rare hematological disease with poor prognosis. We present a case of acute megakaryoblastic leukemia with poor response to cytostatic treatment and a review of the therapeutic attempts in the literature.

### Case Report

A 67-year-old man was admitted to our hospital in 1987 with bleedings, fatigue, and fever. The case history revealed amputation of the right lower leg in 1973, diabetes mellitus type II since 1981, and angina pectoris. At the time of examination the patient was pale and covered with mucocutaneous petechial bleedings. No lymphadenopathy or hepatosplenomegaly was observed. Peripheral blood counts showed a severe pancytopenia, leukocyte count  $0.9 \times 10^9$ /liter with differential fractions of 0.04 band forms, 0.20 neutrophils, 0.72 lymphocytes, and 0.04 blasts. The level of lactate dehydrogenase (LDH) was elevated (Table 1). The bone marrow smears showed a 48% infiltration with

blasts. The blast cells had a high nucleocytoplasmic ratio, moderately fine chromatin, one or two large distinct nucleoli, and scanty blue cytoplasm sometimes forming protrusions and blebs. Ultrastructural criteria and the positivity of the platelet glycoprotein II B/III A complex confirmed the diagnosis of acute megakaryoblastic leukemia. According to recent communications [2], treatment with low-dose cytosine arabinoside 20 mg twice daily s.c. over 21 days was initiated. The subsequent bone marrow examination showed a severe hypoplasia with persistent blasts. In view of the fact that the blasts in the peripheral blood were considerably increased (71%, leukocytes  $0.5 \times 10^9$ /liter), cytostatic treatment with amsacrine and etoposide (amsacrine 120 mg/m<sup>2</sup> days 1–5, etoposide 120 mg/m<sup>2</sup> days 1–5) was restarted. Peripheral blasts disappeared but the patient died of septicemia. Permission for a postmortem examination was not granted.

### Materials and Methods

#### *Bone Marrow Examination and Cytochemical Studies*

Bone marrow smears were treated with May-Grünwald-Giemsa stain. Cytochemical reactions for myeloperoxidase, chloracetate esterase, nonspecific esterase, acid phosphatase, and periodic acid Schiff reaction were performed according to standard techniques [3].

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**Table 1.** Clinical and laboratory findings

	At diagnosis	Terminal
Petechial bleeding	++	+
Lymph nodes	0	0
Liver	0	0
Spleen	0	0
0 = not enlarged		
Hb (g/liter)	77	93
WBC ( $10^9$ /liter)	0.9	0.43
	0.04 bands forms	
	0.20 neutrophils	0.30 neutrophils
	0.72 lymphocytes	0.67 lymphocytes
	0.04 blasts	0.03 blasts
Platelets ( $10^9$ /liter)	11	15
ESR (mm/h)	35	123
SLDH (U/liter)	645	323

SLDH, serum lactate dehydrogenase

#### *Histological and Electron Microscopic Studies*

Posterior iliac crest bone marrow biopsies were obtained using a Jamshidi biopsy needle, fixed in buffered formalin, decalcified using sodium ethylenediaminetetraacetic acid (EDTA), and Paraplast embedded before staining with hematoxylin/eosin, periodic acid-Schiff, and a reticulin method. For electron microscopy small bone marrow particles were fixed in sodium cacodylate-buffered 2% glutaraldehyde for 2 h at room temperature and washed in sodium cacodylate buffer overnight. After dehydration in graded ethanol, the specimen was embedded in Epon 812. The ultrathin sections were stained with lead citrate and uranyl acetate.

#### *Immunocytochemical Stains*

Peripheral blood smears were stained for the presence of platelet glycoprotein II B/III A complex using an immunoperoxidase technique [4].

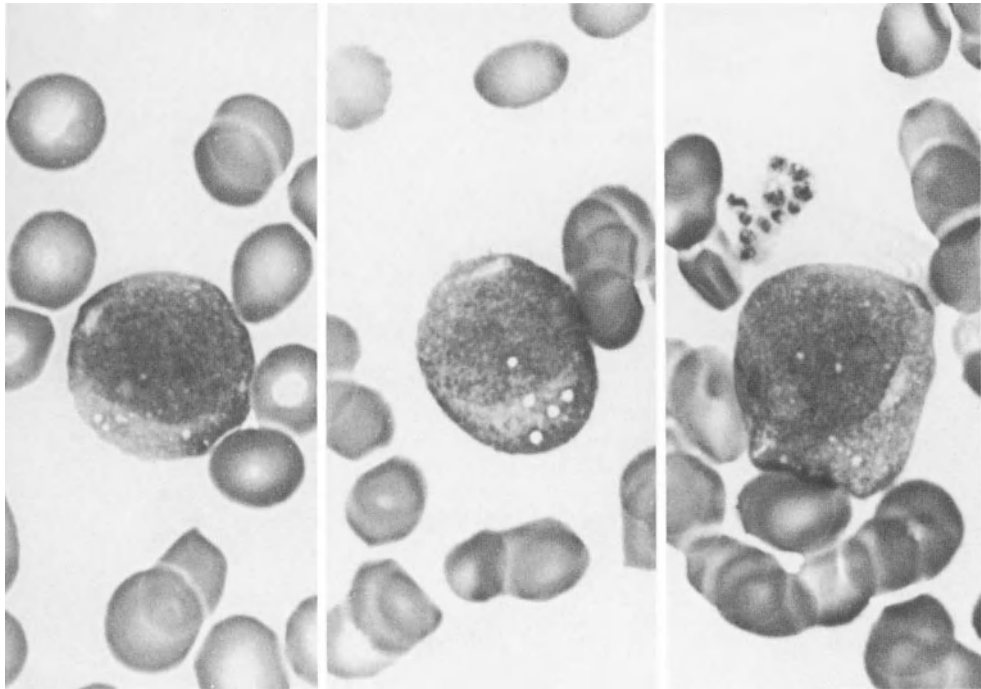
#### **Results**

##### *Bone Marrow Examination, Cytochemistry, and Immunocytochemical Stains*

The bone marrow smears showed an infiltration of undifferentiated blasts of various sizes with a high nucleocytoplasmic ratio, one or two large distinct nucleoli, and scanty blue cytoplasm forming protrusions and blebs. Some cells were vacuolized (Fig. 1). Micromegakaryocytes were seen beside the blasts. Of the cytochemical reactions, only the acid phosphatase reaction was positive. Myeloperoxidase, ASD chloracetate esterase, nonspecific esterase, and periodic acid-Schiff reactions were negative. The platelet glycoprotein II B/III A complex was positive.

##### *Histological and Electron Microscopic Studies*

Bone marrow trephine biopsy showed an infiltration of undifferentiated blasts and an increase in reticulin fibers (Fig. 2a). The nuclei of these blasts were vesicular with prominent nucleoli and sparsely distributed chromatin in semithin sections of plastic-embedded bone marrow (Fig. 2b). Highly immature cells with "blastic" nucleus and a



**Fig. 1.** Undifferentiated blasts with vacuolization. H & E,  $\times 100$

megakaryoblastic differentiation pattern as indicated by very small cytoplasmic progranules and a rudimentary demarcation membrane system were found in ultrathin sections (Fig. 3).

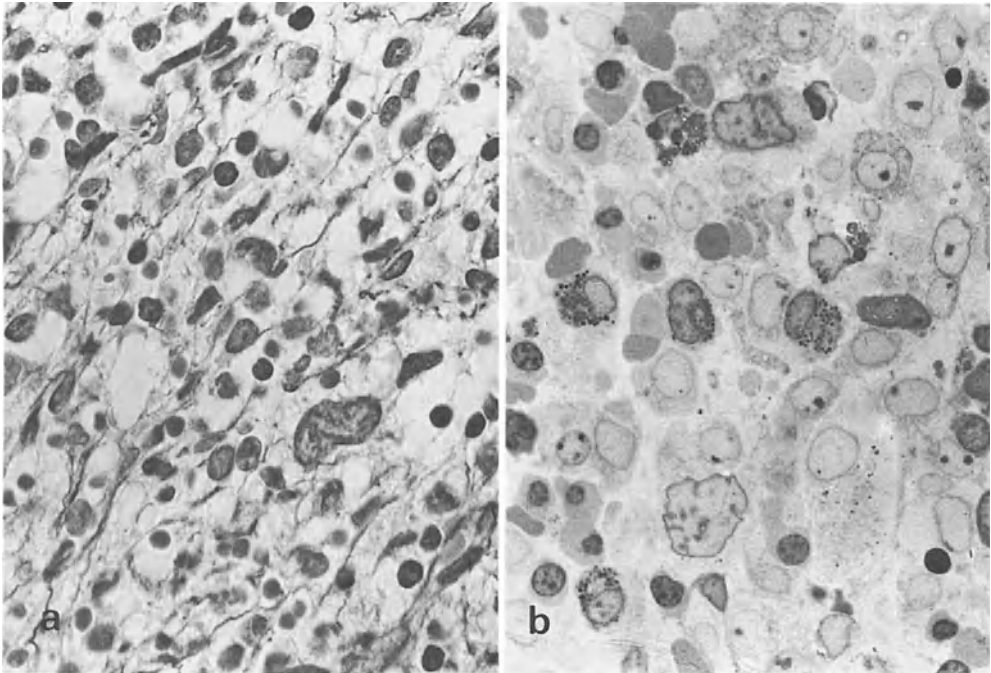
### **Therapeutic Attempts**

From a review of the literature only a small number of cases have been described, with highly varied modes of therapy (Table 2). The actual acute leukemia therapy protocols failed to achieve complete remissions. A remission lasting 5 months has been reported using daunorubicin, cytosine arabinoside and 6-thioguanine [6]. Ruiz-Argüelles et al. presented a series of six cases of acute megakaryoblastic leukemia treated with low-dose cytosine arabinoside in which two patients achieved complete remissions (CRs) of 11 and 15 months [2]. Unfortunately we could not achieve a remission in our reported case. Ming-er Huang et al. have

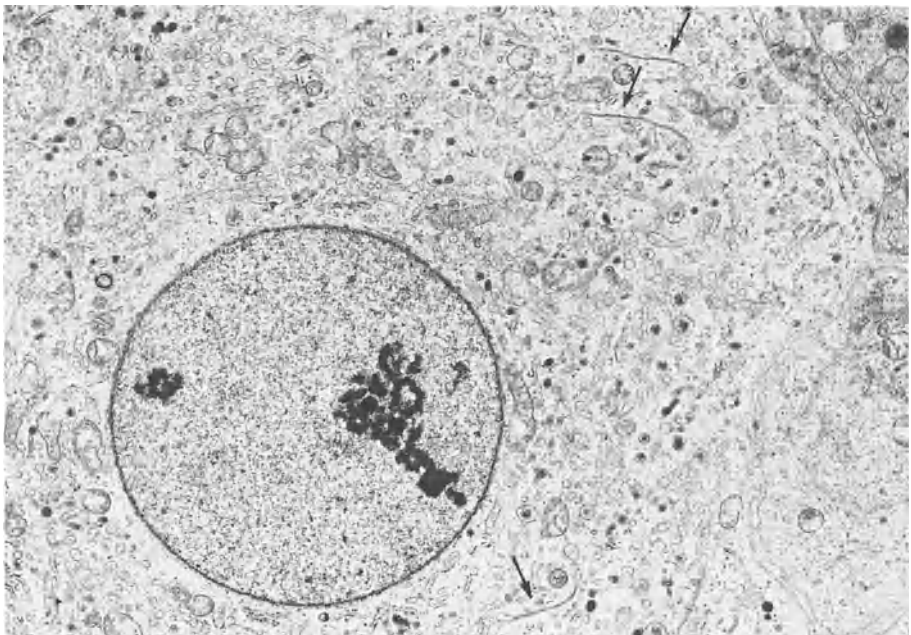
reported one successful bone marrow transplantation. Unfortunately, this patient died 3 months after transplantation from therapy-resistant disseminated aspergillosis in persistent marrow remission with moderately severe graft-versus-host disease.

### **Discussion**

Since 1985 acute megakaryoblastic leukemia has been included as M7 in the FAB classification for acute leukemias [1]. From electron microscopic and immunological methods some of the unclassifiable acute leukemias are now identified as being derived from the megakaryocyte lineage. The incidence of M7 is still unclear. Ruiz-Argüelles et al. suggested that M7 comprises about 8% of all leukemias [2]. Up to now the largest series reported was that of Ming-er Huang et al. from the Mayo Clinic with 12 cases [9]. All patients in this study and in the others listed in Table 2 were treat-



**Fig. 2 a, b.** **a** Bone marrow biopsy with a slight increase in reticulin fibers. Silver stain,  $\times 850$ . **b** Semithin section of plastic-embedded bone marrow. Marked proliferation of immature atypical cells. Epon, azure II – methylene blue,  $\times 850$



**Fig. 3.** Ultrathin section of an immature cell with “blastic” nucleus and a megakaryoblastic differentiation pattern: small cytoplasmic progranules and a rudimentary demarcation membrane system ( $\rightarrow$ ). Lead citrate and uranyl acetate,  $\times 7500$

Table 2. Therapeutic attempts in the literature

Author	Patients		Therapy	Result		Death 1 year after diagnosis and cause	Patients living > 1 year
	Total	Treated		CR	NR		
Den Ottolander et al. [5]	3	3	VCR, Pred, Cylo	∅	∅	Infection 3 Respiratory insufficiencies Hemorrhage	0
Bain et al. [6]	4	2	VCR, Ara-C, Pred, DNR, 6-TG (DAT protocol)	1 (5 months)	1	1 Hemorrhage	1
Bevan et al. [7]	4	1	VCR, Pred	∅	1	3 Bleeding + infection Cardiac failure	1 patient over 5 years in observation without treatment
Mirchandani et al. [8]	3	0	-	-	-	Septicemia 3 Hemorrhage, pneumonia	0
Ming-jeer Huang [9]	12	6	LD Ara-C, Pred, Ara-C, VP-16, MTX, 6-TG, L-Asp	2	10	11 Cause of death not referred	1 patient 65 weeks
Ruiz-Argüelles et al. [2]	6	5	LD Ara-C	2 (11 months, 15 months)	3	4 Cause of death not referred	0
Hruban et al. [10]	3	3	Ara-C, DNR, Cyclo, Amsacrine, VP-16	0	3	2 Septicemia pneumonia	1
Winkelmann et al. [11]	1	1	DNR, Ara-C, HD Ara-C	0	1	1	0
Total	36	21		5	19	28	4

VCR, vincristine; Pred, prednisone; Cyclo, cyclophosphamide; Ara-C, cytosine arabinoside; DNR, daunorubicin; VP-16, etoposide; MTX, methotrexate; 6-TG, 6-thioguanine; L-Asp, L-asparaginase

ed with different acute leukemia protocols with poor results. Two remissions were achieved only with the low-dose cytosine arabinoside (Ara-C) regimen.

The grade of fibrosis might be of importance for the poor response to therapy in acute megakaryoblastic leukemia. This myelofibrosis is considered to be secondary to the proliferation of megakaryoblasts because a platelet-derived growth factor (PDGF) has been found to stimulate fibroblast proliferation [12]. The better results of a milder cytostatic treatment may arise from the differentiation-promotion effect of the low-dose Ara-C regimen.

### Summary

We present a case of acute megakaryoblastic leukemia identified by electron microscopy and platelet-specific antibodies. The histological examination of bone marrow showed distinct myelofibrosis. In accordance with recent communications, low-dose cytosine arabinoside treatment (20 mg twice daily s.c. over 21 days) was initiated. The subsequent bone marrow examination showed a severe hypoplasia with persistent blasts. Amsacrine and VP-16 were given without success. Finally the patient died of septicemia without proof of pathogen uninfluenced by antibiotic and antiseptic therapy 6 weeks after diagnosis. Our case report confirms the poor prognosis of acute megakaryoblastic leukemia.

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## Acute Megakaryoblastic Leukemia (FAB-M7) in an Infant Presenting with Orbital Chloroma and Meningeal Involvement

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### Introduction

Although described as early as 50 years ago [20], acute leukemia of megakaryocytic lineage (AMegL) was only recently added to the French-American-British (FAB) classification as FAB-M7 [2, 3]. To establish the diagnosis for this category, sophisticated techniques like ultrastructural cytochemistry or immunological techniques are required. The limited data available suggest a frequency of 3%–12% in adult acute non-lymphoblastic leukemia (ANLL) [15]. In childhood only 20 cases with a definitive diagnosis of AMegL had been reported up to the year 1986 [5]. To the best of our knowledge the present case is the first in which cranial chloroma was associated with AMegL.

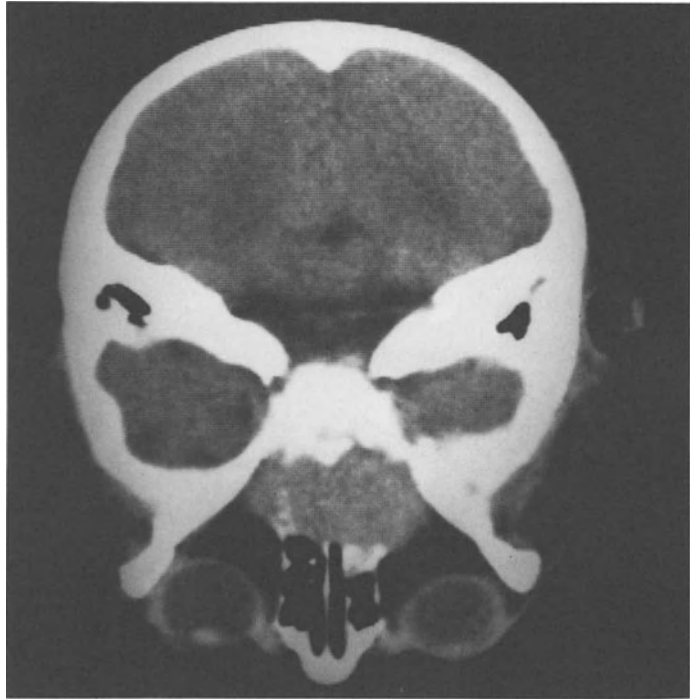
### Case Report

A previously healthy 18-month-old boy presented with a 4-week history of fever, cold, and cough. Physical examination revealed an exophthalmos of the right eye with no other symptoms, especially no lymphadenopathy, no hepatosplenomegaly, and normal neurological findings. A computed roentgen tomogram of the head showed an extended tumor mass ranging from the cribriform plate through the fossa cranii media to the clivus, penetrating into the cavum nasi (Fig. 1). Surgical diagnostic biopsy through

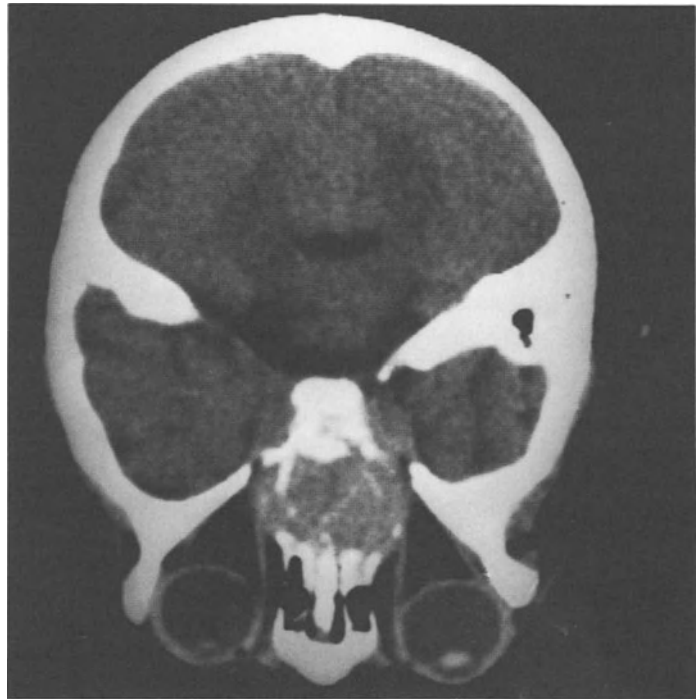
the nasal cavity failed, as pathological studies showed only normal mucous tissue. Blood counts revealed anemia (Hb 7.2 g/dl), a normal platelet count, and moderate leukocytosis. Peripheral blood smears showed no irregularities. The erythrocyte sedimentation rate was elevated to more than 50 mm/h and C-reactive protein was up to 104 mg/liter. Serum chemistry tests, especially catecholamine and beta-human chorionic gonadotrophin (HCG) secretion, were normal except for a lactate dehydrogenase level of 1104 U/liter. Bone marrow aspiration revealed 90% immature myeloid blast cells with pseudopod formation (Fig. 2). Cerebrospinal fluid examination showed 421 cells/ $\mu$ l with 21% blast cells identical in size and staining reactions with the bone marrow blasts. A diagnosis of acute megakaryoblastic leukemia with CNS involvement was made, the retrobulbic tumor mass being interpreted as chloroma. Induction chemotherapy according to the AML-BFM 87 protocols was started with high-dose cytarabine, daunoblastin, and etoposide, including intrathecal administration of cytarabine. The patient achieved only partial remission on day 14, with 15% remaining blasts in the bone marrow. But the cerebrospinal fluid was free from blast cells and the intracranial tumor mass started shrinking. Though consolidation therapy with prednisone, thioguanine, vincristine, adriablastine, cytarabine, and cyclophosphamide was performed, 5% blast cells persisted in the bone marrow. But following three reinduction protocols (regimens I, II: high-dose cytarabine and etoposide; regimen III: high-dose cytarabine and mitoxantrone), mar-

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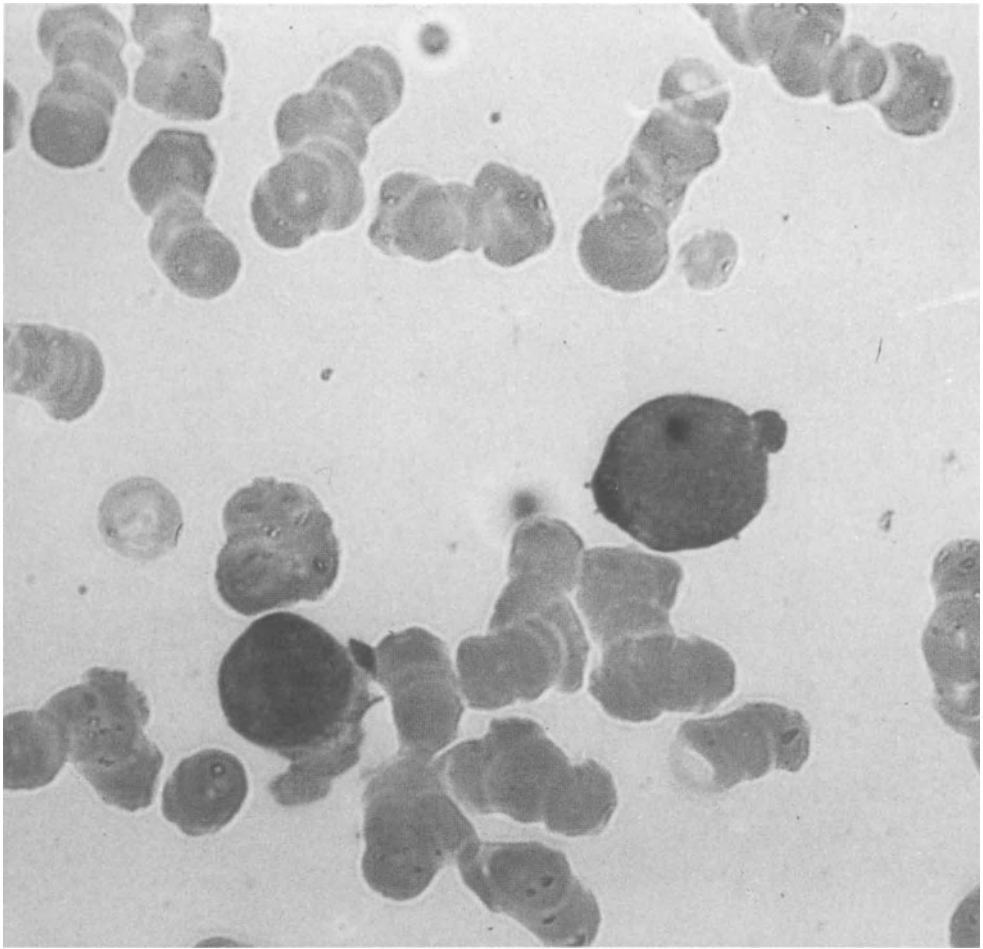


a



b

**Fig. 1 a, b.** Computed roentgen tomogram of the head at time of diagnosis showing the chloroma



**Fig. 2.** Megakaryoblast with typical "budding" of the cytoplasm

row blasts were no longer detectable and cranial irradiation with 24 Gy was performed. At the time of writing, the boy had been in continuous complete remission for more than 14 months while receiving maintenance therapy.

#### *Morphology and Cytochemistry*

No blasts in the peripheral blood smears could be detected. The bone marrow aspirates showed a slightly hypercellular marrow diffusely infiltrated by up to 80% blast cells. Megakaryocytes were not present and

erythro- and granulopoiesis were markedly reduced. The blast cells appeared pleomorphic with one or two prominent nucleoli, round nuclei, and a finely reticulated nuclear chromatin. Auer rods could not be detected. About 10% of the cells demonstrated "budding" of the cytoplasm (Fig. 2). The cytoplasm was agranular and intensively basophilic. Blasts were negative for alpha-naphthyl acetate esterase (NAE), myeloperoxidase (MPO), and periodic acid-Schiff (PAS) activity. Acid phosphatase (AP) was strongly positive, with a granular to patchy distribution of activity.

Blasts isolated from bone marrow aspirates expressed only antigens CD 33 and CDw 42. Typical megakaryocytic antigens such as factor VIII antigen or CD 9 could not be detected. Antigens for the myeloid cell lineage (CD 11c, CD 13, CD 15, Ki M8, VIM 2), for B cells (sIgM, skappa, slambda, CD 19, CD 20, CD 22, CD 23), T cells (CD 7, E-receptor, pan-T, CD 10), and for the pan-leukocyte antigen (CD 45) were negative as well. Cytogenetic analyses of 20 metaphases showed a numerically and structurally normal male karyotype.

### **Discussion**

In the current case the majority of blasts were undifferentiated in appearance and on a morphological basis could not be definitely characterized as either lymphoid or myeloid. Thus morphological diagnosis of AMegL may be confused primarily with the FAB L2 or FAB M1 subtype. Although cytoplasmic blebs, a nonspecific morphological marker previously reported in AMegL, were seen [1, 4, 8], typical megakaryocytic features such as platelet demarcation membranes could not be detected by light microscopy.

In the experience of most authors, routine cytochemistry may be helpful, but is not diagnostic. PAS activity may vary from negative to focal or granular positivity to strongly positive staining. AP reactivity is frequently positive with a localized pattern [2, 8, 19]. MPO activity is generally negative. However, a distinction between megakaryocytes and monocytes can be made, as megakaryocytes have distinct localized alpha-NAE positivity in contrast to the more diffuse cytoplasmic staining of monocytic cells.

Conventional electron microscopy may be useful in identifying differentiation along the megakaryocytic line with characteristic structures, e.g., alpha membranes, but these structures are rarely found in megakaryoblasts [19]. Platelet peroxidase (PPO), determined ultrastructurally by Anderson's technique [10], is exclusively localized on the nuclear membrane and endoplasmatic retic-

ulum. Unfortunately, the PPO reaction is readily inhibited by aldehyde fixatives and for this reason could not be demonstrated in the present case.

While cytogenetic findings were regular in our patient, the non-random involvement of chromosome 21 abnormalities in AMegL-like multiple copies [6, 19, 21], monosomy [9, 14], or constitutional ring chromosome [16] have been reported. Other cytogenetic abnormalities frequently seen in ANLL provide evidence that certain specific translocations [t(8;21) or t(11q)] occur with high frequency in very young children [19].

Recently, monoclonal and polyclonal antibodies have proved to be helpful in diagnosing AMegL. As in this case megakaryoblasts are generally negative for most myeloid and lymphoid markers. The only presence of the CDw 42 and CD 33 antigens – equivalent to glycoprotein Ib and early myeloid progenitor cells, respectively – was taken as proof for making the diagnosis of AMegL. Koike et al. described that reactivity with MoAb MY 9 (CD 33) and MY 10 (CD 34) appears earlier during maturation than platelet-specific glycoproteins such as GP IIb/IIIa antigens [11]. Heterogeneity of surface marker expression among variant patients thus may be related to the level of blast maturation.

On admission the boy presented with a retrobulbar tumor. Though an attempt for taking a biopsy failed, the typical retrobulbar tumor localization in coincidence with ANLL and its shrinking during chemotherapy might be taken as evidence that this solid tumor represented a chloroma. These tumors are extramedullary solid collections of ANLL blasts. In children they typically manifest themselves as unilateral exophthalmos caused by a retrobulbar mass [13]. On rare occasions chloromas may be preceded several months by the onset of leukemia [12]. The occurrence in ANLL varies from 3% to 14% and may be underreported [7], but as far as our knowledge goes no case of AMegL with chloroma has yet been reported.

Incidence of CNS leukemia in ANLL at diagnosis has ranged from 1.8% to 20.7% [13], whereas no data for AMegL have yet been reported. In childhood ANLL it could be demonstrated that CNS leukemia, if

treated with intrathecal chemotherapy followed by 2400 rad cranial irradiation, does not influence either rate of remission induction or the duration of continuous complete remission or survival [17]. This generalization includes patients with chloromas [17].

In AMegL various treatments have been tried [15]. In adults, complete remissions following chemotherapy with conventional induction therapy have been reported, but long-term responses are not common [1]. In some adult cases, low-dose cytarabine proved to be beneficial [18]. On the contrary, children with AMegL seem to respond better to ANLL chemotherapy [5, 6]. But allogeneic bone marrow transplantation may be considered as alternative primary therapy or may be useful if chemotherapy fails [17]. Our patient lacked an HLA-identical sibling and for that reason chemotherapy according to the Berlin-Frankfurt-Münster (BFM) AML protocols was performed. He initially responded with partial remission only, but after an additional reinduction protocol complete remission was achieved. Thus the standard AML BFM 87 treatment protocol seems to be an effective regimen, although our observation period has so far only extended to 14 months.

In summary, we would like to emphasize that, although certain morphological details may lead one to suspect the diagnosis of AMegL, routine cytochemical stains do not contribute to the characterization of smaller, undifferentiated frequently lymphoid-appearing megakaryocytic blast cells. Only the demonstration of antigens or ultrastructural features specific for the megakaryocytic lineage can confirm the diagnosis of AMegL. Greater recognition of AMegL and follow-up of such patients will determine the optimal therapy and long-term prognosis of this uncommon form of leukemia.

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## Risk of Leukemic Transformation in Two Types of Acquired Idiopathic Sideroblastic Anemia

N. Gattermann, C. Aul, and W. Schneider

### Introduction

In 1982 acquired idiopathic sideroblastic anemia (AISA) was included by the French-American-British (FAB) Cooperative Group in their classification of myelodysplastic syndromes (MDSs) [1]. However, since the initial descriptions by Björkman [2], Heilmeyer et al. [3], and Dacie et al. [4], the malignant potentiality of acquired idiopathic sideroblastic anemia has been a matter of debate, with reported rates of leukemic transformation between 0% and 50% (Table 1). This suggests to us that a heterogeneous group of patients is diagnosed as having AISA. In 1978, Heimpele [5] claimed that there may be a "pure" form of sideroblastic anemia (PSA) which shows only signs of dyserythropoiesis on bone marrow examination and has a very low propensity for leukemic transformation.

It would appear reasonable to differentiate this type from the clear myelodysplastic type of AISA, which is characterized not only by erythroid hyperplasia and ineffective erythropoiesis but also by disturbed granulopoiesis and/or megakaryopoiesis. Because of its similarity to the other types of MDS described by the FAB group, the latter disorder should appropriately be called "refractory anemia with ring sideroblasts" (RARS). Our follow-up study of 94 patients with AISA aimed to find out whether a distinction on cytomorphological grounds between PSA and RARS may be of clinical

relevance. On 20 patients we also performed the progenitor cell assay for the granulocyte-macrophage lineage to see whether *in vitro* growth of CFU-GM helps to distinguish between the two proposed types of AISA.

### Materials and Methods

#### *Patients*

All reports between 1972 and 1987 from our bone marrow cytology laboratory were screened for reference to ringed sideroblasts. The respective slides were reevaluated and a provisional diagnosis of AISA was only made when, in keeping with the features proposed by the FAB group, the bone marrow showed normo- or hypercellularity, signs of dyserythropoiesis with at least 15% ring sideroblasts, and no increase in medullary blast count. These patients' records were then traced in the university clinic and at those hospitals that had submitted the slides to our laboratory for evaluation. Special attention was paid to exclusion criteria such as alcoholism, concurrent malignancies, chloramphenicol administration, or treatment with antituberculous drugs. The data presented here include follow-up until 31 May 1988. The patients were either regularly seen in our hematology outpatient clinic, or we regularly contacted their primary care physicians as well as clinicians at local hospitals to obtain information about vital status, causes of death, infections, hemorrhagic complications, etc.

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## *Cytological Distinction Between PSA and RARS*

Morphological abnormalities were examined on May-Grünwald-Giemsa stains of peripheral blood and bone marrow films. For each patient at least 200 erythropoietic and granulopoietic cells, respectively, as well as 25 megakaryocytes were screened for myelodysplastic features as suggested by Bennett et al. [1]. On Prussian-blue staining ring sideroblasts were defined as having at least ten siderotic granules. For a proportion of patients the myeloperoxidase and periodic acid-Schiff (PAS) reactions could also be evaluated. Patients whose bone marrow showed only signs of dyserythropoiesis, while the granulocytic and megakaryocytic series appeared normal, were diagnosed as having pure sideroblastic anemia (PSA;  $n=53$ ). On the other hand, cases with dysplastic features of granulopoiesis and/or megakaryopoiesis in addition to dyserythropoiesis were considered a true myelodysplastic syndrome (refractory anemia with ring sideroblasts, RARS;  $n=41$ ).

### *Granulocyte/Macrophage Colony Assay*

The *in vitro* growth of granulocyte/macrophage progenitor cells (CFU-GM) was assayed using the method described by Iscove et al. [6]. Briefly, mononuclear bone marrow cells (MNCs) obtained by density separation on Ficoll-Hypaque were plated at a concentration of  $1 \times 10^5$  MNCs/ml in semisolid culture medium containing Iscove's modified Dulbecco's medium (IMDM), 1% methylcellulose, 40% fetal calf serum, and 7.5% giant-cell tumor-conditioned medium (GCT; Gibco). All cultures were performed in quadruplicate and scored after 7 and 14 days of incubation at 37°C in a fully humidified 5% CO<sub>2</sub> atmosphere. Colonies were defined as aggregates of more than 40 cells, whereas smaller aggregates (4–40 cells) were counted as clusters. The CFU-GM progenitor cell assay was performed on 10 consecutive patients with PSA and RARS, respectively, and the results compared with those from a control group of 20 persons whose bone marrow was normal on cytological and histological examination.

## *Statistics*

The statistical evaluation of the data was carried out with computer assistance using the program system BMDP [7]. The procedure suggested by Kaplan and Meier [8] was used for estimating the probability of survival and of leukemic transformation. The characteristics of the survival distributions for the two groups of patients (PSA, RARS) were tested by means of nonparametric test statistics [log-rank (Mantel-Cox) test and W-score (Breslow) test]. The incidences of leukemic transformation were compared by the chi-square test with Yates' correction. Wilcoxon's rank-sum test was used to compare colony numbers between the two groups of patients (PSA, RARS).

## **Results**

### *Cytology*

By definition, specimens from patients with PSA did not show significant changes of granulopoiesis or megakaryopoiesis. It must be recognized that on careful examination of the slides one will always find a few granulopoietic or megakaryopoietic cells with morphological abnormalities, even in "pure" sideroblastic anemia. However, these findings are too rare to be considered evidence of dysgranulopoiesis or dysmegakaryopoiesis. Pseudo-Pelger anomalies, hypogranulation of myelocytes, defective myeloperoxidase staining, micromegakaryocytes, and multinucleated megakaryocytes could only be found in patients with RARS and were useful markers for the myelodysplastic form of AISA. With respect to dyserythropoiesis, morphological abnormalities were similar in both groups of patients. However, a positive reaction on PAS-staining of erythroid precursor cells was exclusively seen in patients with RARS. On average, the percentage of ring sideroblasts was higher in PSA, but this difference did not reach statistical significance. No difference between PSA and RARS was noted as to the pattern of iron accumulation within erythroblasts.



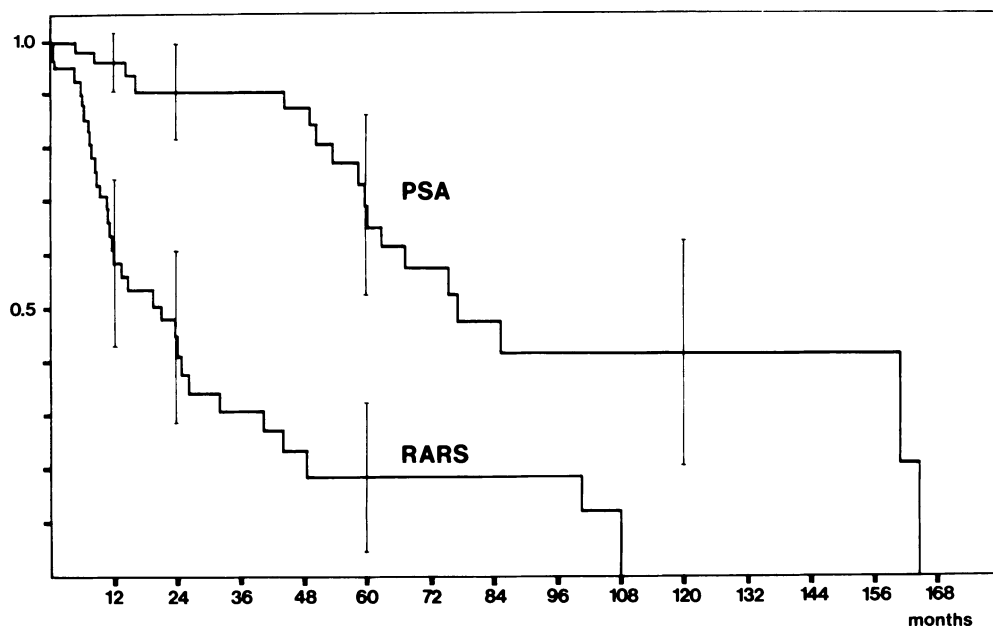


Fig. 1. Cumulative survival rates (Kaplan-Meier plot) of patients with PSA ( $n=53$ ) and RARS ( $n=41$ )

### Cumulative Survival

Kaplan-Meier survival curves are shown in Fig. 1. Follow-up periods ranged up to 164.5 months in PSA and up to 108 months in RARS. One year after diagnosis cumulative survival is 96% for PSA and 58% for RARS. At 2 years (90% vs. 45%) and 5 years (69% vs. 19%) the difference between the two groups is even more striking. Ten years after diagnosis none of the RARS patients is alive, whereas cumulative survival for PSA is still 41%. Survival curves differ significantly on analysis by log-rank test ( $P<0.0005$ ) and W-score test ( $P<0.0005$ ).

### Leukemic Transformation

The cumulative rates of leukemic transformation are shown in Fig. 2. Only one patient who was classified as having PSA subsequently developed acute myeloid leukemia (AML) at 12.6 months of follow-up. Figure 2 thus shows an early plateau at 1.89% for

PSA. None of the PSA patients showed progression to an MDS type of higher malignancy (RAEB, RAEB-T). Prognosis for RARS, on the other hand, was significantly worse ( $P<0.0001$ ); AML developed in 10 out of 41 patients. The Kaplan-Meier estimate yields a 48% cumulative proportion of leukemic transformation less than 4 years after diagnosis (Fig. 2).

### Causes of Death

There were 31 deaths among our 40 patients with RARS. Infections (ten cases) and hemorrhages (eight cases), unrelated to leukemic transformation, were common causes of death in this group. In comparison, there was only one death from infection and one death from bleeding within the PSA group. Causes of death not related to the underlying disease prevailed in patients with PSA, whose life expectancy did not seem to be very much compromised by their hematological disorder.

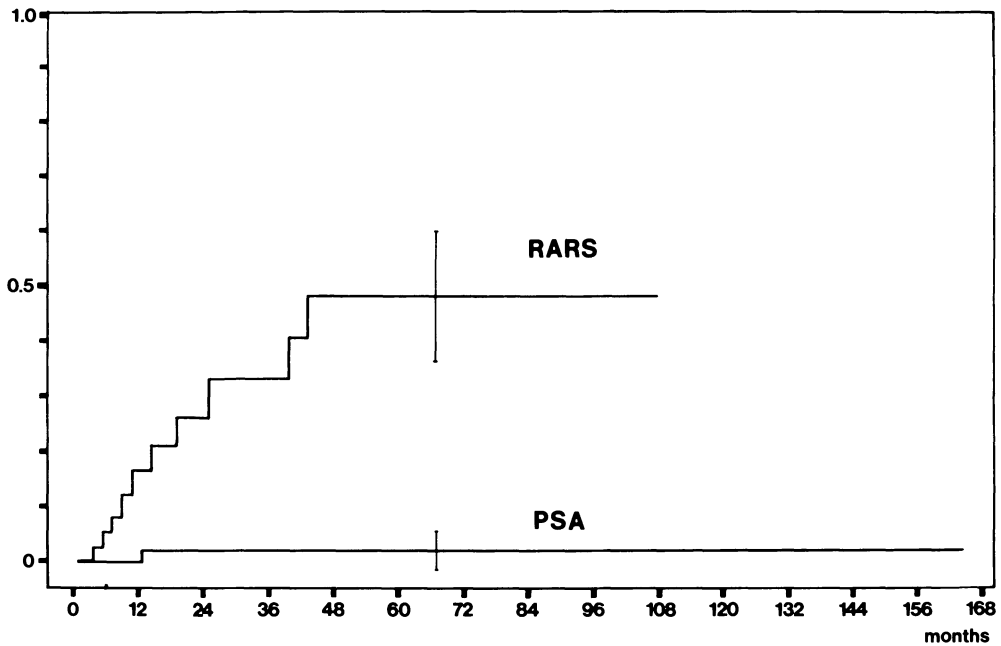


Fig. 2. Cumulative percentage of transformation to acute leukemia (Kaplan-Meier plot) of patients with PSA and RARS

### CFU-GM

Granulocytic colony growth was markedly reduced in RARS (Fig. 3). Compared with controls (day 7:  $111 \pm 72$ ; day 14:  $67 \pm 42$ ) mean colony numbers in RARS were much smaller (day 7:  $11 \pm 14$ ; day 7+8) ( $P < 0.01$ ) on both days). For some RARS patients "zero growth" was noted. PSA patients showed a less impressive yet significant reduction of colony formation (day 7:  $54 \pm 40$  ( $P < 0.05$ ); day 14:  $28 \pm 20$  ( $P < 0.01$ )). The difference between RARS and PSA was significant on day 7 ( $P < 0.05$ ) as well as on day 14 ( $P < 0.01$ ). Macrophage colonies were rare on day 7 but could be evaluated on day 14 of culture. There was no significant difference between PSA ( $45 \pm 42$ ) and the control group ( $60 \pm 52$ ). RARS patients, however, had very low colony counts ( $3.3 + 3.9$ ) as compared with controls ( $P < 0.01$ ). The difference between PSA and RARS was statistically significant ( $P < 0.01$ ).

We found a significant negative correlation ( $r = 0.83$ ;  $P < 0.01$ ) between the number of CFU-GMs in culture and the degree of bone marrow erythropoietic hyperplasia in

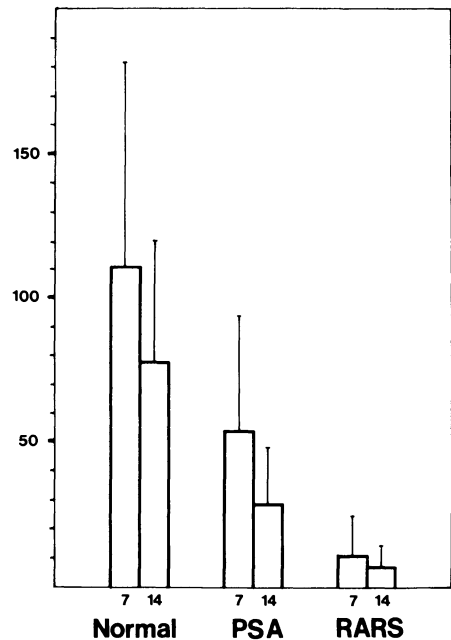


Fig. 3. Number of granulocytic colonies on days 7 and 14 in patients with PSA ( $n = 10$ ), RARS ( $n = 10$ ), and a group of normal controls ( $n = 20$ ). As the CFU-GM assay was performed in quadruplicate, each column represents the mean (+ standard error) of 40 colony counts ( $10 \times 4$ ) for PSA and RARS or 80 counts ( $20 \times 4$ ) for controls

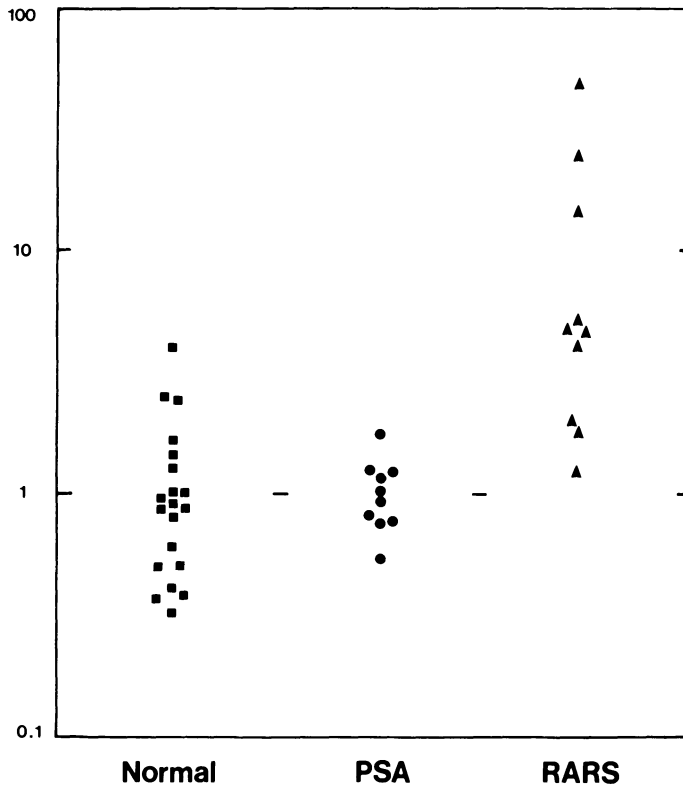


Fig. 4. Ratio (logarithmic scale) of granulocytic clusters and colonies (day 14 of cell culture) in patients with PSA ( $n=10$ ), RARS ( $n=10$ ), and controls ( $n=20$ )

patients with PSA but not with RARS (data not shown). Culture dishes from RARS patients contained mainly small aggregates of cells, thus producing high cluster/colony ratios (median 4.76; mean 11.0), whereas patients with PSA had cluster/colony ratios (0.96; 1.01), which did not differ from those of the control group (0.89; 1.14) (Fig. 4). The difference between RARS and PSA was statistically significant ( $P < 0.01$ ), as was that between RARS and controls.

## Discussion

Our follow-up study of 95 patients with AISA shows that distinction between an erythropoietic (PSA) and a myelodysplastic form (RARS) is indeed of clinical relevance. Though all patients fulfilled the criteria of the FAB classification for MDS, only those

with clearly dysplastic features of granulopoiesis and/or megakaryopoiesis in addition to dyserythropoiesis had a markedly elevated risk of progression to acute myeloid leukemia. The only patient in our series with PSA who later developed AML had slight morphological changes in some of the granulocytic bone marrow cells, which we believed were inadequate as evidence of dysgranulopoiesis.

We think that our follow-up findings can be used to explain at least in part the widely divergent accounts of the risk of leukemic transformation in AISA. Table 1 summarizes the data on the incidence of progression to acute leukemia that we found in the literature. The authors generally do not distinguish between an erythropoietic and a true myelodysplastic form of sideroblastic anemia, but we surmise that the different rates of malignant transformation greatly

**Table 1.** Risk of progression to acute leukemia in patients with acquired idiopathic sideroblastic anemia. Data from the literature

Study	Number of patients	Progression to AML
Greenberg et al. 1976 [9]	10	0 (0%)
Juneja et al. 1983 [10]	16	0 (0%)
Foucar et al. 1985 [11]	22	0 (0%)
Kerndrup et al. 1986 [12]	10	0 (0%)
Weisdorf et al. 1983 [13]	17	1 (4%)
Kushner et al. 1971 [14]	61 <sup>a</sup>	3 (5%)
Mufti et al. 1985 [15]	21	1 (5%)
Heilmeyer et al. 1964 [16]	18	1 (6%)
Cheng et al. 1979 [17]	268 <sup>a</sup>	27 (10%)
Streeter et al. 1977 [18]	17	2 (12%)
Mende et al. 1980 [19]	23	3 (13%)
Kerkhofs et al. 1987 [20]	88	14 (16%)
Beris et al. 1983 [21]	45	8 (18%)
Coiffier et al. 1983 [22]	20	4 (20%)
Lewy et al. 1979 [23]	25	6 (24%)
Björkman 1963 [24]	12	3 (25%)
Reizenstein et al. 1972 [25]	14	4 (29%)
Todd et al. 1986 [26]	56	19 (34%)
Dameshek 1969 [27]	?	ca. 50%

<sup>a</sup> Meta-analysis

depend on how patients are distributed among the two proposed subtypes of the disease. Samples with a low incidence of leukemic transformation presumably contain a high percentage of patients with PSA according to our definition.

Refractory anemia with ring sideroblasts and PSA differ not only in terms of leukemic transformation but also as far as overall survival is concerned. Survival rates are much better for patients with pure sideroblastic anemia, who have less complications from hemorrhage and infection. It is interesting to note that, in follow-up studies of MDS, sideroblastic anemia usually has a comparatively good prognosis. Again, we would like to interpret this as an indication that the statistics of clinical outcome are favorably influenced by a subgroup of patients with PSA.

By demonstrating marked quantitative and qualitative impairment of granulocytic colony growth on the one hand (RARS) and absence of dysgranulopoiesis on the other hand (PSA), our cell culture findings are in

support of splitting up AISA into a true myelodysplastic type (RARS) and a relatively benign form which seems to be largely confined to dyserythropoiesis (PSA).

A moderate decrease in the number of granulocytic colonies was, however, found in PSA (Fig. 4), which we initially did not expect because bone marrow cytology did not show dysgranulopoiesis. It must be taken into consideration, however, that erythropoietic hyperplasia may influence granulocytic colony counts. We found a significant negative correlation between the degree of bone marrow erythroid hyperplasia and the number of CFU-GMs in culture for patients with PSA. Presumably this simply reflects the fact that in erythroid hyperplasia the MNC fraction of the bone marrow aspirate, from which cells are harvested for plating, contains a high proportion of erythroid progenitor cells and corresponding decreased numbers of CFU-GM.

While such "dilution" of CFU-GM by erythropoietic precursors may be responsible for the moderate reduction of colony numbers in PSA, this mechanism cannot account for the markedly depressed growth of CFU-GM in RARS patients, for whom there is no correlation between CFU-GM and erythroid hyperplasia. In addition to very low colony formation in RARS we found the prevalence of small cell aggregates (clusters) typical of the myelodysplastic syndromes. The pathological ratio of clusters and colonies strongly points to an intrinsic impairment of granulopoietic progenitor cells.

While our distinction between PSA and RARS seems to be clinically important and is supported by the cell culture findings, we must further ask whether this distinction is between two different clinical entities or between an early and an advanced stage of the same disease process. In terms of the multi-step leukemogenesis concept one might speculate that PSA represents a first step caused by an insult to a pluripotential stem cell, which manifests itself, for unknown reasons, exclusively as an impairment of erythropoiesis. This stage seems to be rather stable until one or more further attacks on the genetic code produce a myelodysplastic syndrome (RARS) and at the same time lead to an acceleration of genetic instability, thus

predisposing to preleukemic progression. Our data do not allow us to decide whether PSA and RARS are within the spectrum of the same disease process or must be ascribed to different pathogenetic mechanisms. However, we have shown that on morphological grounds we can distinguish between two types of AISA, which differ considerably in terms of survival, risk of leukemic transformation, and findings on CFU-GM bone marrow culture. Because one of the two types (PSA) usually runs a benign course whereas the other (RARS) is a preleukemic condition possibly requiring aggressive treatment [28], we think the distinction is of clinical importance.

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## Treatment of Advanced Myelodysplastic Syndromes: Trend Toward More Aggressive Chemotherapy?

C. Aul and W. Schneider

### Introduction

The myelodysplastic syndromes (MDSs) are acquired disorders of hemopoiesis, probably deriving from the clonal expansion of transformed pluripotent hemopoietic stem cells [1, 2]. In the majority of patients, they have a fatal course within months to years owing to complications of bone marrow failure or transition to acute myeloid leukemia (AML). Prognosis is particularly poor in patients with refractory anemia with excess of blasts (RAEB), RAEB in transformation (RAEB/T), and chronic myelomonocytic leukemia (CMML), who, according to recent studies, have a median survival of only 2.5–17.5 months [3–5].

At present, no generally effective therapy is available for these disorders. Aggressive chemotherapy, which on theoretical grounds should be the most appropriate form of treatment, is usually considered ineffective or even dangerous because of the risk of severe and prolonged bone marrow aplasia. This view is largely based on earlier reports comprising small groups of patients and using somewhat arbitrary doses and combinations of cytotoxic drugs [6–8]. The low remission rates (0%–15%) obtained in these studies compared unfavorably with those reported for patients with de novo AML. Recently, however, more encouraging results have begun to appear. Tricot and Boogaerts [9] treated 15 patients (RAEB, RAEB/T, MDS-AML) with aggressive

chemotherapy and observed a complete bone marrow remission in eight cases. After successful remission induction, all patients initially presenting with chromosomal abnormalities returned to a normal karyotype, suggesting the disappearance of the malignant cell clone. Similar results have been described by Preisler et al. [10] in a series of 11 patients with secondary AML. In view of these findings, it seemed important to us to reevaluate the potential of cytoreductive chemotherapy in MDS patients. The present paper summarizes the data on 19 patients with advanced MDS (RAEB/T, MDS-AML) who were treated with standard AML protocols at the University of Düsseldorf.

### Patients and Methods

#### *Patients*

Between January 1984 and November 1988, 19 patients (13 men, 6 women) with an initial diagnosis of primary MDS were entered into the study. MDS was classified according to the French-American-British (FAB) criteria [11]. The median time from diagnosis to initiation of aggressive chemotherapy was 4 months (range, 1–16 months). Prior to therapy, a repeat bone marrow examination was performed in most cases to exclude the possibility that the bone marrow changes represented an early phase of de novo AML. All patients included in this study had a Karnofsky score of more than 80% and did not suffer from severe cardiac, respiratory, or renal disease. Their morpho-

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logical diagnoses as well as hematological characteristics at the start of chemotherapy are summarized in Table 1. The median age of patients was 55 years (range, 17–65 years). Six cases were treated during the preleukemic phase and 13 after progression of MDS to overt leukemia. Five patients (Nos. 11, 12, 15, 18, 19) had previously been treated with low doses of cytarabine. Of these, only one patient (No. 11) achieved a short-lived remission (6 months).

### *Treatment Protocols*

For remission induction, 17 patients received one cycle of TAD9 consisting of cytarabine (100 mg/m<sup>2</sup> per day by continuous intravenous infusion on days 1 and 2, and 100 mg/m<sup>2</sup> every 12 h by infusion on days 3–5), daunorubicin (60 mg/m<sup>2</sup> i.v. on days 3–5), and 6-thioguanine (200 mg/m<sup>2</sup> per day orally on days 3–9). Two younger patients (Nos. 2, 7) were treated with a double induction regimen in which the initial TAD9 course was followed on day 21 either by a second TAD9 course or a combination of high-dose cytarabine (3 g/m<sup>2</sup> every 12 h by 3-h infusion on days 1–3) and mitoxantrone (10 mg/m<sup>2</sup> i.v. on days 3–5). Supportive care was provided by oral antibiotic (trimethoprim-sulfamethoxazole/colistine or ciprofloxacin) and antimycotic (nystatin) prophylaxis as well as adequate transfusion of blood products. After achievement of CR, six patients received additional consolidation (TAD9) and maintenance (M1–M4) chemotherapy according to the recommendations of the German AML Cooperative Group [12].

### *Response Criteria*

Response to treatment was assessed by both bone marrow examination and peripheral blood counts. Complete (CR) and partial remission (PR) were defined according to Cancer and Leukemia Group B (CALGB) criteria [13]. Early death (ED) was defined as death during the first 6 weeks after the start of treatment, and nonresponse (NR) as failure to achieve CR or PR in patients surviving at least 6 weeks of therapy.

## **Results**

The results of induction therapy are presented in Table 1. Twelve (63%) out of 19 patients entered CR, and 2 patients had a partial response. Early death occurred in two cases and three patients had refractory disease. The median age of the complete responders was 53 years, as compared with 58 years in the group of failures. It is of interest that two of four patients who had unsuccessfully been treated with low-dose cytarabine responded to induction therapy with TAD9. Patient 11, who had already responded to low-dose cytarabine, achieved a second CR with TAD9. Excluding those patients who were treated with a double induction regimen, all complete remissions in this series were obtained after one course of TAD9.

No unusual toxicities of chemotherapy were noted. Sixteen of 19 induction cycles were complicated by episodes of fever ( $\geq 38.5^{\circ}\text{C}$ ) which required systemic antibiotic therapy. Despite these measures, two patients died of pneumonia 10 and 31 days after the start of chemotherapy. The median duration of bone marrow aplasia (leukocytes  $< 1.0 \times 10^9$ /liter) for patients achieving CR was 21 days (range, 6–51 days). Prolonged periods of aplasia (34 and 51 days) were observed in only two patients, one of whom received an intensified preremission chemotherapy (double induction). Except for three cases, morphological analysis of postinduction bone marrow smears revealed complete disappearance of myelodysplastic features.

The duration of CR was variable, ranging from 2 to 36+ months. Of interest, prolonged remissions could only be obtained in patients treated with consolidation and maintenance therapy after induction of CR. In this group, one patient (No. 1) has been in continuous CR for 36 months now, and two other patients (No. 4, 12) achieved a long-lasting remission (23, 29 months, respectively). In contrast, patients who did not receive postremission chemotherapy had a less favorable outcome and all of them relapsed within 1 year of CR.



**Table 1.** Results of aggressive chemotherapy in 19 patients with advanced MDS

Pa- tient	Sex/ age	Diagnosis	Peripheral blood		Bone marrow blasts (%)	Chemotherapy	Aplasia period (days)	Out- come <sup>a</sup>	Consoli- dation/ maintenance therapy	Duration of CR (months)
			Leukocytes (10 <sup>9</sup> /liter)	Platelets (10 <sup>9</sup> /liter)						
1	F/17	RAEB/T	4.7	194	16, Auer +	TAD9	6	CR	Yes	36+
2	F/36	RAEB/T	3.2	135	23, Auer +	TAD9-TAD9	27	CR	Yes	3+
3	M/52	RAEB/T	4.8	14	25, Auer +	TAD9	19	CR	No	2
4	M/54	RAEB/T	9.3	42	26, Auer +	TAD9	24	CR	Yes	29
5	M/56	RAEB/T	21.6	79	24	TAD9	21	CR	No	2
6	M/57	RAEB/T	3.3	13	28	TAD9	25	CR	Yes	5
7	M/32	RAEB-AML	7.4	67	70	TAD9-HAM	51	CR	No	9
8	M/37	RAEB/T-AML	1.6	52	42	TAD9	21	PR		
9	M/44	RAEB/T-AML	6.6	39	40, Auer +	TAD9	34	CR	Yes	1+
10	M/46	RAEB/T-AML	5.3	143	40	TAD9	14	CR	No	5
11	M/54	CMMI-AML	6.2	132	32	TAD9	21	CR	Yes	4+
12	M/55	RAEB/T-AML	0.6	16	45	TAD9	20	CR	Yes	23
13	M/55	RARS-AML	38.9	14	95	TAD9		ED		
14	F/56	RAEB/T-AML	14.9	48	80, Auer +	TAD9	25	NR		
15	M/58	RAEB/T-AML	2.5	40	55	TAD9	14	NR		
16	F/58	RARS-AML	180.0	24	91	TAD9		ED		
17	M/59	RA-AML	1.2	19	59, Auer +	TAD9	21	CR	No	5
18	F/62	RAEB-AML	13.5	52	90	TAD9	31	NR		
19	F/65	RAEB-AML	60.5	7	95	TAD9	11	PR		

<sup>a</sup> Remission criteria according to CALGB

## Discussion

Since the initial description of "preleukemic anemia" by Hamilton-Paterson [14], this syndrome has been diagnosed with increasing frequency, largely as a result of improved morphological criteria. Epidemiological data now suggest an incidence of about 2–3/100 000 people [15]. According to the proposals of the FAB Cooperative Group [11], the MDSs can be separated into five morphological subtypes (RA, RARS, RAEB, RAEB/T, CMML). Prognosis of MDS patients is markedly influenced by the bone marrow blast cell count at the time of diagnosis. Thus, median survival for these disorders progressively shortens from over 30 months in RA to 11 months in RAEB and 5 months in RAEB/T [3–5, 16]. Unfortunately, no treatment modality is known that can clearly alter the natural course of the disease.

Intensive chemotherapeutic protocols as used in de novo AML are usually not recommended for patients with MDS. Such reluctance stems from earlier studies according to which these patients have a low probability of entering remission, but often succumb to complications of chemotherapy-induced bone marrow aplasia [17]. It has been argued, however, that these unfavorable results are at least partly due to a negative selection of patients, inadequate dosage of chemotherapeutic agents, and insufficient supportive measures. The aim of our study, therefore, was to define more precisely the role of aggressive chemotherapy in MDS.

In our trial, 19 MDS patients at different stages of leukemic transformation were treated with standard AML protocols. Apart from the hematological disease, all patients were in good clinical condition and presented with a Karnofsky score of more than 80%. In this series, response to chemotherapy was excellent and 12 patients (63%) entered a complete bone marrow remission. In the majority of cases, the post-remission bone marrow examination revealed no signs of persisting dyshemopoiesis. Including those patients who achieved a partial remission, the overall response rate in our patient population was 74%. These data strongly argue against the assumption that the malignant cell clone in MDS is

generally refractory to cytoreductive chemotherapy.

According to our experience, two variables appeared to be associated with successful remission induction:

1. the presence of Auer rods, which could be demonstrated in 6 of 12 complete responders at the time of diagnosis, and
2. the pretreatment bone marrow blast count. Patients with a comparatively low blast count (RAEB/T) had a much better chance of successful remission induction than those patients whose bone marrow disease had already progressed to overt leukemia. In contrast to previous findings [9], age was not found to have a profound influence on the probability of remission.

Although the majority of our patients were more than 50 years old, aggressive chemotherapy was well tolerated and not associated with an increase in toxicity. Except in two patients we did not observe excessive periods of bone marrow aplasia. These findings suggest to us that, as far as chemotherapy is concerned, MDS may not be all that different from de novo AML.

In this study, marked differences in outcome were found between patients receiving or not receiving postremission chemotherapy. Of four patients entered on a consolidation and maintenance regimen as currently used by the German AML Cooperative Group [12], one has remained in continuous CR for 36 months now and 2 other patients achieved remissions of more than 2 years. On the contrary patients who did not receive further treatment after induction of CR were uniformly characterized by an early relapse of their disease. This preliminary analysis suggests an important role for intensive postremission chemotherapy in prolonging remission and survival in MDS.

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## Chronic Myelomonocytic Leukemia: Clinical Data, Morphological Features, and Outcome in 56 Patients

A. Heyll and G. Derigs

Chronic myelomonocytic leukemia (CMML) is a hematological disorder showing features of both myelodysplastic (MDS) and myeloproliferative syndromes. Due to the proposals of the French-American-British (FAB) group in 1982 [1], precise diagnostic criteria facilitate the grouping of MDS patients according to hematologic parameters. We present the clinical data, morphological features, and outcome in 56 patients diagnosed as having CMML according to the FAB criteria.

### Material and Methods

*Diagnostic Criteria for CMML.* In 48 patients a diagnosis of CMML was established exactly according to the FAB criteria: monocytosis over 1000/ $\mu$ l, blasts in peripheral blood less than 5%, and bone marrow blasts up to 20%. In five cases we accepted a diagnosis of CMML despite peripheral blast counts of more than 5% (6%, 9%, 11%, 12%, and 15%). However, bone marrow blasts did not exceed 20% and thus the most important criterion for the differential diagnosis of refractory anemia with excess blasts in transformation (RAEB-T) or even acute myeloblastic leukemia (AML) was not fulfilled. Another three patients were also diagnosed as having CMML although the absolute monocyte count was in the range of 800–1000/ $\mu$ l. These three patients had relatively low leukocyte counts

(range 4100–4500/ $\mu$ l), so that they did not reach the threshold value although the differential blood count displayed significant monocytosis (18%, 19%, 22%). In addition, other criteria such as splenomegaly, signs of dyshematopoiesis, and increase in bone marrow blast count favored a diagnosis of CMML.

### Methods

In addition to a basic clinical investigation peripheral blood counts and a broad panel of laboratory investigations were carried out by standard techniques. To measure thymidine kinase serum levels, a commercially available radioenzyme assay (Prolifigen Tk-REA, Byk-Sangtec, Diezenbach, FRG) was used. The normal range extends from 0.9 to 4.9 U/ml in our laboratory. Peripheral blood and bone marrow smears of all patients were stained with conventional techniques (Pappenheim, myeloperoxidase (POX), alpha naphthyl acetate esterase ( $\alpha$ -NE), periodic acid-Schiff, Prussian blue) and studied by two independent observers. Cumulative survival from the time of diagnosis and the risk of leukemic transformation were estimated by the Kaplan-Meier method.

### Results

#### *Patient population*

Fifty-six patients diagnosed as having CMML in our institution from 1981 to 1988

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were studied. Men ( $n=36$ ) presented twice as often as women ( $n=20$ ). Median age at time of diagnosis was 74 years; there were only two patients younger than 50 years. In none of the patients was there a history of myelotoxic agents (e.g., alcoholism, drugs).

### *Clinical Findings*

At the time of diagnosis performance status was reduced in 53% of patients. Frequent findings were: splenomegaly (53%), symptoms caused by anemia (50%), hepatomegaly (44%), signs of bleeding (skin, gastrointestinal, hematuria, epistaxis) (43%). Less frequent were: infections (28%), fever (12%), and lymphoma (5%). Of the 27 patients with splenomegaly, in 25% splenic enlargement could only be diagnosed by sonography (sagittal diameter  $>5$  cm). In 50% of these patients the spleen extended less than 10 cm below the left costal margin and 25% showed massive splenomegaly.

### *Hematological Data*

**Blood count.** Ninety-four percent of patients showed absolute monocytosis over  $1000/\mu\text{l}$ , 82% thrombocytopenia, 68% anemia, 60% leukocytosis, and 45% granulocytosis. On peripheral blood examination at least some blasts were found in 27%, Pelger-like anomaly in 18%, nucleated red precursors in 28%, fragmentocytes in 15%, and polychromasia of erythrocytes in 36% of patients. Anemia in most cases was normochromic and normocytic.

**Bone Marrow.** Bone marrow smears were hypercellular in 72% of patients. In 98% of patients the relative amount of monocytes in the bone marrow was  $>3\%$  of nucleated cells (median: 18%, range: 3%–60%). Bone marrow smears stained by the Pappenheim technique often did not reveal the true proportion of monocytes. Thus an alpha-NE stain was evaluated in all cases. Granulocytopoiesis was hypercellular in 93% and showed a shift to immature precursors in 98% of patients. The median percentage of blast cells was 8% (range 2%–19%). Ery-

throcytopoiesis in most cases was decreased ( $<15\%$  erythroblasts in two thirds of patients) and megakaryocytopoiesis was normocellular (53% of patients), hypercellular (25%), or hypocellular (21%). Signs of dys-hematopoiesis were found in all cell lines; only in erythrocytopoiesis were they less frequent than in other MDS subtypes (Table 1).

### *Other Laboratory Findings*

The neutrophil alkaline phosphatase (NAP) index was in the range of 0–10 in 45% of patients, in 55% the index was in the normal range, and one patient showed an NAP index of 239. In concordance with augmentation of medullar iron stores, increased serum ferritin levels of up to 2500 ng/ml were found in 50% of patients. Fifty-five percent showed increased lactate dehydrogenase (LDH) values up to 900 U/liter. Signs of hemolysis were not found. Total serum protein was increased in the range of 8.1–9.9 g/dl in 19% of patients, and the gamma-globulin fraction was elevated in 69% (IgG: 21%–53% of total protein). In two patients a paraprotein was detected, in one case IgM with concomitant immunocytoma in bone marrow, and IgG in the other patient with no symptoms of multiple myeloma. Serum thymidine kinase showed increased levels of up to 190 U/ml in 86% of patients. Lysozyme serum levels were increased up to 100 times of the normal upper limit in all cases investigated. In one patient urine lysozyme was not detectable, in all other cases urine levels were significantly increased without impaired kidney function. In five patients with marked splenomegaly cytogenetic analysis for the Ph<sup>1</sup>-chromosome was negative.

### *Therapy*

Sixteen patients were treated with cytotoxic drugs, 14 of them with low-dose Ara-C. In one case CR was obtained. Ten patients received prednisone with no evidence of response. Kaplan-Meier analysis of survival showed no difference in treated and untreated patients.

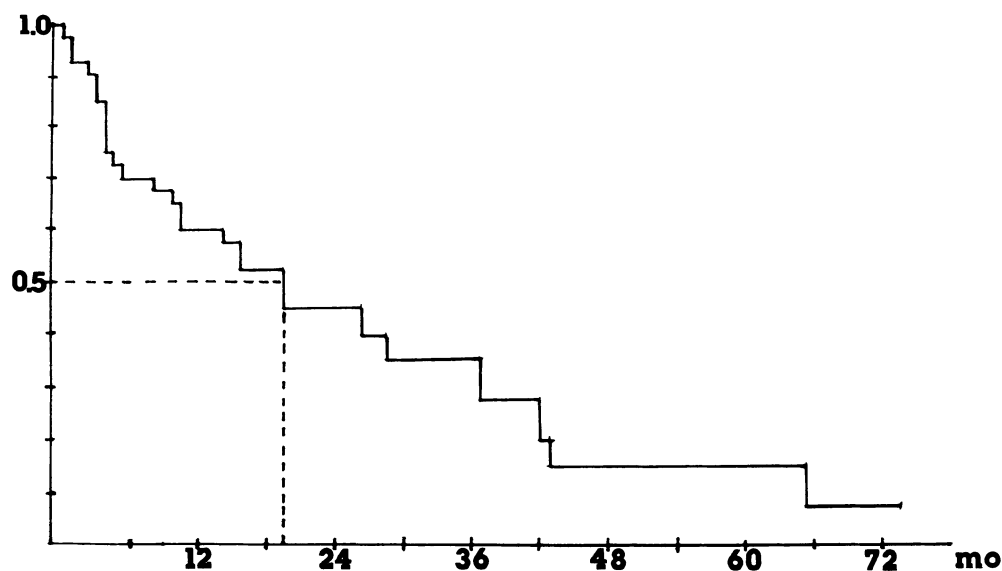
**Table 1.** Signs of dyshematopoiesis

	CMML (n = 56)	Total MDS group (n = 261)
Granulocytopoiesis		
Hypersegmentation	71%	26%
Hypogranulation	50%	56%
Pelger-like anomaly	45%	31%
Myeloperoxidase deficiency	23%	26%
Erythrocytopoiesis		
Sideroblastosis	28%	60%
Nuclear fragments	16%	35%
Megaloblastic transformation	14%	31%
Ringed sideroblasts > 15%	9%	28%
Megakaryocytopoiesis		
Hypersegmentation	43%	24%
Micromegakaryocytes	26%	35%
Large mononuclear megakaryocytes	25%	33%

### *Prognosis and Survival*

At the time of evaluation 28 patients were alive, 26 patients had died, and 2 were lost to follow-up. Most patients died of bleeding and infection. In 47 patients observation time was 1–74 months. Median survival was 19 months; 9 patients lived more than 24 months (Fig. 2). Compared with other MDS subtypes leukemic transformation was ob-

served in only a minority of patients. During the time of observation only four patients progressed to acute leukemia (two AML and two mixed type AL) (Fig. 2). Some authors postulate two forms of CMML: a “myelodysplastic” and a “myeloproliferative” subtype [2]. To study this hypothesis we divided our patients into those with splenomegaly and those with normal spleen size. Both groups comprised about 25 pa-

**Fig. 1.** Cumulative survival of 47 patients with CMML

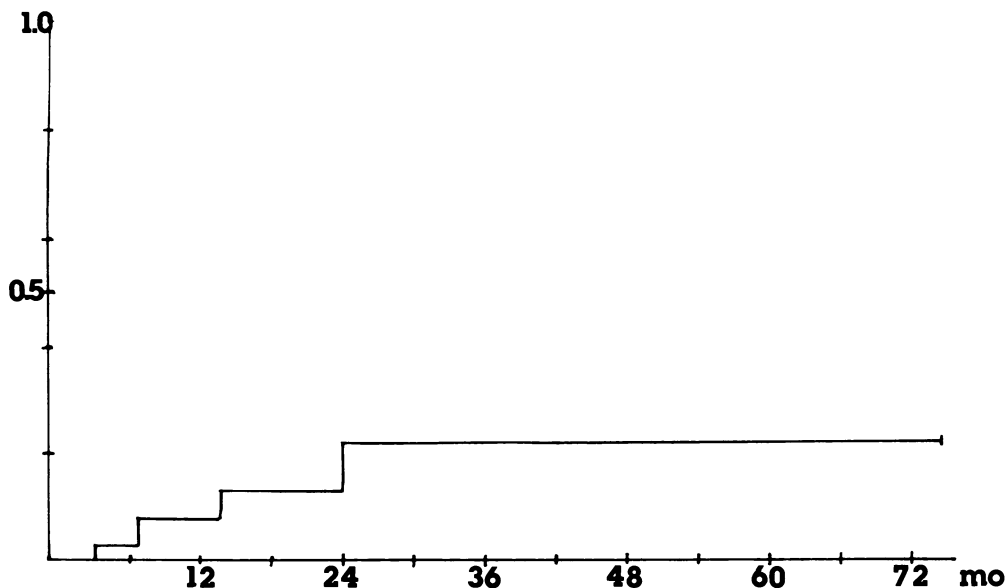


Fig. 2. Cumulative incidence of transformation to acute leukemia

tients. There were no significant differences in leukocyte or monocyte count, percentage of medullar blasts, or NAP index. It was striking, however, that among the nine patients living more than 24 months eight showed splenomegaly.

### Discussion

Besides the hematological features of peripheral and medullar monocytosis, thrombocytopenia, anemia, normal or increased leukocyte counts, hypercellular bone marrow with dominance of granulocytogenesis, a moderate increase of medullar blast count and splenomegaly, other findings such as high lysozyme levels in serum and urine, increased LDH serum levels, and enlargement of gamma-globulin fraction with evidence of paraprotein in some cases have already been reported by several other authors [1, 3–6]. Thymidine kinase showed a distribution similar to that of LDH. Increased activity of both enzymes seems to indicate a poor prognosis. Leukemic transformation was more

frequent in some studies [4], while median time of survival was in the same range. Prognostic value of spleen size is uncertain, as other authors found no prognostic significance [4] or even pointed out splenomegaly as a parameter of poor prognosis [5]. Perhaps the importance of prognostic factors can be specified after a longer period of observation. Compared with other MDS subtypes, CMML differs in monocytosis, normal or increased leukocyte counts, high incidence of splenomegaly, erythropoietic hypoplasia, a lower degree of dysplasia in erythrocytogenesis, and low frequency of leukemic transformation. Therefore it remains a matter of discussion whether at least some CMML cases should be included in the myeloproliferative syndrome as an atypical form of CML [2]. Our data do not confirm this hypothesis as we found no correlation of splenomegaly with leukocyte count or NAP index. Perhaps morphological features are not suitable to distinguish a “myelodysplastic” from a “myeloproliferative” subtype. It may be possible that more sophisticated techniques such as molecular genetics may be successful.

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## Chemotherapy of Acute Myeloid Leukemia of 35- to 60-Year-Old Patients

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### Aim of Study

This study was carried out to assess patients with acute leukemia who were not suited for bone marrow transplantation. Therefore age limits were 35–60 years. The TADVP regimen was compared with ADVP (Table 2) because of repeated doubts concerning the efficacy of thioguanine.

### Patients and Procedure

The results from 62 patients were evaluated. The procedure is shown in Tables 1 and 2.

### Results

Tables 3 and 4 show the results.

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Table 1. 62 patients, procedure

FAB classification	Number	TADVP	ADVP
M1	21	14	7
M2	21	14	7
M3	3	1	2
M4	9	7	2
M5	7	6	1
M6	1	–	1
Total	62	42	20

### Acute Myeloid Leukemia with Bone Marrow Fibrosis

Isolated cases of acute myeloid leukemia (AML) and increase in local trivial reticulin filament do occur. In Dresden, we have so far observed only one patient, a 33-year-old female, with AML FAB M2, who initially showed dense reticulin filament netting and procollagen and collagen fibers *histologically*. Also present were an increase in bone marrow cells, intensifying erythropoiesis with an increase in erythroblasts, granulocytopenia with numerous myeloblasts and promyelocytes, and enlarged megakaryocytosis with atypical cells.

### Bone Marrow Cytology

Poverty of cells, 32% partially atypical blast cells.

**Table 2.** Procedure

## Scheme 1. TADVP

## 1. Induction therapy

– Daunorubicin, 45 mg/m <sup>2</sup> i. v.	day 1–3
– Cytosine arabinoside (Ara-C), 200 mg/m <sup>2</sup> i. v.	day 1–7
– Thioguanine, 100 mg/m <sup>2</sup> p. o.	day 1–7
– Vincristine, 2 mg i. v.	day 1
– Prednisolone, 40 mg/m <sup>2</sup> p. o.	day 1–7

Second cycle: only 2 days daunorubicin and 5 days Ara-C, thioguanine, and prednisolone. Bone marrow puncture

If only PR or NR: second induction program

Cyclophosphamide, 800 mg/m <sup>2</sup> i. v.	day 1+10
Methotrexate (MTX), 20 mg/m <sup>2</sup> i. v.	day 2+5

If no CR: repetition of second program

If CR: consolidation therapy, if PR of NR: maintenance therapy

## 2. Consolidation therapy

Daunorubicin, 45 mg/m <sup>2</sup> i. v.	day 1+2
Ara-C 200 mg/m <sup>2</sup> i. v.	day 1–5
Three cycles, then maintenance therapy	

## 3. Maintenance therapy

A: POM II (1st month)

Vincristine, 2 mg i. v.	day 1
MTX, 0.14 mg/kg bodyweight p. o.	day 1–5
6-Mercaptopurine, 1.4 mg/kg p. o.	day 1–5

B: COTP (2nd month)

Vincristine, 2 mg i. v.	day 1
Cyclophosphamide, 20 mg/kg i. v.	day 1
6-Thioguanine, 3 mg/kg p. o.	day 1–5
Prednisolone, 30 mg/m <sup>2</sup> p. o.	day 1–5

C: DA (3rd month)

Daunorubicin, 45 mg/m <sup>2</sup> i. v.	day 1+2
Ara-C 200 mg/m <sup>2</sup> i. v.	day 1–5

Maintenance therapy for 2 years

## 4. Gnotobiotic procedure during induction and consolidation therapy

Trimethoprim, Sulfamerazin	3 × 0,16 rel. 0,24 g daily
Polymyxin M	8 × 500 000 U daily
Nystatin	6000 000 U daily

## Scheme 2. ADVP

Same procedure as scheme 1, only without thioguanine

**Table 3.** Results therapy

	Number	TADVP	ADVP
Complete remission	13/62 (21%)	8/42 (19%)	5/20 (25%)
Partial remission	16/62 (26%)	13/42 (31%)	3/20 (15%)
Nonresponder	9/62 (15%)	6/42 (14%)	3/20 (15%)
Early death	24/62 (38%)	15/42 (36%)	9/20 (45%)

The early death patients were distributed fairly evenly between all the treating clinics (favorable exceptions: Rostock, none out of four treated patients; Zwickau, one out of five treated patients)

**Table 4.** Results according to classification

FAB classification	Number	CR	PR	NR	Early death
	TADVP+ ADVP	TADVP+ ADVP	TADVP+ ADVP	TADVP+ ADVP	TADVP+ ADVP
M1	14+7	2+2	4+0	2+2	6+3
M2	14+7	4+2	4+0	1+1	5+4
M3	1+2	- 1	- 1	1 -	- -
M4	7+2	- -	4+1	- -	3+1
M5	6+1	2+0	1+0	2+0	1+1
M6	- 1	- -	- 1	- -	- -

### *Peripheral Blood*

Hb, 5.9 mmol/liter, leukocytes 2.2 Gpt/liter with 21% atypical blasts and hiatus leucaemicus; thrombocytes, 42 Gpt/liter. The liver was 4 cm below the costal margin; spleen at costal margin.

No hint of bleeding. The sister of the patient's mother had died from leukemia.

### **Therapy**

This patient was put on the thioguanine-daunorubicin-scheme (with Ara-C), but achieved only partial remission. Continuous blood transfusions were necessary. Distinct neutropenia retarded the treatment. The therapy was mainly oral with thioguanine and mercaptopurine. The spleen was enlarged. After 14.5 months jaundice, cholestatic hepatitis, and bronchopneumonia was present, and the patient died after 15 months.

*Autopsy.* Major hepatosplenomegaly. Myeloblastic-promyelocytic myeloid leukemia with a distinct megakaryocytic component and bone marrow fibrosis. Cholestatic hepatitis was present.

### *Differential diagnosis*

1. Acute myelofibrosis (distinct bone marrow fibrosis, poverty of cells, no hepatosplenomegaly, short clinical course).

2. Megakaryoblastic leukemia FAB M7 (recognized by monoclonal antibodies, dominating megakaryoblast cells in the bone marrow with or without little fibrosis).

### **Discussion**

The still low remission rate of over 35-year-old patients with acute leukemia (47%) correlates with the high rate of early death, especially on the basis of abstention from election of patients. The early death cases are caused by thrombocytopenic bleedings in particular. Some subcenters have not had the facilities to produce thrombocyte concentrates with the bag method and/or cell centrifugation until now. Comparison of TADVP and ADVP shows no significant alterations because of the small number of patients and the slight differences in results. The slightly higher rate of early death under ADVP corresponds with the lower number of partial remissions; but the differences are not significant. The primary therapy of AML will be further improved with TADVP, varied by vepeside in M4, 5, especially aclacinomycin, amsacrine, and other drugs.

# **Acute Lymphoblastic Leukemia in Adults**

## Importance of Long-Term Follow-up in Evaluating Treatment Regimens for Adults with Acute Lymphoblastic Leukemia\*

B. Clarkson, J. Gaynor, C. Little, E. Berman, S. Kempin, M. Andreeff, S. Gulati, I. Cunningham, and T. Gee

### Introduction

A complete report was recently published [1] which describes the results of our experience at Memorial Hospital in the treatment of adults with acute lymphoblastic leukemia during the past 20 years as of December 1986 and compares them with those of other large published series. In this paper I will update our overall experience, summarize the results of our most recent trials, and discuss our future plans.

### Methods

Between 1969 and May 1985, 199 previously untreated adults (> age 15 years) had a diagnosis of ALL, and they were treated with four successive multidrug treatment protocols (L2, L10, L10M, and L17/17M), which have been described in detail in previous reports [1–5]. In all of these protocols except for the earliest L2, the maintenance phase

was designed to try to destroy residual leukemic cells which had remained dormant for long periods prior to resuming proliferation [6–8]. Cranial irradiation was not employed for prophylaxis of CNS leukemia, but we relied on intrathecal or intraventricular methotrexate for this purpose [9, 10]. The date of last follow-up in our recent report [1] was December 1986. Follow-up on these patients now extends through August 1988.

### Results

The induction components of all the protocols were similar and the incidence of complete remission (CR) did not differ significantly among them. Overall 163/199 patients (82%) had CR. Previous analyses showed that the three later protocols had better remission durations and long-term survival compared with the L2, but the differences were not significant [5]. The most recent remission duration curves are shown in Fig. 1. As can be seen, on further follow-up, due to additional relapses and deaths, the L17/17M results are now similar to the earliest (L2) protocol and are worse than those of the earlier L10 and L10M protocols, but the differences are of marginal significance.

We examined the distribution of patients in the different protocols who had adverse prognostic factors as previously identified [1]. There were no significant differences in percentage of patients with Ph<sup>+</sup> ALL, WBC level, or phenotype (except for B cell), but a higher proportion of patients on the L2 and

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\* The results reported here were previously presented at The UCLA Symposia on Molecular and Cellular Biology entitled: "Acute Lymphoblastic Leukemia: Current Controversies, Future Directions," held at Tapatío Springs, Texas, United States, 29 November–2 December 1988; and will be published in the UCLA Symposia on Molecular and Cellular Biology, New Series, Volume 108: *Acute Lymphoblastic Leukemia*. Edited by: Gale RP, Hoelzer D, Alan R. Liss, New York (in press, 1989)

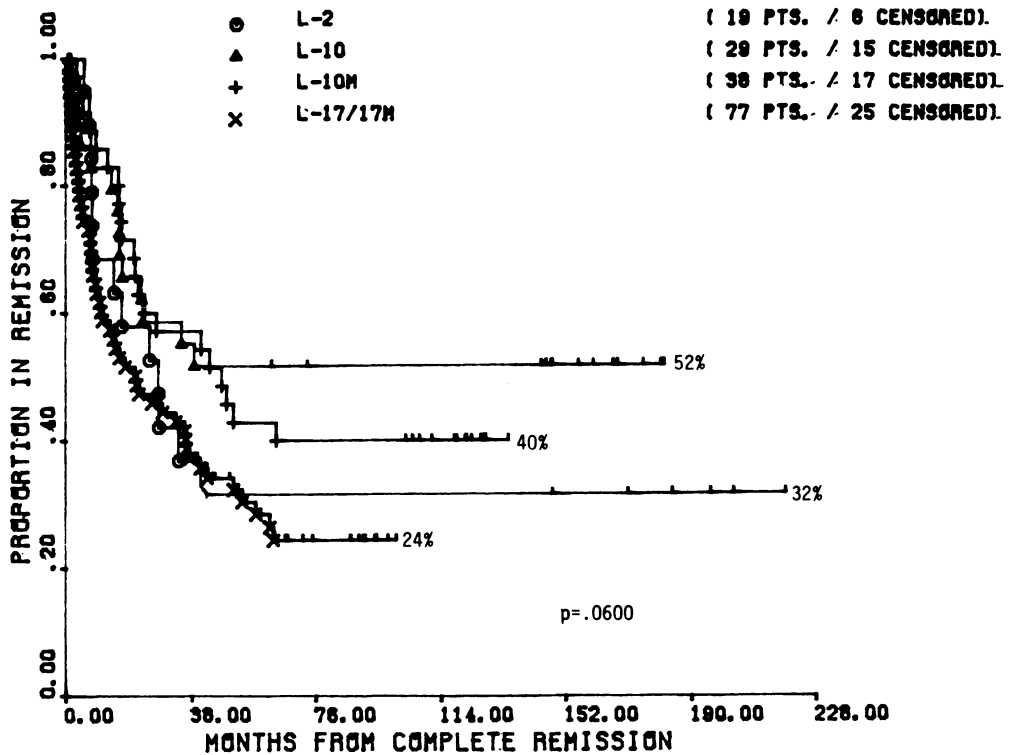


Fig. 1. Remission durations of L2, L10, L10M, and L17/17M protocols

L17/17M protocols had the following poor prognosis features: B-cell phenotype, L3 morphology, CNS involvement at presentation, age > 35 years, and low serum albumin (< 3.5 g%). When patients with these adverse characteristics were excluded from the analyses, the observed differences between the protocols were even less significant.

Another possible explanation for the worse results of the L17/17M protocol is the duration of treatment. The L2 and L10/10M protocols called for 3 years of treatment after achievement of CR; this was arbitrary since the optimum duration of treatment is unknown. The duration of maintenance treatment was shortened to 2.5 years in the L17/17M protocols, and a shorter consolidation phase using daunomycin, cytosine arabinoside, 6-thioguanine, methotrexate, and L-asparaginase was compared with the longer one used in the L10M; the long arm of the L17M was identical to the L10M consolidation phase [5].

Comparison of the results of the two consolidation arms showed a slight advantage for the longer consolidation, but the difference was not significant (Fig. 2). It is not possible to determine whether the longer duration of the "maintenance" treatment on the L10/10M protocols was responsible for the better results compared with the later protocols, but this remains a possible explanation. It should be borne in mind that the treatment schedule used was designed as an eradication regimen rather than as a more traditional maintenance regimen [5], and that therefore other trials which have failed to demonstrate an advantage of prolonged maintenance therapy may not be entirely comparable.

The overall remission duration for the 163 patients achieving CR is shown in Fig. 3; a plateau is reached after 6 years with 35% long-term survivors. Ten patients who died in remission are excluded from this curve, including six treatment-related deaths 1-9

Fig. 2. Remission duration of patients receiving long or short arms of consolidation treatment on the L17M protocol. The first 43 patients were randomized and evenly distributed between the 2 arms. Because there appeared to be no significant difference in the initial analysis, the next 17 patients received the short arm. If only the randomized patients are considered, the short arm is almost identical to that shown in Fig. 2

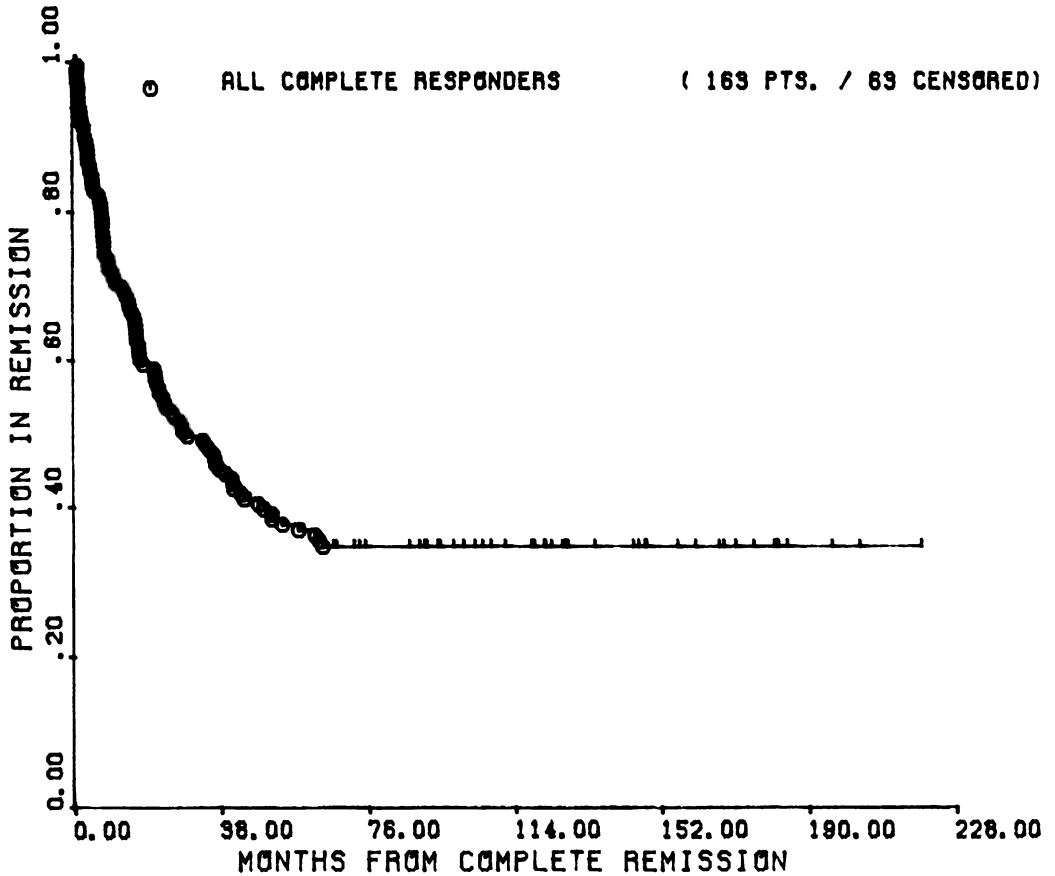
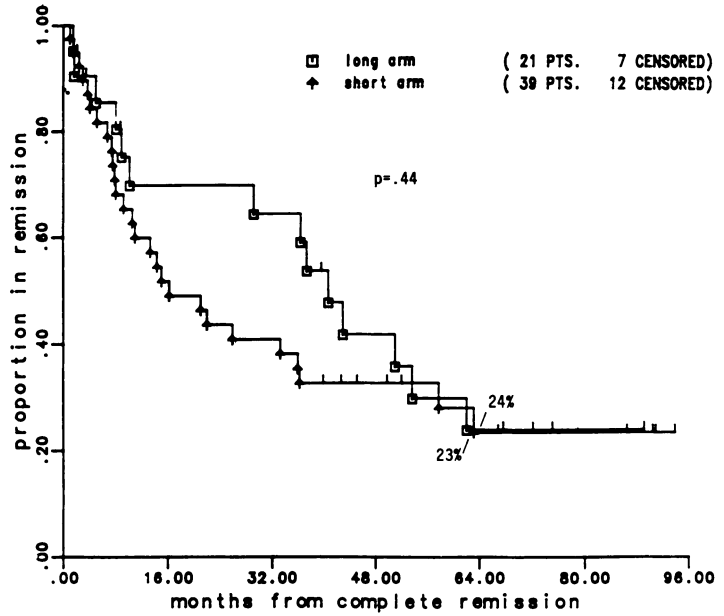


Fig. 3. Remission duration of 163 patients achieving remission

months (mean 3.5 months) after achieving CR (all died of infections and/or hemorrhagic complications) and four late deaths of unrelated causes (i.e., multiple sclerosis, construction accident, stroke, and narcotic

overdosage) after 45–172 months in continuous CR. Three Ph<sup>+</sup> ALL patients who had bone marrow transplants in first remission are excluded at the time of transplant; only one of the three is still alive at 119 months. None of the other patients in this series had transplants in first remission, but 15 had transplants after relapse, as will be discussed below.

The disease-free survival of the 163 patients achieving CR (which includes the 6

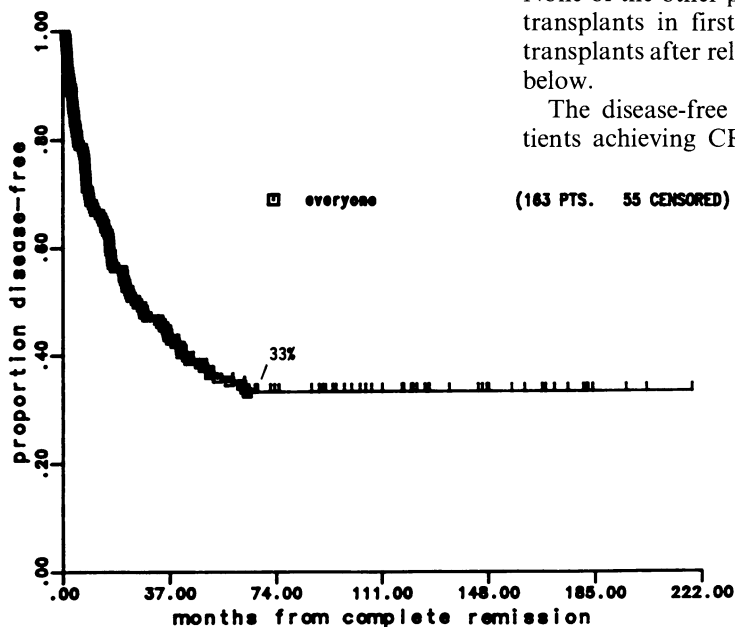


Fig. 4. Disease-free survival of 163 patients achieving remission

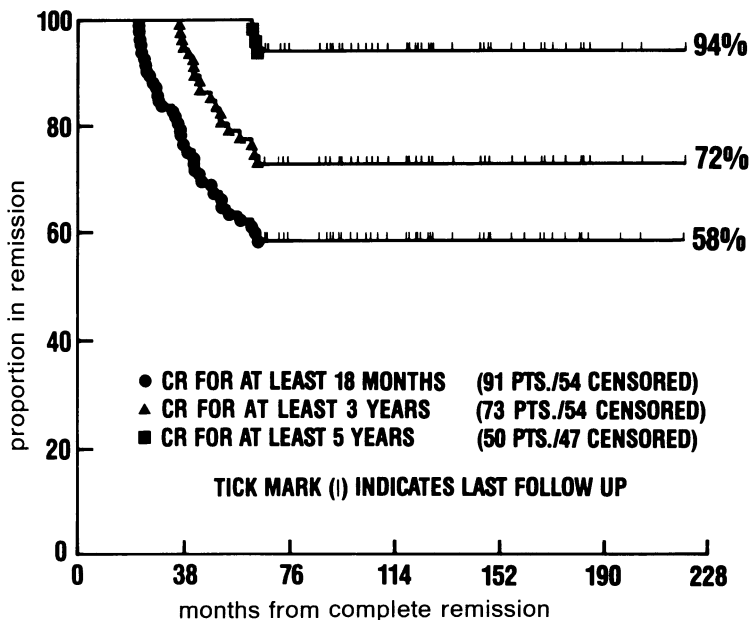


Fig. 5. Subsequent remission duration of patients who had continuous remission durations of 18, 36, and 60 months



early deaths as failures and does not censor the 3 patients transplanted in first CR mentioned above) is shown in Fig. 4; the 4 late unrelated deaths are censored.

Sixty-three of the patients achieving CR had early relapses (within 18 months). Of the 91 patients remaining in continuous CR for 18 months, 42% subsequently relapsed (Fig. 5). Seventy-three patients remained in continuous CR for 3 years, of whom 28% later relapsed. Only 3 of the 50 patients fol-

lowed for 5 years in continuous CR subsequently relapsed, and so far there have been no relapses after 6 years in this series, although a few still later relapses have occurred in previously treated patients with ALL who were retreated with these same protocols or with earlier protocols.

The postrelapse survival of the 63 patients who relapsed while still on treatment and whose remissions lasted less than 18 months was generally quite short, while the postrelapse survival of the 37 patients whose first relapse occurred after 18 months was significantly longer (Fig. 6). Only 19 patients relapsed after being in continuous remission for 3 years, and their postrelapse survival is shown in Fig. 7; about 25% are projected to

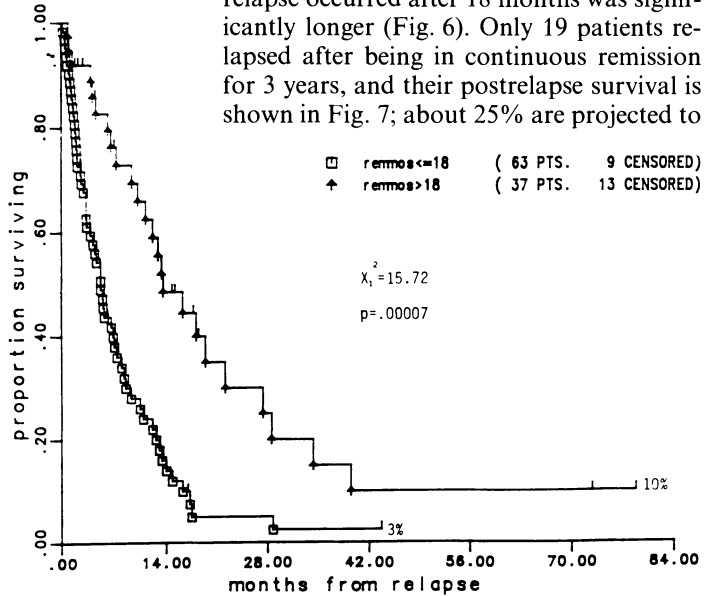


Fig. 6. Postrelapse survival of patients in remission less than and greater than 18 months

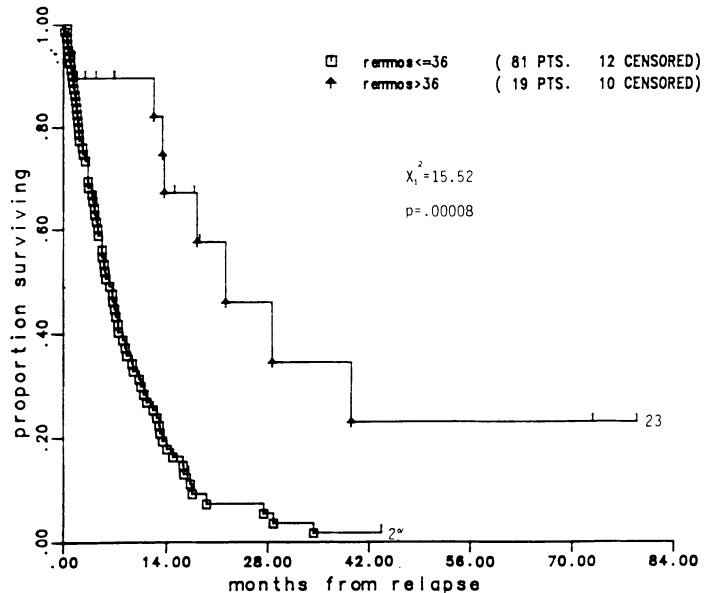


Fig. 7. Postrelapse survival of patients in remission less than and greater than 36 months



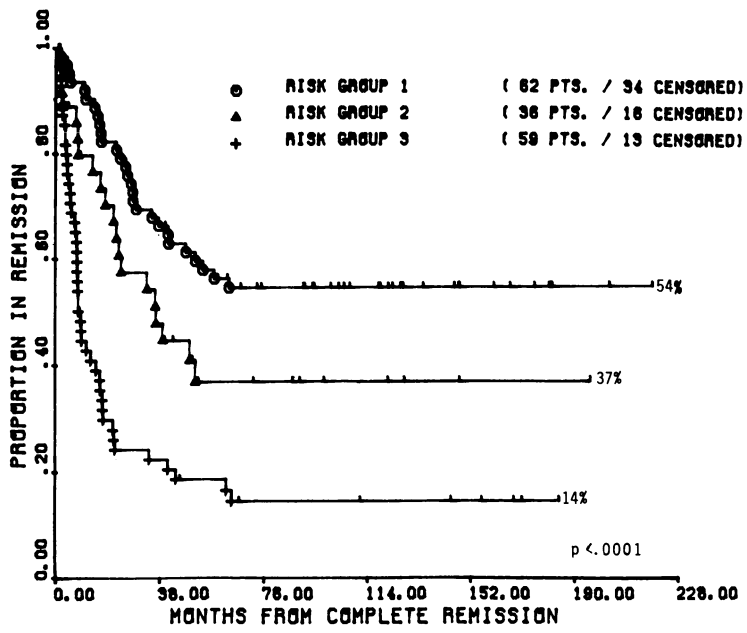


Fig. 8. Remission duration by prognostic group as defined previously [1] and summarized in Table 2

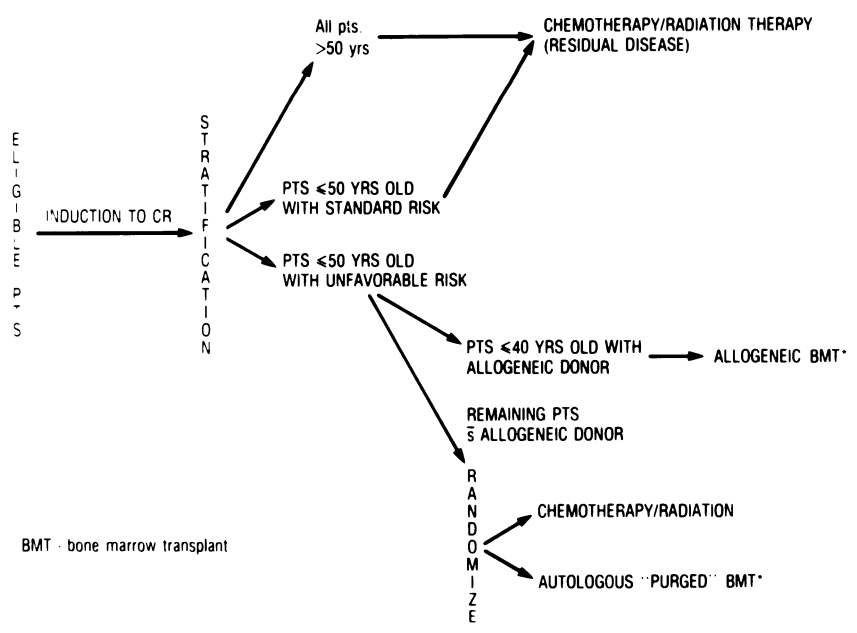


Fig. 9. Schema showing projected patient flow on the L20 protocol

**Table 3.** Adult ALL: Eligibility for randomization to chemotherapy or BMT in first remission on L-20 protocol

Requirements: age 15–20 years  
 Good: Liver (bilirubin < 2 mg%)  
 Renal (creatinine < 2 mg%)  
 Cardiopulmonary, function  
 Performance status

Plus one or more of following:

1. ALL null (CALLA negative) or B phenotype
2. CALLA<sup>+</sup> if pretreatment WBC > 20000
3. CNS involvement at presentation
4. t(4;11), t(8;14), t(9;22), or hypodiploidy

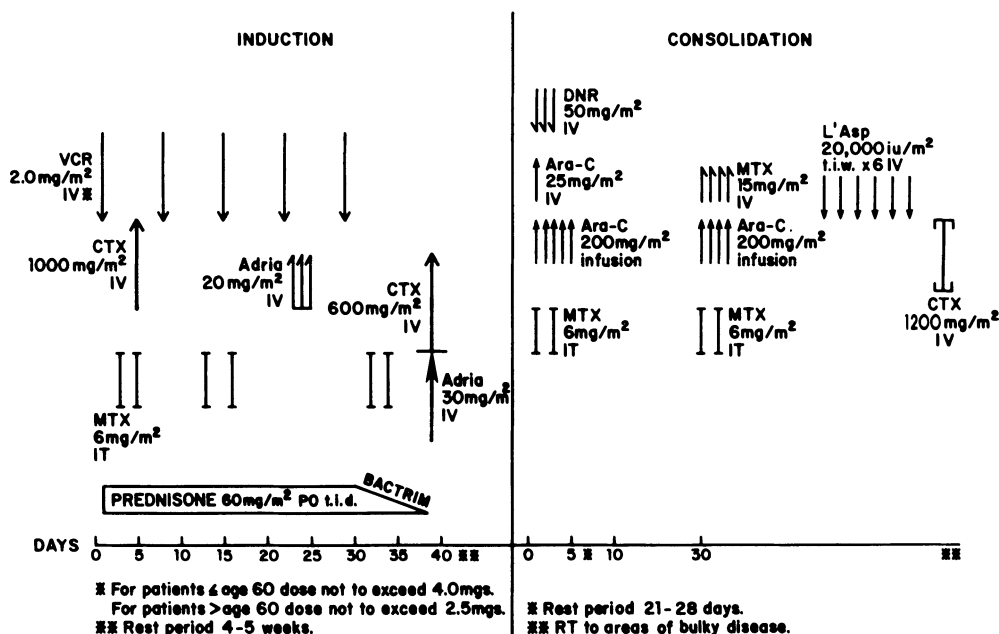
CALLA, common acute lymphoblastic leukemia antigen

The L20 induction and consolidation regimens are shown in Fig. 10; the maintenance or eradication phase was identical to that used in the L10/10M and L17/17M protocols [4, 5]. For reasons which are still unexplained, the early results with the L20 protocol have been disappointing. This was unexpected because the protocol does not differ greatly from our previous protocols. We have not yet analyzed all the possible prog-

nostic factors to see if a higher percentage of patients were in the higher risk groups, but the median age of the first 48 patients entered on the L20 was 39 years compared with 26 years for the 199 patients on the previous protocols; 54% of these 48 patients on the L20 protocol were >35 years old compared with only 32% on the previous combined protocols. There were no significant differences in WBC level, and the same proportion (18%) were Ph<sup>+</sup> as in our previous series.

Patients with Ph<sup>+</sup> ALL were scheduled to have an allogeneic transplant after achieving CR, providing they had a matched donor; autologous transplants were not planned for this group because we have not yet been able to devise a satisfactory method to purge Ph<sup>+</sup> cells selectively.

Of the first 48 patients entered on the L20 protocol who have had sufficient follow-up for evaluation, only 35 (73%) had CRs as compared with an average CR incidence of 82% on the earlier protocols. The remission duration of the 35 patients having CRs is disappointingly short with only about 35% of patients projected to be still in remission at 2 years (Fig. 11). Patients having marrow



**Fig. 10.** L20 protocol induction and consolidation regimens. The consolidation was very similar, but not identical, to the short consolidation arm of the L17M protocol [5]

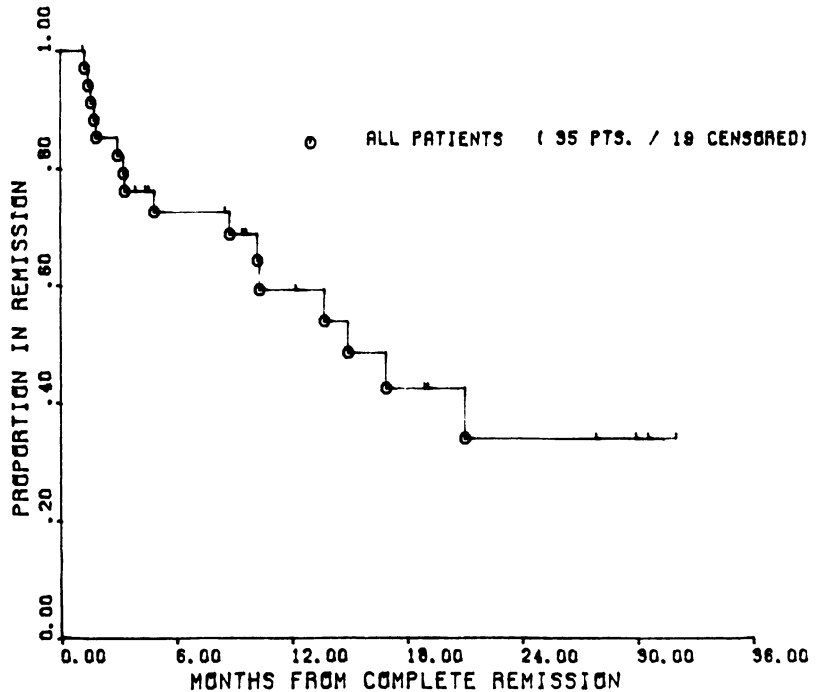


Fig. 11. Remission duration on L20 protocol

transplants in first remission were censored at the time of transplant.

The number of patients who were actually transplanted has been less than anticipated; the reasons for their ineligibility or failure to have a BMT are summarized in Table 4.

Of the 21 poor-prognosis patients 50 years of age or younger, 12 either died during attempted induction, failed to reach CR, or had very short remissions, thus leaving only 9 patients eligible for transplantation. Of these nine patients, one refused, one was improperly not randomized because the cytogenetic results reporting hypodiploidy were not noted early enough, and one Ph<sup>+</sup> patient had no matched donor. Another Ph<sup>+</sup> patient had an allogeneic transplant but later had a CNS relapse and died.

Of the five patients actually randomized, two were randomized to chemotherapy and three to AuBMT. One refused to be transplanted, so only two patients actually had AuBMT, of whom one died of peritransplant complications and the other remains in CR.

## Discussion

The initial results of the L20 protocol have been disappointing, and we are currently designing a new protocol for both standard-risk and high-risk adult ALL patients which will employ more intensive treatment early in the induction phase to obtain rapid cell kill initially since there is suggestive evidence this may be a useful therapeutic strategy in high-risk patients with ALL [1, 12–15]. We are also planning to modify the consolidation treatment to try to enhance its effectiveness.

In general, the results of our previous treatment protocols for adult ALL appear to be comparable to those reported by other investigators [12–20]. The improved early results reported by a few investigators must be regarded with caution until all the risk factors have been analyzed to see if there has been inadvertent selection of patients in more favorable risk groups and until there has been a longer follow-up since later relapses may still occur. The treatment for

**Table 4.** L-20 protocol for ALL: distribution of patient population with reference to consideration for autologous bone marrow transplantation

# Patients entered:	51
— 13   Age > 50 years	
— 38	
— 13   “Standard risk”, age < 50 years	
— 25	
— 4    Age < 50 years “unknown risk”, data inconclusive	
# Poor prognosis:	
— 2    Died during treatment	21
— 19	
— 10   Failures very short CR	
# Poor prognosis eligible for randomization:	9
— 4    Patients not randomized:	
1 Refused randomization (still in CR on chemotherapy)	
1 Never randomized (hypodiploid in CR)	
2 Ph' (+) ALLs:	
1 Allo-BMT; CNS relapse, expired	
1 No match; relapsed at 21 months, expired	
# Poor prognosis patients randomized:	5
3    Patients to L21:	
2 Received ABMT:	
1 Expired	
1 in CR	
1 Refused ABMT and continues in CR on Rx	
2    Patients to chemotherapy:	
1 A & W	
1 Relapsed at 10 months; alive and well on Rx	

adults with ALL remains unsatisfactory since only about one-third of patients are probably being cured with any of the current treatment protocols and better treatment regimens are urgently needed.

## Summary

During the past 20 years, we have treated 250 previously untreated adults (> age 15 years) with acute lymphoblastic leukemia (ALL) with five successive multidrug protocols: L2, L10, L10M, L17/17M, and L20. The L10 and L10M protocols had the highest percentage of long-term (> 5 years) remissions (52% and 40% respectively) compared with the L2 and more recent

protocols (24%–32%); this is partly attributable to a greater prevalence of adverse risk factors among the latter protocols. The overall long-term survival of the first 199 patients with minimum 3 years follow-up is now 31%, with 35% of the 163 patients achieving complete remission (CR) remaining free of relapse for > 5 years. The disease-free survival of the 163 patients reaches a plateau of 33% after 6 years. The percentages of patients subsequently relapsing after remaining in continuous CR for 1.5, 3, and 5 years are 42%, 28%, and 6%, respectively; no relapses have yet occurred after 6 years in this series. Postrelapse survival improved progressively with longer duration of first remission. The results of treatment in second or later remission with either chemo-

therapy or bone marrow transplantation (BMT) were unsatisfactory and there were only a few long-term survivors. Recently we have attempted to select patients at highest risk of early relapse for BMT in first remission, but the number of eligible patients actually having BMTs has been low for a variety of reasons, including early death, failure to reach CR, early relapse, patient refusal, or medical contraindications. Since it appears that only about one-third of adults with ALL are currently being cured, more effective treatment programs are urgently needed.

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## Comparison of Chemotherapy and Autologous and Allogeneic Transplantation as Postinduction Regimen in Adult Acute Lymphoblastic Leukemia: a Preliminary Multicentric Study

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### Introduction

The results of chemotherapy in adults with acute lymphoblastic leukemia (ALL) have improved in recent years but have lagged behind those in children with ALL [1, 2]. The overall probability of achieving complete remission (CR) exceeds 75%, and it now appears that approximately 35% of patients achieving CR can be cured using chemotherapy alone [2, 3]. It is therefore appropriate to study alternative forms of treatment such as allogeneic or autologous bone marrow transplantation (BMT) in adult ALL.

In its first trial, the French group compared diverse modalities of induction and postremission chemotherapy [4]. The best results were achieved in the arm consisting of intensive induction chemotherapy followed by consolidation before maintenance chemotherapy was initiated, with a projected disease-free survival (DFS) of 49% at 3 years. Allogeneic BMT was performed in all patients aged under 40 years having an HLA/MLC-identical sibling; DFS was 53% at 3 years, indicating a slight advantage of BMT over optimal chemotherapy.

It was therefore decided to conduct a second trial comparing the optimal postremis-

sion chemotherapy as defined by the first trial to autologous BMT and allogeneic BMT. Results of this second trial are reported here.

### Patients and Methods

#### *Treatment Protocol*

This trial remained open between May 1985 and December 1986. A total of 164 patients from 33 institutions were registered. Patients were aged 15–60 years, with the morphological and cytochemical diagnosis of ALL, L1 or L2 type according to the French-American-British (FAB) classification [6], and who had not been previously treated.

The protocol schedule is shown in Table 1. In brief, the induction regimen consisted of vincristine, cyclophosphamide (Cytosan), zorubicin (Rubidazole), and prednisone administered over 28 days. If CR was not reached by day 28, salvage therapy using a regimen similar to the consolidation course was immediately started. The postinduction chemotherapy arm began with 3-month consolidation using doxorubicin (Adriamycin), cytosine arabinoside and, asparaginase, followed by central nervous system (CNS) prophylaxis with 18 Gy cranial irradiation and five injections of intrathecal methotrexate. The maintenance chemotherapy was similar to that of the L10 mainte-

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**Table 1.** Treatment protocol

Phase	Drug	Dose	Administered on days
Induction <sup>a</sup>	Cyclophosphamide	400	1, 8, 15
	Vincristine	1.4	1, 8, 15
	Zorubicin	100	1–3
	Prednisone	60	1–15
Remission Consolidation	Doxorubicin	40	1
	Cytosine arabinoside	60	3–7
	Asparaginase	1000	8–12
Maintenance	Prednisone	60	1–8
	Vincristine	1.4	1, 8
	6-Mercaptopurine	60	25–56
	Methotrexate	20	35, 42, 49, 56
	Dactinomycin	1000	56
	Doxorubicin <sup>b</sup>	20	15–17
	Cyclophosphamide <sup>b</sup>	800	15
Carmustine	80	15	

<sup>a</sup> If no CR by day 28, initiation of consolidation treatment

<sup>b</sup> alternate

nance regimen [1], using eight drugs sequentially.

This postinduction chemotherapy regimen was administered to all patients aged 50–60 years achieving CR, while patients aged 15–50 years were randomized to receive either the chemotherapy arm or autologous BMT.

Stem cells were harvested in patients randomized to receive autologous BMT after one (9 patients), two (11 patients), or three (7 patients), courses of consolidation. Autologous BMT was performed using bone marrow stem cells in 22 patients and peripheral blood stem cells in 5. Ex vivo purging of the stem cells was performed in all but one case, using Mafosfamide in 23 patients and monoclonal antibodies plus complement in three cases. Stem cell reinfusion occurred before day 120 of CR in 23 cases and between days 120 and 180 in the other four cases.

All patients in CR aged under 40 years and who had an HLA/MLC-identical sibling received allogeneic BMT after a standard conditioning regimen including cyclophosphamide and total body irradiation. Graft-versus-host disease prophylaxis was free but was generally realized using cyclosporin A.

### Patients

Originally 164 patients were entered in the trial but five were secondarily excluded because of erroneous diagnosis (two cases) or previous treatment (three cases). Initial characteristics of the 159 remaining patients are listed in Table 2.

The overall CR rate in this patient population was 83%, and 133 patients were available for postremission study, including 21 who achieved CR only after salvage therapy. Of these 133 patients, ten were excluded from further study because of excessive induction regimen toxicity (three patients) or major induction protocol violation (seven patients). 17 aged 50–60 years received the postremission chemotherapy regimen without randomization and 39 aged under 40 who had a suitable donor were scheduled to receive allogeneic BMT (median age 24 years). Grafting was performed before day 120 after CR in 32 cases and on day 150 or 155 in two cases; 12 patients received T-depleted BMT. The remaining 67 patients in CR, aged 15–50 years, were randomized to receive either postinduction chemotherapy (32 patients) or autologous BMT (35 patients).

**Table 2.** Patients' characteristics at diagnosis

Male/female ratio	108/51
Age	
Median (years)	33
40–49 (years)	23
50–59 (years)	27
Organomegaly	105
CNS involvement	6
FAB classification	
L1	82
L2	60
Undetermined	17
Hemoglobin < 80 g/l	52
Leukocytes > 50 G/l	54
Evaluable immunologic phenotype	128
T-cell ALL	40
CALLA + ALL	66
Null-cell ALL	22
Evaluable karyotypes	44
Normal	18
Abnormal	27
t(9, 22)	9
t(4, 11)	5

## Results

Statistical analysis was performed in September 1988, with a median follow-up of 30 months. For the whole group DFS was 34% and overall survival 42%. Among the ten excluded patients six have relapsed, and among the 17 patients aged over 50 years 11 had relapsed at the time of analysis. Evolution of these two groups of patients will not be further discussed.

Among the 39 patients eligible for allogeneic BMT, five were not transplanted in first CR due to early relapse (two cases), poor clinical status (two cases), or patient refusal (one case). Two of these patients were still alive at the time of analysis. Thirty-four patients were grafted. Two of these died from the transplant procedure, and 12 relapsed after transplant, including 10 out of the 12 patients having received T-depleted bone marrow.

In the chemotherapy arm, 17 out of 32 patients had relapsed at the time of analysis, three of them within 3 months of achieving CR. All of them subsequently died. Fifteen patients are still in continuing CR.

Among the patients scheduled to receive autologous BMT, eight could not be grafted due to early relapse within 3 months of CR achievement (five cases), poor clinical status (two cases), or patient refusal (one case). Three of these patients are still alive. Twenty-seven patients received autologous BMT.

Nineteen patients have relapsed after transplantation, generally within 8 months; 11 of them subsequently died, and 8 are still in second CR. Eight patients have been in first remission for more than 18 months without any treatment. Overall, 16 out of 27 patients in this group are alive.

No difference appears between the three arms for DFS or survival in terms of whether the starting points were the date of CR or 3 months after CR (the median time lapse between achievement of CR and BMT). No prognostic factors were found for induction of CR. A leukocyte count over 50 g/l and a platelet count under 100 g/l were significantly associated with short CR duration. Immunologic phenotype was analyzed (Table 1); this was not a prognostic factor for CR duration. The presence of chromosomal abnormalities was associated with short CR duration. Only 3 out of 9 patients with t(9; 22) are surviving while all patients with t(4; 11) achieved CR but relapsed and died within 10 months of diagnosis [5].

## Discussion

In this trial the distribution of patient characteristics is similar to that in other reported series [2, 3]. The CR rate is in the upper range of CR observed for adult ALL, and the overall DFS and survival are also quite the same [1, 2, 10].

The three therapeutic arms used in post-remission do not show any statistical differences [11]. No controlled comparison between chemotherapy, allograft, and autograft in first CR have been yet published [7, 8]. In opened studies transplantations, either allogeneic or autologous, give better results, but some bias in selection may occur [9] – the simplest being in the interval between CR and transplantation: the longer interval, the better DFS.

Available data do not allow at this time, to find any difference in adult ALL [10]. The number of allocated patients in each arm is too small in our study. According to the protocol schedule, there were possible variations in the intensity of cytoreduction pre-harvest as well as in the ex vivo treatment of the allogeneic or autologous marrow. In the allogeneic arm, 12 out of 34 patients relapsed, 10 of them having received T-depleted marrow. But, in the preceding trial, only 6 out of 39 patients grafted in first CR relapsed [4].

This is a preliminary study testing the feasibility of this strategy in a multicentric group. The trial may be considered as a pilot study. In December 1986 a new multicentric protocol was started, in which there are now (March 1989) 385 patients. The total number of included patients must be 600. The aim is to ascertain clearly the value of transplantation and of chemotherapy as postinduction treatment in first CR and to define indications. The first interim analysis will be made in October 1989.

Although the rate of CR in adults has improved in recent years, the challenge is now to prolong remission and to cure adults as frequently as children who have ALL.

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## Intensive Chemotherapy for Acute Lymphoblastic Leukaemia in Adults

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### Introduction

High-dose cytosine arabinoside (HD Ara-C) has been shown to induce complete remission in acute lymphoblastic leukaemia (ALL) which is refractory to, or which has recurred following conventional therapy [1–5]. This study was undertaken to determine whether the incorporation of HD Ara-C at a dose of 2 g/m<sup>2</sup> twice daily for 6 days into the treatment previously used at St. Bartholomew's Hospital (OPAL) [6] would improve the prognosis of adults with ALL.

### Materials and Methods

#### 1. Patients (Table 1)

#### Study

Between January 1983 and October 1986, 54 newly diagnosed patients were treated at three centres: St. Bartholomew's Hospital (SBH) in London, Hopital Riuniti in Bergamo, and Hopital San Bortolo in Vicenza. Data on these patients are presented in Table 1.

Table 1. Patient characteristics

		OPAL + H/D ARA-C	OPAL/ HEAV-D
Age (years)	Range	15–57	15–58
	Mean	33	29
	Median	32	25
Blast count (×10 <sup>9</sup> /litre)	Range	0–355	0–435
	Median	5.0	3.4
Phenotype			
C		20	43
“Null”		13	23
B		5	6
T		9	15
“Other”		4	1
Not done		3	23
Total		54	100

#### Controls

The results for 111 newly diagnosed patients treated ‘conventionally’ at SBH between 1972 and 1982 [7] and subsequently to 1986 and 1987 are used for comparison.

#### Diagnosis

The morphological diagnosis was based on May-Grunwald-Giemsa and cytochemical staining of bone marrow smears, which showed infiltration by at least 30% lymphoblasts according to the French-American-

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British (FAB) system [8]. Immune phenotype was allocated according to the following criteria:

C-ALL:

CD10<sup>+</sup>, HLA-DR<sup>+</sup>, CD19<sup>+</sup>, CD20<sup>-</sup>, Tdt<sup>+</sup>, WTI<sup>-</sup>, surface immunoglobulin (sIg)<sup>-</sup>

Null-ALL:

CD10<sup>-</sup>, HLA-DR<sup>+</sup>, CD19<sup>-</sup>, CD20<sup>-</sup>, Tdt<sup>+</sup>, WTI<sup>-</sup>, sIg<sup>-</sup>

T-ALL:

CD10<sup>-</sup>, HLA-DR<sup>-</sup>, CD19<sup>-</sup>, CD20<sup>-</sup>, Tdt<sup>+</sup>, WTI<sup>+</sup>, sIg<sup>-</sup>

B-ALL:

CD10<sup>-</sup>, HLA-DR<sup>+</sup>, CD19<sup>-</sup>, CD20<sup>+</sup>, Tdt<sup>-</sup>, WTI<sup>-</sup>, sIg<sup>+</sup>

### Treatment

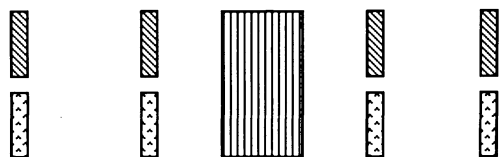
#### Study Patients (OPAL-HD Ara-C)

Details are shown in Fig. 1. Ara-C 2 g/m<sup>2</sup> was given by 3-h intravenous infusion twice daily as soon as the bone marrow had recov-

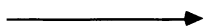
ered from cycle 2 of Adriamycin and vincristine; when there was persistent heavy infiltration it was given sooner. Prednisolone eye drops were prescribed routinely. Intrathecal (i. t.) methotrexate (MTX) was given as additional CNS prophylaxis to the Ara-C as soon as the blasts had cleared from the peripheral blood and with each subsequent cycle of Adriamycin and vincristine. Intrathecal therapy with either MTX or Ara-C was given on alternate months for 2 years. There was no cranial irradiation. Maintenance therapy comprised 6-mercaptopurine daily and cyclophosphamide and MTX weekly to maintain the white cell count below 3 × 10<sup>9</sup>/litre, for a total of 3 years.

### Controls

Patients received either OPAL or HEAV-D (O/H) followed by cranial irradiation and maintenance chemotherapy as previously described [7].



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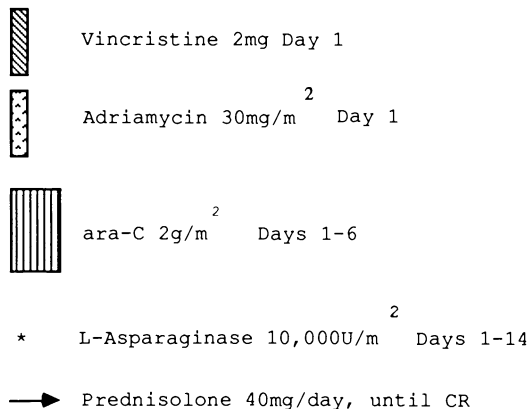


Fig. 1. Treatment programme: OPAL-HD Ara-C

### Supportive Care

Prophylactic, oral, nonabsorbable antibiotics [9] were prescribed from the outset of treatment. Platelet transfusions from single donors were given prophylactically to maintain the platelet count above 20 × 10<sup>9</sup>/litre or if clinically indicated. Fever was assumed to be bacterial in origin and was treated with an aminoglycoside/cephalosporin combination in the first instance.

### Statistical Analysis

Duration of remission and overall survival were plotted using standard life-table methods [10] and compared using the log-rank method [11]. The significance of prognostic factors in determining the achievement of CR was evaluated by logistic regression analysis, whereas duration of CR and overall survival differences were determined using a stepwise linear regression method based on Cox's proportional hazards model [12].

## Results

### Response to Therapy

Complete remission was achieved in 25/54 patients after two cycles of Adriamycin and vincristine with 14 days' asparaginase and prednisolone, and in a further 11/54 following high-dose Ara-C to give an overall CR rate of 36/54 (67%). None of the remissions achieved in patients with *overt* leukaemia at the time of receiving high-dose Ara-C (4/5) were durable.

### Duration of Remission

Eleven out of 36 patients continue in CR between 2.8 and 5.4 years, only two solitary CNS relapses having occurred and three patients having died in CR following HD Ara-C. Two patients both of whom relapsed have been excluded from the analysis of duration of remission; one who never received HD Ara-C because of cerebral haemorrhage with hypofibrinogenaemia following asparaginase, and the other, with B-ALL, because he proceeded in first CR to high-dose therapy with autologous bone marrow sup-

port. The median duration of CR was 1 year. The duration of remission correlated favourably with rapid achievement of CR ( $P=0.03$ ) and there was a trend ( $P=NS$ ) in favour of patients with high blast count ( $>10 \times 10^9/\text{litre}$ ) and T-cell phenotype.

### Survival

The median survival for all 54 patients was 1 year. Three patients died in CR during hypoplasia associated with high-dose Ara-C. Fifteen patients remain alive (11 in first CR, 2 in second CR, 1 in third CR, one in third relapse). The factors correlating unfavourably with survival were B-cell phenotype ( $P=0.006$ ) and advanced age ( $P=0.01$ ).

### Comparison with Historical Controls

The complete remission rate, duration of remission curve and survival curve (Fig. 2) were identical with those achieved previously, if all patients are considered together. Division of patients according to "high"- or "low"-risk category for duration of remission as expected with conventional therapy at SBH [7] and elsewhere [13–17] shows marked differences (Figs. 3, 4). High-risk patients treated with OPAL-HD Ara-C had a significantly better duration of remission

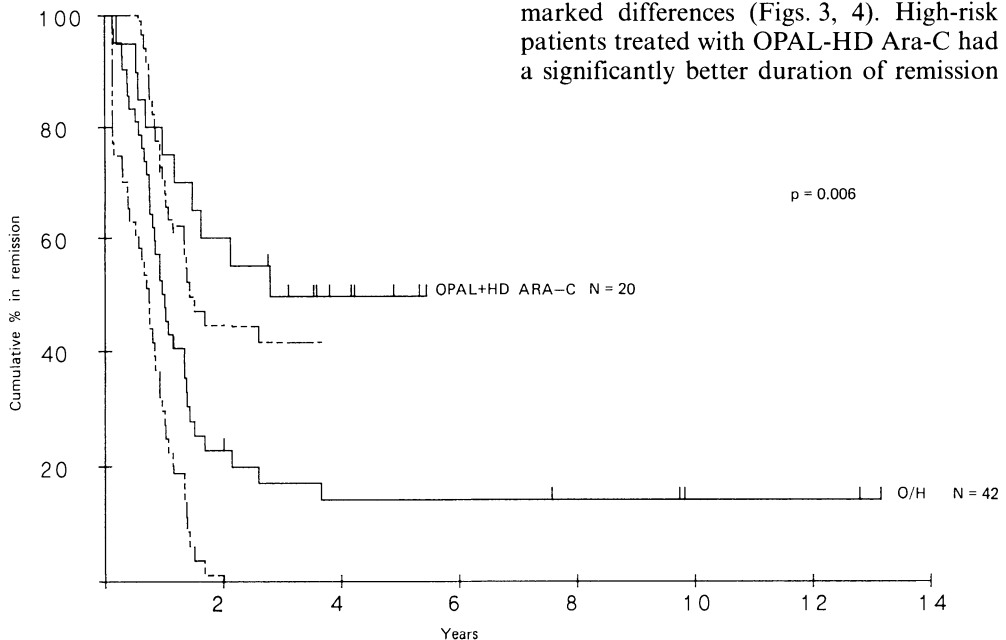
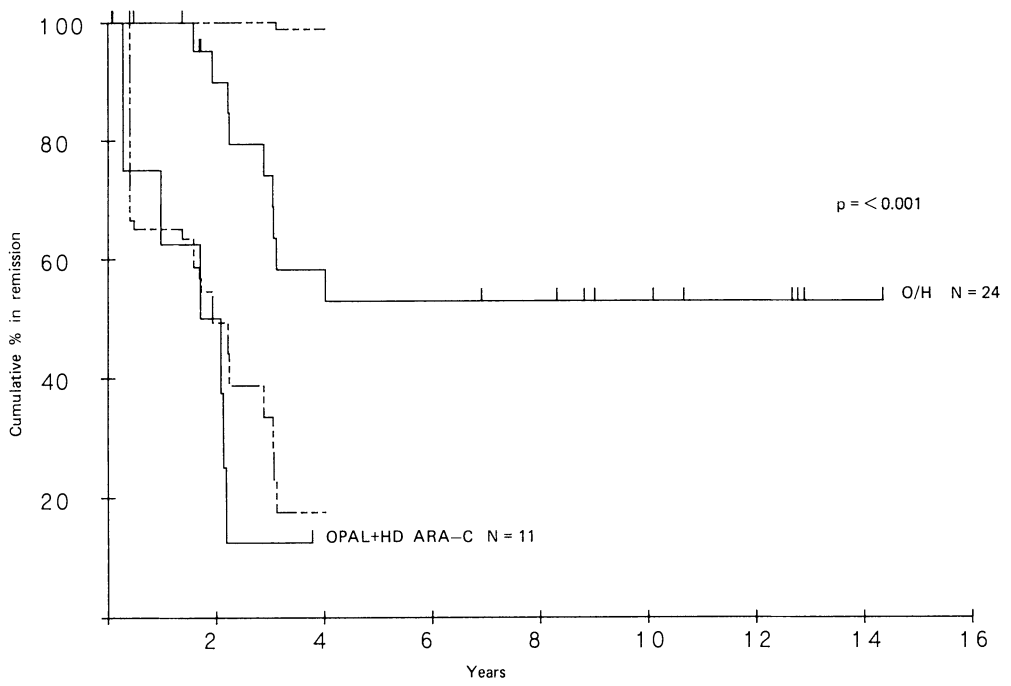
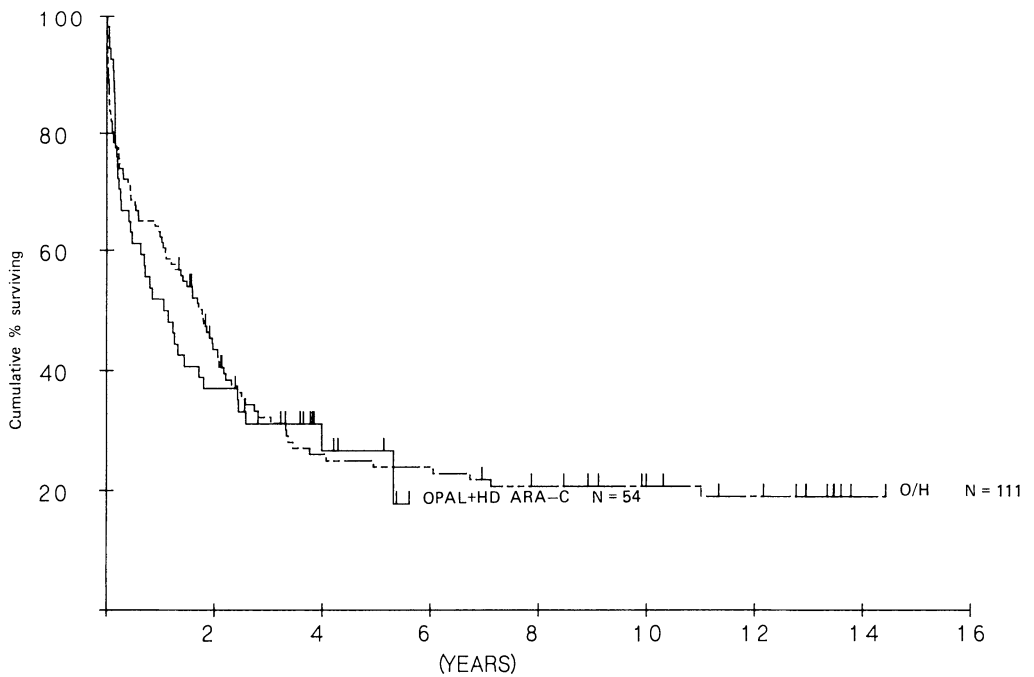


Fig. 2. Overall survival



**Fig. 3.** Duration of remission for high-risk patients, according to therapy O/H vs. OPAL-HD Ara-C



**Fig. 4.** Duration of remission for low-risk patients according to therapy O/H vs. OPAL-HD Ara-C



than those treated with OPAL or HEAV-D ( $P=0.006$ ). In contrast, those anticipated to be at low risk had a worse prognosis with OPAL-HD Ara-C ( $P=0.001$ ).

### *Toxicity of High-Dose Ara-C*

All patients became profoundly neutropenic ( $<0.5 \times 10^9$ /litre) and spent approximately 4 weeks in hospital. Eight of the 11 "early deaths" occurred following high-dose Ara-C and 3 other patients died while receiving the drug as consolidation therapy. Nausea and vomiting were inevitable and more than half developed an erythematous skin reaction, which was most marked on the hands and feet. Some patients complained of ocular discomfort despite the regular use of prednisolone eye drops. Neurological toxicity was manifest as nystagmus (one patient), tremor (two patients), and grand mal fits associated with transient CT scan abnormalities in one patient as described previously [18].

### **Discussion**

This study lends support to the data of others indicating that the prognosis of adults with "high-risk" ALL may be improved by intensification of the early therapy [17, 19]. Whether or not the apparently better duration of remission is a function of HD Ara-C itself is not, however, clear although there is theoretical evidence, at least for T-ALL, that this may be the case [20]. Furthermore, other treatment programmes showing good results for T-ALL include Ara-C, although not at such a high dose [17, 19]. Enthusiasm for these results must be muted. First, those patients with "low-risk" disease (C-ALL, low blast count ( $<10 \times 10^9$ /litre at presentation) had a worse prognosis than previously, possibly due to delays in administering the conventional therapy after HD Ara-C. Secondly, the improvement was only significant in terms of duration of remission. There was only a *trend* in favour of the HD Ara-C group in terms of disease-free survival because of deaths in aplasia possibly related to therapy. In spite of these reservations, the results are encouraging, and at least argue in

favour of individualization of therapy for ALL according to risk factors.

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## Clinical Importance of T-ALL Subclassification According to Thymic or Prethymic Maturation Stage\*

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### Introduction

Several recent reports have described the incidence and clinicopathologic features of childhood and adult T-lineage acute lymphoblastic leukemia (ALL) [1–7]. According to these reports, T-cell ALL accounts for approximately 15% of childhood and slightly over 20% of adult cases of ALL. It occurs more frequently in male adolescents or young adults and is usually associated with a mediastinal mass and high white blood cell count. In contrast to findings in children, in whom T-lineage ALL is usually associated with clinical risk factors and thus has a significantly worse treatment outcome than common ALL [2, 3, 7], recent studies in adult patients with T-cell ALL have shown a remarkable improvement in the prognosis for this subgroups (reviewed in [8]).

Identification of T-cell associated differentiation antigens by monoclonal antibodies (mAbs) has greatly facilitated the diagnosis and immunophenotypic characterization of

T-lineage ALL and has permitted, in most cases, the entry of human T-cell leukemias in a putative differentiation scheme on the basis of their composite cellular phenotypes [9, 10]. Similarly, more recent studies employing specific DNA probes for determination of T-cell receptor (TCR) gene rearrangements revealed a developmental sequence of TCR activation during T-cell maturation analogous to immunoglobulin gene rearrangements during B-cell development [11, 12]. These immunophenotypic and genotypic findings suggest the existence of an ordered hierarchy with respect to T-cell differentiation antigen expression as well as to rearrangement, transcription, and expression of TCR genes; the earliest recognizable step of T-lineage commitment is membrane expression of CD7 and intracytoplasmic accumulation of CD3 (cyCD3) prior to the expression of CD5 and CD2 as well as the rearrangement of  $\gamma$  and  $\beta$  genes [12, 13]. TCR  $\alpha$ -chain gene transcription occurs at a later stage of thymic ontogeny, probably as a critical regulatory event in the surface expression of the TCR-CD3 complex accompanied by the expression of more mature T-cell differentiation antigens (e.g., CD1, CD4, CD8) [12–14].

Despite the abundance of immunophenotypic and genotypic data on T-lineage ALL, very few studies have as yet attempted to evaluate the clinical features and treatment outcome according to stage of thymocyte differentiation. We therefore describe in this report our phenotypic and genotypic results in a large series of patients with adult T-lineage ALL studied as part of the German

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\*\* For the German BMFT ALL/AUL and ALL-BFM study groups.

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prospective study BMFT-ALL/AUL-study, focusing particularly on the biological features of patients with a putative prethymic (E-rosette<sup>-</sup>, E-R<sup>-</sup>) phenotype. Additionally, to further delineate possible biological and clinical differences, we compare immunophenotypic, genotypic and clinicopathologic features of adults with those of the children with pre-T-ALL studied within the ALL-BFM (BFM, Berlin-Frankfurt-Münster) 83/86 trials.

## **Patients and Methods**

### *Patients*

Pretreatment specimens (heparinized bone marrow and/or peripheral blood) were obtained from 927 adults and 1013 children qualified for the BMFT-ALL/AUL or ALL-BFM 83/86 protocols. Classification of ALL subtypes followed the FAB criteria [15]. All patients were treated according to risk-adapted multidrug chemotherapy protocols as described elsewhere [16–18].

### *Methods*

Leukemic cells for immunophenotype determination were isolated by standard Ficoll-Hypaque density-gradient centrifugation. Surface antigen expression was identified by an indirect immunofluorescence assay (IF) and evaluated by an epi-illuminated fluorescence microscope or by flow cytometry as previously described [19, 20]. Rosette assays were performed at 4° C with sheep erythrocytes that were untreated or treated with S-(2-aminoethyl) isothiuronium bromide hydrobromide as described [1]. The following panel of mABs was applied to all cases of adult T-lineage ALL: CD10 (VIL-A1), CD24 (BA-1), CD7 (WT1 or Leu-9), CD1 (OKT6-Na1/34 mixture), CD15 (VIM-D5), and VIM-2. In selected cases, cryopreserved material was available for more detailed analysis of the immunophenotype using a sensitive immunocytochemical method as described elsewhere [21], and a broader panel of mABs permitted detection of all major T-cell differentiation antigens, i.e., CD2 (OKT11), CD5 (Leu-1), cytoplasmic (cy) or

membrane CD3 (UCHT1), CD4 (Leu-3a), and CD8 (Leu-2a) as well as markers of hematopoietic progenitor cells, including CD34 (My10) and OKIa1 (HLA-DR). A comparable panel of mABs was used for phenotype determination in childhood ALL: For cyCD3 (Leu-4), and intranuclear terminal deoxynucleotidyltransferase (TdT) staining, cytospin preparations were fixed in acetone (CD3) or methanol (TdT) and analyzed by indirect IF.

The main reactivity and the source of the above-listed mABs have been previously described [7, 19, 20, 22]. The criterion for marker positivity was expression by at least 20% of the leukemic blast population.

### *Definition of Patient Subgroups*

The criteria for classifying childhood and adult ALL patients as pre-T-ALL were slightly different. Children considered to have the pre-T-ALL phenotype were positive for CD7, CD5 in most cases and TdT and were negative for CD2 as well as CD1, CD3, CD4, and CD8. Adults with a pre-T-ALL phenotype were characterized by expression of CD7 and TdT activity, lack of a receptor for sheep erythrocytes (E-R<sup>-</sup>), and negativity for CD1, CD4, and CD8. Further subclassification of T-ALL patients according to the expression of thymocyte antigen CD1 and more mature T-cell differentiation antigens will be the subject of a separate report.

### *DNA Analysis*

Southern blot analysis was performed by standard methods, as described in detail elsewhere [23, 24].

### *Statistical Analysis*

The  $\chi^2$  test was used to evaluate differences in initial patient characteristics. The Kaplan-Meier method was used to construct the life-tables plotted in Fig. 1, and comparisons of the life-tables were calculated by the Mantel-Cox test [25, 26].

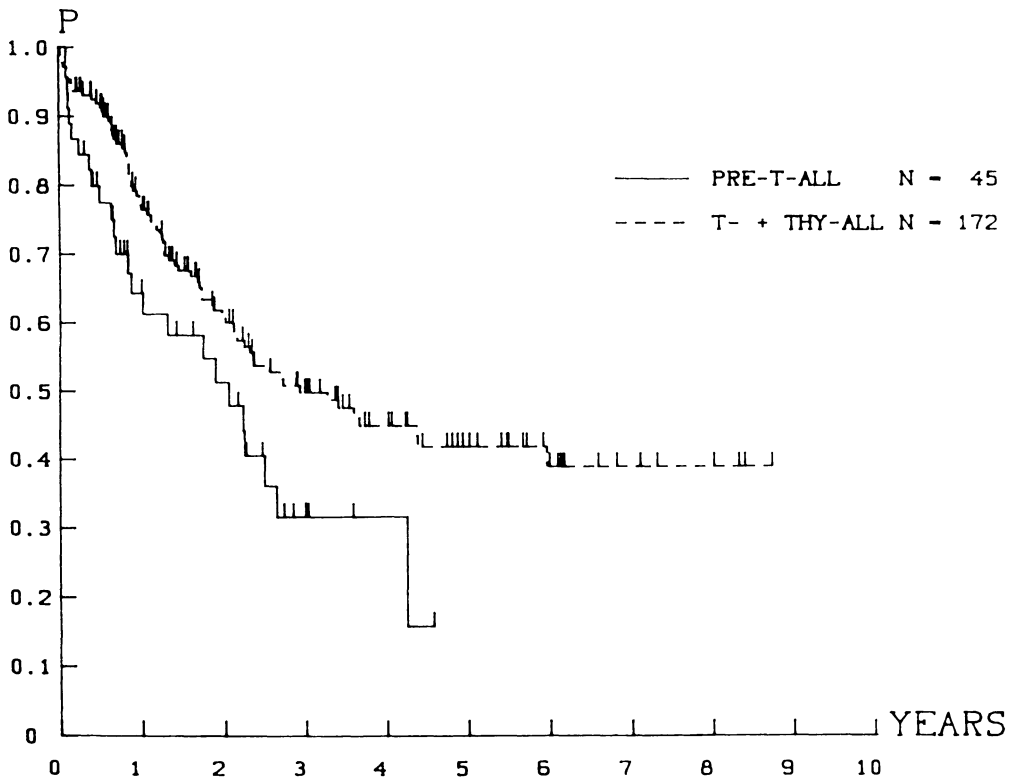


Fig. 1. Survival probability of adult ALL patients with T-lineage ALL in the BMFT-ALL/AUL trial. E-R<sup>-</sup> pre-T-ALL versus E-R<sup>+</sup> T-ALL

## Results

By the end of November 1988, the BMFT-ALL/AUL trials 1-81, 2-84, and 3-87 had recruited 217 patients with T-lineage ALL who had been submitted to satisfactory immunologic marker studies. Results on response to induction chemotherapy and life-table analysis were obtained from this cohort of patients, whereas the comparison of clinicopathologic features shown in Table 1 was based on findings in a slightly smaller subgroup of patients evaluated at an earlier date (November 30, 1987).

Childhood T-lineage ALL patients comprised 138 cases included in the ALL-BFM 83 and 86 trials between October 1, 1983 and December 31, 1988.

## Immunophenotypic and Genotypic Features

E-R<sup>-</sup> T-ALL (pre-T-ALL) was diagnosed in 45 of 217 adult patients (21%) with T-lineage ALL. More extended immunocytochemical studies of immunophenotypic features combined with analysis of TCR gene rearrangements were performed in 28 cases with pre-T-ALL. The phenotypic pattern of these patients is related to TCR  $\beta$  and  $\gamma$  gene configuration in Table 2. All patients had a majority of blast cells displaying CD7 expression; this was always associated with TdT positivity in the pre-T-ALL tested. T-lineage affiliation was further substantiated by cyCD3 expression in 27/28, CD5 positivity in 23/28, and CD2 in 14/28 pre-T-ALL.

**Table 1.** Presenting clinical and hematologic features of childhood and adult T-lineage ALL

Feature	Adult		Childhood	
	T-ALL ( <i>n</i> = 143) No. (%)	Pre-T-ALL ( <i>n</i> = 43) No. (%)	T-ALL ( <i>n</i> = 112) No. (%)	Pre-T-ALL ( <i>n</i> = 26) No. (%)
Age <sup>a</sup>	34 (24)	7 (16)	44 (39)	12 (46)
Male sex	103 (72)	30 (70)	85 (76)	18 (69)
Mediastinal mass	77 (54) <sup>b</sup>	15 (35)	71 (63)	13 (50)
Lymphadenopathy	117 (82) <sup>b</sup>	27 (63)	82 (73)	22 (85)
Hepatomegaly	77 (54)	20 (47)	46 (41)	15 (58)
Splenomegaly	96 (67)	22 (51)	61 (54)	17 (65)
CNS involvement	21 (15)	7 (16)	10 (9)	1 (4)
White blood cells > 30 × 10 <sup>9</sup> /l	91 (64)	23 (54)	86 (77)	19 (73)
Hemoglobin < 8 g/dl	35 (25)	9 (21)	13 (12) <sup>b</sup>	9 (35)
Platelets < 50 × 10 <sup>9</sup> /l	71 (50) <sup>b</sup>	11 (26)	42 (38)	7 (27)
Focal acid phosphatase	77 (66) <sup>c</sup>	15 (46)	99 (88)	21 (81)

<sup>a</sup> Adults > 35 years; children ≥ 10 years.

<sup>b</sup> *P* < 0.05.

<sup>c</sup> Only cases analyzed by central morphology and cytochemistry were considered (116 patients with T-ALL and 33 with pre-T-ALL).

**Table 2.** Immunophenotype of E-R<sup>-</sup> pre-T-ALL in relation to TCR gene configuration

Hematopoietic progenitor antigens				T-cell antigens				
TdT	HLA-DR	CD34	CD10	cyCD3	CD7	CD5	CD2	CD3
I. TCR $\beta$ and TCR $\gamma$ genes in germline								
13/13 <sup>a</sup>	5/14	11/14	5/14	13/14	14/14	9/14	8/14	1/14
II. TCR $\beta$ in germline, TCR $\gamma$ genes rearranged								
3/ 3	0/ 3	2/ 3	2/ 3	3/ 3	3/ 3	3/ 3	1/ 3	2/ 3
III. TCR $\beta$ and TCR $\gamma$ genes rearranged								
10/10	1/11	4/11	5/11	11/11	11/11	11/11	5/11	4/11

<sup>a</sup> Number of patients with > 20% positive blast cells/number of patients studied.

In none of these patients did leukemic blasts disclose any reactivity with mABs recognizing antigens characteristic for more mature intrathymic differentiation stages such as CD1, CD4, CD8. Expression of hematopoietic precursor cell associated antigens was detected in 17/28 (CD34) and 6/28 (HLA-DR) pre-T-ALL cases, providing further evidence of a prethymic level of maturation arrest. As also depicted in Table 2, a considerable proportion of pre-T-ALL retained the germline position for both TCR

$\beta$ - and  $\gamma$ -chain genes, whereas only TCR  $\gamma$  genes were rearranged in three cases, and rearrangement of T  $\beta$  and T  $\gamma$  genes was found in 11 cases of pre-T-ALL. The full documentation of the immunophenotypic and genotypic features of these patients, including TCR  $\delta$  genes analysis, are given elsewhere [22, 24].

CD2<sup>-</sup> (pre-T-ALL) was diagnosed in 26 of 138 children (19%) with T-lineage ALL. Their phenotypic and genotypic characterization disclosed some important differences:

- a) hematopoietic precursor cell associated antigens were expressed in only 1/8 (CD34) and 1/26 (HLA-DR) pre-T-ALL investigated;
- b) rearrangements of TCR  $\beta$  and  $\gamma$  genes were demonstrated in all pre-T-ALL tested ( $n=6$ ).

A similarity to the adult patients was evident in the positivity of CD7 in all cases and of CD5 in 24/26 cases of pre-T-ALL, mostly associated with TdT activity. CyCD3 expression, analyzed in only a small number of patients ( $n=5$ ), was strongly positive in all pre-T-ALL. As defined, CD2 and other T-cell differentiation antigens (CD1, CD4, CD8) were absent in pre-T-ALL.

### *Clinicopathologic Features*

As shown in Table 1, adult pre-T-ALL and E-R<sup>+</sup> T-ALL differed significantly with respect to several clinical features. Adults with pre-T-ALL were less likely to present with a mediastinal mass, lymphadenopathy, and thrombocytopenia. In contrast to these findings, T-lineage immunophenotypic subgroups in childhood were similar in their clinical and hematologic features.

### *Treatment Outcome*

Adults with pre-T-ALL had a lower remission rate (77% versus 85% for T-ALL;  $p=0.11$ ) and a higher mortality rate during induction therapy (13% versus 6% for T-ALL). These differences were more pronounced between patients with E-R<sup>-</sup> and E-R<sup>+</sup>/CD1<sup>+</sup> T-ALL than between those with E-R<sup>-</sup> and E-R<sup>+</sup>/CD1<sup>-</sup> T-ALL (data not shown). Life-table analysis revealed significant differences in the survival time (Fig. 1) as well as the median duration of remission (17.2 months for pre-T-ALL versus 34.2 months for T-ALL;  $p=0.02$ ).

Childhood T-lineage subgroups (pre-T-ALL versus T-ALL) have not as yet shown any significant differences with regard to either the CR rate (92% for pre-T-ALL and 96% for T-ALL) or event-free survival (54% for pre-T-ALL versus 61% for T-ALL patients).

## **Discussion**

According to current knowledge, the heterogeneity of ALL with regard to biological and clinical features can best be explained by leukemia cells arising in the B- or T-lymphocyte progenitor compartments and remaining arrested at discrete, characteristic levels of early B- or T-cell maturation in a predominantly monoclonal expansion [27].

Recent studies have demonstrated that the stage of cell maturation in precursor B-cell ALL has independent prognostic significance and is associated with characteristic biological features [28, 29]. Similar attempts to correlate biological features with clinical findings and treatment outcome in T-lineage ALL classified according to T-cell differentiation stages have been hampered by the relatively small number of patients prospectively studied within multicenter trials.

mAb characterization of surface antigens, performed mostly in childhood T-cell ALL, has not as yet revealed any significant relationships between the clinical features or prognosis and the developmental stage of leukemic T-lymphoblasts [4, 6]. In contrast, studies applying heteroantisera and E-rosette determination for subclassification of T-lineage ALL have provided evidence suggesting clinically relevant differences between E-R<sup>-</sup>/T-antigens<sup>+</sup> and E-R<sup>+</sup>/T-antigens<sup>+</sup> patients [5, 30]. The data that we obtained from a detailed immunophenotypic and genotypic analysis of a large series of adult T-lineage patients confirm and extend the latter observations. By choosing E-R<sup>-</sup> as a prescreening criterion, we were able to identify an immature pre-T-ALL subgroup with characteristic phenotypic (CD7<sup>+</sup>/cyCD3<sup>+</sup>/TdT<sup>+</sup>/CD34<sup>+/-</sup>/HLA-DR<sup>+/-</sup>/CD10<sup>+/-</sup>), genotypic (germline position for TCR  $\beta$  and  $\gamma$  genes in 14/28 patients) as well as clinicopathologic features (significantly lower incidence of mediastinal mass, lymphadenopathy, and thrombocytopenia). The invariant expression of CD7 and cyCD3 in these cases is in line with other recent studies indicating that the earliest phenotypically identifiable stage of T-lineage commitment both in normal and malignant T cells is characterized by surface membrane expression of CD7 and intracellular accumulation of CD3 in the perinuclear

space [12, 13, 31–34]. Further evidence suggesting a prethymic level of maturation arrest was provided by the absence of CD1, CD4 and CD8 antigens in all pre-T-ALL and the frequent expression of hematopoietic precursor cell associated markers such as HLA-DR and CD34.

Although earlier studies have demonstrated a higher incidence of HLA-DR expression in adult than in childhood T-lineage ALL [35], our results on CD34 expression in pre-T-ALL are the first report to indicate the usefulness of this antigen as a precursor cell marker within the T-cell lineage. Consistent with the immature phenotypic features observed in adult pre-T-ALL cases was the frequent occurrence of TCR  $\beta$  and  $\gamma$  genes retaining their germline position. Several recent reports showed that certain T-cell antigens such as CD7, CD2, CD5, and TdT can be expressed prior to TCR  $\beta$  or  $\gamma$  gene rearrangements [36–40], and, based on these findings, the authors speculated upon a functional role of these early T-cell differentiation antigens in the thymic homing process of immature, T-lineage committed precursor cells [39, 41].

Although pre-T-ALL and E-R<sup>+</sup> T-ALL patients had many clinical features in common, there were some significant differences, including the incidence of mediastinal mass, lymphadenopathy, and thrombocytopenia. The lower incidence of extramedullary involvement observed in pre-T-ALL may result from a more pronounced bone marrow homing of pre(thymic)-T leukemia cells. The substantially worse prognosis of the most immature subset within T-lineage ALL (pre-T-ALL) is comparable to the poor treatment outcome in adults or infants with immature early B-cell precursor ALL (null ALL), suggesting that, irrespective of T- or B-cell differentiation, ALL of early primitive lymphohematopoietic cells did not respond adequately to conventional chemotherapy [42, 43]. Noteworthy in this context is a recent study which evaluated clinical and biological features of nine adolescents and adults with CD7<sup>+</sup>, CD4<sup>-</sup>, CD8<sup>-</sup> ALL [44]. This phenotype was associated with distinct clinical features, including male predominance, frequent mediastinal mass, and high white blood cell counts as well as a high treatment failure rate. Importantly, leuke-

mic cells of these patients retained the capacity for multilineage differentiation *in vitro*, consistent with the malignant transformation of an immature pluripotent hematopoietic cell.

Children with pre-T-ALL differed markedly from adult patients with respect to their phenotypic and genotypic features as well as their treatment response data. The leukemic cells very rarely expressed hematopoietic progenitor cell associated antigens and always showed rearrangements of both TCR  $\beta$  and  $\gamma$  genes, in accordance with other studies disclosing that virtually all childhood T-lineage ALL had rearranged TCR genes [45–47]. Furthermore, no unique clinical features or significant differences in clinical outcome were found.

Thus, although slightly different phenotypic criteria were used to distinguish T-lineage ALL subgroups, several important observations emerge from this comparison between childhood and adult pre-T-ALL. The frequent expression of hematopoietic progenitor cell antigens as well as the striking incidence of TCR  $\beta$  and  $\gamma$  genes in germline position suggest an arrest of adult pre-T-ALL at a less mature differentiation stage than in childhood, which may be closely related to the poor prognosis of these patients. This subgroup of phenotypically immature acute leukemias requires further studies involving biological characterization of the leukemic cells and their presumptive normal counterpart as well as prospective evaluation of their clinical features and treatment outcome. It might thus be possible to identify a new group of patients requiring a novel therapeutic approach.

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## Treatment of Adult Acute Lymphoblastic Leukaemia \*

P. Jacobs, L. Wood, and N. Novitzky

### Introduction

The outcome of treating acute lymphoblastic leukaemia (ALL) is better in children than in adults [1], although in both groups results improved when anthracycline antibiotics were added to induction therapy [2], with other agents playing a subsidiary role [3]. In addition, outcome in adults is influenced by trial design [4] and there persists controversy about postremission therapy [5], including the use of bone marrow transplantation [6]. The influence of CNS involvement [7] and the prophylactic treatment of the neuraxis, either directly [8] or with drug schedules that permit the penetration of the spinal fluid, are the subject of continued study [9].

Taking prognostic factors into account [7, 10], patients can be stratified into standard- or high-risk groups and management allocated on a risk-adapted basis that will take into account alternative and more aggressive treatment options [11].

The current experience provides support for the use of intensive postremission chemotherapy in managing adults with ALL.

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### Patients and Ethical Considerations

Eighty-five previously untreated adults with ALL, having a mean age of 24 years (range 10–69 years), were entered on a protocol approved by the University of Cape Town and Groote Schuur Hospital Ethics and Research Committee and after having had all details explained to them participated with informed consent.

### Trial Design and Statistics

Induction was undertaken as far as possible on an outpatient basis, with weekly intravenous infusions of vincristine (2 mg), Adriamycin (25 mg/m<sup>2</sup>), l-asparaginase (10 000 units/m<sup>2</sup>), and daily oral prednisone (1 mg/kg). Consolidation was given immediately after remission had been achieved, using the identical drug regimen for an additional 4 weeks. CNS prophylaxis consisted of 2400 cGy cranial irradiation and concurrently six alternating injections of intrathecal methotrexate (12.5 mg/m<sup>2</sup>) and cytosine arabinoside (30 mg/m<sup>2</sup>).

Maintenance therapy was given for 3 years and comprised titrated doses of daily 6-mercaptopurine (50 mg/m<sup>2</sup>) and weekly methotrexate (20 mg/m<sup>2</sup>) combined with monthly intensification on a random basis to either VAA or COAP, comprising cyclophosphamide (100 mg/m<sup>2</sup> IVI for 25 days), vincristine (2 mg IVI), cytosine arabinoside (100 mg/m<sup>2</sup> IVI for 5 days) and prednisone (100 mg orally for 5 days).

Statistical analysis was undertaken to determine the influence of age, sex, race, mor-

phology, immunophenotyping, total white cell and blast count, the presence of lymphadenopathy, splenomegaly, hepatomegaly, lactic dehydrogenase, bone marrow cellularity and uric acid level on achievement of CR using the chi-squared method with Yates' correction. To determine remission duration these factors were screened for prognostic value using Kaplan-Meier product limit survival curves and compared for statistical significance using the generalized Wilcoxon and Savage techniques [12].

## Results

The CR rate was 69% (59/85 patients). Only the FAB L1 morphology was a predictive factor ( $P=0.048$ ). Twenty-three patients failed, and 12 had primary drug resistance. Median follow-up is 260 weeks; median predicted survival of all patients is 58 weeks and for those who achieved CR it is 104 weeks. Median duration of CR is 70 weeks. Of the prognostic factors for survival only the FAB L1 morphology was significant. Bone marrow relapses occurred in 29 patients and 9 (31%) again achieved CR. There has been CNS relapse in two patients and both have died. Eleven patients continue in CR off therapy, with a median of 152 weeks (range 12–372 weeks).

## Discussion

In order to improve disease-free survival, it is necessary to accurately define the results that can be achieved with a standardized treatment programme and then to identify factors predicting for response and incorporate these into regimens that balance outcome against side effects of therapy. In such a risk-adapted approach more aggressive modalities are appropriate for patients anticipated to have suboptimal remission rates or survival. Our current experience emphasizes four issues.

Firstly, the CR rate of 69% compares reasonably with that reported in recent trials [13] as well as British [14] and Spanish data [15]. Furthermore, analysis shows the consecutive patients entered in the present population-based study have long-term sur-

vivals marginally better than the 20% reported from Britain [14] which more closely approximate the 38% found in Spain over a 13-year period [15]. The only significant factor predicting for achievement of CR was morphology, which is consistent with previously reported data [16], but contrasts with other studies that found a difference [13]. It is uncertain as to whether these observations are influenced by age, since lymphoblasts having the L1 appearance are found more frequently in the younger age groups than with the more heterogeneous L2 subtype.

Similarly, age has its greatest impact on remission duration and survival rather than achievement of this status [7, 13], as found in the present series, although these data are controversial [14]. In addition, involvement of the CNS at diagnosis does not appear to influence achievement of CR [17], although it has been reported as an adverse prognostic feature [7]. The low incidence of CNS relapse in the present series may reflect the adequacy of craniospinal prophylaxis, while prolonged intrathecal drug administration during the 3 years of maintenance is an alternative explanation for this finding.

Secondly, it is noteworthy that of the 23 patients who failed to achieve CR, 12 had primary drug resistance, and it is this subset that needs to be promptly identified and then treated on a risk-adapted basis, which should include the option for bone marrow transplantation [6, 10, 11]. In the present series only the FAB L1 morphology, and not the immunophenotype, predicted for achievement of CR. Of note is our more recent experience (Jacobs; unpublished observations) suggesting that the time taken to achieve remission is the single best index in recognizing patients who should be more aggressively treated, as reported by others [13].

A point of relevance is the generally excellent way in which our patients tolerated chemotherapy when this was administered and monitored by an experienced multidisciplinary medical and nursing team. During induction, 100% of the predicted dose was given exactly on schedule but, as previously reported [13], infection remains a significant complication. In our series, patients who died early with marrow hypoplasia while residual disease was present reflect a subgroup of slow responders and again a reasonable

alternative would be to recognize their high probability of failing therapy and proceed to a salvage programme.

Thirdly, in considering survival among patients who achieved CR the significant prognostic factor was the FAB subtype, where L1 was better than L2, and this is consistent with an earlier observation [16].

Fourthly, 29 patients experienced medullary relapse whilst on maintenance therapy and 9 (31%) achieved a second CR, which is durable in a single individual who underwent allogeneic bone marrow transplantation. These findings again emphasize the need to identify subgroups of patients where more aggressive therapy is justified. Those who relapse after maintenance therapy has been discontinued can be reinduced with the same agents, whereas this event occurring during therapy requires alternative management, and here there is likely to be a role for bone marrow transplantation [18].

It is concluded that this chemotherapy schedule achieves a significant remission rate and durable disease-free survival in which the FAB morphology is a significant predictive factor for good prognosis. The incidence of resistant and relapsing diseases is an argument for further intensifying both induction and postremission therapy, particularly in the subgroup best identified by the slow time taken to achieve CR.

## Summary

Eighty-five consecutive patients with acute lymphoblastic leukaemia (ALL), having a median age of 24 years (range 10–69 years), underwent induction and consolidation chemotherapy with weekly parenteral vincristine, Adriamycin, l-asparaginase and daily oral prednisone (VAAP), followed by standard (CNS) prophylaxis. Maintenance therapy was given for 3 years and consisted of daily 6-mercaptopurine, weekly methotrexate and monthly intrathecal therapy, with drug intensification comprising either vincristine, Adriamycin and l-asparaginase (VAA) or cyclophosphamide, vincristine, cytosine arabinoside and prednisone (COAP). Complete remission (CR) was obtained in 59 patients (69%) and only the French-American-British (FAB) L1 morphology

was a significant predictive factor ( $P=0.048$ ). Twenty-three patients failed to achieve CR and of these 12 had primary drug resistance. Median follow-up is currently 260 weeks, median predicted survival of all patients is 58 weeks and for those who achieved CR it is 104 weeks. Median duration of CR is 70 weeks. Of the prognostic factors for survival, only FAB L1 subtype was significant. Bone marrow relapses occurred in 29 patients, and of these 9 (31%) achieved CR. There has been CNS relapse in two patients and both have died. Eleven patients continue in CR off therapy, with a median of 152 weeks. This regimen is effective, with acceptable toxicity, and a number of patients are potentially cured. The incidence of resistant and relapsing disease is an argument for further intensifying both induction and postinduction therapy.

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## Treatment of Relapsed Acute Lymphocytic Leukemia in Adults

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### Introduction

The treatment of acute lymphoblastic leukemia (ALL) with modern multidrug schedules is highly effective. In the first German multicenter study, 01/81, complete remissions were induced in 272 of 368 patients (73.9%), and in the second trial, 02/84, in 350 of 442 (79.2%) [1]. Though 35% of the patients remain disease free after 7.5 years in the first study and 48% after 3 years in the second, relapses are still frequent. The devel-

opment of effective salvage schedules is warranted. Against this background we have started a protocol to test an intensive induction and consolidation therapy for patients with relapsed ALL/acute undifferentiated leukemia (AUL).

### Patients and Methods

Between May 1986 and June 1988, 33 patients (25 male, 8 female) with a median age of 25.7 years (range 16.7–62.2 years) were admitted to the study after informed consent. The median duration of the preceding complete remission was 26.1 months (range 6–80 months). All patients had ALL/AUL in first bone marrow relapse or combined bone marrow and extramedullary relapses (e.g., testis and/or CNS relapse). Seventeen patients had common ALL, 14 T-ALL, 1 0-ALL, and in 1 no immunotyping was performed. Patients with severe concomitant diseases, without response to platelet transfusions and with severe untreatable complications of leukemia, were not admitted to the study.

The treatment schedule consisted of two induction phases and two different consolidation regimens. Induction-phase I consisted of prednisone (60 mg/m<sup>2</sup> p.o. days 1–21), vindesine (3 mg/m<sup>2</sup> i.v. days 1, 8, 15), daunorubicin (45 mg/m<sup>2</sup> i.v. days 1, 8, 15), *Erwinia* asparaginase (10 000 U/m<sup>2</sup> i.v. days 7, 8, 14, 15), and i.t. methotrexate (MTX) (15 mg days 1, 8). After hemopoietic regeneration, phase II was started with high-dose cytarabine [3000 mg/m<sup>2</sup> 3 h infusion twice days 1–4 (in patients over 50 years 1000 mg/

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m<sup>2</sup>], and etoposide (100 mg/m<sup>2</sup> i.v. days 1–5).

Patients were randomly assigned to consolidation A with dexamethasone (3 × 5 mg/m<sup>2</sup> days 1–5), MTX (1500 mg/m<sup>2</sup> infusion 1 h day 1), teniposide (80 mg/m<sup>2</sup> i.v. days 1–3), cytarabine (300 mg/m<sup>2</sup> i.v. days 1–3), and folinic acid (15 mg/m<sup>2</sup> p.o. q 6 h days 2–4), or to consolidation B with ifosfamide (5000 mg/m<sup>2</sup> infusion 24 h day 1 with mesna), and vindesine (3 mg/m<sup>2</sup> i.v. day 2). Fifteen milligrams i.t. MTX was given on day 1 of consolidation A and B.

## Results

Twenty-one patients (64%) achieved a complete remission (CR), 14 after induction phase I and 7 after induction phase II. One patient had a partial remission, and seven did not respond. Four patients died during induction: two with sepsis, one with *Candida* pneumonia, and one with disseminated intravascular coagulation and bleeding.

Side effects of induction phase I consisted of hematotoxicity with subsequent infections and gastrointestinal toxicity (see table 1). The median duration of granulopenia grade 4 WHO was 17 (3–33) days. Throm-

bopenia grade 4 WHO lasted for a median of 15 (3–29) days. In induction-phase II some patients experienced additional cutaneous, ocular, and hepatic toxicity (see table 2). Granulopenia lasted for a median of 17 (7–32) days and thrombopenia for a median of 11 (2–40) days.

The treatment was discontinued in 23 of the surviving patients after induction due to the following reasons: BMT (8), side effects (2), refractory disease (6), relapse (6), and decision of the physician (1). Therefore the effect of the consolidation therapy cannot yet be evaluated.

The median disease-free survival was 3.6 months (Fig. 1), and the median overall survival was 7.2 months (Fig. 2).

Fifteen (45%) of the patients were given bone marrow transplantation (BMT). Five had an allogeneic or an autologous BMT in CR, three had an allogeneic, and two an autologous BMT after another relapse. Four of eight patients with allogeneic BMT are alive and well. Two died with relapse, one with hepatic failure and one with graft-versus-host disease (GvHD). One of seven patients with autologous BMT is alive and well. Four had a relapse, of whom three have died, and two died from infection.

**Table 1.** Toxicity in 27 cycles of induction phase I

	Grade 0 %	Grade 1 %	Grade 2 %	Grade 3 %	Grade 4 %
Granulopenia	4	0	0	15	81
Thrombopenia	15	7	19	15	44
Bleeding	65	23	4	4	4
GOT/GPT	44	26	19	7	4
Nausea, vomiting	59	15	7	15	4
Mucositis	62	15	12	12	0
Diarrhea	93	4	4	0	0
Pulmonal	89	4	4	0	4
Alopecia	23	8	38	31	0
Local infection	70	15	7	4	4
Sepsis	89	0	4	4	4
Cardiac rhythm	85	4	12	0	0
Cardiac function	96	0	4	0	0
Peripheral neurophils	93	7	0	0	0
Constipation	93	4	0	4	0

GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase

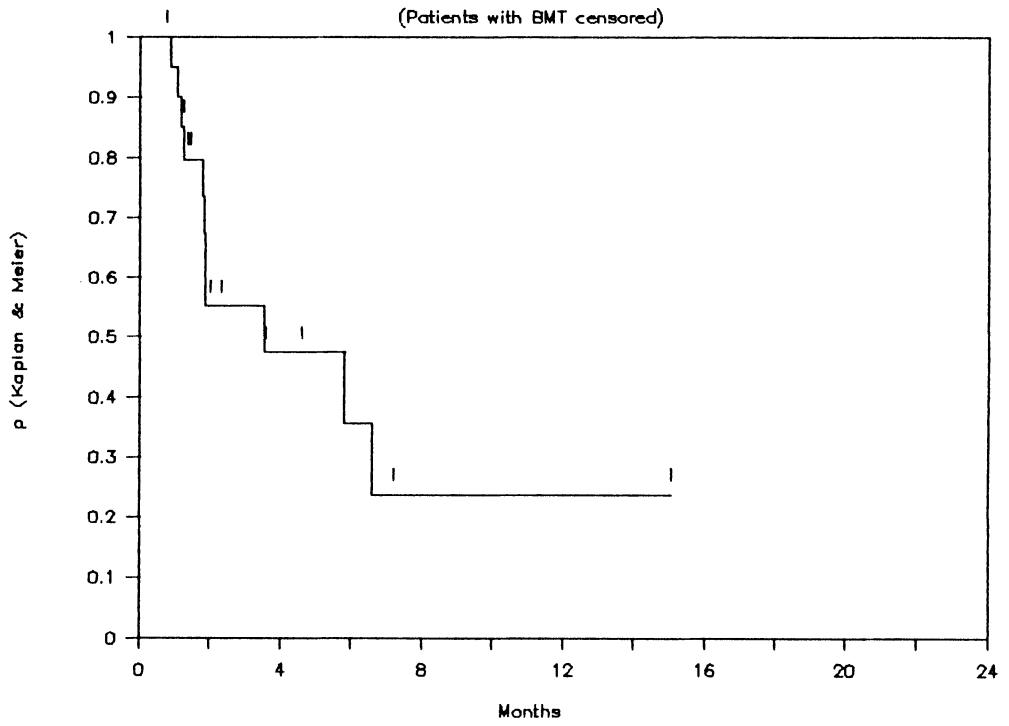


Fig. 1. Disease-free survival after chemotherapy. Patients with BMT are excluded

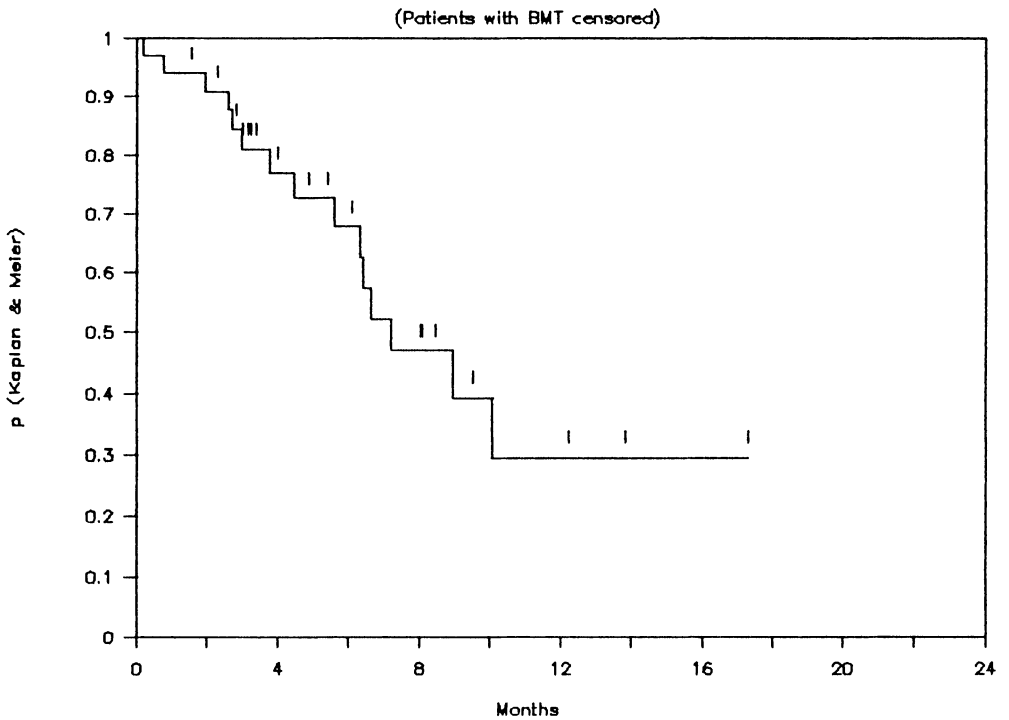


Fig. 2. Total survival after chemotherapy. Patients with BMT are excluded

**Table 2.** Toxicity in 19 cycles of induction phase II

	Grade 0 %	Grade 1 %	Grade 2 %	Grade 3 %	Grade 4 %
Granulopenia	0	0	0	0	100
Thrombopenia	0	0	0	5	95
Bleeding	63	11	21	5	0
GOT/GPT	37	16	32	16	0
Nausea, vomiting	0	17	28	56	0
Mucositis	39	22	6	33	0
Diarrhea	33	17	28	22	0
Pulmonal	89	5	0	5	0
Cutaneous	42	26	26	5	0
Alopecia	21	0	11	68	0
Local infection	59	12	12	18	0
Sepsis	59	0	6	18	18
Fever of unknown origin	76	0	18	6	0
Cardiac rhythm	68	32	0	0	0
Cardiac function	100	0	0	0	0
Eye	68	16	16	0	0
Cerebellar	95	5	0	0	0

GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase

## Discussion

The prognosis of relapsing adult ALL is generally considered to be poor. It is controversial whether a palliative or a curative concept should be favored. There are few therapeutic experiences [2–8] based on small and heterogeneous patient populations. This study was started by the German ALL Multicenter Group to gain experience in a uniformly pretreated patient population.

We achieved a CR rate of 64%, which is superior [6–8] or comparable [5] to those of earlier reports. In recently published studies, high doses of cytarabine have been given in combination with amsacrine (AMSA) [2], AMSA ± VP16 and prednisone [5], or mitoxantrone [3]. The latter study deals with patients with refractory ALL and is not comparable to our data. A report on AMSA ± VP16 and prednisone [5] included only nine patients, of whom seven responded to the treatment. Arlin et al. [2] reported a superior response rate of 75% in 36 patients but have excluded all patients with Ph<sup>+</sup>-positive ALL from their study.

The major problem in relapsing ALL is the limited duration of disease-free survival. There is no study with markedly different results in this respect. The only established procedure to overcome this is allogeneic BMT. The main future challenge is to prolong the remission duration for those patients who do not have an HLA-identical sibling donor.

## Summary

Thirty-three patients with ALL/AUL in first relapse were treated with an induction of prednisone, vindesine, daunorubicin, *Ervwinia* asparaginase, i. t. MTX (phase I), high-dose cytarabine, and etoposide (phase II). Twenty-one (64%) achieved a complete remission, one a partial remission. Side effects of induction-phase I were predominantly hematological with subsequent infections and gastrointestinal toxicity. In phase II some patients had additional cutaneous, ocular, and hepatic toxicity. The treatment efficiently induced remissions with tolerable toxicity in relapsed ALL. The

disease-free survival, however, needs to be improved.

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# **Acute Lymphoblastic Leukemia in Children**

## Results and Significance of Six Randomized Trials in Four Consecutive ALL-BFM Studies

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### Introduction

In addition to all the biological properties and clinical features of individual patients with acute leukemia, the quality of therapy itself has evolved as the most relevant predictive factor for cure or failure. Leukemia is still a life-threatening condition and needs adequate management, irrespective of the phenotypic or genotypic characterization of the leukemic clone.

During the past three decades, our knowledge of optimizing therapy for acute lymphoblastic leukemia (ALL) has increased by leaps and bounds. Most improvements have been introduced empirically: some had a significant impact on outcome; others were hardly recognized. In order to avoid specific risks and side effects, changes in structure, composition, dosage, and timing of therapeutic regimens have been imperative and have been the consequence of therapy results. Some of these changes may have had obvious disadvantages for outcome or remained prognostically unrecognized because of the countereffects of other

modifications. Only randomized trials – if properly performed, supported with an adequate number of patients, critically analyzed and correctly interpreted – give unequivocal information. On the other hand, randomized trials are difficult to assess and may be ethically delicate. Only highly motivated and disciplined cooperative groups may cope with these difficulties. From an ethical point of view, some major questions cannot be asked, which certainly is a regrettable limitation of the method. In general no major therapy changes in randomized trials are possible, if a significant difference in results is anticipated, no matter how relevant the question may be. Thus major (positive) modifications of therapy design have usually been introduced uncontrolled by randomization. But these elements of progress also opened new doors and initiated questions to be answered in subsequent (randomized) trials.

During the decade 1976–1986, the BFM study group asked six questions in four consecutive randomized trials. Written consent by parents and/or patients was demanded, because optional decisions for the other therapy arm also had to be accepted. Thus, not all patients could be included in the randomized trials. It was the intention of the study design to create randomized therapy arms with similar outcome.

### Patients and Trial Questions

Between October 1976 and September 1986, a total of 1793 patients under the age of

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18 years were recruited in four consecutive BFM trials. In trial ALL-BFM 76, 158 patients were enrolled, in trial ALL-BFM 79, 325 patients, in trial ALL-BFM 81, 633 patients, and in trial ALL-BFM 83, 677 patients. The trials included all infants, patients with B-type ALL, hybrid leukemias, and all lymphoma-like syndromes, providing they met the requirement of more than 25% relative bone marrow involvement with leukemic cells. In the four trials a total of 1864 patients were enrolled for randomization, 380 patients in trials ALL-BFM 76 and ALL-BFM 79 and 1464 patients in trials ALL-BFM 81 and ALL-BFM 83. In the latter two trials the study design required two randomizations; thus the number of patients randomized was greater than the total number recruited.

The six questions to be answered by randomization in these four trials are as follows:

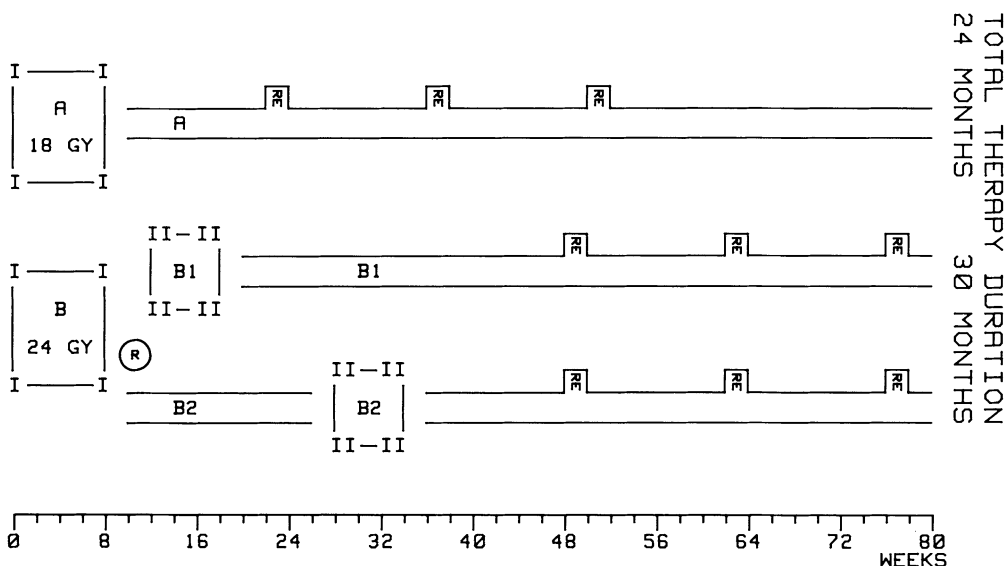
1. Trials ALL-BFM 76 and ALL-BFM 79 (Figs. 1, 2): Patients with a *risk index* of  $\geq 3$  (35% of all recruited patients, predominantly defined by a white blood count of  $\geq 25000/\text{mm}^3$ ) were randomized

to receive intensive reinduction therapy by *protocol II early* or *late* after achievement of remission [2, 3]. Question: Is the outcome different with respect to event-free interval (EFI)?

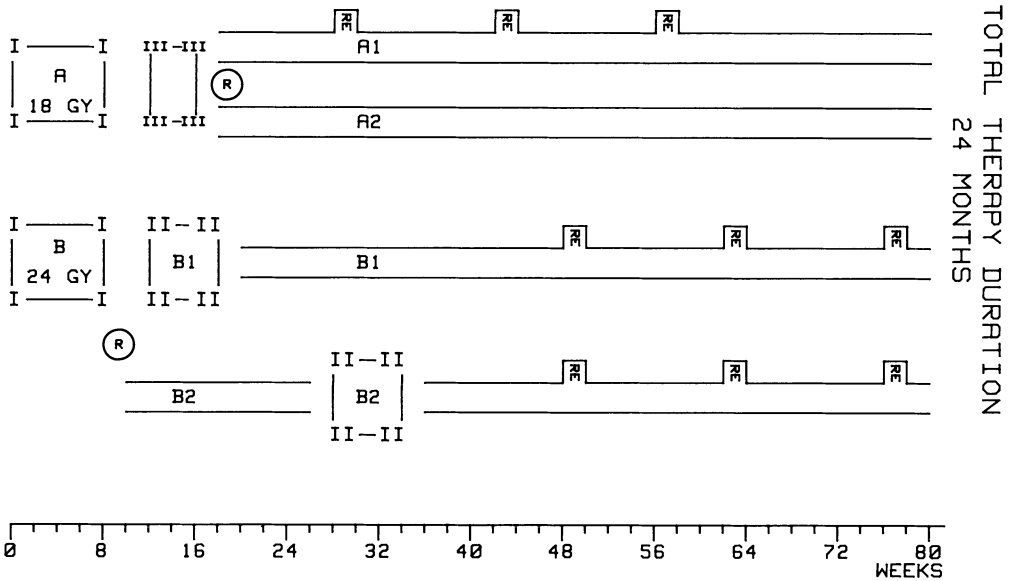
2. Trial ALL-BFM 79 (Fig. 2): Patients with a *risk index* of  $\leq 2$  (65% of patients with standard risk features) *received* or *did not receive* three pulses of *prednisone/vincristine* during the 1st year of remission [3]. Question: Do the two branches differ with respect to EFI?

3. Trial ALL-BFM 81 (Fig. 3): Patients with a *risk factor* of  $\leq 1.2$  (standard risk, 60% of all recruited patients) received either *cranial irradiation* (during protocol I, 18 Gy) or intermediate-dose *methotrexate* (after protocol I,  $4 \times 0.5 \text{ g/m}^2$  body surface) [5, 12]. Question: Do both branches differ with respect to EFI, especially in events concerning the CNS?

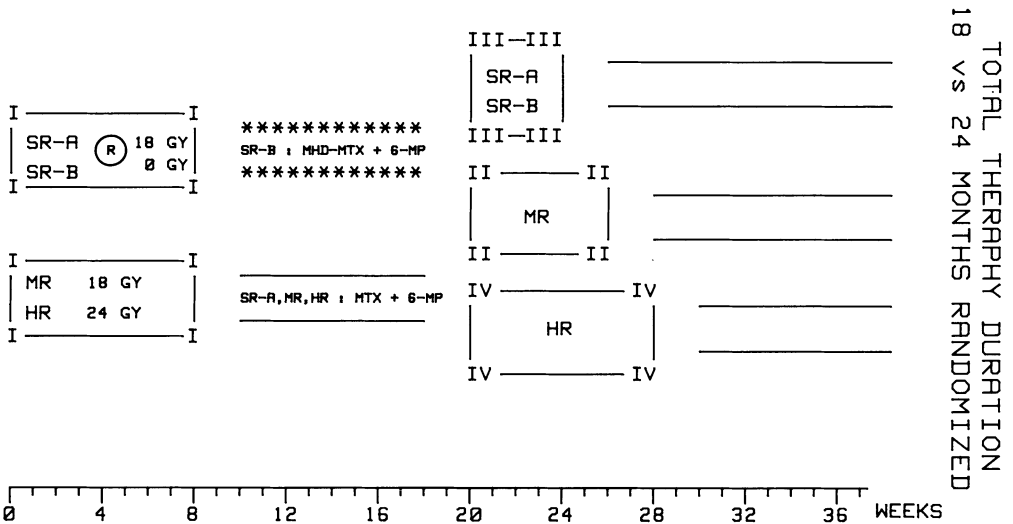
4. Trial ALL-BFM 83 (Fig. 4): Patients with a *risk factor* of  $\leq 0.8$  (standard risk low, 25% of all recruited patients) *received* or *did not receive* intensive reinduction therapy with *protocol III* after achievement of remission [5, 11]. Question: Do both branches differ in respect to EFI?



**Fig. 1.** Design for therapy trial ALL-BFM 76. Group *A* patients, risk index 2 or less; group *B* patients, risk index 3 or more. Risk index (sum of points):  $\text{WBC} \geq 25000/\text{mm}^3$ , 3 points; CNS disease at diagnosis, 2 points; thymic mass, 1 point; focal acid phosphatase positivity, 1 point; negative PAS reaction, 1 point; age at diagnosis  $< 2$  or  $\geq 10$  years, 1 point; significant extranodal tumor, 1 point. *RE*, reinduction pulses with prednisone/vincristine. *I*, protocol I; *II*, protocol II. *Randomization (R) branch B1 vs. branch B2*

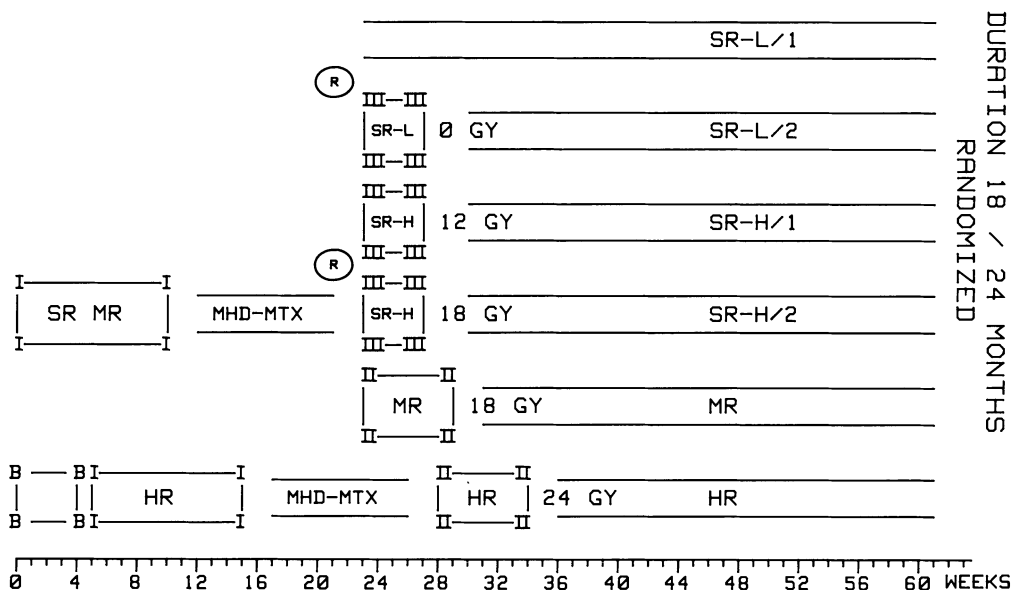


**Fig. 2.** Design for therapy trial ALL-BFM 79, III, protocol III. Other information as in Fig. 1. Randomization (R) branch A1 vs. branch A2 and branch B1 vs. branch B2



**Fig. 3.** Design for therapy trial ALL-BFM 81. Risk factor (RF) =  $0.2 \log(\text{blasts} + 1) + 0.06 \times \text{liver} + 0.04 \times \text{spleen}$  (organ size in centimeters below costal margin). Standard risk (SR),  $RF < 1.2$ ; medium risk (MR),  $1.2 \leq RF < 1.7$ ; high risk (HR),  $RF > 1.7$ . MTX, methotrexate; 6-MP, 6-mercaptopurine; MHD-MTX, intermediate dose MTX. IV, protocol IV. Other information as in Figs. 1, 2. Randomization (R) branch SR-A vs. SR-B





**Fig. 4.** Design for therapy trial ALL-BFM 83. Information as in Fig. 3. *SR-L*, standard risk low; *SR-H*, standard risk high. *B*, therapy element as given in B-type ALL/NHL. Randomization (*R*) *SR-L/1* vs. *SR-L/2*, *SR-H/1* vs. *SR-H/2*, and 18 vs. 24 months duration of therapy

5. Trial ALL-BFM 83 (Fig. 4): Patients with a risk factor of 0.8–1.2 (standard risk high, 30% of all recruited patients) received either 12 Gy or 18 Gy cranial radiation following protocol III [3, 5, 11]. Question: Is the low dose (“minimal effective dose”) equivalent to the standard dose in respect to EFI, especially events concerning the CNS?
6. Trials ALL-BFM 81 and ALL-BFM 83 (Figs. 3, 4): Patients in complete continuous remission at 18 months were or were not exposed for another 6 months of continuation therapy with oral methotrexate and 6-mercaptopurine (total duration of therapy 18 vs. 24 months) [10]. Question: Is the EFI different in both branches?

Thus all questions are related to intensity or quality of therapy, four to intensity of chemo- or radiotherapy (questions 2, 4, 5, 6), one to quality of chemotherapy (question 1), and one to the interaction of both (question 3). For analysis, standard methods including the Kaplan-Meier plot and the log-rank test were used.

### Answers to the Trial Questions

In trial ALL-BFM 76, the uncontrolled introduction of a new therapy element – protocol II – for patients with increased risk for therapy failure generated a dramatic improvement in EFI [2, 9, 10] if compared with the results of the historical Berlin pilot study [8], performed between 1970 and 1976 (Fig. 5,  $P=0.001$ ). In the Berlin pilot study all patients were only exposed to protocol I, and did not receive protocol II. Improvement of EFI has been interpreted to be caused exclusively by the introduction of this new therapy element [9]. In order to enroll a substantial number of patients to answer trial question number one, the randomization of early or late exposure to protocol II was not only used in trial ALL-BFM 76, but was continued in the subsequent trial ALL-BFM 79 [2, 3]. Since both trials differed only in one minor modification (duration of maintenance therapy, Figs. 1, 2), the answer is rather unambiguous (Fig. 6). Kaplan-Meier plots for both branches are superimposable for up to 3

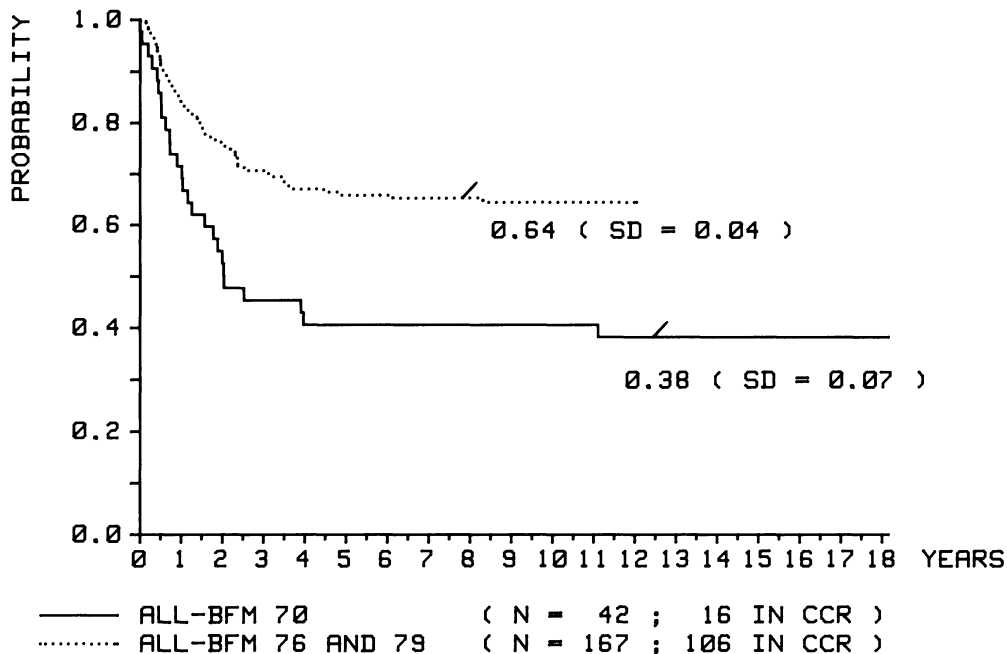


Fig. 5. Event-free interval as related to exposure to protocol II,  $P=0.001$

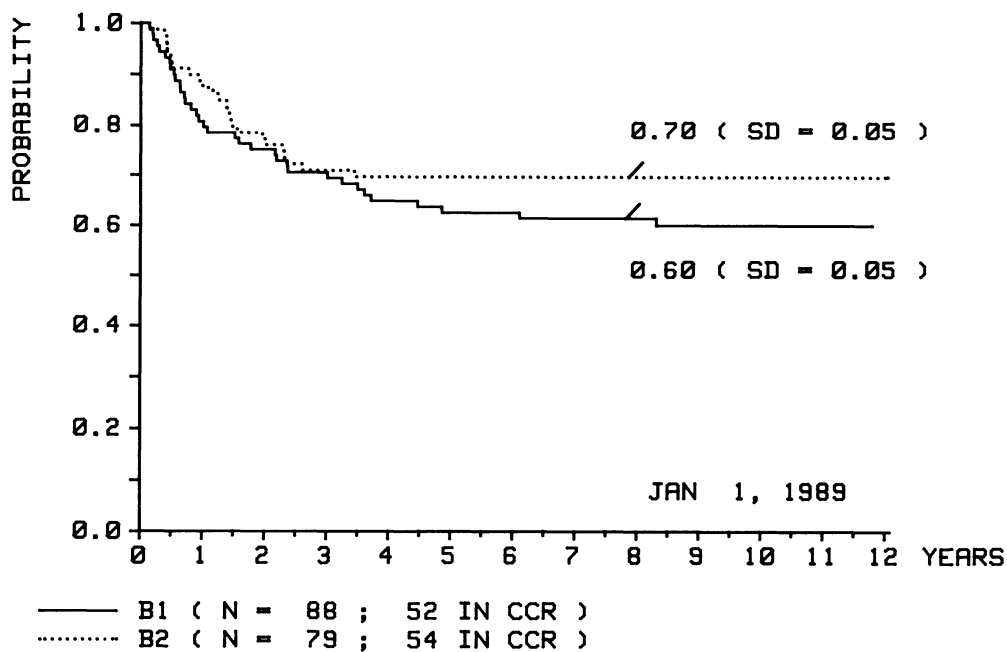


Fig. 6. Event-free interval as related to early (B1) or late (B2) application of protocol II,  $P=0.24$  (randomization)

years of follow-up and subsequently differ only slightly. Thus, exposure to protocol II late in remission could have been preventive nonsignificantly ( $P=0.24$ ) for late events. This information was utilized in trial ALL-BFM 81 with late rather than early application of protocol II since additionally late application of protocol II resulted in less toxicity [10].

Traditionally, reinduction pulses with prednisone/vincristine are used during maintenance therapy. The purpose of the second trial question was to quantify these pulses in the context of the BFM study design, which for the first time used systematically intensive front-line therapy. The morbidity of these pulses was significant at that time. Due to repeated exposure to corticosteroids the pulses were often followed by *Pneumocystis pneumonia*, thus being a target for a randomized trial question (Fig. 2). Probably, enforced by the efficacy of front-line induction therapy, patients with standard-risk features (risk index  $\leq 2$ ) did not benefit from these three pulses as demonstrated in Fig. 7 ( $P=0.44$ ). This result prompted us to omit this treatment element in subsequent trials [3].

The question of the best mode of CNS prevention is still unsettled [1, 4, 10, 13]. Most investigators consider it to be appropriate to substitute cranial irradiation by a pharmacologically suitable drug, if an almost equal efficacy can be predicted [1, 6]. In spite of cranial irradiation, the risk of CNS disease increases with the increase of tumor burden at diagnosis. Thus in trial ALL-BFM 81 [12] only patients with a lower tumor load (risk factor  $\leq 1.2$ ) were qualified for this randomization (Fig. 3). In this randomized trial, intermediate-dose methotrexate was not an adequate substitute for cranial irradiation. This result is not highly significant (Fig. 8,  $P=0.08$ ), if all patients of this subset are considered and all events evaluated. CNS events, however, were significantly more frequent in branch SR-B (standard-risk B, no cranial irradiation) if compared with branch SR-A (standard-risk A, cranial irradiation), as demonstrated in Fig. 9 (3 vs. 19 events,  $P=0.0003$ ). Furthermore, only patients with a risk factor of  $0.8 - \leq 1.2$  contributed to this negative result, whereas patients with a risk factor of  $\leq 0.8$  were, in respect to CNS events, equally protected by cranial irradiation and methotrex-

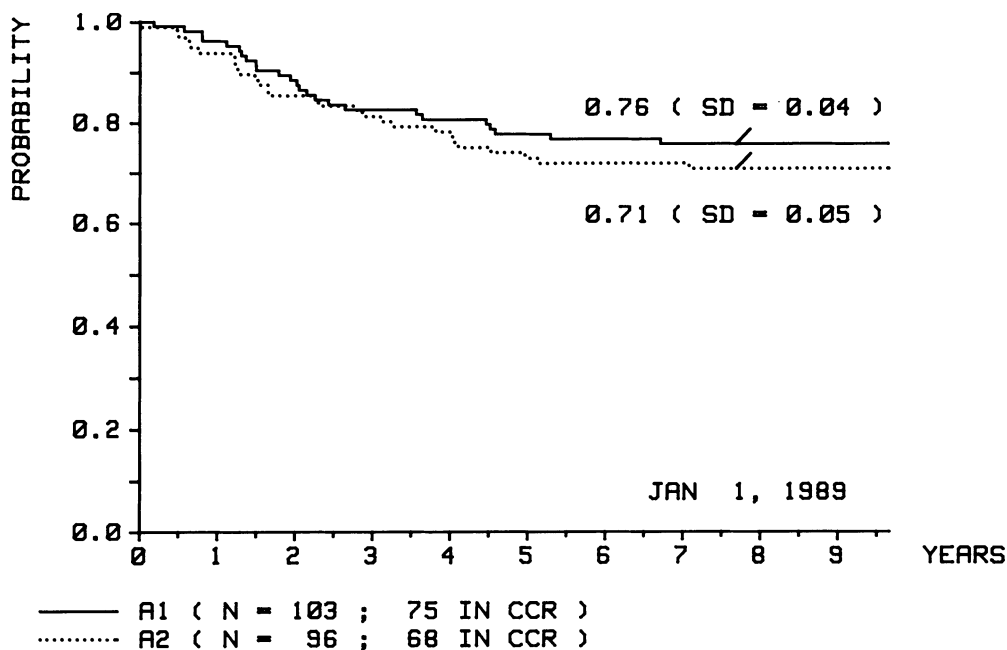


Fig. 7. Event-free interval as related to prednisone/vincristine pulses (A1) or no pulses (A2),  $P=0.44$  (randomization)

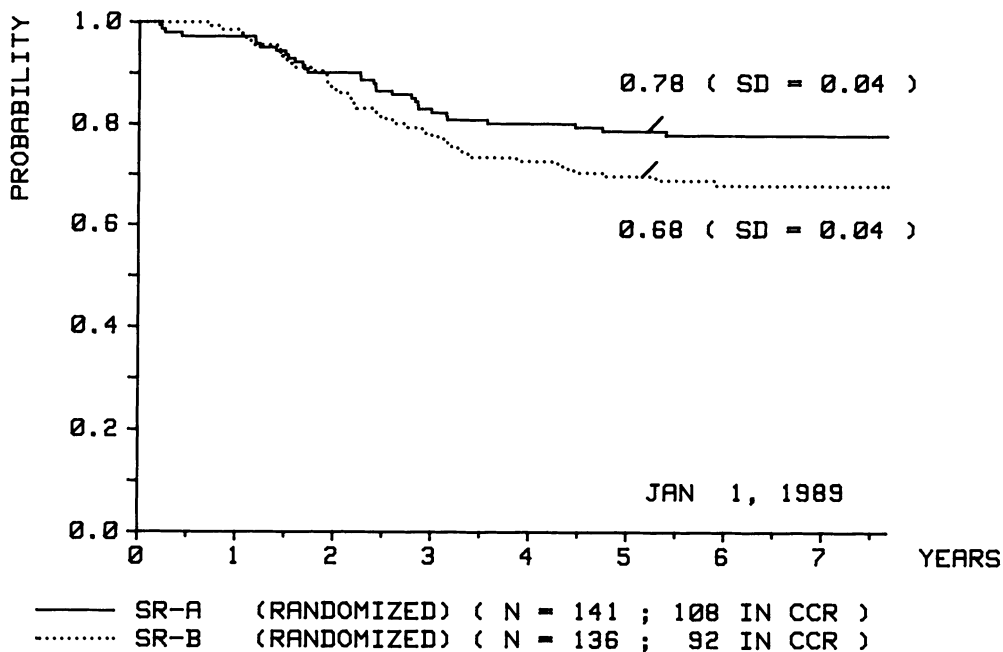


Fig. 8. Event-free interval as related to cranial irradiation (SR-A) or intermediate-dose methotrexate (SR-B) for all events,  $P=0.08$  (randomization)

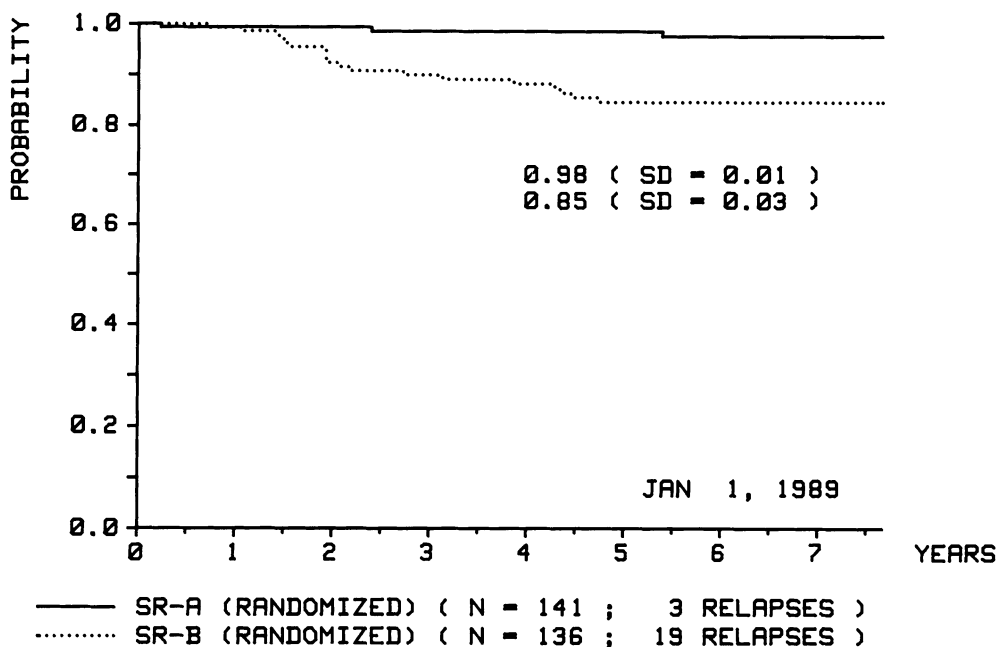


Fig. 9. Event-free interval as related to cranial irradiation (SR-A) or intermediate-dose methotrexate (SR-B) only for all CNS events;  $P=0.0003$  (randomization)

ate (1 vs. 3 events,  $P=0.30$ ). In the subsequent ALL-BFM 83 trial for the endangered subset of patients, this information led us not to omit cranial irradiation, but to avoid it for patients with a risk factor of  $<0.8$ . Alternatively, the argument is well accepted that intermediate-dose methotrexate is inappropriate as a substitute for cranial irradiation, but that higher doses would have been protective.

Patients with a low tumor load at diagnosis (in the setting of the BFM trials approximately 25%–30%) have been considered to be critically overtreated and should not be exposed to intensive reinduction therapy [11]. In these patients the risk/benefit ratio is very delicate, since overtreatment in a group of patients with a 75% chance of cure must be balanced against treatment-induced toxicity, morbidity, and even mortality. Thus the question in trial ALL-BFM 83 for patients with a risk factor of  $<0.8$  was focussed on the benefit of protocol III (Fig. 4). It was anticipated that this element of therapy was unnecessary. Unfortunately, the group of patients who did not receive protocol III as an intensive reinduction were critically disadvantaged compared with the

complementary group (Fig. 10,  $P=0.007$ ). This negative result was only recognized after the trial had been closed in September 1986, since both therapy branches obviously did not differ during the first 2 years of follow-up. The hypothesis that more therapy in this patient group will not necessarily generate a better result was strikingly disproved. At the same time, most of the events in this subset occurred after discontinuation of maintenance therapy, which may still offer a major chance of second-line rescue to these patients. If one evaluates the BFM experience of all trials in which protocol III has been used (risk index  $\leq 2$ , risk factor  $0 \leq 1.2$ , patients of the Berlin pilot study included), the superiority of intensive reinduction compared with no reinduction is evident (Fig. 11,  $P=0.01$ ), irrespective of other therapeutic modifications in these trials over the years (Henze et al., this volume).

In question five of trial ALL-BFM 83 [11] the dose of cranial irradiation was randomized as outlined in Fig. 4. Additionally, in this trial intermediate-dose methotrexate (four times  $0.5 \text{ g/m}^2$  body surface between protocols I and III) had been introduced uncontrolled by randomization as a conse-

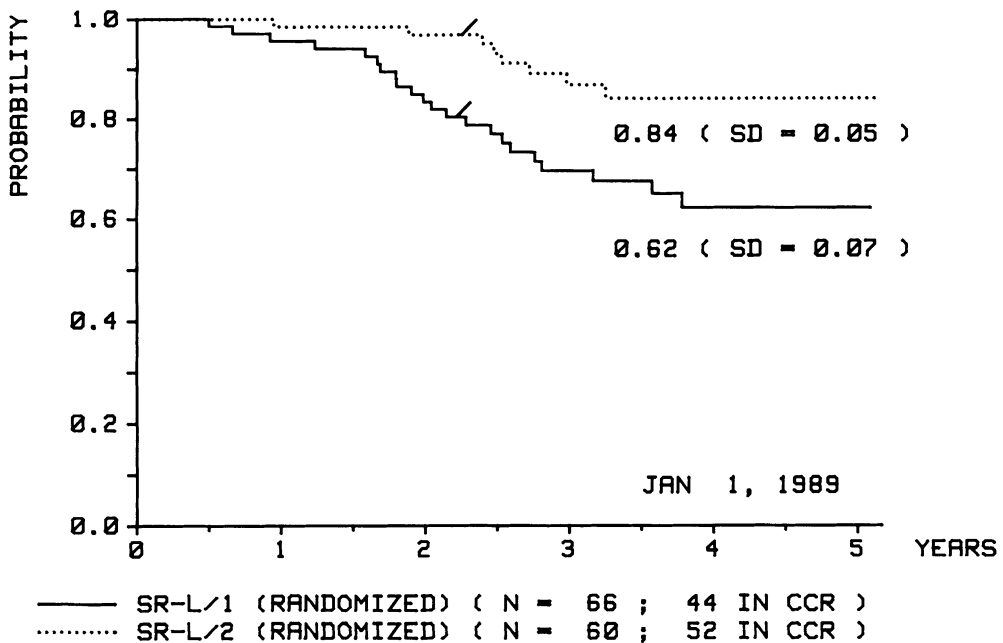


Fig. 10. Event-free interval as related to no protocol III (*SR-L/1*) or exposure to protocol III (*SR-L/2*),  $P=0.007$  (randomization)

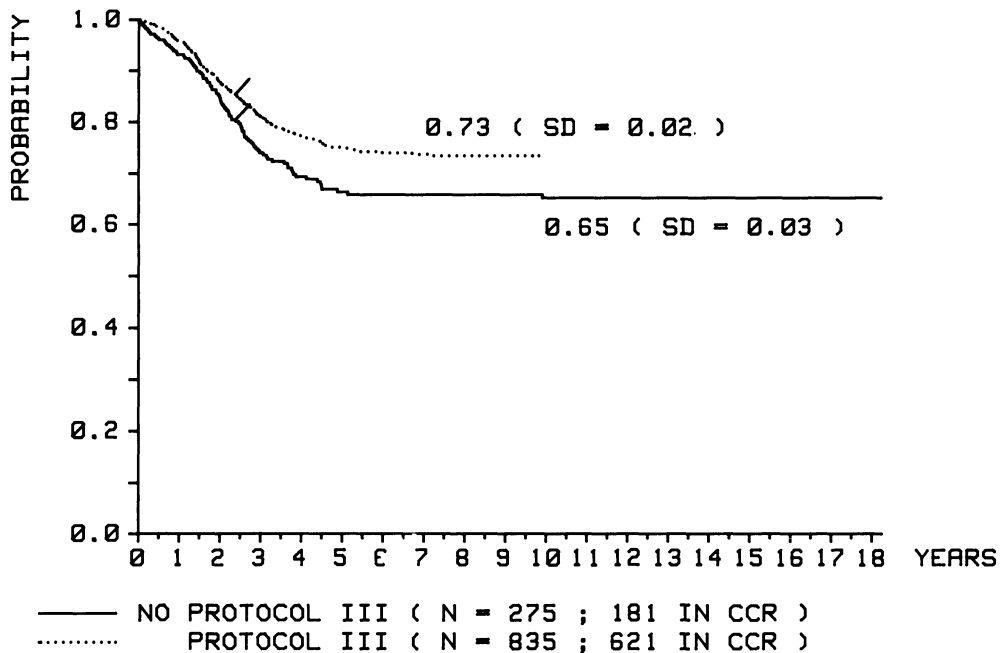


Fig. 11. Event-free interval as related to no protocol III or exposure to protocol III in four BFM trials and the Berlin pilot trial,  $P=0.01$

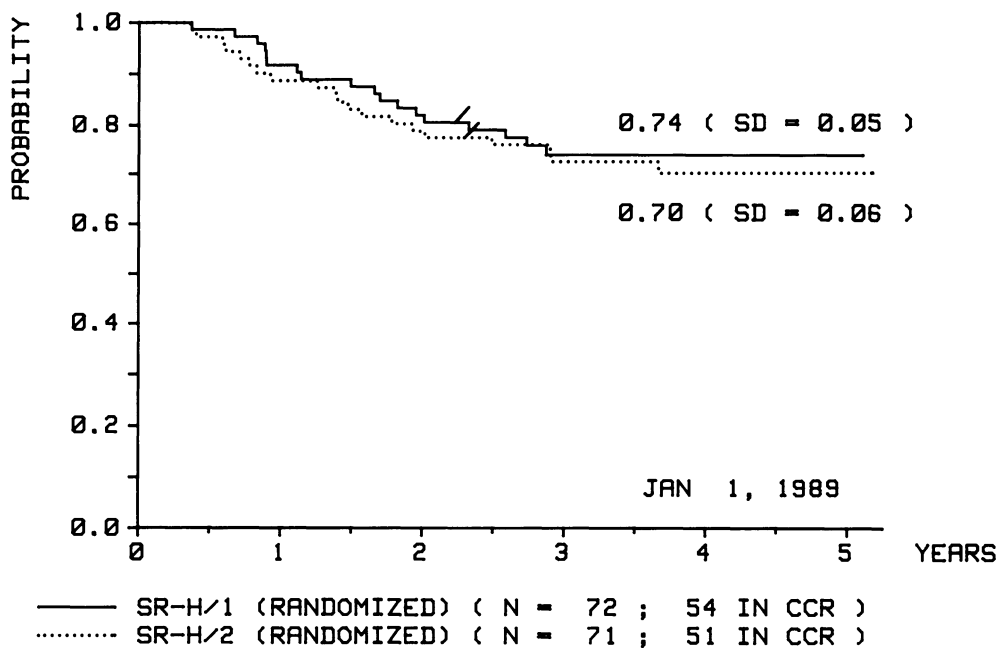


Fig. 12. Event-free interval as related to 12 Gy cranial irradiation (SR-H/1) or 18 Gy cranial irradiation (SR-H/2),  $P=0.7$  (randomization)

quence of new information at that time [1, 6]. Thus, the trial result must be interpreted cautiously, if one has this introduction in mind, which by itself may have a higher potential for extracompartmental disease prevention. In the setting of this trial, low-dose cranial irradiation with 12 Gy was as effective as the standard dose (18 Gy, Fig. 12,  $P=0.7$ ). In future the “minimal effective dose” of 12 Gy may offer the opportunity to protect even patients with a higher risk of CNS disease successfully. This dose should certainly be less effective in inducing brain damage or second CNS tumors.

Despite the three randomizations in trials ALL-BFM 81 and ALL-BFM 83 concerning front-line therapy, another question for patients recruited in both trials has been posed, provided patients had been in continuous remission after an 18-month total duration of therapy. The antileukemic efficacy of maintenance therapy with respect to the rate of long-term survivors is virtually unknown, since after the impact of intensive front-line therapy no randomized trials for duration of treatment have been carried out. Therapy duration in many trials fluctuated

between 2 and 5 years [1–3, 7]; never before has therapy been limited to 18 months (Figs. 3, 4).

Figure 13 shows the randomization of 746 patients in both trials. After a median duration of these trials of 6 years and all patients being no longer on maintenance therapy for at least 6 months, the difference between both branches is unimpressive, but nevertheless recognizable ( $P=0.04$ ). For all further subsets of patients, which may be characterized (both trials analyzed separately, different strategy groups, sex, immunophenotypes), this difference disappears more or less with  $P$  values between 0.05 and 0.5. The incidence of events in the 24-month branch is only three-fourths of that of the 18-month branch; thus continuation of maintenance therapy for another 6 months in the 18-month branch would have avoided approximately 25 relapses. Again, these patients may have a major opportunity to be treated successfully with any effective second-line therapy. This result logically raises the question of whether a therapy duration of more than 24 months may be even more effective.

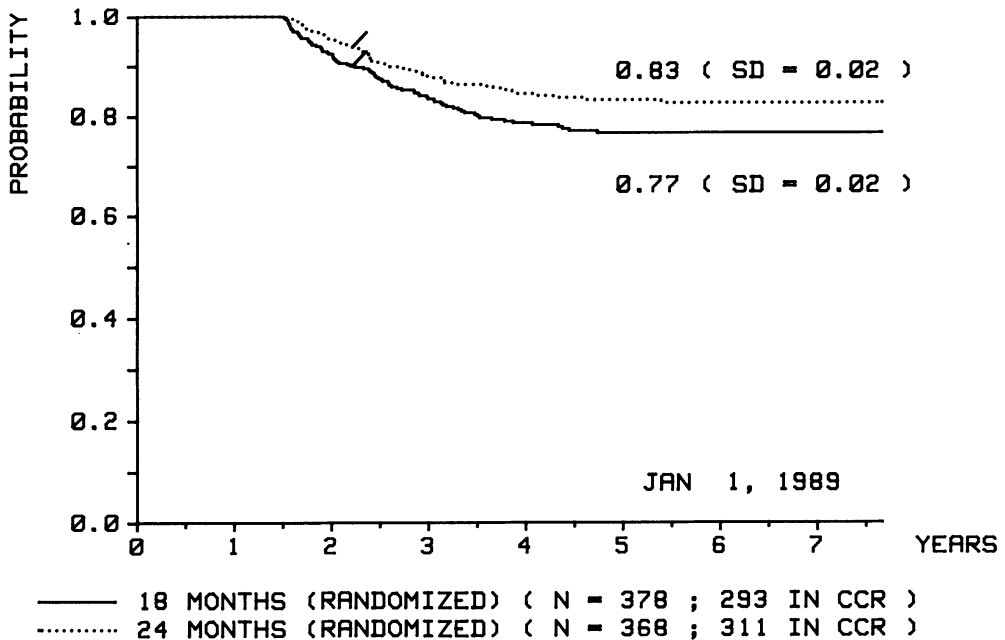


Fig. 13. Event-free interval as related to total duration of therapy of 18 months or 24 months,  $P=0.04$  (randomization)

## Conclusions

The answers to the six questions raised in the four ALL-BFM trials are unequivocally in favor of the simple statement that in general more intensive treatment or longer therapy exposure was superior with respect to EFI [9, 10]. The hope that less therapy, if more appropriately applied, is equally as or even more effective is in vain, at least in our experience. None of the therapy protocols presently in use can claim to be optimal with respect to efficacy and safety, certainly including those being used in the BFM trials. If one analyzed all randomized therapy branches with favorable results separately, one might conclude ALL to be a curative disorder at a level of almost 80%, the actual rate in the BFM trials being only at a level of 70%, however. In spite of the difficulties of randomized trials, their results give – if critically evaluated and interpreted – invaluable information superior to that obtained by other methods.

As anticipated in three of six trial questions, the difference in therapy intensity or quality was not expressed as a difference in EFI. Thus, therapy branches with less hazards are more favorable (late protocol II, no reinduction pulses, low-dose cranial irradiation). The unexpected differences in three trial results are of ethical concern, yet comprise important information. None of the trial answers were available at the time the trial was closed for further entries. Cranial irradiation proved to be more effective than intermediate-dose methotrexate. Nevertheless, this potent drug – if administered at a different dosage and/or at different times – is to be preferred, considering the late effect of ionizing radiation on the child's brain. Most significant, however, was the information that in patients with a minimal tumor burden not using intensive reinduction therapy provoked an inferior outcome. This was not to be anticipated at the time, but was compatible with the hypotheses that

- a) tumor load is not the only predictor for prognosis and
- b) minimal residual disease may be overcome by higher therapeutic intensity.

The latter argument fits also to trial question six: longer therapy exposure may pro-

duce a negative blast cell turnover, which is more frequently followed by the nonreappearance of the neoplastic clone – no matter which mechanism may additionally have been active. To repeat the phrasing of the introduction: leukemia in childhood is a life-threatening condition and needs adequate management.

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## Cytogenetics of Childhood Acute Lymphoblastic Leukemia in Multicenter Trials\*

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### Introduction

Cytogenetic analysis and molecular genetics of leukemic blast cells have increased our knowledge of the pathogenesis of leukemias and solid tumors by revealing a correlation between chromosomal abnormalities and malignancy [1]. Several consistent aberrations have been described, which can be used as an additional tool in diagnosis of hematological disorders [2–5]. The appearance of these chromosomal changes, such as hyperdiploidy with over 50 chromosomes or translocations, furthermore even seems to have an independent prognostic value [6–12]. We, therefore, wish to present the results of a 5-year cytogenetic study, performed on bone marrow and blood samples of children with acute lymphoblastic leukemia (ALL), all treated in one of the Federal German Republic multicenter therapy trials, BFM or CoALL.

### Materials and Methods

Bone marrow samples, mostly received by mail (80%–90%), were washed twice in RPMI 1640 medium and then either prepared directly or incubated in RPMI 1640 + 20% FCS for a 24-h culture including a methotrexate (MTX) synchronization for 17 h. The cell suspension was then brought to hypotonic solution (KCl, 15 min) and fixed in methanol-acetic acid (3:1). After being washed six to eight times, the cells were dropped on a cold wet slide to spread the metaphases. G-banding was performed after a trypsin pretreatment (10–15 s) 3–5 days later.

### Results

From January 1984 to December 1988, 1180 bone marrow and blood samples of children with ALL (869 at diagnosis and 311 at relapse) were sent in by 72 hospitals in the Federal Republic of Germany. Chromosomal analysis was successful in 672 cases (57%), and of these a normal karyotype was found in the leukemic cells of 279 children (41.5%). In the majority of the patients, however, the leukemic karyotype was structurally and/or numerically altered. Whereas hypodiploidy was a very rare phenomenon ( $n=23$ ; 3.4%), a hyperdiploid karyotype was found in 212 children. In 72 cases (10.7%) the chromosome number of the leukemic cells ranged between 47 and 50, whereas in the bone marrow of 140 patients (20.8%) over 50 chromosomes/cell could be

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counted. A pseudodiploid karyotype, exhibiting only structural aberrations, appeared in 158 cases (23.5%) (Fig. 1). By comparing cytogenetic data with the immunophenotype, common ALL could be distinguished from B-, T-, and null-ALL (Fig. 2): A pseudodiploid karyotype ap-

peared in only 27% of the aberrant cases in c-ALL, whereas in the other three groups this abnormality ranged from 70% in B-ALL and 78% in T-ALL to up to 94.7% in null-ALL, with an average of 82.0%. On the other hand, a hyperdiploid karyotype with over 50 chromosomes was found only in one child with T-ALL, whereas nearly half of the c-ALL patients (48%) had this numerical aberration in their leukemic karyotype.

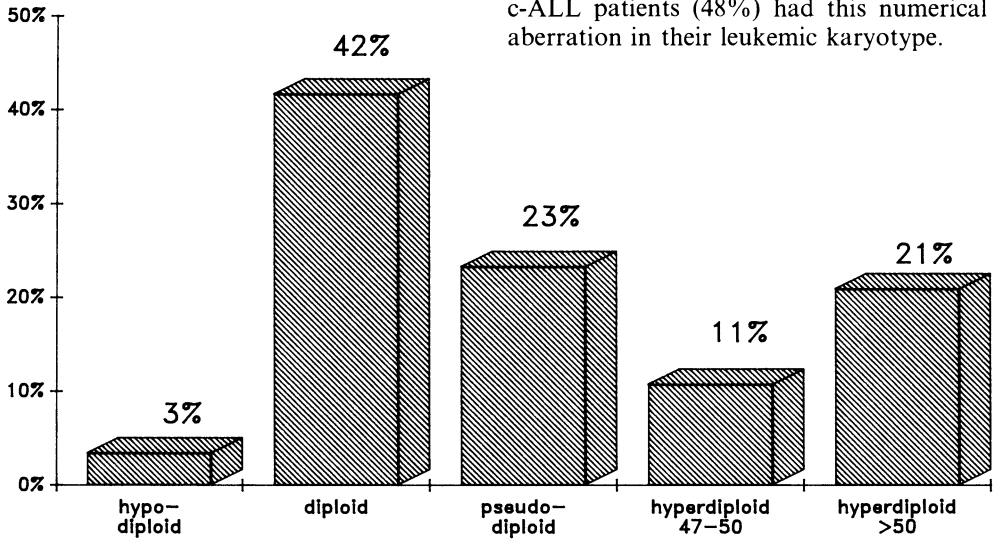


Fig. 1. Frequency of ploidy groups in children with ALL ( $n=672$ )

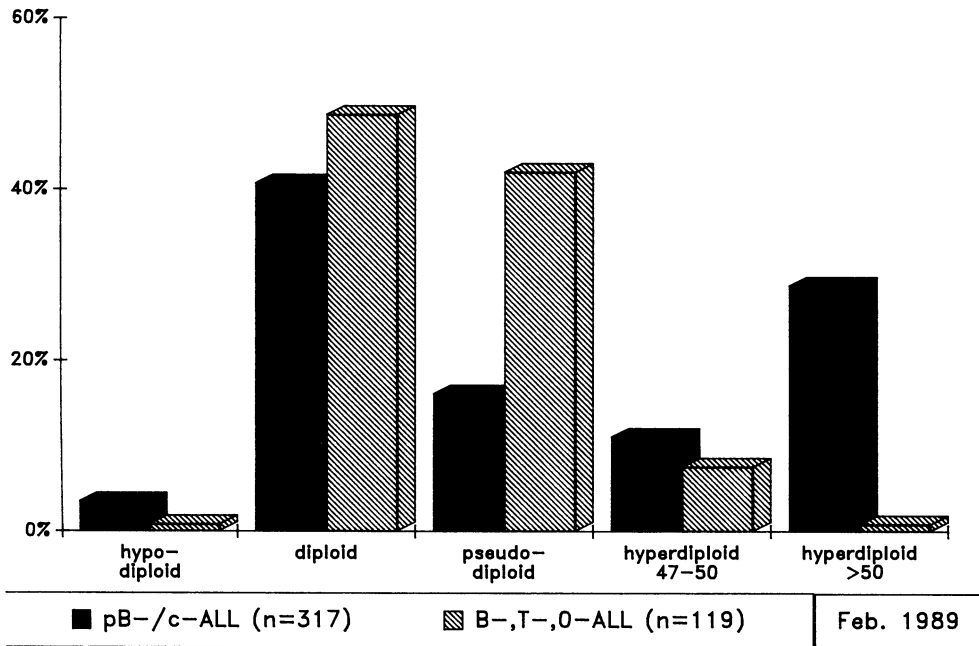


Fig. 2. Frequency of ploidy groups in bone marrow samples of children with different immunophenotypes of ALL at diagnosis ( $n=429$ )

**Table 1.** Frequency and distribution of consistent chromosomal aberrations in the different immunophenotypic subgroups of ALL (therapy studies ALL-BFM 83, ALL-BFM 86, and CoALL 85)

	c/pB	B/NHL	pT/T	0	Mixed
t(1;19)(q13;p13)	11	–	–	–	–
t(4;11)(q21;q23) <sup>a</sup>	–	–	–	10	5
t(8;14)(q24;q32)	–	17	–	–	–
t(9;22)(q22;q11)	10	–	–	–	–
t(11;19)(q23;p13)	–	–	–	–	1
14q11 aberrations	–	–	13	–	–
del(6q)	13	1	6	1	–
del(9p)	6	–	3	1	–
del(12p)	3	–	–	–	2

<sup>a</sup> +1 patient with ANLL M5

The majority of the consistent chromosomal aberrations were closely correlated to specific subgroups of ALL (Table 1). The translocations t(1;19) and t(9;22) appeared only in the pre-B/common ALL (c-ALL), whereas t(8;14) was typical of B-cell ALL and lymphomas. The two variant forms of this aberration, t(8;22) and t(2;8), known to be very rare in children, were never encountered. T(4;11), characteristic of the most immature B-cell precursor ALL (CD10<sup>-</sup>, ppB, or null-ALL), was also found in mixed leukemia as well as in one patient with an ANLL-M5. Aberrations with breakpoint 14q11, the gene locus of *TCR*  $\alpha$  and  $\delta$ , were only seen in patients with T-cell ALL. The precise chromosome aberrations of this group were: t(11;14)(p13;q11), t(1;14)(p32;q11), t(10;14)(q24;q11), inv(14)(q11q32), and del(14)(q11) (Fig. 3a, b).

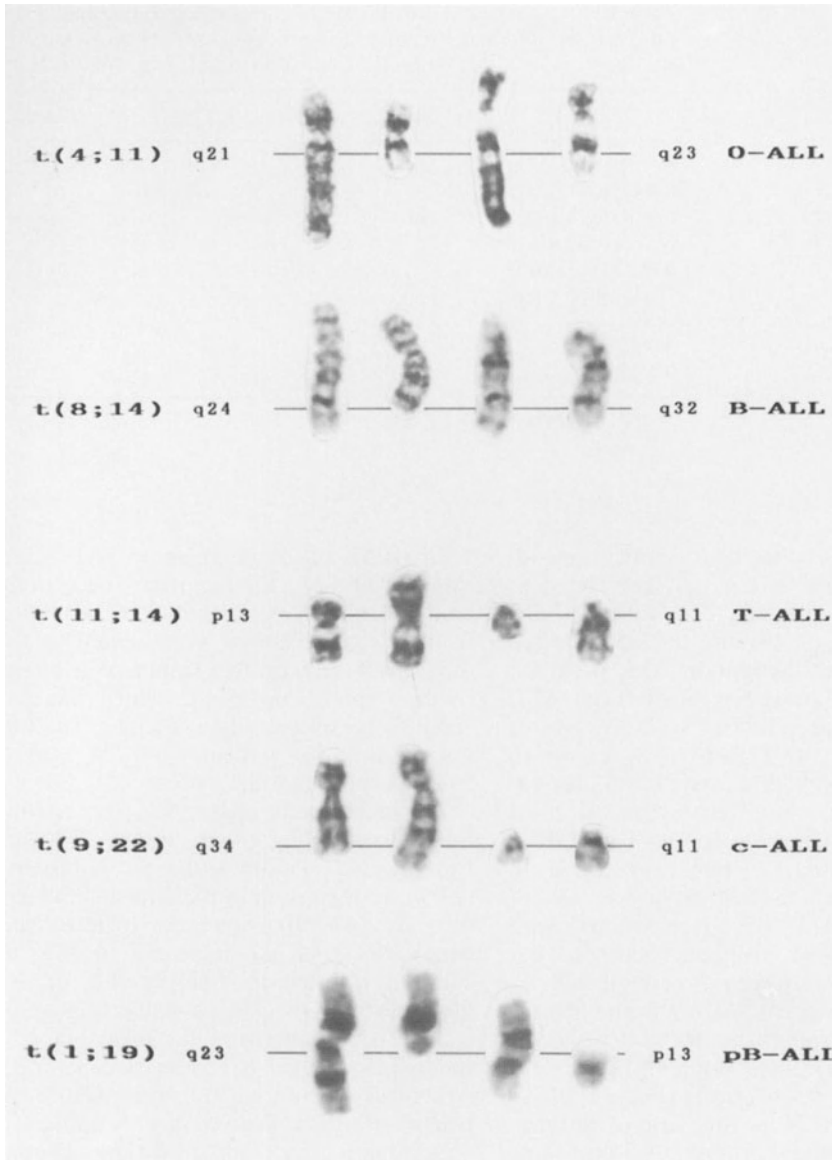
The deletions of the long arm of chromosome #9 and #12, respectively, were not as specific, but were found in all immunological subgroups except B-cell ALL. Besides these structural aberrations, a numerical karyotype abnormality was found to be highly specific for one subgroup, i.e., hyperdiploidy with chromosome numbers over 50 only appearing in common ALL (except one case of T-ALL) (Fig. 2).

To find out the prognostic value of cytogenetic analysis in ALL, we compared only children who were uniformly treated by the same therapy protocol, the multicenter trial

ALL-BFM 83. Bone marrow and blood samples of 154 children were successfully analyzed. Twelve patients were excluded from this group: seven were treated by the different B-ALL protocol and five belonged to the hypodiploid group, which was too small to be statistically evaluated. The follow-up time for patients in CCR ranged from 24 to 62 months.

The incidence of relapse was very similar in children having either diploid (28%) or hyperdiploid (>50) leukemic karyotype (29%). In the group of patients with hyperdiploidy (47–50), however, relapse incidence was 37% and increased to 44% in children with pseudodiploidy (Fig. 4). By life-table analysis (Kaplan-Meier) a significant difference between the probability of event-free survival (EFS) in patients with structural aberrations and those who have either a normal karyotype or only numerical abnormalities ( $P < 0.001$ ) can be demonstrated (Fig. 5a).

By evaluating (Kaplan-Meier analysis) the other ploidy groups, no significant difference between children with normal karyotype and those with hyperdiploidy >50 can be seen. Both have a relatively good prognosis, with an EFS of over 70%. On the other hand, patients with a pseudodiploid karyotype or hyperdiploidy with 47–50 chromosomes have a poorer EFS, and the difference between diploid/hyperdiploid >50 and pseudodiploid karyotype is significant ( $P < 0.005$ ) (Fig. 5b).



**a**

**Fig. 3a, b. a** Consistent chromosomal aberrations in ALL; **b** variant forms of 14q11 aberrations

### Discussion

This study confirms that there are specific chromosomal translocations for defined subgroups of ALL [1]. Deletions of the long arm of chromosome #6 and of the short arm of #9 and #12, however, were encountered in more than one subgroup of ALL, and often considered to be secondary

changes. On the other hand, chromatid breaks of specific translocations often occur at the location of specific genes and the rearrangement might therefore have led to a distinct malignancy. In T-cell ALL, e.g., variant forms of 14q aberrations were found, always involving the 14q11 band, where the genes of T-cell receptors  $\alpha$  and  $\delta$  are located [13].

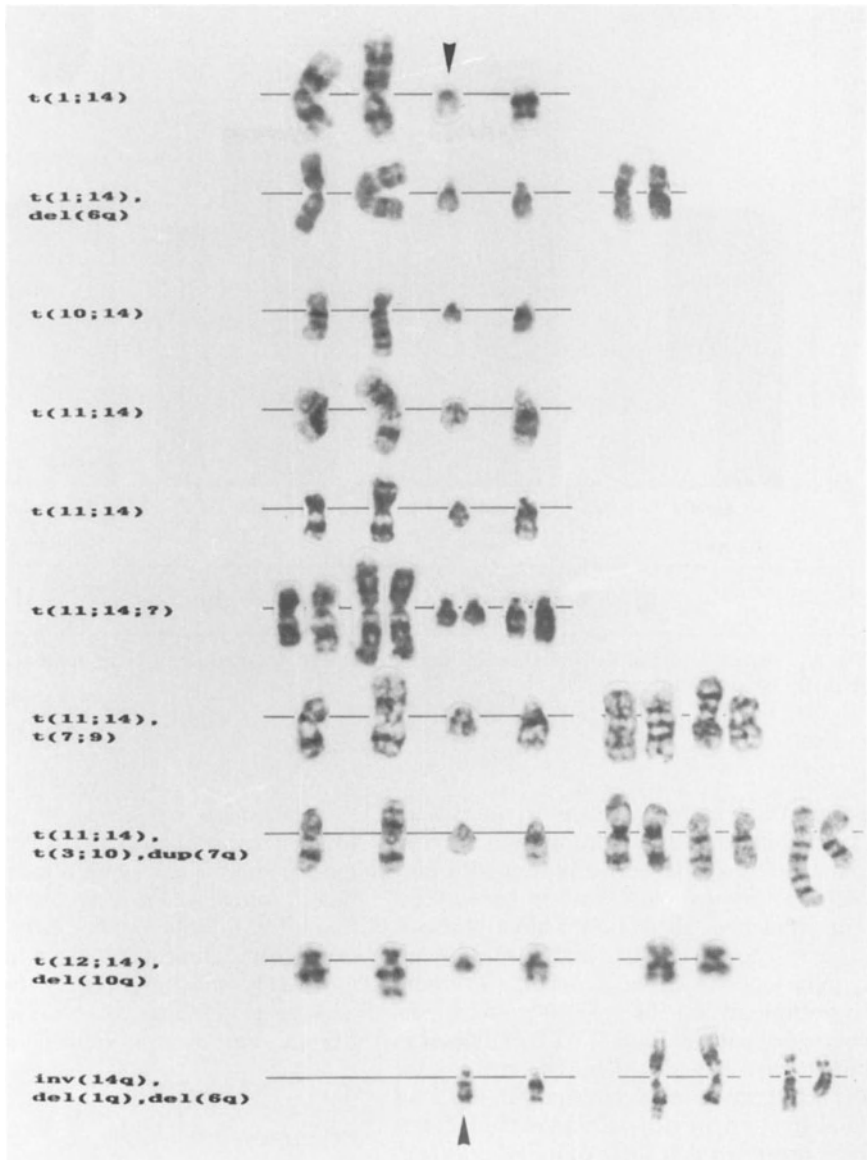
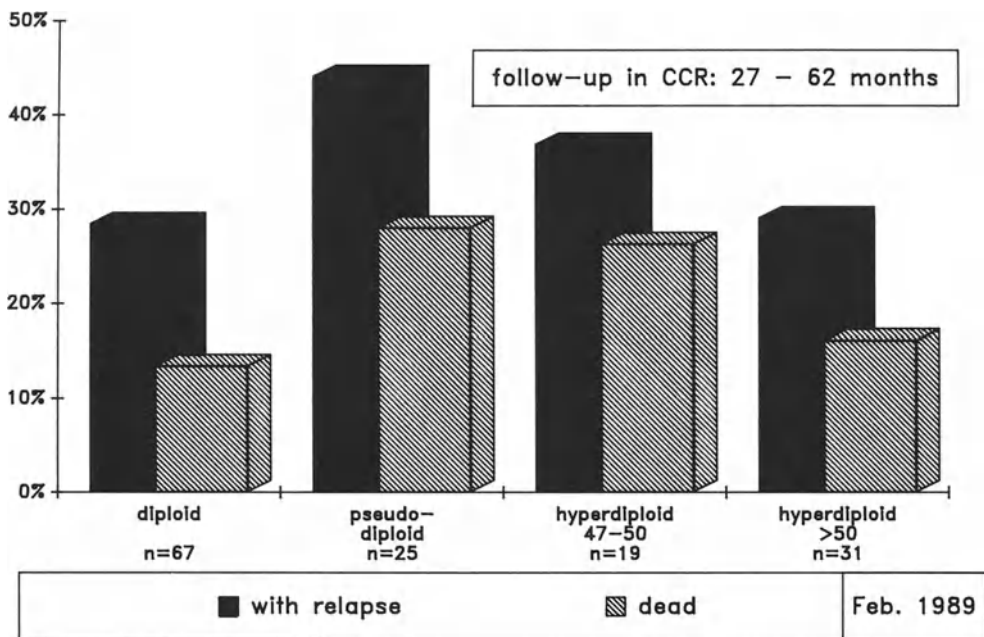


Fig. 3b

In nearly all cases these consistent structural abnormalities appeared in cells with a pseudodiploid karyotype. This group probably indicates a poor prognosis [14]. Kaplan-Meier life-table analysis demonstrates significant differences between this group and those of patients with normal or hyperdiploid (> 50) karyotype. Our results thus confirm recently published results of other groups, who also found this poorer

EFS in patients with pseudodiploidy in their leukemic cells [14–16].

The best rate for EFS (over 75%) was found in patients with over 50 chromosomes. This group differs significantly from those with pseudodiploidy and hyperdiploidy with less than 50 chromosomes, but is nearly at the same level as in children with a normal karyotype. These results correspond with those of another European



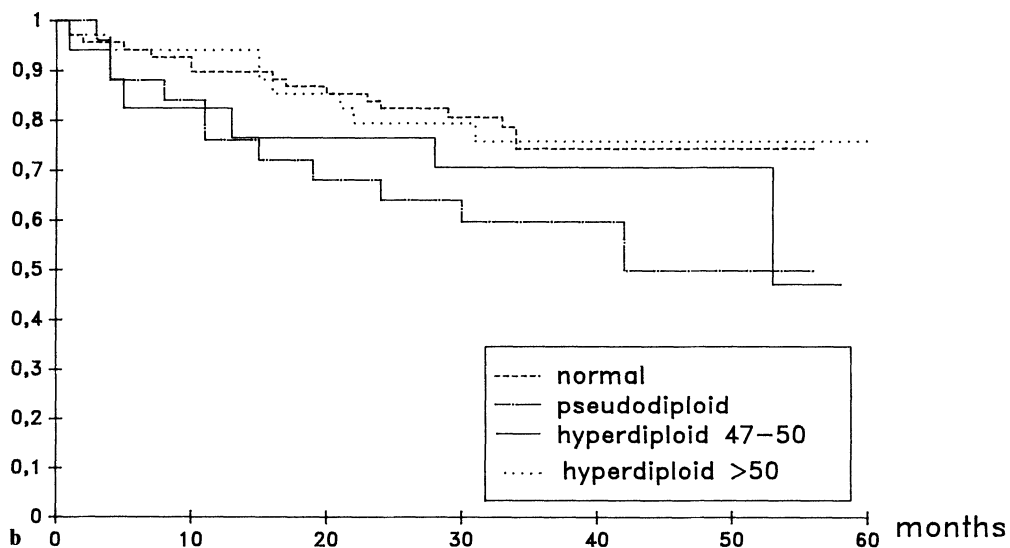
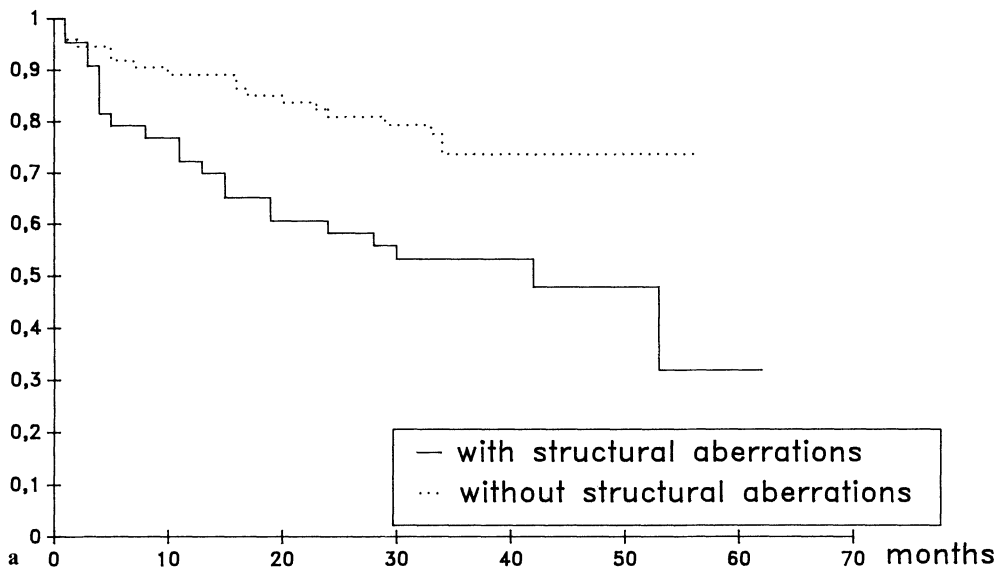
**Fig. 4.** Frequency of relapses in patients with non-B-cell ALL and different karyotypes (therapy study ALL-BFM 83;  $n=142$ )

group [16], but are in contrast to those of an Australian group [15], who showed a significant difference between children with normal and hyperdiploid ( $>50$ ) karyotypes. This numerical aberration is closely associated with common ALL, and was found only once in T-ALL. Pui et al. [14] also found hyperdiploidy ( $>50$ ) only in a small percentage of patients with T-ALL and never in patients with B-cell ALL. The reasons for the better outcome of children with this kind of aberration are not yet understood. It has been proposed that these cells are more sensitive to drugs because of a longer S-phase [17], or might be more sensitive to corticosteroids [18, 19]. Kaplan-Meier analysis established the poorest prognosis (only 30% EFS) for children having any structural aberration in their leukemic karyotype (regardless of clinical factors), and there may be no difference between random or non-random rearrangements [11]. It remains to be seen, however, whether very aggressive chemotherapy can overcome the resistance of these characteristically DNA-altered cells.

*Acknowledgment.* We wish to thank all colleagues from other German Pediatric Centers for supplying us with bone marrow and blood samples. Furthermore we wish to thank Dr. J. Hofmann and Mrs. C. Kirstein for patients' clinical data from the BFM and CoALL groups, respectively. For technical assistance we thank Miss S. Gräf, Miss A. Maurer, and Miss S. Vaupel.

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**Fig. 5 a, b.** Life-table analysis by Kaplan-Meier. **a** Probability of CCR according to karyotype group (43 children with and 73 children without structural aberrations). **b** Probability of CCR according to karyotype group (normal,  $n=68$ ; pseudodiploid,  $n=25$ ; hyperdiploid 47–50,  $n=17$ ; hyperdiploid >50,  $n=34$ )

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## More is Better! Update of Dana-Farber Cancer Institute/Children's Hospital Childhood Acute Lymphoblastic Leukemia Trials \*

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### Introduction

In October 1973, we instituted a series of clinical trials for the treatment of childhood acute lymphoblastic leukemia (ALL). Each protocol was based on the results of its predecessor. Several questions were addressed in a randomized fashion to determine whether long-term event-free survival (EFS) could be improved by:

1. the addition of an anthracycline to vincristine and prednisone during induction (protocol 73-01) [1–4];
2. prolonged, high-dose asparaginase therapy (protocol 77-01) [5];
3. a non-cross-resistant intensification regimen [cyclophosphamide, cytosine arabinoside, vincristine, prednisone (COAP)] (protocol 80-01) [6]; and
4. high-dose methotrexate during remission induction (protocol 81-01) [7].

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In the latter program, we treated prospectively patients on "risk-group-specific" regimens. Patients with T-cell ALL were excluded from protocols 77-01 and 80-01 and treated with a T-cell ALL-specific protocol [8].

We report here the updated outcome data for each of these programs and discuss findings that persist, or have changed, over time.

### Patients and Methods

All consecutively diagnosed patients ages newborn to 18 years old were included in these programs. Since 1981, patients with mature B-cell ALL (surface immunoglobulin positive ALL), about 1% of all new patients, were excluded from protocol entry. Also beginning in 1981, a group of coinvestigators at seven other institutions began entering their patients with childhood ALL on Dana-Farber Cancer Institute-based protocols.

Each of the protocols to be updated has been previously reported [1–8]. Protocol 73-01 accrued patients from October 1973 to May 1977, and randomized the anthracycline induction therapy between October 1973 and December 1974. Protocols 77-01 and 80-01 were conducted between June 1977 and December 1979, and in the calendar year 1980, respectively. We accrued patients onto protocol 81-01 from January 1981 to May 1985, and conducted the induction methotrexate dose randomization between May 1981 and December 1983.

The patient population was divided into two risk groups: standard-risk (SR) and

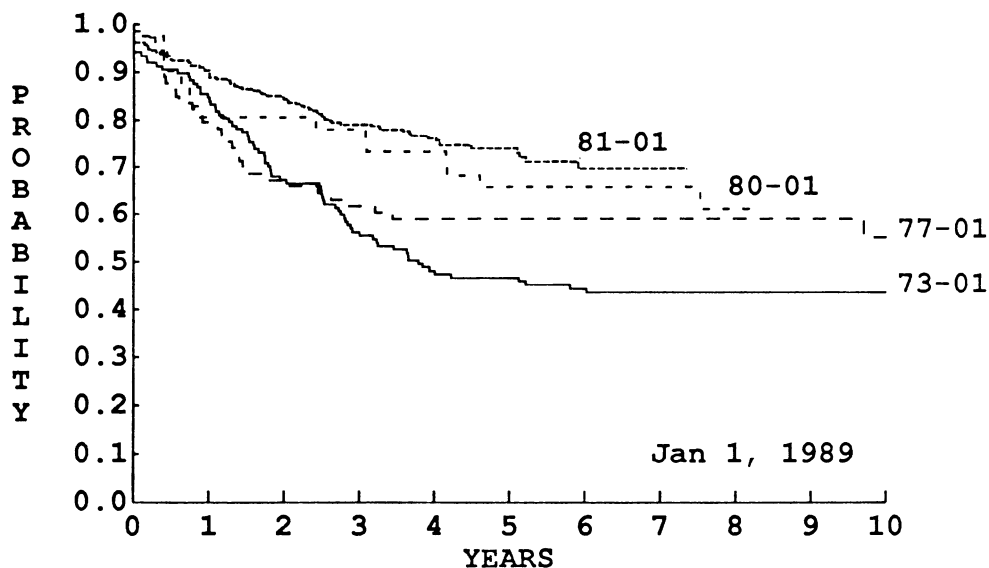
high-risk (HR). SR criteria included age 2–9 years, WBC <20000/mm<sup>3</sup>, and absence of T-cell markers, CNS involvement, or mediastinal mass. HR criteria encompassed all other patients. These risk groups were prospectively assigned beginning in 1981, and, for purposes of comparative data analysis, were retrospectively assigned to previously treated patients. All patients diagnosed between 1973 and 1980 received the same therapy irrespective of risk group designation.

The primary end point for evaluation of treatment effectiveness for the population of newly diagnosed patients with childhood ALL was event-free survival (EFS). EFS was calculated for all eligible patients who entered the treatment protocol and events included induction failures, induction deaths, leukemic relapses, remission deaths, and deaths from second primary tumors, whichever occurred first. A second end point, leukemia-free survival (LFS), was also calculated to estimate the effect of treat-

ment on leukemia-specific events. LFS was calculated for all eligible patients who entered the treatment protocol, but events included only induction failures (failure to control leukemia) and leukemic relapses. Estimated EFS and LFS percentages were calculated by the Kaplan-Meier method [9], and standard errors were estimated using Greenwood's formula [10]. Analysis of randomized groups included patients who received the assigned treatment. Analyses of "more" versus "less" intensive regimens also included additional patients who were directly assigned to receive treatment.

### Results

Figure 1 shows the overall EFS of protocols 73-01, 77-01, 80-01, and 81-01 as of January 1989. There was a progressive improvement in EFS between programs. This improvement was not due to changes in proportion of patients in either the standard- or high-risk group.



	STUDY	CCR	FAIL	TOTAL	MEDIAN
—	73-01	60	77	137	3.8
- - - -	77-01	41	32	73	10.3
. . . . .	80-01	26	15	41	UNDEF
- · - · -	81-01	214	75	289	UNDEF

Fig. 1. Event-free survival for consecutively entered children with ALL. CCR, continuous complete remission; FAIL, any relapse, death in remission, or induction failure; Median, median time to failure

**Table 1.** Long-term follow-up of randomized trials

Protocol			%LFS	P value	%EFS	P value	Median F/U (yrs)
73-01	↔	VP (N=15) VPA (N=30)	47±13 } 69±9 }	0.13	47±13 } 67±9 }	0.20	12.4
77-01	↔	NO ASP (N=36) ASP (N=28)	43±9 } 77±8 }	0.02	31±11 } 71±9 }	0.03	9.4
80-01	↔	NO COAP (N=13) COAP (N=9)	91±9 } 86±13 }	0.77	77±12 } 53±17 }	0.45	7.9
81-01	↔	LD MTX (N=40) HD MTX (N=38)	64±9 } 91±5 }	0.01	64±9 } 82±6 }	0.16	5.2

V, vincristine; P, prednisone; A, anthracycline; ASP, asparaginase; EFS, event-free survival; LFS, leukemia-free survival; COAP, cyclophosphamide + V + P + cytosine arabinoside; LD MTX, conventional-dose methotrexate; HD MTX, high-dose methotrexate; F/U, follow-up

We determined whether the results of our randomized clinical trials remained stable over prolonged periods of follow-up (Table 1). We have previously reported that patients who were randomized to receive either vincristine and prednisone, or those two drugs plus an anthracycline (either doxorubicin or daunorubicin), for remission induction therapy did not have statistically significant differences in EFS [3]. However, when the EFS of all of those who received the two-drug combination was compared with that of all of those who received the three-drug combination, the difference was statistically significant; at a median follow-up of 12.4 years, EFS for the two- and three-drug combination groups was  $37\% \pm 5\%$  and  $67\% \pm 9\%$ , respectively ( $P=0.007$ ) (Table 2).

Of the randomized patients in protocol 77-01, 36 did not receive asparaginase intensification and 28 did. We found that our initial observation, showing the advantage of intensive asparaginase [5], persisted over a long period; with a median of 9.4 years, EFS for those who did not and those who did receive asparaginase intensification were  $31\% \pm 11\%$  and  $71\% \pm 9\%$ , respectively ( $P=0.03$ ) (Table 1). The difference remained statistically significant when all patients were evaluated (those randomized in addition to those assigned to the asparaginase intensification later in the trial) (Table 2).

Interpretation of the protocol 80-01 randomization was complicated by the small number of patients. Only 13 patients were randomized not to receive the COAP intensification and 9 patients to receive it. The difference in EFS between the two arms was attributed to the high incidence of morbidity, especially infectious morbidity and mortality in the COAP-treated group. Our evaluation of LFS, a measure used to control for fatal complications of treatment, showed no difference in leukemia control between the two regimens (Tables 1, 2).

Protocol 81-01 results have been reported [7] and updated [11]. Of the 78 patients randomized, 40 received conventional-dose methotrexate ( $40 \text{ mg/m}^2$ ) and 38 high-dose methotrexate (either  $4 \text{ g/m}^2$  or  $33 \text{ g/m}^2$ ) as part of remission induction. Statistically significant differences were found between the groups for LFS, but not for EFS (Tables 1, 2). None of the remission deaths that occurred in the high-dose methotrexate group could be attributed to the methotrexate itself.

To evaluate whether more intensive chemotherapy resulted in superior EFS and LFS, we evaluated all patients (randomized and nonrandomized) treated on protocols 73-01, 77-01, 80-01, and 81-01. The children were divided into two groups: those who received the less intensive and the more intensive chemotherapy regimen in each pro-

**Table 2.** Outcome by treatment with less and more intensive chemotherapy

Protocol		%LFS	P value	%EFS	P value
73-01	↙ Less (N=107)	38 ± 5	0.005	37 ± 5	0.007
	↘ More (N= 30)	69 ± 9		67 ± 9	
77-01	↙ Less (N= 36)	43 ± 9	0.006	31 ± 11	0.02
	↘ More (N= 36)	79 ± 7		72 ± 7	
80-01	↙ Less (N= 30)	77 ± 8	0.95	66 ± 9	0.62
	↘ More (N= 11)	78 ± 14		53 ± 15	
81-01	↙ Less (N=230)	68 ± 5	0.02	64 ± 4	0.13
	↘ More (N= 38)	91 ± 5		82 ± 6	
Overall	↙ Less (N=403)	57 ± 3	0.0002	53 ± 3	0.005
	↘ More (N=115)	80 ± 4		71 ± 4	

“Less” and “more” intensively treated group definitions are in text

tocol (Table 2). Definitions for “more” intensive were as follows: protocol 73-01 – three-drug induction; protocol 77-01 – asparaginase intensification; protocol 80-01 – COAP intensification; and protocol 81-01 – high-dose methotrexate induction. Of note is that SR patients in protocol 81-01 did not receive doxorubicin intensification [7]; if they received high-dose methotrexate induction, they were included in the “more” intensive group, and vice versa. For the four protocols, the overall LFS and EFS were statistically significantly better for the more intensively treated patients than for the less intensively treated group; LFS 80% ± 4% vs. 57% ± 3%, and EFS 71% ± 4% vs. 53% ± 3%, respectively ( $P=0.0002$ ,  $P=0.005$ , respectively) (Table 2).

Event-free survival and LFS gradually improved for SR patients with each new program and are summarized in Tables 3 and 4. Of note was the finding that no CNS relapses occurred in SR patients who were treated with 2400 cGy cranial irradiation and intrathecal methotrexate (i.t. MTX). CNS relapses were first found after the cranial irradiation dose was lowered to 1800 cGy. We also observed late relapses (occurring at greater than 5 years from diagnosis) in SR patients. There were 56 SR males and 43 SR females evaluable for relapse at greater than 5 years. In protocols 73-01, 77-01, and 80-01, 3/31 relapses (10%) were late, compared with protocol 81-01, wherein 4/14 (29%) were late. Five of the late relapses occurred in male patients, four

**Table 3.** Standard-risk treatment results

Protocol	N	Four-year %EFS	Median years F/U	# BM relapses	# isolated CNS relapses	# combined CNS relapses	Cranial RT dose (cGy)
73-01	51	51 ± 7	11.9	24	0	0	2400
77-01	24	79 ± 8	9.3	3	0	0	2400
80-01	14	86 ± 9	7.6	1	0	0	2400
81-01	110	88 ± 3	4.8	9	2	5	1800

EFS, event-free survival; RT, radiotherapy; F/U, follow-up; BM, bone marrow; CNS, central nervous system

**Table 4.** Long-term outcome for randomized standard-risk patients

Protocol		%LFS	P value	%EFS	P value
73-01	↔ VP VPA	(N=10) 40±15 (N=13) 67±14	} 0.26	40±15 62±13	} 0.42
77-01	↔ NO ASP ASP	(N=10) 70±14 (N=12) 100		} 0.07	
80-01	↔ NO COAP COAP	(N= 6) 100 (N= 2) 100	} 0.00		100 0
81-01	↔ LD MTX HD MTX	(N=16) 77±13 (N=15) 100		} 0.11	77±13 93± 6
Overall (all SR)	↔ Less More	(N=144) 64± 5 (N= 44) 90± 5	} 0.008		57± 7 75± 7

For abbreviations, see Table 1

in the bone marrow, and one in the testes; the two females relapsed in the marrow. Thus, it appeared that intensification resulted in improved survival for most SR patients. However, there might be a subgroup of these patients who require a different therapeutic approach.

We assessed the outcome of the four randomized studies in the SR patients (Table 4). In protocol 73-01, with a median follow-up of 12.4 years, there were ten relapses (all in the marrow): six of the ten were in the two-drug group and four of the ten in the three-drug group. There was one remission death in the latter group, and, of the 23 patients, 12 remained in continuous complete remission.

In SR patients on protocol 77-01, with a median follow-up of 9.3 years, there were three relapses, all in the marrow and all in the group who did not receive the asparaginase intensification. There were three remission deaths, one in the no asparaginase group and two in the asparaginase group. Sixteen patients have remained in continuous complete remission. In protocol 80-01, with a median follow-up of 7.9 years, there were no relapses in SR patients, two remission deaths (both in the COAP group), and the other six patients have remained in continuous complete remission.

Of the 31 SR patients on protocol 81-01 who were randomized to receive conven-

tional-dose or high-dose methotrexate, 16 received conventional-dose and 15 high-dose methotrexate. Based on this low number of patients, differences between the groups for EFS or LFS were not statistically significant (Table 4).

High-risk (HR) EFS also improved with each new program and is summarized in Tables 5 and 6. There were no statistically significant differences in CNS LFS in HR patients who received 2800 cGy or 2400 cGy, each with i. t. MTX. Relapses, especially in the bone marrow, remained the major source of treatment failure. Most relapses occurred between 1 and 3 years from diagnosis (median time of relapse was 1.2 years), and only 2/107 relapses in HR patients occurred after 5 years. Induction failure occurred in 14 HR and only 1 SR patient during these trials.

Outcome for HR patients based upon randomizations is summarized in Table 6. Although the only statistically significant difference found was an improved LFS in patients who received high-dose methotrexate as part of induction in protocol 81-01, when examined as a whole, HR patients treated with more intensive regimens had an improved LFS (75%±5%) and EFS (69%±5%) when compared with those HR patients treated with the less intensive regimens (LFS 54%±4% and EFS 51%±4%,  $P=0.008$  for LFS and  $P=0.02$  for EFS).

**Table 5.** High-risk treatment results

Protocol	<i>N</i>	Four-year %EFS	Median years F/U	# BM relapses	# isolated CNS relapses	# combined CNS relapses	Cranial RT dose (cGy)
73-01	86	45 ± 5	11.2	28	4	3	2400
77-01	49	49 ± 7	9.2	16	3	3	2400
80-01	27	69 ± 9	7.7	3	0	0	2400
81-01	179	69 ± 4	4.5	25	11	2	2800

For abbreviation see Table 3

**Table 6.** Long-term outcome for randomized high-risk patients

Protocol		%LFS	P value	%EFS	P value
73-01	↙ VP	( <i>N</i> = 5)	0.54	60 ± 22	0.54
	↘ VPA	( <i>N</i> = 17)			
77-01	↙ NO ASP	( <i>N</i> = 26)	0.11	31 ± 10	0.08
	↘ ASP	( <i>N</i> = 16)			
80-01	↙ NO COAP	( <i>N</i> = 7)	0.84	57 ± 19	0.54
	↘ COAP	( <i>N</i> = 7)			
81-01	↙ LD MTX	( <i>N</i> = 24)	0.03	58 ± 10	0.22
	↘ HD MTX	( <i>N</i> = 23)			
Overall (all HR)	↙ Less	( <i>N</i> = 259)	0.008	51 ± 4	0.02
	↘ More	( <i>N</i> = 71)			

For abbreviations, see Table 1

This was also demonstrated in SR patients, for whom more intensive therapy resulted in LFS of 90% ± 5% and EFS 75% ± 7%, compared with less intensive treatment, LFS 64% ± 5% and EFS 57% ± 7%. The LFS difference was statistically significantly different, *P* = 0.008.

Updated results of the T-cell protocol as of January 1989 showed that, of 13 evaluable patients, EFS was 15% ± 10%, with a median follow-up of 10.3 years.

## Discussion

We believe that our outcome results for childhood ALL are good and at least comparable to the best contemporarily conduct-

ed trials [4, 12]. Moreover, our findings substantiate long-held views that more intensive chemotherapy results in better long-term disease control. Some observations, such as the incidence of late relapses, require long periods to assess. However, it is noteworthy that all statistically significant observations from previous reports remain significant with longer follow-up.

Although we are concerned that more CNS relapses occurred in SR patients as cranial irradiation doses were reduced, the data of others [13, 14], who have successfully treated SR patients without cranial irradiation, are encouraging.

High-risk outcome data have progressively improved, but bone marrow relapses continue to be the major cause of treatment

failure. These relapses have occurred despite our ongoing efforts to intensify both remission induction and intensification therapy. Alternatives for more intensive chemotherapy are use of reinduction treatment, late intensification, longer duration of therapy, different drugs (e.g., epipodophyllotoxins), higher doses of drugs (e.g., methotrexate or cytosine arabinoside), or combinations of the above. Others have advocated first-remission bone marrow transplantation for certain very high risk patients (e.g., those with t9;22 or t4;11). We are currently considering reinduction therapy and late intensification. Longer duration of therapy might be a rational alternative for some patients, especially SR males who remain at risk for relapse for prolonged periods.

We believe that attempts to diminish morbidity, especially in SR patients, must be carefully considered. Above all, we must not compromise the possibility of cure to diminish non-life-threatening complications.

### Summary

Between 1973 and 1985, 553 children with childhood acute lymphoblastic leukemia were treated on Dana-Farber Cancer Institute/Children's Hospital, Boston, protocols. The programs featured intensive remission induction therapy, CNS treatment with cranial irradiation and intrathecal drugs, doxorubicin intensification with or without asparaginase, and 2–2½ years of conventional continuation therapy. There has been progressive improvement in event-free survival for each successive program. Leukemia control concerns pertain to:

1. late relapses (at > 5 years) in "standard-risk" patients;
2. an increased incidence of CNS relapses, especially in "standard-risk" patients, as preventative treatment is reduced in intensity; and
3. bone marrow relapses in "high-risk" patients.

Comparisons of patients receiving the more intensive arm of each protocol with those receiving the less intensive arm support the hypothesis that more intensive chemotherapy results in improved event-free survival.

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## Treatment of Acute Lymphoblastic Leukemia: Protocol Fralle 83–85

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From the early 1950s to the mid-1970s all acute lymphoblastic leukemias (ALLs) in childhood were treated according to a unique protocol. It became evident that some parameters had prognostic significance: age, sex, white blood cell count, lymphoma syndrome, initial meningeal involvement, mediastinal mass, treatment efficacy, and more recently immune markers and cytogenetics [8]. In 1974 we were the first to tailor the treatment according to initial prognostic factors: treatment was increased by one-third in high-risk patients, and decreased by one-third in low-risk patients [3].

In June 1983 a new regimen (Fralle 83) was designed in a French multicenter study to treat ALL in children according to risk factor. It was based on the following:

1. In leukemias with good prognosis it is possible to decrease the treatment and to avoid skull irradiation [2].
2. The usefulness of early consolidation with intermediate-dose methotrexate has been demonstrated by Moe et al. [4] and with a multiagent regimen by Riehm et al. [5].
3. In relapsing patients previously heavily treated an association of amsacrine and cytosine arabinoside had given 60% complete remission, suggesting a powerful activity of these two drugs [7].
4. We have reported good results of monthly pulses in very high-risk patients [6].

One can identify three groups of patients: low-risk patients, intermediate-risk patients, and high-risk patients.

### Low Risk Patients

#### *Definition*

Low-risk patients were patients aged between 2 and 10 years with a WBC count of less  $15\,000/\text{mm}^3$  and hemoglobin less 10 g%, with no large tumor or mediastinal mass. All the preceding criteria were required. Patients with persistent leukemic cells in blood or more than 50% in bone marrow at day 15 were subsequently excluded and moved to intermediate risk.

#### *Aims*

The protocol aimed at using a nontoxic regimen without anthracycline in induction, with intermediate-dose methotrexate and intrathecal treatment for CNS prophylaxis without irradiation. Maintenance was randomized between 2 and 3 years.

#### *Protocol*

Induction was vincristine  $2\text{ mg}/\text{m}^2$  or vindesine  $4\text{ mg}/\text{m}^2$  days 1, 8, 15, and 22; prednisone  $40\text{ mg}/\text{m}^2$  per day for 28 days; and asparaginase  $1000\text{ U}/\text{kg}$  per day days 29–38. CNS prophylaxis was intermediate-dose methotrexate  $500\text{ mg}/\text{m}^2$  in a 4-h infusion with folinic acid rescue and 12 intrathecal

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injections of methotrexate. Maintenance phase was daily 6-mercaptopurine and weekly methotrexate with monthly pulses (during the 1st year) with vincristine or vindesine and prednisone.

### Results

Among 136 patients, 23 were subsequently moved to the intermediate-risk category. Complete remission rate is 99%. Twenty-two patients experienced a relapse, 13 in bone marrow, 6 in CNS, 1 in the testis, and 2 mixed. Ninety patients are in CR, and disease-free interval at 54 months is  $72\% \pm 5\%$  (Fig. 1).

### Conclusions

The results are similar to those with other nontoxic protocols but there are too many CNS relapses (5%), which can be avoided by higher-dose methotrexate. Intermediate-dose methotrexate gives only a borderline CSF concentration ( $0.1 \mu\text{mol}$ ) and for too short a period [1]. Thirteen percent of pa-

tients experienced a bone marrow relapse which could have been prevented by increasing induction, by consolidation, or by a better selection of patients according to cytogenetic or immune markers. At present there is no statistical difference between patients treated for 2 or 3 years but a longer follow-up is needed.

### Intermediate-Risk Patients

#### Definition

Intermediate-risk patients were children, younger than 2 or older than 10 years, children with a WBC count of less  $100\,000/\text{mm}^3$ , children without mediastinal mass, and failure patients at days 14 or 28 in a low-risk protocol.

#### Aims

The aims were to use an aggressive induction with five drugs including high-dose anthracycline and to combine three methods of CNS prophylaxis.

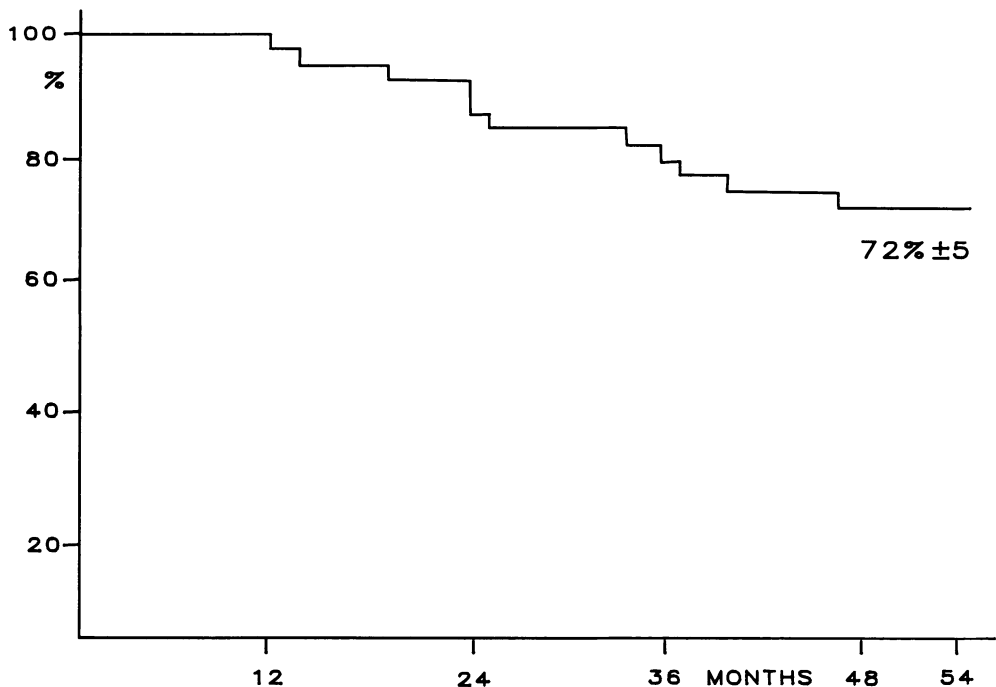


Fig. 1. Disease-free interval in low-risk patients

## Protocol

Induction therapy used one course of prednisone 100 mg/m<sup>2</sup> days 1–5, daunorubicin 80 mg/m<sup>2</sup> days 2–3–4, vincristine 2 mg/m<sup>2</sup> or vindesine 4 mg/m<sup>2</sup> day 1, cyclophosphamide (Cytoxan) 600 mg/m<sup>2</sup> day 2, and asparaginase days 7–16. Urate oxidase, intravenous hydration, and alkalinization were instituted prior to beginning chemotherapy in an attempt to prevent the development of metabolic imbalance secondary to the rapid tumor lysis. Consolidation was started at day 25 with four courses of intermediate-dose methotrexate, followed by folinic acid rescue. Cranial irradiation takes place at the end of consolidation with a dose of 18 Gy. Maintenance was 6-mercaptopurine and methotrexate for 3 years with vincristine or vindesine plus prednisone pulses monthly the 1st year, and every 3 months the 2nd year. All patients received 12 intrathecal injections of methotrexate, 6 during the first 2 months and 6 during the rest of the first year. (Dosage of methotrexate: 0–12 months, 6 mg; 1–2 years, 8 mg; 2–3 years, 10 mg; >3 years, 12 mg.)

## Results

Among 220 patients, 205 achieved a complete remission (CR rate 93%), 43 patients experienced a relapse, 37 in bone marrow, 3 in CNS, and 3 in the testis. One hundred and sixty-two are in CR, and disease-free interval is 62% ± 7% at 54 months (Fig. 2).

## Conclusions

There is a very low rate of CNS relapse (less than 2%) but all patients had received intrathecal methotrexate, intermediate-dose methotrexate, and skull irradiation. At the present time there is no deleterious effect of the combination but a longer evaluation is necessary to assess completely this conclusion. Probably higher dose methotrexate could replace skull irradiation. There is a very low rate of testicular relapses which can be explained by the use of intermediate-dose methotrexate. The results of this group could be increased by a better selection of patients or a more aggressive consolidation.

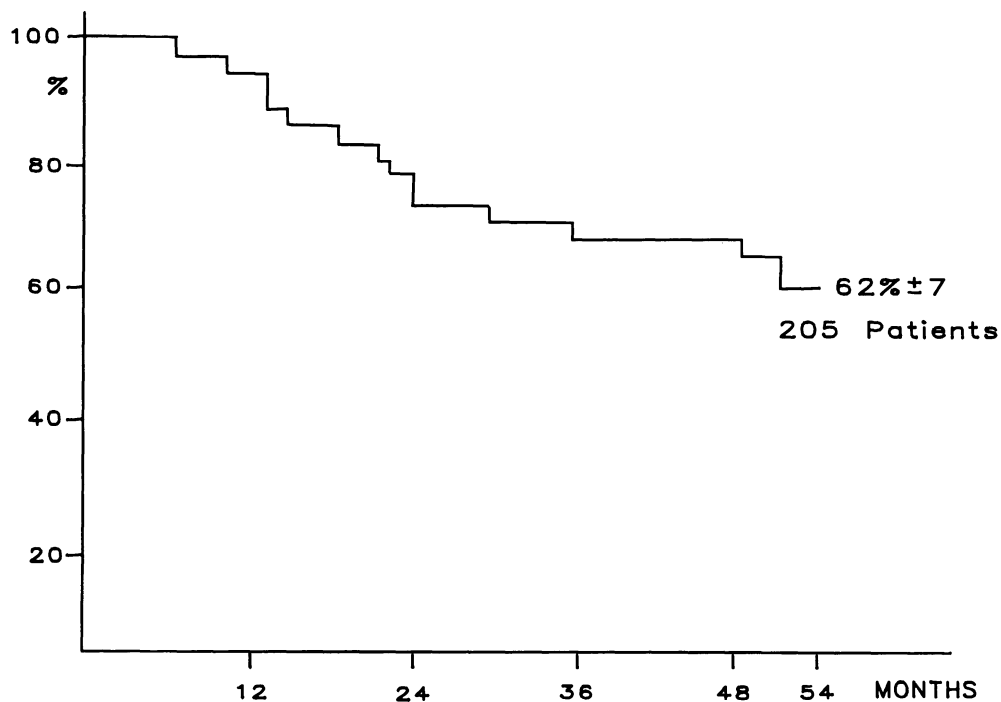


Fig. 2. Disease-free interval in intermediate-risk patients

## High-Risk Patients

### Definition

High-risk patients were patients with a WBC count over  $100\,000/\text{mm}^3$ , with mediastinal mass, or with initial CNS involvement. Patients with a WBC over 50 000 and hemoglobin over 10 g were also included.

### Aims

The aims of the protocol were to use a five-drug induction similar to the former regimen, followed by two courses of amsacrine and cytosine arabinoside for consolidation, to use heavy monthly pulses instead of permanent maintenance, to irradiate the testis in half the boys and to test whether it improved survival.

### Protocol

Induction was the same as in the intermediate-risk regimen. Consolidation was two courses of amsacrine  $75\text{ mg}/\text{m}^2$  day 1 and

cytosine arabinoside  $1\text{ g}/\text{m}^2$  push followed by  $50\text{ mg}/\text{m}^2$  subcutaneously every 12 h for 4 days. Patients received skull irradiation (18 Gy) and 12 intrathecal methotrexate, 6 during the first couple of months and 6 during the 1st year. Boys were randomized with or without testicular irradiation.

Maintenance was monthly pulses always with cytosine arabinoside for 5 days and asparaginase for 5 days with vincristine or vindesine and Cytosan, or VM26 or daunorubicin and Cytosan. Daunorubicin was omitted after 6 months.

### Results

One hundred and seventy patients were treated and 150 (88%) achieved complete remission. Sixty-three patients experienced a relapse, 55 in bone marrow, 2 in testis, and 6 in CNS. Eighty-seven patients are in CR. Disease-free interval is  $48\% \pm 4\%$  at 54 months (Fig. 3). But according to the initial presentation, DFI is 28% (Fig. 4) for patients with hyperleukocytosis over 100 000 and without mediastinal mass, and 56%–67% (Fig. 5) for patients with hyperleukocytosis and a mediastinal mass. Two testicular relapses occurred in nonirradiated patients. Bone marrow relapses were no more frequent in patients with or without irradiation of the testis.

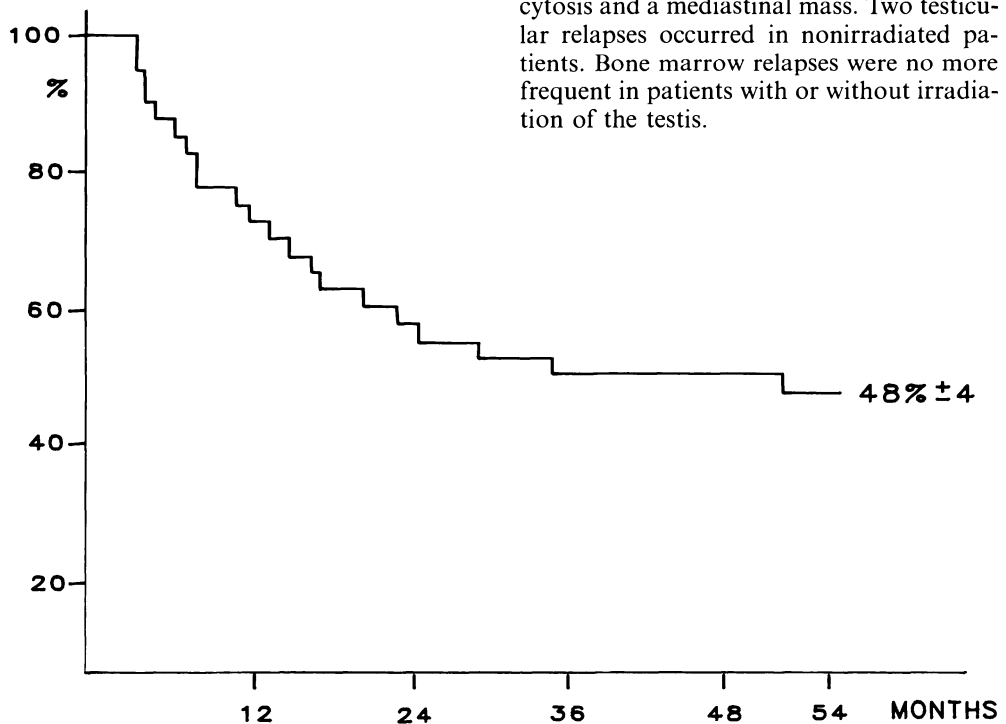


Fig. 3. Disease-free interval in high-risk patients

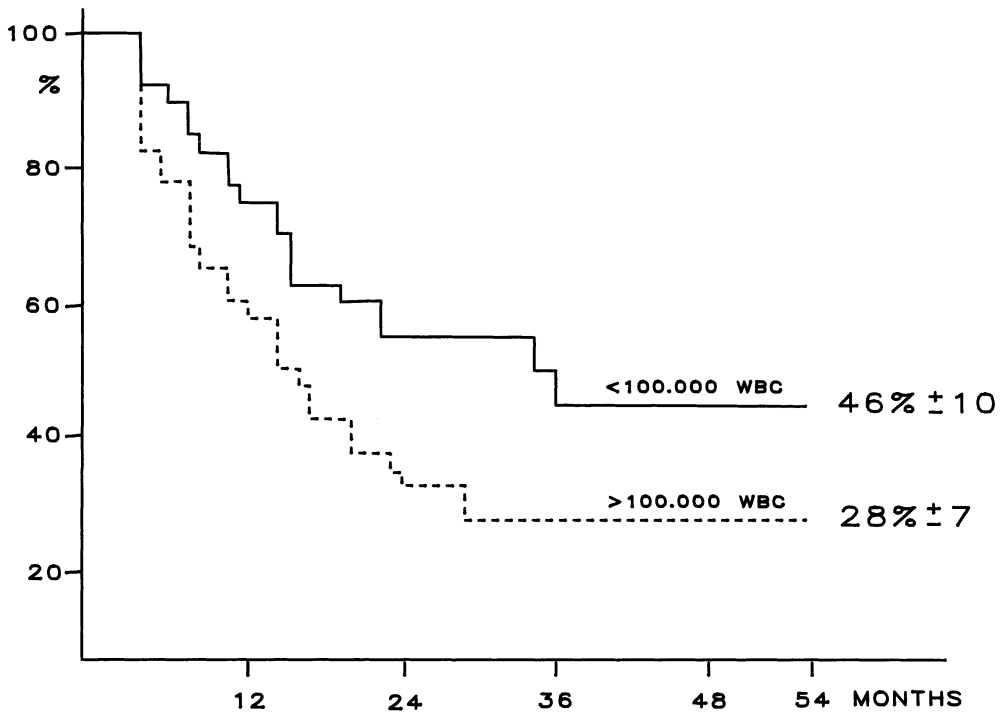


Fig. 4. Disease-free interval in high-risk patients without mediastinal mass

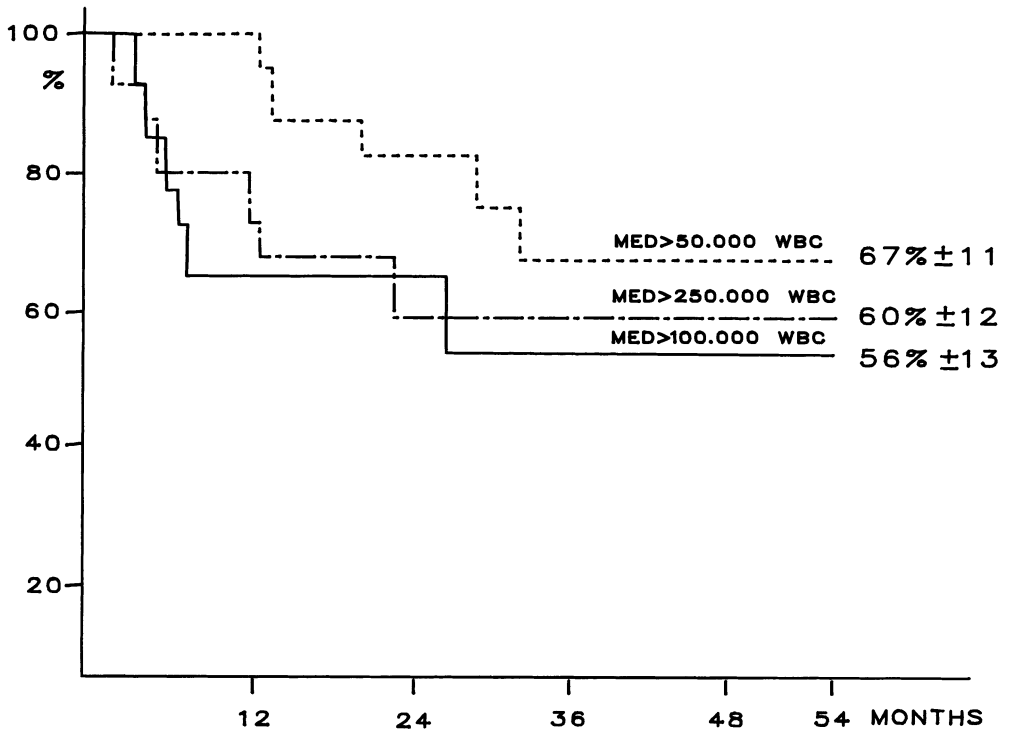


Fig. 5. Disease-free interval in high-risk patients with mediastinal mass

## Conclusions

The disease-free interval at 48% is similar to other protocols for very high risk patients but the results must be interpreted according to the presence or not of a mediastinal mass. This protocol with monthly pulses gives a high cure rate in patients with a thymic mass but is inadequate for patients with high hyperleukocytosis and without mediastinal mass. In other words in this protocol a mediastinal mass improves the prognosis of patients with hyperleukocytosis. Testicular irradiation does not seem useful but needs further study.

## Overall Results and Conclusions

Among 503 patients included in the protocol 340 are always in complete remission with a mean follow-up of 3½ years. There is no statistical difference between patients receiving vincristine or vindesine. There was a very low rate of CNS relapse except for patients not receiving skull irradiation. Intermediate-dose methotrexate (500 mg/m<sup>2</sup>) appeared an effective dosage to prevent testicular relapse but inadequate to prevent CNS relapse. Monthly pulses are a good maintenance regimen for patients with a mediastinal mass. Patients with high-risk leukemia without mediastinal mass need an increased consolidation and bone marrow transplantation in first remission should be considered in patients with poor outcome, for example those with translocation t(4;11) or t(9;22). A better selection of bad-risk patients should be the rule, and cytogenetics and immune markers must be included in the initial parameters.

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## Dutch Childhood Leukemia Study Group: Early Results of Study ALL VI (1984–1988)

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### Introduction

The Dutch Childhood Leukemia Study Group (DCLSG) protocol ALL VI was designed as a nationwide study to treat children with non-high-risk acute lymphoblastic leukemia (ALL) in a uniform protocol. More than 98% of all children with leukemia in the Netherlands are registered by the DCLSG [1]. Previous protocols in the Netherlands used cranial irradiation as CNS prophylaxis [2]. Since long-term risks for cerebral function became apparent, in study ALL VI cranial irradiation has been replaced by three courses of medium-dose methotrexate (MDMTX; 2 g/m<sup>2</sup>) combined with intrathecal MTX, followed by seven intrathecal injections with triple chemotherapy [MTX, cytosine arabinoside (ARA-C), prednisolone] [3, 4]. Furthermore, oral administration of dexamethasone was used instead of prednisone since data became available that this resulted in a lower incidence of meningeal relapse [5]. The feasibility of this treatment was first evaluated in a pilot study, which, however, still employed prednisone instead of dexamethasone.

In study ALL VI risk factors were prospectively studied. The treatment results of protocol ALL V, designed for the same category of patients [2], served as historical control.

### Patients and Methods

#### *Patients*

From 1 December 1984 until 30 June 1988, 206 newly diagnosed patients fulfilled the criteria (see below) to enter the study. Sixteen patients were not treated according to this protocol (2 of these have relapsed so far), so 190 patients were entered into the study. Fifty-two patients were entered into the pilot study from February 1984 until 30 November 1984.

#### *Diagnostic Procedures*

The diagnosis of ALL was made in the participating pediatric clinics, followed by confirmation on bone marrow, blood and CSF samples in the central laboratory of the DCLSG. French-American-British (FAB) typing and immunophenotyping were also performed centrally. An initial CSF specimen was also evaluated in the central laboratory of the DCLSG [6]. Bone marrow and CSF were examined every 14 weeks during as well as after chemotherapy.

#### *Criteria*

Children with ALL, age 0–15 years, excluding those with more than  $50 \times 10^9$ /liter white blood cells, with mediastinal mass, with initial CNS involvement, or with B-ALL. The resulting non-high-risk group includes 70% of all children with ALL in the Netherlands.

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The risk factor was determined prospectively in the same way as in the BFM ALL studies [6, 7]. A bone marrow relapse was diagnosed when more than 20% blasts were present in the bone marrow aspirate. A CNS relapse was diagnosed when in two successive CSF specimens, with an interval  $\geq 24$  h, cytological lymphoblasts were present and confirmed in the second specimen by immunophenotyping [8]. All bone marrow specimens and the second CSF specimen were evaluated in the Central Laboratory of the DCLSG in the Hague.

### Protocol

The outline is shown in Fig. 1. It consists of a 6-week induction period (dexamethasone, vincristine, L-asparaginase), followed by three courses of medium-dose methotrexate (MDMTX, 2 g/m<sup>2</sup>) and a 2-year period of maintenance chemotherapy. In the ALL VI pilot study prednisone was used instead of dexamethasone. The MDMTX was given as one-fifth push and four-fifths continuous infusion over 24 h, at 36 h citrovorum factor rescue was started and adapted for age [ $5 \times 5$  mg (<2 years),  $5 \times 10$  mg (2–5 years) or  $5 \times 15$  mg ( $\geq 6$  years), orally]. Intrathecal therapy with MTX was given twice during induction and three times with MDMTX. Intrathecal treatment was continued with triple chemotherapy once every 14 weeks during the 1st year of maintenance.

### Statistical Analysis

All 190 patients were entered in the EFS analysis. The starting point was the date of complete remission (CR). Patients not achieving CR were counted as failures at time 0 for the EFS analysis. End point was the lack of achievement of CR after 6 weeks of treatment, relapse, or death in CR. The Kaplan-Meier evaluation was used to estimate the proportion of patients in EFS. All results were updated as of 1 December 1988.

### Results

Patient characteristics are shown in Table 1. The median follow-up period for the 190 study patients is 95 weeks (range 0–197+ weeks). Of these, 43% had a BFM risk factor of over 0.8 (Table 1). Most patients had common ALL or pre-B ALL. The cytogenetic data are described elsewhere in this volume (R. Slater et al.). Complete remission was achieved in 184 patients (97%), there was only one nonresponder after 6 weeks of treatment; five patients (3%) died of initial infection or toxicity. The overall results of the pilot study and study are given in Table 2. The results of ALL VI (with ALL V as historical control) in terms of EFS are given in Fig. 2. The estimated EFS at 2 years is 92.7% (SE, 2%) and at 3 years 80% (SE, 5.4%) with 80 and 22 patients at risk, re-

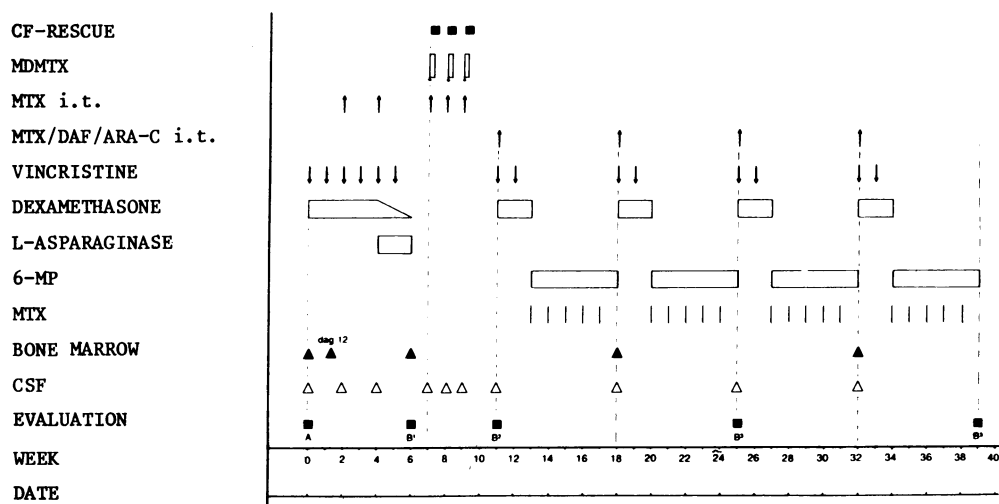


Fig. 1. Schematic representation of study ALL VI. Maintenance is continued until 116 weeks; i.t. triple chemotherapy every 7 weeks until week 62

**Table 1.** Patient variables in the DCLSG study ALL VI at diagnosis

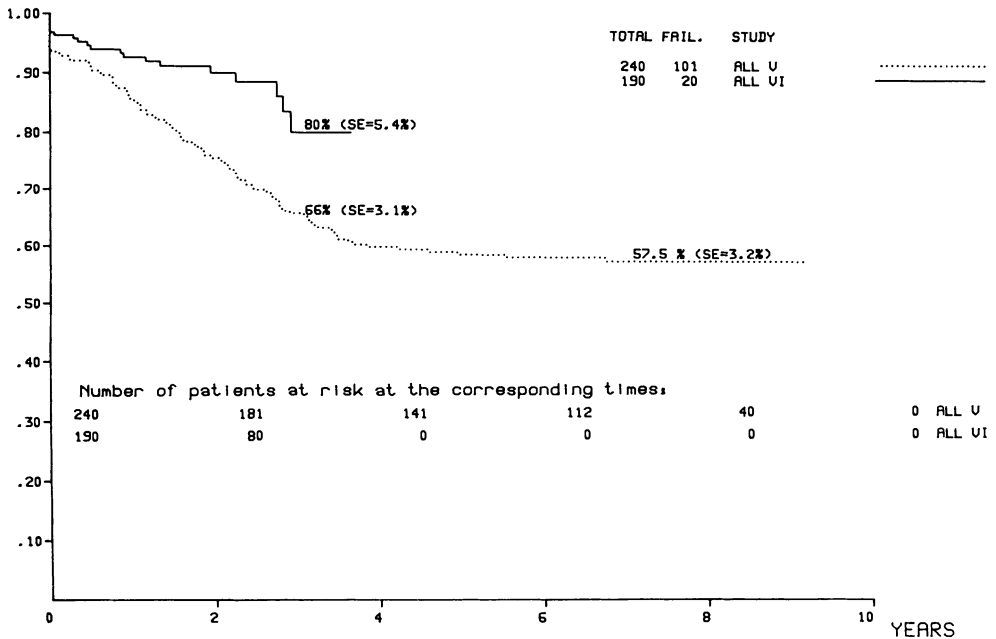
Variable	Category	Total (%)
Age (years)	<1	1 (0.5)
	1	9 (4.7)
	2-9	158 (83.2)
	10-15	22 (11.6)
Sex	Boy	98 (51.6)
	Girl	92 (48.4)
White blood cell count (10 <sup>9</sup> /liter)	<10	133 (70.0)
	<25	43 (22.6)
	<50	14 (7.4)
Risk factor ("BFM")	<0.8	101 (56.7)
	<1.2	62 (34.8)
	<1.7	15 (8.4)
	Inevaluable	12 -
Immunology	Common ALL	127 (66.8)
	Pre-B ALL	25 (13.2)
	AUL	5 (2.6)
	Not available	33 (17.3)
Total		190 (100.0)

BFM, Berlin-Frankfurt-Münster group; ALL, acute lymphoblastic leukemia; AUL, acute undifferentiated leukemia

**Table 2.** Data of the ALL VI pilot study and study proper. The pilot study used prednisone in induction and maintenance, the study dexamethasone. In other respects the protocols were identical

	ALL VI pilot	ALL VI study
Number of patients	52	190
No remission	1	6
Early death	1	4
Death hypoplasia	0	1
No response	0	1
Complete remission	51	184
Failure	16	14
Bone marrow relapse	8	8
Central nervous system	6	1
Testicular relapse	2	1
Death in remission	0	4
Event-free survival	36	170
Follow-up (weeks)	148-204	0-197
Follow-up, median (weeks)	176	95

Probability



**Fig. 2.** Kaplan-Meier curves of EFS in the DCLSG studies ALL V and ALL VI. The EFS estimations at 3 and 8 years as well as the corresponding standard errors (SE) based on the Greenwood Formula are given in the graph

spectively. The ten relapses encountered so far were mainly in the bone marrow (eight patients). Isolated CNS relapse and testicular relapse each occurred in one patient.

The toxicity of the dexamethasone periods seemed to be more pronounced than with prednisone in the previous protocol, although theoretically the doses were equivalent (6 mg/m<sup>2</sup> and 40 mg/m<sup>2</sup>, respectively). The Cushing effect and psychological effects seemed to be more pronounced, also diabetes in the second half of induction was more often seen. During induction nausea and vomiting occurred in 38% of the patients, diarrhea in 31%, and liver function disturbances in 58%.

During the MDMTX period 33% of the patients suffered from mucositis (mostly WHO grade I, only two patients with grade IV). Liver function disturbances occurred in 52%, renal function disturbances in 12%, and all were reversible. During the MDMTX and maintenance period, four patients (2%) were lost due to toxicity. Two of them died from septicemia, one patient was found dead without apparent cause (autopsy not performed), and one patient died from fungal abscess.

## Conclusions

The early results of the ALL VI protocol for non-high-risk patients are encouraging. The EFS at 3 years is 80% (SE, 5.4%), compared with 66% (SE, 3.1%) for the previous protocol, ALL V. Especially the occurrence of extramedullary relapses has been diminished substantially. Only one CNS relapse and one testicular relapse have been recorded after a median follow-up of 95 weeks. However, the prolonged intrathecal therapy in this protocol might just have the effect of postponing the occurrence of CNS relapses. Three different treatment modifications may have contributed to this apparent improvement. Firstly, dexamethasone was given during induction and maintenance instead of prednisone. Others have demonstrated that this substitution results in a lower incidence in CNS relapses [5]. Secondly, MDMTX has been shown to be about equally effective in preventing CNS relapse as 1800 cGy cranial irradiation and in-

trathecal MTX for 3 weeks [9]. In the latter study from St. Jude, however, less MTX (1 g/m<sup>2</sup>) was given more often (15 × instead of 3 ×) than in our study. Thirdly, intrathecal triple chemotherapy was administered according to the same lines as in the Pediatric Oncology Group (POG) study [4]. It is not possible to judge the relative contribution of each of the three approaches separately. Apart from their improved protection against extramedullary relapses, MDMTX infusions have also been shown to decrease the number of bone marrow relapses [9]. The optimum dose for MDMTX infusions is still controversial. The dose used in our study, 2 g/m<sup>2</sup>, seems appropriate and is currently also under study in the St. Jude protocols [9].

In summary, the aims which were set for the ALL VI protocol, a more effective, less toxic treatment scheme for the treatment of children with non-high-risk ALL, seem to have been realized. Prolonged follow-up is necessary for definitive conclusions, however.

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## Results of Acute Lymphoblastic Leukemia Therapy in Childhood: GDR-Experiences 1981–1987

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Over the past 14 years the GDR Hematology and Oncology Working Group has conducted a series of therapeutic trials for childhood acute lymphoblastic leukemia (ALL). In this period the prognosis of childhood ALL has been improved by intensive combination chemotherapy. In 1981 we decided to adopt a modified Berlin-Frankfurt-Münster group (BFM) protocol ALL-VII 81 [1, 2]. In this article, we review the results of this treatment strategy with respect to the importance of some conventional risk factors.

### Materials and Methods

#### Patients

A total of 524 children, without prior treatment, suffering from ALL were entered into a multicenter controlled randomized study between 1 September 1981 and 31 December 1987 (Table 1).

#### Treatment

Patients were divided into three risk groups by calculating a risk factor (RF) on the basis of the number of initial leukemic cells and liver and spleen enlargement: standard- (SR), medium- (MR), and high-risk (HR) groups (Table 2). The treatment protocol of this study has been described [3]. For prophylactic CNS therapy in the SR group, pa-

**Table 1.** Patient characteristics

	<i>n</i>	%
Patients, total	524	100
Median age at diagnosis (years)	4	10/12
< 2 years	52	10
≥ 10 years	85	16
Boys	283	54
Girls	241	46
White blood cell count:		
≤ 25 × 10 <sup>9</sup> /liter	358	68
> 25 × 10 <sup>9</sup> /liter	166	32
> 50 × 10 <sup>9</sup> /liter	103	20
> 100 × 10 <sup>9</sup> /liter	56	11
Enlargement of liver	156	30
Enlargement of spleen	132	25
CNS involvement	28/522	5
Mediastinal mass	56/523	11
Kidney involvement	24/504	5
Bone infiltration	61/511	12
Lymph node enlargement	289/524	55
Acid phosphatase positive	90/507	18

tients were randomized to receive cranial irradiation (18 Gy) and intrathecal methotrexate (MTX) or a medium dose of MTX (MDMTX: 500 mg/m<sup>2</sup>) and intrathecal MTX. This randomization was stopped in 1986. The reason for this decision was an unacceptably high CNS failure rate in the MDMTX group. Thirty-one patients received additional CNS irradiation after MDMTX with 18 Gy (7–36 months after MDMTX). Therefore the SR-B group was reduced to only 43 patients. The number of patients with MDMTX and 18 Gy CNS ir-

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**Table 2.** Therapy results

	Total = 100%		SR = 65%		MR = 29%		HR = 6%	
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
Patients, total	524	100	342	100	150	100	32	100
Deaths before remission	21	4	12	4	7	5	2	6
Complete remission	503	96	330	96	143	95	30	94
Deaths in remission	21	4	16	5	4	3	1	3
Lost to follow-up	6	1	3	1	2	1	1	3
Lost by BMT	6	1	–	–	–	–	6	19
Relapses, total	142	27	79	23	46	31	17	53
BM	74	14	42	12	23	15	9	28
CNS	20	4	11	3	4	3	5	16
CNS + BM	23	4	12	4	9	6	2	6
Testes	9	2	6	2	3	2	–	–
Testes + BM	8	2	3	1	5	3	–	–
Mediastinal mass	1	0.2	1	0.3	–	–	–	–
Mediastinal mass + BM	2	0.4	1	0.3	–	–	1	3
Mediastinal mass + LN	1	0.2	1	0.3	–	–	–	–
Testes + bone	1	0.2	–	–	1	0.7	–	–
Testes + CNS + BM	1	0.2	–	–	1	0.7	–	–
Bone + BM	2	0.4	2	0.6	–	–	–	–
BM involvement	110	21	60	18	38	25	12	38
CNS involvement	44	8	23	7	14	9	7	22
Testes involvement	19	4	9	3	7	5	–	–
In first remission	328	63	232	68	91	61	5	16
Alive ( <sup>a</sup> = 3 after BMT)	388	74	271	79	105	70	12 <sup>a</sup>	38
Life-DFS (SD)	0.57 (0.03)		0.59 (0.04)		0.58 (0.04)		0.30 (0.09)	
Table-EFI (SD)	0.59 (0.03)		0.61 (0.04)		0.61 (0.05)		0.32 (0.09)	
Analysis-RFI (SD)	0.62 (0.03)		0.65 (0.04)		0.63 (0.05)		0.33 (0.10)	

Time of observation 13–88 months SR = MR/HR: *P* = 0.001

BM, bone marrow; LN, lymph nodes

radiation increased by 1986/1987 to 70 (SR-C group), and 187 patients received 18 Gy CNS irradiation (SR-A). For the duration of maintenance therapy, patients were randomized after 78 weeks to receive MTX and 6-mercaptopurine (6-MP) for another 6 months or a late intensification (protocol III). Patients with B-ALL were excluded from this study.

#### Statistical methods

The Kaplan and Meier method was used to construct life tables and curves, and the Mantel-Haenszel-test was used to compare life tables [4, 5].

#### Results

Therapy results are summarized in Table 2. Induction therapy according to protocol I [1] led to a complete remission (CR) rate of 96% (SR-patients 96%, MR patients 95%, HR patients 94%). Twenty-one children (4%) died within the first 4 weeks of therapy without having achieved remission. Causes of death were infections (six) and cerebral bleeding (five). Ten patients did not respond to therapy, and 21 (4%) died in the phase of first CR, mostly from infections.

The probability of event-free survival for 524 patients was 57% ± 3%. Of 503 patients who had achieved CR, 142 (27%) relapsed (Table 2), 23% in the SR group, 31% in the

MR group, and 53% in the HR group. The probability of DFS is 59% for SR patients (representing 65% of all patients), 58% for MR patients (29% of all patients), and 30% for HR patients (6% of all patients, Fig. 1, Table 2). Comparing the three regimens for CNS prophylaxis in SR patients, SR-A (CNS irradiation), SR-B (MDMTX), and SR-C (MDMTX plus CNS irradiation), p-CCR is more favorable in patients if MDMTX and irradiation with 18 Gy is given as a preventive measure; EFI is 72% for SR-C patients, 62% for SR-A patients, and 57% for SR-B patients.

Five CNS relapses in 31 patients (16%) with delayed irradiation in the SR-C group occurred (Fig. 2). Only 6 relapses with CNS involvement of 187 SR-A patients (3%) were observed. There was no relapse with testes involvement in SR patients with MDMTX.

With respect to the conventional-risk factors in this study, regression analysis revealed that the prognosis for patients with white blood cell counts above 50000/mm<sup>3</sup> ( $P=0.001$ ), with mediastinal mass ( $P=0.01$ ), for infants less than 1 year of age ( $P=0.025$ ), and for children younger than 2 years and

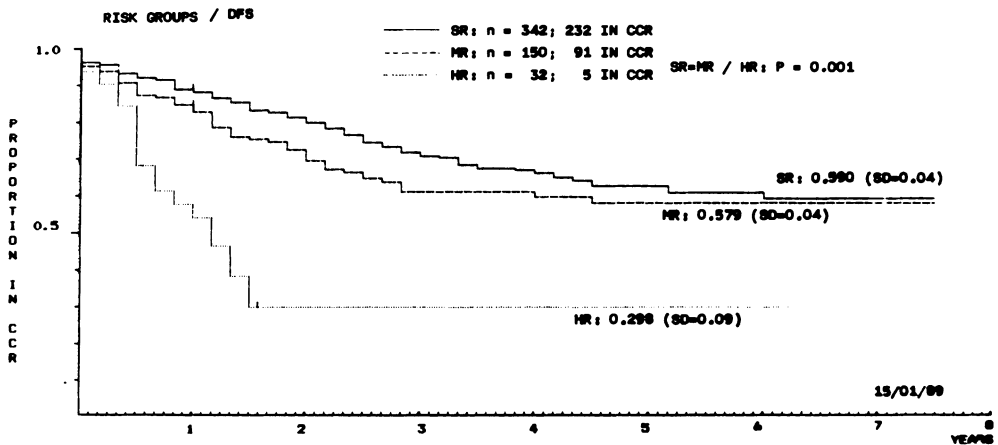


Fig. 1. Probability of disease-free survival for three subgroups ( $P$  values: SR vs. HR <0.001; MR vs. HR <0.001)

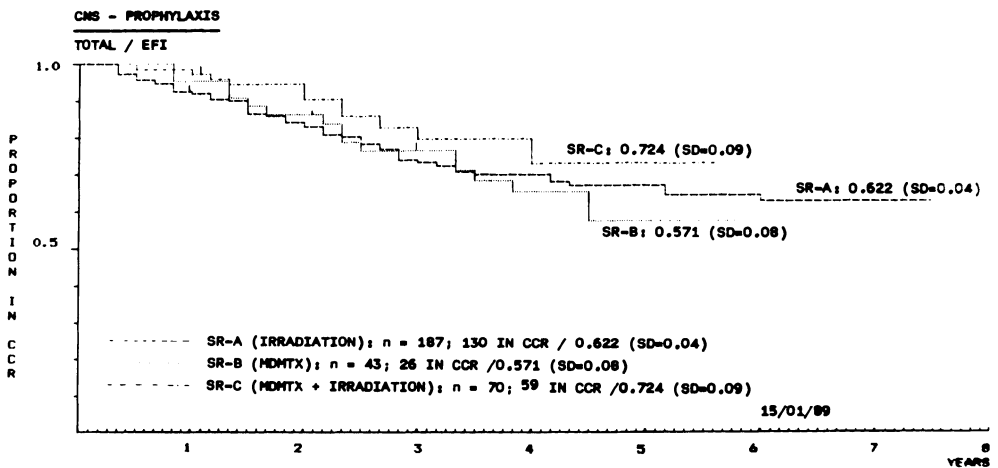


Fig. 2. Probability of event-free interval for standard-risk patients with different CNS prophylaxis

**Table 3.** Conventional risk factors and disease-free survival

Risk factors	In CCR/ <i>n</i>	DFS	SD	<i>P</i>
Age < 1 year	5/ 14	0.230	0.135	0.025
≥ 1 years	323/510	0.580	0.028	
< 2 and ≥ 10 years	71/137	0.476	0.054	0.01
2 to < 10 years	257/387	0.603	0.032	
Sex: boys	168/283	0.523	0.039	0.10
girls	160/241	0.622	0.038	
White blood cell count				
< 50 GPT/liter	287/421	0.611	0.031	0.001
≥ 50 GPT/liter	41/103	0.412	0.052	
Mediastinal mass: positive	26/ 55	0.472	0.075	0.01
negative	301/468	0.582	0.029	
Primary CNS involvement:				
positive	12/ 28	0.454	0.104	0.001
negative	316/494	0.581	0.028	
Enlargement of liver ≥ 5 cm	84/156	0.510	0.050	0.10
< 5 cm	244/368	0.596	0.033	
Enlargement of spleen ≥ 5 cm	72/132	0.551	0.049	0.10
< 5 cm	256/392	0.576	0.033	
Acid phosphatase: positive	49/ 90	0.539	0.057	0.10
negative	269/417	0.581	0.031	

$P > 0.05$  = not significantly different

older than 10 years of age ( $P=0.01$ ) was significantly worse (Table 3). CNS disease of diagnosis, hepatomegaly, splenomegaly, and sex were not significant predictors of disease-free survival. The results of two groups with different treatment durations

(18 months plus protocol III versus 24 months) are shown in Fig. 3. The probability of event-free interval for the 18-month group (60%) is no different from that for the 24-month group (59%).

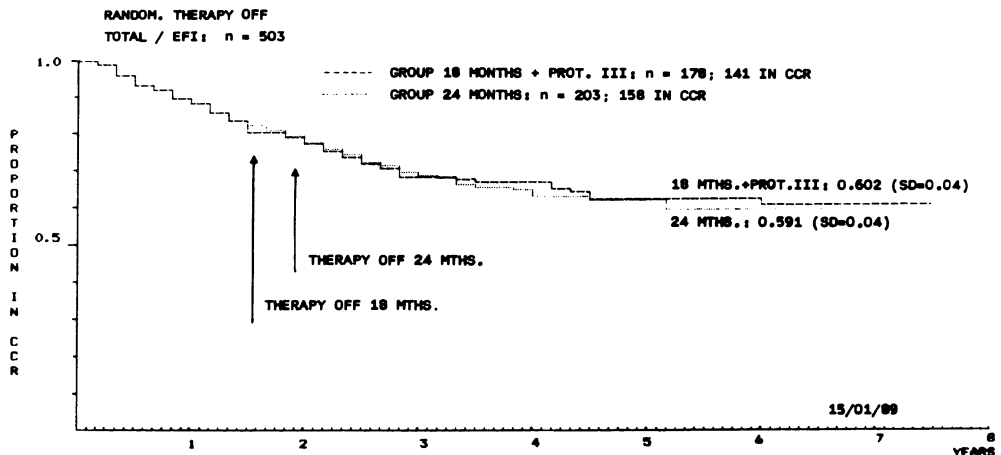


Fig. 3. Probability of event-free interval for two sugroups with different treatment durations



## Conclusions

1. The BFM treatment strategy for acute lymphoblastic leukemia in childhood improved our results in a study from 1981 to 1987 to a probability of disease-free survival of about 60%. As in other groups the high lethal toxicity in the beginning of the study decreased markedly [6].
2. Although conventional-risk factors lost their prognostic significance in varying degrees during the evolution of risk-adapted intensive therapy [2], the most important significant prognostic factor in this study was the leukocyte count (tumor burden) above 50 000/mm<sup>3</sup> ( $P < 0.001$ ).
3. Intermediate-dose MTX could not efficiently protect standard-risk patients from CNS leukemia.

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## Impact of Early Intensive Reinduction Therapy on Event-Free Survival in Children with Low-Risk Acute Lymphoblastic Leukemia

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### Introduction

Since 1970, the basic concept of the Berlin-Frankfurt-Münster (BFM) study group trials for childhood acute lymphoblastic leukemia (ALL) has been intensified and prolonged induction and consolidation chemotherapy [9, 10]. In 1976, a trial was started with the aim of improving the prognosis of patients with high risk for relapse features, mainly characterized by an elevated  $WBC \geq 25000/mm^3$ . After successful remission induction these patients received an intensive reinduction protocol of 6 weeks duration early in remission, i.e., during the first 6 months after diagnosis, which increased the event-free survival (EFS) by about 30% compared with the historical control group of study ALL BFM 70 [3].

In the subsequent trial ALL BFM 79, the concept of early intensified reinduction therapy was introduced for standard-risk patients in the form of "protocol III," a treatment course of 4 weeks duration. Initial analyses of this trial failed to detect an effect on EFS by the addition of therapy compared with the preceding ALL BFM 76 study [5]. Subsequent follow-up studies, however, suggested that there might still be a difference in outcome in favor of patients who had received protocol III. Therefore, in

study ALL BFM 81 treatment was not changed for standard-risk patients, and again the results were not conclusive. Hence, in the subsequent ALL BFM 83 trial the decision was made to randomize the patient group with the lowest risk (LR) for relapse to receive or not to receive protocol III.

The purpose of the present paper is to analyze and to reevaluate the treatment results with respect to this kind of early intensified treatment in low-risk ALL.

### Patients and Methods

Out of 685 patients with non-B ALL registered for the ALL BFM 83 study, a total of 199 children were assessed to be at low risk for treatment failure (standard risk low: SR-L). The risk for relapse was estimated by a risk factor [7] according to the equation:

$$RF = 0.2 \cdot \log(BI + 1) + 0.06 \cdot L + 0.04 \cdot S$$

with  $BI$  = absolute number of leukemic cells in the blood/ $mm^3$ ,  $L$  = enlargement of the liver (centimeters below the costal margin), and  $S$  = enlargement of the spleen (centimeters below the costal margin).

Low-risk patients were those with an  $RF < 0.8$ . Patient characteristics are given in Table 1. These children were randomized in one of two treatment arms SR-L1 or SR-L2. In all children therapy was started with protocol I with the drugs prednisolone, vincristine, daunorubicin, and L-asparaginase (induction), followed by cyclophosphamide, cytarabine, mercaptopurine (6-MP), and i.t. methotrexate (consolidation). Radiotherapy

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**Table 1.** Low-risk patient characteristics: study BFM ALL 83

	SR-L1	SR-L2
Total number patients	104	95
Boys	47 (45.2%)	49 (51.6%)
Girls	57 (54.8%)	46 (48.4%)
Age <1 year	1	1
1 ≤ 10 years	80 (76.9%)	69 (72.6%)
≥ 10 years	23 (22.1%)	25 (26.3%)
White blood cell count < 50 000/mm <sup>3</sup>	104	95
Hemoglobin > 8 g/dl	43 (41.3%)	37 (38.9%)
Immunology		
CALLA	81 (77.9%)	72 (75.8%)
preT/T	0	4
Null	2	1
AUL	1	0
NE	20	18

CALLA, common acute lymphoblastic leukemia antigen; AUL, acute undifferentiated leukemia NE (n.e.?), not evaluated

to the (CNS) was not applied in this patient group.

After protocol I, the children received an 8-week course of 6-MP and four doses of i.t. methotrexate (MTX) and i.v. MTX at 500 mg/m<sup>2</sup> each, given as a 24-h infusion with two routine doses of leucovorin of 15 mg/m<sup>2</sup> at 48 and 54 h. In treatment arm SR-L1, the course of intermediate-dose MTX was followed by oral maintenance therapy with daily 6-MP (50 mg/m<sup>2</sup>) and weekly MTX (20 mg/m<sup>2</sup>). In limb SR-L2, the children were treated with protocol III,

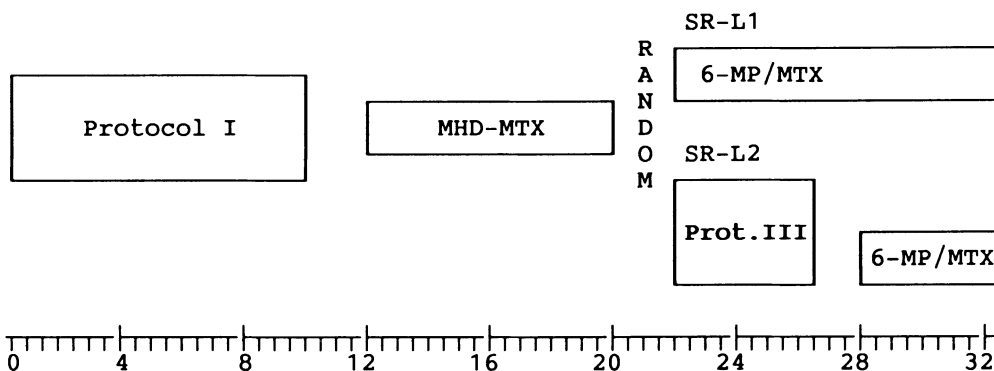
consisting of oral dexamethasone (10 mg/m<sup>2</sup> per day for 14 days), vincristine, and adriamycin (days 1 and 8 at doses of 1.5 mg/m<sup>2</sup> and 30 mg/m<sup>2</sup> each, respectively) and four doses of L-asparaginase (10 000 U/m<sup>2</sup>), followed by a second phase of treatment with oral thioguanine (50 mg/m<sup>2</sup> per day for 14 days), two 4-day cycles of cytarabine (days 3–6 and 10–13 at doses of 75 mg/m<sup>2</sup> per day), and two doses of i.t. MTX. After the end of this protocol, the same maintenance regimen was started as described for arm SR-L1 patients.

The study design for LR patients is outlined in Fig. 1. There was a second randomization scheduled for all patients of the study at 18 months of continuous complete remission (CCR), which is not mentioned in the schema. Half of the patients were to have their treatment continued up to a total of 24 months, whereas in the remaining patients treatment was stopped at 18 months of CCR.

Diagnostic procedures and the criteria for response and remission were the same as reported elsewhere [4]. Life-table estimates were established by the Kaplan-Meier method.

## Results

All patients achieved complete remission after the induction phase of protocol I (Table 2). There was one child who died in CCR as a consequence of toxicity in arm SR-L1. Relapse rates are clearly different between both arms, with 33/104 in SR-L1 and 15/95 in



**Fig. 1.** Treatment design for low-risk patients of study ALL BFM 83

**Table 2.** Treatment results in low-risk patients: study BFM ALL 83

	SR-L1	SR-L2
Total number patients	104	95
Complete remission achieved	104	95
Death in CCR	1	0
Relapsed	33 (31.7%)	15 (15.8%)
Isolated BM	19 (18.3%)	9 (9.5%)
Isolated CNS	3 (2.9%)	3 (3.2%)
BM/CNS	9 (8.7%)	1 (1.1%)
BM/test	1	2
Other	1	0

SR-L1: without protocol III  
 SR-L2: with protocol III

SR-L2. The incidence of isolated bone marrow relapse is twice as high in SR-L1 compared with SR-L2. Surprisingly, there is also a marked difference between the CNS relapse rates in both groups, the majority of which were simultaneously located in the bone marrow in arm SR-L1.

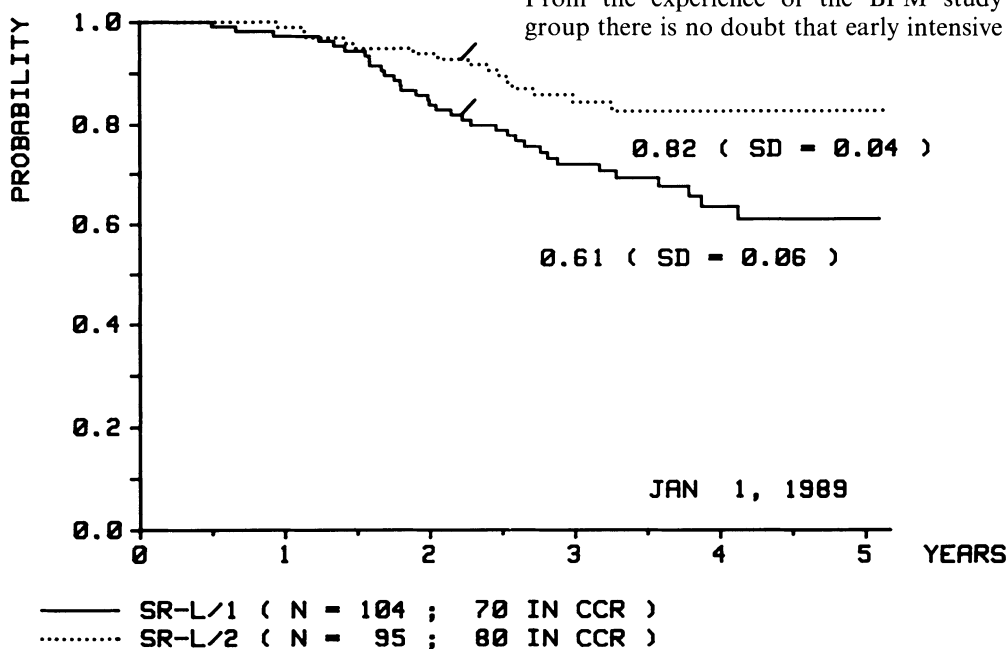
Life-table estimates with respect to the probability of an event-free interval of 5 years yield 0.61 and 0.82 for all patients of

treatment regimens SR-L1 and L2, respectively (Fig. 2). Considering only the fractions of children who were randomized, the results are identical and the difference between both treatment arms is statistically significant at  $P < 0.01$  (Fig. 3). Figure 4 shows the probability of being in CNS remission and again there is a difference of borderline significance ( $P = 0.05$ ) between both arms.

In order to evaluate the observed effect of protocol III in this randomized study in a larger number of patients, the risk factor was retrospectively calculated for children of five subsequent BFM studies and the patients grouped into those who had additionally received protocol III within their treatment course and those who did not. The life-table estimates yield a 20% difference in long-term results in favor of the patients who were additionally treated early in remission (Fig. 5). This analysis is restricted to patients who experienced at least a period of 10 weeks of CCR (earliest time for start of protocol III).

### Discussion

From the experience of the BFM study group there is no doubt that early intensive



**Fig. 2.** Kaplan-Meier plot for all patients treated in either arm SR-L1 or SR-L2; diagonal: last follow-up

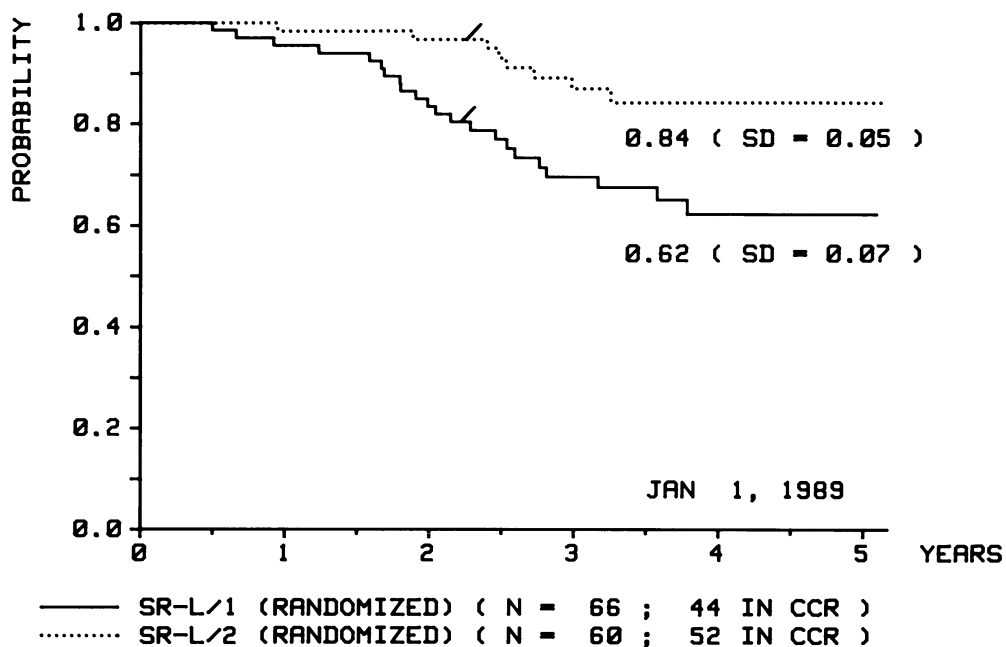


Fig. 3. Kaplan-Meier plot restricted to patients who were randomly allocated to arms SR-L1 or SR-L2

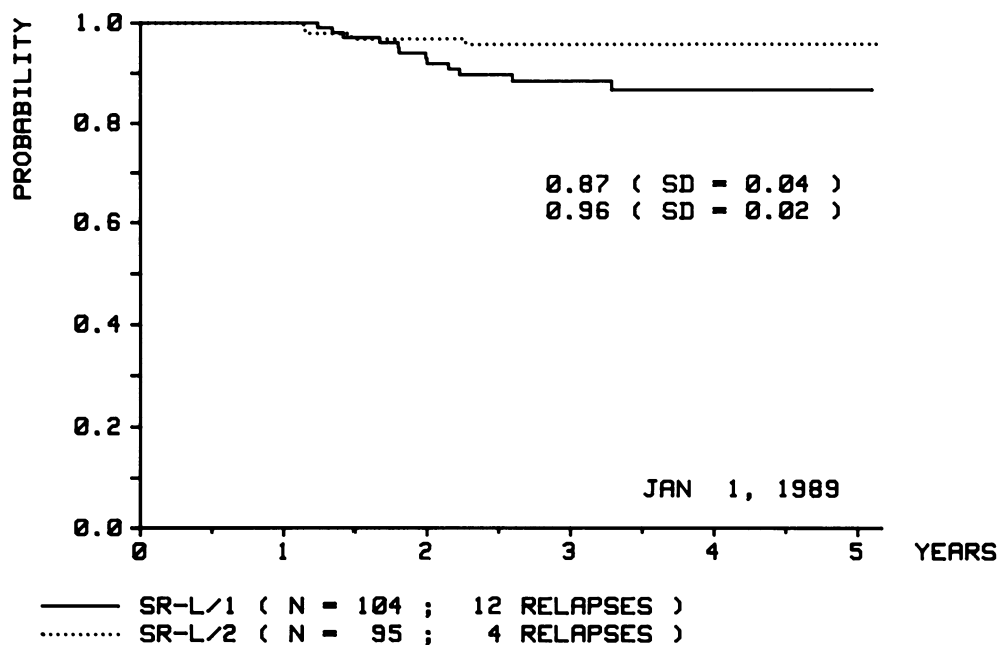


Fig. 4. Kaplan-Meier plot for CNS relapse-free interval; only patients with isolated or combined CNS relapses are considered; all other events are excluded

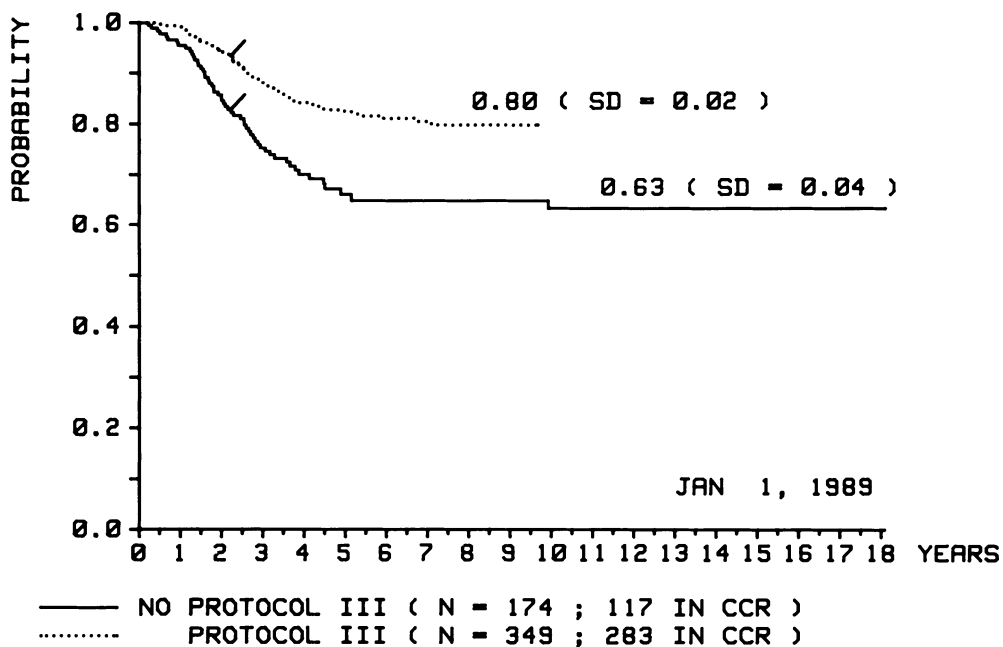


Fig. 5. Kaplan-Meier plot for low-risk patients from five subsequent BFM trials. The risk factor was retrospectively assessed and the patients grouped into those who received reinduction treatment with protocol III and those who did not

treatment has been the most important factor influencing long-term EFS. A variety of attempts were reported in the literature aimed at improving the prognosis of children with ALL by intensification procedures during maintenance therapy, such as reinduction pulses with prednisolone and vincristine [8, 11] or the design of cyclic rotational regimens [6, 12]. Also the introduction of high-dose MTX [1], mainly for the prevention of CNS relapses, which was performed by a number of study groups, has to be mentioned in this context. Recently, promising results were published obtained with the continuous administration of asparaginase and for high-risk patients additionally given adriamycin [2].

In the BFM trials, the introduction of a second intensive treatment protocol of 6 weeks duration produced major progress in the prognosis of high-risk patients [3]. Hence, there is good evidence meanwhile that intensification of therapy during maintenance may result in better long-term results, probably depending on the time sched-

ule and the degree of intensification. The conclusion in 1982 drawn from the analyses of study ALL BFM 79 proved obviously false. With longer periods of observation and based on a larger number of patients there is now no longer doubt that the addition of a 4-week reinduction protocol did improve the prognosis. The life-table curves show a stable plateau for both treatment groups and the calculated difference is as high as 20%.

From the randomized patients of study ALL BFM 83 there is evidence that the addition of protocol III not only reduced the number of systemic relapses but also of – mostly combined – CNS relapses. Usually, CNS relapses tend to occur with prolonged periods of hematological remission as they apparently need a certain time span to emerge. Since no radiotherapy was included in the study design one would rather have expected the opposite, i.e., a higher frequency of CNS relapses in the treatment arm with longer hematological remission durations. One explanation for this finding

would be that it is only a result by chance. On the other hand, one could argue that dexamethasone and the two i.t. injections of MTX could have had an effect on residual leukemic cells in the CNS, as well, and consequently have led to a lower risk for reseed- ing of the marrow. A third explanation might be that the cells responsible for the CNS relapse originated from the bone marrow and spread subsequently to the meninges.

At present, it is impossible to obtain a definite answer on the question of the inter- relationship between marrow and CNS re- lapses but the observations demonstrate again that hematological and CNS remis- sion durations are not independent from each other.

In summary, the results substantiate that a seemingly minor change of the treatment regimen was followed by a major improve- ment in outcome. The effect is to be ob- served relatively late and becomes evident at about 3–4 years of CCR. We conclude, therefore, that study results should not be evaluated prematurely in order to avoid conclusions which may prove false after more time has elapsed.

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## Improved Prognosis for Childhood Acute Lymphocytic Leukemia with Very High White Blood Cell Count ( $>100/\text{nl}$ ) with Rotation of Non-Cross-Resistant Drug Combinations\*

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### Introduction

Among several initial prognostic factors in acute lymphoblastic leukemia (ALL), i.e., age, hepatosplenomegaly, immunologic subtype, and chromosomal abnormalities [1–4], an elevated white blood count (WBC) at diagnosis has remained the single most important adverse prognostic factor in many studies. In spite of the markedly improved overall prognosis for children with ALL, patients with a WBC  $>100/\text{nl}$  still have a risk of relapse of 50% or more [2, 5–7].

### Patients and Methods

In a cooperative Federal German Republic treatment study for childhood ALL (COALL-03-85), 284 patients age 1 month to 18 years were entered between 1 January 1985 and 1 August 1988. The patients were considered to belong to the high-risk group if one or more of the following parameters were present: initial WBC  $\geq 25/\text{nl}$ , T-ALL, null-ALL/acute undifferentiated leukemia (AUL), age  $\geq 10$  years. Of 147 patients with high-risk criteria, 40 patients were considered to be at very high risk of relapse because of an initial WBC of  $>100/\text{nl}$ .

The treatment protocol is shown in Fig. 1 and Table 1. ALL high-risk patients were randomized to receive six mostly non-cross-resistant drug combinations either in slow rotation with a change of the drug combinations every 4–6 weeks or in rapid rotation with a change of the drug combinations every 2–3 weeks. In the latter arm the whole sequence of the combinations was repeated; thus all patients received the same total amount of drugs within the same period. In the first 2 years of the study high-dose cytosine arabinoside (Ara-C) and asparaginase (ASP) were given only to patients with a WBC  $>50/\text{nl}$ ; since February 1987 all patients received this combination, which had turned out to be one of the least toxic.

Treatment of presymptomatic CNS leukemia consisted of cranial irradiation with 24 Gy and nine doses of intrathecal methotrexate, four during irradiation and five during the first 10 weeks of treatment. Maintenance therapy consisted of daily 6-mercaptopurine p.o. and weekly methotrexate p.o. up to 2 years from the time of diagnosis. Low-risk patients received a separate less intensive protocol.

### Results

The initial characteristics of the group of very high risk patients with a WBC  $>100/\text{nl}$  are shown in Table 2. Within the randomized groups there was an even distribution in age, immunological subtype, presence of initial CNS disease, mediastinal mass, and massive hepatosplenomegaly. In the rapid

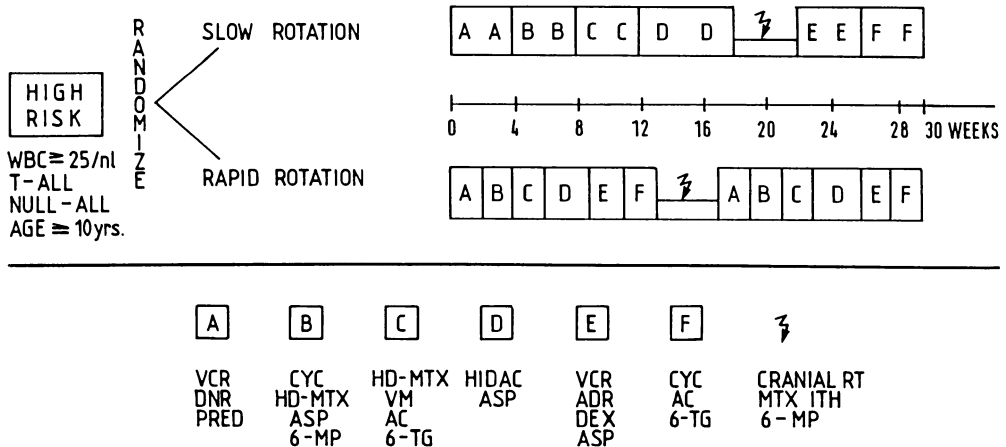
For the <sup>c</sup>ALL Study Group  
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\* Supported by the Hamburger Landesverband für Krebsbekämpfung



**Table 1.** Drug doses and abbreviations

	Day	Drug		Dose
<b>A</b>	1+8	VCR	Vincristine	1.8 mg/m <sup>2</sup>
	1+8	DNR	Daunorubicin	36 mg/m <sup>2</sup>
	1-14	PRED	Prednisone	60 mg/m <sup>2</sup>
<b>B</b>	1	CYC	Cyclophosphamide	900 mg/m <sup>2</sup>
	2	HD-MTX	High-dose methotrexate	1000 mg/m <sup>2</sup> 24-h infusion
	4+6	ASP	Asparaginase	45000 U/m <sup>2</sup>
	1-7	6-MP	6-Mercaptopurine	100 mg/m <sup>2</sup>
<b>C</b>	1	HD-MTX	High-dose MTX	as in B
	3	VM	Teniposide	165 mg/m <sup>2</sup>
	3	AC	Cytarabine	300 mg/m <sup>2</sup>
	1-7	6-TG	6-Thioguanine	100 mg/m <sup>2</sup>
<b>D</b>	1-3	HIDAC	High-dose Cytarabine	3000 mg/m <sup>2</sup> q 12 h x 4
	3+5	ASP	Asparaginase	45000 U/m <sup>2</sup>
<b>E</b>	1+8	VCR	Vincristine	1.8 mg/m <sup>2</sup>
	1+8	ADR	Adriamycin	30 mg/m <sup>2</sup>
	9+11	ASP	Asparaginase	45000 U/m <sup>2</sup>
	1-14	DEX	Dexamethasone	10 mg/m <sup>2</sup>
<b>F</b>	1	CYC	Cyclophosphamide	900 mg/m <sup>2</sup>
	2-5	AC	Cytarabine	90 mg/m <sup>2</sup>
	1-7	6-TG	6-Thioguanine	100 mg/m <sup>2</sup>



**Fig. 1.** Treatment protocol COALL-03-85 for high-risk patients

rotation arm the mean WBC was higher; 6/19 patients had a WBC > 300/nl opposed to 4/21 in the slow rotation arm. Of the 40 patients 1 was a late responder, i.e., a remission was not achieved at day 28 but after an additional 4 weeks of treatment. Two patients with a WBC of 350 and 820/nl were

not in remission by day 56 and were considered to be non-responders. Both achieved a short-lived remission later but died in relapse shortly thereafter.

As of 1 September 1988 (median follow-up 24 months) there were 4 relapses 11, 14, 23, and 29 months after diagnosis (Table 3).

**Table 2.** Initial characteristics of high-risk patients with a WBC >100/nl

	Slow rotation	Rapid rotation
Number of patients	21	19
Boys	14	10
Age < 1 year	1	1
> 10 years	5	6
WBC, mean (nl)	202	280
range	(108-400)	(105-950)
Mediastinal mass	7	4
CNS involvement	2	2
Liver/spleen > 5 cm	9	9
C-ALL	8	7
T-ALL	9	10
Null-ALL/AUL	2	2

**Table 3.** Clinical results in high-risk patients with a WBC >100/nl

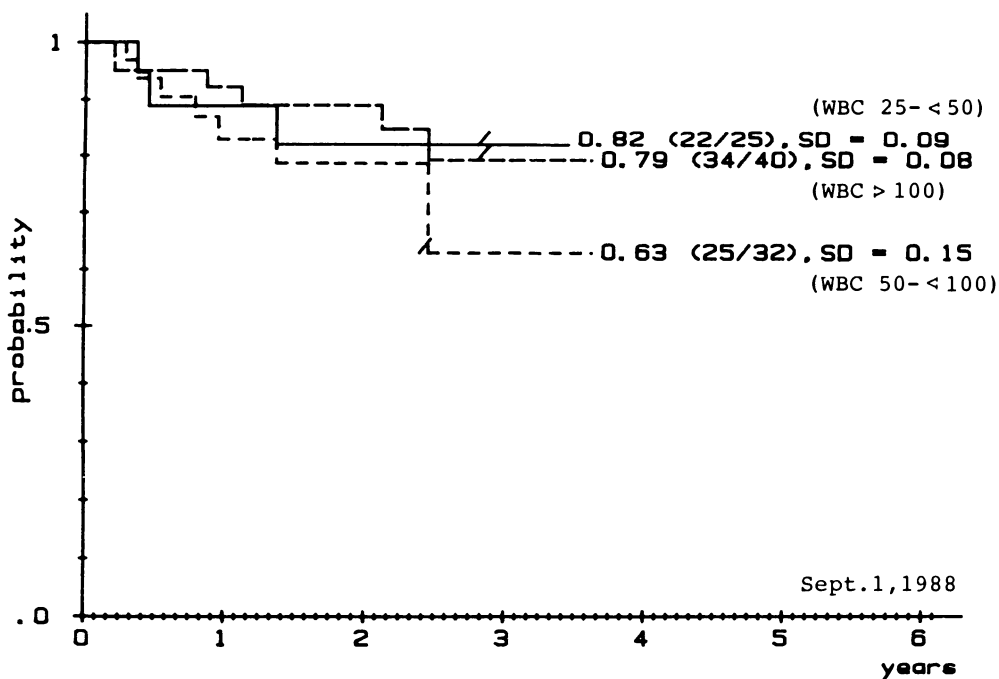
	Slow rotation	Rapid rotation
Number of patients	21	19
Late responder (> day 28, < day 56)	3	3
Nonresponder	-	2
Induction death	-	-
Remission death	-	-
Relapses	-	4
Bone marrow	-	3
CNS	-	1

All relapses so far were in the rapid rotation arm. Two relapsed patients had an initial WBC >300/nl. For the total group of the patients with a WBC >100/nl the event-free survival rate estimated by the life-table method is 0.79 (SD=0.08) at 45 months (Fig. 2), which is similar to those of the total high-risk group and also the low-risk group (Fig. 3).

Chemotherapy was generally reasonably well tolerated, the main side effects being stomatitis from methotrexate and infectious complications. However, no patient was lost due to the complications of chemotherapy.

### Discussion

As in other malignancies also in ALL the presence of a large tumor burden increases



**Fig. 2.** Event-free survival rate in high-risk patients according to the WBC

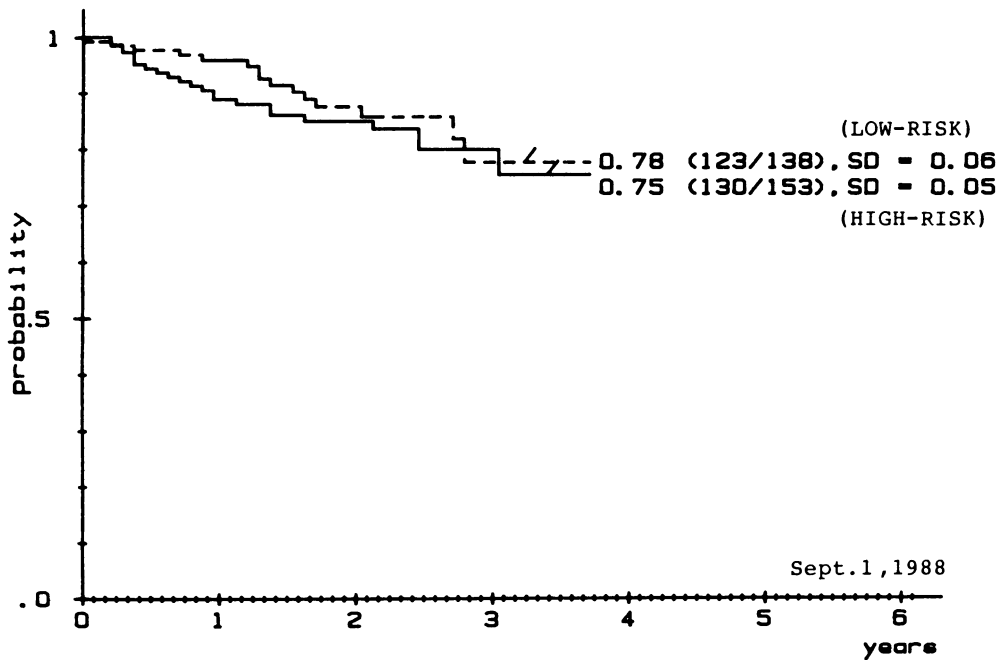


Fig. 3. Event-free survival rate of high-risk patients compared with low-risk patients

the risk for the emergence of resistant cell lines which are considered to arise from spontaneous mutations [8–10]. It is reasonable to assume that the diversity of the malignant clones might be best countered by a diversity of drug combinations. According to the model of Goldie and Coldman, a therapeutic advantage could be expected if no drug combination is given over a prolonged period but is rather alternated with other non-cross-resistant drugs [11, 12].

In the present study for high-risk patients six different drug combinations were used and no combination was given for longer than 6 weeks. In the rapid rotation arm the combinations were even already changed after 2–3 weeks. From the present results it appears that in patients with a WBC >100/nl the rapid rotation arm offers no advantage over the slow rotation arm and may even ultimately turn out to be inferior. However, the mean WBC in this group was higher (280 vs. 208/nl) and four out of six events occurred in children with a WBC >300/nl. Nevertheless, also in the total group of high-risk patients there is a trend toward better results in the slow rotation

arm: relapse-free interval at 45 months in 74 patients is 0.89 vs. 0.66 in the rapid rotation arm ( $P=0.17$ ). This suggests that longer exposure to a drug combination may be necessary to achieve optimal results.

Although the results in our group of poor prognosis patients with a WBC >100/nl still have to be considered preliminary, the event-free survival rate of 0.79 compares favorably with other studies [2, 5–7] and emphasizes the fact that treatment remains the most important prognostic variable.

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## Prognosis of Initial CNS Involvement in Acute Lymphoblastic Leukemia Childhood

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### Aim of the Study

The introduction of an effective prophylaxis of the CNS involvement represented great progress in the treatment of acute lymphoblastic leukemia (ALL), since until some years ago CNS involvement was considered to be incurable once it became manifest. In the Berlin-Frankfurt-Münster BFM-ALL studies isolated CNS relapses occur relatively seldomly, but manifestation of a CNS-leukemia by the time of diagnosis is still considered a risk factor for subsequent relapses [1]. We analyzed the prognosis of the initial CNS involvement in 41 children from four multicenter ALL therapy studies of our Arbeitsgemeinschaft für Pädiatrische Hämatologie und Onkologie of the GDR. In the GDR-ALL-VII (81) study, which is a slightly modified ALL-BFM 81-protocol, we also investigated the patients' characteristics with an initial CNS involvement in comparison with the total group of patients of this study in order to discover associations with other risk factors. Finally, we tried to find out whether the signs of the initial CNS manifestation are important for the prognosis. The question for the prognostic risk of a "smoldering" meningeal leukemia was of special interest, i.e., the findings of leukemic

blasts in the CSF without elevation of the CSF cell count.

### Patients and Methods

The evaluation covered all *patients*, who were treated according to protocols of the GDR-ALL studies, including the early death cases and nonresponders. Up to 20 November 1988 we evaluated: study ALL-IV, 111 children from the years 1976–1977; studies V (low-risk patients) and VI (high-risk patients according to the Memphis criteria), 267 children from the years 1978–1981; and study VII, 525 children, who were admitted from June 1981 to December 1987 as new cases of ALL.

The following diagnostic criteria were used for the *diagnosis of the initial CNS involvement*:

1. Leukemic blast cells in the CSF when diagnosing ALL. For that purpose cytological preparations of the CSF were made from all children according to the sedimentation chamber method of Sayk [2] or were produced by means of cytocentrifugation and assessed with panoptic staining according to Pappenheim. Patients with a normal amount of cells ( $\leq 5 \times 10^6$ /liter), but characteristic blast cells in the CSF, were considered to have a "smoldering" form of an initial meningeal leukemia.
2. Children with neurological signs (cranial nerve paresis and/or considerable cerebral compression) or the pathological-anatomical verification of a CNS leukemia in early death cases.

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The *treatment* of the patients according to the protocols ALL-IV (76) and ALL-V corresponds fundamentally with the Memphis studies VII and VIII. The GDR-ALL-VI study (78) (only for high-risk patients according to the Memphis risk criteria) corresponds with the LSA<sub>2</sub>-L<sub>2</sub> protocol (for details see [3]). The GDR-ALL-VII (81) study is a slightly modified ALL-BFM-81 study (for details see [4]).

To all patients with initial CNS involvement generally methotrexate was administered intrathecally (ITMTX) once a week for 4 weeks parallel with the induction therapy. After that the patients in studies IV, V, and VI in most cases received cranial irradiation (24 Gy) during the so-called consolidation phase (between the 5th and 12th week after the treatment began) combined with further ITMTX administration, and in a smaller number of cases craniospinal irradiation or intrathecal administration of radiogold colloid. The patients in study ALL-

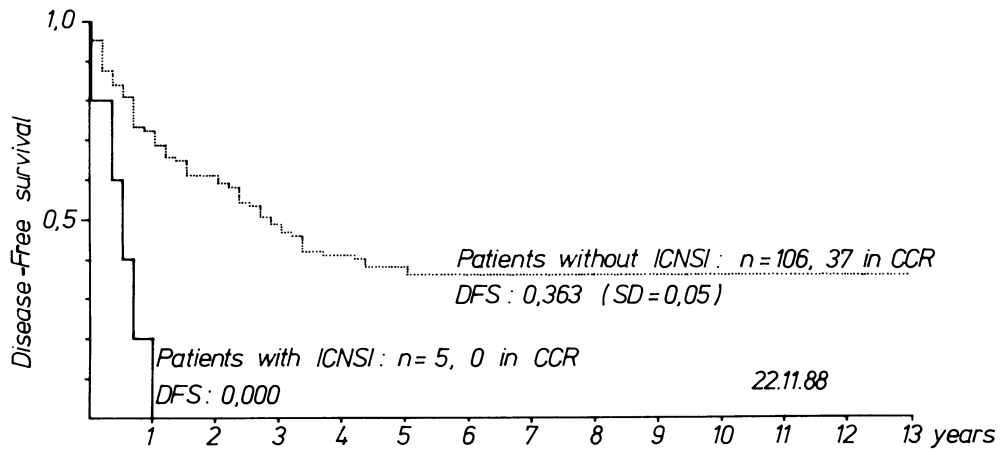
VII (81) normally received a cranial (30 Gy) and spinal (24 Gy) irradiation during the I/2 phase. As maintenance, ITMTX was administered to the patients in study IV every 12 weeks, and to those in studies V, VI, and VII every 8–12 weeks for 3 years.

## Results

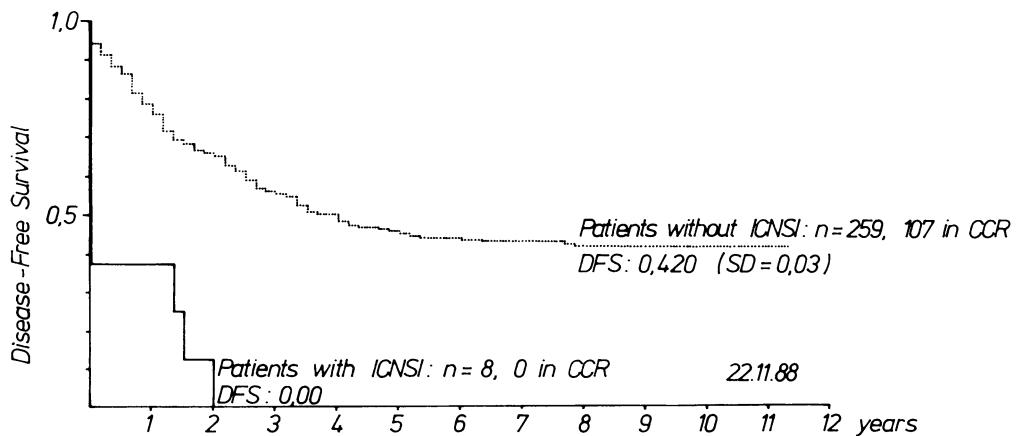
The influence of the initial CNS involvement on the duration of the first remission is given in Table 1. Figures 1–3 are based on the life-table analysis by Kaplan and Meier [5]. Of the children with ALL and initial CNS involvement, all five GDR-ALL-IV-study patients have died after 1 year and all eight patients in studies ALL-V and ALL-VI have died after 2 years. On the other hand, the disease-free survival (DFS) rate for 28 patients with initial CNS involvement in study ALL-VII is 0.51 (SD=0.10) after 5.5 years and does not differ significantly

**Table 1.** Influence of initial CNS involvement (ICNSI) on duration of the first remission in four ALL therapy studies: GDR-ALL-IV (76) and V (78), which are essentially derived from Memphis studies VII and VIII; GDR-ALL-VI (78), according to the LSA<sub>2</sub>-L<sub>2</sub> protocol, used by us for patients with a high risk of relapse after the Memphis criteria; and GDR-ALL-VII (81), according to a slightly modified ALL-BFM 81 study

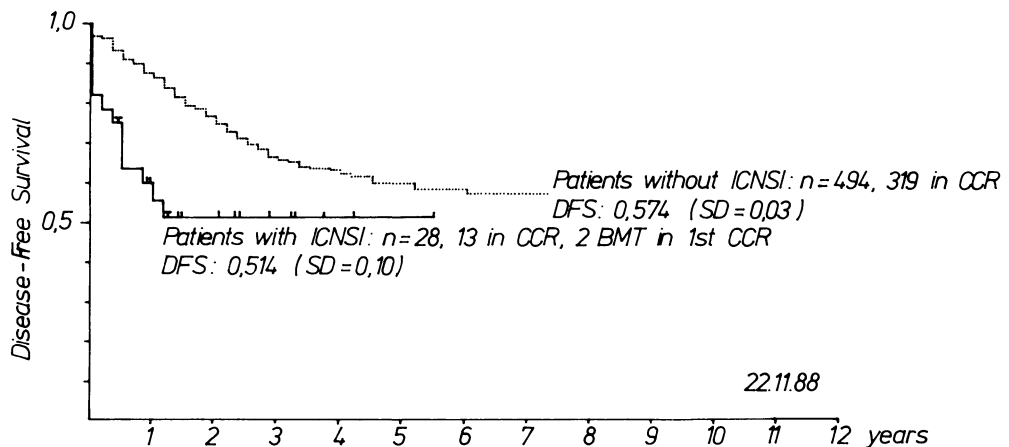
	Study IV (76)				Study V + VI (78)				Study VII (81)			
	ICNSI				ICNSI				ICNSI			
	Positive		Negative		Positive		Negative		Positive		Negative	
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
Patients, total	5	100	106	100	8	100	259	100	28	100	494	100
Death prior to remission	1	20	5	5	5	63	13	5	5	18	14	3
Complete remission achieved	4	80	101	95	3	37	246	95	23	82	480	97
Death in remission	–	–	3	3	1	13	8	3	2	7	19	4
Lost in remission	–	–	2	2	–	–	3	1	2	7	8	2
Relapses, total	4	80	59	56	2	25	128	49	6	21	134	27
Relapse sites:												
BM	2	40	46	43	2	25	71	27	4	14	69	14
CNS	1	20	5	5	–	–	29	11	–	–	19	4
CNS + BM	1	20	4	4	–	–	9	3	2	7	21	4
Others	–	–	4	4	–	–	19	7	–	–	25	5
In CCR	0	0	37	37	0	0	107	41	13	46	319	65
Surviving after relapse	0	0	8	8	0	0	33	13	2	7	55	11
Life-table analysis												
DFS	0.000		0.363		0.000		0.420		0.514		0.574	
(SD)			(0.047)				(0.031)		(0.100)		(0.029)	



**Fig. 1.** Probability of disease-free survival in patients with (—) and without (---) initial CNS involvement in the GDR-ALL-IV study (76)



**Fig. 2.** Probability of disease-free survival in patients with (—) and without (---) initial CNS involvement in the GDR-ALL-V and VI studies (78)



**Fig. 3.** Probability of disease-free survival in patients with (—) and without (---) initial CNS involvement in the GDR-ALL-VII study (81)

from the DFS rate of the 494 patients without initial CNS involvement (0.57; SD=0.03).

In all studies the rate of early death cases and non-responders is very high in children with initial CNS involvement. Out of the first relapses of the patients who achieved remission, only one child (study IV) suffered from an isolated CNS relapse. Among the first relapses, isolated bone marrow relapses (study IV, 2/4; V and VI, 2/2; VII, 4/6) and combined BM-CNS relapses (IV, 1/4; VII, 2/6) dominated. In all the studies the CNS involvement proves not to be the main prognosis-limiting factor, but the initial CNS involvement correlates with a lower remission rate in comparison with the complementary patients without CNS involvement (study IV, 80% vs. 95%, V and VI, 37% vs. 95%; VII, 82% vs. 97%). And while all patients

with initial CNS involvement in studies IV–VI finally died from a relapse – with the exception of one child, who died in remission – the relapse rate of patients in remission with an initial CNS involvement in study VII (6/23=26%) is approximately at the same level as in patients without initial CNS involvement (134/480=28%).

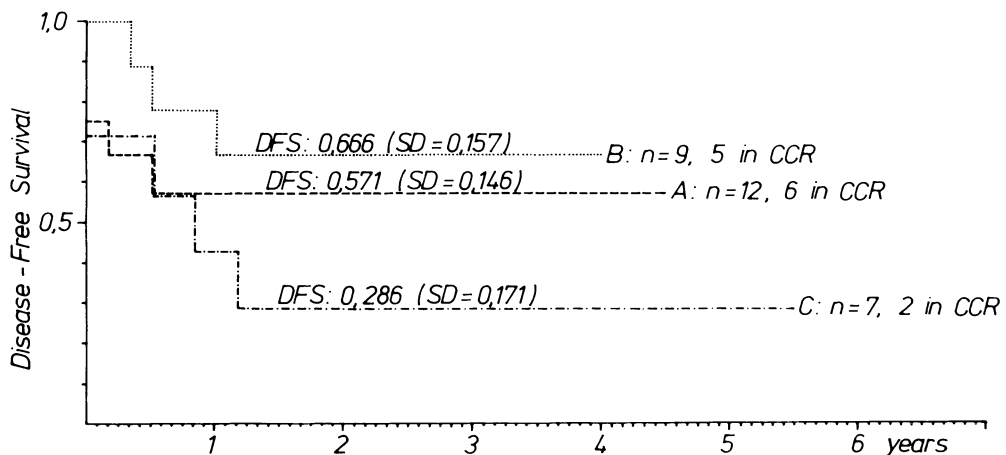
As can be seen from Table 2, among the patients with initial CNS involvement are significantly fewer children with a BFM risk factor [6] of less than 1.2 and leukocyte values of less than  $25 \times 10^9$ /liter; on the other hand, there are significantly more patients with a risk factor above 1.7, with leukocyte values above 25, 50, and  $100 \times 10^9$ /liter, with essential enlargement of the liver and spleen, and with a mediastinal mass.

A differentiated analysis of the subgroups of our patients with an initial CNS involve-

**Table 2.** Comparison of patient characteristics in study ALL-VII: total group of the ALL-VII study versus the group of patients with initial CNS involvement (ICNSI)

	ALL study VII				P
	Patients total		Patients with ICNSI		
	n	%	n	%	
Patients	525	100	28	5	
Risk factor					
< 1.2 = standard risk	342	65	13	46	0.05
1.2–1.7 = medium risk	150	29	9	32	NS
> 1.7 = high risk	33	6	6	21	0.01
Age at diagnosis					
< 2 years	53	10	6	21	NS
> 10 years	85	16	7	25	NS
White blood cells count (Gpt/liter)					
≤ 25	358	68	13	46	0.025
> 25	167	32	15	54	0.025
> 50	103	20	14	50	0.001
> 100	56	11	9	32	0.001
Platelet count (Gpt/liter)					
< 10	75	14	4	14	NS
≥ 10 < 20	88	17	6	21	0.05
≥ 20	361	69	18	64	NS
Enlargement of the liver ≥ 5 cm	157	30	14	50	0.025
Enlargement of the spleen ≥ 5 cm	133	25	15	54	0.01
Mediastinal mass	56	11	8	29	0.01
Dead patients	123	25	13	46	





**Fig. 4A–C.** Probability of disease-free survival in patients with initial CNS involvement differentiated for three subgroups: **A** Patients with elevated CSF cell count ( $> 5 \times 10^6$ /liter) and lymphoblasts in the CSF; **B** Patients with lymphoblasts in CSF but normal CSF cell count ( $\leq 5 \times 10^6$ /liter); **C** Patients with neurological signs or CNS involvement demonstrated by postmortem examination

ment regarding associated risk factors seems questionable to us because of the low number of patients. Figure 4 shows a life-table analysis of the three subgroups with the following results: In group A of the 12 patients (6 in CCR), where the investigation of the CSF revealed an elevated cell count and lymphoblasts, the DFS is 0.571 (SD = 0.146). In group B of nine patients (five in CCR) with lymphoblasts in the CSF and normal cell count it is 0.666 (SD = 0.157) and thus does not differ significantly from group A. Group C of seven patients (two in CCR), in which a CNS involvement was diagnosed on the basis of neurological signs or postmortem investigation, has a significantly lower DFS of 0.286 (SD = 0.171) as expected.

### Discussion and Conclusions

1. The therapy according to BFM studies has much improved the prognosis of the initial CNS involvement in ALL in childhood. The DFS rate for all non-B-ALL patients of our ALL-VII (81) study in total is significantly lower than that of the ALL-BFM 81 study [1] (0.56 vs. 0.69). On the other hand, in patients with initial CNS involvement the DFS rate is about at the same level as in all BFM studies [1]

together (0.51 vs. 0.48) and not significantly different from the patients without initial CNS involvement in our ALL-VII study (0.51 vs. 0.57).

2. In initial CNS involvement the remission rate is reduced in comparison with patients without initial CNS involvement (ALL-VII: 82% vs. 97%), but the relapse rate of the patients in remission is about at the same level (26% vs. 28%), although in the group of children with initial CNS involvement are found significantly less standard-risk patients and significantly more high-risk patients. Also, hyperleukocytosis, essential liver and spleen enlargement, and mediastinal tumors were significantly more often found in our patients with initial CNS involvement.
3. The initial smoldering meningeal leukemia has only a trend toward a higher DFS rate (0.666 vs. 0.571) without significance in the life-table analysis. This reinforces our opinion, gained from casuistics, that this form of CNS involvement also requires an efficient CNS therapy.

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## Central Nervous System Relapse Prevention in 1165 Standard-Risk Children with Acute Lymphoblastic Leukemia in Five BFM Trials

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### Introduction

In children with acute lymphoblastic leukemia (ALL), relapse in the CNS is still a major limiting factor for cure [1]. CNS relapse prevention by cranial irradiation plus intrathecal methotrexate [2], while adequately protective for most patients, is stigmatized by considerable neurological and other sequelae [3]. For standard-risk ALL children (60% of all patients), the BFM study group has addressed the necessity of cranial irradiation in a risk-adapted and randomized fashion in trials ALL-BFM 81 and 83. Together with the historical controls of the preceding trials ALL-BFM 70, 76, and 79, the overall as well as the CNS relapse incidence in these trials is presented here.

### Patients and Methods

In the ALL-BFM trials 70, 76, 79, 81, and 83, remission was achieved in 1143 out of 1165 standard-risk patients (98%) by an intensive induction regimen (protocol I), followed by maintenance therapy with daily 6-MP and weekly MTX orally. Modified versions of the induction regimen (protocol II and III) were used for reinduction. In the first three ALL-BFM trials, CNS prophylaxis for all children consisted of cranial irra-

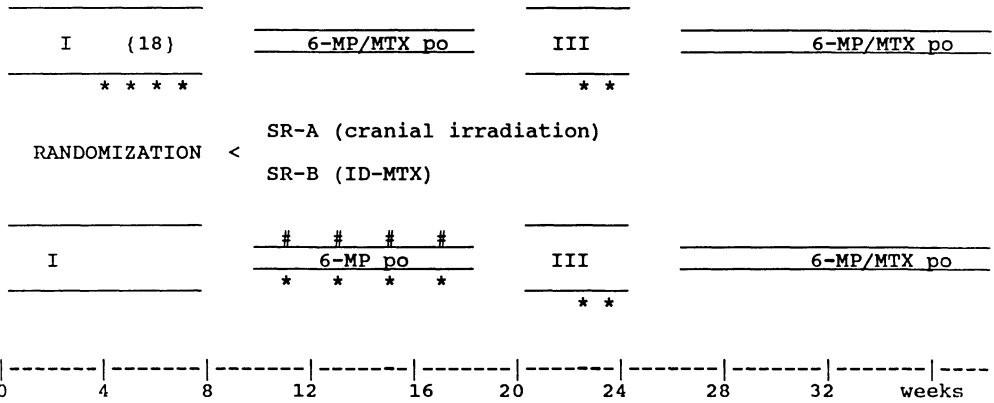
diation (18 or 24 Gy, reduced in patients below 3 years of age) plus four to six injections of intrathecal methotrexate (with the exception of a small group of patients in 1970/1971, who were given 8.5 Gy craniospinal irradiation plus i.t. MTX).

In ALL-BFM trials 81 and 83, patients were stratified according to a RF value [4] calculated from the number of leukemic blast cells/mm<sup>3</sup> peripheral blood [*B*], and the enlargement of liver [*L*] and spleen [*S*] at the time of diagnosis (centimeters below the costal margin) using the equation [4]

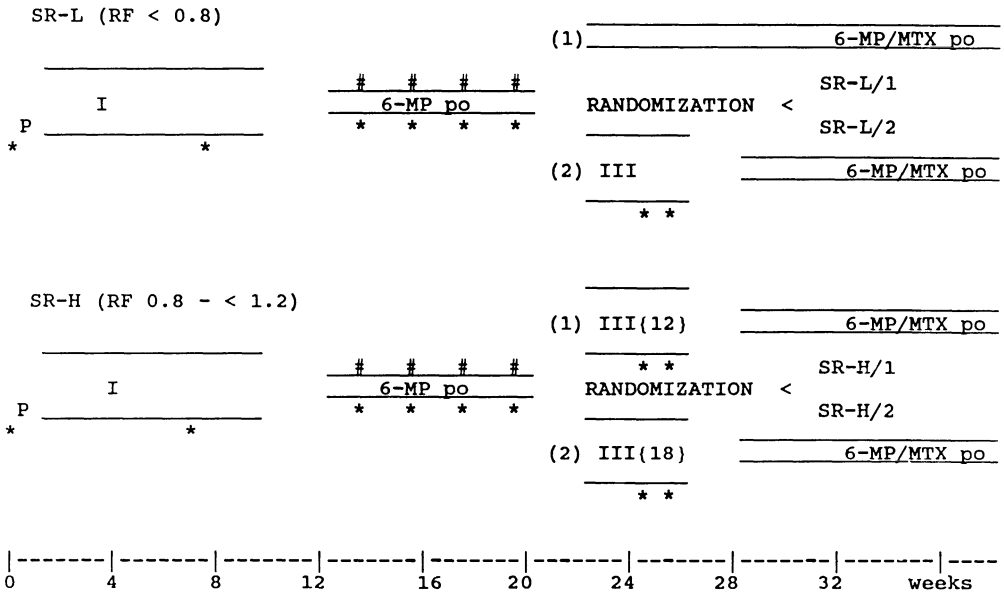
$$RF = 0.2 \times \log(B + 1) + 0.06 \times L + 0.04 \times S$$

Children with an RF < 1.2 were assigned to the standard-risk group, which comprised 60% of all patients. In trial ALL-BFM 81 (Fig. 1), all standard-risk children (RF < 1.2) received reinduction therapy (protocol III) and were randomized for either 18 Gy cranial irradiation (SR-A) or four 24-h infusions with intermediate-dose (0.5 g/m<sup>2</sup>) MTX during an 8-week period between protocol I and III (SR-B). ID-MTX was given to all children in trial ALL-BFM 83 (Fig. 2). In this trial, standard-risk patients were further subdivided into standard-risk low (SR-L) and standard-risk high (SR-H) children, the cutoff RF value between the two groups being 0.8. SR-L patients (RF < 0.8) were not irradiated, and the effectiveness of reinduction therapy with protocol III was tested by randomization (SR-L/1, without; SR-L/2, with protocol III). SR-H patients (RF 0.8 – < 1.2) were given reinduction therapy and randomized for cranial radiotherapy (RT) at doses of 12 Gy (SR-H/1) or 18 Gy (SR-H/2). For reasons of comparison, RF values

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**Fig. 1.** Trial ALL-BFM 81, standard-risk patients (RF < 1.2). *I, III*, protocols I, III; *P*, 7-day prednisone prephase; [12], [18], cranial irradiation with 12 Gy, 18 Gy; #, intermediate-dose MTX (0.5 g/m<sup>2</sup> within 24 i. v.); \*, MTX intrathecally (12 mg in children ≥ 3 years, 10/8/6 mg below 3/2/1 years of age)



**Fig. 2.** Trial ALL-BFM 83, standard-risk patients (RF < 1.2)

were calculated retrospectively for all patients enrolled in the preceding trials ALL-BFM 70, 76, and 79. Children with B-ALL were excluded from the data presented here.

### Results

In children with RF < 0.8, the relapse rate was in the range 14.3%–22.9% in three out

of four treatment strategy groups (SR-A, SR-B, SR-L/2) of the trials 81 and 83, and in all arms of the 70, 76, and 79 trials (Table 1). Differences between these arms were not significant. Only children in the 83:SR-L/1 group (who received neither radiotherapy nor reinduction therapy) had a significantly ( $P=0.01$ ) increased relapse rate (31.7%). Twelve of the 33 relapses (36.4%) observed in 83:SR-L/1 involved the CNS, whereas in

**Table 1.** ALL-BFM trials 70, 76, 79, 81, and 83: standard-risk patients (absolute numbers of patients)

Trial arm	Complete remission	Relapses	Isolated CNS relapses	Relapses with CNS involvement	Fraction of relapses with CNS involvement
Children with RF <0.8					
70, 76: A	70	16	2	3	0.19
76: B, 79	103	18	1	3	0.17
81: SR-A	77	11	0	1	0.09
81: SR-B	79	18	1	3	0.11
83: SR-L/1	104	33	3	12	0.36
83: SR-L/2	95	15	3	4	0.27
Children with RF 0.8 ≤ 1.2					
70, 76, 79	201	56	10	14	0.25
81: SR-A	103	26	4	5	0.19
81: SR-B	98	35	11	22	0.63
83: SR-H/1	84	20	2	3	0.20
83: SR-H/2	100	24	2	3	0.13

the randomization control group 83:SR-L/2 CNS involvement occurred in less than 26.7% of relapses ( $P=0.05$ ). The results of children with RF <0.8 in all other arms were statistically similar those of the 83:SR-L/2 group. The rate of isolated CNS relapses was not affected significantly.

In irradiated children with an RF between 0.8 and 1.2, the incidence of relapses involving the CNS was in the range of 3%–7%. The rate of isolated CNS relapses was between 2% and 5%. Differences between individual treatment arms did not reach significant levels. However, a dramatic increase in CNS relapses was seen in children with RFs between 0.8 and 1.2 of the 81:SR-B (no RT) subgroup: Out of 98 patients, 22 experienced a relapse involving the CNS. Eleven of these relapses were restricted to the CNS. These increases were of statistical significance ( $P=0.007/0.04$ , respectively). In the 81:SR-B subgroup with RF between 0.8 and 1.2, the CNS was involved in 63% of all relapses, whereas in all other arms the percentage of CNS involvement was in the range between 13% and 25%.

## Discussion

The data of the five ALL-BFM trials 70–83 presented here demonstrate the importance

of initial disease features for assigning ALL children to less toxic therapeutic regimens without increasing overall and CNS relapse rates. In the ALL-BFM trials 81 and 83, an RF value was calculated from leukemic blasts/mm<sup>3</sup> peripheral blood, liver, and spleen size. Children with an RF <0.8 did not require irradiation as long as reinduction therapy and intermediate-dose MTX were given. This could be shown directly by randomization (SR-L/1 vs. SR-L/2) in the ALL-BFM 83 trial. When reinduction therapy (SR-L/1) was omitted, the relapse rate increased from 15.8% to 31.7%.

Children with an RF between 0.8 and 1.2 could not be protected by ID-MTX and reinduction therapy alone. Substitution of irradiation resulted in an increase of CNS relapses (isolated and combined) from 5% to 22% (randomization SR-A vs. SR-B in trial ALL-BFM 81). The dosage of irradiation, however, could be lowered from 18 to 12 Gy (randomization ALL-BFM 83:SR-H/2 vs. H/1) without negatively affecting the relapse rate in any compartment. It has to be noted that this was tested in children receiving ID-MTX plus reinduction therapy (protocol III).

These findings gain importance with regard to reports about adverse late side effects of cranial irradiation, ranging in severity from mild neurological and endocrine

dysfunction to progressive necrotizing encephalopathy [5–7] and secondary neoplasms [8–10]. In humans who had received cranial irradiation for reasons other than neoplastic disease (ringworm of the scalp), a strong relation was found [11] between the incidence of brain tumors and the amount of irradiation administered, with the relative risk approaching 20 after estimated doses of 2.5 Gy. We show here that it is possible to abandon or substantially reduce radiotherapy by administration of intermediate-dose MTX (0.5 g/m<sup>2</sup>) plus reinduction therapy. High-dose MTX (5 g/m<sup>2</sup>), as given in the ongoing ALL-BFM 86 trial, might further reduce the necessity of radiotherapy.

### Summary

In treatment of childhood ALL, prevention of CNS relapse by cranial irradiation is followed by considerable long-term sequelae. In the three ALL-BFM (Berlin-Frankfurt-Münster) trials 70, 76, and 79, employing radiotherapy (8.5 Gy craniospinal, 18 or 24 Gy cranial irradiation) in all arms, the incidence of CNS relapses (isolated and combined) in standard-risk patients (SR, 60% of all children) was consistently <6%. A risk factor (RF) calculated from absolute blast number, liver, and spleen size at diagnosis was used to stratify patients in the subsequent trials ALL-BFM 81 and 83. In ALL-BFM 81, SR patients (RF < 1.2) were randomized to receive 18 Gy cranial irradiation or intermediate-dose i.v. methotrexate (ID-MTX) (4 × 0.5 g/m<sup>2</sup>). In ALL-BFM 83, the SR group was further subdivided into group SR low (SR-L, RF < 0.8) and group SR high (SR-H, RF 0.8–< 1.2). SR-L patients received no irradiation, and were tested by randomization for the effectiveness of an intensive reinduction regimen (protocol III). SR-H patients were randomized for 12 or 18 Gy.

The results were as follows: In patients of both trials with RF < 0.8, radiotherapy could be replaced by ID-MTX plus protocol III. Without protocol III, relapses increased from 15.7% to 31.7%. Concomitantly, the fraction of relapses with CNS involvement increased from 26.7% to 36.4%. However, SR patients with RF between 0.8 and 1.2

could not be protected by reinduction alone (isolated/overall CNS relapse rate with irradiation, 4%/5%; without irradiation, 11%/22%). Dosages of 12 and 18 Gy were found to be equally protective.

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## Effective Prevention of Central Nervous System Leukemia with Intrathecal Methotrexate and Intrathecal Methotrexate, Cytosine Arabinoside, and Hydrocortisone in Childhood Acute Lymphocytic Leukemia

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### Introduction

Early attempts to prevent CNS leukemia employed 500–1200 rad craniospinal (CS) irradiation early in remission. However, the frequency of CNS leukemia was similar to that seen if no preventative therapy was given. The combination of 2400 rad cranial irradiation plus intrathecal (i.t.) methotrexate (MTX) proved highly effective. The value of 2400 rad CS radiation to prevent CNS leukemia was also determined in a controlled study. Therefore, 2400 rad CS irradiation and 2400 rad cranial irradiation plus simultaneous i.t. MTX seemed equally effective for prevention of CNS relapse [1]. In view of the fact that spinal irradiation might impair growth in height and also limit marrow reserve, cranial irradiation plus i.t. MTX was indicated as the preferred method of CNS preventative therapy. With this method, the likelihood of primary CNS relapse is 10% or less [2]. The dosage of 1800 rad cranial irradiation plus i.t. MTX for a standard-risk patient has been proven to be as effective as 2400 rad and to be less toxic to the CNS. The use of chemotherapy alone to prevent CNS leukemia has been studied by Freeman et al., utilizing simultaneous i.v. intermediate-dose MTX (500 mg/m<sup>2</sup> with one-third as a push dose and the remaining two-thirds as a 24-h infusion) and i.t. MTX three times at 3-week intervals following the remission of induction [3]. Others have attempted to

control CNS disease with periodic i.t. therapy alone with comparable results and less toxicity to the CNS [4, 5], but a randomized study by the Children's Cancer Study Group indicated clearly the inferior results obtained in a group of patients (group IV) receiving i.t. therapy as the sole prevention of CNS leukemia [6]. In our previous series, CNS leukemia interrupted continuous complete remission in 16% of patients in the standard-risk category receiving 1800 rad cranial irradiation plus simultaneous i.t. MTX given immediately following the attainment of complete remission [7]. This high incidence of CNS relapse was unacceptable and changes had to be made. The option of using i.t. chemotherapy was chosen and the results are reported here.

*Complete remission bone marrow (M-1)* is defined as a cellular marrow demonstrating satisfactory hematopoiesis and containing 5% or less lymphoblasts plus stem cells. A hypocellular marrow specimen containing mainly peripheral blood cells is considered unsatisfactory and not a remission marrow.

*Complete remission* means that the patient is free from symptoms and physical findings attributable to leukemia, the peripheral blood contains 500 mm<sup>3</sup> or more neutrophils, and 75 000/mm<sup>3</sup> or more platelets, and the marrow demonstrates complete remission status. Granulocytopenia and thrombocytopenia obviously secondary to therapy and reversible by dosage adjustment do not terminate remission.

*Continuous complete remission duration* is the time between the first complete remission marrow and first evidence of marrow relapse, whether hematologic, CNS, or visceral.

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*Relapse* is defined as the reappearance of leukemia as manifested by a partial remission marrow, persistent or progressive to a relapse marrow, or by the appearance of CNS leukemia, or visceral infiltration or any symptoms or signs definitely attributable to leukemia.

*Continuous hematologic remission duration* is the time between the first complete remission marrow and first evidence of marrow relapse. Thus, hematologic remission duration includes periods with leukemia in the CNS or other sites outside the marrow.

*Time to CNS leukemia* is the period between the first complete remission marrow and the first evidence of CNS leukemia.

*Survival* is the period between diagnosis of leukemia and death.

*Early death* refers to patient dying within the 1st month from diagnosis and not receiving a full induction course of therapy.

## Evaluation of Patients

Confirmation of the diagnosis of ALL always preceded the institution of specific therapy. The initial evaluation includes physical examination and anthropometric values and laboratory and radiological studies. All pertinent analyses and studies are repeated according to the phase of therapy. The presence of CNS leukemia is determined by the presence of leukemic blasts, regardless of number, in the CSF; and the presence of thymic enlargement is determined on plain chest X-rays. A diagnosis of testicular leukemia is tissue proven when leukemic cells are found in samples obtained through either percutaneous fine-needle aspiration or surgical biopsy. These procedures are performed in the presence of testicular enlargement and/or induration. Patients with B-cell ALL are assigned to a separate treatment program.

## Outline of Therapy

The outline of therapy and starting dosages of drugs utilized are shown in Table 1. Patients failing to attain remission in 6 weeks received asparaginase (Asp) and arabinosyl cytosine (Ara-C) for 2–4 weeks. If failure

persisted, the patients received teniposide (VM-26) and Ara-C for 2–4 weeks. Patients who develop CNS leukemia during maintenance therapy phase receive an i.t. combination of MTX, Ara-C, and hydrocortisone (HC) weekly for six doses and then monthly until at least 1 year of a second CR is attained. The doses of i.t. MTX, Ara-C, and HC are shown in Table 1. For these patients, before stopping all therapy, the cranium receives 2400 rad given in 14 fractions over a period of 24 days and through portals including the cervical spine; and the spine is treated through posterior fields interlacing at portal junctions. Dosage of spinal irradiation is limited to 1800 rad given in 14 fractions.

For testicular leukemia, 2000 rad irradiation is given in ten sessions over a period of 2 weeks. Radiation is given to testis or testes according to tissue-proven involvement with leukemic cells. Radiation is given to full thickness of the testis involved and using an anterior portal.

All therapy is scheduled to be discontinued after 24 months of CCR or 12 months of the second CR following treatment for isolated extramedullary relapse as described above.

## Patients

Eighty consecutive patients, 6 months to 13 years old (median, 5 years) entered this study from 1 January 1984 to 31 December 1986 [8]; 54 were boys and 26 were girls (Fig. 1). The initial peripheral white blood cell count ranged from 300 to 1 040 000/mm<sup>3</sup> with a median of 18 600/mm<sup>3</sup> (Fig. 2). Fourteen of the patients had counts greater than 100 000/mm<sup>3</sup>, 10 had counts between 50 000 and 99 999/mm<sup>3</sup>, 10 had counts between 25 000 and 49 999/mm<sup>3</sup>, 11 had counts between 10 000 and 24 999/mm<sup>3</sup>, and 35 had counts less than 10 000/mm<sup>3</sup>. Seventeen patients had thymic involvement (one also had CNS and testicular involvement), two had isolated CNS leukemia, and four had testicular infiltration at the time of diagnosis. Sixty-nine patients had L-1 and 11 had L-2 FAB morphology. Patients with L-3 FAB morphology were treated according to another protocol. Bone marrow cytogenetic



**Table 1.** Outline and drug dosages of childhood acute lymphocytic leukemia (ALL-KFSH-84 protocol)

## Phase I – induction of remission (6 weeks)

Prednisone p. o.	40 mg/m <sup>2</sup> /day × 28 days only
Vincristine i. v.	1.5 mg/m <sup>2</sup> /week × 6 doses (days 1, 7, 14, 21, 28, 35)
Daunomycin i. v.	25 mg/m <sup>2</sup> /week × 4 doses (days 1, 7, 14, 21)
VP-16 i. v.	250 mg/m <sup>2</sup> /week × 4 doses (days 1, 7, 14, 21)
Cyclophosphamide i. v.	600 mg/m <sup>2</sup> /week × 2 doses (days 28, 35)
Methotrexate i. t.	12 mg/m <sup>2</sup> /week × 6 doses (days 1, 7, 14, 21, 28, 35)
Note: Vincristine i. v.	2.0 mg maximum dose/injection
Methotrexate i. t.	12 mg maximum dose/injection in all phases of therapy

## Phase II – 1-year maintenance chemotherapy (54 weeks)

6-Mercaptopurine p. o.	75 mg/m <sup>2</sup> /day
Methotrexate p. o.	40 mg/m <sup>2</sup> /week
Methotrexate i. t. <sup>a</sup>	12 mg/m <sup>2</sup> every 4 weeks

## Phase III – reinduction chemotherapy during maintenance phase (4 weeks)

## Subphase A

VM-26 i. v.	165 mg/m <sup>2</sup> twice a week × 4 doses
Ara-C i. v.	300 mg/m <sup>2</sup> twice a week

## Subphase B

Asparaginase	10 000 units/m <sup>2</sup> twice a week × 4 doses
Ara-C	300 mg/m <sup>2</sup> twice a week

## Phase IV-2nd-year maintenance chemotherapy (54 weeks)

6-Mercaptopurine p. o.	50 mg/m <sup>2</sup> /day
Methotrexate p. o.	25 mg/m <sup>2</sup> /week
(Methotrexate i. t. <sup>a</sup>	12 mg/m <sup>2</sup> /dose)
(Ara-C	30 mg/m <sup>2</sup> /dose) every 8 weeks
(Hydrocortisone (HC)	30 mg/m <sup>2</sup> /dose)

## Phase V – late intensification phase (4 weeks)

Prednisone p. o.	40 mg/m <sup>2</sup> /day × 28 days only
Vincristine i. v.	1.5 mg/m <sup>2</sup> /week × 4 doses
Adriamycin i. v.	40 mg/m <sup>2</sup> /every 2 weeks × 2 doses

## Backup chemotherapy for patients failing to achieve remission in the first 6 weeks

## Schedule 1

## Simultaneous:

Ara-C i. v.	300 mg/m <sup>2</sup> twice a week × 4–8 doses
Asparaginase i. v.	10 000 units/m <sup>2</sup> twice a week × 4–8 doses

If failure occurs again,

## Schedule 2

## Simultaneous:

VM-26 i. v.	165 mg/m <sup>2</sup> twice a week × 4–8 doses
Ara-C i. v.	300 mg/m <sup>2</sup> twice a week × 4–8 doses

## Contingency plans for extramedullary relapses during maintenance phase

1. CNS leukemia – combination of i. t. methotrexate 10 mg/m<sup>2</sup>, Ara-C 30 mg/m<sup>2</sup>, and HC 30 mg/m<sup>2</sup>/week × 4–6 doses, then monthly if patient achieves a second complete remission
2. Testicular leukemia (biopsy proven) – radiotherapy to the involved testicle(s), 2000 rad (ten fractions)

<sup>a</sup> Oral methotrexate is omitted the week i. t. methotrexate is given

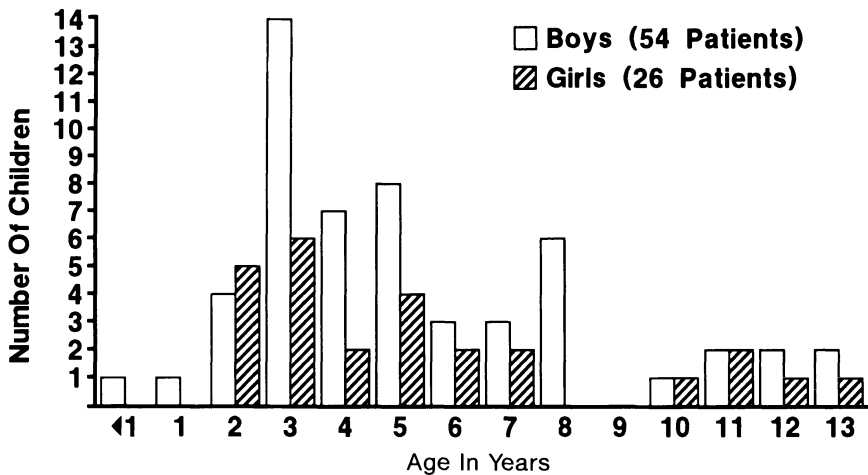


Fig. 1. Initial peripheral WBC/mm<sup>3</sup> of 80 children

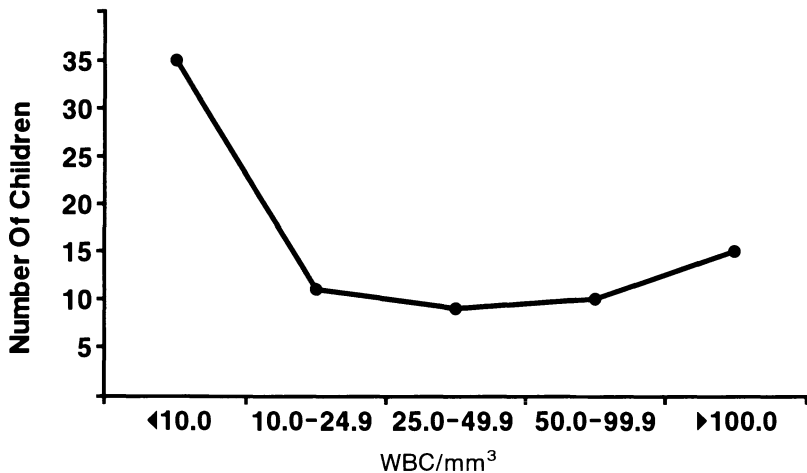


Fig. 2. Age and sex of 80 children

studies were successfully performed in 45 of the patients and normal clones were observed in 31 of them. Fourteen patients had abnormal clones.

## Results

The clinical outcome of the 80 patients is depicted in Table 2 (results from 1 January 1984 through 31 December 1988). The actuarial graphic representation of the continuous complete remission duration is depicted

in Fig. 3. The minimum follow-up for all surviving patients is 24 months. Thirty-seven patients (61%) remain in continuous complete remission from 23 to 60 months (median, 33 months). The clinical and laboratory features of the three patients who experienced CNS relapse are shown in Table 3.

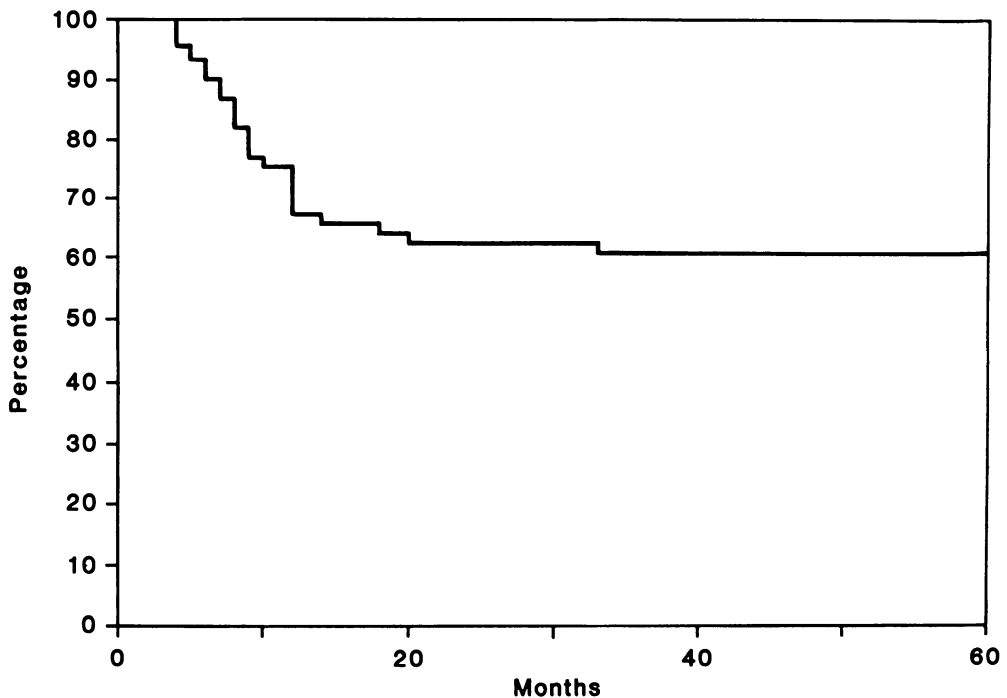
The first patient developed CNS leukemia at 9 months into remission, was retreated, but relapsed in the bone marrow 4 months later. He attained subsequent remission, received bone marrow allogeneic graft twice, and has been well now without evidence of

**Table 2.** ALL-KFSH-84 study, 1 January 1984 to 31 December 88: current outcome

# Patients	80
Receiving induction	0
Induction failures	14
Left	3
Died	9
Failed	2
Attained CR	66
Refused therapy	5
Maintenance	61
Died in CR	1
Relapses	23
Bone marrow	17
Testicular	1
Bone marrow and testicular	1
Central nervous system	3
Nodal	1
Taken off therapy	38
Off therapy	34
Still on therapy	3
Continuous CR	37
Relapses off therapy	4
Bone marrow	3
Test (Unil)	1

leukemia for 8 months. The second patient developed CNS leukemia at 20 months into remission, was retreated, but relapsed in the bone marrow 1 month later. She achieved subsequent remission, received bone marrow allogeneic graft, but presented with hematological relapse 7 months later. She was retreated with chemotherapy and attained a subsequent complete remission, which is being maintained on combination chemotherapy for 2 months. The third patient developed CNS relapse at 14 months into remission, was retreated, and attained a second complete remission which lasted until she received therapeutic CS irradiation and had all therapy stopped afterwards. This patient has remained well without evidence of disease for 2 months. Despite the fact that the follow-up period for these three patients is still short, two significant points need to be stressed:

1. the incidence of CNS leukemia in this study is 2.8% (one of the lowest ever reported) and



**Fig. 3.** Percentage of continuous complete remission of 61 patients – 1 January 1984 through 31 December 1988

**Table 3.** Clinical and laboratory features of the three patients developing CNS leukemia

#	Sex	Age (years)	WBC/mm <sup>3</sup>	CNS	Thymus	Gonads	FAB	Phenotype	Karyotype
1	Boy	3	102000	0	0	0	L-1	CALLA	Failure
2	Girl	3	2100	0	0	0	L-1	CALLA	46, XX
3	Girl	7	1040000	0	0	0	L-2	CALLA	t(11,19)

CALLA, common acute lymphoblastic leukemia antigen

2. these patients are long-term survivors, with two of them off Rx for 8 months (post bone marrow transplant) and 2 months (following late CS irradiation).

Three additional patients had CNS leukemia at diagnoses (CSF leukemic pleocytosis): one died during the induction of remission phase; one had bone marrow relapse at 7 months; and one had thymic infiltration at diagnosis in continuous complete remission for 30 months and off therapy for 6 months. Two other patients in continuous complete remission for 23 months developed seizures during the induction of remission phase, and two others had seizures during the maintenance phase. Neurologic workup of these four patients failed to show any abnormality, and they remain neurologically normal.

## Discussion

In this study we elected to use i.t. MTX throughout the 6 weeks of the induction of remission phase and periodically at monthly intervals during the 1st year of maintenance of remission therapy. The prevention of CNS leukemia during the 2nd year of treatment was scheduled using the combination of i.t. MTX, Ara-C, and hydrocortisone every 8 weeks. These agents enter the venous circulation and provide systemic as well as meningeal antileukemic effects. Intrathecal therapy is more widely and readily available than expert radiation therapy, especially in underdeveloped countries. It is also less expensive and avoids the encephalotoxic, hematosuppressive, growth-inhibitory, and carcinogenic effects of radiation. Radiation also has two potential disadvantages: (1) chronic CNS functional impairment does occur in some patients treated and (2) the

delivery of cranial and craniospinal irradiation limits the dosage of parenteral MTX one can safely administer during continuation therapy without inducing the emergence of crippling leukoencephalopathy [1, 2, 9].

Finally, CNS radiation can be administered more safely and probably with more efficacy to the child who has received the i.t. therapy and who experiences isolated CNS meningeal relapse than to the child who has previously received irradiation. Such a case is well documented by patient number 3, who remained in continuous hematological remission for the duration of maintenance phase and had craniospinal irradiation prior to the cessation of all therapy. Currently, the four most effective methods of preventing meningeal relapse are:

1. cranial irradiation with simultaneous i.t. MTX;
2. craniospinal irradiation;
3. intravenous intermediate-dose MTX; and
4. intrathecal therapy with MTX and/or MTX, Ara-C with/without hydrocortisone [1, 3–6, 10–12]

We elected to use the fourth method, combining i.t. MTX with Ara-C and hydrocortisone. It is of paramount importance to indicate that the significant results in preventing CNS leukemia are due to two undeniable factors:

1. efficacy of an intensive and well-tolerated i.t. chemotherapy and
2. intensive combination chemotherapy, which resulted in a 61% incidence of continuous complete remission for 23–60 months (median, 33 months).

It is our opinion that the clinical and laboratory parameters at presentation used as indicators of risks at treatment failures may be

neutralized by using a more intensive and effective chemotherapy program. Universally, near-half of the children with ALL still fail to current forms of therapy. Based upon actual biological and pharmacological knowledge, more intense or modified forms of therapeutic strategies are fully warranted. The worst prognostic factor in childhood ALL may be represented by the utilization of inadequate forms of treatment.

*Acknowledgment.* We would like to express our gratitude to Ms. Lynn Sutton for her secretarial support in typing this manuscript.

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## Risk of CNS Relapse After Systemic Relapse of Childhood Acute Lymphoblastic Leukemia

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### Introduction

During the past 5 years, in the German Berlin-Frankfurt-Münster (BFM) study group a standard treatment has been established for relapse of childhood acute lymphoblastic leukemia (ALL) [1]. In study ALL-REZ BFM 85 remission rates were improved compared with the preceding study ALL-REZ BFM 83. Furthermore, by use of methotrexate (MTX) given at a dose of 1 g/m<sup>2</sup> for 36 h there is good evidence for more effective control of bone marrow leukemia (G. Henze et al., this volume). No clear improvement was found, however, in terms of long-term event-free survival of these children, so far, because of an inappropriate high incidence of second extracompartmental relapses, especially in the CNS. The purpose of this report is to evaluate the impact of secondary CNS relapses on the prognosis of children with first isolated marrow relapse and the interrelationship between the duration of systemic disease control and the risk of subsequent extracompartmental leukemia.

### Patients and Treatments

The subject of our analysis was 72 patients of the multicentre relapse studies ALL-REZ BFM 83 ( $n=29$ ) and BFM 85 arm M ( $n=43$ ), who were treated for first isolated bone marrow relapse (bone marrow blasts > 25%, no other sites involved). Distribution of age, sex, and duration of first remission were not substantially different in either group. The proportion of early relapses (during initial treatment or within 6 months thereafter) was slightly higher in study 85M (Table 1).

**Table 1.** Characteristics of patients with bone marrow/non-CNS relapse treated in ALL relapse studies BFM 83 and 85

ALL-REZ study	BFM 83	BFM 85M
Total number	29	43
Median age (years)	8 2/12	7 3/12
Male/female	17/12	22/21
Early/late relapse <sup>a</sup>	12/17	22/21

<sup>a</sup> Early relapse = relapse during or within 6 months after the end of chemotherapy

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Treatment consisted of at least eight blocks of multidrug chemotherapy (Fig. 1) followed by maintenance with daily oral 6-thioguanine (50 mg/m<sup>2</sup>) and biweekly intravenous MTX (50 mg/m<sup>2</sup>) up to a total duration of 2½ years. Patients with early relapse additionally received an intensive remission

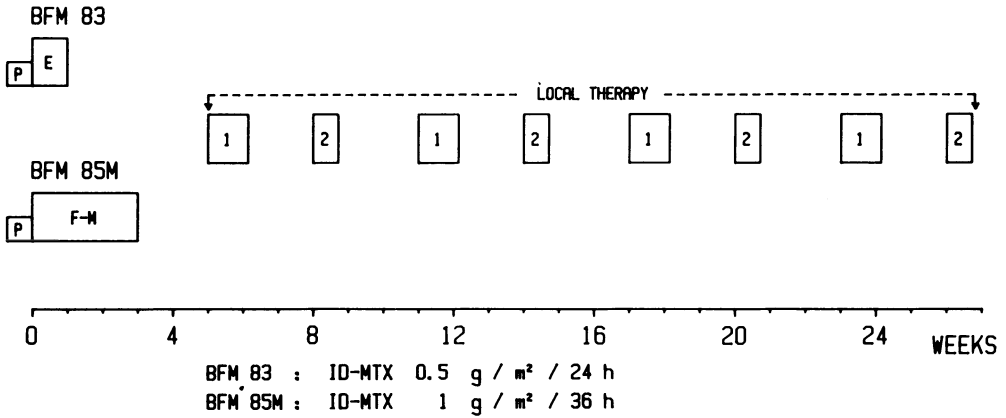


Fig. 1. Study design of relapse studies ALL-REZ BFM 83 and 85

induction protocol E in study 83 and F-M in study 85M (Fig. 2). In all treatment courses the administration regimen of intermediate-dose MTX was different in both studies, being 0.5 g/m<sup>2</sup>/24 h and 1 g/m<sup>2</sup>/36 h, respectively, in otherwise identical chemotherapy blocks R1 and R2 (Fig. 3).

### Statistical Methods

Life-table results were estimated using the Kaplan-Meier algorithm. As efficiency of treatment in avoiding further relapse was the main aspect of analysis, patients with non-response and early death were excluded

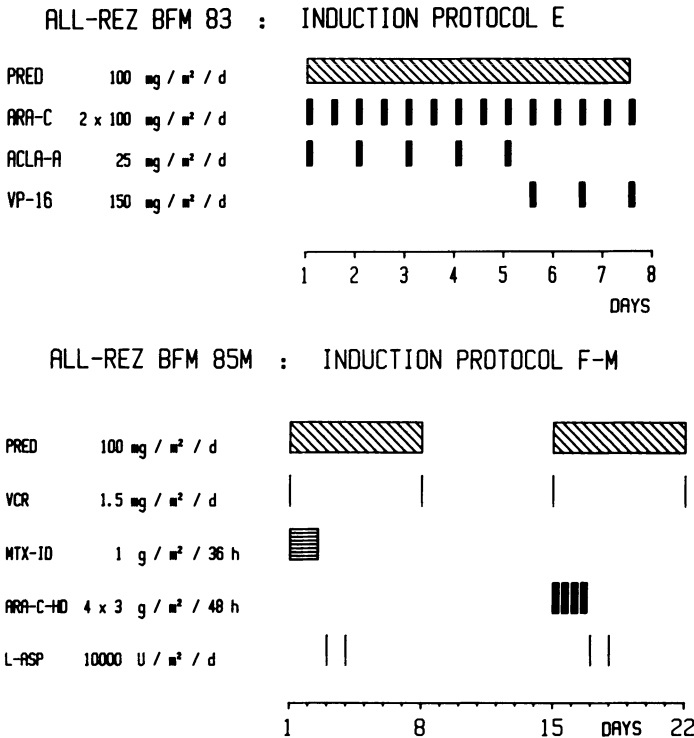
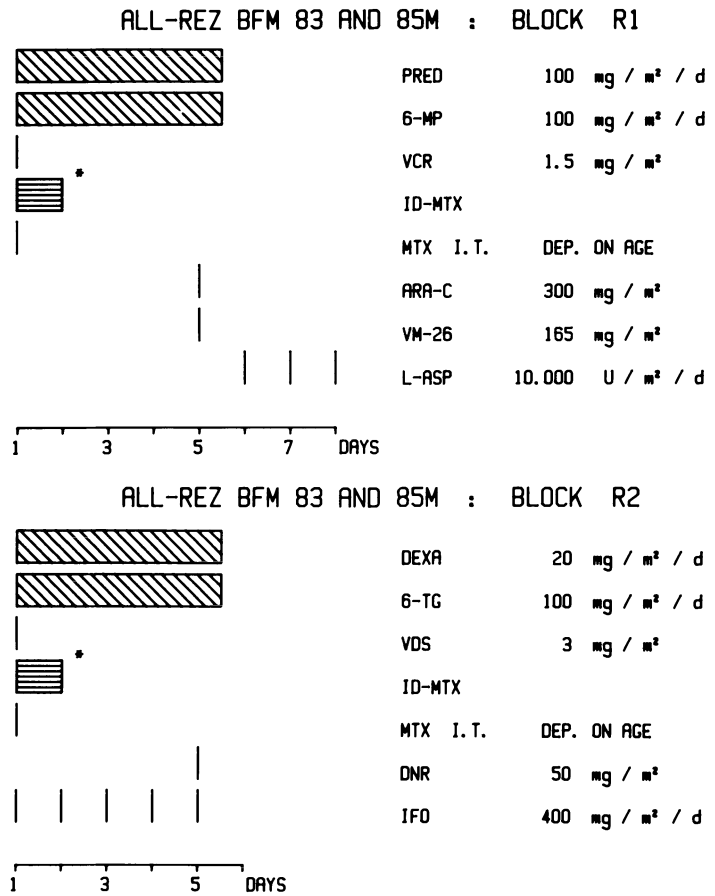


Fig. 2. Induction protocols E and F-M for patients with early bone marrow relapse in studies ALL-REZ BFM 83 and 85



**Fig. 3.** Standard block elements of relapse treatment in studies ALL-REZ BFM 83 and 85

\*) BFM 83 : ID-MTX 0.5 g / m<sup>2</sup> / 24 h  
 BFM 85M : ID-MTX 1 g / m<sup>2</sup> / 36 h

and events not associated with control of leukemia were excluded (patients were counted as lost to follow-up).

### Results

Apparent though not statistically significant differences could be observed in terms of duration of second hematological remission (bone marrow relapse-free interval: BM-RFI) between both treatment protocols (Fig. 4). In study 83, second systemic relapses occurred earlier than in study 85M. Most of the events were observed during the second half of the 1st year after the start of second CR. In study 85M, the cascade of

second systemic relapses is flatter, and the probability of remaining in second hematological remission at 3 years is about 20% higher than in study 83.

In contrast, the risk of experiencing a second adverse event in the CNS is higher for patients of study 85M (Fig. 5). Nearly all CNS relapses occurred between 18 and 24 months of second CR, and the pattern is identical to CNS relapses during front-line treatment of ALL. Thus, the event-free survival is not different in both studies, as a consequence of an increase of extracompartment leukemia as the second event in spite of improved systemic disease control in study 85M.



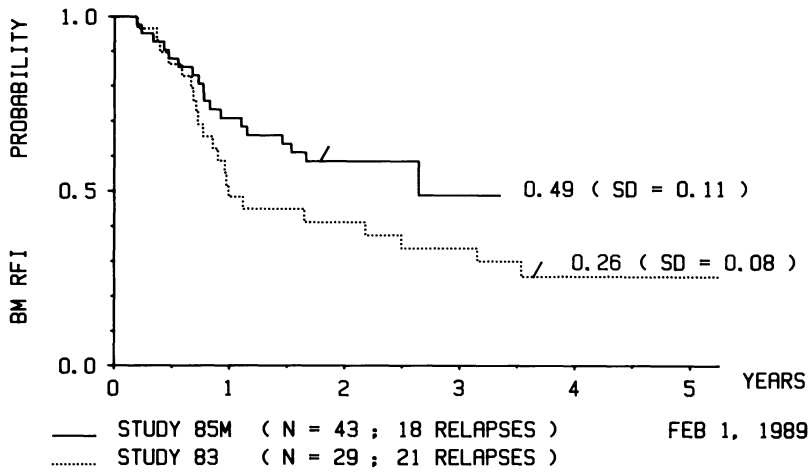


Fig. 4. Probability of continuous second hematological remission after bone marrow relapse without CNS involvement

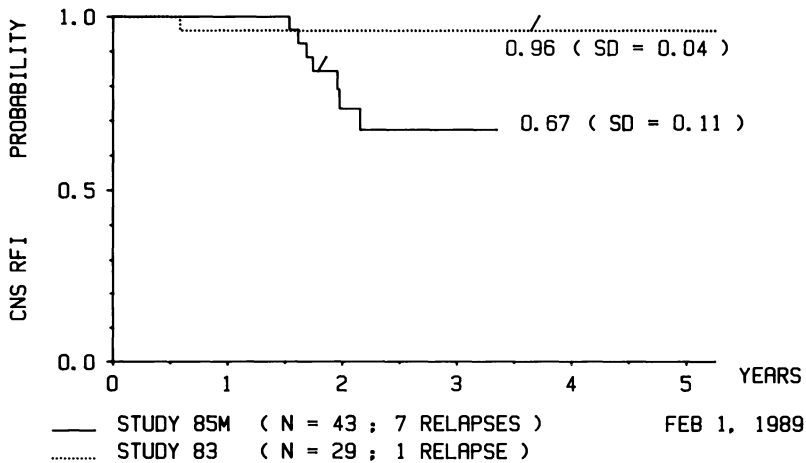


Fig. 5. Probability of preventing subsequent CNS relapse after successful reinduction treatment for bone marrow relapse without CNS involvement

## Discussion

When designing the treatment protocol, we believed that the combination of intrathecal and intravenous MTX given in each course of treatment for a total of eight courses within about 6 months would be sufficient for the prevention of secondary CNS leukemia in patients with isolated marrow relapse. Except for the introduction of protocol F for patients with early marrow relapse in study 85, which included additional-

ly high-dose cytosine arabinoside (Ara-C) as a component active in the CNS, the only difference between both studies was the MTX administration regimen. The increase of the dose from 0.5 to 1.0 g/m<sup>2</sup> and the prolongation of the infusion period from 24 to 36 h apparently led to a better control of systemic disease. Paradoxically, the improved quality of second hematological CR remains the only explanation for the increased rate of treatment failures in the CNS, as CNS relapses need a longer time

span to emerge. Hence, the majority of patients in study 83 were not subject to possible relapses in the CNS because of earlier and more frequently occurring bone marrow relapses. Similar findings have already been reported by other investigators, however, in much less intensively pretreated patients [2, 3].

Undoubtedly, children with isolated marrow relapse need a second preventive treatment for subclinical CNS leukemia. The question is whether or not chemoprophylaxis alone is sufficient in the face of the fact that i.t. and i.v. MTX have become a constituent of almost all front-line protocols. As in our series, prolonged treatment for 6 months was not capable of effectively preventing CNS disease. Perhaps successful chemoprophylaxis would be possible by more extensive use of other drugs such as high-dose Ara-C in combination with VP-16 and/or additional dexamethasone. The safest method would probably be a second

course of radiotherapy in previously irradiated patients or a first course in nonirradiated children. As relapse of leukemia is usually associated with a poor prognosis, CNS prophylaxis by radiotherapy would be justified in spite of its possible late side effects.

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## Incidence and Clinical Implications of Acute Hybrid Leukemia in Childhood\*

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### Introduction

Acute leukemias are thought to arise from a single abnormal progenitor cell in which expression of differentiation is restricted to either myeloid or lymphoid pathways [1]. Recently, however, this concept has been challenged by the fact that the availability of monoclonal antibodies (moAbs) to lineage-associated surface antigens and their use to characterize immature leukemic cells demonstrate an increasing amount of multilineage differentiation and phenotypic ambiguity (reviewed in [2]). Furthermore, application of molecular biological techniques has revealed that clonal rearrangements of the Ig heavy-chain (*IgH*) or T-cell receptor (*TCR*) genes occur in leukemic cells of the "inappropriate" lineage (reviewed in [3]).

Depending on the interpretation of these phenomena (leukemic or normal), different terms have been used, suggesting either aberrant gene expression due to the leuke-

mogenic event ("lineage infidelity") [4] or malignant transformation of a progenitor cell capable of differentiating into, or expressing characteristics of, more than one lineage ("lineage promiscuity") [5]. In addition, other more descriptive designations have been proposed: biphenotypic leukemia [6], acute "mixed-lineage" leukemia [7], or hybrid acute leukemia [8].

Very few studies, however, have as yet prospectively evaluated the clinical implications of acute leukemias with lymphoid and myeloid features. The reported incidence ranged from between below 1% to 20% in acute myeloid leukemia (AML) coexpressing lymphoid-associated surface antigens [2, 9, 10]; and from 5% to over 30% of patients in whom acute lymphocytic leukemia (ALL) was diagnosed by standard criteria exhibited blast-cell reactivities with myeloid markers [3, 10–13]. These discrepancies are probably due mainly to the distinct patient populations studied (e.g., children versus adults), the different myeloid- or lymphoid-associated markers analyzed, and the inhomogeneous criteria used for identification of these cases. In the present study, detailed immunophenotypic analyses were therefore performed on leukemic blast cells from a large series of consecutively diagnosed children with ALL or AML with the following aims:

1. to assess prospectively the incidence and clinical relevance of acute leukemias displaying lymphoid and myeloid features and
2. to correlate "mixed-lineage" surface antigen expression with cytogenetic and gene rearrangement analyses.

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## Patients and Methods

This study included 434 children with ALL and 91 with AML whose bone marrow (BM) or peripheral blood (PB) samples contained >80% blast cells. All patients were studied at diagnosis and entered on ALL-BFM 86 (BFM, Berlin-Frankfurt-Münster) or AML-BFM 87 protocols.

Classification of ALL/AML subtypes followed the French-American-British (FAB) criteria [14]. Leukemic cells were isolated by standard Ficoll-Hypaque density-gradient centrifugation. Surface antigen expression was identified by an indirect immunofluorescence (IF) assay as previously described [15] and evaluated by an epi-illuminated fluorescence Zeiss microscope or by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA, United States).

A panel of moAbs to B-cell (CD19/20/24), T-cell (CD2/3/5/6/7/8), non-lineage-restricted (CD10/HLA-DR/TdT) and myeloid-lineage-associated antigens (CD13/33/VIM-2) was used for phenotype determinations in children with ALL, whereas AML patients were analyzed for myeloid (CD13/14/15A/33/w41/VIM-2/glycophorin A), non-lineage-restricted (CD10/HLA-DR/TdT), T-lineage (CD2/4/7), and B-lineage-associated (CD19) antigens. The moAbs used in these studies are described elsewhere [15, 16].

For cytoplasmic IgM (cyIgM), CD3 (cyCD3), and intranuclear terminal deoxynucleotidyl transferase (TdT) staining, cyto-spin preparations were fixed in acetone (cyIgM, cyCD3) or methanol (TdT) and analyzed by an indirect IF assay using fluorescein isothiocyanate (FITC)-conjugated goat antimouse Ig antiserum (Dakopatts, Hamburg, FRG) or by a TdT assay kit (P.L. Biochemicals, Milwaukee, WI, United States).

The criterion for marker positivity was expression by  $\geq 20\%$  of the blast cells (surface antigens) or intracytoplasmic/intranuclear detection (cyIgM, cyCD3, TdT) in  $\geq 10\%$  of the leukemic cells. Cytogenetic analysis and gene rearrangement studies were performed by standard methods as described in detail elsewhere [15, 16]. Chi-square tests were used for comparison of differences among subgroups.

## Results

Coexpression of myeloid antigens (My<sup>+</sup> ALL) was observed in 24 of 434 patients (5.5%) with ALL. A high percentage of My<sup>+</sup> ALL patients were <1 year of age (29%) and presented with a WBC  $\geq 50 \times 10^9$ /liter (42%). No significant differences in the complete remission (CR) rate

**Table 1.** Comparison between My<sup>+</sup> and My<sup>-</sup> ALL patients with respect to clinical features and treatment response

	My <sup>+</sup> ALL (n=24)	My <sup>-</sup> ALL (n=410)	P
Age (years)			
<1	7	15	
1-9	13	305	0.0001
$\geq 10$	4	90	
WBC ( $\times 10^9$ /liter)			
<50	14	334	0.01
$\geq 50$	10	76	
Complete remission (percentage of evaluable patients)	87.5	97	NS <sup>a</sup>
Probability of complete continuous remission <sup>b</sup>	0.53 (SD=0.20)	0.74 (SD=0.04)	NS

<sup>a</sup> NS, not significant

<sup>b</sup> According to life-table analysis (Kaplan-Meier)

**Table 2.** Immunophenotype, karyotype, and genotype of My<sup>+</sup> ALL patients

Immunophenotypic subgroup	n	Karyotype	Gene rearrangement <sup>a</sup>				
			Cμ	Cκ	Tβ	Tγ	Tδ
CD10 <sup>-</sup> pre-pre-B ALL	8	Normal, n=2 t(4;11) n=2	7 <sup>b</sup> /7	1/7	1/7	2/7	3/6
Common ALL	7	Normal, n=2	6/6	2/4	4/5	3/5	2/2
Pre-B ALL	3	Normal, n=1	2 <sup>b</sup> /2	1/1	1/2	1/2	2/2
T-ALL	1		0/1	0/1	1/1	1/1	1/1

<sup>a</sup> Patients with rearranged Ig or T-cell receptor genes/patients analyzed

<sup>b</sup> Multiple rearranged fragments (pre-pre-B ALL, n=2; pre-B ALL, n=1)

and the duration of event-free survival (EFS) have as yet been found between My<sup>+</sup> and My<sup>-</sup> ALL (Table 1). Of children with My<sup>+</sup> ALL (vs. 12.9% with My<sup>-</sup> ALL), 41.7% were qualified for the intensified “experimental group” protocol, mainly on the basis of their poor response to corticosteroids.

Table 2 shows the distribution of the immunological subgroups as well as the genotypic features of My<sup>+</sup> ALL patients with a predominant lymphoid commitment according to their composite immunophenotype. B-cell precursor ALL was diagnosed in 18 of 19 children. Patients with common or pre-B ALL more frequently displayed CD13 and/or CD33 positivity, whereas VIM-2 co-expression was mainly identified in children with a CD10<sup>-</sup> pre-pre-B ALL phenotype. Cytogenetic studies were successfully carried out in seven of these patients, and only

two patients with CD10<sup>-</sup> pre-pre-B ALL and coexpression of myeloid antigens (VIM-2/CD15A) exhibited specific chromosomal rearrangements [t(4;11)]. All patients with B-cell precursor ALL showed *IgH* gene rearrangements, and there were relatively frequent cross-lineage rearrangements of *TCR* genes, especially in the common ALL subgroup.

Five of 434 patients (1%) disclosed ambiguous morphological and/or phenotypic features which did not allow a definite single-lineage association (Table 3). Interestingly, patient No. 5 showed myeloperoxidase (MPO) positivity and structural aberrations (+8) consistent with the diagnosis of AML, whereas the immunophenotype (CD7<sup>+</sup>, CD5<sup>+</sup>, cyCD3<sup>+</sup>) as well as *TCR-β* and *TCR-γ* gene rearrangement suggested T-lineage ALL.

**Table 3.** Summary of morphological and genotypic features in five patients with acute leukemia and ambiguous immunophenotypes

Patient No.	Age (years)	FAB	POX (%)	Immunophenotype	Karyotype	Gene configuration				
						Cμ	C	Tβ	T	Tδ
1	1	L1/L2	1	CD19 <sup>+</sup> /CD10 <sup>+</sup> /VIM-2 <sup>+</sup> /CD13 <sup>+</sup>	Normal	ND	ND	ND	ND	ND
2	10	L2	1	CD19 <sup>+</sup> /CD10 <sup>+</sup> /CD33 <sup>+</sup> /CD4 <sup>+</sup>	Normal	ND	ND	ND	ND	ND
3	0.5	L2	0	CD19 <sup>+</sup> /TdT <sup>+</sup> /VIM-2 <sup>+</sup> /CD15 <sup>+</sup>	ND	R	ND	G	R	ND
4	0.7	L1/L2	3	CD19 <sup>+</sup> /(CD10 <sup>+</sup> )/CD13 <sup>+</sup> /CD33 <sup>+</sup>	ND	R	G	G	G	G
5	5	L1/M1	3	CD7 <sup>+</sup> /CD5 <sup>+</sup> /VIM-2 <sup>+</sup> /(CD13 <sup>+</sup> )	47, XX, +8	G	G	R	R	ND

ND, not done; R, rearranged; G, germline

**Table 4.** Hematological and clinical characteristics of AML patients with T-lymphoid features and/or TdT positivity

	Total <sup>a</sup> (n = 91)	CD7 <sup>+</sup> (n = 15)	CD2 <sup>+</sup> (n = 13)	CD4 <sup>+</sup> (n = 18)	TdT <sup>+</sup> (n = 17)
Age (years)					
<2	17	3	2	4	1
2 < 10	36	6	2	5	7
≥10	38	6	9	9	9
WBC ( $\times 10^9$ /liter)					
< 10	30	5	2	5	3
10 ≤ 100	47	8	8	9	10
≥ 100	14	2	3	4	4
FAB subtype					
M1/M2/M3	37	7	6	3 <sup>b</sup>	10 <sup>b</sup>
M4/M5	45	5	5	14	3
Complete remission (%) <sup>a</sup>	76	62	58	82	75

<sup>a</sup> Total number of protocol patients

<sup>b</sup>  $P < 0.05$  (FAB subtypes M1/M2/M3 vs. M4/5)

<sup>c</sup> Percentage evaluable patients

Table 4 summarizes clinical, morphological, and treatment-response data for AML patients with T-lymphoid features and/or TdT activity. Of 91 children with AML, between 14% (CD7) and 20% (CD4) showed simultaneous expression of one or more T-lineage-associated antigens, and 19% were TdT positive. Blasts from four children co-expressed the T-cell-associated surface antigens CD7 and CD2 and were TdT positive, but cyCD3 was not present in any of them. CD10 or CD19 antigens were detected in only two children with AML who disclosed morphological evidence for dual populations of leukemic blasts. No significant differences were observed between these subgroups with regard to age and WBC, whereas the CR rate was slightly lower for CD7<sup>+</sup> and CD2<sup>+</sup> AML cases. Increased TdT activity was usually detected in FAB M1–M3 subtypes, whereas CD4 expression was strongly associated with M4/M5 subtype.

## Discussion

Our prospective analysis of a large series of children with ALL revealed myeloid antigen expression in 5.5%, a frequency much lower

than previously reported for children or adults with ALL [2, 12]. Based on the composite immunophenotype, the majority of My<sup>+</sup> ALL patients could be affiliated with B-cell precursor ALL, and surface antigen expression generally correlated with the genotype. These findings are compatible with results in adult ALL, demonstrating a higher incidence of mixed myeloid/lymphoid antigen expression in B-cell precursor ALL [12]. Our own data (coexpression of myeloid antigens in 16 of 119 consecutively diagnosed adults with ALL; unpublished results) substantiate a higher incidence of My<sup>+</sup> ALL in adults, probably corresponding with the greater frequency of immature phenotypic subsets (e.g., CD10-negative pre-pre-B ALL, pre-T ALL) in these patients [17, 18]. Although no significant differences in the CR rate and the duration of EFS have as yet been found between children with My<sup>+</sup> and My<sup>-</sup> ALL, it is noteworthy that we were able to identify a high incidence of My<sup>+</sup> ALL in patients with unique clinical (e.g., aged <1 year, high WBC, poor response to treatment) and biological features (predominantly CD10-negative pre-pre-B-ALL phenotype and chromosomal translocations involving 11q23) [16].

T-lymphoid features were encountered in approximately 15%–20% of childhood AML patients. A recent report described a similar incidence of childhood AML with T-lymphoid features [19]; and, based on the immunophenotypic (coexpression of CD2, CD7, and cyCD3) as well as clinical features (older age, higher WBC, higher frequency of lymphadenopathy, poor response to therapy) of these patients, the authors concluded that this leukemia represents a distinct biological and clinical entity. Children with AML and T-lymphoid features in the ALL-BFM study 87 have not as yet shown significantly different clinical characteristics, and their response to induction chemotherapy was only slightly worse. Expression of CD10 and CD19 antigens is very unusual in classical AML [9, 10, 20]. Interestingly, patients with Philadelphia chromosome-positive (Ph<sup>1</sup>) acute leukemia and a Ph<sup>1</sup>-positive novel leukemia cell line have recently been reported to show concomitant expression of myeloid and CD10/CD19 antigens [10, 21, 22]. Two children in our series showed these features, but, unfortunately, cytogenetic data were not available.

Attempts to correlate the TdT activity in AML with morphology and prognosis have yielded conflicting results. In some recent studies, TdT has been associated with predominantly monocytic leukemia [20, 23, 24], while other reports have suggested an association with immature types of myeloblastic leukemia [25, 26]. In our experience, increased TdT activity was more frequently detected in cases showing features of granulocytic differentiation. In agreement with previous studies [26, 27], we did not observe significant correlations between the TdT positivity and CR rate; and, in 117 children studied within the AML-BFM study 83, TdT positivity was not predictive of an inferior prognosis (unpublished results).

Based on their findings of a high incidence of *IgH* gene rearrangement in TdT<sup>+</sup> AML, Foa et al. postulated a molecular B-lineage orientation of the neoplastic clone in most patients with TdT<sup>+</sup> AML [28]. Our results (data not shown) as well as the observations of others [29], however, have demonstrated considerable heterogeneity with regard to gene rearrangement in the subgroup of childhood or adult TdT<sup>+</sup> AML (e.g., the

*IgH* gene is rearranged in only a part of these patients).

In conclusion, our prospective study on childhood ALL reveals a low incidence of myeloid antigen coexpression. Apart from biologically and clinically defined entities (e.g., infant ALL), it remains to be seen whether children with My<sup>+</sup> ALL represent a clinically significant subgroup which might benefit from alternative treatment protocols. In accordance with other recent reports, children with AML disclose a relatively high incidence of T-lymphoid features. Further studies, however, with expanded diagnostic approaches, including cytogenetic analyses and new molecular biological methods as well as longer follow-up times, are clearly needed to determine whether these children form a distinct biological and clinical subset of acute leukemias.

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## **Supportive Care in Acute Leukemias**

## Prevention of Infection in Acute Leukemia

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### Introduction

It is well accepted that severe infections are the main cause of death in patients with acute leukemia undergoing remission induction chemotherapy. The correlation of the increasing incidence of life-threatening infections and the duration of profound neutropenia thus limits the efforts of aggressive antileukemic treatment. However, granulocytopenia seems to be the most important although not the only factor contributing to the high susceptibility of these patients to severe infections. The damage of the epidermal and mucosal barriers, deterioration of humoral defense in terms of antibody and complement production, deterioration of cellular immunity in terms of a compromised monocyte-macrophage system, as well as malnutrition and occasionally splenectomy are patient-related factors with regard to the different virulence of pathogens colonizing the patient. Before we focus on approaches for prevention of infection by antimicrobial drugs, some basic rules should be remembered which should be strictly attended to although they might be disregarded occasionally:

1. Adequate patient accommodation
2. Avoidance of nebulizers, urinary catheters, air-conditioning systems, plants, uncooked food, reconstructional work
3. Careful disinfection of hands and clean clothing by nursing staff and physicians

4. No taking of blood specimens from fingertips or earlobes
5. Most careful handling of skin injuries especially central venous catheters

Apart from these general measures a great effort has been made over 20 years to improve prevention of infection by prophylactic oral administration of antibiotics, in which nonabsorbable as well as absorbable drugs have been used in combination with nonabsorbable antimycotics such as amphotericin B or nystatin. In controlled studies [3–12, 14–17], advantages of prophylactic antimicrobials compared with placebo could be demonstrated with regard to the incidence of bacteremia and in some cases also to mortality (Table 1).

However, recent studies by Kurrle et al. [11] and Dekker et al. [2] comparing different drug regimens for prevention of infection without a control group showed higher rates of bacteremia and mortality which are similar to those of former placebo groups. Additionally, in recent years the overall mortality of leukemia patients has been more likely to decrease due to improved antimicrobial intervention therapy with a high efficacy against microorganisms associated with exceptionally high lethality such as *Pseudomonas* spp.

The increasing incidence of bacteremias and of other infections in these recent studies, however, does not mean a falsification of earlier data but seems to be related to the widespread use of more invasive instrumentation such as central venous catheters or *Ommaya* reservoirs and more aggressive an-

**Table 1.** Rates of bacteremia and mortality in controlled (top) and comparative (bottom) studies for infection prevention

Author	Year	N	Drugs	Bacteremia/Mortality (%)	
				Treatment group	Control group
Storring et al. [15]	1977	95	FRACOL	13/0	37/14
Gurwith et al. [8]	1979	111	COT	0/3	17/15
Sleijfer et al. [14]	1980	105	Various	6/0	15/17
Kauffman et al. [10]	1983	55	COT	7/0	31/27
Guiot et al. [7]	1983	33	NEOCOL	13/0	41/6
Gualtieri et al. [6]	1983	58	COT	17/3	38/14
EORTC-IATPG [4]	1984	342	COT	12/?	19/?
Karp et al. [9]	1987	68	NOR	11/17	36/9
Kurrle et al. [11]	1986	140	NEOCOL	18/7	–
			COTCOL	2/10	–
Dekker et al. [2]	1987	56	COTCOL	22/7	–
			CIP	8/7	–

FRA, framycetin; COL, colistin/polymixin; COT, cotrimoxazole (trimethoprim-sulfamethoxazole); NEO, neomycin; NOR, norfloxacin; CIP, ciprofloxacin

tileukemic chemotherapy such as high-dose cytosine arabinoside or high-dose methotrexate, leading to prolonged neutropenia and severe damage of the mucosa.

Apart from the improvement of empirical broad-spectrum antimicrobial therapy such as the sequential protocol of the Paul-Ehrlich-Gesellschaft study group (which will be presented by Dr. H. Link), we have tried to reduce the incidence of fever and infections by the introduction of more effective and better-tolerated drug regimens for prophylaxis. In the past decade, cotrimoxazole (trimethoprim-sulfamethoxazole) with or without colistin (or polymixin) was most often used for this purpose [2–4, 6–8, 10–12, 14, 16–18]. However, since cotrimoxazole has little or no activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, coagulase-negative staphylococci, *Enterococcus*, group A streptococci, and some strains of *Klebsiella* and *Serratia* [1] and is associated with a marked rate of side effects, it appeared useful to look for therapeutic alternatives such as modern fluoroquinolones. Their broad antimicrobial activity including *Pseudomonas aeruginosa* and *Staphylococcus aureus* makes sub-

stances like norfloxacin or ciprofloxacin promising for the prevention of infection (Table 2).

### Study I: Ciprofloxacin or Norfloxacin for Prevention of Infection

First we studied the tolerance, decontamination efficacy, and drug levels of norfloxacin and ciprofloxacin, each in two different dosages and in combination with oral non-absorbable antimycotics [13]. Ciprofloxacin in a dosage of 500 mg every 12 h appeared to be most effective and yet well tolerable. Investigation of quinolone concentrations in serum and saliva on days 5 or 6 and days 13 or 14 showed that our study patients had only about 50% of the drug levels of normal volunteers.

#### Design

1. Randomized prospective multicenter study
2. Treatment groups: CIP 1000 = 500 mg ciprofloxacin every 12 h; CIP 500

**Table 2.** In vitro activity of norfloxacin and ciprofloxacin (MIC 50% and 90% in mg/liter). (From Fernandes [5])

	Norfloxacin	Ciprofloxacin
<i>Escherichia coli</i>	0.06/0.12	<0.03/ <0.03
<i>Klebsiella</i> spp.	0.12/0.5	0.06/0.12
<i>Enterobacter cloacae</i>	0.06/0.12	<0.03/0.06
<i>Serratia marcescens</i>	0.12/1.0	0.12/0.25
<i>Citrobacter freundii</i>	0.06/0.5	<0.03/0.25
<i>Proteus</i> spp.	<0.03/0.12	<0.03/0.06
<i>Salmonella</i> spp.	0.03/0.06	0.03/0.03
<i>Hemophilus influenzae</i>	<0.03/0.06	<0.03/ <0.03
<i>Pseudomonas aeruginosa</i>	0.5/2.0	0.25/1.0
<i>Staphylococcus aureus</i>	0.5/1.0	0.25/0.5
Coagulase-negative staphylococci	0.5/0.5	0.25/0.25
<i>Enterococcus faecalis</i>	4.0/8.0	2.0/4.0
<i>Streptococcus pneumoniae</i>	8.0/16	1.0/2.0
<i>Bacteroides</i> spp.	> 64/ > 64	16/ > 64

= 250 mg ciprofloxacin every 12 h; NOR 800 = 400 mg norfloxacin every 12 h; NOR 400 = 200 mg norfloxacin every 12 h; each in combination with nonabsorbable antimycotics

3. Participating units: Essen-Werden (FRG), Groningen (NL), Rotterdam (NL), Ulm (FRG), and Utrecht (NL)

#### Results (n = 51 patients)

1. All regimens well tolerated
2. Effective gut decontamination
3. Low incidence of microbiologically documented infections
4. Predominance of gram-positive cocci
5. Drug levels in serum and saliva about 50% those of healthy volunteers
6. Occasional gram-negative rod colonization and single *Pseudomonas* infections in norfloxacin treatment groups
7. Single cases of gastrointestinal intolerance and allergic reactions in norfloxacin treatment groups

#### Study II: Cotrimoxazole + Colistin Versus Ciprofloxacin

Encouraged by the observations of the Utrecht group which demonstrated cipro-

floxacin superior to cotrimoxazole plus colistin [2], we decided to undertake a larger prospective randomized study to compare the prophylactic efficacy of ciprofloxacin with that of the standard regimen of cotrimoxazole plus colistin, both groups in combination with oral amphotericin B or nystatin. Only patients with acute leukemia in first or second remission induction who had not been included earlier in the study and who did not exhibit signs of hepatic or renal dysfunction were eligible for randomization.

#### Design

1. Randomized prospective multicenter study
2. Treatment groups: CIP = 500 mg ciprofloxacin every 12 h; COTCOL = 960 mg cotrimoxazole + 200 mg colistin every 8 h; each in combination with nonabsorbable antimycotics
3. Participating units: Essen-Werden (FRG), Groningen (NL), Hannover (FRG), Münster (FRG), Nijmegen (NL), and Rostock (GDR)
4. Exclusion criteria: age < 18 or > 65 years, no aggressive remission induction intended, second or higher relapse, former inclusion in the study, hepatic or renal dysfunction, known allergy to one study drug, and patient not willing to participate

### Preliminary Results of Study II

Out of 292 randomized patients, 236 were evaluable (120 for the CIPRO group; 116 for the COTCOL group). Fifty-six patients had to be excluded from evaluation, mostly because of early deaths within the 1st week under study or because of contraindications which appeared only when the patients were already randomized. Age and sex distribution, types and stages of the underlying disease as well as the drugs for aggressive chemotherapy were equally distributed between both treatment groups. Hematological and chemical laboratory parameters also showed no marked differences. Fever and/or systemic antibiotic therapy at the time of admission were present in 33 patients in the CIPRO and in 29 patients in the COTCOL group. A total of 7545 treatment days (4069 CIPRO, 3476 COTCOL) were evaluated. Fever, defined as an axillary temperature of or above 38°C was observed in 17% of treatment days in the CIPRO group compared with 13% in the COTCOL group. The median time to the onset of fever was 12 days in patients with CIPRO and 15 days in patients with COTCOL.

The number of acquired infections was significantly higher in the CIPRO treatment group: 108 infections in 66 out of 120 patients versus 66 infections in 46 out of 116 patients in the COTCOL group. Also the type of acquired infections in the CIPRO group was more unfavorable with respect to septicemias and pneumonias as well as to minor infections such as urinary tract or oropharyngeal infections and also to the incidence of unexplained fever (FUO) (Tables 3, 4). As expected, gram-positive bacteria dominated by far in cases of documented major and minor infections. Streptococci and staphylococci were found most often in blood cultures, whereas only single cases of gram-negative septicemia were observed in both treatment groups. Remarkably, we had four cases of proven *Pneumocystis carinii* pneumonia in patients not receiving cotrimoxazole. On the other hand, we did not observe a marked incidence of fungal infection in either treatment group. A total of 3519 samples of oral washings and of feces were cultured for surveillance. Potential pathogens were found in 40% of cultures in

**Table 3.** Incidence of acquired infections in study II

	CIP-RO	COT-COL	Total
No. of patients without acquired infection	30	40	70
No. of patients with FUO only	24	30	54
No. of patients with at least one documented infection	66	46	112
One infection	39	29	68
Two infections	17	14	31
Three infections	6	3	9
Four infections	3	0	3
Five infections	1	0	1
Total No. of infections	108	66	174
Total No. of patients	120	116	236

**Table 4.** Type of acquired infections in study II

	CIP-RO	COT-COL	Total
Total No. of major infections	66	38	104
Septicemia	30	14	44
Lower respiratory tract	33	23	56
Infection with bacteremia	1	1	2
Gastrointestinal tract	2	0	2
Total No. of minor infections	42	28	70
Oropharynx	13	10	23
Upper respiratory tract	6	6	12
Gastrointestinal tract	1	1	2
Urinary tract	7	2	9
Skin and soft tissue	8	9	17
Others	7	0	7
Fever of unknown origin (FUO)	40	16	56
Total	148	82	230

the CIPRO and in 42% of cultures in the COTCOL group. Gram-positive cocci also dominated in both oral washings and in feces with only single cultures positive for gram-negative enterobacteria and *Pseudomonas* spp., but we found a remarkably high rate of colonization with *Acinetobacter* spp. However, these nonfermenters seemed to be restricted to surveillance cultures and did not appear as causative germs in sep-

ticemias or other cases of documented infections.

The time to bone marrow recovery measured at a granulocyte count at or above 1000/mcl was not different in the two treatment groups. With respect to the outcome of antileukemic therapy there was no difference between both groups. The mortality due to infections was higher in the CIPRO group: 8 out of 120 (6.7%) versus 2 out of 116 (1.7%) patients, with a remarkably low mortality rate in the overall study patients. To our surprise, the incidence of allergic reactions and other adverse effects could not be reduced in the CIPRO group compared with the COTCOL group. The patients' compliance appeared to be equal in both treatment groups, as far as this could be objectively observed.

### Summary and Conclusions

In a randomized study comparing cotrimoxazole plus colistin with ciprofloxacin, each in combination with nonabsorbable antimycotics, the incidence of major infections in terms of septicemias and pneumonias as well as of minor infections and episodes of unexplained fever (FUO) was higher in patients treated with ciprofloxacin. In cases of microbiologically documented infections, gram-positive cocci dominated by far. In surveillance cultures of oral washings and of feces, gram-negative enterobacteria were only rarely detected; however, large numbers of cultures were positive for *Acinetobacter* species. There were four cases of documented *Pneumocystis carinii* pneumonia in patients not receiving cotrimoxazole. The incidence of documented mycotic infections as well as the detection of fungi in surveillance cultures was similar in both treatment groups. A decrease in the number of adverse events, especially of allergic reactions, could not be achieved by the administration of ciprofloxacin. *In conclusion*, cotrimoxazole plus colistin in combination with nonabsorbable antimycotics remains the standard regimen for prevention of infection in patients with acute leukemia undergoing aggressive remission induction therapy. A detailed analysis of study II will be prepared for publication.

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## The Febrile Neutropenic Patient: Newer Options for Empirical Therapy

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It has become well accepted that the onset of fever in a neutropenic patient requires the prompt initiation of empirical antimicrobial therapy. The main goal of empirical antibiotics is to protect against the early morbidity and mortality associated with untreated bacterial infections in the neutropenic population.

Qualities considered essential for empirical regimens are

(a) a broad spectrum of activity, encompassing gram-positive and gram-negative organisms (including *Pseudomonas aeruginosa*),

(b) the use of bactericidal drugs that achieve bactericidal levels in both serum and tissue,

(c) efficacy against virulent organisms, even in the absence of neutrophils, and

(d) an acceptable toxicity profile.

Historically, in order to accomplish these goals, it has been necessary to use combinations of antibiotics. Most empirical combination-antibiotic regimens have consisted of an aminoglycoside at "the core", usually in combination with an extended-spectrum or "anti-pseudomal" beta-lactam antibiotic, and often including some additional gram-positive coverage provided by a first-generation cephalosporin, antistaphylococcal penicillin, or vancomycin (Table 1) [1].

Given the fact that aminoglycoside-based combination regimens have provided a time

honored profile of effective empirical coverage, one might appropriately ask if there are any valid reasons to reconsider their use in this population. Indeed, a number of factors support the search for alternative regimens, and include:

(a) *a change in the pattern of bacterial isolates* – while gram-negative organisms are still important pathogens requiring appropriate antibiotic coverage, many centers are witnessing the re-emergence of gram-positive organisms as the most frequently encountered isolate;

(b) *increasing aminoglycoside resistance* – with the increased use of aminoglycosides, some institutions are encountering a concomitant rise in aminoglycoside-resistant gram-negative organisms;

(c) *concerns about toxicities* – as potentially toxic agents are increasingly employed in the management of many underlying malignancies, valid concerns arise about additive toxicities (particularly nephrotoxicity) of the aminoglycosides. Examples include the potential for added nephrotoxicity with platinum-based compounds or cyclosporin;

(d) *changes in the spectrum of neutropenic patients* – when many of the initial empirical regimens were developed, they were directed mainly toward patients with hematological malignancies that were treated with earlier developed cytotoxic regimens. Subsequently, refinements in the delivery of cytotoxic therapy and supportive care, coupled with the development of novel antineoplastic strategies, have resulted in a more heterogeneous population at risk for infection. The clinician now frequently encounters neu-

**Table 1.** Aminoglycoside-based combination regimens

Aminoglycoside + Antipseudomonal $\beta$ -lactam $\pm$ Additional antigram-positive		
Gentamicin	Extended spectrum	Isoxazolyl-Penicillin
Tobramycin	penicillin	Nafcillin
Amikacin	Carbencillin	Oxacillin
Netilmicin	Ticarcillin	or
	Azlocillin	1st Gen. Cepalosporin
	Mezlocillin	Cephalothin
	Piperacillin	Cephazolin
	or	or
	3rd Generation cephalosporin	Glycopeptide
	Ceftazidime	Vancomycin
	Cefoperazone	Teicoplanin
	or	
	Monobactam	
	Aztreonam	

tropenic patients with solid tumors as well as hematological malignancies. This often results in significant variations in the degree and duration of neutropenia, as well as in additional risk factors.

(e) *Concerns about cost* – the cost of antibiotic regimens is an increasingly important and relevant issue. The cost of combination regimens may be relatively high, not only because of the costs of the antibiotics per se, but also because of additional factors such as staff time for drug preparation, cost of apparatus for intravenous administration, and monitoring of antibiotic levels (e.g., aminoglycosides, vancomycin). Most importantly, certain newer antibiotic developments have provided us with viable alternatives to the more traditional, aminoglycoside-based combination regimens [2, 3]. At the National Cancer Institute, United States, we have been particularly interested in the concept of single-agent empirical therapy, or “monotherapy”.

At the present time, three classes of currently available antibiotics include potential candidates for monotherapy – the third-generation cephalosporins, the carbapenems (of which impipenem is the prototype), and the quinolones [3]. At the National Cancer Institute, we have developed a series of clinical studies designed to assess agents from each of these classes in this setting. The monobac-

tams (of which aztreonam is the prototype) are another relatively new class of antibiotics being evaluated for efficacy in neutropenic patients. This discussion will focus on the potential clinical utility of these agents for empirical therapy in febrile granulocytopenic cancer patients.

### The Third-Generation Cephalosporins

While many third-generation cephalosporins are available, most do not represent appropriate agents for single-agent empirical therapy, primarily because of a lack of activity against *Pseudomonas aeruginosa* [4, 5]. Of the third-generation cephalosporins currently available in the United States, only cefoperazone and ceftazidime have any significant activity against *P. aeruginosa*, and of these, ceftazidime’s activity is greatest.

The first trial of monotherapy conducted at the National Cancer Institute was designed to determine whether a single broad-spectrum beta-lactam could be as effective as a standard combination regimen for initial empirical therapy [6]. In that study, patients with fever (one oral temperature  $\geq 38.5$ , or three temperatures  $\geq 38$  in a 24-h period) and granulocytopenia ( $\leq 500$  granulocytes and band forms per  $\mu\text{l}$ ) underwent a standard initial evaluation, and then were

**Table 2.** Outcome of 550 febrile neutropenic episodes randomized to monotherapy or to combination antibiotic therapy (from [6])

	Regimen	
	Monotherapy (ceftazidime)	Combination therapy (cephalothin, gentamicin, carbenicillin)
Number of episodes	282	268
Success without modification <sup>a</sup>	175 (62%)	180 (67%)
Success with modification <sup>b</sup>	93 (33%)	78 (29%)
Failure <sup>c</sup>	14 (5%)	11 (4%)

<sup>a</sup> Successful treatment, not requiring any additions to or changes in the initial antibiotic regimen

<sup>b</sup> Successful treatment, requiring some addition to or change in the initial antibiotic regimen

<sup>c</sup> Death due to infection

randomized to receive either a combination regimen consisting of cephalothin, gentamicin, and carbenicillin ("KGC"), or ceftazidime alone.

In our judgment, the most important objective with respect to effectiveness of an empirical regimen is its impact on survival of the patient through the neutropenic episode. Moreover, it is during the initial 72 h, before initial culture results are available, that the therapy is "truly" empirical. Accordingly, in this trial, we evaluated patients with respect to success of therapy (alive vs. dead), at 72 h after entry, and also for the remainder of the neutropenic episode. In addition, we grouped patients according to whether their initial workup (including cultures) revealed a documented infection or a fever of undetermined origin.

The results, based on 550 patient episodes, indicate that monotherapy with ceftazidime compares favorably to the combination regimen. Survival at both the early and later evaluation time points was the same for each group. The overall results also show comparable efficacy of the two regimens (Table 2). Approximately two-thirds of the episodes in both groups were successfully treated for the entire duration of their granulocytopenia, without requiring any changes in their initial regimen. Another one-third of the episodes required some change or modification (such as addition of an antibacterial, antifungal, or antiviral drug). In the neutropenic patient, modifica-

tions of the initial empirical regimen may be required in a number of clinical settings in order to assure a successful outcome (Table 3). Finally, an equally low number in both randomization groups (about 5%) died of infection. None of the deaths were attributable to a specific deficiency in one regimen that was not present in the other (i.e., an organism sensitive to one regimen but resistant to the other). In addition, the average time to initial defervescence was equivalent for those receiving monotherapy and those treated with combination antibiotics.

Two subgroups of patients were identified in this study, that appeared to require more frequent modifications of the initial regimen in order to achieve a successful outcome: those presenting with a documented source of infection to account for the initial fever, and those having relatively protracted periods of granulocytopenia ( $\geq 1$  week). Importantly, however, the need for modification in these subgroups was identical regardless of whether the patient was treated initially with monotherapy or with combination therapy. In this study, these modifications were not considered a failure of the primary regimen per se, but instead were reflective of the limitations of virtually any antibiotic(s) in treating patients who are at particularly high risk for development of secondary infections.

Several authorities have raised concerns regarding the use of ceftazidime as a single agent for fever and neutropenia. These have

**Table 3.** Possible modifications of initial empirical therapy

● Breakthrough bacteremia	If gram-positive isolate (e. g., <i>S. epidermidis</i> ), add vancomycin. If gram-negative isolate (i. e., presumably resistant), switch to new regimen.
● Catheter-associated infection	Add vancomycin for resistant gram-positive organisms.
● Severe oral mucositis or necrotizing gingivitis	Add specific anti-anaerobic agent (e. g., clindamycin or metronidazole).
● Esophagitis	Trial of oral clotrimazole or I.V. amphotericin B, and/or acyclovir.
● Pneumonitis – diffuse or interstitial	Trial of trimethoprim-sulfamethoxazole and erythromycin (plus broad spectrum antibiotics if neutropenic).
● Pneumonitis – new infiltrate in a granulocytopenic patient receiving antibiotics	Granulocyte count rising – watch and wait. Granulocytopenic – biopsy, lavage, or empirical therapy.
● Perianal tenderness/infection	Add antianaerobic agent.
● Persistent fever and granulocytopenia	Empirical amphotericin B.

included the lack of synergy against documented gram-negative infection, lack of activity against certain gram-positive isolates, poor anti-anaerobic activity, and the potential for development of resistance [7–13]. Some of these concerns are purely theoretical, while others are based on unique experiences limited to specific centers.

One issue that has been the subject of continued debate is whether vancomycin should be included routinely in initial empirical regimens, particularly if single-agent therapy is used. Supporting the argument for routine use of vancomycin is the observation that gram-positive organisms have been increasing in incidence, and now comprise the majority of isolates at many centers. Many of these (such as enterococci and the coagulase negative staphylococci) are inadequately covered by many empirical regimens. Conversely, it has been argued that since many of these organisms are of relatively low virulence and are often inhibited by the antibiotics (even “suboptimal” antibiotics), vancomycin may be safely withheld until the gram-positive isolate has been identified microbiologically.

One recent study randomized patients between a vancomycin- and a non-vancomycin-containing regimen and showed that the incidence of secondary gram-positive infections was reduced in the van-

comycin containing group [8]. There also appeared to be less of an amphotericin B “requirement” in the group that received vancomycin as part of the initial regimen. However, there was no difference in morbidity related to gram-positive infections between the two groups, and all of the gram-positive infections in the non-vancomycin group were successfully treated by its addition when the organism was identified and reported by the microbiology laboratory.

A retrospective analysis from the National Cancer Institute, in contrast, indicated that there was no excess morbidity in delaying the institution of vancomycin by waiting for either a microbiological or clinical indication for its use [14]. One hundred percent of the primary gram-positive isolates (from cultures obtained after institution of antibiotics) were treated successfully with this pathogen-directed approach. Of the three patients with secondary gram-positive isolates that were scored as failures, only one represented a true microbiological failure, with persistently blood cultures for *E. faecalis* after 2 days of vancomycin. This patient was treated successfully following a change to ampicillin and gentamicin. The other two patients died of noninfectious causes while receiving vancomycin and, since they did not represent true successes, were scored conservatively as failures. There

was a greater proportion of secondary gram-positive infections in the group initially receiving monotherapy (ceftazidime) compared to combination therapy (16 of 282 patients compared with 6 of 268,  $P_2=0.04$  by chi-squared test), but all of these were treated successfully by subsequent addition of vancomycin.

Another study randomizing patients between a vancomycin- and non-vancomycin-containing regimen also demonstrated more gram-positive infections in the latter group, but in eight of nine of these there was no excess morbidity reported due to delay in institution of the vancomycin until after the organism was recovered [15]. Interestingly, the conclusion drawn from this study was that vancomycin should be used routinely in empirical regimens. The weight of the recommendations rested on a single case of fatal gram-positive sepsis that occurred in a patient not receiving vancomycin, although the organism was susceptible to the antibiotic regimen that the patient was receiving at the time (ticarcillin-clavulanate plus amikacin). Also, clearing of this organism occurred only after institution of another beta-lactam (cefotaxime) in combination with vancomycin.

Clearly, an important factor to be considered in the selection of components for empirical regimens is the sensitivity pattern of organisms encountered at a given institution. For example, while vancomycin may

not be a necessary component of empirical regimens at most centers, its routine use is clearly appropriate at institutions with a high incidence of methicillin-resistant *S. aureus*.

### The Carbapenems

Imipenem is a relatively new agent, and the prototypic member of the carbapenem class of antibiotics. It is provided in fixed combination with cilastatin, an inhibitor of renal dehydropeptidase, which is an enzyme that rapidly degrades imipenem. The spectrum of activity compared to ceftazidime is reviewed in Table 4. Of note is that in addition to good in vitro activity against the important aerobic gram-negative pathogens, imipenem has improved activity against gram-positive organisms (including most enterococci) and also against most of the clinically important anaerobic bacteria (including *B. fragilis* and *clostridia* spp.).

With respect to the potential use of imipenem as a single agent for empirical therapy, two questions are relevant. First, will it be as effective as ceftazidime for empirical monotherapy and, second (and perhaps more importantly), will its broadened activity translate into any improved clinical efficacy?

An initial small, nonrandom study suggested that imipenem may be useful and ef-

**Table 4.** Spectrum of antibacterial activity

	Ceftazidime	Imipenem
Effective coverage (in vitro)	Gram negative aerobes (good, including <i>P. aeruginosa</i> ) Gram positive bacteria (moderate; not enterococci)	Gram negative aerobes (good, including <i>P. aeruginosa</i> ) Gram positive bacteria (good, including enterococci) Anaerobes
Deficiencies	Methicillin resistant staphylococci Enterococci <i>P. maltophilia</i> <i>P. cepacia</i> Anaerobes <i>Listeria</i>	Methicillin resistant staphylococci <i>P. maltophilia</i> <i>P. cepacia</i>

**Table 5.** Outcome of 126 febrile neutropenic episodes randomized to monotherapy with ceftazidime or imipenem (from [18])

	Regimen	
	Ceftazidime	Imipenem
Number of episodes	65	61
Success without modification <sup>a</sup>	42%	52%
Success with modification <sup>b</sup>	54%	44%
Failure <sup>c</sup>	5%	3%

<sup>a</sup> Successful treatment, not requiring any additions to or changes in the initial antibiotic regimen

<sup>b</sup> Successful treatment, requiring some addition to or change in the initial antibiotic regimen

<sup>c</sup> Death due to infection

fective as monotherapy [16]. Early results of two randomized studies appear to corroborate its efficacy in this setting (one comparing it to an aminoglycoside-containing combination, and another, being performed at the National Cancer Institute, comparing it to monotherapy with ceftazidime) [17, 18]. Preliminary results of the NCI trial are presented in Table 5. Interestingly, neither of these studies appears to demonstrate superior efficacy. Three potential drawbacks to the use of imipenem are:

(a) a relatively high incidence of the development of resistant *P. aeruginosa*,

(b) its potential to decrease the seizure-threshold in patients with central nervous system pathology, and

(c) its potential to cause significant nausea and vomiting in some patients.

In the ongoing NCI trial, approximately one-third of patients receiving imipenem report significant nausea, and about one-third of the patients experiencing nausea have required discontinuation of the drug. In contrast, only about 2% of patients receiving ceftazidime report associated nausea, with none requiring discontinuation.

## The Quinolones

The fluoro-quinolones are agents with a broad spectrum of activity that encompasses

the majority of pathogens encountered in neutropenic patients. Their activity against most aerobic gram-negative organisms (including *P. aeruginosa*) and staphylococci (including methicillin-resistant strains) is excellent. However, they have only moderate activity against many streptococci and are devoid of activity against all of the clinically important anaerobic infections.

The appropriate role for the quinolones in the neutropenic patient has yet to be defined. Because of their relatively poor activity against certain gram-positive organisms, they should probably not be used for empirical single-agent therapy. They may, however, be useful for completion of therapy in patients who initially respond to intravenous antibiotics, and who have had either a fever of undetermined origin, or a susceptible bacterial isolate. At the National Cancer Institute, we are currently randomizing such patients (if they are still neutropenic after 3–7 days of i.v. antibiotics) to either continue the i.v. therapy or to switch to oral ciprofloxacin. Clearly, the identification of low-risk patients that could be switched to oral therapy might be of practical benefit.

Another potential use for the quinolones that has received recent attention is for oral prophylaxis of bacterial infections in patients with prolonged neutropenia. Thus far, the published data suggest that the incidence of gram-negative infections in patients receiving quinolones may be decreased. Despite this, however, none of the studies has shown a decrease in infection-related death, and two have shown some increase in gram-positive infections [19–22]. At the present time, the clinical usefulness of the quinolones as prophylactic agents has not been definitively established. Importantly, their overuse may lead to significant quinolone resistance, thus vitiating all beneficial potential.

## Aztreonam

Aztreonam is a relatively new, synthetic beta-lactam antibiotic of the “monobactam” class. Although it shares certain structural features with other beta-lactams, its monocyclic structure provides a unique microbiological spectrum and clinical profile. It can

only be used intravenously, and has a pharmacological and toxicity profile similar to other beta-lactam antibiotics.

Aztreonam has a relatively narrow, highly specific spectrum of activity directed against the aerobic gram-negative bacteria. Susceptible organisms include most enteric gram-negative rods, as well as *P. aeruginosa*. In contrast, aztreonam has no significant activity against any of the clinically important gram-positive aerobic organisms or against anaerobes. A number of studies in non-neutropenic patients have shown aztreonam to be effective for treating serious gram-negative infections. On the other hand, there is very limited data with respect to its use in the neutropenic population. One early study suggests that it may provide effective empirical coverage for febrile neutropenic patients when combined with vancomycin (in order to provide gram-positive coverage [23]). More data will be needed, however, in order to clarify its utility for single-agent gram-negative coverage in this population. Until this information is available, addition of an aminoglycoside is warranted if aztreonam is selected for use.

A particularly useful feature of aztreonam is its apparent lack of cross-reactivity to the other beta-lactams in patients who have penicillin or beta-lactam allergies [24]. It is in this setting that it may be most useful – specifically for the patient with a significant allergy to beta-lactams in whom therapy with an antipseudomonal beta-lactam antibiotic is still desirable or required. An appropriate empirical regimen for this group of patients might include a combination of vancomycin, aztreonam, and an aminoglycoside.

## Conclusion

It is well established that the onset of fever in the granulocytopenic patient mandates the expeditious institution of empirical antibiotic therapy. Traditionally, combination regimens have been employed in order to achieve the desired properties of empirical therapy. They have usually consisted of an aminoglycoside combined with an antipseudomonal penicillin, and have often included an anti gram-positive beta-lactam. Howev-

er, the development of newer broad spectrum antibiotics that can achieve high bactericidal concentrations has provided potential alternatives to the traditional combination regimens. These include non-aminoglycoside-containing combinations and certain agents used alone. With respect to monotherapy, the third-generation cephalosporins and the carbapenems have been most extensively studied, each offering agents of potential clinical usefulness. In addition, studies are underway to help determine the most appropriate role for the fluoro-quinolones in neutropenic patients. Finally, the development of the monobactams appears to provide the clinician with an antipseudomonal agent that can be safely used in beta-lactam allergic patients. Again, it should be emphasized that there are a variety of appropriate options for empirical antibiotic management, and that there is no single best approach. The selection of a specific regimen should depend on many factors, including insititutional sensitivity patterns, individual and institutional experience, and clinical parameters.

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## Supportive Care of the Marrow Transplant Recipient: The Seattle Experience\*

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Bone marrow transplantation is a worldwide activity involving more than 250 transplant centers in over 40 countries [5]. Since its clinical introduction almost 20 years ago, the indications for transplantation have increased as the long-term results have improved [33]. Following marrow ablation with supralethal doses of total body irradiation and/or chemotherapy, hematopoietic and immunologic reconstitution is achieved via proliferation of engrafted marrow donor stem cells [36]. The ultimate success of the procedure depends, in large measure, upon supportive care of the transplant recipient. The following report reviews aspects of this special care.

### Supportive Care in the Early Transplant Period

#### *Effects of Chemoradiotherapy and Marrow Harvest*

The pretransplant preparative conditioning with high-dose cytotoxic therapy causes the

side effects of nausea, vomiting, diarrhea, and alopecia. Oral mucositis is often the most problematic toxicity and increasing doses of total body irradiation are significantly associated with increased oral pain [7]. Patient-controlled opioid infusions ameliorate oral pain while reducing narcotic-related toxicities. Pain is also observed in the marrow donor at the sites of multiple aspirations for marrow harvest. Typically donors use analgesic medications for a median of 3 days after marrow aspiration but report less than complete relief with standard oral acetaminophen and codeine analgesia [10].

A modified right atrial catheter is inserted in each transplant recipient to assure venous access. Development of multilumen Hickman catheters has aided the supportive care of patients and allowed uninterrupted infusion of blood products, antibiotics, and hyperalimentation.

Recently a system for grading toxicities of pretransplant preparative regimens has been reported [3]. Older patients, patients transplanted during advanced stages of leukemia, and those with HLA-nonidentical donors are more likely to experience increased degrees of regimen-related toxicity. This is especially true within the 1st month of transplant when several factors may amplify toxicity. For example, veno-occlusive disease may impair hepatic function, alter hepatorenal hemodynamics, and precipitate acute renal failure [14, 39]. Essential supportive care components such as amphotericin or cyclosporine administration may have to be attenuated or discontinued in the face of multiorgan failure. Although mortality is

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high after mechanical supportive care for organ failure, no characteristics accurately predict survival [9].

### *Hematopoietic Recovery and Transfusion Support*

Marrow transplantation may be performed across “minor” blood group incompatibilities (0 donors for A or B recipients) without adverse effect. Transplantation across “major” ABO incompatibilities requires either removal of red cells from the donor marrow or reduction of isohemagglutinin titers from the recipient. In a recent review of 140 transplants in which major ABO incompatibility existed between donor and recipient, it was found that plasma immunoadsorption and whole blood immunoadsorption were less efficient in removing antibody than plasma exchange [4]. However, immunoadsorption techniques resulted in less platelet consumption than plasma exchange. Since ABO antigens are not expressed on marrow stem cells, graft rejection was not influenced by blood group incompatibilities.

Graft failure can be influenced by HLA incompatibility of marrow donor and recipient [1]. Risk factors associated with graft failure include HLA incompatibility and a positive crossmatch for antidonor lymphocytotoxic antibody. In patients with no prior alloimmunization receiving marrow from HLA-identical siblings, graft failure increases after *in vitro* depletion of marrow T-cells in an attempt to prevent graft-versus-host disease (GVHD). This risk appears reduced with use of higher doses of pretransplant total body irradiation [13]. Maximal cell yield during the marrow harvest, increased pretransplant immunosuppression, and “selective” marrow T-cell removal are potential approaches to decrease graft failure associated with T-cell depletion.

Blood products are routinely irradiated following transplantation to prevent lymphoid proliferation which might produce GVHD. Most centers administer 12–30 Gy irradiation to blood products until the donor marrow becomes fully functional. The average transplant recipient requires 10–20 units of red cells and approximately 150 units of platelets. If alloimmunization and

refractoriness to random donor platelets develop, partial or fully HLA-identical family members are used for platelet support. In a retrospective analysis of 264 patients with severe aplastic anemia undergoing allogeneic transplantation, the most important factors correlating with development of platelet refractoriness were the presence of lymphocytotoxic antibodies and the number of platelet units transfused before referral for transplantation [11]. When given HLA-compatible platelets, only 7% of patients refractory to random donor transfusions failed to show reasonable posttransfusion platelet increments.

In most patients transfusion support is discontinued 60–100 days after transplant. However, persisting thrombocytopenia after allogeneic marrow grafting is associated with poor patient survival. Measurements of platelet and fibrinogen kinetics and antiplatelet antibodies indicate that some patients develop autoimmune thrombocytopenia in association with GVHD [2]. Immunosuppressive treatment of acute or chronic GVHD may lead to reduced platelet destruction.

Considerable attention has been given to the administration of hematopoietic growth factors to speed marrow graft recovery. Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) can accelerate myeloid recovery after autologous marrow transplantation [20]. This could lead to a reduction in the cost of care due to earlier discharge from the hospital. Current studies are aimed at enhancing the effectiveness by using combinations of hematopoietic growth factors.

### *Immunodeficiency and Infection*

The kinetics of posttransplant immune reconstitution have been recently reviewed [12]. Investigations have been conducted in an attempt to accelerate immune recovery after marrow grafting [38]. The use of thymic epithelial grafts or administration of thymic hormones such as thymopentin or thymosin fraction 5 did not prevent infection or hasten recovery of T-cell function. In contrast, passive antibody prophylaxis with high-dose immunoglobulin appears promis-

ing [26]. In five controlled studies, infection developed in 30% of immunoglobulin recipients and 43% of controls. In four controlled trials, interstitial pneumonia developed in 17% of immunoglobulin recipients and 43% of control patients. Moreover, immunoglobulin recipients have fewer episodes of septicemia [21]. Compared with no prophylaxis, high-dose intravenous immunoglobulin appears to lessen the probability of local infection, septicemia, and acute GVHD [32].

Despite impressive immunodeficiency in the immediate posttransplant period, most patients do not succumb to infections. The fact that fewer than 5% of patients die of infectious complications during the period of granulocytopenia reflects the efficacy of antibiotic support. In a controlled study of patients with aplastic anemia, patients randomized to laminar airflow (LAF) room isolation and enteric decontamination had significantly fewer infections compared with those given prophylactic granulocyte transfusions from a single family member or to patients randomized to conventional rooms with hand washing and mask precautions [19]. The incidence of acute GVHD was reduced in patients transplanted in LAF rooms and survival was improved among patients with aplastic anemia. Similar controlled trials have been conducted in patients with hematologic malignancies. The incidence of septicemia was significantly lower in patients with leukemia randomized to LAF isolation, gastrointestinal decontamination, and prophylactic systemic antibiotics [22]. However, there was no significant effect on survival or the incidence or severity of GVHD. It is possible that total body irradiation given to patients with leukemia (but not given to patients with aplastic anemia) could be sufficiently emetic to preclude successful enteric decontamination with nonabsorbable oral antibiotics.

Such studies underscore the interrelated and time-dependent relationship of opportunistic infections, recovery of immune function, and GVHD [15]. Cytomegalovirus (CMV) infection can be life-threatening and occurs more frequently in CMV antibody seropositive patients, in older patients, and in those experiencing acute GVHD [16, 17]. Prior reports have demonstrated a case fa-

tility of 85% in patients developing CMV pneumonia; however, recent investigations suggest that a combination of ganciclovir and CMV hyperimmune globulin may be of benefit.

Prevention of CMV pneumonia is of critical importance. Although 50% of the North American population harbors latent CMV as evidenced by antibody seropositivity, CMV seronegative patients with seronegative marrow donors can be effectively protected from CMV infection by exclusive use throughout the transplant of blood products from CMV seronegative donors [6]. In a prospective randomized study, 1 of 32 seronegative patients developed CMV infection during seronegative blood support compared with 8 of 25 seronegative patients receiving unscreened blood ( $P < 0.007$ ). In that study, CMV immune globulin did not appear to prevent CMV infection. Likewise, prophylactic human leukocyte interferon did not appear to alter the incidence of CMV infection when administered intermittently for 80 days after transplantation [18].

#### *Acute Graft-Versus-Host Disease*

Since infection is often the terminal event in patients with uncontrolled GVHD, regimens which are less effective in preventing GVHD would be expected to increase infection and decrease survival after allogeneic marrow transplantation. This hypothesis was recently tested in a group of 16 patients transplanted for advanced-stage malignancies [29]. Deletion of all posttransplant immunosuppression in these patients given unmodified marrow from HLA-identical siblings led to 100% incidence of grade II–IV acute GVHD. Nonrelapse mortality was increased compared with historical controls given a 102-day course of methotrexate after transplant. Most deaths resulted from interstitial pneumonia and infection.

Acute GVHD is presumed to be the consequence of engrafted immunocompetent lymphoid cells reacting with disparate transplantation antigens located on host cells of the skin, liver, and intestinal tract. T-lymphocytes appear vital in the genesis of GVHD and provide the rationale for in vitro depletion of marrow T-cells in an attempt to prevent this complication. Although the

incidence of acute GVHD does decrease after T-cell depletion, benefits are offset by more frequent marrow graft failure and recurrent leukemia [13].

In vivo prophylaxis with immunosuppressive agents appears of key importance in preventing GVHD. A combination of methotrexate given intermittently for 11 days after transplant together with cyclosporine given for 180 days after transplant is superior to either drug used alone in preventing GVHD and results in decreased infection and improved survival [25]. However, renal and hepatic toxicity may limit the dose or schedule of drug administration and impair the efficacy of prophylaxis. Moreover, even though the incidence of acute GVHD is lowered, there is no apparent alteration in the incidence of chronic GVHD.

### **Supportive Care in the Late Transplant Period**

#### *Effects of Chemoradiotherapy*

The late effects of pretransplant conditioning have been recently reviewed [28]. Preparative regimens containing total body irradiation preclude recovery from gonadal failure [24]. The probability of having ovarian failure was 0.35 after preparation with cyclophosphamide alone compared with a probability of 1.00 for patients receiving cyclophosphamide plus total body irradiation ( $P < 0.0001$ ). In addition, endocrine failure may be observed in children who become long-term survivors after conditioning with chemotherapy and total body irradiation [23]. Abnormal thyroid function and growth hormone deficiency have been reported. Supportive care of these patients includes prompt identification of the endocrinopathy and appropriate hormone replacement.

#### *Immunodeficiency and Infection*

Long-term survivors without chronic GVHD remain remarkably free of infection and have evidence of return of normal immune function [12]. In contrast, patients with chronic GVHD have persisting abnormalities of B- and T-cell regulation. Chronic

GVHD patients frequently develop pneumococcal, staphylococcal, and streptococcal bacteremias and sinopulmonary infections [27]. Supportive care with prophylactic trimethoprim-sulfamethoxazole appears of benefit in these patients.

#### *Chronic Graft-Versus-Host Disease*

Chronic GVHD is a disorder with protean manifestations resembling several naturally occurring autoimmune diseases [27]. Recently, a syndrome of bronchodilator-resistant airflow obstruction resembling bronchiolitis obliterans has been reported in these patients [8]. Without immunosuppressive treatment, fewer than 20% of patients with clinical extensive chronic GVHD survive with Karnofsky performance scores greater than 70% [27]. We conducted a double-blind randomized trial comparing prednisone and placebo (group I,  $n = 63$ ) to azathioprine (1.5 mg/kg per day) and prednisone (group II,  $n = 63$ ) given as early treatment of extensive chronic GVHD [30]. Patients with platelet counts less than 100 000/ $\mu$ l received prednisone alone (group III,  $n = 38$ ). All three groups received similar doses of prednisone (1 mg/kg qod) and prophylactic trimethoprim-sulfamethoxazole (1 double strength (DS) p.o. bid). The double-blind nature of the study allowed analysis of the contribution of cytotoxic treatment to the frequency of late infections. For groups I, II, and III the respective incidence of infection was: disseminated varicella zoster, 11%, 24%, 34%; bacteremia, 6%, 11%, 34%; and interstitial pneumonia 5%, 14%, 18%. Nonrelapse mortality estimates were: 21% in group I, 40% in group II, and 58% in group III (I vs. II  $P = 0.003$ ; I vs. III  $P = 0.001$ ). Actuarial survival 5 years after transplantation was 61% in group I, 47% in group II, and 26% in group III (I vs. II  $P = 0.03$ ; I vs. III  $P = 0.0001$ ). Thus, early treatment with prednisone alone resulted in fewer infections and better survival than prednisone and azathioprine in standard-risk chronic GVHD. Treatment did not influence the rate of recurrent leukemia in any of the three groups. Treatment with prednisone alone was less effective in high-risk patients with thrombocytopenia. The reason

for this increase in the rate of infection was unclear, since granulocyte counts were normal in all groups.

We reasoned that GVHD-associated immunosuppression was the cause of increased rates of infection in patients with high-risk chronic GVHD. In these patients with persisting thrombocytopenia, we next studied oral cyclosporine (6 mg/kg q 12 h qod) combined in an alternating-day regimen with prednisone (1 mg/kg qod) and daily trimethoprim-sulfamethoxazole [31]. After 9 months of therapy complete response rates with this combination regimen were twofold higher than in the previous study with prednisone alone. Moreover, long-term survival was increased by twofold.

A major cause of treatment failure (especially among patients transplanted during relapse) is recurrence of malignancy following transplantation. In general, syngeneic and autologous transplant recipients have higher relapse rates than allogeneic recipients developing GVHD [37]. This apparent graft-versus-leukemia effect of allogeneic donor marrow is called adoptive immunotherapy and has been studied in a variety of transplantable tumor models. Recent updates of the Seattle experience confirm a graft-versus-leukemia effect in man [35]. Development of either acute or chronic GVHD was associated with improved long-term survival in patients with acute lymphoblastic leukemia transplanted in relapse and chronic myelogenous leukemia transplanted in blast crisis. Current studies are aimed at manipulating the posttransplant immunosuppressive support in an attempt to amplify a graft-versus-leukemia effect and improve disease-free survival in patients with advanced-stage malignancies [34].

## Summary

It is now almost 2 decades after the first successful human marrow transplants from HLA-identical siblings for the treatment of life-threatening hematologic diseases. Results have improved, especially for patients transplanted earlier in the course of disease. However, major problems remain in supporting patients through the transplant. More effective and less toxic conditioning

regimens are needed. Acceleration of hematopoietic and immunologic reconstitution by use of various cytokines holds promise for decreasing infectious morbidity and mortality. Improved regimens to control acute and chronic GVHD and prevent opportunistic infections will play a major role in the advancement of supportive care of the marrow transplant recipient.

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## Management of Fungal Infection in Neutropenic Patients with Fluconazole

K. W. Brammer

### Introduction

Fluconazole is a new orally absorbed anti-fungal azole which is soluble in water. It has a half-life of 24–30 h in humans [1], and is being evaluated in the treatment of localized and systemic fungal infections using a simple once daily oral or intravenous dosing regimen. Early clinical results with fluconazole have demonstrated it to be effective in the treatment of candidosis of the oropharynx, oesophagus, urinary tract and a variety of deep tissue sites at a daily dosage of 50–100 mg/day [2–6]. There are also reports of efficacy in the treatment of cryptococcal meningitis [7–9]. The ready penetration of fluconazole into the CSF is highly relevant in this context [10].

The convincing demonstrations of efficacy against systemic and mucosal fungal infections in patients, combined with an emerging profile of excellent toleration and safety, encouraged us to initiate a programme to evaluate fluconazole prophylaxis in patients at high risk of fungal infection. This paper summarizes the results of an interim analysis of a comparative prophylactic study in patients undergoing a period of induced neutropenia during the course of their treatment for haematological disease. The comparison was with oral polyene antifungals (amphotericin B or nystatin), preparations commonly used to combat fungal infection in this type of patient.

### Patients and Methods

Patients with acute leukaemia, lymphoma or aplastic anaemia, about to receive treatment (e.g. cytostatic therapy, radiotherapy) which was expected to render them temporarily neutropenic (less than 1000 neutrophils/mm<sup>3</sup> blood), were entered into this multi-centre study. They were randomized equally at each participating centre to receive treatment, orally, with either fluconazole (50 mg daily) or polyene (amphotericin B, at least 2 g daily of nystatin, 4 × 10<sup>6</sup> IU daily). Dosing was scheduled for 28 days or until the patient's neutrophil count recovered to near normal values.

Routine specimens for microscopy and mycological culture were taken from the mouth, nose, genitals, stool or rectum, and urine before chemotherapy/radiotherapy and then at least weekly for the duration of antifungal prophylaxis.

Alternative antifungal therapy was to be considered if a proven infection developed or if persistent fever (unresponsive to antibacterial therapy) occurred. The need to initiate alternative antifungal therapy, normally with intravenous amphotericin B, was considered to be a failure of antifungal prophylaxis. The incidence of mycologically documented fungal infection was determined as a definite indicator of prophylaxis failure.

All patients were closely monitored for possible side effects and a full range of haematological parameters and clinical biochemistry tests were carried out on blood and urine samples taken at least once weekly.

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The protocol was approved by a European multinational ethical review committee and, where necessary, by the relevant local ethics committee. All patients gave their informed consent to take part in the study.

## Results

At the time of this interim analysis data were available for a total of 248 patients entered at 12 different centres in France, Germany, Norway, Sweden and the United Kingdom. Of these, 126 patients were randomized to fluconazole prophylaxis and 122 to oral polyenes. Both groups were comparable in terms of their demographic details and the degree of neutropenia achieved (Table 1). Thirty-three patients in the fluconazole group and 25 in the polyene group received bone marrow transplants.

**Table 1.** Patient demographic and other relevant characteristics

Characteristics	Treatment group	
	Fluconazole (n = 126)	Polyenes (n = 122)
Sex (male, female)	71, 55	64, 58
Mean age (years)	48.0	46.9
Mean weight (kg)	67.1	67.5
Median baseline neutrophil count (/mm <sup>3</sup> of blood)	1456	2410
Median minimum neutrophil count (/mm <sup>3</sup> of blood)	236	306
Patients receiving bone marrow transplants	33	25

Fluconazole prophylaxis consisted of a single 50-mg oral capsule given once daily for a mean duration of 25.3 days. Prophylaxis with the polyenes, amphotericin B and/or nystatin took into account the existing practice in each participating unit and the availability of relevant formulations of the two agents in the country concerned. However, at least the specified minimum daily

dose was given, in divided doses at a frequency of at least four times daily, for a mean duration of 28.2 days.

Failure of prophylaxis, as defined by the need to initiate alternative antifungal therapy, occurred in 33 patients in the fluconazole group and 53 patients in the polyene group (Table 2). Four of the 126 patients in the fluconazole group and 3 of the 122 patients in the polyene group were unevaluable. Fluconazole therefore provided more effective prophylaxis than polyenes ( $P < 0.05$ , Fisher's exact test).

**Table 2.** Incidence of suspected and mycologically documented fungal infections

Result of antifungal prophylaxis	Treatment group	
	Fluconazole	Polyenes
No. of patients evaluable	122	119
No. of suspected fungal infections <sup>a</sup>	33 (27%) <sup>b</sup>	53 (45%) <sup>b</sup>
No. of proven fungal infections	1 (0.8%)	17 (14.3%)
Nature of mycologically documented infections		
Fluconazole group	1 <i>Aspergillus</i> pneumonia	
Polyene group	1 <i>Mucor</i> sinusitis 4 Candidaemias 12 Oropharyngeal candidososes	

<sup>a</sup> Suspected fungal infection = alternative antifungal treatment initiated

<sup>b</sup> Significance,  $P < 0.05$  (Fisher's exact test)

The reduction in incidence of mycologically documented fungal infection was even more strikingly in favour of fluconazole. Only 1 patient in the fluconazole group, compared with 17 in the polyene group, had culture-confirmed infections. It is noteworthy that there were no documented infections due to *Candida* in the fluconazole group whereas there were 16 in patients receiving polyene prophylaxis. In four of these cases there was proven systemic invasion, i.e. candidaemia.

An analysis of the surveillance cultures from the five routinely monitored sites showed only one significant difference in the rates of culture conversion. Conversion of negative faecal culture at baseline to positive at the end of treatment was more common in the fluconazole treatment group. The fungi cultured were usually *Candida* species or related yeasts, however, and there was no evidence that this increased colonization led to infection since all the documented *Candida* infections occurred in the polyene treatment group.

Toleration and safety of the study treatments was difficult to assess because of the severity of the patients' underlying illnesses and the adverse effects commonly associated with the large number of drugs being taken concomitantly, e.g. cytostatic drugs. Study drug treatment was discontinued in seven patients due to side effects thought to be possibly associated with the antifungal prophylaxis. Five patients discontinued fluconazole, due to skin rash (2), abdominal pain (2) and an allergic reaction (1) and two patients discontinued polyenes, due to abdominal pain (1) and vomiting (1).

Haematological abnormalities were present in virtually all patients because of the nature of their underlying diseases and the effects of treatment (cytostatic chemotherapy). Abnormalities in clinical biochemistry tests, possibly related to the study treatments, occurred in similar numbers of patients in each group (14 in the fluconazole group and 12 in the polyene group). As a precautionary measure with a well-absorbed investigational drug, fluconazole was discontinued in five of these patients.

## Discussion

Profound neutropenia has long been known to predispose patients to systemic, life-threatening infections. Since this condition is usually only transient, e.g. most commonly associated with the therapy of acute leukaemia and certain other malignancies, the period of risk is predictable and can be monitored by routine haematology. Bacterial infection, the major risk in neutropenic patients, is countered by routine prophylaxis and aggressive therapeutic use of an-

tibiotics at the first signs of infection, e.g. pyrexia. Increasing efficiency in the management of bacterial infection during the 1970s has resulted in the emergence of fungi, especially *Candida* species, as an important infection risk and a common cause of death in such patients [11].

The limited choice of effective antifungal drugs, all of which have limitations in terms of efficacy or safety, has delayed the development of a satisfactory method of combating fungal infection in neutropenic patients. The most common practice is to give patients oral preparations of the polyene antifungals, nystatin and amphotericin B, prophylactically during the period of neutropenia. These preparations are largely unabsorbed, the objective being to reduce yeast colonization of the alimentary tract and reduce the risk of systemic invasion. It has been reported that high doses of nystatin, at least  $4.5 \times 10^6$  IU daily, are required to reduce *Candida* colonization of the digestive tract [12]. This observation provided the basis for the minimum dosage of polyenes specified in the present study. Despite the widespread use of such regimens there has been no convincing evidence to demonstrate that they reduce the incidence of systemic invasion by *Candida* yeasts.

In this study fluconazole, given as a single 50-mg capsule daily, is proving significantly more effective than oral polyenes in reducing the incidence of either suspected or mycologically documented fungal infections, especially those caused by *Candida* species. Fluconazole is well absorbed after oral administration and distributes widely into body tissues and fluids [1, 10], indicating that the systemic presence of an antifungal may be necessary for optimum antifungal prophylaxis. Isolation of *Candida* in surveillance cultures from various body sites, especially the stool, has not correlated with invasive or symptomatic mucosal infection in this series of patients. The generally excellent toleration and safety profile of fluconazole observed in this study supports its use prophylactically in patients at risk of severe fungal infection.

Fluconazole has the added advantage of being water soluble at both acidic and neutral pH so that its absorption from the digestive tract is not affected by reduced gastric

acidity (Pfizer Central Research, data on file). Absorption is also unimpaired in patients following total body irradiation and bone marrow transplantation [13]. Furthermore, in patients who have difficulty in taking oral medication it may be given intravenously. These features, combined with the convenience of once daily dosing, enhance its suitability for prophylactic use.

The single mycologically documented fungal infection in the fluconazole treatment group, an *Aspergillus* pneumonia, is an uncommon but significant problem in the neutropenic patient. Dissemination of the infection is associated with a high fatality rate. It is not clear from this study whether this single case of aspergillosis represents either a total or partial failure of the 50-mg daily dosage to prevent this type of infection. Results obtained with higher daily dosages of fluconazole, currently being evaluated prophylactically in other similar studies, may provide the answer to this question.

## Summary

Fluconazole is a new orally absorbed antifungal azole which is effective in the treatment of mucosal and systemic infections caused by *Candida*, cryptococci and other fungi. In view of its favourable efficacy, safety and pharmacokinetic profile it was considered appropriate to evaluate its use prophylactically in patients undergoing a period of neutropenia. Two hundred and forty-eight patients receiving chemotherapy and/or bone marrow transplantation for the treatment of acute leukaemia, lymphoma or aplastic anaemia, and expected to be rendered temporarily neutropenic, have been entered into an ongoing multicentre comparative clinical study to compare the prophylactic efficacy of 50 mg daily oral fluconazole with that of widely used regimens of oral polyenes. The incidence of suspected fungal infection was less in the fluconazole group (27%) than in the polyene group (45%), the difference being statistically significant ( $P < 0.05$ ). Only one of the suspected infections in the fluconazole group was confirmed mycologically compared with 17 in the polyene group. Fluconazole prophylaxis was well tolerated and it therefore of-

fers a promising new approach to the management of fungal infection in the neutropenic patient. Further studies are warranted to define the optimum dosage for use in this situation.

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## Prevention of Bacteremias Caused by $\alpha$ -Hemolytic Streptococci by Roxithromycin in Patients Treated with Intensive Cytotoxic Treatment

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### Introduction

Microorganisms that frequently cause infections in patients with severe and prolonged granulocytopenia are endogenous gram-negative bacilli belonging to the normal flora of the alimentary tract [1]. Patients treated with cytotoxic drugs with a high degree of mucosal toxicity such as amsacrine and high-dose cytosine arabinoside or conditioning regimens for bone marrow transplantation are at high risk for bacteremias caused by  $\alpha$ -hemolytic streptococci, microorganisms not commonly recognized as opportunistic pathogens [2–5]. We have shown that ciprofloxacin is highly effective in prevention of infection as well as colonization by gram-negative bacilli in granulocytopenic patients [6, 7]. However, infections caused by gram-positive bacteria, especially  $\alpha$ -hemolytic streptococci and *Staphylococcus epidermidis* (often associated with the presence of indwelling catheters), have remained a problem. In the present study we added oral roxithromycin [8], a well-absorbed macrolide, to our prophylactic regimen during the granulocytopenic days with the highest degree of mucosal damage.

### Patients and Methods

Forty-five adult patients were evaluated, 15 patients receiving intensive consolidation therapy for acute leukemia with amsacrine and high-dose cytosine arabinoside, 15 patients receiving the same regimen for refractory leukemia, and 15 patients being treated with cyclophosphamide and total body irradiation followed by autologous bone marrow transplantation for malignant lymphoma. All patients received prophylactically ciprofloxacin 500 mg twice daily and antifungal prophylaxis with amphotericin B 400 mg four times daily [6]. Roxithromycin 150 mg twice daily was added to ciprofloxacin for 10 days, from the 3rd day after discontinuation of cytotoxic therapy.

*Microbiological Surveillance.* Quantitative cultures of feces and oral washing were performed at admission and thereafter once a week. Antimicrobial susceptibility was tested by an agar diffusion method on Isosensitest agar (Oxoid, Basingstoke, England) using Neo-sensitabes (Rosco, Taastrup, Denmark). Susceptibility of gram-positive bacteria to roxithromycin was tested with disks (Oxoid) consisting of 15  $\mu$ g roxithromycin.

### Results

#### *Patient Characteristics* (Table 1)

Forty-five patients were evaluated. There were 30 patients treated for acute leukemia

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**Table 1.** Clinical data of patients

	ABMT	CONS	REFR
Patients, <i>n</i>	15	15	15
Mean age (years)	38	37	48
Granulocytes < 500/ $\mu$ l median days	25	18	20
Days with fever (%)	8	11	21
Central venous catheter, <i>n</i>	15	11	12

ABMT, autologous bone marrow transplantation; CONS, consolidation therapy; REFR, refractory leukemia

and 15 patients underwent autologous bone marrow transplantation. Patients spent a median of 16 days with granulocytes <100/ $\mu$ l. In 39 of 45 patients central venous catheters were present.

#### *Acquired Infections and Microbiological Documentation (Table 2)*

There was a total of 15 acquired infections, 7 of them bacteriologically documented with 6 bacteremias. No infections caused by  $\alpha$ -hemolytic streptococci were seen during the period when roxithromycin was included in the prophylactic regimen. In one patient a bacteremia caused by  $\alpha$ -hemolytic streptococcus occurred after discontinuation of roxithromycin. This patient suffered from persisting mucosal ulcerations. These data

were compared with results obtained in previously treated patients receiving ciprofloxacin alone for infection prevention. These patients were comparable with regard to the underlying disease and cytotoxic regimens to the group of patients in the present study (data not shown). In those 80 historical control patients, 16 bacteremias (20%) caused by  $\alpha$ -hemolytic streptococci occurred. In two patients a respiratory distress syndrome occurred. All 16 isolated strains of  $\alpha$ -hemolytic streptococci were resistant to ciprofloxacin, with a minimal inhibitory concentration (MIC)  $\geq 4$  mg/liter.

To exclude the possible influence of roxithromycin on the effectiveness of ciprofloxacin in elimination of gram-negative bacteria from the alimentary tract, surveillance cultures were performed during the entire period of prophylaxis. During the prophylactic treatment no colonization by gram-negative bacilli occurred. Administration of the ciprofloxacin together with roxithromycin did not lead to overgrowth by yeast and no localized *Candida* infections were observed. The combination of ciprofloxacin and roxithromycin was very well tolerated and no allergic skin reactions or other side effects were observed.

#### **Discussion**

Antimicrobial prophylaxis directed against "endogenous" potentially pathogenic gram-

**Table 2.** Acquired infections and microbiological documentation in patients receiving roxithromycin in addition to ciprofloxacin for infection prophylaxis

	Ciprofloxacin + roxithromycin	Ciprofloxacin <sup>a</sup>
Patients, <i>n</i>	45	80
Acquired infections	15 (6)	47 (20)
Bacteriologically documented	7 (6)	23 (20)
<i>Streptococcus epidermidis</i>	6 (5)	5 (4)
Alpha-hemolytic streptococcus	1 (1) <sup>b</sup>	16 (16)
Gram-negative bacilli	0	2
Clinically documented	8	24
Unexplained fever	8	16

( ) number with bacteremia

<sup>a</sup> Historical control patients receiving ciprofloxacin alone

<sup>b</sup> Infection after discontinuation of roxithromycin

negative bacilli plays an important role in the supportive therapy in granulocytopenic patients [6, 9–12]. In previous studies we have shown that ciprofloxacin was very effective in prevention of infections caused by gram-negative bacilli [6, 7]. However, gram-positive infections caused by *Staphylococcus epidermidis* and  $\alpha$ -hemolytic streptococci remained a problem [2–5]. Many infections caused by  $\alpha$ -hemolytic streptococci are frequently related to intensive cytotoxic treatment (amsacrine, high-dose cytosine arabinoside, and conditioning regimens for bone marrow transplantation) and could be severe. Intensive cytotoxic treatment leads not only to profound granulocytopenia but it often also leads to ulcerations of the oropharynx and alimentary tract, which provide entry for streptococci with subsequent invasion of the bloodstream. A life-threatening situation resembling adult respiratory distress syndrome and/or shock can occur [3, 5, 13]. In order to decrease infections caused by  $\alpha$ -hemolytic streptococci, we have added roxithromycin (a new macrolide antibiotic) to our ciprofloxacin prophylactic regimen during the expected days with the highest degree of mucosal damage and bone marrow aplasia. Because we had the previous experience of a relatively high incidence of bacteremia caused by  $\alpha$ -hemolytic streptococci, we felt it would be rather hazardous in our hospital to study the combination of ciprofloxacin and roxithromycin in a controlled fashion, i.e., including a control group of patients receiving ciprofloxacin alone. The clinical data of 45 patients from this study who received ciprofloxacin plus roxithromycin were comparable to a historical group of 80 patients receiving ciprofloxacin alone, and, therefore, we have used this group of patients as a control. Our present study showed that the addition of roxithromycin to ciprofloxacin prevented infections, especially bacteremias caused by  $\alpha$ -hemolytic streptococci. During the days patients received combined drugs neither gram-negative infections nor infections caused by  $\alpha$ -hemolytic streptococci were observed. Roxithromycin prevented no infections caused by *S. epidermidis*, which were usually catheter associated. No antagonistic interaction between roxithromycin (which affects protein synthesis) and

ciprofloxacin was observed. This was concluded from serum bactericidal titers and the results from surveillance cultures (data not shown).

On the basis of our results we conclude that oral roxithromycin prevents bacteremias caused by  $\alpha$ -hemolytic streptococci in patients receiving intensive cytotoxic therapy for hematological malignancies.

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## Hepatosplenic Candidiasis in Acute Leukemias

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Infections are now the main cause of death in treatment of acute malignant disease. The spectrum of pathogens involved is showing a growing tendency toward mycotic and other opportunistic infections. Systemic fungal infections are a major problem, particularly in the treatment of hematological disease [1–3].

Between January 1980 and May 1988 we diagnosed systemic mycosis in 40 cases from a patient population with hematological diseases. In 26 patients, systemic mycosis was confirmed histologically on tissue obtained in vivo or postmortem, while the others displayed a clear elevation of serum *Candida* antibodies by at least two titer steps or repeated positive blood cultures.

Of the 40 patients, 14 (9 male, 5 female) had candidiasis of the liver and spleen. The average age of these patients was 40 years (range, 19–77 years). All the patients were suffering from acute leukemia, two of them from recurrence. Ten had acute myelocytic leukemia, and four acute lymphoblastic leukemia. All patients had been treated for their underlying disease with effective aplasia-togenic chemotherapy. In the severe granulocytopenic phase induced by cytostatic medication, the patients developed septic fever of up to 39°C, for which systemic treatment with different antimicrobials was administered. The indication for such treatment was generally empirical. However, the antimicrobial therapy did not bring about

defervescence. During the septic phase of the disease, the patients were seriously ill and confined to bed.

Monitoring with weekly cultures of oral and anal smears and of sputum, stool, and midstream urine showed growth of yeast colonies at three different locations on average. Thoracic X-ray revealed pulmonary infiltrates in eight patients during the aplasia phase. Bronchoscopy to identify the pathogens was performed in four of the eight patients, with fungal pneumonia being demonstrated in three.

After the granulocyte count had returned to normal in the peripheral blood, multiple circular foci were imaged in the liver and spleen by ultrasound and computed tomography. The general clinical condition had distinctly improved after the renewed rise in granulocyte numbers. When the first ultrasound and computed tomography procedures confirming the liver and spleen foci were performed, defervescence had already been achieved in two of our patients while a further six were only subfebrile. The ultrasound and computed tomography investigations revealed circular, almost echo-free foci 0.5–2 cm in diameter and isolated target-like rounded foci 1–3 cm in diameter. The echogenic potential of the generally enlarged livers and spleens was reduced. Computed tomography was performed in 11 of the 14 patients. Scanning without contrast medium revealed the multiple foci in the same size and distribution as hypodense areas. After bolus administration of contrast medium, filling of these foci did not match that of the adjacent parenchyma. In six patients these

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findings prompted further investigation in the form of laparoscopy, which on macroscopic examination revealed multiple, circular, yellowish foci on the surface of the enlarged liver. Histological examination of the selectively obtained liver biopsy material showed fungal mycelia in four patients. In the other two patients, the biopsy material displayed necrotic zones and multiple, small, granulomatously demarcated abscesses, suggesting mycosis.

In the course of the disease, pathologically elevated serum levels of liver enzymes were ascertained in 13 of 14 patients. Typically, the enzymes indicative of cholestasis were already elevated before demonstration of the liver and spleen foci by ultrasound or computed tomography. In 11 of the 13 patients, elevation of gamma-GT and AP started in the granulocytopenic, antimicrobial-resistant, septic phase of the disease and was thus an early indicator of hepatic involvement in the *Candida* septicemia.

The focal lesions on the liver and spleen were reduced in size and number by systemic antifungal therapy. The patients were treated with intravenous antifungal therapy for an average of 44 days (range 19–86 days). The total mean dosage administered was 1300 mg (range, 340–2400 mg) amphotericin B and 230 g (range 24–540 g) 5-flucytosine.

The liver and spleen candidiasis was cured in 12 patients. One patient died of generalized *Candida* septicemia while in complete remission of leukemia. One patient achieved temporary defervescence under the systemic antifungal therapy, but liver and spleen abscesses demonstrable by ultrasound persisted, as did pathologically elevated liver enzymes. The patient died 2 months later as a result of recurrence of leukemia and the re-activated *Candida* septicemia.

## Discussion

Candidiasis of the liver and spleen appears to be a special form of the disease found in immunosuppressed patients with relatively good immune defenses. To date, 73 patients with liver and spleen candidiasis have been described, mainly in the literature from English-speaking areas [4, 5].

All the patients described in the literature and our own patients had aggressive malignant tumors and they were all suffering from antimicrobial-resistant, septic fever. In our patients and in 68 of the 73 published cases the liver-spleen candidiasis had been preceded by high-grade granulocytopenia, triggered either by the underlying hematological disease or by chemotherapy. The candidiasis of the liver and spleen was therefore not manifested until the blood count had been normalized.

Twenty-five of the 59 published cases with confirmed candidiasis of the liver and spleen who were treated with systemic antifungal treatment died. This is a mortality rate of 42%. Compared with the data in the literature, the early mortality rate in our patient population is much lower at 14%. Only 2 of the 14 patients died of generalized candidiasis. The lower mortality rate in our patients is possibly due to the empirical early use of systemic antifungal agents. A further possible cause might be the intravenous antifungal treatment combining amphotericin B and 5-flucytosine. Most of the patients described in the literature on candidiasis of the liver and spleen were treated with amphotericin B alone.

In conclusion, focal hepatosplenic candidiasis has been recognized with increasing frequency in the last ten years. This form of systemic candidiasis has so far been found exclusively in patients with aggressive malignant tumors treated with effective cytostatic chemotherapy. Antimicrobial-resistant fever, which persists despite normalization of the blood picture, and multiple, almost echo-free or hypodense foci up to 2 cm in diameter on the liver and spleen which are demonstrable by ultrasound and computed tomography are typical of the clinical picture. If the patients are in complete remission of the underlying disease when the liver and spleen candidiasis is demonstrated, and if infiltration of the liver and spleen by the underlying disease can be ruled out, then in our opinion the features described are so characteristic that candidiasis of the liver and spleen can be diagnosed without recourse to histological confirmation.

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## Effect of Antifungal Therapy on Hematological Recovery After Intensive Antileukemic Chemotherapy

M. E. Essink, W. Hiddemann, M. von Eiff, Th. Büchner, and J. van de Loo

### Introduction

Systemic fungal infections are diagnosed at an increasing frequency during intensive chemotherapy for acute leukemias. This observation may be due to earlier diagnoses but also to more intensive antileukemic and antimicrobial chemotherapy. Hence, antifungal therapy is applied to patients with fever of unknown origin after nonresponse to antibiotic combination treatment. The other use of antifungal agents, however, may be hampered by a possible myelosup-

pressive effect, especially of 5-flucytosine, which may be more pronounced following intensive antileukemic treatment.

In order to define the possible myelosuppressive action of antifungal treatment, a retrospective analysis was performed in 87 patients with acute myeloid leukemia. Twenty-two of these patients were treated with amphotericin B and 5-flucytosine (5-FC) for proven or suspected fungal infections. Hematopoietic recovery was measured by the time from the start of chemotherapy to the rise of granulocytes above  $500/\text{cm}^3$  and thrombocytes above  $20000/\text{cm}^3$ . Antileukemic therapy consisted of the TAD-9 protocol combining thioguanine, cytosine arabinoside (Ara-C), and daunorubicin and/or

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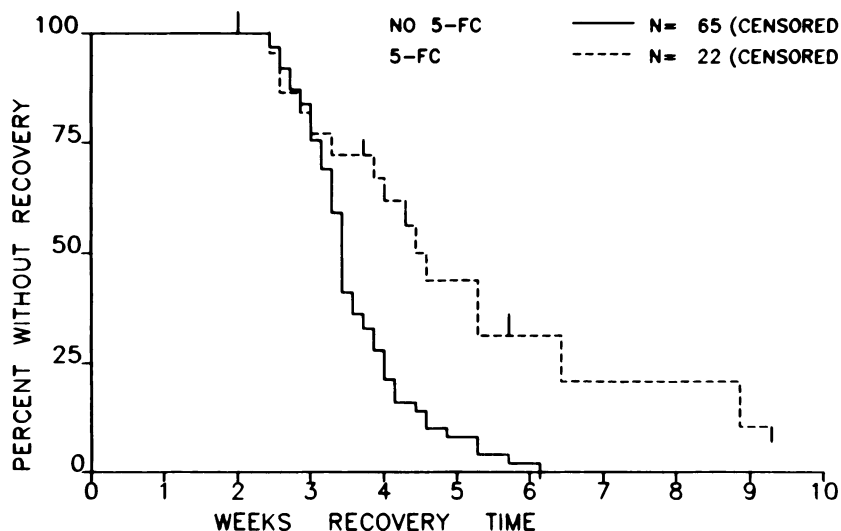


Fig. 1. Kaplan-Meier plot for time to recovery in patients with and without antifungal therapy

the combination of high-dose Ara-C and mitoxantrone (HAM). In 5 of the 22 patients receiving systemic antifungal treatment with 5-flucytosine and amphotericin B *Candida albicans* infections were verified by culture and/or positive serological testing. These patients received the combination of Amphotericin B and 5-FC until the resolution of infection over a median of 13 days (11–33 days). The remaining 17 patients received empirical antifungal therapy for a median of 18 days. The mean dose per day was 34 mg for amphotericin B and 10 g for 5-FC.

**Results** (Table 1, Fig. 1)

Time to recovery was significantly longer in patients receiving *systemic antifungal therapy* at 29 days as compared with 24 days for patients *without antifungal treatment*. Forty percent of these patients needed more than 5 weeks for the recovery of normal blood counts as compared with 5% in the patients without antifungal therapy. The small number of cases did not allow the evaluation of recovery time and 5-FC doses. For the group of 17 patients receiving empirical antifungal therapy, the mean 5-FC dose was 50 mg, with a median time to recovery of 28

**Table 1.** Time to recovery in patients with and without antifungal therapy

	Number patients	Median TR	Range TR
A	22	29	17–65
B	65	24	14–45

A, antifungal therapy; B, no antifungal therapy; TR, time to recovery (days)

days (17–45 days). The other five patients with proven fungal infections received a mean 5-FC dose of 125 g with a median time to recovery of 30 days (21–65 days).

**Conclusion**

These data strongly suggest that 5-flucytosine and amphotericin B therapy causes a prolongation of the recovery times of hematopoietic cells in patients with acute myeloic leukemia after intensive chemotherapy. Although a direct effect of the fungal infection on hematopoiesis cannot be excluded, a direct myelosuppressive effect of the applied antifungal combination is the most probable cause for the observed prolonged recovery.

## Preliminary Results of Treatment with Itraconazole in Patients with Systemic Fungal Infections

H.-H. Wolf, R. E. Scharf, M. Arning, and W. Schneider

Patients with acute leukemias undergo a high risk of lethal systemic fungal infections during prolonged periods of granulocytopenia. While amphotericin B is still the most effective antimycotic drug, its use is limited by anaphylactoid, hepatotoxic and/or nephrotoxic side effects. Imidazole antimycotics, such as miconazole and ketoconazole, lack activity against aspergillus infections [1]. The new triazole derivative itraconazole has shown its efficacy in treatment of fungal infections due to *Candida* and *Aspergillus* species [2, 3, 6]. Here we report our experiences with itraconazole in six patients aged 24 to 47 years suffering from acute myelogenous ( $n=5$ ) or acute lymphoblastic leukemias ( $n=1$ ), and microbiologically proven ( $n=4$ ) or clinically suspected ( $n=2$ ) systemic aspergillus or candida infections.

### Patients

Following cytostatic therapy all these patients suffered from high fever during the phase of bone marrow aplasia despite systemic multidrug antibiotic therapy. Blood cultures were negative. There were no signs of viral infections or *Pneumocystis carinii* pneumonia. In five patients chest roentgenograms showed mostly interstitial segmental pneumonias. Four patients underwent bronchoscopy; *Aspergillus* species were

identified in three, and candida in one. One patient could not undergo bronchoscopy because of thrombocytopenia. In the remaining patient no microorganisms could be identified during induction therapy of acute myelogenous leukemia. As bone marrow aplasia was prolonged and fever persisted despite of antibiotic therapy, the possibility of an underlying systemic fungal infection was considered.

### Previous Treatment

All patients were treated with oral amphotericin B for intestinal decontamination from the first day of hospitalization. When there was evidence of a fungal infection, we started parenteral therapy with amphotericin B and 5-flucytosine (Table 1). Therapy with amphotericin B was discontinued because of hyperthermia, shock, or cerebral edema in four patients and hepatotoxic or nephrotoxic side effects in the remaining two. In four patients amphotericin B was administered for 1–3 days only (Table 1). Two of the patients were treated with miconazole for 5 and 41 days combined with 5-flucytosine for 4 and 24 days, respectively. In both patients pneumonic infiltrations persisted. Two other patients were treated with amphotericin B in therapeutic dosages. One patient developed serious side effects, such as vomiting, hypotension and cerebral edema after 10 days of treatment. The other patient sustained a rise in serum creatinine after 32 days of amphotericin B therapy. During this time his pneumonia decreased but did not remit completely.

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**Table 1.** Previous antimycotic treatment in patients with acute myelogenous or lymphoblastic leukemias

Patient	Sex	Age (years)	Species	Pneumonia	Previous treatment (days)		
					Amph. B	5-FC	Miconazole
1	m	45	aspergillus	+	1	24	41
2	f	36	not identified	+	1	4 + 14	5
3	f	37	aspergillus	+	32	30	—
4	m	47	aspergillus	+	3	2 + 4	—
5	m	33	candida	+	2	2	—
6	f	23	not identified	—	10	5	—

### Treatment with Itraconazole

Itraconazole is only available for oral administration. As its resorption rate depends on the gastric pH, the daily dosage was divided into three to four portions given by meal [4]. We administered 600 mg itraconazole per day for 5 days, and subsequently reduced to 400 mg per day for approximately 12 days. For further therapy patients received a mean daily dosage of 200–400 mg. The duration of therapy ranged between 14 and 165 days; the total dosage per single patient was between 4.6 g and 45.8 g. Itraconazole was administered without additional antimycotic drug in four patients. In two patients also treated with 5-flucytosine for 4 and 14 days, pneumonic infiltrations

developed. One of the patients received previous treatment with miconazole and 5-flucytosine for 5 and 4 days, respectively, because of an amphotericin B-induced allergy.

### Results

All patients treated with itraconazole recovered from fever, and the five with pneumonia improved markedly (Table 2). After 165 days of therapy one patient suffering from candida pneumonia demonstrated no radiological residua. He received itraconazole as a single antimycotic drug in a total dosage of 45.6 g. Amphotericin B could not be administered because of serious hepatic disorders.

**Table 2.** Treatment with itraconazole following discontinuation of therapy with amphotericin B in patients with acute myelogenous or lymphoblastic leukemias

Patient	Side effects of amphotericin B			Itraconazole		Remittance of fever	Radiological findings	Follow-up
	anaphylactoid	hepatotoxic	nephrotoxic	Duration of treatment (days)	Total dosage (g)			
1	+	—	—	43	19.8	+	mostly improved	lethal pulmonary bleeding
2	+	—	—	60	24.6	+	mostly improved	pulmonary segmentectomy
3	—	—	+	83	28.2	+	completely recovered	cured
4	+	—	—	32	11.0	+	completely recovered	cured
5	—	+	—	165	45.8	+	completely recovered	cured
6	+	—	—	14	4.6	+	—	cured

One patient with extended aspergillus pneumonia improved during treatment with amphotericin B and 5-flucytosine for 18 days. Because of nephrotoxic side effects, therapy was changed to itraconazole. After 2 months of treatment (total dosage: amphotericin B 1160 mg, 5-flucytosine 112.5 g, itraconazole 16.6 g), aspergillus was undetectable in the bronchial lavage and transbronchial biopsy. As there were large caverns in both upper lobes, we continued itraconazole treatment after discharge. The total dosage was 28.2 g over 83 days.

Another young woman who had developed pneumonia during miconazole treatment received an itraconazole dosage of 24.6 g. Clinical symptoms corresponded to pulmonary mycosis. Bronchoscopy was contraindicated because of thrombocytopenia. She underwent pulmonary segmentectomy for persistent interstitial pneumonia after 60 days of treatment with itraconazole. During bone marrow aplasia following reinduction chemotherapy because of relapsing acute myelogenous leukemia, she developed no signs of inflammation.

Another patient receiving long-term miconazole therapy because of aspergillus pneumonia improved following itraconazole (total dosage 19.8 g, duration of treatment 43 days). However, this patient died of bleeding from a great pulmonary artery.

Another patient with acute lymphoblastic leukemia acquired pulmonary aspergillosis during induction chemotherapy. He improved without complications following early combined itraconazole treatment with 5-flucytosine. The remaining patient with fever and amphotericin B-induced allergy improved completely following itraconazole.

### Side Effects

All patients who received a mean daily dosage of > 300 mg demonstrated a transient elevation of serum gamma-glutamyltransferase (GGT) ranging from 70 to 404 U/l. Serum concentrations of more than 130 U/l were seen only in patients with pre-existing liver dysfunction. In four of six patients serum alkaline phosphatase (AP) increased slightly (graduation I according to WHO). No elevation of serum bilirubin was

seen. One patient receiving itraconazole because of amphotericin B-induced hepatic dysfunction showed a rise of serum GGT and AP, but serum bilirubin decreased. After reducing the itraconazole dosage below 300 mg per day, all laboratory findings returned to normal. No gastrointestinal or neurological side effects were seen. A delayed rise in peripheral platelet count in two patients was probably due to partial remission of AML and not to the side effects of itraconazole.

### Conclusions

In patients with systemic fungal infections amphotericin B should be administered. In our patients itraconazole treatment was initiated in those who did not tolerate amphotericin B because of its anaphylactoid, hepatotoxic, or nephrotoxic side effects. We administered itraconazole for several months without severe complications, and five of six patients with fever and pneumonia improved significantly. Combined therapy with 5-flucytosine seems to be useful [5]. As the number of our patients is still small, the results are preliminary and therefore require further clinical investigation.

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## Lymphocyte Contamination in Leukocyte-Depleted Red Cell and Platelet Concentrates Obtained by Filtration

N. Müller, M. Gummelt, S. Osskop, and Ch. Schlake

Several of the major complications of blood transfusion result from white cell contamination of blood components, such as non-hemolytic febrile reactions, refractoriness to platelet transfusion, and virus transmission [1, 2, 5, 7]. There is evidence that mainly lymphocyte-contaminated blood products are the major cause of alloimmunization and refractoriness of the recipients [3, 6]. Repeated transfusion of platelet concentrates leads to HLA alloimmunization in 40% of patients [3]. The management of alloimmunized patients represents a difficult and expensive challenge and any means by which the rate of alloimmunization can be reduced is to be welcomed. Therefore, blood component therapy aims to transfuse standardized blood products as purely as possible in a very selective way in order to provide optimal therapeutic efficacy and minimal side effects.

At present, white-cell-depleted red cells and platelets for transfusion can be prepared by a filtration procedure [5, 8]. With this generally accepted process, it is possible to remove most of the white cells, but it should be noted that some patients could potentially become immunized due to the remaining lymphocytes, and these dividing donor cells may represent a subclinical graft-versus-host reaction [9]. Therefore, it could be of interest to investigate and evaluate different filter systems for their white cell removal capacity with special regards to the residual

lymphocyte contamination of packed red blood concentrates (RBCs) as well as random-donor platelet concentrates.

### Material and Methods

This study evaluated the filtration of packed RBCs and platelets through filter systems. RBCs stored <4 days and >20 days were filtered through Erypur-G 2 (Organon Teknika, Akzo), containing cellulose-acetate fiber, and Sepacell R 500 (Diamed/Asahi), containing nonwoven polyester fiber. Platelet concentrates were filtered by the Imugard IG-500 (Terumo), containing cotton wool fiber as well as a pilot study through the Erypur systems.

Red cell concentrates were conventionally prepared from 450 ml whole blood using triple-bag systems with CPDA-1 (Baxter) buffycoat-poor by standard methods, containing an average of  $1.68 \pm 0.60 \times 10^9$  leukocytes,  $0.8 \pm 0.17 \times 10^9$  lymphocytes/unit. A total of 20 RBCs per filter system were evaluated. Platelet concentrates (PCs) were also prepared routinely from 450 ml whole blood using triple-bag systems with CPDA-1 (Baxter). PCs prepared in our processing laboratory contain in average of  $73.50 \pm 26.44 \times 10^9$  platelets/unit,  $0.337 \pm 0.231 \times 10^9$  leukocytes and  $0.284 \pm 0.187 \times 10^9$  lymphocytes suspended in 50 ml plasma. All PCs were stored on a rotator (Helmer Labs) (6 rpm) at  $22^\circ \pm 0.5^\circ \text{C}$  for 1–2 days. Using standard pooling procedures, six AB0-identical concentrates were pooled into a 600-ml transfer bag. A total of 28 platelet pools

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were investigated. Cell counts of sample before and after filtration were performed automatically by Sysmex CC-180 (TOA) and manually using a standard counting chamber. Slides were also prepared from the units to ascertain the percentage lymphocytes versus granulocytes remaining after the procedures.

Bio-Rad's quantigen T- and B-cell assays were performed for the identification and enumeration of the lymphocyte subpopulations, and T and B cells. Lymphocytes were isolated by density gradient centrifugation, incubated at 37°C to elute cytophilic Ig. These cells were then incubated with immunobeads in order to label all cells present. Yellow-brown immunobeads bind to and rosette B cells; colorless immunobeads bind to and rosette T cells. A rosette is defined as any cell that has three or more immunobeads bound to the cell surface. Macrophages are phagocytic cells which contaminate density gradient preparations of lymphocytes. They are identified by their ability to ingest both types of immunobeads. The remaining unlabeled cells represent the null cell population.

## Results

Table 1 shows the effectiveness of the cellulose-acetate and nonwoven-polyester-fiber-filter in providing white-cell-poor packed RBCs. Both techniques easily satisfy current standards for WBC and lymphocyte removal as well as RBC recovery on the condi-

tion that only freshly (<4 days of storage) and one unit is processed. The good efficiency of WBC removal did not appear to be obtained by filtering two packed RBCs or one unit of RBCs more than 20 days old. Thereby, lymphocyte counts (>90% T cells) above the critical immunogenic load could be observed.

In Table 2 the effectiveness of the cotton-wool filtration of pooled PCs is shown. The percentage recovery of filtered platelets varied from 55% to 82%. The residual lymphocytes in the filtered units were  $0.0464 \pm 0.0204 \times 10^9/\text{unit}$ . Removing white cells with the cellulose acetate filter was also a highly efficient method, but far fewer platelets were recovered in the filtered products, so that this system is no longer applied.

## Discussion

Blood processing using filter systems may be useful and practical as a routine method for the preparation of white-cell-poor RBCs. With this process it is possible to remove the white cells, including lymphocytes, at the lower end of the critical antigenic load for leukocytes ( $0.25 \times 10^9/\text{unit}$ ) or the critical immunogenic load ( $0.02 \times 10^9/\text{unit}$ ) [5, 6] on the condition that only freshly (<4 days of storage) and one unit/filter is processed. Thereby, these filtration methods appear to decrease alloimmunization and in vivo lymphocyte stimulation. But it should be kept in mind that most transfusion services

**Table 1.** Cell distribution (range/mean  $\pm$  SD) in packed RBCs prepared by the cellulose-acetate fiber and nonwoven-polyester-fiber filter

Age filtration (units)	Cellulose acetate fiber							Nonwoven polyester fiber				
	WBC (mm <sup>3</sup> )	Lymphocytes					WBC (mm <sup>3</sup> )	Lymphocytes				
		T	B	O	T <sub>4</sub>	T <sub>8</sub>		T	B	O	T <sub>4</sub>	T <sub>8</sub>
days 1 <4	0/50	0/0	0/0	0/0	0/0	0/0	0/40	0/0	0/0	0/0	0/0	0/0
2	60/425	22/23	0/1	0/2	17/19	3/6	190/1775	20/188	1/10	1/14	17/135	3/56
	%	90.67	1.33	1.30	78.67	21.33	%	90.40	1.88	5.70	78.60	21.40
	$\pm$	2.31	0.31	1.30	8.33	8.33	$\pm$	6.73	1.93	2.95	7.60	7.60
days 1 >20	12/490	4/44	0/2	1/6	3/32	1/12	40/410	36/46	0/1	1/3	28/40	6/8
	%	90/65	3.55	6.80	72.88	27.12	%	91.00	2.25	6.75	81.75	18.25
	$\pm$	2.82	2.26	1.19	1.93	1.93	$\pm$	1.41	0.35	1.06	6.01	6.01

**Table 2.** Cell distribution in random pooled platelet concentrates (mean  $\pm$  SD) prepared by a cotton wool filter system compared with nonfiltered components

	Platelets ( $\times 10^9/\mu$ )	WBC ( $\times 10^9/\mu$ )	Lymphocytes ( $\times 10^9/\mu$ )					
			Total	T	B	O	T <sub>4</sub>	T <sub>8</sub> cells
Filtered	47.10	0.048	0.0464	0.0216	0.0179	0.0069	0.0192	0.0024
	$\pm 8.32$	$\pm 0.033$	$\pm 0.0204$	0.0097	0.0094	0.0013	0.0086	0.0011
			%	80.91	4.46	15.62	88.88	11.11
			$\pm$	3.50	2.39	1.80	2.46	2.14
Non-filtered	3.59	0.337	0.284	0.212	0.044	0.028	0.113	0.099
	$\pm 26.44$	$\pm 0.231$	$\pm 0.187$	0.138	0.030	0.019	0.075	0.064
			%	74.65	15.49	9.86	53.30	46.70
			$\pm$	3.69	3.02	1.18	2.72	4.64

have most units of RBCs that are more than 4 days old.

On providing white-cell-depleted platelets, the cotton wool filter looks better than the cellulose acetate filter, but the platelet recovery is poor and the lymphocyte contamination remains above the level of the critical immunogenic load. The function of the filtered platelets has not been studied.

In conclusion, transfusion of filtered blood components may prevent major complications, such as nonhemolytic febrile transfusion reactions [1, 2, 5], but it does not seem to be the definitive answer. It may, however, be possible that lymphocyte contamination may cause immunization and a refractory state in multiple transfused patients [3, 9]. Therefore, it is also commendable to irradiate the filtered blood components to prevent the proliferation of the host cells, when transfusing filtered blood to immunocompromised patients [4].

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## Effects of Verapamil on Anthracycline-Induced Cardiomyopathy: Preliminary Results of a Prospective Multicenter Trial

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### Introduction

The use of anthracyclines and anthrachelones in antineoplastic treatment is limited by the possible induction of irreversible cardiomyopathy [1, 2, 6, 7, 9]. In 1976, Daniels et al. observed a protective effect of subcutaneously administered verapamil in white rabbits [4]. Müllerleile et al. reported a beneficial effect of low-dose oral verapamil in 22 patients with different tumors treated with doxorubicin [10]. The aim of this study was to determine whether verapamil can protect against anthracycline cardiomyopathy without altering the clinical efficiency of the antineoplastic regimen in patients with acute myeloid leukemia (AML) [8, 10–12].

### Methods

All patients included had documented AML and were aged between 18 and 60 years.

None had contraindications against anthracycline treatment, and none had previous irradiation or chemotherapy. Chemotherapy was administered according to the protocol of the AML COOP study group Münster 1986 [3] with double induction and consolidation chemotherapy. The patients were randomized in a group with ( $3 \times 40$  mg p.o. daily) and without accompanying verapamil treatment. Cardiological checks including physical examination, ECG, echocardiography with measurement of left ventricular shortening fraction (LVSF), and multigated nuclear scans with measurement of left ventricular ejection fraction (LVEF) were performed before induction and 3 weeks after consolidation chemotherapy.

Serum levels of verapamil and norverapamil were measured in the verapamil group on days 2, 14, and the last day of verapamil treatment [5] (M. Eichelbaum, Stuttgart). The cardiologists and nuclear medicine physicians were blinded to the patient treatment group, but the hematologists and patients were not.

### Results

Between July 1986 and January 1989, 64 patients from three different hospitals (Dept. of Medicine, Evangelisches Krankenhaus, Essen-Werden; Dept. of Hematology, University of Münster; Medical Clinic II, University of Kiel) entered the study. Their characteristics are shown in Table 1. Both treatment arms are balanced in terms of sex, age, pretreatment cardiological findings, and antineoplastic chemotherapy. Thirty

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**Table 1.** Patient characteristics

	Verapamil	
	Yes	No
Total number	30	34
Male	14	17
Female	16	17
Age		
Mean	41.3	41.3
Range	21–59	19–59
Findings at check 1		
Hemoglobin (g/liter)	98 ± 19	99 ± 13
Heart rate	85 ± 13	93 ± 13
Systolic blood pressure	121 ± 12	126 ± 17
Diastolic blood pressure	73 ± 9	76 ± 10
LVSF (mm)	35.8 ± 5.5	39.0 ± 6.0
LVEF (%)	54.8 ± 5.9	56.3 ± 6.4
Chemotherapy		
TAD/TAD/TAD <sup>a</sup>	17	13
TAD/HAM/TAD <sup>b</sup>	15	19
Patients evaluable for checks 1 and 2	13	17
Patients still in study	6	2
Deaths during therapy	6	8
Chemotherapy not completed <sup>c</sup>	3	5
Protocol violation	2	2

<sup>a</sup> See Büchner et al. [3], ≅ 540 mg daunorubicin/m<sup>2</sup>

<sup>b</sup> See Büchner et al. [3], ≅ 360 mg daunorubicin/m<sup>2</sup> + 30 mg mitoxantrone/m<sup>2</sup>

<sup>c</sup> Reasons: BMT (3 ×) severe infection (2 ×) No response after induction chemotherapy (3 ×)

patients have been evaluated for pre- and posttreatment cardiological checks so far, and eight patients have not yet completed consolidation chemotherapy. Eighteen of the 30 patients showed a posttreatment decrease in LVSF and/or LVEF of more than 10%. Differences between the verapamil and nonverapamil group are given in Figs. 2 and 3, differences between the two chemotherapy regimens (TAD × 3 – TAD/HAM/TAD) in Figs. 4 and 5. Table 2 shows different degrees of posttreatment LVSF reduction in the verapamil and nonverapamil group.

One patient in the nonverapamil group with a more than 40% decrease in LVSF developed severe congestive heart disease

(NYHA IV). All differences observed were not statistically significant.

Mean serum levels of verapamil were 34.6 ng/ml on day 2, 17.2 ng/ml on day 14, and 10.9 ng/ml on the last day of verapamil treatment. In two patients of the verapamil group, no serum verapamil could be measured; they were excluded from further evaluation (see Table 1, protocol violation).

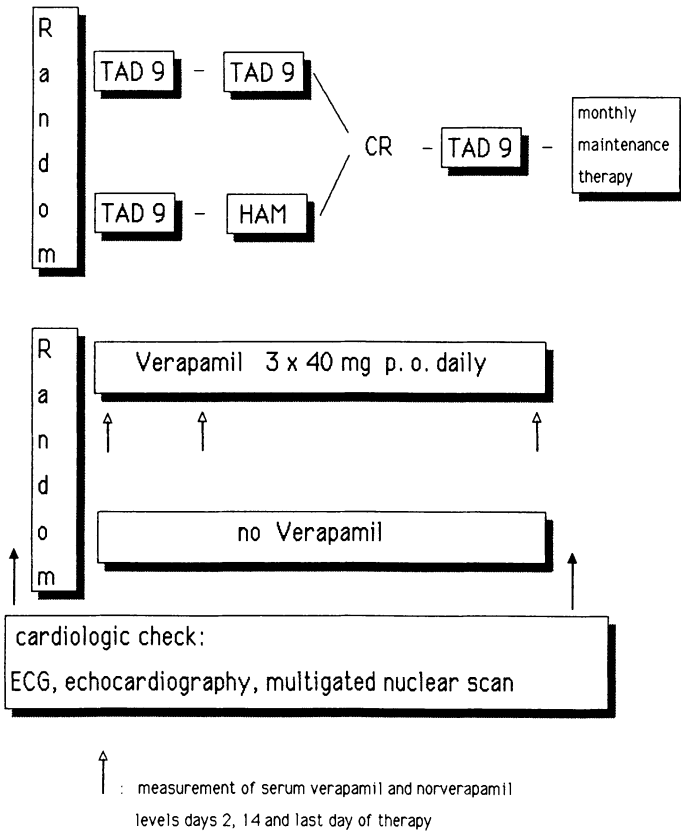
## Discussion

The data from this prospective randomized trial did not show a difference in cardiotoxicity between both chemotherapy arms. The decrease in LVSF and LVEF was similar, indicating equivalent toxicity for the TAD and HAM regimens. So far, a statistical difference in cardiotoxicity between the verapamil and nonverapamil group has also not been observed. There are fewer patients with a severe (>30%) reduction of LVSF in the verapamil group, but the number of patients investigated is still too small.

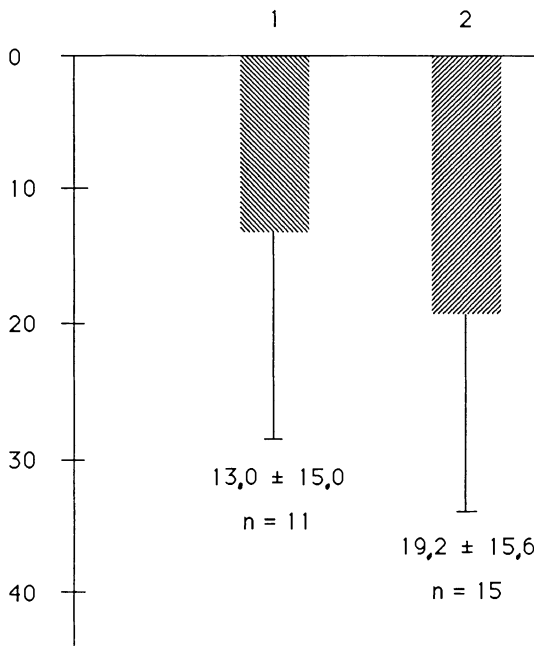
**Table 2.** Percentage decrease shorting fraction (LVSF)

% decrease	Verapamil	
	Yes	No
<10	7	6
10–20	0	1
20–30	2	3
30–40	1	2
>40	1	3

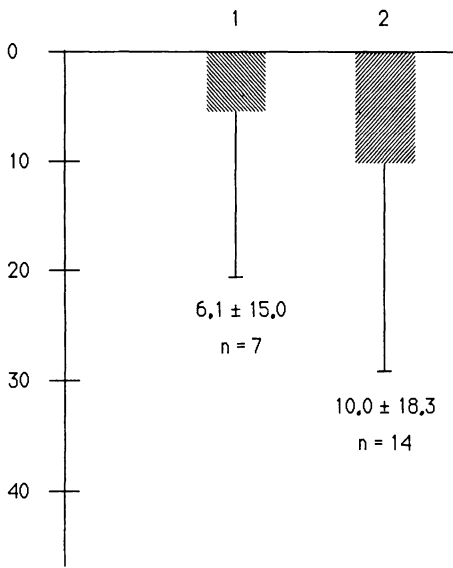
Although only one patient developed severe cardiomyopathy, 60% had a significant reduction in LVSF and LVEF after consolidation chemotherapy. Modern concepts of AML treatment increasingly include bone marrow transplantation as a therapeutic option. In patients with relapse, aggressive chemotherapy protocols with cardiotoxic drugs [i.e., high-dose cytosine arabinoside (Ara-C)/mitoxantrone] have been effective. This underlines the necessity for cardiac monitoring of AML patients as well as the necessity for reducing cardiotoxicity of the antineoplastic chemotherapy.



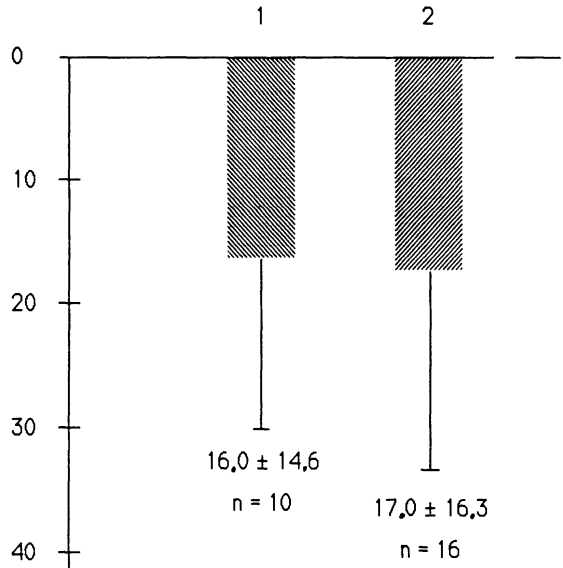
**Fig. 1.** Study design



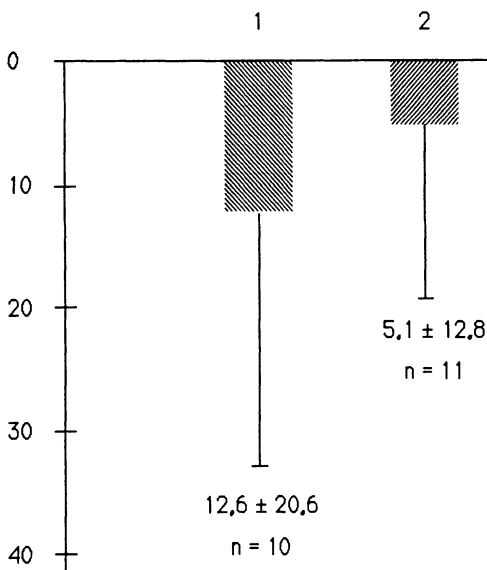
**Fig. 2.** Percentage decrease of shortening fraction. 1, verapamil; 2, no verapamil



**Fig. 3.** Percentage decrease of ejection fraction. 1, verapamil; 2, no verapamil



**Fig. 4.** Percentage increase of shortening fraction. 1, TAD/TAD/TAD; 2, TAD/HAM/TAD



**Fig. 5.** Percentage increase of ejection fraction. 1, TAD/TAD/TAD; 2, TAD/HAM/TAD

### Summary

Previous investigations in animals and one retrospective study in man suggest that verapamil can prevent anthracycline-induced cardiomyopathy. In the following study, pa-

tients with acute myeloid leukemia (AML) treated with double induction and consolidation chemotherapy (AML COOP study 1986, [3]) were randomized in a group with and without accompanying low-dose oral verapamil treatment. Since July 1986, 64 patients have been included. Thirty patients have been evaluated for pre- and posttreatment cardiological investigations. So far, no significant difference in cardiotoxicity has been observed either between the verapamil and nonverapamil group or between the two induction chemotherapy regimens (TAD/TAD – TAD/HAM).

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## Prospective Study on the Influence of Disease or Treatment on Pituitary Function in 31 Children with Acute Leukemia and Non-Hodgkin's Lymphoma

U. Mittler, K. Mohnike, U. Kluba, G. Kröning, W. Kapitza, V. Aumann, and R. Röpneck

### Introduction

The number of long-term survivors of childhood acute leukemia and non-Hodgkin's lymphoma (NHL) has steadily increased over the past decades. Most of these patients are approaching adolescence and adulthood. This stresses the necessity for thorough investigation to establish the long-term effects both of the disease and of its treatment. With this in mind our studies have especially focused on endocrine sequelae to assess the quality of life of children who can be considered cured from a malignant systemic disease.

### Patients and Methods

#### Group 1

To assess the effects of chemo- and radiotherapy on the endocrine system, 31 children (13 girls, 18 boys) with acute leukemia and non-Hodgkin's lymphoma (3 AML, 24 ALL, 4 NHL) were investigated. The median age at onset of disease was 5 years 6 months (range, 2–16 years). Twenty-five patients were in prepuberty, 5 in puberty, and 1 post puberty. All children were treated according to modified Berlin-Frankfurt-Münster (BFM) protocols (protocol VII of the GDR leukemia group). This therapy consisted of prednisolone, vincristine

(VCR), daunorubicin (DNR) or adriamycin (ADR), asparaginase (ASP), cyclophosphamide (CP), cytarabine (Ara-C), 6-mercaptopurine (6-MP), and 6-thioguanine (6-TG) during induction and consolidation, and 6-MP and methotrexate (MTX) during remission maintenance in acute lymphocytic leukemia (ALL); it lasted 2 years.

Children suffering from NHL were given similar cytostatic treatment. The therapy in patients with AML included prednisolone, 6-TG, VCR, ADR, Ara-C, and CP during induction and consolidation and 6-TG, Ara-C, and ADR during remission maintenance and was stopped after 2 years. Twenty-nine patients received CNS prophylaxis consisting of intrathecal injections of MTX and cranial irradiation with a dose of 18 Gy. Before treatment (0), during induction (1), cranial irradiation (2), 4–6 weeks later (3), and during maintenance therapy (4/5), the following hormone values were estimated: basal luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin, T3 and T4, thyroid-stimulating hormone (TSH) basal and 30 min after thyroid-releasing hormone (TRH) (5 µg/kg). All hormone levels were established by radioimmunoassays.

#### Group 2

In addition, we performed a retrospective growth study in 35 children (16 girls, 19 boys) with ALL ( $n=28$ ) or NHL ( $n=7$ ). The median age of this group at the onset of disease was 5 years. Patients were treated

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according to protocol VII or LSA2L2 [10]. Twenty-eight patients received cranial irradiation with 18 Gy, but in 7 intrathecal injection of colloidal gold-198 was given for CNS prophylaxis. Standing height was measured at regular intervals, in most cases by the same experienced person.

These values were compared with the data of the second Zurich longitudinal study (Prader et al., Züricher Longitudinalstudie, personal communication) taken as the reference standard. Height standard deviation score (SDS) were calculated for comparison between children of different age and sex. As a second parameter, height velocity was calculated using the same standards as Prader et al. Bone age was estimated according to standards of Greulich and Pyle by the same experienced person.

### *Statistical Methods*

The results were analyzed by the sign test of Dixon and Mood, the Wilcoxon matched-pair signed rank test, and the U-test of Mann and Whitney. The level of significance was set at  $P < 0.05$ .

## **Results**

### *Group 1*

Basal and TRH-stimulated TSH were within normal limits, except in three patients, who had occasionally elevated levels (Fig. 1). According to T4 as well as T3 all cases were euthyroid. Seven patients had a single elevated prolactin level, but in three patients moderate hyperprolactinemia was repeatedly found, in part already before treatment. At least in 18 out of 119 single determinations prolactin levels were between 15.5 and 32.0 ng/ml, far from those found in prolactinoma (Fig. 2). No preference of the higher prolactin levels for a certain point in our time schedule was found. Basal LH and FSH were normal according to the stage of puberty. In 18 prepubertal children repeated measurements showed normal LH and FSH levels, but with an increasing tendency after cranial irradiation (Fig. 3a, b).

### *Group 2*

Figure 4 shows the mean SD-score (SDS) for height in children with ALL/NHL treated according to different schedules. Protocol LSA2L2 used in the past resulted in a permanent height reduction, but within the normal range of  $\pm 2$  SD ( $P < 0.01$ ). In contrast, the mean SDS for height in children treated with protocol VII declined only during the intensive period of treatment. A catch-up growth occurred during maintenance therapy. Three years after diagnosis the previous height centile was regained ( $P < 0.01$ ). After 4–5 years, the height SDS of a subgroup of patients ( $n=9$ ) was greater than at diagnosis. This might be due to a random selection of taller children (height SDS at the start in this group:  $\pm 0.1$  SD) or early puberty. No differences in height SDS were found between pubertal versus prepubertal children and between different kinds of CNS prophylaxis (cranial irradiation with 18 Gy or colloidal gold-198). During the more intensive period of treatment a significant decrease in height velocity (Fig. 5) was observed ( $P < 0.01$ ), but already during the second half-year after starting therapy height velocity was normalized in both girls and boys treated with protocol VII ( $P < 0.05$ ). In contrast, growth during the LSA2L2 protocol was characterized by a continuous lower velocity rate several years after diagnosis. Bone maturation was not influenced during treatment, although in a few patients there was either a minor retardation or an acceleration of bone age at diagnosis.

## **Discussion and Conclusions**

Chemo- and radiotherapy in children with ALL may lead to transient elevations of TSH in a few patients, but according to the T4 and T3 data there is no proof of a permanent thyroid dysfunction. TSH elevations are found as a sign of latent hypothyroidism. At the time of investigation in the GDR, a iodine deficiency was common.

Moderate hyperprolactinemia may be observed before and during therapy of patients with malignant hematological diseases. Therefore this endocrine abnormality can be due to acute leukemia and NHL itself as a

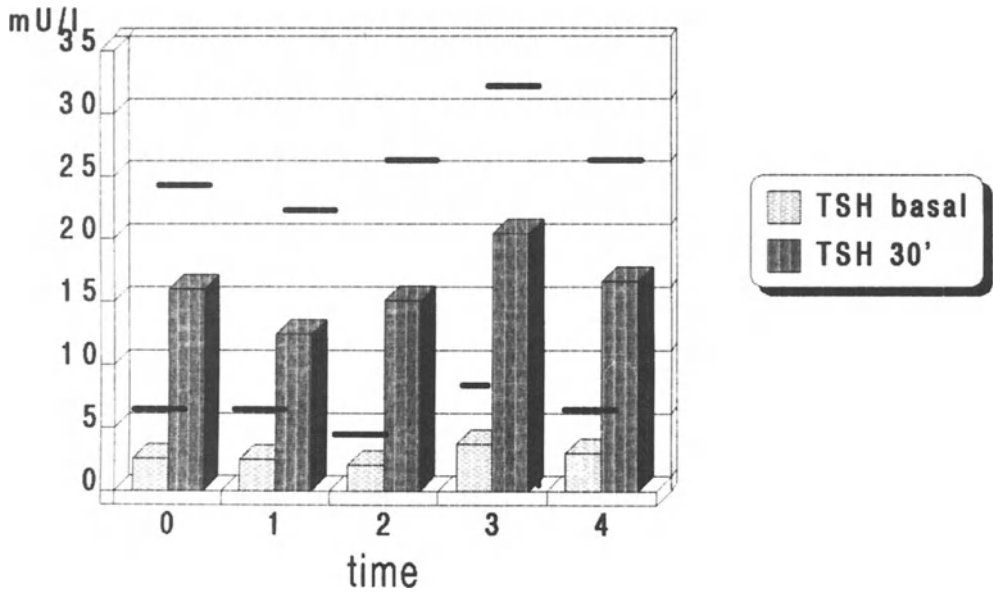


Fig. 1. Mean levels of basal and TRH stimulated TSH in 31 patients with acute leukemia and NHL (horizontal bar, SD)

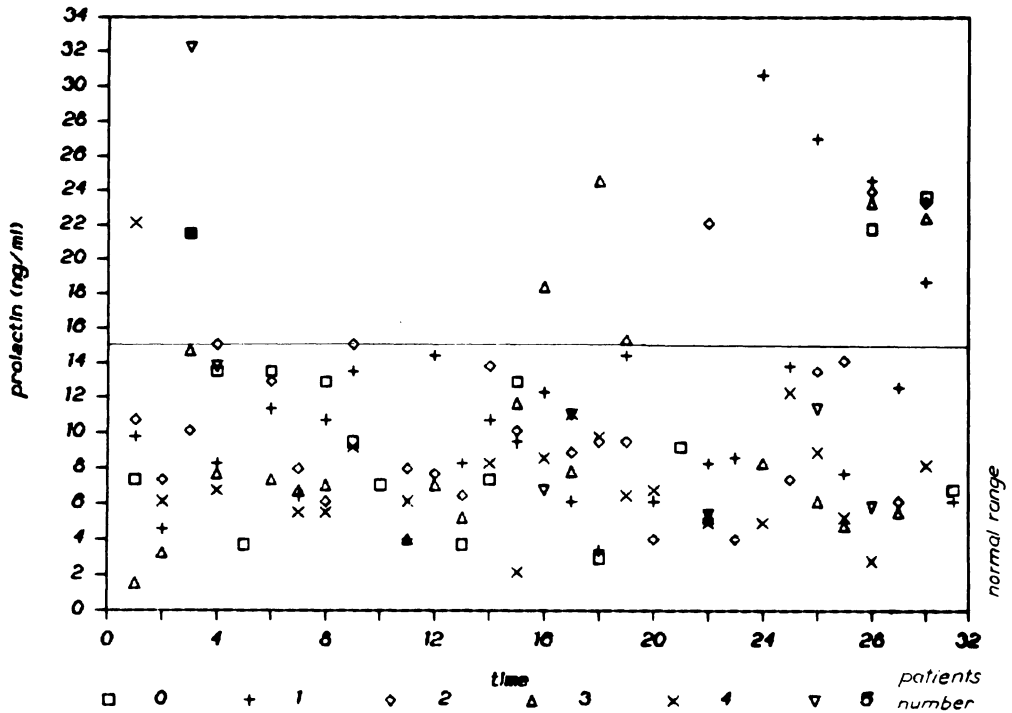
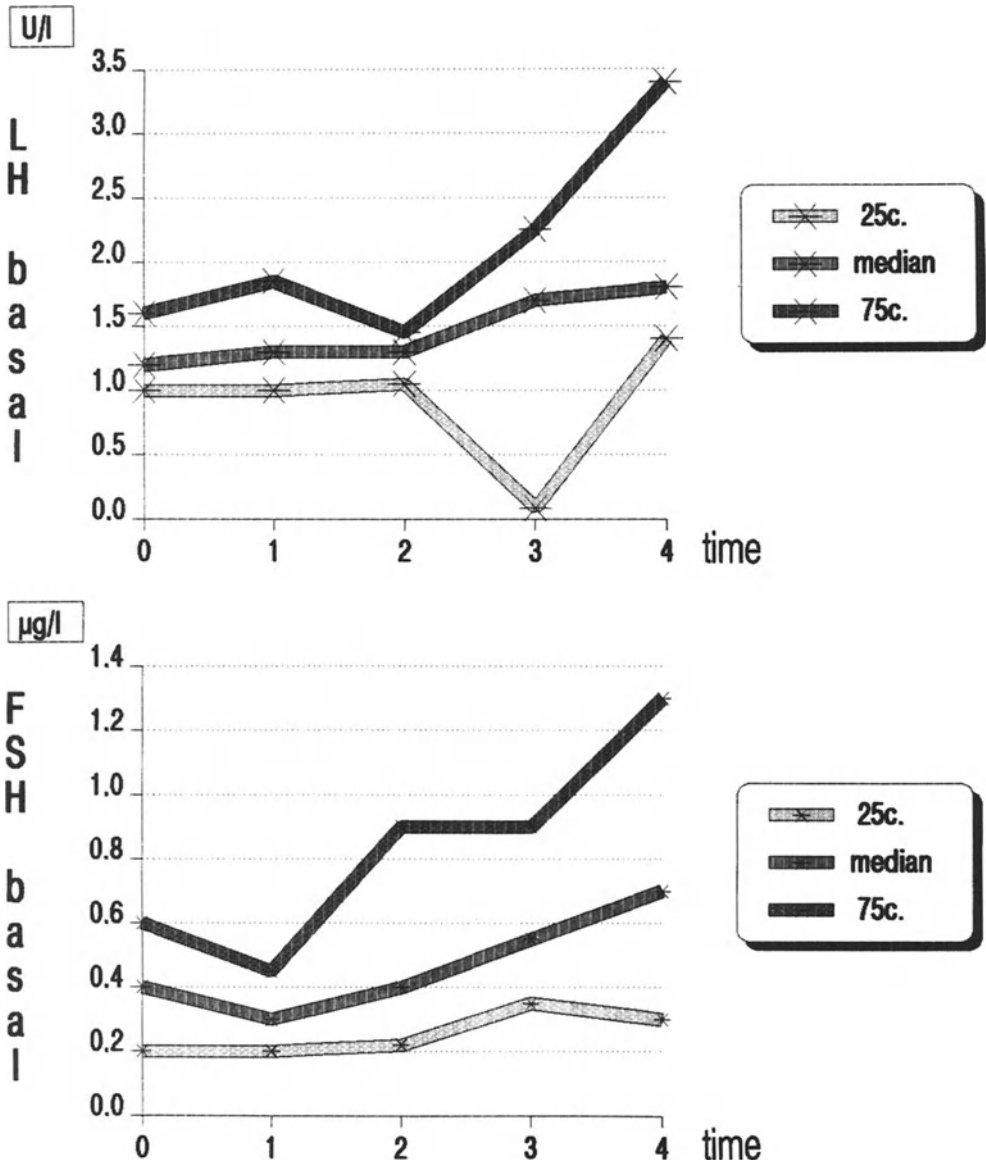


Fig. 2. Prolactin levels in 31 patients with acute leukemia and NHL



**Fig. 3 a, b.** a Basal LH in 18 prepubertal children with acute leukemia and NHL. b Basal FSH in 18 prepubertal children with acute leukemia and NHL

systemic disease [5, 7]. Increased prolactin levels are known from a broad spectrum of endocrine and nonendocrine disorders [8]. Therefore, prolactin is an unsuitable parameter to estimate the side effects of antileukemic therapy on the endocrine system. The tendency to higher basal LH and FSH levels shortly after cranial irradiation in pre-

pubertal children may reflect premature activation of the hypothalamic-pituitary axis.

Leiper et al. [3] and Wheeler et al. [9] reported on precocious and premature puberty in 28 out of 233 children with ALL. According to bone age in our patients, such an early priming of puberty could not be observed.

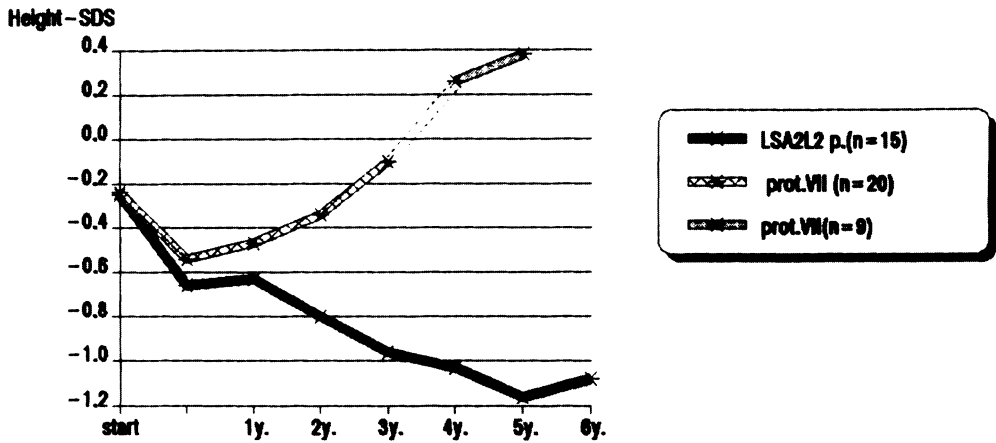


Fig. 4. Height-SD in 35 children with ALL/NHL: comparison of two different treatment schedules

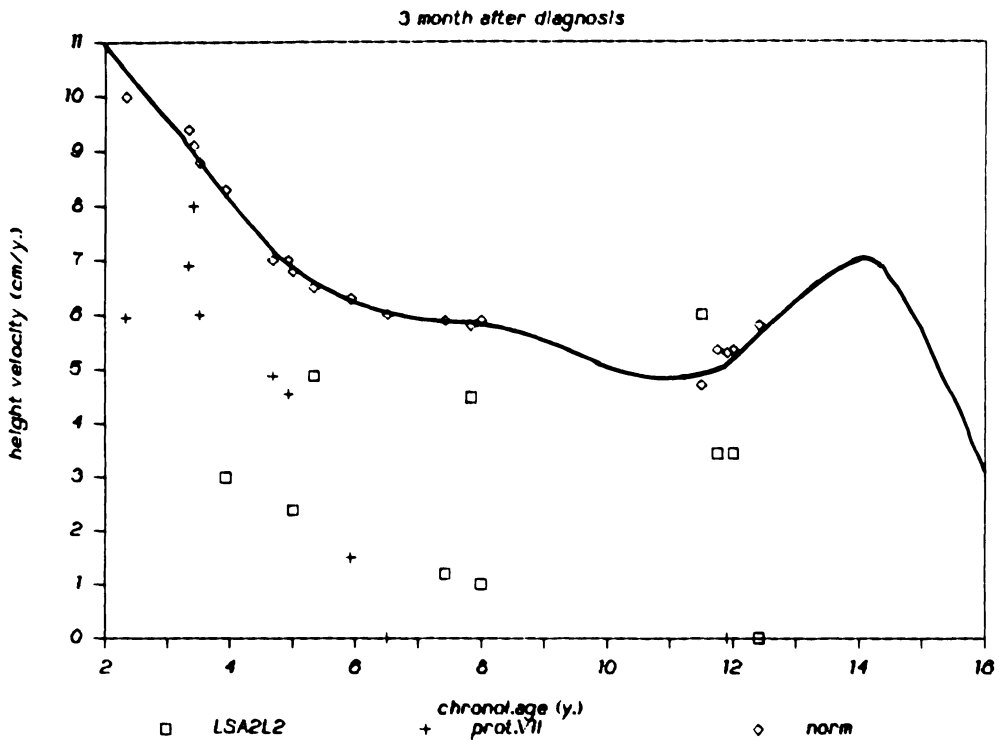


Fig. 5. Height velocity in 19 boys with ALL/NHL compared with the data of the Second Zurich longitudinal study

Previous studies on children treated for ALL have shown a height reduction [4]. Shalet et al. [6] reported normal growth despite abnormalities of growth hormone secretion. Controversy about the clinical im-

portance exists after the paper of Kirk et al. [2], who described greater inhibition of growth, at both short- and long-term follow-up. Therefore some authors concluded that growth hormone treatment in these pa-

tients is necessary. Our study has shown that continual intensive cytostatic treatment is more important for growth than other factors. A loss in height-SDS was not observed during a moderate cytostatic maintenance therapy, i.e., the recent protocol VII. Although growth velocity during induction and consolidation phase was seriously impaired in almost all cases, catch-up growth occurred in the 2nd half-year after diagnosis during maintenance therapy. One can speculate that this was partly due to acceleration, but the bone age data contradicted this opinion. Over a period of 5 years, height-SDS in protocol VII compared with LSA2L2 was significantly higher. Recently published data [1] showed a negative influence on growth by a cranial irradiation with 24 Gy in contrast to a dose of 18 Gy. Prophylactic cranial irradiation with 18 Gy in our patients did not affect growth during the first 5 years after diagnosis. However, a smaller final height due to early maturation in some patients can only be excluded after cessation of growth.

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## Incidence of Aseptic Osteonecrosis Following the Therapy of Childhood Leukemia

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### Introduction

Aseptic osteonecroses following the treatment of adult patients with Hodgkin's and non-Hodgkin's lymphomas have been reported to occur with an incidence of 0.12%–10% [2, 7]. Corticoid therapy as well as field irradiation of joints is thought to represent major pathoetiological factors. The serious complication of osteonecroses after therapy has also been described in childhood leukemia [4, 8]. But little is known about the incidence. Also, antileukemic protocols prescribe corticoids for several weeks at most and irradiation to the spinal cord and/or skull only, if at all. We studied 551 children with acute leukemia, treated at the University Children's Hospital at Münster, retrospectively, for the occurrence of aseptic osteonecrosis.

### Patients

From 1971 to 1985, 441 children with acute lymphatic leukemia (ALL) and 110 children with acute myelogenous leukemia (AML) were treated in cooperative therapy studies DAL and BFM (Berlin-Frankfurt-Münster). The patients' data were reviewed for osteonecrotic bone lesions up to December 1988. The diagnoses of affected patients were reexamined by using the original X-ray pictures, and their case histories prior to the

occurrence of the osteonecrosis were analyzed. The doses of the various corticoid derivates were calculated by conversion to their prednisone equivalent.

### Results

Aseptic osteonecroses were found in 6 of the 551 children, i.e., 1.09%. All patients had been inactive or immobilized prior to or during corticoid medication (Table 1). Except in one child (L.B., corticoids for 1 year) the corticoid therapy lasted 4–5 weeks at most. At diagnosis, all children had been treated conservatively with protected weight bearing. Five children had had ischial weight-bearing braces (Thomas splint's) and one child (L.B.) a wheelchair and elbow crutches later on. The children still alive are free from pain and there is no need for any orthopedic support.

### Discussion

Osteonecroses in patients with malignant lymphomas and leukemias occur between 2 and 156 months after onset of therapy [2, 6, 7]. Corticoids in chemotherapy protocols are thought to be a major pathoetiological factor. They are known to increase the fat cell mass and cause microfat embolism, leading to ischemia within bone and bone marrow [10]. But the corticoid effect in patients with renal transplantation is still the subject of controversy. Cruess [1] found an increased rate of osteonecroses with an aver-

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Table 1. Data of patients with osteonecroses

Patient	Therapy	Prednisone equivalent (g/m <sup>2</sup> )	Localization of osteonecrosis	Months after onset of therapy	Immobilization	Outcome, 1988 (survival in years)
J. W.	AML-BFM 78	1.9	Femoral head	8	Several weeks of inactivity early in therapy, exclusion of psoas bleeding	Died of AM, relapse (3)
T. H.	ALL-BFM 81	4.2	Femoral head Humeral head Femoral condyle	14 14 21	At diagnosis, due to pain, relieving posture of shoulder joint later affected by osteonecrosis	First remission (5+), secondary arthrosis
L. B.	ALL-DAL	20.5	Femoral condyles Femoral heads	15 30	Forearm crutches and pelvic cast due to abacterial inflammation of knee joint during extended corticoid treatment, for 4.5 months	First remission (5+), restitution
P. H.	ALL-DAL ALL-BFM 79 ALL-BFM 81	5.0 3.9 3.9	Femoral heads	109 58 14	Pain of hip joint with relieving posture, at the times of relapse	Fourth remission, secondary arthrosis
M. M.	ALL-BFM 81	4.0	Femoral head	13	Splintage and lower leg cast for hip dysplasia and pes adductus, until 3 months before diagnosis	First remission (5+), secondary arthrosis
A. T.	ALL-BFM 79 ALL-CNS ALL-relapse	5.0 3.8	Femoral condyle	36 12	Several weeks of inactivity and phlebotrombosis early in therapy, exclusion of osteonecrosis by scintigraphy	Died of ALL relapse (6)

Conversion factor of dexamethasone, × 7



age prednisone dose exceeding 32 g, whereas others found them to be rather associated with renal osteodystrophy at the time of transplantation [3]. They were also occasionally found in children with ALL prior to the initial treatment [5]. The authors discuss leukemic infiltrates in the vascular adventitia.

In our patients with leukemia, the corticoid doses were clearly lower than those mentioned by Cruess [1], except in one child. They were comparable to those reported in the literature on oncological patients with osteonecroses [2, 6, 7].

It is quite conspicuous that all of our patients were immobilized or inactive before or during corticoid treatment for several weeks at least. A splintage for hip dislocation is known to increase the risk of femoral head necrosis [4]. The patient L.B. with the highest corticoid dosis also had had the longest time of immobilization.

Inactivity, especially in childhood, leads to atrophy of bones with restricted blood circulation due to the absence of muscular contractions [9]. It may be assumed that the short-term administration of corticoids in antileukemic protocols alone does not lead to osteonecrosis. Patients, however, who were immobilized before or during therapy seem to be at a higher risk. A therapeutic attempt with non- or partial weight bearing, as in our patients, seems to be indicated, because little is known about the long-term results of surgical intervention in childhood. Nevertheless, the further course has to be watched carefully.

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## Osteoporosis in Children with Leukemia: A Potentially Debilitating Anomaly?\*

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### Introduction

Osteoporosis in children with leukemia can affect quality of life if it is not recognized in time. Only few reports have addressed this problem so far. Roentgenological alterations in the skeleton of children with acute lymphoblastic leukemia (ALL), compatible with loss of bone mass, can be found at diagnosis [1]. Some children even have collapsed vertebrae before leukemia treatment has been started. At least two major components of ALL treatment can cause further loss of bone mass: corticosteroids [2] and methotrexate [3]. The role of other chemotherapeutic agents is less clear. From 1987 we have estimated the presence of osteoporotic changes, studying bone mineral content in children with ALL.

### Patients

Bone mineral content (BMC) was assessed in 19 children with ALL (Table 1): nine girls, age at diagnosis 4.3–12.7 years (median 7.2 years), and ten boys, age at diagnosis 0.9–14.3 years (median 9.8 years). At the time of BMC assessment, 16 patients were in first remission, two in second and one in third remission. All children were on therapy. Criteria for the intensity of the treatment were the peripheral white blood cell count

(WBC) and the presence of a mediastinal mass at diagnosis (standard risk group: WBC  $< 50 \times 10^9$ /liter, no mediastinal mass; high-risk group: WBC  $\geq 50 \times 10^9$ /liter and/or mediastinal mass). Figure 1 shows the time of BMC in relation to the stage of treatment.

### Methods

Bone mineral content was measured by means of  $^{125}\text{I}$  single-photon absorptiometry, scanning the radius of the nondominant side at two-thirds the distance from the olecranon to the styloid process. Each reading is the mean of four consecutive determinations [4].

### Results

The results of BMC measurements for 19 children with ALL are given in Fig. 2. For comparison, reference data of healthy age-matched children are also shown [5].

### Conclusions

The distribution of the results of the BMC assessments was not as expected in healthy children. A decreased BMC was seen in 14 patients (8 girls and 6 boys) at  $\geq 2$  months after diagnosis. No association between the decreased BMC and duration or intensity of treatment could be found. Younger patients showed the most striking decreased BMC. This is consistent with previous reports, sug-

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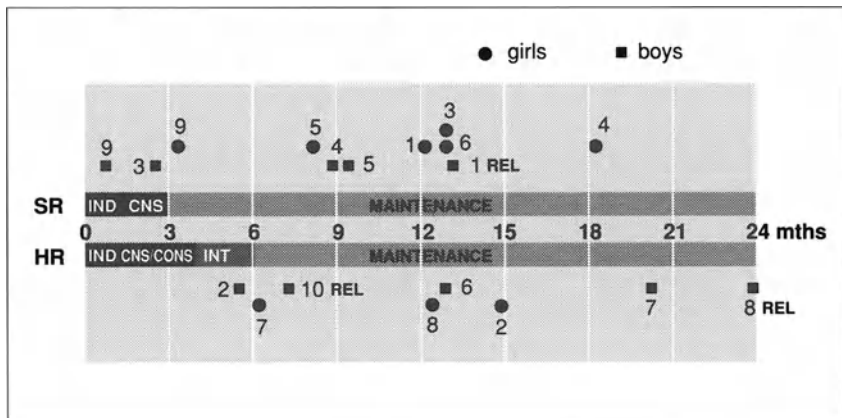
\* This study was supported by the Foundation for Pediatric Oncology Groningen (SKOG).

**Table 1.** Characteristics of 19 children with ALL

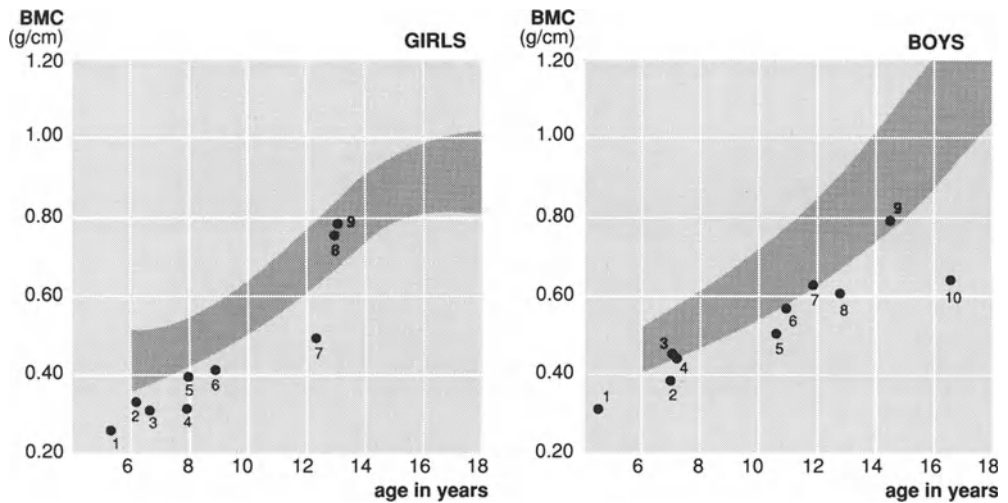
Patient No.	Age at diagnosis (years)	Age at BMC assessment (years)	Duration of treatment (months)	Risk group	Cranial irradiation (24 Gy)
Girls					
1	4.3	5.3	12	SR	—
2	4.9	6.2	15	HR	+
3	5.5	6.6	13	SR	—
4	6.3	7.8	18	SR	—
5	7.2	7.9	8	SR	—
6	7.7	8.8	13	SR	—
7	11.7	12.3	7	HR	+
8	11.8	12.9	13	HR	+
9	12.7	13	3	SR	—
Boys					
1	0.9	4.6	13 (45) <sup>a</sup>	SR REL	+
2	6.4	7	7	HR	+
3	6.8	7.1	3	SR	—
4	6.5	7.2	9	SR	—
5	9.8	10.6	10	SR	—
6	9.8	11	14	HR	+
7	10.3	11.9	19	HR	+
8	4	12.8	22 (106) <sup>a</sup>	SR REL	—
9	14.3	14.5	2	SR	—
10	13.1	16.6	8 (42) <sup>a</sup>	SR REL	—

SR, standard risk; HR, high risk; REL, relapsed patient

<sup>a</sup> Time from initial diagnosis to BMC assessment in parentheses



**Fig. 1.** Time of bone mineral content assessment and treatment schedule. (.), girls; [], boys. The numbers refer to the patient number in Table 1. SR, standard risk; IND, induction: vincristine, dexamethasone, L-asparaginase; CNS, CNS-prophylaxis: high-dose methotrexate, leucovorin rescue, intrathecal chemotherapy MAINTENANCE, 5 weeks 6-mercaptopurine, methotrexate, alternated with 2 weeks vincristine, dexamethasone (identical in SR and HR). HR, high risk; IND, induction: vincristine, dexamethasone, daunorubicin; CNS, CNS prophylaxis: cranial irradiation (24 Gy), intrathecal chemotherapy; CONS, consolidation: L-asparaginase, vincristine, dexamethasone, daunorubicin, 6-mercaptopurine; INT, intensification: cyclophosphamide, cytosine-arabioside; REL, relapse treatment, not specified



**Fig. 2 A, B.** Results of bone mineral content assessment in 19 children with ALL. The *numbers* refer to the patient number in Table 1. Girl No. 6 had multiple vertebral fractures; boy No. 6 had fractures of forearm and ankle. Both had in addition roentgenological evidence of loss of bone mass

gesting that the most severe roentgenographic skeletal alterations at the time of diagnosis of ALL occur in children younger than 5 years [6]. The results of this study suggest that a considerable number of children with ALL suffer from osteoporosis; either because of the primary disease or due to current ALL treatment strategies. However, these preliminary data need to be confirmed in a prospective study.

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Translation for ref. 6:  
Skeletal changes in children with acute lymphoblastic leukemia

## Principles of Supportive Psychological Care for Patients Undergoing Bone Marrow Transplantation

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### Preconditions

Patients undergoing bone marrow transplantation (BMT) are in a psychological dilemma: On the one hand they intensely hope for cure from a life-threatening disease; on the other they have to bear the stress of the procedure and its consequences, including the threat of an untimely death. They have to balance the challenges of the treatment and their personal resources in order to master the situation. Most patients are able to maintain their psychological balance, but some do not cope as well and show signs of psychological distress. From the small number of studies conducted on psychological aspects of BMT it is known that psychological disturbance during the period of intensive care in most cases is associated with medical complications, which magnify negative feelings [1]. It is also known that most of the patients with no or only minor complications who survive the period of intensive care do quite well on average, but some develop dysfunctions in cognitive, emotional, sexual, social, or other areas of life in the long run [2, 3]. Risk factors for psychic disorders under or after BMT are as yet widely unknown.

Psychological care for BMT patients should aim at the prevention of such disturbances occurring either during the period of intensive care or afterwards. We are con-

vinced that generally it should emotionally equip the patient to counterbalance the distress of treatment and its consequences. This is what we mean by *supportive* care.

Supportive care can be provided by relatives, nurses, physicians, and mental health professionals. At the Department of Bone Marrow Transplantation Essen two mental health professionals from the Department of Medical Psychology are on duty for the psychological care of BMT patients in a consultation-liaison service. All patients, regardless of their psychological state, have an initial interview with the mental health specialist within 2 days after admission for BMT. Permanent contact is provided from admission to discharge by visiting the patient at least every 2nd day and helping them cope with whatever psychological problems arise.

### Strategies

To date no investigations on useful strategies for the psychological care of BMT patients are to be found in the published literature. The principles suggested here have been derived from the experience gained from the psychological care of patients with chronic diseases – mostly patients with chronic renal failure – as well as from our own experience with BMT patients. Even though there are a number of differences between patients under BMT and patients with chronic diseases, there are also a number of psychological similarities: generally neither patients with chronic diseases nor

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BMT patients suffer from primary psychological disorders; also, in both patient groups thinking is dominated by the somatic reactions, and the awareness of psychological problems seems to be lower than in healthy individuals. On the other hand, the acuteness of the threat of death, the relatively short time of treatment, and problems provoked by specific treatment modalities are special features of the psychological situation of BMT patients.

Continuous psychological care for chronically ill patients in a liaison service in contrast to consultation as a "task force" is held to be essential by many authors (e.g. [4]). Resolving psychological crises is difficult in most cases and requires some time, which is usually not available under BMT. A positive and already established relationship with the patient is necessary for effective psychological intervention in crisis situations under medical treatment (cf. [5]).

Many of the psychological problems under BMT develop as a consequence of an imbalance between demands and coping capabilities. Psychological care for BMT patients therefore has to focus on adjustment problems which arise. These may emerge from the apprehension of possible, but uncertain, future events or from the anticipation of actual future events. Anxiety and depression may also stem from past experiences or conflicts actualized by the present or anticipated situation.

Furthermore, BMT may lead to psychic traumatization if patients cannot cope. Even not necessarily recognized in the intensive care unit, such experiences may hinder readaptation and rehabilitation.

Supportive psychological care for BMT patients is thus characterized by three aims:

1. Preservation – Helping the patient to maintain normal psychological functioning
2. Adjustment – Helping the patient to keep up with the demands of the BMT procedure
3. Prevention – Helping the patient to prevent long-lasting traumatization

## Principles of Intervention

The basic principle of psychological care for BMT patients is to reduce psychological distress under BMT as much as possible and to avoid long-lasting psychological trauma by helping them to cope with the stress experienced in the BMT situation. From this basic principle some more specific principles can be derived. If a patient has a history of psychic disorder, we suggest not touching on preexisting problems. If the patient has reached some level of stability, there is no need to strive for personality change since medical problems are dominant. The only exception to this rule is if these conflicts negatively affect treatment necessities to a greater extent.

A patient with a generalized anxiety syndrome had been under psychiatric treatment before entering the hospital for BMT. We did not work on the anxiety syndrome until it became obvious that irrational manifest anxiety of irradiation emerged.

Psychological problems arise if certain medical interventions actualize a premorbid conflict. It is not useful to deal with these underlying conflicts in the BMT situation because working on such conflicts leads to temporary psychic destabilization even in somatically healthy individuals. Supportive care, on the contrary, implies helping the patient cope with the problematical situation which has emerged.

One female patient had a history of manifest claustrophobia and was afraid of moving into the plastic isolator. She was treated by a systematic desensitization technique without uncovering potential preexisting conflicts.

If a problem occurs or is anticipated which may interfere with treatment, psychological support is indicated. Reduced medication compliance due to pain, conditioned nausea, or taste aversions is the most frequent indication for psychological care. Also, interaction with relatives sometimes gives rise to problems which may interfere with patient discipline. Alterations of physical appearance are in some instances a source of concern, especially to adolescent patients, and help is required in order to prepare for life outside the BMT ward. Rather frequently a parent of an adolescent patient cares for the

patient very intensely, which may lead to feelings of overprotection on the part of the patient. Recognizing that this behavior is also motivated by the parent's own anxieties may reduce interpersonal tensions.

During the whole time of treatment patients have to keep up with changing treatment requirements, symptoms, and complications. Due to these strains a high level of discipline, power, and stability is demanded from the patients. Psychological functioning may be challenged as time goes by. Patients therefore need steady support and reinforcement to maintain psychological functioning. In some instances patients feel exhausted and doubt their own strength. This may happen even for trivial reasons. In our experience it is helpful to refer to abilities which the patient has employed successfully in former situations. We sometimes use suggestive techniques to strengthen these effects and to improve patient self-esteem.

A patient with depression emerging during aplasia and stomatitis could hardly verbalize his feelings. A suggestive technique was employed by telling stories of analogue situations, which are known to everybody, have a relaxing context, and end with a resolution of the problem. For example, the story of a Sunday walk which turns out to be much longer and strenuous than expected, but eventually leads to the goal initially desired, can be helpful in facilitating catharsis of negative feelings.

The intervention principles suggested may be summarized as follows:

1. Postpone preexisting conflicts as long as they do not interfere with treatment.
2. Do not uncover unconscious conflicts in order to avoid additional psychological distress.
3. Deal with problems, which may interfere with treatment or lead to traumatization.
4. Try to maintain psychological functioning.
5. Activate personal and internal resources.

## **Methods of Intervention**

### *Consultation*

Because they are in an isolation unit, patients do not have the chance to seek information from and share experiences with fel-

low patients. We have found that a lot of help and encouragement can be provided for patients by communicating coping efforts which we have seen other patients employ successfully. Although the focus of consultation lies in disease-related issues, we try to be sufficiently informed about public sources of financial and social help, so that organizational problems do not become too great a burden for the patient.

### *Nondirective Verbal Techniques*

In a situation, where it does not seem feasible to work on long-lasting conflicts or to strive for a major personality or behavioral change, a patient-centered approach which leaves the choice of issues to be dealt with primarily to the patient him- or herself seems appropriate. Therapeutic goals can be the clarification of diffuse feelings and the facilitation of cathartic effects.

### *Behavior Modification Techniques*

Often, however, it is not so much the vital threat itself which causes distress, but rather the specific treatment elements like radiation, swallowing medication, and nausea. Conditioned responses to chemotherapy, like nausea and vomiting, for example, have been successfully treated by behavior modification techniques such as systematic desensitization [6]. Compliance problems seem to be sensitive to operant or self-monitoring procedures. (By operant procedures positive consequences of a behavior are emphasized or some kind of reinforcement is introduced. With self-monitoring techniques complex behavior is subdivided into simple steps, which are scheduled over time; keeping up with the schedule is less demanding and the attainment of a distant goal is facilitated.)

### *Suggestive Techniques*

Suggestive procedures such as hypnosis, progressive muscle relaxation, and other relaxation techniques have also been found to be helpful in treating conditioned responses and fear.

## **Difficulties of Psychological Care for BMT Patients**

Patients under BMT are faced with a double challenge: On the one hand, it is important for their psychological well-being to maintain their personal autonomy while depending on medical intervention. On the other hand, they should be able to accept (psychological) help from others, which for some patients amounts to an admission of their own weakness. Some of the patients deny outright having any psychological problems at all although these are obvious and may even interfere with the treatment regimen. The fact that patients are very dependent on people around them may lead to a sense of helplessness and in consequence to overly regressive behaviors which are a particular burden to the staff. The mental health professional's efforts to deal with the emotional impact of the physical strains of the treatment are impeded by the patients' preoccupation with bodily concerns and their heightened awareness of sensations and functions. Controlled studies on the necessity and differential proceedings of psychological care for patients under BMT would be very helpful to the professional working with BMT patients. As already mentioned above, so far no comprehensive theory on the psychological processes to be found in patients under BMT has been developed and

therefore no therapeutic strategies specifically relating to the work with these patients exist. Controlled studies, however, are rare because adequate control groups are lacking and study designs cannot be freely chosen for ethical and treatment-related reasons.

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## Fibronectin in Stomatitis Therapy of Leukemic Children

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Fibronectin (FN) is a glykoprotein found in plasma [1, 2] and in cell-surface membranes [2]. It occurs in both soluble and insoluble forms. FN has been intensively studied because of its frequent interactions with cells and macromolecules. It may influence a number of physiological processes including phagocytosis [3, 4] remodeling during embryogenesis and wound healing [5]. The concentration of plasma FN is altered in certain disease states. Low FN levels have been seen in sepsis [6–9], burn injury [6, 7, 10], and acute lymphoblastic leukemia (ALL) in adults [11, 12]. In our previous study [13], we noticed that the plasma FN level in patients with ALL prior to chemotherapy was significantly lower than during remission. A significant fall in plasma FN level was found in patients with viral or bacterial infections undergoing chemotherapy, and a subsequent increase in plasma FN level when the infection was treated successfully. Changes in FN concentration related to stage of disease, clinical condition, and subsequent complications as presented above have encouraged us to start clinical trials of this drug in substitutive treatment of patients with acute leukemia.

### Material and Methods

Eleven children with ALL and stomatitis during chemotherapy (six girls, five boys)

aged between 6 and 16 years were studied. All of them received FN-rich plasma (cryoprecipitate) as well as antibiotics and antimycotic drugs. Cryoprecipitate was given on days 1, 3, and 5. Fibronectin levels were measured before and after each infusion and also on the 3rd day after the last infusion, by the method of Mancini et al. [14] using specific antisera against human plasma FN. The control group consisted of 12 ALL children (eight girls, four boys) aged between 8 and 14 years, with stomatitis during chemotherapy, who did not receive cryoprecipitate. The results were statistically evaluated with Student's *t*-test.

### Results

Mean plasma FN levels before and after each cryoprecipitate infusion are shown in Table 1. We found a significantly higher FN level after the second and the third infusion of cryoprecipitate ( $0.02 > P > 0.01$ ) and also 3 days after the last infusion ( $0.05 > P > 0.01$ ). Mean time of recovery from stomatitis ( $x=8$  days) in children who received FN-rich plasma (cryoprecipitate) was shorter than that in the control group ( $x=15$  days).

### Discussion

Cytotoxic drugs used in the treatment of leukemia are considered injurious to the oral mucosa. Many of the drugs appear to have a direct effect on the mucosa by interfering

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**Table 1.** Mean fibronectin plasma levels

	Before one cryoprecipitate infusion	After one cryoprecipitate infusion	Before two cryoprecipitate infusions	After two cryoprecipitate infusions	Before three cryoprecipitate infusions	After three cryoprecipitate infusions	Three day after three cryoprecipitate infusions
Number of patients	10	11	11	9	9	9	4
Mean plasma FN level ( $\mu\text{g}/100\text{ ml}$ )	202.7	294.4	269.4	289.7	260.9	321.9	307.8
Median	206.3	247.5	285.0	300.0	192.5	307.5	318.7
Standard deviation	85.2	121.8	110.8	69.5	117.5	99.4	53.8

with the replication of epithelial cells, which makes the oral epithelium more vulnerable to different types of trauma [15–17]. In addition, myelosuppressive and immunosuppressive effects of the cytotoxic drugs as well as the disease itself may indirectly favor the development of oral mucosal lesions, by lowering the resistance of the tissue to infection [15]. The occurrence of different oral mucosal lesions in patients with ALL has been reported to range from 29% to 94% [15, 18, 19]. It is inevitable that the presence and extent of mucosal lesions varies during different phases of treatment, reflecting the intensity of chemotherapeutic treatment and the degree of myelosuppression [15]. In our previous study [13] we reported changes in FN concentration in ALL children related to the stage of disease and subsequent complications. Low fibronectin levels during infection and during the first weeks of chemotherapy were reported earlier by Choate and Mosher [11], Boughton [20], and others. Good results achieved after using FN-rich plasma in sepsis [6] and FN properties have encouraged us to start clinical trials of FN in substitutive treatment of ALL patients. Our preliminary results from using FN in the treatment of ulcerative stomatitis as a complication of chemotherapy in children with ALL are very promising and encouraging for the further use of FN-rich plasma.

## Conclusion

Fibronectin-rich plasma (cryoprecipitate) given to leukemic children is useful as supportive therapy in stomatitis which complicates chemotherapy.

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## **Treatment of Relapse and Pharmacokinetics**

## **Karyotype of Leukemia Cells Consistently Predicts for Response to Therapy and Survival Following Salvage Therapy in Acute Myeloblastic Leukemia**

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### **Introduction**

Following the introduction of anthracyclines and cytosine arabinoside (Ara-C) into clinical practice in the late 1960s, response rates of 50%–80% in previously untreated patients with acute myeloblastic leukemia (AML) have been obtained [1, 2]. Despite a variety of postremission treatments the remission duration remains 12–15 months, with 20%–25% of patients staying in long-term disease-free state and possibly being cured [3, 4]. One-third of the patients who failed to achieve an initial complete remission do so because of persistence of leukemia cells [5], and 75%–80% of patients who achieve a remission have recurrence of leukemia [3, 4]. Both of these patient populations are eligible for salvage therapy.

A variety of new treatment approaches have been studied following the discovery of the activity of amsacrine [6, 7], mitoxantrone [8, 9], new anthracyclines [10, 11], and the use of agents such as Ara-C and VP-16 in new doses and schedules [12–16]. Comparison of the results of these studies is difficult because of different doses and schedules or variations in supportive care. However, a major component of responsiveness may be the inherent biology of the leukemic cells. Our first attempt to evaluate the association of prognostic characteristics

and response to initial salvage therapy (S-1) or survival following salvage therapy discovered the association existing between prognosis and duration of initial complete remission, age of the patient, hepatic function, cellularity of the bone marrow, and the white blood cell count [17]. Subsequent analysis showed that the karyotype of leukemic cells was strongly associated with remission duration [18].

This study addressed the prognostic factors noted for 243 patients with banded karyotypes treated between 1974 and 1985, and also for a more recent group of 116 patients treated between 1986 and 1988. The consistency of the ability of prognostic factors to predict response to initial salvage therapy and survival was compared in the two patient populations.

### **1974–1985**

Two hundred and forty-three patients who had received all of their treatment under the direct supervision of the Department of Developmental Therapeutics (later Department of Hematology at the M. D. Anderson Cancer Center) during the period 1974 to December 1985 were evaluated. Cytogenetic analysis of the mitoses of the leukemic cells were attempted on all patients (97% of patients entering the institution during the study period). Those patients who failed to achieve a complete remission with initial remission induction therapy were classified as primary refractory and those who had recurrence of disease after achieving remission

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as being relapsed. Different salvage protocols were used in this patient population for their initial salvage treatment and have been assigned to eight treatment categories. The first category includes Ara-C given at low (10–25 mg/m<sup>2</sup> per day) or conventional doses (50–200 mg/m<sup>2</sup> per day) alone or combined with vincristine and prednisone (OAP), plus Adriamycin (AdOAP), rubidazone (ROAP), AMSA (AMSAOAP), daunorubicin (DOAP), or cyclophosphamide (COAP), or combined with thioguanine (Ara-C+TG). Category 2 includes high-dose Ara-C (3 g/m<sup>2</sup> q 12 h) alone in 4- to 12-dose schedules, or combined with mitoxantrone (DHAD+HD Ara-C). Category 3 includes the anthracyclines (rubidazone, Adriamycin, Adriamycin-DNA complex, daunorubicin, and 4'-epi-Adriamycin). Category 4 includes amsacrine alone or combined with *cis*-platin or amsideine alone. Category 5 includes piperazinedione and total body irradiation (PIP-TBI), or cyclophosphamide, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), and VP-16 (CBV), plus autologous bone marrow rescue. Category 6 includes PIP-TBI, or CBV plus allogeneic transplant. Category 7 includes miscellaneous combination regimes and category 8 includes miscellaneous single agents, not included in categories 1–4. None of the regimens in categories 7 and 8 had more than 10 patients entered on study [19]. The majority of patients received initial remission induction therapy with AdOAP, ROAP, or AMSA-OAP, with some patients receiving ASMA combined with six doses of high-dose Ara-C. Patients were continued on maintenance chemotherapy for 9–18 months, usually Ara-C in conventional doses combined with other drugs. Salvage therapy was initiated when patients had more than 25% absolute marrow leukemic infiltrate (defined as percentage cellularity × percentage blasts + promyelocytes : 100). Twenty-one patients had treatment initiated with less than 30% blasts in the bone marrow because they were being entered on bone marrow transplantation protocols and were treated at the first sign of relapse, or because of patient preference, or the development of life-threatening pancytopenia. Patients who had long initial remissions and relapsed off-therapy were usually treated

with the front-line remission induction regimens for AML active at the time which usually included conventional-dose Ara-C or high-dose Ara-C. Patients who relapsed on maintenance regimens which included Ara-C were usually entered on phase I–II studies with new agents, except during the phase when high-dose cytosine arabinoside was being studied. Karyotype analysis at the time of relapse had been systematically applied only in the latter period of the study. Therefore the cytogenetic characteristics used for analysis were obtained prior to the start of the initial front-line chemotherapy. There were eight cytogenetic categories considered: inversion 16 (inv16), translocation between chromosomes 8 and 21 (t8q;21q), translocation between chromosomes 15 and 17 [t(15;17)], diploid (including 45X, -Y), -5/-7, +8, miscellaneous, or insufficient metaphases [19].

### Results of 1974–1985 Population

Eighty (33%) of the 243 patients achieved a complete remission. The median survival was 18 weeks, with 20% of patients being alive at 1 year and 8% at 2 years. Six patients died in CR (three from infection, one from hemorrhage, and two from second malignancies), and one was lost to follow-up.

Some of the prognostic factors associated with response to initial salvage therapy are illustrated in Table 1. A strong association between duration of the patient's initial complete remission and response to S-1 therapy was noted. Most patients who had stayed in remission for more than 52 weeks achieved a second remission with results comparable to those of initial treatment. There is also a strong association between age and response with more patients under the age of 50 years achieving a complete remission with a steady decline in response rate from that point on. Additional factors found to be associated with response to treatment were a history of antecedent hematologic disorder prior to start of initial induction therapy, enlargement of liver or spleen, performance status of 3 or 4 (Zubrod scale), presence of Auer rods, ≥5% blasts or progranulocytes in the peripheral blood, and elevated serum bilirubin and/or lactic

**Table 1.** Comparison of prognostic factors for response to first-salvage AML

Characteristics	1974–1985 vs. 1986–1988		
	Value	1974–1985 (243 patients) Proportion CR	1986–1988 (116 patients) Proportion CR
Age (years)	< 50	0.42	0.38
	50–64	0.23	0.26
	≥ 65	0.15	0.14
Duration of initial CR (weeks)	< 52	0.19	0.23
	≥ 52	0.60	0.42
Serum lactic dehydrogenase (U/100 ml)	< 225	0.48	0.29
	225–600	0.29	0.32
	> 600	0.14	0.16
Performance status (Zubrod)	0–2	0.35	0.31
	3–4	0	0.07
Auer rods	No	0.27	0.18
	Yes	0.41	0.48
White cell count ( $\times 10^3/\mu\text{l}$ )	< 25	0.35	0.32
	≥ 25	0.23	0.12
Alkaline phosphatase (U/100 ml)	< 85	0.39	0.43
	≥ 85	0.28	0.18
Bilirubin (mg%)	< 1	0.36	0.31
	≥ 1	0.16	0.15

dehydrogenase level. The outcome according to treatment category is illustrated in Table 2. The most favorable results were obtained in patients who received allogeneic or autologous transplantation followed by those who received Ara-C-based regimens.

AMSA and anthracyclines were associated with lower response rate and the miscellaneous combinations of drugs or miscellaneous single-drug regimens had the lowest response rates.

**Table 2.** Outcome of first-salvage treatment in AML by treatment category and treatment period

Treatment category	1974–1985 CR/total (%)	1986–1988 CR/total (%)
Conventional-dose Ara-C	25/62 (40)	2/11 (18)
High-dose Ara-C <sup>a</sup>	21/52 (40)	23/64 (36)
Anthracyclines	6/28 (21)	1/3 (33)
Amsacrine	12/38 (32)	0/1 (–)
Mitoxantrone ± VP-16	3/5 (60)	4/14 (29)
Miscellaneous single agents	1/22 (5)	0/7 (–)
Miscellaneous combinations	2/19 (11)	0/10 (–)
Autologous transplant	5/9 (56)	0/1 (–)
Allogeneic transplant	5/8 (63)	3/5 (60)

<sup>a</sup> Single agent or combined with mitoxantrone or daunorubicin

## Karyotype

The highest response rates were noted in patients with *inv16* or *t(15;17)*, where two-thirds of the patients achieved CRs. The only other abnormality with a response rate higher than that of the diploid patient group was the *t(8;21)* group. Patients with miscellaneous abnormalities and trisomy of chromosome number 8 had response rates slightly lower than the diploid group, whereas the response rates in patients with *-5/-7* were exceptionally low. Multivariate analysis was undertaken and it was found that the duration of initial complete remission, age of the patient, and serum lactic dehydrogenase factors provided the best fit for predicting response to initial salvage therapy. After evaluating the effect of treatment and cytogenetics, no significant change in the ability of the model to predict for response was noted [19]. The logistic regression model was then applied retrospectively to the patient population from which it was derived, which provided a good fit for the data (Table 3).

A similar analysis of the prognostic factors significantly associated with survival following initial salvage therapy was undertaken. Duration of initial complete remission, age, presence of an antecedent hematologic disorder, presence of infection, performance status, white blood cell count, per-

centage of blasts and progranulocytes in the blood, presence of Auer rods, serum albumin, alkaline phosphatase, serum glutamic oxaloacetic transaminase (SGOT), lactate dehydrogenase (LDH), glucose, and bilirubin levels were all associated with response. A proportional hazards model was derived and it was found that the best combination of factors to describe the observed survival was the serum bilirubin level, initial CR duration, age of the patient, percentage of blasts and progranulocytes in the blood, and serum alkaline phosphatase. Evaluation of the effect of treatment and cytogenetics did not result in a significant change in the maximized log-likelihood. The proportional hazards model derived provided a good fit for the data in the patient population from which it was derived (Fig. 1). A confounding factor in the analysis of this study was the strong correlation between the duration of initial complete remission and karyotype of patients. The longest CR durations were noted in patients with *inv16*, *t(15;17)*, and *t(8;21)*. The diploid patients had intermediate remission durations and those with other karyotypic changes had the shortest remission durations. The response rate according to both the duration of initial CR and karyotype is presented in Table 4.

The conclusion drawn from this analysis was that two major determinants were pre-

**Table 3.** Predictive model for response to initial salvage therapy in AML

$$\text{Probability of CR in first salvage} = \frac{e^x}{1 + e^x}$$

$$x = -0.94 + 1.8 \text{ 1 (duration of first CR} - 1.33) - 0.90 \text{ (age} - 1.57) - 0.67 \text{ (LDH} - 1.87)$$

Duration of first CR 1 = < 52 weeks, 2 = ≥ 52 weeks

Age 1 = < 50 years, 2 = 50–62 years, 3 = ≥ 65 years

LDH (U/100 ml) 1 = < 225<sup>a</sup>, 2 = 225–599, 3 = ≥ 600

Retrospective application of model to patient population used to derive model

Probability of CR (first salvage)	No. of patients	Observed CR (%)	Expected No. of CRs	O/E ratio
≤ 0.15	78	9 (11)	7.7	1.17
0.16 – 0.25	58	14 (24)	14.1	0.99
0.26 – 0.65	58	23 (40)	24.2	0.95
> 0.65	49	34 (69)	35.0	0.97

<sup>a</sup> Normal < 225



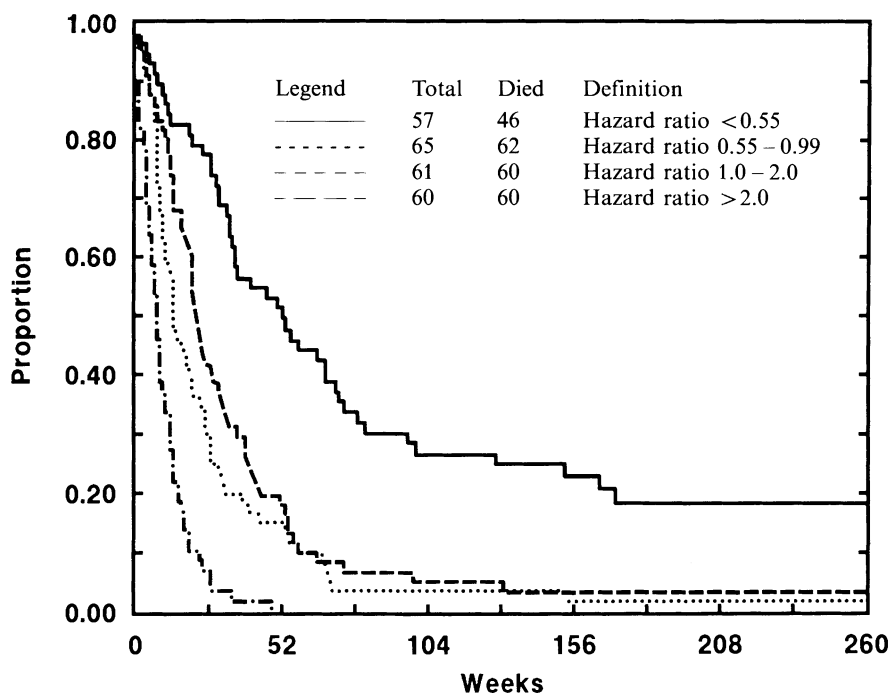


Fig. 1. Test of proportional hazards model for survival in first-salvage AML

Table 4. Bivariate analysis of response to first-salvage therapy by duration of first CR and karyotype for the Periods 1974–1985 and 1986–1988

Karyotype	1974–1985		1986–1988	
	First CR < 52 weeks CR/total (%)	First CR ≥ 52 weeks CR/total (%)	First CR < 52 weeks CR/total (%)	First CR ≥ 52 weeks CR/total (%)
inv16	1/2 (50)	10/15 (68)	2/3 (68)	4/4 (100)
t(15;17)	3/4 (75)	6/9 (68)	3/4 (75)	3/4 (75)
t(8;21)	2/11 (18)	6/9 (68)	2/4 (50)	2/4 (50)
Diploid (45X, -Y)	17/73 (23)	20/40 (50)	10/30 (33)	5/13 (39)
Miscellaneous	7/31 (23)	3/3 (100)	1/16 (6)	0/4 (0)
+ 8	0/10 (0)	3/3 (100)	1/10 (10)	0/2 (0)
- 5/- 7	1/20 (5)	0/1 (0)	0/13 (0)	0/2 (0)
Insufficient Metaphases	0/10 (0)	1/2 (50)	0/3 (0)	0/0

dictive of response to initial salvage therapy. The first was the sensitivity of the cells to chemotherapy from a general point of view as manifested by the duration of initial complete remission and, secondly, the tolerance of the host therapy as manifested predominantly by age and various parameters of organ function. Karyotype was associated both with age and initial CR duration.

#### Results in 1986–1988

During the 3-year period 1986–1988, 116 patients have undergone salvage therapy. During that time 11 patients were treated with conventional-dose Ara-C either alone or combined with anthracyclines. The majority of the patients received high-dose Ara-C either as 3 g/m<sup>2</sup> every 12 h alone or com-

bined with mitoxantrone (10 patients), continuous infusion Ara-C 1.5 g/m<sup>2</sup> per day over 4 days either alone (21 patients) or combined with daunorubicin (11 patients), or intermediate-dose Ara-C 0.5 g/m<sup>2</sup> over 2 h every 12 h for 12–21 doses (22 patients). A total of 75 patients received these regimens. Six patients received either allogeneic (5 patients) or autologous transplant (1 patient), 3 had anthracyclines as single agents, one had AMSA as a single agent, and 24 received a miscellaneous group of salvage therapies. Fourteen of these patients received mitoxantrone plus VP-16. The overall complete remission rate is very similar to that of the earlier patient group, with 33 out of 116 patients (28%) achieving complete remission. A slightly higher mortality during initial salvage therapy was also noted. The prognostic factors which are associated with response to salvage therapy were, by and large, similar to those in the earlier study. The duration of initial complete remission was again strongly associated with survival although those with longer remission durations had a somewhat lower response rate than in the earlier study. Age was again associated with response to treatment, as were a history of antecedent hematologic disorder (AHD), hepatomegaly, splenomegaly, performance status, presence of Auer rods, white blood count, alkaline phosphatase, bilirubin, and serum LDH. The comparison of the proportions of patients achieving a complete remission by these prognostic characteristics in the two-patient populations is shown in Table 1.

In addition, a strong association between karyotype and response to salvage therapy was again noted (Table 4). The best response rate was again noted in patients with inv16 and t(15;17). These were somewhat superior to those for the t(8;21) and the diploid

groups. A much lower response rate was noted in the trisomy 8 and miscellaneous groups of patients, where only 2/32 patients achieved a complete remission compared with approximately 25% in the earlier study. None of the 16 patients with -5/-7 achieved a remission.

### Prospective application of model to predict response to initial salvage therapy and survival

The models for response to treatment and proportional hazards models for survival developed in the 1974–1985 patient population have been compared in 116 subsequent patients. Overall, there was an effective stratification of the patients as shown in Table 5 and Fig. 2. Sorted into three approximately equal groups, only 5 of the 40 patients (14%) with a probability of responding to initial salvage of less than 14% achieved a complete remission, whereas 13 (28%) out of 44 patients with a probability of responding between 14% and 30% responded, and 15 (44%) of 32 patients with a probability of response greater than 30% responded (Table 5). There was a tendency for the poorer prognosis patients to exceed their expectation and for those with a better expectation not to match their expectation.

There was a strong correlation between the duration of initial complete remission rate and karyotype. Twelve (52%) of 23 patients with inv16, t(15;17), and t(8;21) had an initial CR duration greater than 1 year. This is in contrast to 13 (30%) of 43 diploid patients and 8 (16%) of 50 in the other karyotypic groups (Table 4). When the complete remission rate according to karyotype and initial CR duration is compared, even patients whose initial CR duration was less

**Table 5.** Prospective test of model of predict response to first-salvage therapy in AML

Probability of CR	No. patients	Observed CRs No. (%)	Expected CR	O/E ratio
<0.14	40	5 (13)	3.0	1.64
0.14 – 0.30	44	13 (30)	9.8	1.33
>0.30	32	15 (44)	19.3	0.78

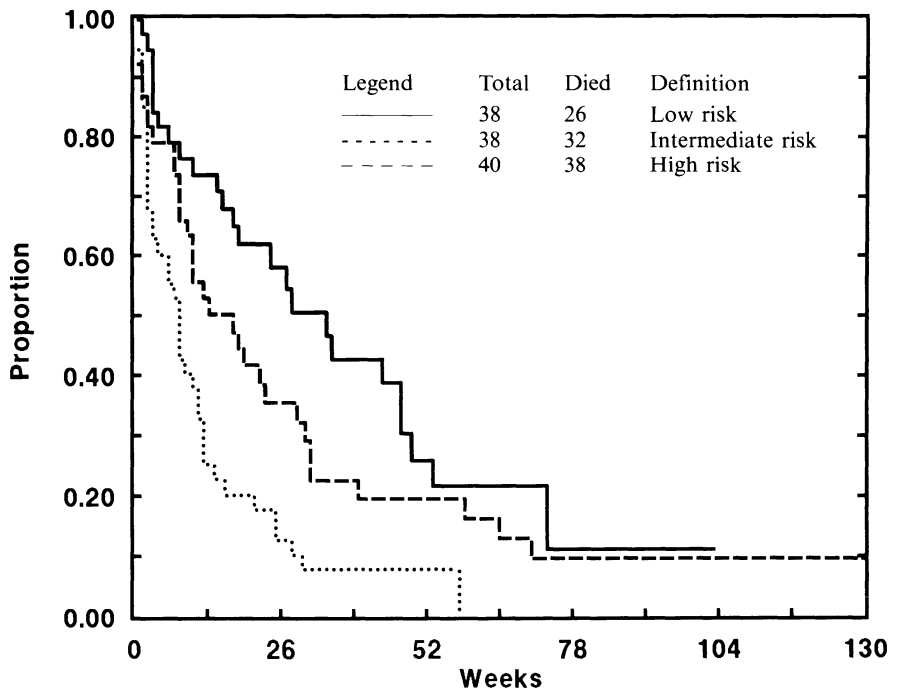


Fig. 2. Prospective test survival model in first-salvage AML

than 1 year who had favorable karyotypes had a CR rate above 50%. In similar fashion the diploid patients and other cytogenetic categories showed no difference in complete remission rate according to the duration of initial CR (Table 4). This suggests that the karyotype is a stronger predictor than the duration of initial remission for the probability of response to initial salvage therapy.

There were 23 patients with favorable karyotypes [inv16, t(15;17), t(8;21)], 43 with diploid karyotypes, and 50 with unfavorable karyotypes. The probability of response to first-salvage therapy has been analyzed according to the probability of achieving com-

plete remission on first salvage for the most favorable 23 patients predicted by the model developed from the 1974–1985 patient population and also for the next best 43 and the least favorable 50 patients. The cytogenetic pattern stratified patients more clearly than the model (Table 6).

In similar fashion, the karyotype was a strong predictor of survival. The majority of patients who remained free of disease long-term are in the subset of patients with favorable karyotypes (Fig. 3). Again, if the 23 patients with the most favorable predictions for survival (using the proportional hazards model derived from the 1974–1985 patient

Table 6. Ability of regression model to predict response to first-salvage therapy in AML compared with the single variable of karyotype

Karyotype	No. of patients	CRs No. (%)	Probability of CR–first salvage	No. of patients	CRs No. (%)
inv16, t(15;17), t(8;21)	23	16 (70)	>0.45	23	11 (48)
Diploid	43	15 (35)	0.20 – 0.45	43	15 (35)
Other	50	2 (4)	<0.20	50	7 (14)

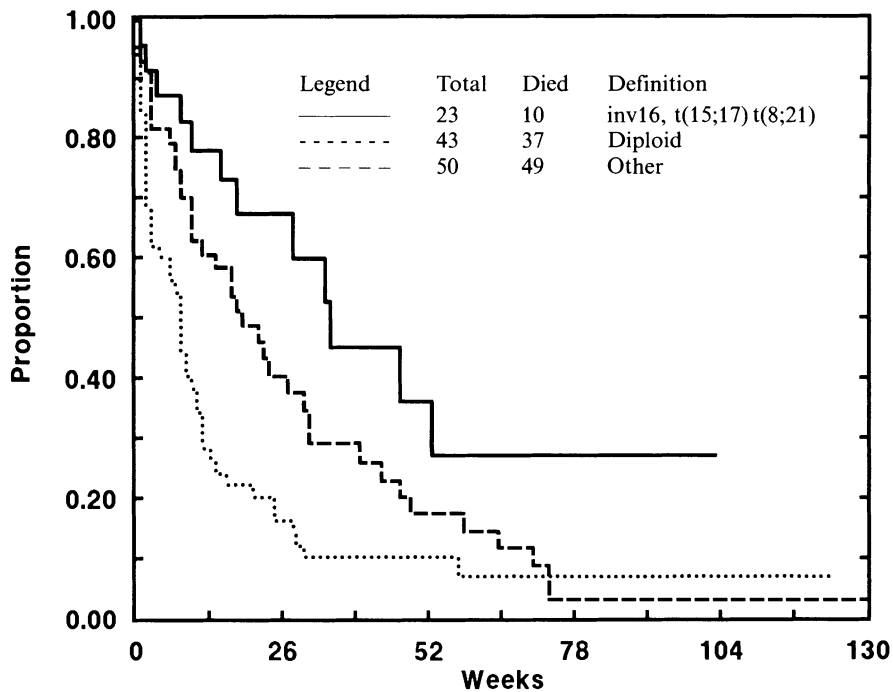


Fig. 3. Survival of test group by karyotype (1985–1988)

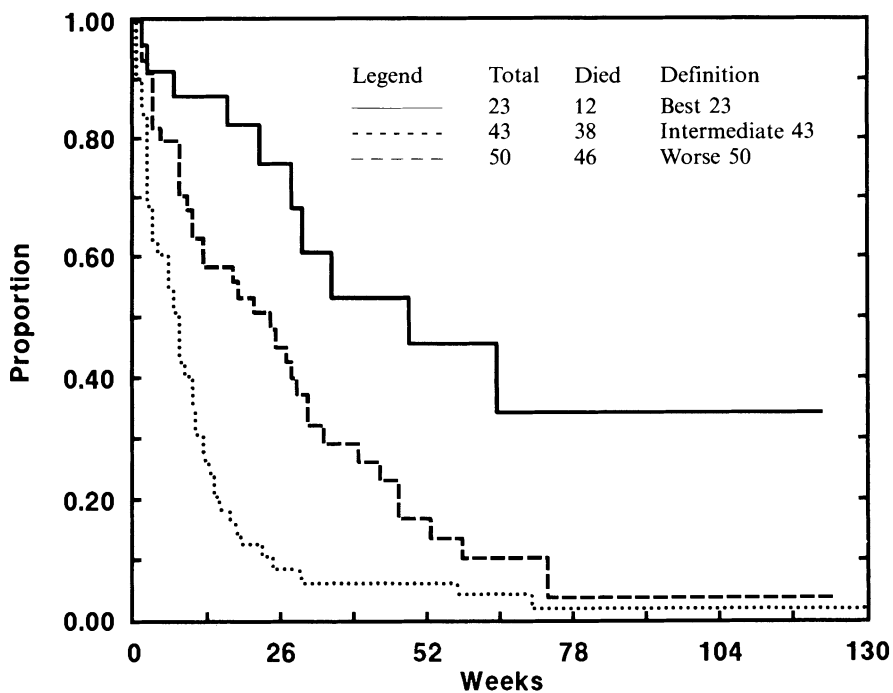


Fig. 4. Prospective test survival model in first-salvage AML

population) are identified and compared with the next 43 patients and the worst 50 patients to correlate numerically with the distribution in the cytogenetic categories, the cytogenetic pattern as a single variable is able to predict as strongly as the multivariate model (Fig. 4). This analysis suggests that the karyotype of the patients' leukemic cells at the time of initial diagnosis is the single most powerful predictive factor for response to salvage therapy and for survival after salvage therapy and is equal to the combination of factors developed from the 1974–1985 patient group.

#### *Interrelationship of Initial CR Duration and Karyotype*

When the two patient populations, 1974–1985 and 1986–1988, are grouped together it is apparent that, while the responsiveness of inv16 and t(15;17) is unaffected by duration of initial remission, that of the t(8;21) and the diploid patients is favorably influenced by duration of initial CR. This also seems to apply to those patients with trisomy 8 and miscellaneous karyotypes. In this group relatively few patients remained in remission for longer than 1 year. The results are unfavorable for the -5/-7 group regardless of remission duration (Table 4).

#### **Discussion**

Previous studies in untreated patients with AML have shown that a variety of prognostic factors are consistently reproducible over long periods [18, 20]. These factors appear to apply to a variety of treatment regimens although most rely on the use of Ara-C as a major drug. Age, performance status, organ function (in particular liver and kidney), infection status, hemoglobin level, platelet count, and fibrinogen level are all consistently associated with response to front-line remission induction therapy [18, 20]. Our studies and more recently those of others have confirmed that cytogenetics are a consistent predictor of outcome [18, 21, 22]. In our studies the cytogenetic pattern was the most striking factor predicting for response to remission induction therapy [18]. The cy-

togenetic pattern is also strongly predictive for remission duration and patients with a t(15;17) and inv16 have long initial CRs compared with diploid and t(8;21) patients who are intermediate and those with adverse karyotypes, who have very short remission durations. This is particularly so for the -5/-7 group.

Prognostic factor models to predict for response to remission induction therapy [18–20], remission duration [18], and survival [23] have been developed. The former two models have been applied to the design and analysis of a clinical trial [24]. This clinical trial showed that the models accurately predict for the probability of patients achieving a complete remission and staying in remission. The models which have been developed with karyotype have been applied prospectively and validated in an independent patient population [18]. Thus, multivariate analytical procedures can lead to the development of models which can be used in design and analysis of clinical trials.

Patients who fail front-line induction therapy or who relapse after having achieved an initial remission and are candidates for initial salvage therapy have greater heterogeneity. They enter the first salvage programs having had a variable intensity of drug exposure and a variety of complications secondary to the recurrence of the disease and the drug programs which have been administered. Despite this, as we have shown, models can be developed for response and survival from a patient population treated over a 10-year time span [18] and applied prospectively to a newer patient group. The models give a good stratification of response categories. Even using simple clinical characteristics and patient history with some characteristics of the leukemia cell, these patients can be stratified into risk groups.

In studies by others and ourselves the duration of initial complete remission has consistently been associated with the probability of obtaining a second remission [15, 18, 19]. In our initial analysis, the strong interrelationship between this factor and cytogenetics which existed [19] was not surprising in view of the strong correlation of cytogenetics with initial CR duration detected in front-line patients [18]. In the present study

the association of karyotype and initial CR duration was explored. Even in the group with short initial CR duration, patients who had the karyotypes inv16 and t(15;17) had a good response. The duration of initial CR was most strongly predictive for the probability of achieving a response to salvage therapy and those who were diploid or had t(8;21), whereas those with adverse karyotypes had a dismal response to treatment regardless of the duration of their first remission. It is not surprising that the vast majority of patients with adverse karyotypes had very short remission durations. It is apparent from this study that most of the active protocols demonstrated their activity in those with karyotypes inv16, t(15;17), t(8;21), and diploid. Clinically significant activity was not noted for any drug program (including those with high-dose Ara-C) in patients with adverse karyotypes.

Comparison of salvage studies in the literature is extremely difficult as most report on small patient populations that have received the treatment being studied. They also come from a variety of treatment backgrounds, some patients having had treatment discontinued and being unmaintained prior to relapse and other having continued on treatment. Most protocols have stringent exclusion criteria for organ dysfunction and performance status. In addition, there is the element of patient selection due to physician impression. If the physician has the impression that a patient is refractory to Ara-C they will presumably not be entered on studies that use Ara-C as a major component. In addition, autologous bone marrow transplantation programs have the difficulty of requiring patients to be in remission for a substantial period to have their bone marrow aspirated and stored and a reasonable period after the bone marrow is stored to indicate a relatively low concentration of residual leukemic cells. Prognostic factor models can be used to a degree to account for these components of treatment heterogeneity. It would seem prudent for future reports of clinical trials in salvage therapy for AML to at least include the denominator of patients from which the patient population was derived, the duration of their initial complete remission, whether the patients were maintained or unmaintained, and how

long they were off maintenance treatment before the study.

The greatest need for new agents is in the group of patients with adverse karyotypes and to a lesser degree the diploid group of patients. Patients who have a diploid karyotype and short remission duration have an unsatisfactory response to salvage therapy. It would seem that, in the adverse karyotype patient population, new agents which have reached the phase II level should be explored as there is an urgent need for new drugs that are active in this patient population. Patients undergoing second and subsequent salvage therapies can also be studied to develop expectations of outcome so that these patients are not denied access to active protocols solely because of the fact that they have received two treatment regimens previously.

The present prognostic factor analyses are admittedly crude, relying on incompletely understood clinical characteristics and only a few biologic characteristics of the leukemic cells. More sophisticated analyses of cellular biologic characteristics and pharmacokinetic parameters are necessary for more accurate predictions of response. In addition, while we emphasize the importance of complete remission, two other outcomes, namely dying during salvage therapy and being resistant to treatment, must be addressed. It would seem useful in future studies to develop predictive factors for mortality during treatment protocols so that an estimate as to whether patients are being under- or over-treated can be obtained.

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## Proposal for the Classification of Relapsed and Refractory Acute Myeloid Leukemias as the Basis for an Age-Adjusted Randomized Comparison of Sequentially Applied High-Dose Versus Intermediate-Dose Cytosine Arabinoside in Combination with Mitoxantrone (S-HAM)

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### Introduction

In spite of the substantial improvement of first-line treatment in adult patients with acute myeloid leukemia (AML) and a gradual increase in long-term remissions, the majority of patients still relapse with their disease and ultimately die due to drug-resistant leukemia. More effective antileukemic therapy is therefore warranted and a variety of new drugs or new applications and dose ranges of established agents have been explored in clinical phase I and II studies. From these investigations cytosine arabinoside (Ara-C) obviously emerged as the most active single agent when administered at doses between 1.0 and 3.0 g/m<sup>2</sup> over 4–6 days [1–7]. A more detailed analysis, however, reveals a substantial interstudy variation of response rates ranging from 11% to 70% even when considering only studies using the original Herzig regimen of 12 single doses of 3.0 g/m<sup>2</sup> high-dose (HD) Ara-C [1–

8]. This discrepancy can only be explained by differences in patient selection and eligibility criteria [9, 10]. Based on the standardized first-line treatment of the German multicenter AML trials 81 and 85 [11, 12], it was the aim of the present study to analyze the response to salvage therapy at relapse and possibly to identify patient subgroups with sustained sensitivity against conventional chemotherapy or refractoriness against standard regimens. These criteria may serve as a guide for the selection of the most appropriate therapy at relapse and also for the decision to enter patients into trials with investigational drugs. Based on the classification derived from these data, preliminary results of an age-adjusted comparison of high-dose versus intermediate-dose Ara-C in combination with mitoxantrone are also presented.

### Patients, Protocols, and Methods

The present analysis comprises 110 patients who relapsed after first-line treatment by TAD-9 (thioguanine, Ara-C, daunorubicin) induction therapy with subsequent randomization for monthly maintenance therapy or no further treatment in remission [11]. Salvage therapy at first relapse consisted of a repetition of the identical TAD-9 regimen. In addition, 12 patients were included who received the TAD-9 protocol at second re-

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lapse. The following parameters were evaluated for their significance on response to the salvage TAD-9 treatment: duration of first remission, age, prior assignment to maintenance therapy or no treatment in remission, French-American-British (FAB) subtype, WBC, number of circulating leukemic blasts, serum lactate dehydrogenase (LDH), and presence of DNA aneuploidy as detected by flow cytometry. Differences between these variables were analyzed by the chi-square test.

Based on the results of this evaluation a subsequent trial was initiated in patients relapsing from the German multicenter trial AML 85 [12]. This study is based on the sequential application of high-dose Ara-C and mitoxantrone (S-HAM) and consists of an age-adjusted comparison of high-dose versus intermediate-dose Ara-C [13]. According to the S-HAM protocol, Ara-C is given on days 1, 2, 8, and 9 and mitoxantrone is administered uniformly at 10 mg/m<sup>2</sup> per day while the doses for Ara-C were randomly assigned as follows: patients younger than 60 years of age received either 3.0 g/m<sup>2</sup> or 1.0 g/m<sup>2</sup> Ara-C per dose while older patients were randomized to either 1.0 g/m<sup>2</sup> or 0.5 g/m<sup>2</sup> per single dose (Fig. 1).

In order to avoid the selection of patients with different prognoses, the randomization was balanced for the following stratification criteria:

1. Type of first-line treatment
2. Primary resistance against induction therapy
3. Duration of the preceding remission
4. Number of relapse

Antileukemic response was judged according to Cancer and Leukemia Group B (CALGB) criteria and side effects were evaluated by WHO definitions.

### Results

The 122 patients who were evaluated for response to the TAD-9 salvage therapy had a median age of 48 years (range 19–74 years). One hundred and ten patients were in first and 12 in second relapse. Further characteristics are listed in Table 1. Analysis of the pretherapeutic parameters revealed a significant correlation between the duration of the first remission and the response to TAD-9 therapy at relapse. Of 35 patients relapsing within the first 6 months, 11 (31%) achieved a second complete remission as

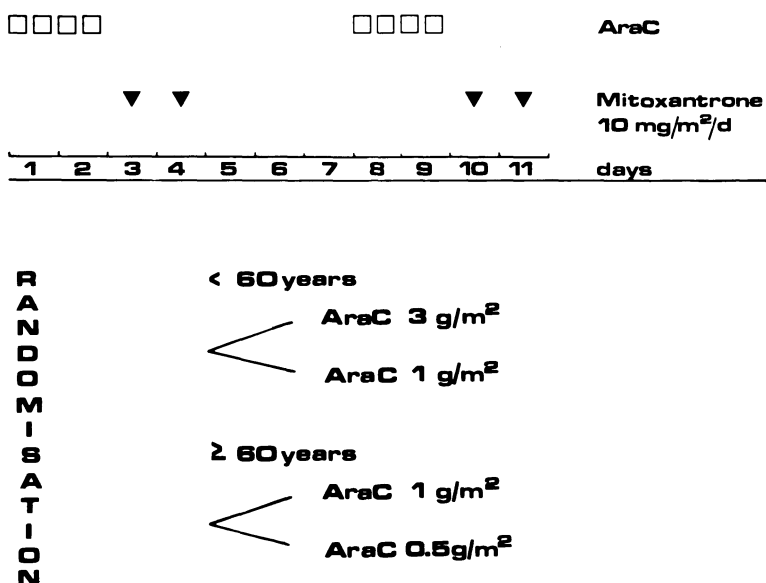


Fig. 1. Design of the age-adjusted randomized comparison of intermediate- versus high-dose Ara-C on the basis of the S-HAM regimen

**Table 1.** Pretreatment patient characteristics

FAB subtype	M1	26
	M2	34
	M3	15
	M4	15
	M5	17
	M6	8
	Unclassified	7
WBC	1200–99000 mm <sup>3</sup> (median 4650)	
Circulating blasts	0–86130 mm <sup>3</sup> (median 1920)	
Serum LDH	90–2540 U/liter (median 245)	
DNA aneuploidy	14/32 (44%)	
Age > 60 years	19	
Age < 60 years	103	
Duration of first remission		
< 6 months	35	
7–12 months	38	
> 12 months	37	
Maintenance chemotherapy		
Yes	25	
No	97	

compared with 2 (58%) of 38 patients with remission durations of 7–12 months and 22 (60%) of 37 cases with preceding remissions of more than 12 months duration ( $P < 0.05$ ) (Table 2). None of the other variables was found to be of prognostic relevance for the response at first relapse (Table 3). However, patients in second relapse had a substantially lower remission rate as compared with first-relapse cases (CR 25% vs. 50%,  $P < 0.05$ ). Including patients with primary nonresponse to induction therapy, these data allow to identify patients with a chance

**Table 2.** Relationship between response and duration of first remission

Duration of first remission	CR <i>n</i> (%)	ED <i>n</i> (%)	NR <i>n</i> (%)
≤ 6 months	11 (31%)	5 (14%)	19 (55%)
	$P < 0.05$		
7–12 months	22 (58%)	4 (11%)	12 (31%)
	NS		
> 12 months	22 (60%)	5 (14%)	10 (26%)

**Table 3.** Relationship between pretreatment characteristics and response rate (CR)

Pretreatment characteristics and response			
		% CR	<i>P</i>
FAB subtype	M1	40%	NS
	M2	36%	
	M3	60%	
	M4	20%	
	M5	40%	
	M6	33%	
	Unclassified	54%	
WBC	< 4650	52%	NS
	> 4650	32%	NS
LDH	< 245	43%	NS
	> 245	36%	NS
DNA index	1.0	50%	NS
	+1.0	29%	NS
Age	< 60	44%	NS
	> 60	33%	NS
Maintenance	Yes	33%	NS
	No	41%	NS

of less than 30% of obtaining a second complete remission by conventional chemotherapy and who can thus be considered as refractory against standard cytostatic therapy. These criteria include the following cases:

1. Nonresponse against induction therapy
2. Early relapse within the first 6 months
3. Relapse after 6 months remission duration with nonresponse against a reinduction attempt by standard induction therapy
4. Second and subsequent relapses

The age-adjusted comparison of high-dose versus intermediate-dose Ara-C in patients at relapse or with primary nonresponse was based on these criteria and randomization was stratified accordingly. At present, 65 patients have been entered into the study from 14 participating centers in Germany and Austria. Fifty patients have completed the S-HAM cycle. Of these, seven patients had to be excluded because of preceding myelodysplastic syndromes, protocol violation, patient refusal or relapse after bone marrow transplantation. Of the remaining 43 patients, 33 were younger than 60 years of age and 10 were older. Overall 19 (49%) of 39

completely documented patients achieved a complete remission. Nine patients were non-responders and 11 patients died within the first 6 weeks of chemotherapy due to infectious complications, fatal bleeding, or drug-resistant leukemia (early death). At this early stage of the study no differences in response rates were observed between the different Ara-C doses in both age groups (Tables 4, 5). Median time to the recovery of blood counts to more than 20 000 thrombocytes/mm<sup>3</sup> and more than 500 granulocytes/mm<sup>3</sup> was 27 days (range 24–56 days); median time to complete remission was 38 days (range 28–63 days). The predominant side effects during S-HAM therapy consisted of nausea and vomiting, mucositis, mild liver enzyme elevations, diarrhea, and skin reactions. Of the 19 patients achieving a complete remission, 2 underwent subsequent bone marrow transplantations, 5 relapsed within 1–5 months, and 10 are in ongoing remission at 2<sup>+</sup>–6<sup>+</sup> months.

**Table 4.** Treatment results in patients above 60 years of age

Ara-C dose	CR	ED	NR	n
1.0 g	1	1	3	5
0.5 g	3	1	2	6
Total	4	2	5	11

**Table 5.** Treatment results in patients below 60 years of age

Ara-C dose	CR	ED	NR	n
3.0 g	7 (54%)	5 (38%)	1 (8%)	13
1.0 g	8 (53%)	4 (27%)	3 (20%)	15
Total	15 (54%)	9 (32%)	4 (14%)	28

## Discussion

The present evaluation was performed in an attempt to improve the characterization of AML at relapse and to develop a proposal for the definition of refractoriness against

conventional chemotherapy. This approach might help to select the most appropriate treatment at relapse and may also provide the basis for a better definition of the eligibility criteria for clinical-phase I and II studies in advanced acute leukemias [9, 10]. Furthermore, it may facilitate interstudy comparisons. From this analysis the duration of first remission clearly emerged as the most important determinant for response to salvage therapy. Patients who experienced relapses within 6 months had a significantly lower remission rate than patients with later occurring relapses. This finding corresponds with previous reports by the British MRC study group and by Keating and coworkers from the MD Anderson Hospital, Houston [14, 15]. Other factors with prognostic significance at first diagnosis such as WBC, serum LDH, FAB subtype, cytogenetic abnormalities, and DNA aneuploidy had no significant impact on the response at first relapse. Hence, duration of first remission is essential for the clinical management of patients at relapse and allows the following definition of refractoriness against conventional chemotherapy:

1. Primary nonresponse against induction chemotherapy
2. Relapse within the first 6 months
3. Later occurring relapse with nonresponse against another reinduction attempt by standard chemotherapy
4. Second and subsequent relapses

Patients falling into these groups have a less than 30% chance of responding to conventional antileukemic treatment and are therefore candidates for experimental chemotherapy in clinical-phase I/II studies. Patients with leukemic recurrences after 6–12 months, however, are still sensitive to standard therapy. They are not refractory to conventional treatment and should therefore not be entered on new protocols with unproven antileukemic activity.

Taking these considerations into account and stratifying for the above-mentioned criteria, the current age-adjusted multicenter trial in relapsed AML in Germany addressed the question of intermediate-versus high-dose Ara-C in a prospective randomized comparison on the basis of the previously established S-HAM regimen [13]

Pharmacokinetic investigations seem to indicate that this issue is settled already in favor of intermediate doses of Ara-C in the range of 500–1000 mg/m<sup>2</sup> per single administration. These doses were shown to saturate the intracellular enzyme deoxycytidine kinase, transforming Ara-C into its active form Ara-CTP [16, 17]. Hence, higher doses may not result in an increased anti-leukemic activity but more pronounced side effects only. Various mechanisms of cellular resistance against Ara-C, however, such as an increase of the Ara-CTP-inactivating enzyme cytidine deaminase, a reduction of Ara-CTP incorporation into the DNA molecule, or more effective repair of Ara-C-induced DNA damage may require administration of higher doses of Ara-C for optimal antileukemic activity [18–23]. This thesis is supported by two successive studies by Willemze et al. in refractory acute myeloid leukemias. In the first trial, 10 of 20 patients achieved a complete remission after treatment with 12 doses of 3.0 g/m<sup>2</sup> Ara-C, whereas in a subsequent study none of 8 patients responded to 12 doses of 1.0 g/m<sup>2</sup> Ara-C [24]. A similar difference was not observed on the other hand by Herzig and coworkers when comparing 2.0 versus 3.0 g/m<sup>2</sup> Ara-C both given twice daily for 6 days [25].

The present study is the first trial addressing this question in ways of randomized comparison. However, at this early stage results are too preliminary to provide an answer and further patient recruitment and longer follow-up is needed for a final conclusion. Thanks to the joint efforts of the participating centers, it may be possible in the near future to accurately define the dosis of Ara-C which can be administered safely with acceptable toxicity without losing antileukemic activity.

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## Pharmacologically Directed Design of Leukemia Therapy\*

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Most nucleoside analogues require intracellular metabolism, usually to the 5'-triphosphate, to produce cytotoxicity. This reflects one mechanism of action of these drugs: inhibition of DNA synthesis through effects on enzymes whose natural substrates are nucleotides. Strong correlations have been demonstrated between cytotoxicity in experimental systems and the cellular pharmacology and pharmacodynamics of nucleotides of nucleoside antimetabolites [1, 2]. Recently, analytical procedures used in experimental studies, principally high-pressure liquid chromatography, have been adapted for investigations of nucleoside analogue metabolites in human leukemia cells during therapy [3].

The biologic activity of arabinosyl cytosine (Ara-C), a nucleoside antimetabolite effective in the treatment of adult acute myelogenous leukemia (AML) and also useful against a variety of other hematologic diseases, exhibits an absolute dependency on phosphorylation and accumulation of the active nucleotide Ara-C 5'-triphosphate (Ara-CTP). Investigations of the plasma

pharmacology of Ara-C have failed to demonstrate any relationships with clinical response. This can be explained by the fact that there are few correlations between the plasma pharmacology of Ara-C and the pharmacokinetics of Ara-CTP in leukemia cells [4].

In contrast, strong relationships have been demonstrated between clinical response and the pharmacokinetics and pharmacodynamics of Ara-CTP in leukemia cells of patients receiving high-dose Ara-C therapy (3 g/m<sup>2</sup> administered over 2 h every 12 h) for acute leukemia in relapse [3, 5]. In these patients the half-life of Ara-CTP elimination from cells, the trough (12-h) concentration, and the total intracellular exposure to Ara-CTP (the area under the accumulation times the retention curve, AUC) were each correlated with the likelihood of achieving a complete remission. No correlation was observed with peak intracellular Ara-CTP concentration, which was greater than 300  $\mu$ M for all patients. As a measure of the pharmacodynamic activity of Ara-CTP, there was a strong linear trend linking the extent of inhibition of DNA synthesis at 12 h with the likelihood of response.

### Prognostic Significance

After pretreatment clinical variables had been accounted for, knowledge of these pharmacokinetic factors added significant prognostic information [6]. This provided a rationale for manipulating the dose rate and schedule of Ara-C administration in pa-

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tients whose Ara-CTP pharmacokinetics were inconsistent with response to an every-12-h schedule.

### Therapeutic Index

Intracellular Ara-CTP pharmacokinetics were similar in bone marrow and blood [7]. Marrow mononuclear cells from patients with solid tumors accumulated less Ara-CTP and eliminated it more rapidly than did the leukemia cells of the patients whose relapsed leukemia subsequently responded to high-dose Ara-C. In contrast, the pharmacokinetics of Ara-CTP in the leukemia cells of patients whose acute leukemia subsequently failed to respond to high-dose Ara-C treatment were similar to the Ara-CTP kinetics observed in normal marrow. These results may represent the pharmacologic basis for the responses to high-dose Ara-C therapy.

### Combination Chemotherapy

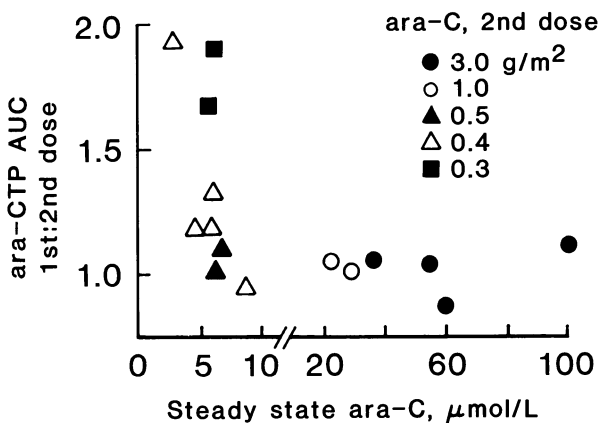
Because leukemia cells of responding patients retained DNA synthesis-inhibiting levels of Ara-CTP for a longer time than did cells from normal marrow, administration of a DNA-damaging agent after Ara-CTP in normal marrow had declined to levels no longer inhibitory to DNA synthesis may result in a selective inhibition of DNA repair in leukemia cells over normal cells. The integrity of DNA in circulating leuke-

mia cells was not changed by infusion of high-dose Ara-C [8]. However, studies of combinations of Ara-C plus mitoxantrone demonstrated that the presence of Ara-CTP in leukemia cells may synergistically increase mitoxantrone-induced DNA damage in these cells during therapy [8]. Additional work is necessary to define better the relationships among combination drug infusions, DNA damage, and clinical response.

### Pharmacologic Rationale for Intermediate-Dose Ara-C

The accumulation of Ara-CTP by leukemia cells remained linear for more than 1 h after the end of a 2-h high-dose Ara-C infusion, suggesting that the infusion rate (1.5 g/m<sup>2</sup> per hour) produced plasma Ara-C concentrations in excess of those required to saturate Ara-CTP accumulation [9, 10]. Serial infusion of 3 g/m<sup>2</sup> followed 12 h later by infusion of 1 g/m<sup>2</sup> resulted after each dose in an Ara-CTP AUC ratio of about 1 in circulating leukemia cells (Fig. 1). A stepwise decrease of the second, lower Ara-C dose to 0.3 g/m<sup>2</sup> administered over 2 h indicated that 0.5 g/m<sup>2</sup> given over 2 h was sufficient to saturate Ara-CTP accumulation in most patients (Fig. 1). The plasma Ara-C concentration achieved during the 0.5 g/m<sup>2</sup> infusion was about 10 μM, a value that correlates well with those demonstrated to saturate Ara-CTP accumulation in fresh leukemia cells *in vitro* [11].

**Fig. 1.** Relationship between the relative ability of circulating leukemia cells to accumulate and retain Ara-CTP after serial infusions of unequal Ara-C doses and the steady-state concentrations of Ara-C observed during the lower-dose infusion. For each patient, the ratio of the cellular Ara-CTP AUC values were plotted against the steady-state concentration of Ara-C in the plasma during the second, lower dose for each patient. The initial dose was 3.0 g/m<sup>2</sup> when the second dose was 3.0 or 1.0 mg/m<sup>2</sup>; the initial dose was 1.0 mg/m<sup>2</sup> when the second dose was 0.5, 0.4, or 0.3 mg/m<sup>2</sup>



## Clinical Trial of Intermediate-Dose Ara-C

This study provided the pharmacokinetic rationale for a phase I–II trial of “intermediate-dose” Ara-C in adult patients with relapsed myelogenous leukemia in which the number of doses of 0.5 g/m<sup>2</sup> administered over 2 h at 12-h intervals was escalated. Dose-limiting toxicity (prolonged myelosuppression, gastrointestinal effects, skin rash) occurred after administration of 15–18 doses. Central nervous system toxicity was markedly reduced compared with a 3-g/m<sup>2</sup> schedule. The response rates with the 0.5-g/m<sup>2</sup> and 3-g/m<sup>2</sup> doses were similar, supporting the critical role of Ara-CTP in determining response to Ara-C. The reduced CNS toxicity of intermediate-dose Ara-C permits exposure of the leukemia cells to Ara-CTP concentrations that equal those achieved with high-dose Ara-C but are of increased duration [12]. The characteristics of reduced toxic effects and increased time of exposure to intensive Ara-C therapy may lead to increased efficacy of combinations of intermediate-dose Ara-C and other active antileukemia drugs.

## Optimizing Cellular Ara-CTP During Continuous Infusions

We hypothesized that the steady-state concentration of intracellular Ara-CTP (Ara-CTP<sub>ss</sub>) in leukemia cells is proportional to the dose rate of Ara-C during continuous infusion [13]. To evaluate this hypothesis, adults with AML in relapse were treated with two sequential schedules of serially increasing dose rates over a total of 36 h. Schedule I consisted of serial infusions of 250, 500, and 750 mg/m<sup>2</sup>, each given over 12 h. Subsequently, different patients entered on schedule II received 500, 1000, and 1500 mg/m<sup>2</sup> serially, each given over 12 h. Steady-state levels of Ara-CTP were achieved within 4 h after beginning Ara-C infusion and, in separate studies of a single Ara-C dose rate of 1500 mg/m<sup>2</sup>, were shown to be maintained beyond 36 h. Four patients treated with schedule I and two patients treated with schedule II showed a linear dose rate-dependent increase of Ara-CTP<sub>ss</sub> at all three dose rates. The leukemia cells of

one patient on schedule I and of two patients on schedule II showed a dose rate-dependent Ara-CTP<sub>ss</sub> increase only at the first two dose levels, whereas no increase or a lower Ara-CTP<sub>ss</sub> was observed at the third dose rate. The Ara-CTP<sub>ss</sub> of one patient on schedule II did not change. These results suggest that there is a proportionality between the continuous infusion dose rate of Ara-C and the Ara-CTP<sub>ss</sub> in circulating leukemia cells within the dose range of 250–1000 mg/m<sup>2</sup> over 12 h. The absolute rate of Ara-CTP accumulation varied greatly among patients so that there was marked heterogeneity in the Ara-CTP<sub>ss</sub> levels after a 12-h infusion. As with Ara-CTP pharmacokinetics after intermittent infusion of Ara-C, it is likely that Ara-CTP<sub>ss</sub> levels have prognostic value [14]. This opens the possibility that pharmacologic determinations may be used to redirect the Ara-C dose rate to achieve a desired Ara-CTP<sub>ss</sub> concentration in leukemia blasts during therapy.

## Summary

The ability to accumulate and retain the active metabolite of Ara-C varies widely among patients. Our studies demonstrate a significant correlation between clinical response and the pharmacokinetics of Ara-CTP in leukemia cells during therapy. Knowledge of the cellular pharmacology of Ara-CTP has been used to optimize dose rates and to design combination treatment schedules. An understanding of the cellular pharmacodynamics of other drugs is likely to be a useful parameter for planning treatment protocols.

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## Treatment of Relapsed or Refractory Acute Leukemia: Comparison of Two Different Regimens

G. Marit, P. Cony, F. Duclos, M. Puntous, A. Broustet, and J. Reiffers

### Introduction

The treatment of relapsed or refractory acute leukemia (AL) remains problematic. The use of high-dose cytosine arabinoside (HD Ara-C) appears promising [1] and its association with M-amsacrine (M-AMSA) has achieved some encouraging results [2]. Such an association permits one to achieve a substantial number of complete remissions (CRs) but the median duration of these CRs remains short and other protocols including new drugs such as mitoxantrone alone [3], or in combination with other drugs are needed [4–6]. The association of mitoxantrone and etoposide (VP-16) appears to be active in acute nonlymphoblastic leukemia [4]. We report here the results observed in relapsed or refractory AL with two consecutive but different regimens combining either HD Ara-C-M-AMSA or mitoxantrone-VP-16.

### Materials and Methods

Fifty-eight patients with acute leukemia [acute non-lymphoblastic leukemia (ANLL), 43; acute lymphoblastic leukemia (ALL), 15] were entered into the two consecutive studies. Thirty-four patients received a combination of M-AMSA 150 mg/m<sup>2</sup> · day, i.v., during 5 days and HD Ara-C 3 g/m<sup>2</sup> every

12 h, in a 1-h infusion, 4 doses on the first 2 days (group I), while the 24 other patients received a 5-day course of chemotherapy associating VP-16 (80 mg/m<sup>2</sup> · day in continuous infusion) with mitoxantrone (12 mg/m<sup>2</sup> · day, i.v.) (group II). The characteristics of the patients of the two groups are summarized in Tables 1 and 2.

Seventeen patients had refractory AL and fulfilled the following criteria [6]:

1. Total absence of response to standard chemotherapy
2. Failure to reach CR after two courses of combination chemotherapy
3. Relapse within 6 months after achieving CR
4. Refractory to salvage therapy following relapse

Two of the refractory patients had received an autologous transplantation (group I: one in second CR, group II: one in first CR) and relapsed 3 months after achievement of CR.

Among the 41 relapsed patients, 7 had been previously transplanted in first CR (6 cases) or in second CR (1 case) with either autologous (group I, one; group II, two) or allogeneic (group I, one; group II, three) grafts. Response to chemotherapy was evaluated according to Cancer and Leukemia Group B (CALGB) criteria [7]. Duration of response was counted from the day of diagnosis of CR to the day of bone marrow relapse. Extrahematological toxicity was graded according to the World Health Organization criteria [8], except for Ara-C toxicity, which was graded according to the Herzig criteria [1].

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**Table 1.** Characteristics of patients with ANLL

	Relapsed		Refractory	
	Group I (n=18)	Group II (n=14)	Group I (n=9)	Group II (n=2)
Sex (M/F)	11/7	7/7	4/5	1/1
Median age (years)	35 (16-58)	45.5 (6-62)	33 (19-52)	39.5 (33-46)
Median duration of first CR (months)	14.5 (7-43)	15.5 (6.5-57)	-	-
Previous treatment				
Chemotherapy	17	12	9	2
Transplantation	1	2	-	-

**Table 2.** Characteristics of patients with ALL

	Relapsed		Refractory	
	Group I (n=4)	Group II (n=5)	Group I (n=3)	Group II (n=3)
Sex (M/F)	3/1	4/1	2/1	1/2
Median age (years)	26 (18-49)	20 (20-28)	32 (19-33)	19 (19-21)
Median duration (months) of				
First CR	17.5 (12-89)	17 (7-72)	18	-
Second CR	13.5 (3-24)	36	-	-
Previous treatment				
Chemotherapy	3	2	2	2
Transplantation	1	3	1	1

## Results

### *Response to Treatment*

The results are summarized in Table 3 for the ANLL and Table 4 for ALL. Three patients died during aplasia (group I, one patient with refractory ANLL; group II, two patients with relapsed ALL) and were thus not evaluable for response. For the patients with relapsed ANLL, the CR rate is higher in group I (66%) than in group II (28%) ( $P < 0.025$ ). The median duration of CR was similar in both groups (group I, 4.5 months; group II, 5.5 months). However, of the

group II patients, only one patient is currently alive in CR 6 months after achievement of CR, while 2 of the group I patients are alive in CR, 31 and 42 months after achievement of CR. These latter two patients received an autologous blood stem cell transplantation. In relapsed ALL, the number of CRs was similar for both groups and the duration of CR was short (group II, 6 months; group I, 2 months). None of these patients are currently alive in CR. Of the patients with refractory AL, none of the group II patients achieved CR, while 3 of the 11 evaluable group I patients achieved CR (ANLL, 2/8; ALL, 1/3), but relapsed within

**Table 3.** Response to treatment: Patients with ANLL

	Relapsed		Refractory	
	Group I (n=18)	Group II (n=14)	Group I (n=9)	Group II (n=2)
Complete remission				
Number	12	4	2	0
Median duration (months)	4.5 (1–40)	5.5 (3.5–8)	2.5 (2–3)	–
Hypoplastic deaths	0	0	1	0
Failure	6	10	6	2
Patients alive in CR				
Number	2	1	1	0
Duration (months)	31–42	6	33	–

**Table 4.** Response to treatment: Patients with ALL

	Relapsed		Refractory	
	Group I (n=4)	Group II (n=5)	Group I (n=3)	Group II (n=3)
Complete remission				
Number	2	3	1	0
Median duration (months)	2 (1–3)	6 (2.5–7)	1.5	–
Hypoplastic deaths	0	2	0	0
Failure	2	0	2	3
No. of patients alive in CR	0	0	0	0

3 months following CR. Only one of these patients who failed to respond to chemotherapy and who underwent an allogeneic transplantation is alive in CR, 33 months after transplant.

#### *Hematological Toxicity*

In those patients achieving CR, the median time to recover more than 500 polymorphonuclear cells/mm<sup>3</sup> was 23 days (range, 15–36 days) in group I and 34 days (range, 21–58 days) in group II. Platelet recovery (more than 50 000/mm<sup>3</sup> without platelet transfusion) occurred after a median duration of 18 days (range, 10–30 days) in group

I and 45 days (range, 16–35 days) in group II. All patients showed signs of sepsis (fever and/or chills) either during chemotherapy or during the subsequent aplastic phase, and blood cultures were positive in six cases in group I and in eight cases in group II. Three hypoplastic deaths were observed: one in group I related to a septic shock (*Pseudomonas aeruginosa* bacteremia), two in group I, both related to hemorrhage.

#### *Extrahematological Toxicity*

Extrahematological toxicity in the two groups of patients is summarized in Table 5. The gastrointestinal system was the most af-

**Table 5.** Extrahematological toxicity (HD Ara-C M-AMSA/VP-16 mitoxantrone)

	Toxicity grade				Total Nos. of patients
	1	2	3	4	
Nausea-vomiting <sup>a</sup>	3/12	23/3	–	–	26/15
Stomatitis <sup>a</sup>	6/11	2/5	0/2	–	8/18
CNS <sup>b</sup>	8/0	2/0	–	–	10/0
Liver <sup>b</sup>	3/0	2/0	5/0	–	10/0
Skin <sup>b</sup>	5/0	4/0	–	–	9/0
Diarrhea <sup>b</sup>	7/0	1/0	–	–	8/0
Eye <sup>b</sup>	6/0	1/0	–	–	7/0
Pulmonary <sup>a</sup>	–	–	–	–	–
Cardiac <sup>a</sup>	–	–	–	–	–

<sup>a</sup> According to the WHO criteria

<sup>b</sup> According to the Herzig criteria

ected in group I, while stomatitis was the most common side effect observed in group II. No cardiac or pulmonary (related to high-dose Ara-C) side effects were reported in either group I or group II.

## Discussion

The results obtained with the combination of HD Ara-C and M-AMSA were similar to those previously observed in our unit [9] and in other studies [2, 10], with an overall CR rate of 63% in relapsed AL and 27% in refractory AL. It is interesting to note that:

1. Three of the five patients who had previously received HD Ara-C as consolidation treatment after achievement of first CR responded well and achieved CR.

2. It was possible, for the patients achieving CR, to collect a sufficient number of peripheral blood stem cells for autologous transplantation.

The results obtained in ANLL with the association of VP-16-mitoxantrone were worse than those observed with HD Ara-C-M-AMSA and in other studies [4, 11]. Ho et al. observed a CR rate of 42% [4]. The mode of administration of VP-16 was not the same, and this may explain the difference. The rationale for the use of VP-16 by continuous infusion was based on the results of two studies [5, 12]. Letendre et al. [12] published results of a phase I study of VP-16

and M-AMSA for the treatment of refractory AL in whom VP-16 was administered by bolus injection. One of their conclusions was that the cytotoxicity of the two drugs could be enhanced if VP-16 were administered by continuous infusion. This hypothesis seemed to be confirmed by the study of Tschopp et al. [5], in which VP-16 was administered by continuous infusion at a lower dose (80 mg/m<sup>2</sup>) than in the previous study. In our study, the same mode of administration and dosage of VP-16 was used; however, mitoxantrone, which is similar to AMSA and is also an intercalating agent, was used. The difference observed in remission rate may result from the lower dose of VP-16 used in our study.

However, this regimen seems to be at least as efficient as the association of HD Ara-C M-AMSA in the treatment of relapsed ALL but the number of patients is too small to draw any conclusions. The toxic effects of these two regimens were not excessive, and the number of hypoplastic deaths was low for the two groups. In conclusion, the association of HD Ara-C M-AMSA seems to be a more effective regimen for the treatment of relapsed ANLL than the combination of mitoxantrone-continuous infusion VP-16. However, no significant difference was observed in our study for refractory ANLL and relapsed or refractory ALL. Further studies are needed to confirm these preliminary results.

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## BFM Group Treatment Results in Relapsed Childhood Acute Lymphoblastic Leukemia

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### Introduction

At the first Symposium on Acute Leukemias in Münster, 1987, we reported on preliminary results obtained in two subsequent trials on relapsed childhood acute lymphoblastic leukemia, ALL-REZ BFM 83 and 85 [1]. The present paper is an update of the results after both studies have been closed for patient entry and follow-up periods have reached 5½ and 3½ years, respectively.

### Patients and Treatment

Between June 1983 and March 1987, 221 children and adolescents with their first relapse of ALL were enrolled on two subsequent BFM trials, studies 83 ( $n=91$ ) and 85 ( $n=130$ ). All children had received intensive multidrug front-line therapy as used in the German BFM and COALL trials [2–6]. Patients characteristics are given in Table 1. Except for a larger number of patients with marrow relapse in study 85, patient populations of both studies are comparable.

By definition, early relapses were those which occurred during or within 6 months

**Table 1.** Patient characteristics of two BFM relapse studies

	ALL- REZ 83	ALL- REZ 85
Patients	91	130
Median age at relapse (years)	8.3	7.4
Girls	36%	33%
Early relapse	58%	57%
BM involvement	64%	85%
CNS involvement	33%	23%
BM transplant	13%	13%

after the end of front-line treatment. The remainder were termed late relapses. Bone marrow (BM) relapse was diagnosed in the presence of  $\geq 25\%$  bone marrow blasts without other manifestations of leukemia (isolated BM relapse) or if there was proven extramedullary leukemia and at least 5% blast cells could be detected in the marrow (combined BM relapse). Accordingly, isolated extramedullary relapses were those with histologically or cytologically proven leukemia at any site except for the marrow.

The patients were subdivided into three different strategic groups, A, B, and C, with respect to the presumed intensity and duration of treatment, necessary for remission induction, consolidation, and maintenance. Group A patients (early BM relapse) were treated with a special induction protocol (E in study 83, F in study 85) because they were expected to need more aggressive initial

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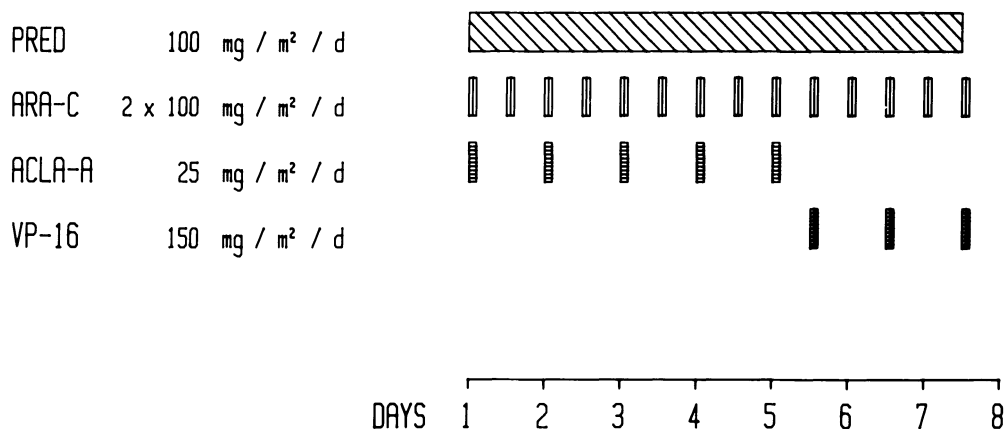


Fig. 1. Induction protocol for group A patients (early bone marrow relapse) of study ALL-REZ BFM 83: *PRED*, prednisone; *ARA-C*, cytarabine; *ACLA-A*, aclarubicin; *VP-16*, Etoposide

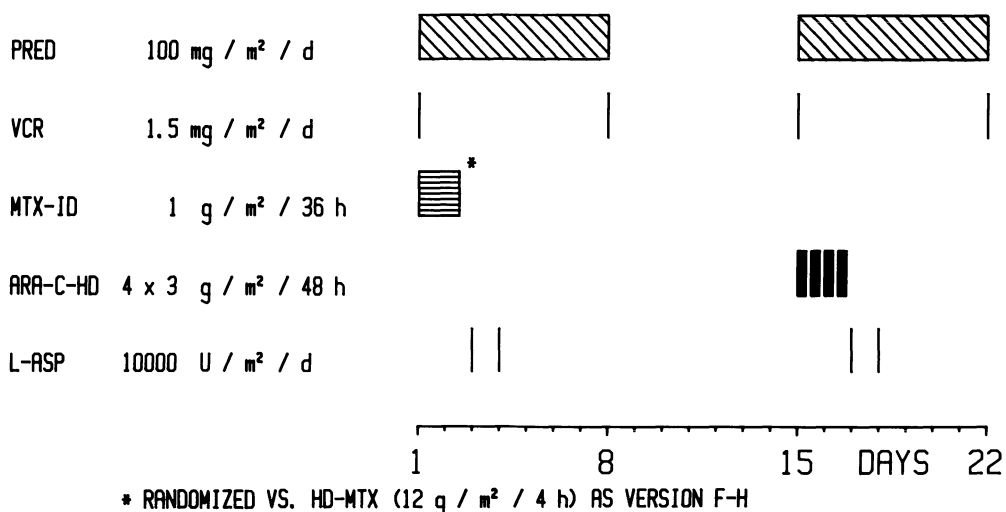
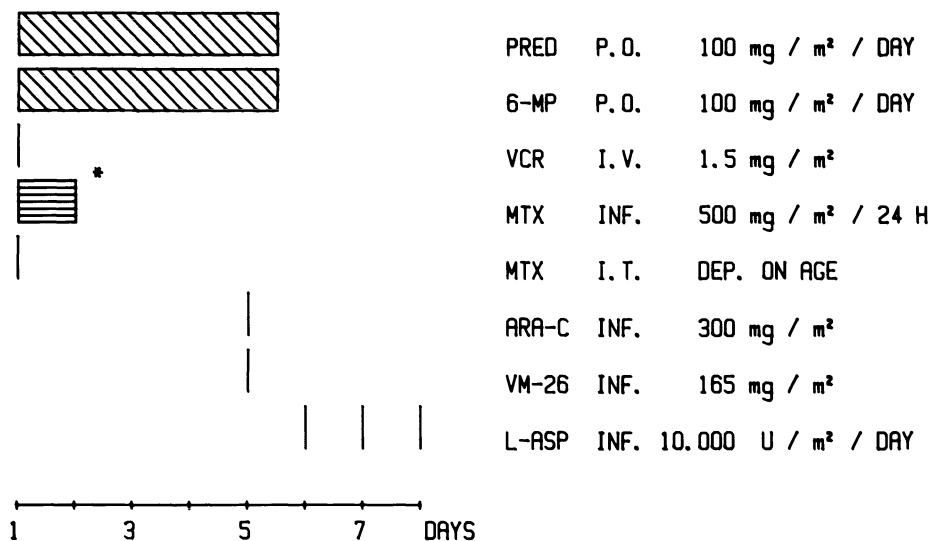


Fig. 2. Induction protocol version M for group A patients (early bone marrow relapse) of study ALL-REZ BFM 85: *PRED*, prednisone; *VCR*, vincristine; *MTX-ID*, intermediate-dose methotrexate (1 g/m<sup>2</sup> 36 h infusion, leucovorin 15 mg/m<sup>2</sup> 48 and 54 h), *ARA-C-HD*, high-dose cytarabine; *L-ASP*, L-asparaginase. Version H: high-dose methotrexate (12 g/m<sup>2</sup> 4 h infusion, 12 doses leucovorin at 15 mg/m<sup>2</sup> each starting at 24 h after start of MTX)

treatment (Figs. 1, 2). Induction was followed by eight courses of multidrug chemotherapy, four courses of R<sub>1</sub> and R<sub>2</sub>, each (Figs. 3, 4). In patients with late marrow relapse (group B), treatment consisted of eight R-blocks without a separate induction. Group C patients received four R-blocks in study 83 and six such courses in study 85, supplemented by radiotherapy to involved extramedullary sites.

In study 83, the treatment elements contained i.v. methotrexate (MTX) at a dose of 500 mg/m<sup>2</sup> 24 h infusion rescued by two doses of leucovorin (15 mg/m<sup>2</sup>) at 48 and 54 h. In study 85, patients were randomly allocated to one of two arms, M or H. The dose of MTX in arm M was 1 g/m<sup>2</sup> 36 h infusion followed by two doses of leucovorin (15 mg/m<sup>2</sup>) at 48 and 54 h, whereas in arm H MTX was given at a dose of 12 g/m<sup>2</sup>

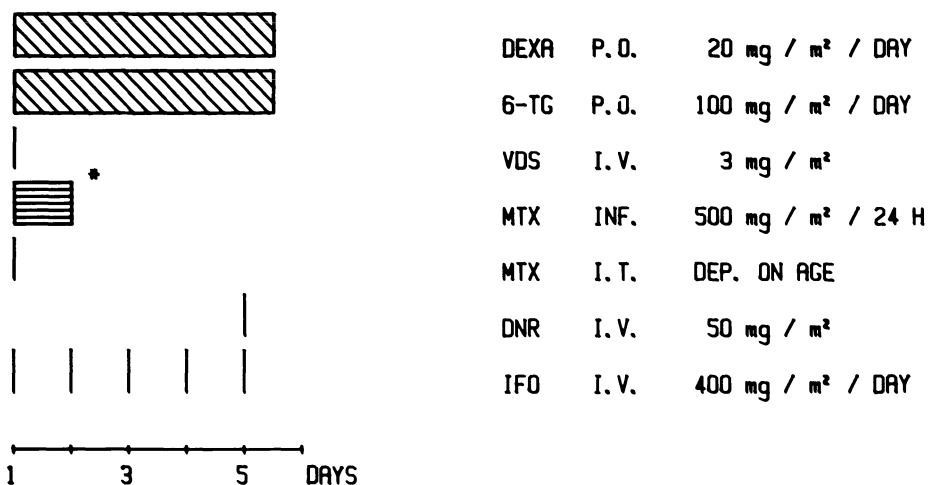




\*) BFM 85 : RANDOMISATION

HD-MTX	INF.	12 g / m <sup>2</sup> / 4 H
ID-MTX	INF.	1 g / m <sup>2</sup> / 36 H

Fig. 3. Treatment element R1 of studies 83 and 85: *PRED*, prednisone; *6-MP*, mercaptopurine; *VCR*, vincristine; *ARA-C*, cytarabine; *VM-26*, teniposide; *L-ASP*, L-asparaginase; *MTX*, methotrexate; study 83: 500 mg/m<sup>2</sup> 24 h infusion with two doses of leucovorin at 48 and 54 h; study 85, randomized 1 g/m<sup>2</sup> 36 h (arm M) versus 12 g/m<sup>2</sup> at 4 h (arm H) with leucovorin doses as described in Fig. 2



\*) BFM 85 : RANDOMISATION

HD-MTX	INF.	12 g / m <sup>2</sup> / 4 H
ID-MTX	INF.	1 g / m <sup>2</sup> / 36 H

Fig. 4. Treatment element R2 of studies 83 and 85: *DEXA*, dexamethasone; *6-TG*, thioguanine; *VDS*, vindesine; *DNR*, daunorubicin; *IFO*, ifosfamide; *MTX*, methotrexate at the same regimens as explained in Fig. 3

4 h infusion rescued by 12 doses of leucovorin (15 mg/m<sup>2</sup> q 6 h) from 24 h after the start of MTX administration. Arm H was prematurely stopped during the course of the study, as the results were rather inferior to those obtained with arm M.

Remission was to be maintained with daily oral 6-thioguanine (50 mg/m<sup>2</sup>) and bi-weekly i.v. MTX (50 mg/m<sup>2</sup>) for 2 years (groups A, B) or 1 year (group C). The treatment design is shown schematically in Fig. 5.

## Results

Except for group A patients in study 83, the remission rates are in the range of 90% for all subgroups (Table 2). The results in group A patients of study 83 were characterized by both a relatively high number of patients who did not respond to therapy or died during the first 28 days of treatment (early death) due to toxic side effects of protocol E.

Comparing the event-free survival (EFS) of group A patients from both studies, there is a statistically significant difference at  $P < 0.05$  (Fig. 6). However, in both studies the median duration of second complete remission (CR) is still below 1 year for these patients.

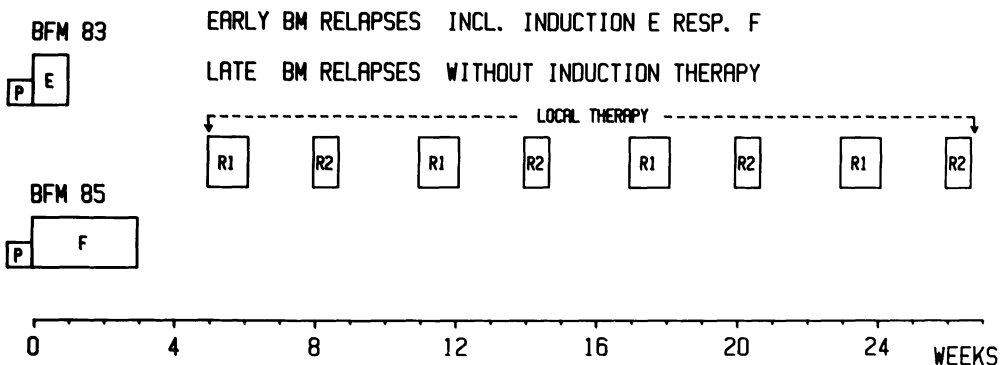
For children with isolated extramedullary relapse (group C), there is good evidence for an improved outcome in study 85 (Fig. 7), although the difference is not statistically

**Table 2.** Remission rates in subgroups of two BFM relapse studies

	N	ALL-REZ BFM: Remission rates		
		No second CR		Second CR achieved
		ED	NR	
<b>Early BM relapse</b>				
BFM 83	28	4	6	18 (64%)
BFM 85	59	1	6	52 (88%)
<b>Late BM relapse</b>				
BFM 83	30	0	2	28 (93%)
BFM 85	53	2	1	50 (94%)
<b>Other sites</b>				
BFM 83	33	2	0	31 (94%)
BFM 85	18	0	0	18 (100%)

significant ( $P = 0.08$ ). The EFS curves for children with late marrow relapse (group B) show a tendency for longer second remission duration in study 85 (Fig. 8). As shown in Table 3, however, the reasons for failure are markedly different in both studies. Whereas second bone marrow relapses terminated CR in most of the patients of study 83, about 50% of relapses in study 85 were located in the CNS, partially in combination with systemic recurrence.

Concerning the randomization of the MTX administration regimen, there was no clear difference between the remission rates



**Fig. 5.** Design of studies ALL-REZ BFM 83 and 85. P, prednisone, cytoreductive phase of 5 days. Patients with early bone marrow relapse received protocols E or F for remission induction followed by eight R-blocks. In patients with late bone marrow relapse, treatment was started with the first R1 block without separate induction therapy (eight blocks). Patients with extramedullary relapse received four R blocks in study 83 and six in study 85

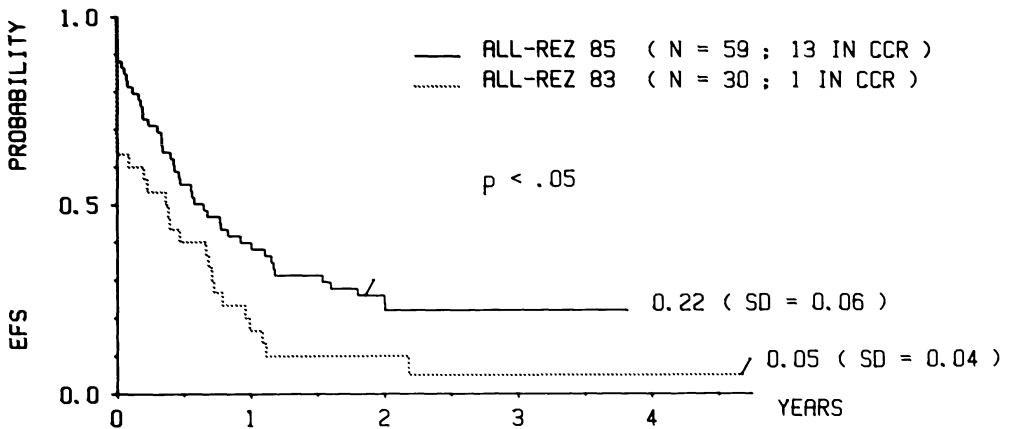


Fig. 6. Probability of EFS in children with early bone marrow relapse of studies 83 and 85. /, last follow-up

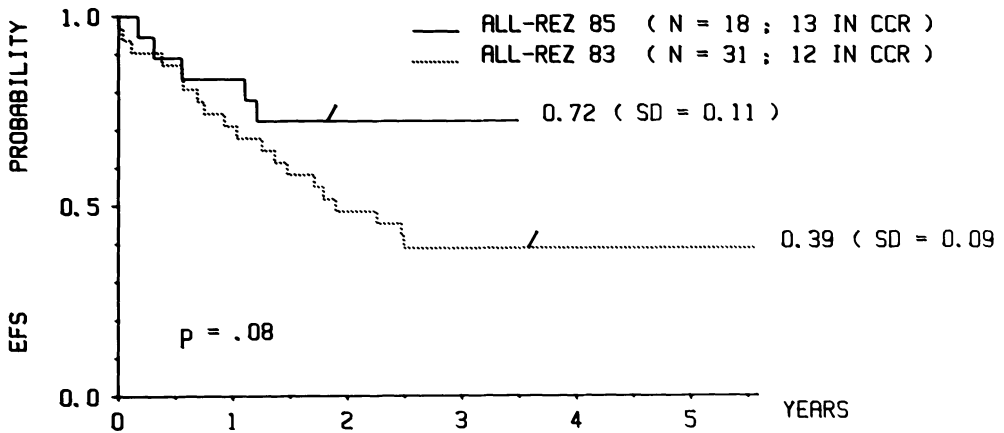


Fig. 7. Probability of EFS in children with extramedullary relapse of studies 83 and 85. /, last follow-up

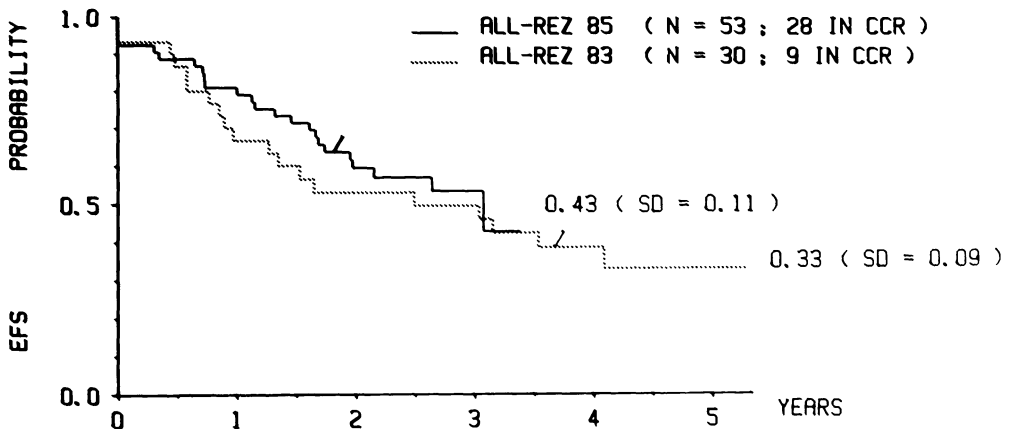


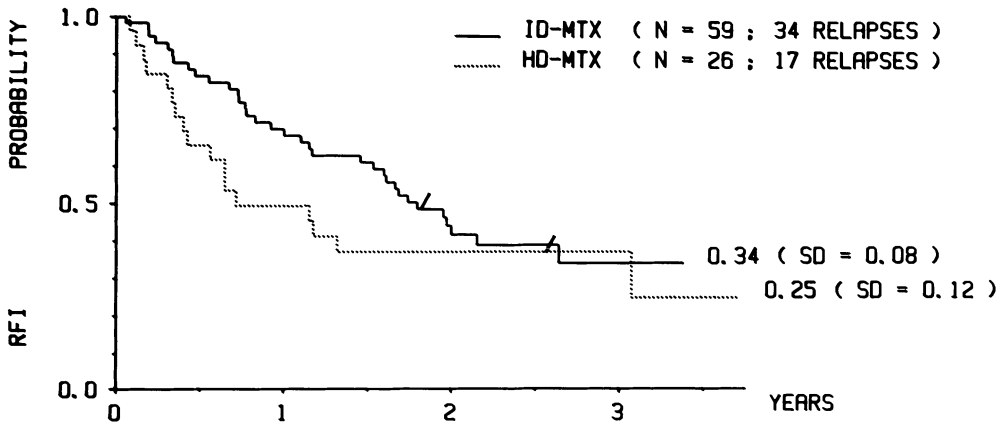
Fig. 8. Probability of EFS in children with late bone marrow relapse of studies 83 and 85. /, last follow-up

**Table 3.** Treatment results and subsequent relapse sites in children with late bone marrow relapse

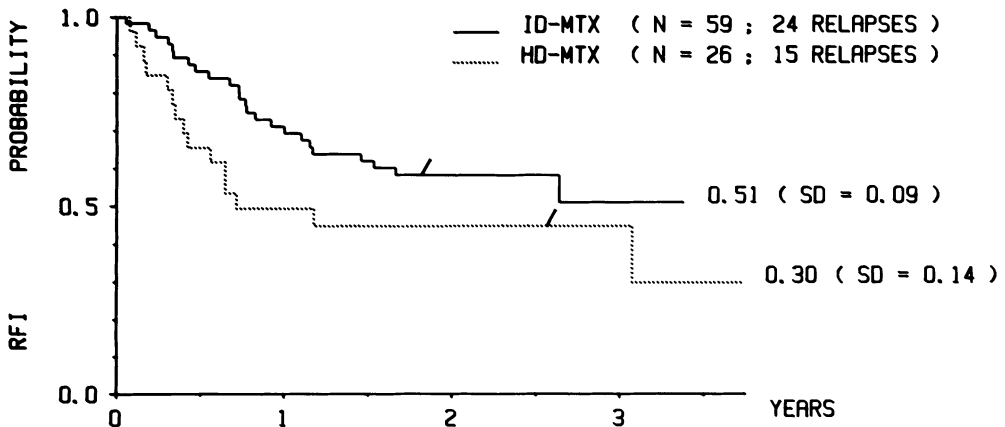
	ALL-REZ 83 <i>n</i> (%)	ALL-REZ 85 <i>n</i> (%)
Number of patients	30	53
Achieved CR	28 (93.3)	49 (92.5)
Death in CR	4	–
Relapses	13	20
Isolated BM	11	8
Isolated CNS	0	9
Isolated testes	1	0
BM/CNS	1	3

in arm H (95%) and arm M (90%). Comparing the duration of the relapse-free interval (RFI), however, patients with BM relapse of arm M experienced markedly longer remission durations (Fig. 9). This difference is even better seen in Fig. 10, which compares both treatment groups for the duration of second hematological remission.

The strongest predictors for poor outcome of relapsed children were found to be time of BM relapse (first remission duration < 18 months) and immunology, with T-cell disease being a very poor prognostic factor. The Kaplan-Meier plots for the different prognostic groups are shown in Fig. 11.



**Fig. 9.** Comparison of the duration of second complete remission between children with bone marrow relapse treated according to arms M or H in study 85. /, last follow-up



**Fig. 10.** Comparison of the duration of second hematological remission between children with bone marrow relapse treated according to arms M or H in study 85. /, last follow-up

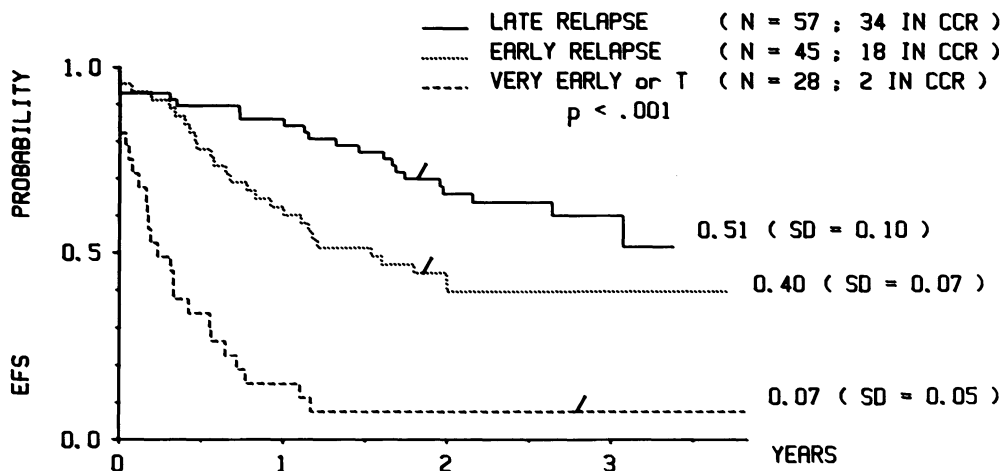


Fig. 11. Probability of EFS in three different prognostic groups defined by time of first relapse and/or immunological phenotype. *Late*, non-T-ALL relapsed beyond 6 months after the end of front-line therapy. *Early*, non-T ALL relapsed up to 6 months after the end of front-line therapy but beyond 18 months duration of first CR. *Very early or T*, relapse before 18 months duration of first CR or T-ALL relapsed at any time. /, last follow-up

## Discussion

Recently, several reports have been published addressing the question which treatment should be given to children with relapsed ALL, chemotherapy, or bone marrow transplantation (BMT) [7–9]. However, for the majority of patients chemotherapy remains the only alternative as they lack a donor for BMT. Hence, at the present time both treatment modalities are no real alternatives, because a second remission has to be obtained before BMT and the duration of second CR must be sufficiently long until a place for BMT becomes available. Thus, results reported on BMT are based on a highly selected group of patients and are not comparable to those of the multicenter BFM relapse trials, where no selection was made. Therefore, we are certainly in need of more effective chemotherapeutic approaches.

No substantial progress has been made during the past few years in the development of new drugs. But there are some new aspects concerning known drugs when used in different regimens, such as the combination of high-dose Ara-C with VP-16 or, as shown in our study, different schedules of MTX administration or the concept of the R-

blocks as elements for successful reinduction even in heavily pretreated patients.

In study 85, about 50% of children attained a second remission as soon as after one course of chemotherapy and the overall remission rate is >90%. As our results show, the problem of CNS relapse becomes evident with sufficiently long periods of second hematological remission, and preventive treatment is required to overcome CNS leukemia (R. Fengler et al., this volume). On the other hand, we can certainly conclude that we have not yet learned to use known chemotherapeutic agents in an optimal way.

Successful retreatment of children with relapsed ALL is obviously possible with chemotherapy, except for the subgroup of patients with very early relapse and/or T-cell disease. For these children, BMT in second remission is indicated if possible.

Another aspect of the obtained results concerns the implication on front-line treatment. In newly diagnosed patients, the risk of relapse can be assessed by a number of pretreatment characteristics and, accordingly, a relatively large proportion of children with high risk of relapse features will represent the study population of trials for relapsed ALL. In the face of a 90% remission

rate in the presence of clinically overt leukemia, as achieved with study ALL-REZ BFM 85, the introduction of treatment elements for relapsed ALL into front-line protocols should be considered. Possibly this would offer the chance to prevent even more relapses and thus not the need to treat them. However, even with successful retreatment for 30% of relapsed patients, cure of ALL can be achieved in 80% of affected children.

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# **Bone Marrow Transplantation**

## Marrow Grafting for Acute Leukemia: Results and Future Treatment Strategies\*

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### Introduction

Allogeneic marrow transplantation has been used increasingly to treat patients with various hematological diseases. A survey by the International Bone Marrow Transplantation Registry estimated the number of transplants carried out through the year 1987 to be in the order of 20 000 [6]. Most of these transplants (80%) have been for therapy of malignant hematological diseases. The current manuscript will review results of and future treatment strategies in marrow transplantation for acute leukemias in Seattle.

There have been remarkable advances in marrow transplantation since the early 1970s when treatment was restricted to patients with advanced leukemias, and disease-free survival was only in the order of 15% [23]. An important step was taken in the mid-1970s when marrow grafting was applied earlier in the course of the patient's disease, during the first remission in patients with acute nonlymphoblastic leukemia (ANL) and in second or subsequent remission in patients with acute lymphoblastic leukemia (ALL) [24, 25]. Patients with ANL

grafted in first remission have shown superior survival compared with those given chemotherapy (50% vs. 20% actuarial survival with the longest surviving patients at 11 years) [2]. Patients with ALL given grafts in second or subsequent remission have shown disease-free survival of approximately 35%, whereas similar patients undergoing chemotherapy have all died of recurrent disease within 3½ years of the initiation of therapy [9]. Also, patients with ANL in early first relapse, incurable by chemotherapy, have shown 5-year disease-free survivals in the order of 30%, equal to or better than seen in patients grafted in second remission of ANL [8].

Despite impressive improvements in the results of marrow transplantation for acute leukemia, major problems and complications remain [2, 8, 9, 14, 15, 23–25]. These are shown in Table 1. Depending on the stage of the disease at which the transplant is carried out, relapse of leukemia has accounted for 22%–75% of treatment failures. Significant acute graft-versus-host disease (GVHD) is seen in 30%–45% of patients, and it has a case fatality rate of approximately 50%. It is responsible for 10%–25% of treatment failures. Conditioning regimen-related toxicity and bacterial or fungal infections during the early period of pancytopenia result in 5%–10% of deaths. Fatal interstitial pneumonias often accompany acute GVHD or may be the result of drug and radiation toxicity. For results of marrow transplantation to improve, progress in each of these problem areas is needed.

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For the Seattle Bone Marrow Transplant Team of the Division of Clinical Research, Fred Hutchinson Cancer Research Center, and the Department of Medicine, University of Washington School of Medicine, Seattle, Washington, USA

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**Table 1.** Complications and survival after HLA-identical marrow transplantation

Disease	ALL				ANL		
	First CR	Second CR	Third CR	Second + Rel	First CR	Second + CR	First Rel
% Five-year disease-free survival	54	35	30	18	50	25	30
% Relapse	35	45	58	75	22	45	31
% Grades II–IV acute GVHD			35			30–45	
% Chronic GVHD			25			25–35	
% Interstitial pneumonia <sup>a</sup>			15			15–35	
% VOD			7			28	
% Bacterial + fungal infections							
During first 3 months							
Before engraftment				20			
After engraftment				12			
After first 3 months				20			
% Graft failure				<1			
% Secondary malignancies				5			

ALL, acute lymphoblastic leukemia; ANL, acute nonlymphoblastic leukemia; CR, complete remission; Rel, relapse; GVHD, graft-versus-host disease; VOD, veno-occlusive disease of the liver

<sup>a</sup> Includes both idiopathic and cytomegalovirus interstitial pneumonia

### Conditioning Regimens To Reduce Relapse

The ideal conditioning program for marrow transplantation both completely eradicates the underlying malignant disease and suppresses host immunity sufficiently to prevent rejection of allogeneic grafts without fatal toxicity. Such a regimen, unfortunately, does not exist. The most commonly used conditioning regimens in Seattle include cyclophosphamide (Cy) and 12–15.75 Gy fractionated total body irradiation (TBI) [2, 8, 9, 14, 15, 23–25]. These regimens are not sufficient to eradicate leukemia in all cases as evidenced by the data presented in Table 1. Furthermore, these regimens are accompanied by a high incidence of graft failure in patients given either T-depleted HLA-identical or non-T-depleted HLA-nonidentical marrow grafts [1, 10]. Finally, while inadequately serving their purpose, these regimens have at least a 5%–10% mortality from associated toxicities. Results of recent uncontrolled studies have suggested advantages with newer programs. The Seattle

team has attempted to combine additional chemotherapy with TBI and to optimize TBI itself. One regimen has included busulfan (6.9–8.7 mg/kg over 4 days) and Cy (49–67 mg/kg over 2 days) combined with 12 Gy fractionated TBI [12]. Disease-free survival of 40% at 18 months has been seen among 33 patients grafted for the treatment of advanced leukemia. Another regimen involved the use of high-dose cytosine arabinoside (3 g/m<sup>2</sup> every 12 h for 12 doses) combined with 12 Gy fractionated TBI with or without 60 mg/kg Cy in 29 patients with advanced leukemia [13]. Although there was considerable early toxicity with this regimen, 40% disease-free survival at 2 years has been observed. Results of these pilot studies are sufficiently encouraging to warrant formal phase III studies comparing these regimens with more established regimens for efficacy. Conventional Cy can also be combined with 16 Gy TBI when given as 200 cGy twice daily fractions or 14.4 Gy TBI when given as 120 cGy three times daily fractions (unpublished).

However, for all approaches involving systemic chemotherapy and TBI, the limits of nonhemopoietic toxicity have been reached and, barring unexpected developments, no quantum improvements can be anticipated from these approaches.

The most efficient method to eradicate leukemia would be to use agents designed to interact specifically with leukemic cells. The method approaching this ideal most closely is the use of monoclonal antibodies directed against antigens expressed on leukemic cells. It is known that monoclonal antibodies injected *in vivo* can concentrate on leukemic cells; however, the antileukemic effect is limited, in part due to the fact that some leukemic cells lack target antigens, and in part because some cells, though coated by antibody, may not be killed by it. Attempts are being made to link antibodies to toxins such as the ricin A chain for more effective tumor cell kill; however, there have been problems with this approach. Also in progress are attempts at attaching monoclonal antibodies to short-lived high-energy radioactive isotopes which deposit most of their energy within a 1- to 2-mm radius. In this fashion, leukemic cells expressing the target antigens will be killed, as will the neighboring cells, which may be antigen negative. Of course, this approach would ablate normal marrow cells, and subsequent marrow "rescue" would be needed. Initial studies in a canine model have shown appropriate antibody isotope conjugates to localize preferentially in marrow and spleen and, to a lesser extent, also in lymph nodes [3-5]. The amount of isotope in the marrow compared with that in other organs achieves ratios of 20:1 or better. The otherwise fatal marrow aplasia caused by radiolabeled antibodies can be reversed by infusion of cryopreserved autologous marrow 8 days after isotope injection, at a time when very little radioactivity is left. Combinations of chemotherapy, TBI, and radiolabeled antibodies are being explored for their ability to prepare dogs for T-cell-depleted marrow grafts. It is likely that refinements of this approach, particularly the use of high-energy beta-emitting isotopes with short linear energy transfers, will result in less toxic but more efficient conditioning programs, not only providing better elimination of leukemia but also ame-

liorating the problem of graft failure in patients with T-cell-depleted or HLA-nonidentical marrow grafts.

### **Prevention of GVHD**

A critical issue is the prevention of GVHD without simultaneously increasing the risk of graft failure and of recurrent leukemia. GVHD prevention has customarily involved postgrafting immunosuppression. In many patients, immunosuppressive drugs can be discontinued 3-6 months after transplantation when a stable state of graft-host tolerance has been reached. Omission of immunosuppressive drugs in patients given unmanipulated marrow has caused a high incidence of acute GVHD and transplantation-related death [22]. Controlled randomized trials have shown the drugs methotrexate and cyclosporine to be equivalent with regard to their ability to prevent acute GVHD [18]. A combination of methotrexate and cyclosporine is significantly better than either drug alone in preventing acute GVHD [17]. However, a survival advantage with the drug combination has only been achieved in patients transplanted for aplastic anemia [16], preleukemia, or chronic myelocytic leukemia [19], while in patients transplanted for ANL in first remission [19] the advantages achieved by the reduction in acute GVHD incidence have been offset by a higher relapse rate. Even with this effective regimen, chronic GVHD continues to be seen. Another way to reduce the incidence of acute GVHD has been to remove T cells from the marrow by immunological or mechanical means. T-cell depletion techniques result in a reduction in the number of infused T cells by 1-3 logs. In this way, most differentiated lymphocytes causing GVHD would be eliminated, and the immune system returned to an early prenatal state. New stem cell-derived T cells would accept the host antigenic environment as "self" and become tolerant to it. Nearly all clinical studies to date have shown a significant reduction in acute GVHD in recipients of T-depleted marrow grafts, providing convincing evidence for a favorable effect of T-cell depletion on GVHD [7, 10]. However, the new reduction in acute GVHD was achieved at

the price of substantial increases in graft rejection and leukemic relapse.

Given that graft rejection and leukemic relapse almost uniformly lead to death, an improvement in survival has not been realized by the use of T-cell-depleted marrow grafts. Nevertheless, the significant decrease in the incidence of acute GVHD suggests that the technique of T-cell depletion is promising provided that the risks of graft rejection and relapse can be lessened. One way of achieving this aim would include improvement of pretransplant conditioning programs which better eradicate immune cells of host type as well as malignant cells. As discussed above, this aim may be achievable through better use of currently available chemoradiation therapy and through innovative approaches using antibody isotope conjugates in addition to chemoradiation therapy.

### Graft-Versus-Leukemia Effect

Animal studies and retrospective analyses of clinical trials have strongly suggested that some of the apparent cures seen after marrow transplantation for leukemia may have been the result of a "graft-versus-leukemia effect" directed at histocompatibility antigens and perhaps also leukemia-associated antigens present on the patient's leukemic cells [reviewed in 21, 26, 27]. As already discussed above, patients given T-cell-depleted marrow grafts experience less GVHD but also have a higher incidence of leukemic recurrence than those given unmanipulated marrow. Similarly, at least among patients grafted for ANL in first remission, the use of methotrexate/cyclosporine has resulted in a reduction in acute GVHD which has been accompanied by an increase in subsequent leukemic relapses. To evaluate a possible adoptive immunotherapy component of allogeneic marrow transplantation, we conducted a randomized trial of manipulating GVHD prophylaxis in patients with advanced hematological malignancies [20]. We compared results in patients given either standard methotrexate, an abbreviated course of methotrexate, or standard methotrexate plus viable donor buffy coat cells in addition to the marrow inoculum. Both pa-

tients with abbreviated methotrexate or additional donor buffy coat cells had significant increases in the incidence of moderately severe to severe GVHD, but the rates of recurrent leukemia did not differ significantly among the groups. Five-year actuarial disease-free survival was 41% for patients given standard methotrexate, 30% for patients given short methotrexate, and 24% for patients given additional buffy coat cells. We concluded that abbreviating methotrexate prophylaxis or adding donor buffy coat cells increased acute GVHD and nonrelapse mortality without altering recurrent leukemia. Thus, these manipulations did not appear to augment the graft-versus-leukemia component of allogeneic marrow transplantation.

### Prevention or Treatment of Interstitial Pneumonia

Among the most serious complications during the first 3–4 months after marrow grafting are interstitial pneumonias (reviewed in [11]). *Pneumocystis carinii* infection, formerly the cause of about 10% of all interstitial pneumonias, is now being prevented by prophylactic trimethoprim sulfamethoxazole. By far the most serious infection is cytomegalovirus (CMV) infection. While often asymptomatic and manifested only by viral excretion in the urine or by increasing antibody titers, CMV activation can develop into pneumonia. CMV pneumonia has a case fatality rate of approximately 85%. Patients who are seronegative before transplant can be protected from infection by the use of CMV-negative blood products after transplant. Immunoprophylaxis using CMV immunoglobulin has been controversial. Interferon, acyclovir, adenine arabinoside, and an acyclovir derivative, dihydroxymethyl-ethoxymethylguanine (DHPG), are not effective in treating CMV pneumonias. However, the use of DHPG has significantly reduced the amount of virus in the lung tissues. DHPG may be beneficial when given along with CMV immunoglobulin in treating established CMV pneumonia. Idiopathic interstitial pneumonia was seen in approximately 13% of patients given single-dose

TBI, but the incidence has declined to 3% with the use of fractionated TBI.

### Conditioning-Related Toxicity and Early Infections

Conditioning regimen-related toxicity may be reduced with the use of more directed therapy, such as the radiolabeled monoclonal antibodies described above. There is also evidence that the use of certain recombinant human hematopoietic growth factors such as G-CSF and GM-CSF are able to shorten the period of granulocytopenia after transplantation, and this may reduce the incidence of early infections and result in a modest improvement of survival, in the order of 5%.

### Conclusions

In the early 1970s, marrow transplants were restricted to patients who had advanced acute leukemia. Since then, the technique has been shown to be beneficial and even curative for patients treated earlier in the course of their disease. In younger patients, marrow grafting is the treatment of choice for any leukemia which has relapsed at least once and for ANL in first remission. For patients who have ANL in first remission, the risk of early death from transplant complications must be weighed against the benefit of long-term cure.

Despite impressive improvements in transplant results, major problems remain. These include acute and chronic GVHD, recurrence of leukemia, graft failure in patients given T-depleted or HLA-nonidentical grafts, infections associated with prolonged immunodeficiency, and toxicity resulting from the conditioning programs. Major advances are needed in the area of more effective and less toxic conditioning programs. The use of short-lived radioactive isotopes linked to monoclonal antibodies holds much promise in this regard. More effective conditioning programs are likely to reduce drastically the problems of leukemic relapse and graft failure. They may permit a broader application of T-cell depletion to prevent acute and chronic GVHD, thus ex-

tending marrow grafting to include less well matched family member donors and even unrelated donors. As combinations of immunosuppressive drugs such as methotrexate and cyclosporine have already decreased the incidence of acute GVHD, the use of recombinant hematopoietic growth factors might lessen the risk of early infections. CMV infections will remain a problem until effective antiviral drugs have been developed.

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## Bone Marrow Transplantation from Histocompatible Sibling Donors for Patients with Acute Lymphoblastic Leukemia

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Between March 1977 and September 1988, 150 patients with acute lymphoblastic leukemia (ALL) were treated with myeloablative radiochemotherapy followed by allogeneic bone marrow transplantation (BMT). Prophylaxis to prevent graft-versus-host disease consisted of methotrexate (MTX)/prednisone (PSE), cyclosporine A (CSA)/PSE, or CSA/PSE/MTX. Fifty patients were in first complete remission (CR) at the time when preparation for BMT was begun (median age, 25 years; range, 1 infant was 1.8 years and 49 adults aged 16–41 years); 49 patients were either in second or third CR (19 years; 3–48 years) and 51 patients were in relapse (22 years; 5–45 years). Median follow-up for these patients is now in excess of 4 years. Actuarial disease-free survival (DFS) rates for the three groups of BMT recipients are

61%, 45%, and 19%. Actuarial relapse rates are 14%, 41%, and 64%, respectively. These data are summarized in Table 1.

In summary, selected patients, i.e., those with poor risk factors such as high blast count, and/or chromosomal translocations, and/or delayed response to adequate induction chemotherapy, and/or those with extramedullary leukemia, and/or those older than 30 years are candidates for BMT during first CR [1]. Nevertheless, a substantial number of patients can be saved if BMT is carried out during second or third CR. Only a small percentage of patients, 19% in our series, are treated successfully after their disease has reached advanced stages. However, new preparatory regimens may allow for improved long-term, disease-free survival also for patients with advanced ALL [2, 3].

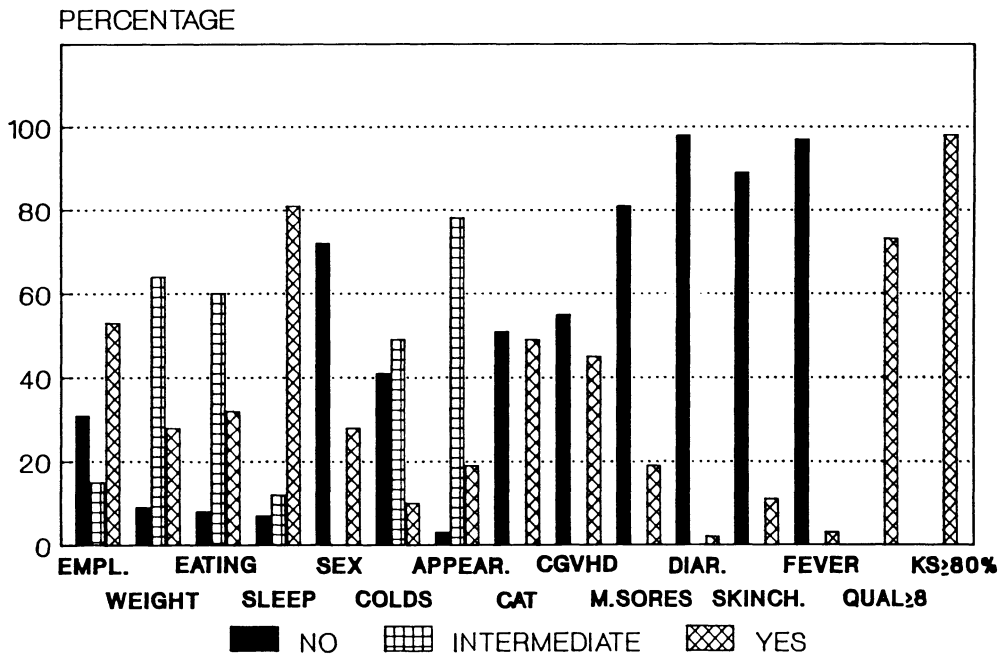
With the increasing success of clinical BMT, resulting in a substantial number of long-term survivors, the need to assess the quality of life of BMT recipients has arisen. We have analyzed 13 different aspects of

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**Table 1.** Bone marrow transplantation for acute lymphoblastic leukemia

Remission status (at BMT)	No. of patients	Median age/range (at BMT)	Actuarial DFS	Actuarial relapse rate	Median age/range of survivors (at BMT)
First CR	50	25 years (1.8; 16–41)	61% (at 9 years)	14%	23 years (1.8; 16–36 years)
Second and third CR	49 (40 and 9)	19 years (3–48)	45% (at 10 years)	41%	19 years (3–36 years)
Relapse	51	22 years (5–45)	19% (at 12 years)	64%	21 years (5–35 years)



**Fig. 1.** Quality of life analysis on the 70 surviving BMT recipients of this study in 150 patients with ALL. Median 4.2 years (range 0.3–12 years) Skinch, skinchanges; EMPL, employment

daily life using standardized questionnaires. BMT recipients are interviewed every 3 months by an experienced nursing specialist. At the end of the interview the patient is asked to rate his/her quality of life on a scale of 1–10, 10 being the best. Finally, a performance score is assigned based on the information obtained during the interview and on the information received from the local physicians who are involved in the patients' follow-up. Figure 1 illustrates the results of a recent survey of the 70 surviving patients who have been part of the BMT trial for ALL described in this publication. With a median follow-up of more than 4 years, 75% of these patients rank their quality of life as 8 or better [4].

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## Allogeneic Bone Marrow Transplantation in Childhood Leukemia: Results and Strategies in the Federal Republic of Germany

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### Introduction

The treatment of acute leukemias in childhood has been very successful in the Federal Republic of Germany. Multicenter trials for both, acute lymphoblastic leukemia (ALL) and acute nonlymphoblastic leukemia (AML), have existed for more than 10 years now and since about 1980 almost 90% of children have been treated within the two BFM trials [1, 2] or the COALL trial [3]. Intensive induction therapy has led to results which belong to the best cure rates so far achieved in these childhood malignancies. An overall disease-free survival of 70% for ALL and at least 50% for AML has been documented. For ALL, conventional chemotherapy is able to cure even a distinct part of those children where late relapses occur (>1/2 year after the end of maintenance therapy). This is not true, however, for children suffering from ALL with early relapses and those with AML for whom the outcome of therapy following a relapse is very unfavorable. These good results have been achieved in Germany earlier than in most other countries. Over many years the intensity of the German chemotherapeutic protocols was thought to be too high by many groups but during recent years the value of this aggressive therapeutic approach has been largely accepted.

Since the indication for allogeneic bone marrow transplantation (BMT) must be

seen in comparison to the results of conventional chemotherapy, the number of transplanted children is rather low in this country. Only few children have been transplanted in 1st complete remission (CR). Recently, BMT has even been included in the treatment protocols of primary disease as well as of relapses.

From the described situation, it follows that most children transplanted so far were resistant to chemotherapy or had at least relapsed once after extremely intensive chemotherapy with a high chance of relapsing again after BMT. The analysis of the results of BMT of all seven German centers aims to answer the question whether the prognosis of BMT in acute leukemias in this country is worse than that in other countries.

For chronic myelogenous leukemia the situation is different: There is no conventional treatment of this disease and BMT is the therapy of choice.

### Methods

In all centers, procedures of treatment before BMT (chemotherapy and radiotherapy) as well as prophylaxis and therapy of graft-versus-host reaction have changed several times over the years. Recently, a tendency toward more unified procedures can be noticed. All transplanted patients below the age of 18 years are included in the report. The majority of the patients reported here were isolated in laminar airflow rooms and the procedures for prophylaxis of infections

Bone Marrow Transplantation Teams at the Universities of Tübingen<sup>1</sup>, Berlin<sup>2</sup>, Essen<sup>3</sup>, Hannover<sup>4</sup>, Kiel<sup>5</sup>, München<sup>6</sup>, and Ulm<sup>7</sup>, FRG

**Table 1.** Allogeneic bone marrow transplantations in patients <18 years in Germany 1975–1988

Disease	Berlin	Essen	Hannover	Kiel	Munich	Tübingen	Ulm	Total
ALL	4	2	4	19	53	36	13	131
AML		1	3	10	16	12	7	49
CML				5	4	13	3	25
AML/ALL				1	1			2
MDS				1	2	1	1	5
NHL	1			2			2	5
Total	5	3	7	38	76	62	26	217
SAA		1		3	23	10	10	47
Neuroblastoma		1	1		2	8		12
Other solid tumors					3	2		5
Other				1				1
Total	5	5	8	42	104	82	36	282

MDS, myelodysplastic syndrome; SAA, severe aplastic anemia

were very similar. One exception was the use of cytomegalovirus (CMV) hyperimmunoglobulin which was initiated at different times. Standard procedures were used for the statistical evaluation of the results.

## Results

### General Data

In Table 1 all allogeneic BMTs performed between 1975 and 1988 in the seven participating centers are summarized. Only the patients with immunodeficiencies are omitted. One hundred and thirty-one children with ALL, 50 with AML, and 25 with CML have been transplanted and 12 children with mixed type of acute leukemia, non-Hodgkin's lymphoma, and myelodysplastic syndrome. Table 2 shows that, during the first 8 years (1975–1982), less than 10 patients were transplanted/year, whereas during the past 5 years with one exception more than 30 patients have been transplanted/year. Since 1983, 24 of the 25 patients with CML have been treated. It was also during this period that 33 of 35 transplantations took place with bone marrow from a nonidentical donor (Table 3). All patients transplanted during the second period were included in the primary intensive chemotherapy protocols. This means that patients transplanted during this period would probably have the

**Table 2.** Bone marrow transplant frequency 1975–1988

Year	N	%	Cumulative
1975	2	0.92	0.92
1976	1	0.46	1.38
1977	2	0.92	2.30
1978	3	1.38	3.69
1979	6	2.76	6.45
1980	5	2.30	8.76
1981	9	4.15	12.90
1982	9	4.15	17.05
1983	19	8.76	25.81
1984	31	14.29	40.09
1985	36	16.59	56.68
1986	36	16.59	73.27
1987	23	10.60	83.87
1988	35	16.13	100.00
Total	217	100.00	

highest incidence of relapses. Various treatment modalities before transplantation were used (Table 4). As can be seen, some of the regimens, such as those including VP16, have only been used during recent years. The same is also true for the prophylaxis of graft-versus-host disease (Table 5).

About half of the children died, a leukemic relapse occurring in about half of these children. In Table 6 the causes of death in those children who died from complica-

**Table 3.** Information about the donors used in 217 patients with leukemia or related diseases, 1975–1988

HLA status	<i>N</i>	%
Identical	138	63.59
Different	35	16.13
Syngeneic	8	3.69
NI	36	16.59
Total	217	100.00

Donor	<i>N</i>	%
Parents	19	54.29
Sibling	7	20.00
Grandparents	2	5.71
Unrelated donor	4	11.42
NI	3	8.58
Total	35	100.00

Mismatch	<i>N</i>	AG
1 Antigen	13	4 A 4 B 2 DR 3 NI
2 Antigens	10	6 A, B 1 A, DR 3 NI
Haploidentical	6	
Phenotypical identical	5	
NI	1	
Total	35	

NI, no information

**Table 4.** Pretreatment of BMT for leukemias and related diseases

Conditioning regimen	1975–1988		1983–1988	
	<i>N</i>	%	<i>N</i>	%
CYC+TBI	123	56.68	107	59.44
VP16+TBI	36	16.59	36	20.00
BAC+TBI	21	9.68	1	0.56
CYC	14	6.45	14	7.78
CYC+BC+TBI	8	3.69	8	4.44
BU+CYC	5	2.30	5	2.78
VP16+BU	4	1.84	4	2.22
Other	6	2.76	5	2.78
Total	217	100.00	180	100.00

CYC, cyclophosphamide; BAC, BCNU, Ara-C, cyclophosphamide; BC, buffy coat; BU, busulfan; TBI, total body irradiation

**Table 5.** Graft-versus-host disease prophylaxis used in BMT for leukemias and related diseases

GvHD-prophylaxis	1975–1988		1983–1988	
	<i>N</i>	%	<i>N</i>	%
CSA, MTX	47	21.66	47	26.11
CSA	40	18.43	40	22.22
MTX	32	14.75	22	12.22
MTX, ATG	32	14.75	11	6.11
T-cell depletion	27	12.44	27	15.00
CSA, PRED	12	5.53	12	6.67
No prophylaxis	9	4.15	5	2.78
CSA, ATG	9	4.15	9	5.00
CSA, MTX, PRED	7	3.23	7	3.89
MTX, PRED	1	0.46		
No information	1	0.46		
Total	217	100.00	180	100.00

ATG, antithymocyte globulin; CSA, cyclosporin A; MTX, methotrexate; PRED, prednisolone

**Table 6.** Causes of death in the 56 children who died of complications of BMT (total death rate, 119/217)

Complications	1975–1988		1983–1988	
	<i>N</i>	%	<i>N</i>	%
Acute GvHD	10	17.86	10	22.72
Take failure	9	16.07	8	18.18
Bacterial infections	9	16.07	6	13.64
IP	8	14.29	3	6.82
Fungal infections	6	10.71	5	11.36
Chronic GvHD	5	8.92	4	9.09
ARDS	2	3.57	2	4.55
Encephalopathy	2	3.57	2	4.55
HUS	2	3.57	2	4.55
CMV sepsis	1	1.79		
Liver failure	1	1.79	1	2.27
Cardiac failure	1	1.79	1	2.27
Total	56	100.00	44	100.00

ARDS, acute respiratory distress syndrome; HUS, hemolytic uremic syndrome; IP, interstitial pneumonia

tions after BMT have been summarized. It can be seen that 25.8% of the children died from complications related to BMT, which is comparable to the rate reported for other groups of patients. In only 6.7% was the death related directly to graft-versus-host disease (4.6% acute and 2.3% chronic GvHD) and only nine children (4.2% of all transplanted patients) died from interstitial pneumonia or generalized CMV infections. During the past 6 years most centers have used CMV hyperimmunoglobulins for prophylaxis and only three children transplanted during this time died from interstitial pneumonia (1.7% of 180 children). This is an extremely low rate compared with the 15%–20% incidence reported from other countries [4].

### Results of Transplantation

#### Total Group

A total of 217 children up to the age of 18 years were transplanted between 1975 and 1988. Thirty-seven were treated during the first 8 years, a time when physicians in Germany were still very reluctant about BMT. So it can be assumed that this group included mostly children with poor prognosis. In addition, lack of experience of the new

teams might have been a problem. As can be seen from Fig. 1, there is no difference in long-term disease-free survival between the group of the past 6 years (36%) and the total group (34%). Therefore, for further evaluations, we mainly used the total group for calculations. Nothing can be said yet about the influence of primary treatment since not enough information is available and the numbers are still very small. There is a distinct tendency that younger children (<10 years) survive better but the difference is still not statistically significant ( $P=0.19$ ) (data not shown). In Fig. 2 the DFS curves for all children with leukemia transplanted in the FRG are depicted.

#### Acute Lymphoblastic Leukemia

The estimated disease-free survival rate for 131 children who have been transplanted at various stages of their disease has been calculated to be 33% (Fig. 2). In Fig. 3 the DFS curves for the children transplanted at different stages of their disease are depicted. The DFS for children transplanted in second CR is 44% in contrast to those transplanted in a later remission (22%), during relapse, or as a nonresponder (19%). The difference is statistically highly significant ( $P=0.004$  and  $0.001$ , respectively). The results for children transplanted in second CR

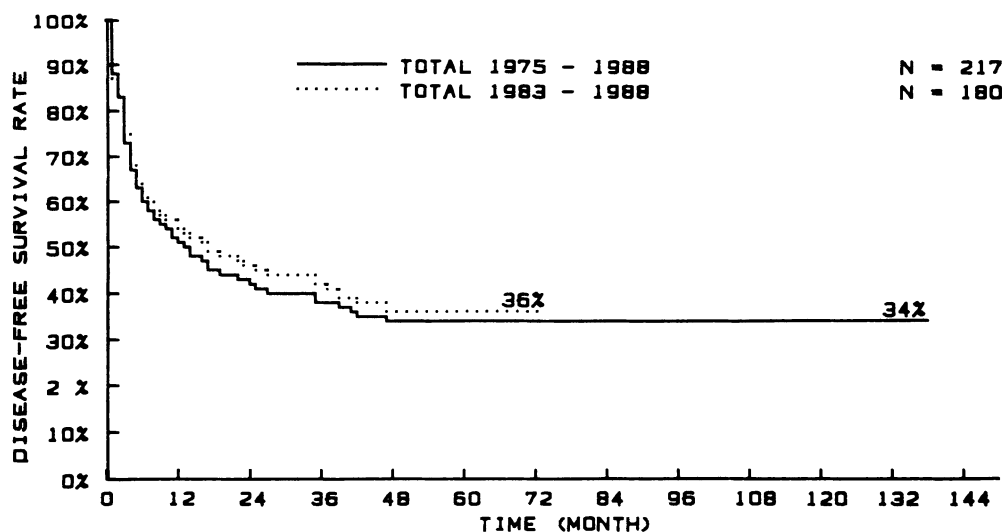


Fig. 1. Disease-free survival rate of all children given transplants during the two periods 1975–1988 and 1983–1988

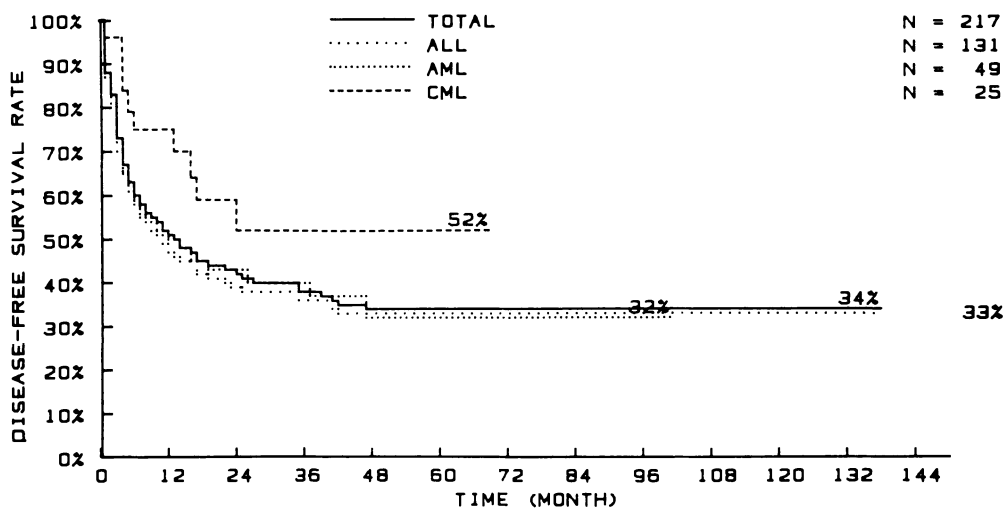


Fig. 2. Disease-free survival rate of all children with leukemia given transplants in the FRG between 1975 and 1988

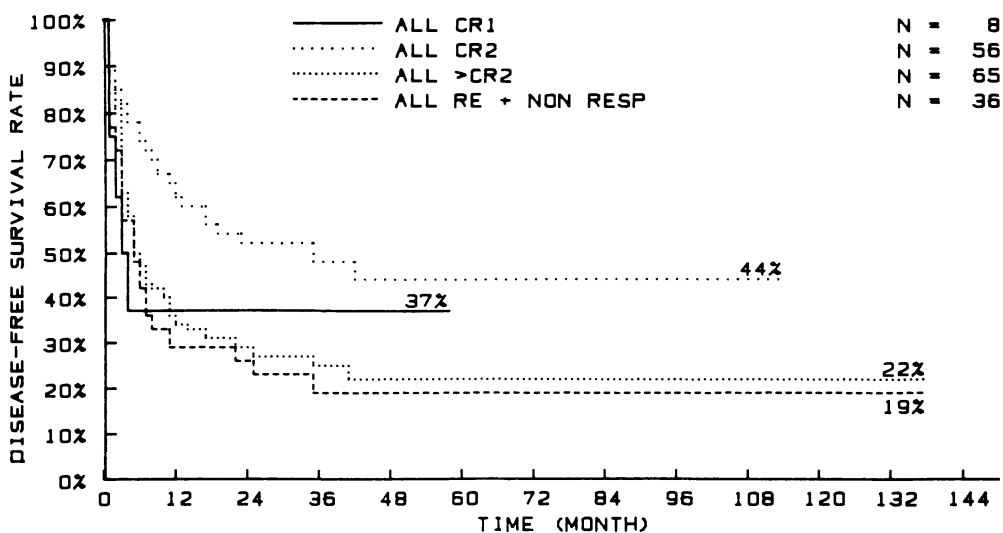


Fig. 3. Results of BMT in 129 children with ALL. The group of patients who were in relapse (RE) or not responding to chemotherapy at the time of BMT are included in the group, which is defined to be >CR2

are even better when in this group the patients without an HLA-identical sibling are excluded (DFS for ALL, second CR, identical sibling donor is 51%).

The chance of relapse in children after BMT for ALL is about 50% (EFI, 49%) (Fig. 4). The results of BMT in those patients transplanted with marrow of a non-identical donor (Table 3) are worse than in

those using an HLA-identical sibling (18 nonidentical donors, DFS=19%; 89 identical donors, DFS, 31%;  $P=0.063$ ). The numbers are too small to decide whether there is a difference between a one- or two-antigen mismatch.

In ALL there is a distinct age influence; children below the age of 10 years have a DFS of 56% compared with those older

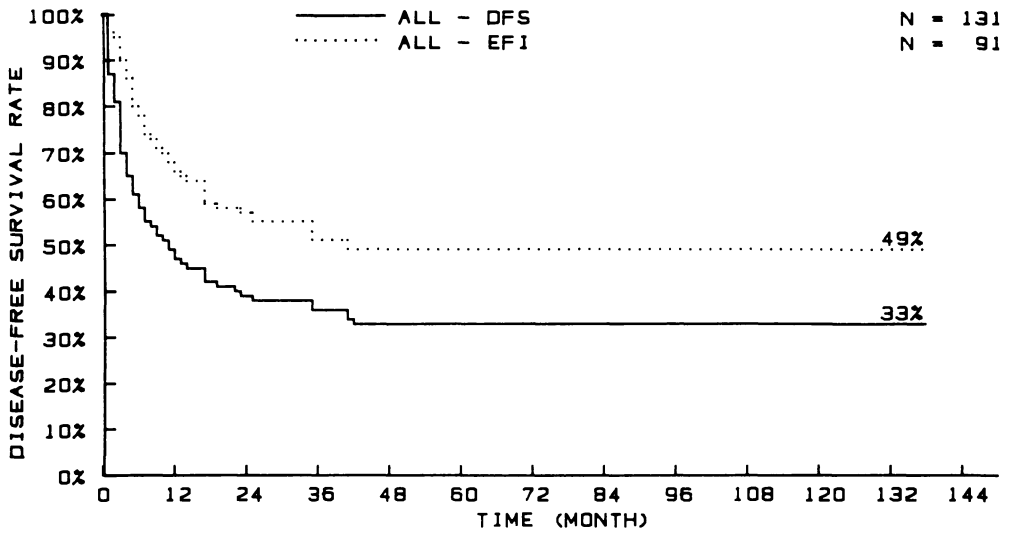


Fig. 4. Disease-free survival and EFI of 131 children transplanted for ALL

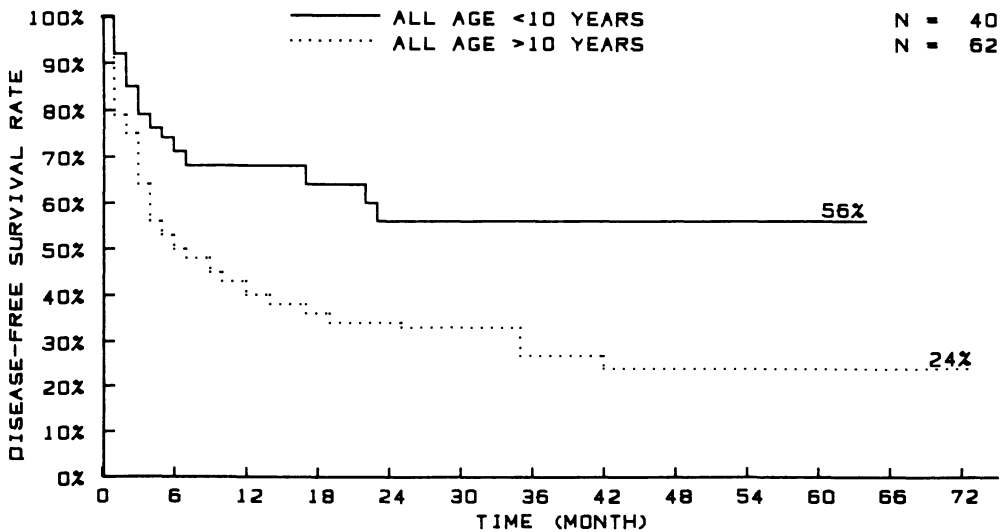


Fig. 5. Difference of DFS of children with ALL given transplants below or above the age of 10 years

than 10 years ( $P=0.016$ ) (Fig. 5). In addition, the preconditioning regimen containing VP16 and TBI seems to be more effective in preventing relapse (Fig. 6). But it has to be said that the numbers of patients transplanted with this regimen are still rather small ( $n=19$ ).

#### Acute Nonlymphoblastic Leukemia

In Fig. 7 the DFS of 49 patients with AML is depicted (32%) and compared with the EFI of 36 patients for whom the exact results are available. The DFS in 29 children who received marrow from an HLA-

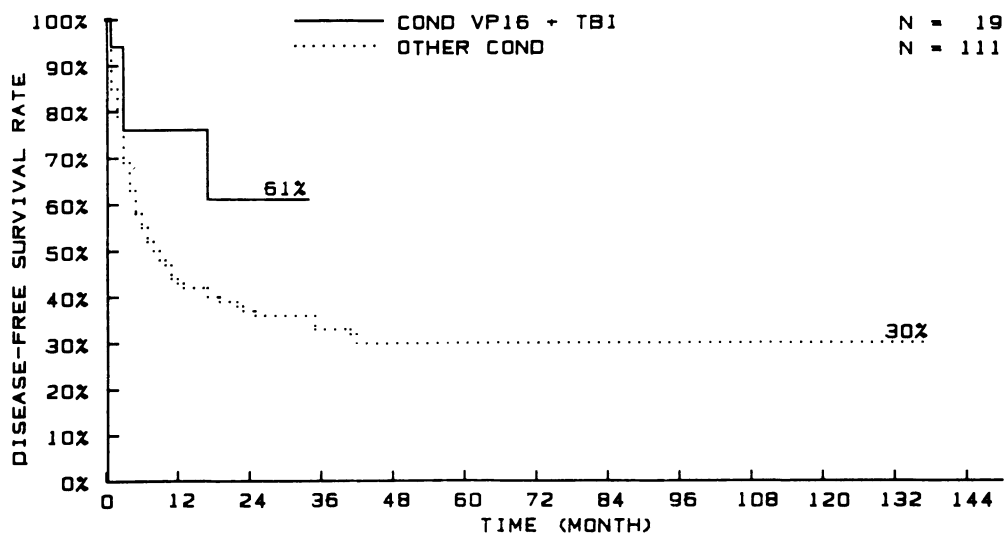


Fig. 6. Disease-free survival of children with ALL comparing the conditioning regimen VP16+TBI with all other regimens

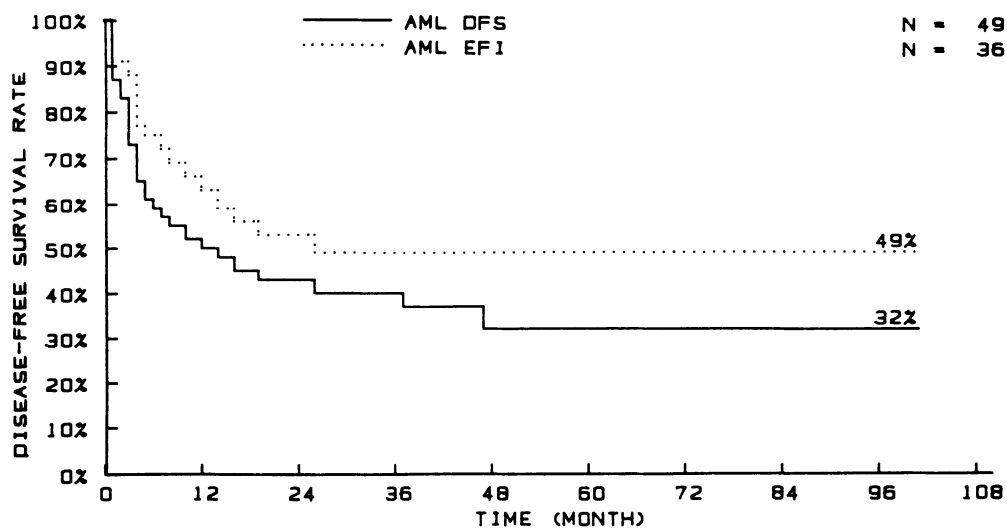


Fig. 7. Disease-free survival and EFI of 49 children given transplants because of AML

matched sibling is 36%. About 50% of the children relapsed again, a number which is identical to that in children transplanted for ALL. Even though the number is rather small, there is a distinct difference between those transplanted in first CR (DFS = 48%), those transplanted in second CR (DFS = 28%,  $P=0.046$ ), and those transplanted

during a later remission, relapse, or as non-responder to chemotherapy (DFS = 20%) (Fig. 8).

#### Chronic Myelogenous Leukemia

In Figs. 9 and 10 the results of BMT in children with CML are summarized. Only the

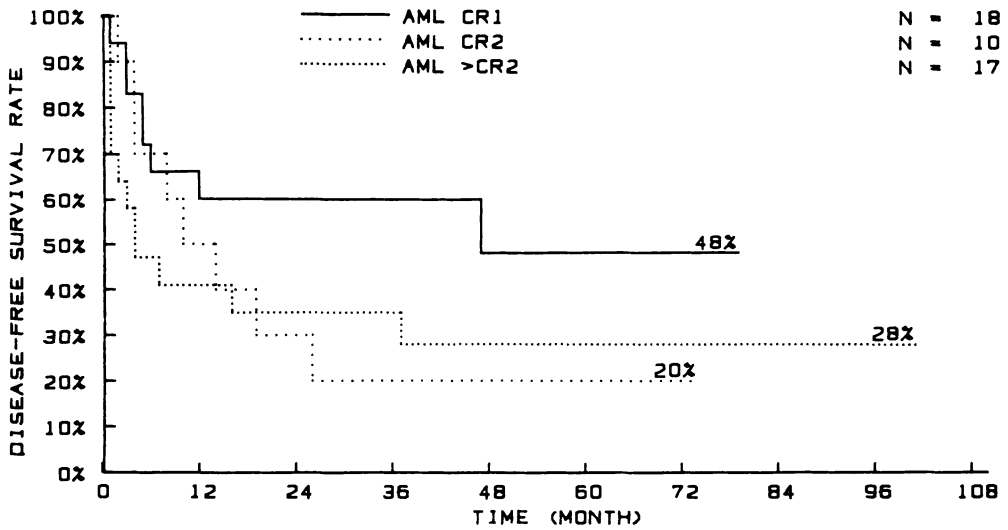


Fig. 8. Disease-free survival of 45 children with AML depending on the stage of the disease at the time of BMT

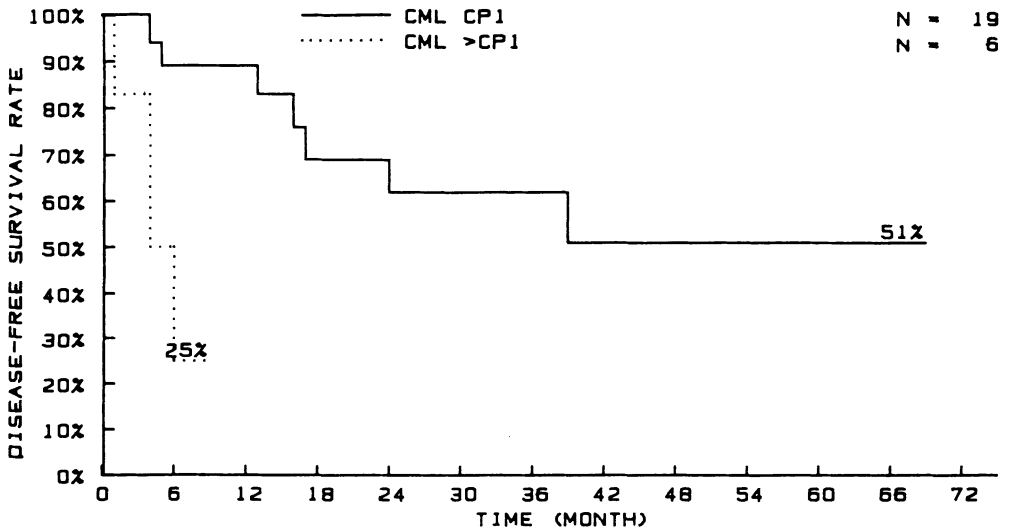


Fig. 9. Results of BMT in 25 children with CML in first chronic phase and at a later stage of their disease

data of 25 children could be analyzed. The DFS is 51% for those children transplanted in their first chronic phase but only 25% for those children transplanted at a later stage of their disease (only six children are in this group). No difference of DFS – as yet –

could be shown for the children when looking at the donor situation. Independent of the fact of whether the donor is HLA identical or not, the DFS is about 50%. But the numbers again are very small.



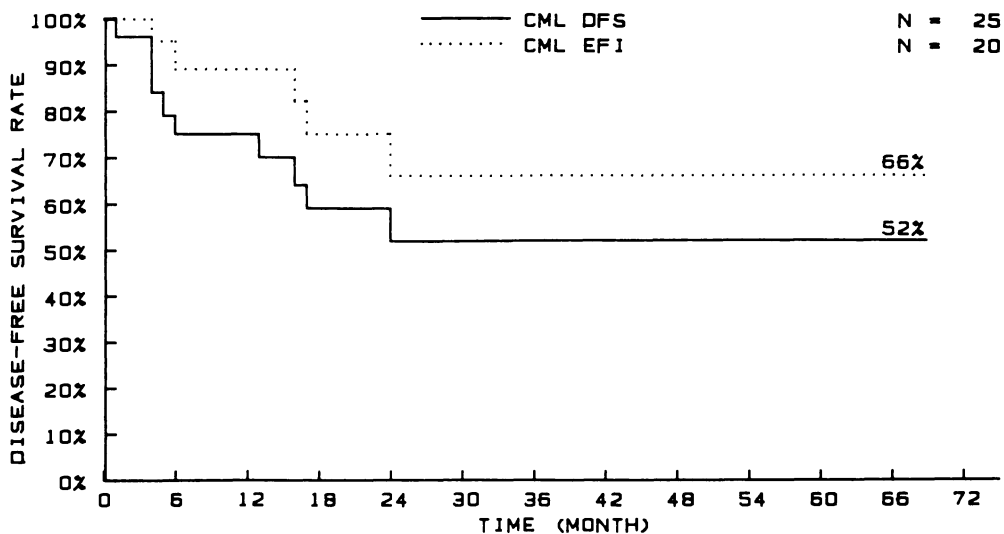


Fig. 10. Disease-free survival and EFI in 25 children transplanted because of CML

### T-Cell Depletion

Only a small number of patients ( $n=20$ ) received a T-cell-depleted marrow. The evaluation of outcome in 11 patients with ALL and 5 patients with AML does not show a significant difference. In the group of children with ALL, the rate of death during the 1st year after transplantation is high compared with the group transplanted with undepleted marrow, which suggests a higher incidence of engraftment problems.

### Discussion

The treatment of acute leukemias in children has a very high standard in the Federal Republic of Germany. Ninety percent of all children with acute leukemias are treated within multicenter trials [1–3]. The disease-free survival of all children with ALL is 70% and even for the group with high risk 50% [1]. For AML the DFS rate for all patients is about 60% (80% for the low-risk and 40% for the high-risk group) [2]. During recent years, even the relapse therapy in ALL has been standardized and this has led to good results for children with a late relapse [4]. Similar results have been reported

by others [5]. All this has to be considered in the discussion of the question of whether a child should receive a BMT. Since 1975, when the first report of BMT in acute leukemia appeared [6], transplantations have been performed in Germany. Until 1982 the number of transplanted patients was, however, very low and it is only since that time that more than 30 allogeneic bone marrow transplantations have been performed every year. The problems of graft-versus-host-disease are similar in all centers. The prophylactic measures have changed over the years and are very heterogeneous. Cyclosporin A is used with increasing tendency (120/175 transplantations performed between 1983 and 1988). T-cell depletion has been used mainly by two centers. A reduction of GvHD and an increase in rejections and relapses has been seen in these centers as reported by others [7]. Cytomegalovirus infections are a severe problem in BMT and the incidence is reported to be between 5% and 35% [8, 9]. Among the various complications in Seattle over the period of 5 years, the incidence of CMV pneumonia was 16%. In most German centers CMV hyperimmunoglobulins are used extensively and a very low incidence of pneumonia has been reported from one center

[10]. In the total group only 3 out of 180 patients died from CMV-related complications between 1983 and 1988.

Our results of BMT in ALL confirm many already reported facts. In second CR the DFS of the total group ( $n = 56$ ) is 44% at a follow-up time of 70 months (51% for those who had an identical sibling donor,  $n = 32$ ). The center in Seattle reports for children a similar DFS for ALL in second CR (40% at 4 years) [11], whereas from New York a DFS of 64% at 61 months is reported [12]. The important point for us is that our results are comparable in spite of the fact that in most cases the front-line therapy was very intensive. The results of BMT in ALL at a later stage of the disease are not satisfying (DFS = 17%). Comparable to other data the incidence of relapses was high [13]; the EFI for children with ALL after successful BMT was 54% at 72 months. Better results might be achieved with a regimen described by Blume et al. (this volume) using VP16 and fractionated TBI. In our small series the DFS was 61% at 30 months. Significantly better is the rate of DFS for children below 10 years of age compared with the older children ( $P = 0.016$ ). Our experience of BMT in first remission is small. For nine patients with an initial blast-count of more

than 90 000/ $\mu$ l and/or a null-ALL the DFS is 89% at 100 months. This is better than the results of conventional chemotherapy. For children relapsing during maintenance therapy – especially during the 1st year – there seems to be no survival chance without BMT (see Henze et al., this volume). Our present strategy for BMT in children with ALL is summarized in Table 7. For BMT in first CR it has yet to be shown that results with BMT are better than those achieved with conventional chemotherapy.

Bone marrow transplantation in AML is performed worldwide frequently in first CR [12, 14–16]. DFS rates of 50%–60% are reported, which are comparable to our results with chemotherapy [2]. In our small group of BMT in first CR the results are not as good but most of them were high-risk cases. The group in Minneapolis reports a DFS rate of 26% for their high-risk patients after BMT [17]. This could support the idea that the risk factors are identical for chemotherapy and BMT. We still have to show whether this is true within the frame of our trials. Following BMT in second CR, the results are not as good as reported by others [16]. In our small group the relapse rate was 50%, it being the main single cause of death. This is higher than in other reports

**Table 7.** Indication for BMT in children with acute leukemias at the time of the meeting within the BFM trials

ALL	First CR:	Steroid poor response (>1000 blasts/ $\mu$ l at day 8) Nonresponse (no CR at the end of protocol I) Late response (no CR after first half of protocol I; CR at the end of protocol I) AUL (cALLA negative, TdT negative) Certain translocations (e.g., t(4:11))
	Second CR:	Early relapse (6 months after end of maintenance therapy) Late bone marrow relapses
	>Second CR	All (Relapse: following two unsuccessful treatment courses)
AML	First CR:	High-risk group (DFS <40%) FAB M1 Auer negative FAB M2 leukocytes >20 000/ $\mu$ l FAB M4 eosinophils in bone marrow <3% FAB M5 all FAB M6 ? FAB M7 all Myelodysplastic syndromes
	Second CR:	All

cALLA, common acute lymphoblastic leukemia antigen; TdT, terminal deoxynucleotidyltransferase

and might be due to the very intensive front-line therapy.

In Table 7 our present strategy for BMT in AML is summarized. Again we have to prove that this strategy is of value for the children. The results of BMT in acute leukemias in this country enable us now to decide for most patients whether they will benefit by this therapeutic strategy. TBI is something which should be avoided in a growing organism. But BMT is, at the present time, the only chance for a distinct group of children. The fact that 90% of the children are treated within multicenter trials which include BMT means that most children in this country will get the best therapy at present available.

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## Allogeneic and Autologous Bone Marrow Transplantation in Acute Leukemia: The Essen Experience \*

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Chemo-/radiotherapy followed by bone marrow transplantation from sibling donors has been used successfully in many centers to treat patients with leukemia in whom primary chemotherapy failed or who were at high risk for relapses after therapy. However, only 30%–40% of patients have a matched allogeneic marrow donor. Therefore several groups initiated programs of autologous bone marrow transplantation with or without attempts to remove residual leukemic cells from the transplant by *in vitro* purging methods.

From December 1975 to February 1989, 312 bone marrow transplantations were performed at Essen University. We report on 128 patients with acute leukemia who were treated by bone marrow transplants from HLA/MLC-identical sibling donors. In addition, our experience with 25 autologous transplantations in first remission of acute myeloblastic leukemia (AML) will be discussed.

### Patients and Methods

Between December 1975 and February 1989, 94 patients with AML and 34 patients with acute lymphoblastic leukemia (ALL) received an allogeneic bone marrow graft

from HLA/MLC-identical siblings. In AML patients (49 females, 45 males) the median age was 29 years (14–49 years). The median interval from diagnosis to bone marrow transplantation was 7 months (1–21 months) in first remission, 19.5 months (6–95 months) in second remission, and 9.5 months (4–30) in first relapse or after second remission.

In ALL (14 females, 20 males) the median age was 23 years (7–44 years). The median interval from diagnosis to bone marrow transplantation was 7 months (1–16) in first remission, 28.5 months (7–59 months) in second remission, and 26 months (5–75 months) in first relapse or after second remission.

Twenty-five patients (12 females, 13 males) with AML in first complete remission (CR) were treated with cryopreserved autologous marrow. The median age was 39 years (16–53 years). The median interval from diagnosis to remission was 40 days (16–157 days), 6 months (4–19 months) from remission to transplantation, and 13 days (9–51 days), from marrow harvest to transplantation.

The cryopreservation technique used has been described elsewhere [1]. No attempts were made to remove by *ex vivo* separation techniques residual malignant cells in the autologous or T cells in the allogeneic setting. The conditioning regimen before allogeneic bone marrow transplantation consisted of total body irradiation (TBI) and high-dose cyclophosphamide (120 mg/kg administered over two consecutive

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days). Four different TBI schedules were employed over the period covered by this analysis:

1. 8.6 Gy single-dose TBI delivered from a linear accelerator ( $n=35$ ) (dose rate 12 cGy/min)
2. 8.6 Gy single-dose TBI delivered from a cobalt-60 source (dose rate 18 cGy/min) ( $n=6$ )
3.  $4 \times 2.5$  Gy fractionated TBI (linear accelerator) over 4 days (dose rate 12 cGy/min) ( $n=14$ )
4.  $4 \times 2.5$  Gy fractionated TBI with lung shielding (lung dose  $4 \times 2.0$  Gy) over 4 days (cobalt-60 source, dose rate 4 cGy/min) ( $n=56$ ) [2]

Fourteen AML patients received busulfan 4 mg/kg on each of four consecutive days and cyclophosphamide 120 mg/kg administered over two consecutive days. Two AML patients and one ALL patient received over four consecutive days 200 mg/kg cyclophosphamide. Two patients with AML received etoposide 60 mg/kg in conjunction with cobalt-60-fractionated TBI. In the autologous setting all patients were pretreated with busulfan (4 mg/kg per day  $\times$  4 days) and cyclophosphamide (60 mg/kg per day  $\times$  2 days).

Strict gnotobiotic care was given to all patients using laminar airflow isolators or ultraclean barrier nursing rooms [3]. Total gastrointestinal decontamination was attempted giving non-absorbable antimicrobials and antibiotics and autoclaved food. Most patients received cytomegalovirus (CMV) hyperimmunoglobulins as well as CMV-negative blood products. For prophylaxis of acute graft-versus-host disease (GvHD) patients were either treated with methotrexate (MTX) intermittently ( $n=78$ ) [4] or given a short course of MTX in combination with cyclosporine ( $n=50$ ) [5]. Manifest GvHD was treated with corticosteroids, ATG, or a monoclonal T-cell receptor antibody [6]. Steroids, azathioprine, or cyclosporine were given as treatment of chronic GvHD.

## Results

### *Allogeneic Transplantation*

In AML patients, 30 out of 57, 10 out of 16, or 1 out of 21 survived when the transplant was grafted in first CR or second CR, or in the first relapse and after second CR. The Kaplan-Meier estimates of event-free survival were  $50\% \pm 7\%$  by 6 years in first CR, and  $42\% \pm 14\%$  by 4 years in second CR. In ALL, 8 out of 14, 4 out of 12, and 3 out of 8 survived when transplanted in first CR, second CR, or in relapse and more advanced stages of the disease. The probability of event-free survival was  $56\% \pm 13\%$  by 4 years in first CR,  $31\% \pm 14\%$  by 6 years in second CR, and  $29\% \pm 17\%$  by 4 years in advanced disease.

The Kaplan-Meier estimates of relapse probability by 5 years were  $7\% \pm 4\%$  in first CR, and  $58\% \pm 10\%$  in more advanced stages of AML or ALL. The main cause of death after allogeneic transplantation was interstitial pneumonia. Out of 128 patients, 31 (24%) died from this complication. A strong influence of the GvHD prophylaxis was demonstrated. Among those patients who received MTX intermittently for 3 months, 33% developed interstitial pneumonia. When the combination of a short course of MTX and cyclosporine was given as GvHD prophylaxis, only 12% of the patients died from interstitial pneumonia. The actuarial incidence of GvHD in patients at risk was 30%. Only 7% had acute GvHD grade III–IV. The prophylactic effect of the two different immunosuppressive regimens used did not differ in patients with acute leukemia.

### *Autologous Transplantation*

Out of 25 patients with AML grafted during first CR with autologous marrow, 21 are still alive, and 16 relapse free. The Kaplan-Meier estimates were  $81\% \pm 9\%$  for survival and  $58\% \pm 11\%$  for event-free survival by 3 years.

The probability of leukemic relapse was  $37\% \pm 11\%$  by 3 years. The median observation time of the patients surviving event free was 16 months. Only two patients died from

transplant-related complications, one by septicemia and one by fungal pneumonia.

## Discussion

In AML as well as in ALL more than half of the patients showed event-free long-term survival if allogeneic transplantation was performed during first remission. Two years posttransplant autologous transplantation during first remission also provided more than 50% event-free survival probability in AML patients. Autologous bone marrow transplantation was followed by lower early morbidity and mortality than allogeneic transplantation. The smoother peritransplantation course in the patients with autologous grafts is probably due to the absence of post-transplant immunosuppressive therapy, of GvHD, and of the lower incidence of infections. However, it should be noted that in AML the risk of leukemic relapse after autologous transplantation was significantly higher than after allogeneic transplantation. For comparison with the results of conventional chemotherapy, one has to take into account that the median remission duration before allogeneic or autologous bone marrow transplantation in our AML/first CR patients was half a year. More prospective trials and retrospective evaluations by matched-pair analyses are needed to obtain better knowledge of the curative potential of the different therapeutic measures.

Using two different immunosuppressive regimens we saw the same low risk of GvHD. MTX as well as the combination of MTX and cyclosporine protected 70% of the patients at risk. The low risk of GvHD in our patients could be due to the strong gnotobiotic care [3].

After prolonged administration of MTX, significantly more interstitial pneumonias were observed than after a short course of MTX in combination with cyclosporine.

The toxic side effects of MTX as well as the different immunomodulation by cyclosporine are possible reasons. Interstitial pneumonia often had a fulminant course, it was always lethal, and it is the main cause of death after allogeneic transplants. In only 12% of the cases cytomegalovirus was detected as the causative agent.

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## Role of Cytokines and Major Histocompatibility Complex Antigens in Graft-Versus-Host Disease: In Vitro Studies Using T-Cell Lines and Keratinocytes or Hemopoietic Targets\*

Ch. Huber and D. Niederwieser

### Introduction

Allogeneic bone marrow transplantation (BMT) for most oncologists still represents a means by which more aggressive tumor therapies can be applied. Immunologists in contrast would consider this approach a special form of immunotherapy. This view in man is mainly supported by the finding of reduced leukemic relapse rates in the context of clinical manifestation of graft-versus-host disease (GvHD) [1]. In this article, we provide further experimental evidence that allogeneic BMT across minor histocompatibility antigen barriers leads to generation of specific killer cells, which lyse host hemopoietic cells, and to the induction of enhanced production of endogenous cytokines, which are meaningful with respect to antitumor defense.

### Material and Methods

#### *Patients*

Patients transplanted with allogeneic bone marrow from their HLA-identical and mixed leukocyte culture (MLC)-negative siblings for treatment of hemopoietic malignancy were investigated. The conditioning regimen consisted of ultrahigh chemothera-

py with cyclophosphamide followed by hyperfractionated total body irradiation. Prophylactic immunosuppression consisted of cyclosporin A. Skin biopsies were obtained prior to transplantation from recipients and their donors to establish long-term keratinocyte cultures. Peripheral blood mononuclear cells (PMNCs) were also harvested and cryopreserved before transplantation. Recipient PMNCs were collected after transplantation at the time of hemopoietic reconstitution when total PMNCs exceeded 1000 cells/ $\mu$ l.

#### *Establishment of T-Cell Lines Specific for Host Tissue Cells*

IL-2-dependent lines were established from posttransplant recipient T cells restimulated with pretransplant PMNCs and supplemented with interleukin-2 (IL-2)-containing T-cell soups (Lymphokult, Biotest, Frankfurt, FRG). Cultures were established in Costar macrowells until cluster formation was visible. They were then transferred to tissue culture flasks, expanded by twice weekly restimulation with the original stimulator cell, and maintained with the above IL-2-containing T-cell soups. Maximum time for expansion was 3 weeks. Lines were characterized by phenotypic analyses using a panel of monoclonal antibodies directed against the various T-cell surface-associated structures. They were also characterized for their functional capacities to recognize in the proliferative assay or to destroy recipient pretransplant hemopoietic or keratinocyte targets. PMNCs and keratinocytes of donor

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origin served as controls. In addition, such T-cell lines were also used to study the segregation patterns of functional lysis and MHC-antigen phenotypes involved in the patient's families. MLC and cell-mediated lysis (CML) assays were performed as previously described [2].

#### *Measurement of Endogenous Cytokine Levels and Beta-2 Microglobulin*

Endogenous cytokine and beta-2 microglobulin levels were measured daily from sera during the posttransplant period using commercially available radioimmunoassays specific for interferon (IFN)-gamma (Centocor, Malvern, United States), tumor necrosis factor (TNF)-alpha, IL-2 (Medgenix, Brussel, Belgium), and beta-2 microglobulin (Pharmacia, Uppsala, Sweden).

## **Results**

#### *Establishment and Characterization of T-Cell Lines Specific for Host Tissue Cells*

T cells harvested from the peripheral blood of patients subsequent to allogeneic BMT were restimulated with the patients' own pretransplant PMNCs. In the presence of IL-2-containing T-cell supernatants, lines with unique specificity for host cells were recovered from all patients exhibiting GvHD. Such lines when tested in a CML assay specifically killed the host's pretransplant hemopoietic cells. They, however, failed to lyse posttransplant host or donor PMNCs. Lines exhibiting specificity for host pretransplant PMNCs were further tested in family studies and in a large population of HLA-typed donors.

These investigations clearly demonstrated that the recognition of the putative minor histocompatibility (HA) antigen on pretransplant hemopoietic cells is restricted by class I MHC antigens. The view of crucial involvement of self-MHC-class I antigens in the recognition of minor HA-positive hemopoietic cells was further supported by antibody-blocking studies. In these tests polyclonal or monoclonal antibodies against common specificities on class I MHC com-

pletely blocked lysis, whereas antibodies against common determinants on class II MHC failed to inhibit.

#### *Relation of Differential Reactivity of Host-Specific T-Cell Lines for Various Tissues to Different Constitutive Expression of Class I MHC Antigens*

T-cell lines described in the previous paragraph were tested against host pretransplant PMNCs and keratinocytes. Target cells were untreated or preincubated with various concentrations of recombinant IFN-alpha or IFN-gamma for 72 h. Cells were either used as targets in CML assay or were analyzed for MHC-antigen expression after staining with the appropriate monoclonal antibodies. We observed that PMNCs bearing the appropriate minor HA were readily lysed whereas keratinocytes of the same derivation were resistant. Pretreatment of resistant keratinocyte targets with rIFN-gamma increased both their class I MHC-antigen expression and susceptibility to lysis. Thus it appears that only tissues with a high constitutive expression of class I MHC antigens are susceptible to lysis by minor HA-specific and class I MHC-restricted cytotoxic T-lymphocytes.

#### *Association of Allogeneic BMT with Elevated Levels of Endogenous IFN-Gamma*

Sera of patients undergoing allogeneic BMT were daily assessed for endogenous levels of cytokines. Results indicated that endogenous IFN-gamma levels and in some cases also TNF-alpha and IL-2 levels increased subsequent to transplantation, reaching peak levels around the time of hemopoietic reconstitution, during acute GvHD or interstitial pneumonitis.

## **Concluding Remarks**

Our search for the induction by allogeneic BMT of immune mechanisms with potential significance for host-antileukemia defense produced the following results.

First, cytotoxic T-cells of donor origin with specificity for certain host tissues ex-



panded in the recipient, in particularly in those patients presenting with clinical signs of GvHD. Such T cells recognized minor HA antigens on hemopoietic targets in the context of self-MHC-class I antigens. Although lysis of normal host hemopoietic cells by such an effector mechanism does not prove its efficacy to kill host leukemia cells, it does, however, represent a likely candidate to explain the reduced incidence of leukemic relapse in GvHD patients. Secondly, we demonstrate the induction of endogenous production of IFN-gamma and other cytokines, as a consequence of allogeneic BMT. Numerous *in vitro* studies have shown the direct antitumor activity of cytokines. Such a mechanism might also operate *in vivo*.

Third, our data clearly demonstrate that cells with low constitutive expression of class I MHC antigens will not be lysed by host-specific killer cells. If clonogenic leukemic precursor cells also exhibit such features, induction of enhanced expression of class I MHC antigens by IFN-gamma

might render them susceptible to lysis by cytotoxic T cells. Such a view has also been formally demonstrated in experimental animal systems [3]. We are presently concentrating on formally establishing the significance of all these mechanisms in the control of growth of autologous leukemic cells.

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## Autologous Bone Marrow Transplantation in Acute Myeloid Leukemia in First Remission: First Dutch Prospective Study

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### Introduction

Since it has become possible to induce complete remissions in most patients with acute myeloid leukemia (AML), cytotoxic therapy has been applied during complete remission in efforts to eradicate residual neoplasm. Modern chemotherapy may produce 20%–30% survival at 3 years in adults [1–7]. In adult patients with AML in first remission between 20 and 40 years of age, HLA (human leukocyte antigen) identical bone marrow transplantation (allo-BMT) has yielded approximately 40% disease-free survival [8–10]. In clinical practice, the applicability of allo-BMT is restricted by complications of graft-versus-host disease (GvHD), stringent age criteria, and the limited availability of HLA-compatible family donors. Marrow ablative chemo- and radiotherapy for eradicating residual AML in combination with autologous bone marrow transplantation (auto-BMT) has been pursued in recent years as an alternative therapeutic modality [11–15]. Transplantation of marrow harvested during complete remission from the patient him- or herself is not associated with the typical immunobiological complications of GvHD and postgraft immunodeficiency. It can therefore be applied to adults up to approximately 60 years of age. Initial pilot

studies have indicated that auto-BMT in patients with AML in first complete remission may result in 50% continuous complete remissions [11–15]. However, prospectively controlled trials have not critically assessed the value of auto-BMT in patients with AML. Here we present the results of the Dutch prospective study that had the objective of comparing the results of auto-BMT and allo-BMT following high-dose chemotherapy and total body irradiation.

### Materials and Methods

#### Study Design

This study by the Dutch Hemato-Oncology Working Party "HOVON" was open for entry between November 1984 and June 1987. Previously untreated patients with de novo AML classified according to the criteria of the French-American-British (FAB) Committee were entered [16], when they were 15–60 years of age. One or two cycles of chemotherapy of daunomycin (3 days – 45 mg/m<sup>2</sup> per day) and cytarabine (7 days Ara-C – 200 mg/m<sup>2</sup> per day) ( $n=88$ ) or cycles of thioguanine (7 days – 100 mg/m<sup>2</sup> p.o. q12 h), cytarabine (7 days – 100 mg/m<sup>2</sup> i.v. q12 h), and daunomycin (60 mg/m<sup>2</sup> i.v. days 5, 6, and 7) ( $n=29$ ) were administered to achieve complete remission.

After complete remission one subsequent consolidation cycle of the same agents was given, though with 1 day of daunomycin only. Thereafter patients were to proceed to auto-BMT or allo-BMT. After autologous

Study by the Dutch Hemato-Oncology Group "HOVON"

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marrow collection, an intermittent course of chemotherapy (amsacrine 150 mg/m<sup>2</sup> i.v. day 1; cytarabine 3 g/m<sup>2</sup> q h i.v. on days 1 and 2) was given to allow time for qualitative evaluation of the marrow harvest. Auto-BMT candidates in continuing remission then proceeded with cyclophosphamide (60 mg/kg i.v. on days -4 and -3) and total body irradiation (8 Gy to the midline of the body with partial lung shielding to reduce the dose to the lungs to 7.0 Gy, on day -1) and reinfusion of autologous bone marrow (on day 0) unless they did not satisfy the eligibility criteria of transplantation (e.g., insufficient marrow cell collection, sensitization to platelet transfusion, refusal) [12]. No antileukemic therapy was given posttransplant.

Patients in complete remission but meeting the criteria for allo-BMT (age less than 46 years, availability of HLA-matched sibling donor) were transplanted in the individual centers according to institutional protocols. They were conditioned with cyclophosphamide 60 mg/kg on 2 days and total body irradiation ( $n=10$ ). In certain cases the preparative combination of cyclophosphamide/total body irradiation was preceded by cytarabine 1 g/m<sup>2</sup> i.v. q12 h on 2 days ( $n=9$ ) or etoposide (350 mg/m<sup>2</sup> i.v. on each of 2 days) ( $n=1$ ) [17]. One patient received busulfan (4 days 4 mg/kg orally) and cyclophosphamide (4 days 50 mg/kg i.v.) as the preparative regimen [18]. Cyclosporin was administered postgrafting as GvHD prophylaxis [17].

Allogeneic bone marrow grafts were depleted of T cells after pretreatment with complement-mediated CD3 antibody lysis ( $n=4$ ) [19], following E-rosette depletion ( $n=10$ ) [17] or soybean agglutinin E-rosette sedimentation ( $n=6$ ) [20]. One patient received a T-cell nonmodified marrow allograft.

### *Statistical Analysis*

Data were analyzed as of 1 September 1988. Median duration of follow-up of these patients was 30 months at that time, with a minimum follow-up of 15 months. Survival and relapse-free survival probabilities were calculated according to the actuarial method of Kaplan and Meier [21]. Patients who

died and those with a relapse were regarded as failures in the relapse-free survival curves. All other patients were excluded at the time of last follow-up. The log-rank test was used to compare the groups of auto-BMT allo-BMT patients with respect to (relapse-free) survival and freedom of relapse [22].

## **Results**

### *Patient Population*

One hundred and seventeen patients aged between 15 and 60 years (median age, 43 years; range, 16–60 years) were enrolled in the study, of whom 90 (i.e. 77%) attained complete remission following induction chemotherapy. Thirty-two of these complete responders (36%) received marrow ablative therapy followed by reinfusion of autologous bone marrow. In 21 other cases (23%), a genotypically HLA-matched allo-transplant was carried out, so that 59% of complete responders were transplanted.

### *Autologous and Allogeneic Bone Marrow Grafts*

The median age of the patient group submitted to auto-BMT was 40 years and the age of the allo-BMT series 30 years. The median times that elapsed between diagnosis and the day of BMT were identical for auto-BMT and allo-BMT recipients, i.e., 5.6 and 5.0 months.

### *Outcome of Auto-BMT and Allo-BMT*

Actuarial survival for all registered patients of the study is 30% at 3 years. Among the two transplant groups of complete responders AML relapse was seen in the following proportions of cases: 17/32 (auto-BMT) and 6/21 (allo-BMT). Mortality (19/32) among the auto-BMT patients was mainly due to relapse of AML ( $n=16$ ) and in three cases related to the toxicity of the procedure. The causes of death in the allotransplanted cases (7/21) were distributed over relapse of AML ( $n=3$ ) and pneumonitis/infections ( $n=4$ ). Currently, 12 auto-BMT patients, 11 allo-BMT patients, and also 3 of the 37 non-

BMT patients remain at risk and survive free of disease. Survival after allo-BMT was significantly better than after auto-BMT. Three-year overall survival is 37% after auto-BMT and 66% after allo-BMT (log-rank test,  $P=0.05$ ). The relapse-free survival rates at 3 years are 35% and 51% respectively (log-rank test  $P=0.12$ ). Because of the differences of age between auto-BMT and allo-BMT patients, the influence of age on determining the somewhat less favorable results of auto-BMT was assessed separately but age appeared not to have prognostic significance for outcome of auto-BMT.

## Discussion

Relapse-free survival after auto-BMT is approximately 50% in several retrospective studies [11–15]. A critical evaluation of this treatment modality has been hampered by the fact that auto-BMT was applied to patients in whom complete remission had previously been established and accrual of these patients was not prospectively controlled. Therefore, the relative value of auto-BMT has not yet been critically assessed. In the study presented herein 117 patients were followed from diagnosis onwards, and complete responders were to undergo auto-BMT or allo-BMT, depending on the specific eligibility criteria of either BMT modality. An important figure from the results of these investigations is that approximately 60% of all remission patients ultimately had access to either auto-BMT or allo-BMT.

Projected survival at 3 years of patients consolidated with high-dose chemotherapy and total body irradiation followed by allo-BMT was significantly better (66%) than that of auto-BMT recipients (37%,  $P=0.05$ ). The prognosis of the patients not undergoing BMT after complete remission was extremely poor. Half of this group were patients with an early relapse or early death. The other half of cases with no BMT were due to refusal or medical contraindications. The latter patients had a 3-year relapse-free survival of only 9%. This may suggest that auto-BMT has a beneficial effect on survival.

Recurrence of AML was significantly more frequent after auto-BMT (at 3 years:

60%) than after allo-BMT (at 3 years: 34%,  $P=0.03$ ) and represented the predominant cause of failure of the former treatment. Two possible origins of AML relapse following auto-BMT can be considered, i.e., AML in the host surviving the intensified pretransplant therapy or contaminating AML cells in the marrow autograft from which the disease reemerges following reinfusion. Because the probability of relapse of AML after auto-BMT is similar to that seen after syngeneic BMT [26], we believe that residual leukemia in the body probably represents the main source of AML recurrence. If this assumption is correct, it would be necessary to develop more effective means of AML cytoreduction prior to auto-BMT [28]. The best chemotherapy programs in adult patients with AML approximate the results of allo-BMT, certainly if the selection of younger patients is taken into account [32–34]. Long-term survival for 20- to 50-year-old patients is 30%–40% after allo-BMT [9, 10] and is 30% after chemotherapy [4–7]. The fact that auto-BMT apparently offers about 35% survival, establishes it as a potentially useful alternative treatment option for patients with AML.

## Summary

We have prospectively compared the values of autologous BMT (auto-BMT) and allogeneic marrow transplantation (allo-BMT) in patients (age 15–60 years) with acute myeloid leukemia (AML) who attained complete remission (CR) following remission-induction therapy. In 90/117 cases CR was reached. In 32 of those complete responders auto-BMT was undertaken and in 21 eligible cases HLA-matched allo-BMT. AML relapse was the predominant cause of failure after auto-BMT (17/32). The incidence of relapse after allo-BMT was 6/21. Patients treated with auto-BMT and allo-BMT have an overall survival of 37% and 66% at 3 years posttransplant ( $P=0.05$ ). Survival of the nongrafted complete responders is less than 10%. Allo-BMT in adult patients with AML in first complete remission provides a superior outcome when directly compared with the results of auto-BMT.

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## Autologous Bone Marrow Transplantation for Acute Leukemia in Remission: An Analysis of 1322 Cases

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This survey is the last to be conducted using computer questionnaires compiled by our department in 1980. At the time of writing, modified computer forms and the program to be used for new data have been sent to all the teams in the study. The survey aims to see whether the results of previous analysis (influence of pretransplant intervals and evidence in favor of marrow purging in AML CR1) could still be verified.

### Material and Methods

Fifty-four teams reported a total of 1322 cases (see Appendix A). Distribution according to diagnosis and status was as follows: *acute lymphocytic leukemia (ALL)*: 560; CR1 standard risk (SR), 172; high risk (HR), 78; CR2 SR, 173; HR, 41; others (CR > 2 and PR), 96. Median age of population, 15 years (1–55 years); children (<15 years), 43%; adults >45 years, 3%. Thirteen patients had a Philadelphia chromosome (CR1, 8; CR2, 4, CR3, 1). Marrow was purged in 55% of patients in CR1 SR (30% monoclonal antibodies), 79% CR1 HR and CR2 SR, 85% CR2 HR (17% monoclonal antibodies). *Acute myeloblastic leukemia (AML)*: 723; CR1 SR, 448; HR, 72; CR2 SR, 145; HR, 14; others, 44. Median age 35 years (1–65 years); children, 14%; adults >45 years, 15%. Marrow was purged

in 29% patients in CR1 SR (21% mafosfamide), 32% in CR1 HR, 42% in CR2 SR, and 64% in CR2 HR. Marrow purging was more frequently used in children than in adults (43% vs. 32%). *Others*: 39. Seventy percent of patients with ALL and 67% with AML experienced at least one complication. Liver veno-occlusive disease was the only reported complication, with a higher incidence in AML than in ALL (4.3% vs. 1.6%  $P < 0.05$ ). All studies: disease-free survival (DFS) and disease-free probability (DFP) in various subcategories, influence of French-British-American (FAB) classification, pretransplant regimens, pretransplant intervals, and marrow purging were performed following the same guidelines as for previous EBMT surveys (Bone Marrow Transplantation 1988, 3, Suppl. 1, pp 39–41; Experimental Hematology To-day 1988 in the press). The median follow-up was 30 months (1–107 months).

### Acute Myeloblastic Leukemia

1. The DFSs were (Fig. 1)  $36\% \pm 4\%$  at 7 years in patients autografted in CR1 SR,  $33\% \pm 6\%$  at 3 years in CR1 HR,  $30\% \pm 5\%$  at 4 years in CR2 SR, and  $28\% \pm 13\%$  at 7 months in CR2 HR ( $P < 0.001$ ). In CR1 SR patients DFSs in relation to the FAB classification were (Fig. 2): M1,  $54\% \pm 6\%$ ; M2,  $40\% \pm 5\%$ ; M3,  $52\% \pm 11\%$ ; M4,  $26\% \pm 7\%$ ; M5,  $30\% \pm 9\%$  ( $P = 0.03$ ) at 4 years. In the same group, DFSs in relation to pretransplant regimens were (Fig. 3): University College Hospital (London),  $61\% \pm 9\%$ ; TBI,  $42\% \pm 5\%$  at 50 months; BAVC,  $44\%$

For the EBMT  
Presented by F. Lemoine, Department of Hematology, Hôpital Saint Antoine, Paris, France

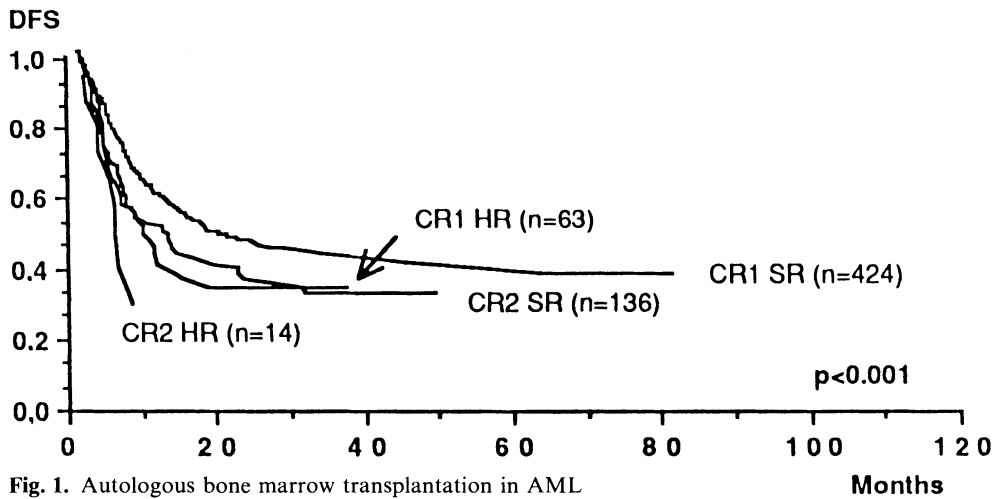


Fig. 1. Autologous bone marrow transplantation in AML

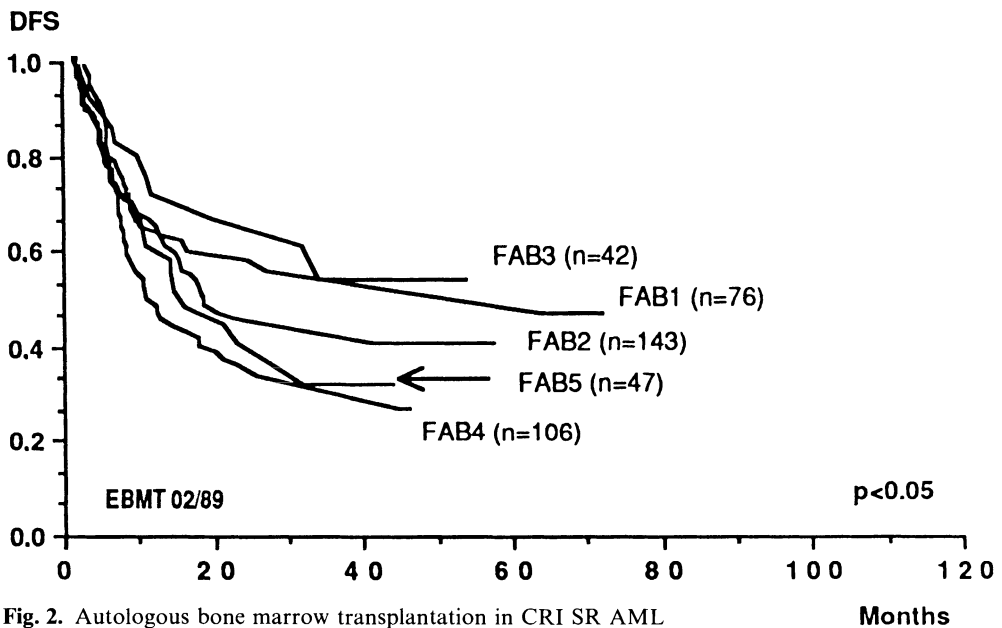


Fig. 2. Autologous bone marrow transplantation in CR1 SR AML

$\pm 10\%$  at 34 months; and busulfan + cyclophosphamide,  $43\% \pm 9\%$  at 17 months.

2. Long pretransplant intervals were associated with better DFSs in patients given transplants in CR1 (Fig. 4). With regard to the interval from remission to transplant, DFSs at 60 months were  $28\% \pm 6\%$ ,  $38\% \pm 4\%$ ;  $46\% \pm 6\%$ , and  $56\% \pm 8\%$  for inter-

vals of  $< 3$  months (92 patients), 4–6 months (232 patients), 7–9 months (96 patients), and  $> 9$  months (58 patients);  $P = 0.01$ .

3. Marrow purging with mafosfamide was associated with better DFP than no purging in patients in CR1 SR with regard to: (a) the whole population: purge ( $n = 125$ ) versus no



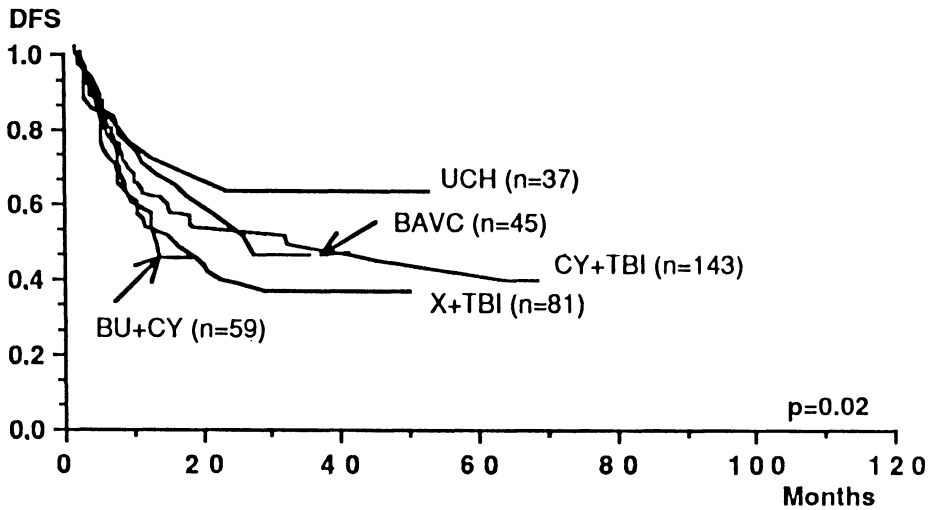


Fig. 3. Autologous bone marrow transplantation in CRI SR AML: influence of pretransplant regimen

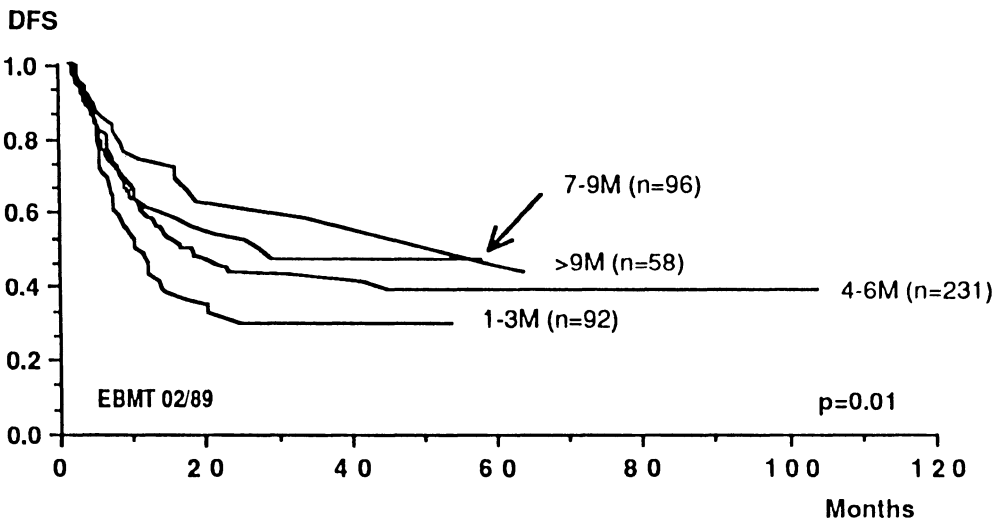


Fig. 4. Autologous bone marrow transplantation in CRI AML: influence of the interval from remission to transplant

purge ( $n = 303$ ),  $60\% \pm 5\%$  vs.  $39\% \pm 5\%$  at 5 years ( $P = 0.02$ ) (Fig. 5); (b) patients grafted before January 1988; and (c) patients grafted before January 1987: purge versus no purge,  $63\% \pm 6\%$  vs.  $37\% \pm 6\%$  at 5 years ( $P < 0.01$ ). This population was extensively studied last year (NC Gorin, P Aegerter et al., for the EBMT: Autologous BMT for acute myelocytic leukemia: decreased incidence of relapse associated with

marrow purging. Submitted for publication) and it is important to note that the previous observation on the beneficial effect of marrow purging remains valid after an additional follow-up of 1 year.

When separately considering marrow purging with mafosfamide at standard dose and purging with mafosfamide at doses individually adjusted versus no purge, DFP was better for purging with adjusted doses

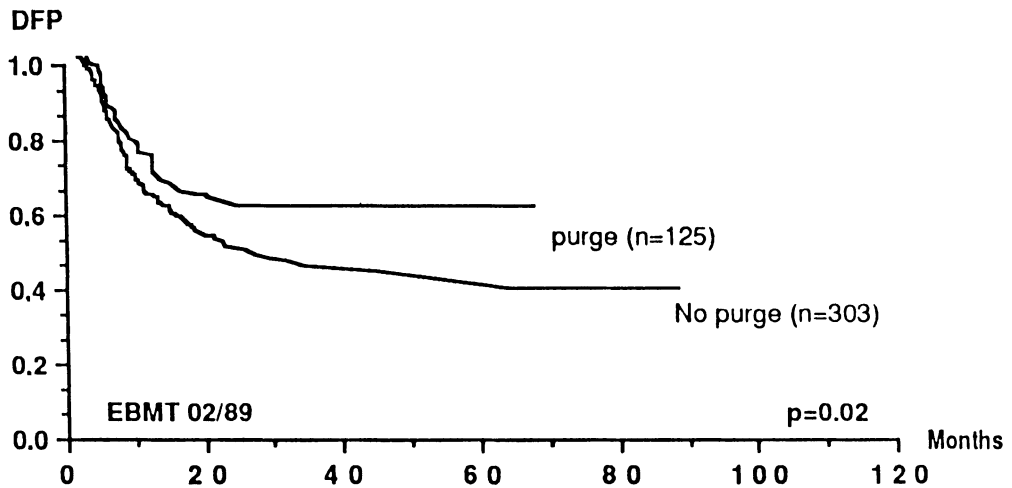


Fig. 5. Autologous bone marrow transplantation in CRI SR AML: influence of marrow purging

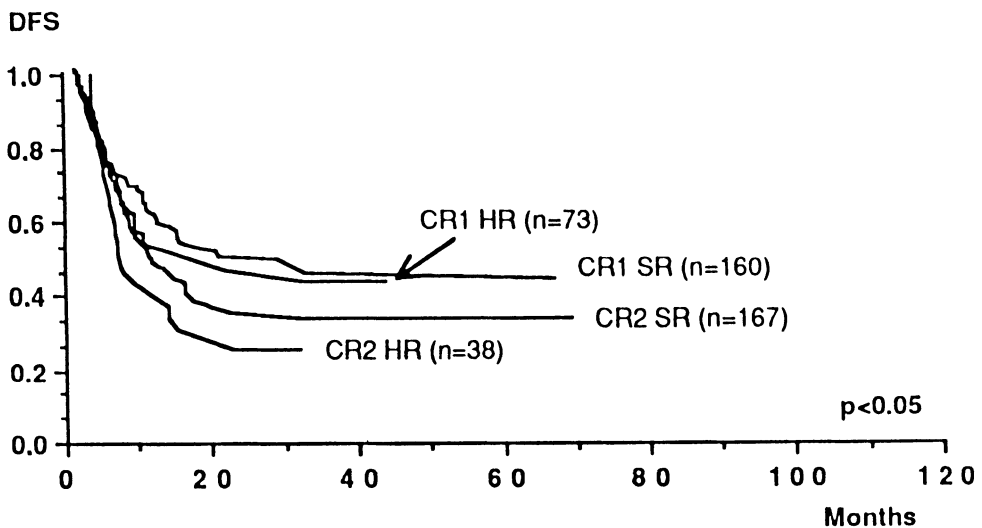


Fig. 6. Autologous bone marrow transplantation in ALL

( $n=36$ ),  $79\% \pm 8\%$ , than a standard dose ( $n=53$ ),  $47\% \pm 8\%$ , and no purge ( $n=303$ ),  $42\% \pm 4\%$  at 5 years;  $P=0.02$ .

### Acute Lymphocytic Leukemia

The DFSs for patients autografted in CR1 SR (Fig. 6) ( $n=160$ ), CR1 HR ( $n=73$ ), CR2

SR ( $n=167$ ), and CR2 HR ( $n=38$ ) were  $42\% \pm 5\%$  at 5 years,  $41\% \pm 7\%$  at 40 months,  $31\% \pm 4\%$  at 5 years, and  $23\% \pm 8\%$  at 30 months, respectively ( $P<0.05$ ). The corresponding DFPs were  $47\% \pm 5\%$ ;  $46\% \pm 7\%$ ,  $36\% \pm 5\%$ , and  $25\% \pm 8\%$  ( $P<0.01$ ). For patients autografted in CR2, survival was better in children ( $42\% \pm 6\%$  at 52 months) than in adults ( $20\% \pm 8\%$  at

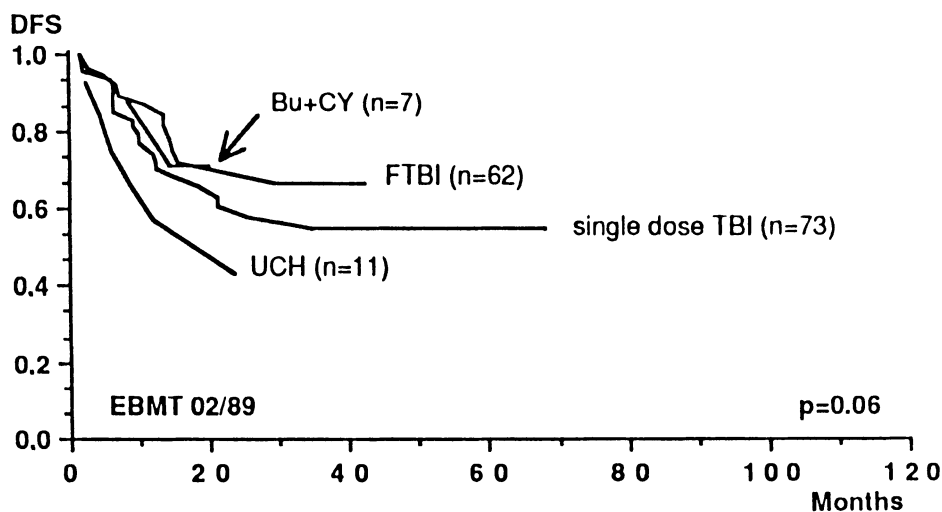


Fig. 7. Autologous bone marrow transplantation in CR1 SR ALL: influence of pretransplant regimens

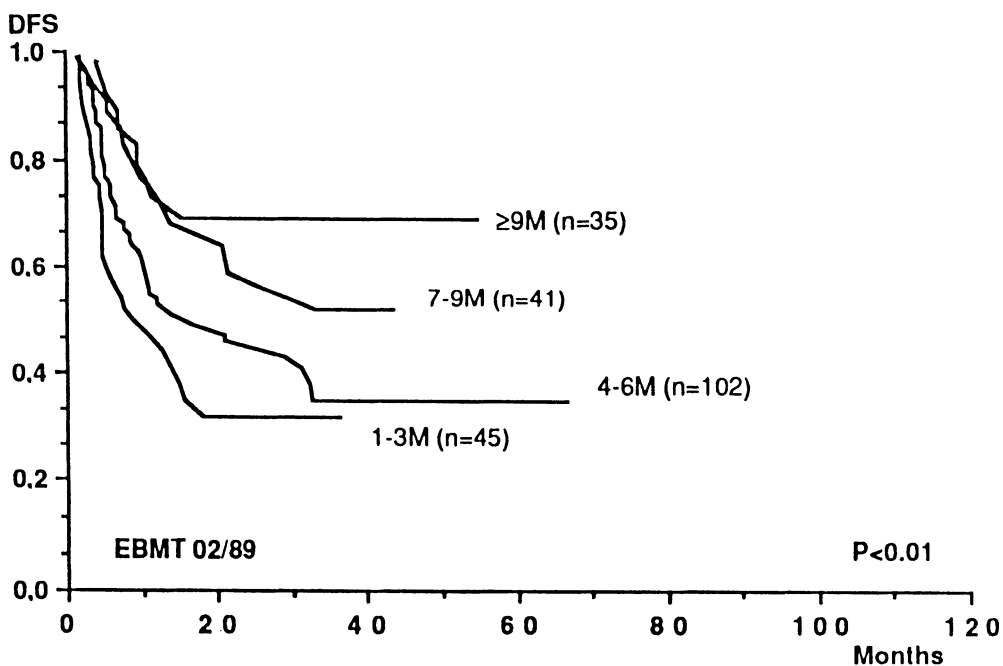


Fig. 8. Autologous bone marrow transplantation in CR1 ALL: influence of the interval from remission to transplant

34 months) ( $P < 0.05$ ). We compared the survival of CR1 SR patients receiving fractionated total body irradiation (FTBI;  $n = 62$ ) with those receiving single-dose TBI ( $n = 73$ ): the figures were  $65\% \pm 8\%$  and  $50\% \pm 7\%$  at 40 months, respectively

( $P = NS$ ) (Fig. 7). Long-term intervals from remission to transplant were associated with better DFSs (Fig. 8). In CR1 ALL, the DFSs were  $30\% \pm 7\%$  at 3 years for an interval of 1–3 months,  $34\% \pm 6\%$  for 4–6 months,  $51\% \pm 10\%$  for 7–9 months, and

**Appendix A.** Autologous bone marrow transplantation for acute leukemia (February 1989). List of institutions reporting data

Team	Coordinator	Number of patients
Rome, Italy	Meloni	117
Paris, St. Antoine, France	Gorin	109
Heidelberg, FRG	Korbling	88
Bloomsbury, UK	Goldstone	83
Besançon, France	Herve	75
Marseille, France	Maraninchi	52
Genova, Italy	Carella	49
Uppsala, Sweden	Simonsson	46
Leipzig, GDR	Helbig	39
Glasgow, Scotland	Burnett	39
Parma, Italy	Rizzoli	37
Lyon, France	Souillet	30
Tours, France	Colombat	28
Bordeaux, France	Reiffers	27
Utrecht, Holland	Verdonck	25
Royal Free Hospital, UK	Prentice	25
Milan, Italy	Polli	23
Rotterdam, Holland	Lowenberg	21
Nijmegen, Holland	De Witte	21
Pavia, Italy	Alessandrino	20
Brussels, Belgium	Ferrant	20
Pesaro, Italy	Porcellini	20
Birmingham, UK	Franklin	19
London, UK	Barret-Poynton	18
Barcelona, Spain	Brunet Mauri	17
Nantes, France	Harousseau	16
Pescara, Italy	Torlontano	16
Turin, Italy	Aglietta	15
Leiden, Holland	De Planque	14
Ulm, FRG	Wiesneth	14
Barcelona, Spain	Ortega	14
Padua, Italy	Colleselli	13
Newcastle, UK	Proctor	13
Trieste, Italy	Andolina	12
Rome, Italy	De Laurenzi	11
Hotel Dieu, France	Zittoun	11
Bern, Switzerland	Brun del Re	10
Nancy, France	Witz	10
Amsterdam, Holland	Vaan Leewen	10
Bolzano, Italy	Coser	9
Nice, France	Gratecos	8
Geneva, Switzerland	Chapuis	8
Birmingham, UK	Milligan	8
Firenze, Italy	Ferrini Rossi	8
Bologna, Italy	Visani-Tura	8
Vienna, Austria	Hinterberger	7
Innsbruck, Austria	Huber	7
La Pitie Paris, France	Leblond	6
Mondor Paris, France	Vernant	6
Rotondo, Italy	Greco	6
Lund, Sweden	Bekassay	5
St. Etienne, France	Freycon	3
Cochin, France	Belanger	2
Milan, Italy	De Cataldo	2
Beijing, China	Cao Lung	1
Gemelli, Italy	Leone	1
Total		1322

67% ± 9% at 50 months for an interval >9 months ( $P < 0.01$ ). There was a trend in favor of marrow purging with mafosfamide at adjusted levels in patients in CR1 HR: individually adjusted mafosfamide ( $n = 17$ ), DFP 75% ± 12% at 30 months; standard mafosfamide ( $n = 15$ ), 46% ± 14%; monoclonal antibodies ( $n = 20$ ), 47% ± 12% at 18 months; no purge ( $n = 22$ ), 60% ± 18% at 1 year ( $P = 0.13$ ).

## Conclusions

Results of previous surveys hold true globally:

1. CR1 SR AML: DFS from 30% to 61% according to the FAB classification and the nature of the pretransplant regimen, with a positive selection bias for patients

- transplanted late (>9 months) and a favorable impact of marrow purging. Best results were observed in the CR1 SR subgroup for varieties M1, M2, and M3, transplanted with marrow purged with mafosfamide at adjusted levels, or following the UCH (University College Hospital, London) polychemotherapy regimen.
2. CR1 SR ALL: DFS 42% at 5 years, with a possible beneficial effect (not demonstrated) of fractionated TBI over single-dose TBI.
  3. Future directions of evaluation and/or clinical research within the EBMT include: (a) randomized study on marrow purging for ABMT in AML CR2 (study of feasibility in progress), (b) evaluation of peripheral blood stem cells (PBSCs) and PBSC + marrow autografts, and (c) evaluation of hemopoietic growth factors and IL2 post-ABMT.

## Role of Autologous Bone Marrow Transplantation in Acute Leukemia

K. A. Dicke<sup>1</sup>, M. J. Evinger-Hodges<sup>2</sup>, J. A. Spinolo<sup>2</sup>, and V. Spencer<sup>2</sup>

### Treatment of Acute Leukemia

With conventional-dose chemotherapy, the prognosis of adult acute leukemia varies with morphological type from 25% long-term disease-free survival (DFS) in acute myelogenous leukemia (AML) to 40% in acute lymphocytic leukemia (ALL). For this reason, we would like to discuss the role of autologous bone marrow transplantation (ABMT) separately for each disease modality.

### Acute Myelogenous Leukemia

From published data there is evidence in support of AML as a clonal disease. The leukemic cell population appears to escape the normal growth regulatory mechanism at either the stem cell level or at a more differentiated level such as the myeloblast and promyelocyte and begin proliferating in an uncontrolled manner. Such unresponsiveness to normal growth regulatory mechanisms is intrinsic to the leukemic cell and is most likely not due simply to a change in the feedback regulatory system of the cell. Numerous chromosomal abnormalities have been described for AML many of which in-

volve specific gene rearrangements or deletions. Based on such chromosomal markers, prognostic factors have been identified to predict the rate as well as the durability of response to therapy. One such a model is that described by Keating et al. [1]. This model predicts the probability of achieving remission (PR) and the probability of staying in remission for 1 year (PCR1). Using this model we are able to predict that the remission durability in patients with the -5, -7 chromosome deletion is short (PCR1 < 0.6% or 60%) whereas inversion 16 or translocation 15,17 has a high probability of staying in remission for 1 year (PCR1 > 0.6 or 60%). The DFS in patients with an inversion 16 or a 15,17 translocation leukemic cell clone is approximately 40% whereas the majority of patients with the -5, -7 leukemic cell population survive less than a year.

Variability in prognosis is a pitfall for any treatment modality to be tested on its effectiveness since patient selection may heavily influence the outcome of the study. To avoid the influence of patient selection, such studies must either be performed separately for each leukemic subpopulation or be performed under strict randomization.

### *Auto-BMT in AML CR1*

For the reasons mentioned above, the European ABMT data in AML collected and presented by Dr. Gorin [2] at this meeting have restricted value. The patients described were transplanted in numerous institutions and the criteria for patient entry at these

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various centers are not known. Dr. Gorin has reported a 32% long-term survival in AML patients transplanted in CR1 with unpurged marrow harvested within 6 months after achieving CR. Patients treated with cyclophosphamide and total body irradiation (TBI) rescued with marrow cleansed in vitro with mafosfamide had a 56% 3-year DFS. When comparing the treatment of these two patient groups, purging may be the difference in the ability to achieve increased DFS; however, the time of marrow harvest may also influence the prognosis. As a result, multivariate analysis of the data is necessary to evaluate purging.

Dr. Lowenberg et al. reported the first randomized study in CR1 AML comparing conventional-dose therapy to remission intensification with cyclophosphamide + TBI and unfractionated marrow, shortly after consolidation treatment [3]. Randomization was done immediately after achieving complete remission. The outcome of these two groups was not significantly different. Time of marrow harvest was early after remission induction (within 2–3 months) and the intensity of the consolidation program was moderate – most likely leaving significant numbers of leukemic cells in the marrow at the time of harvest.

Our study of ABMT in CR1 AML was originally designed as a randomized study comparing intensification of remission with ABMT versus conventional-dose therapy [4]. At the beginning of the study, however, the patients refused randomization so that patients were entered in the ABMT arm on a voluntary basis. The treatment schedule has been outlined in Table 1. The time interval between onset of CR and marrow harvest was –3 months, and between CR and ABMT, 6 months. Remission was induced by amsacrine, cytosine arabinoside (Ara-C), vincristine, and prednisone; intensification occurred by high-dose (HD) Ara-C 12–15 g/m<sup>2</sup> and amsacrine, 250–350 µg/m<sup>2</sup>. To harvest sufficient numbers of marrow cells, patients were treated after intensification with three courses of AD-OAP followed by Cytoxan, 6 g/m<sup>2</sup>; 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), 300 µg/m<sup>2</sup>; and VP-16: 750 mg/m<sup>2</sup> (CBV). The CBV treatment scheme is shown in Table 2. Two days after the last dose of VP-16, unpurged marrow

**Table 1.** High-dose CBV-ABMT in relapsed Hodgkin's disease

Drugs	Day						
	-6	-5	-4	-3	-2	-1	0
Cyclophosphamide 1.5 g/m <sup>2</sup>	x	x	x	x			
BCNU 300 mg/m <sup>2</sup>	x						
VP-16 125 mg/m <sup>2</sup> q12 h × 6 doses	x	x	x				
ABMT							x

**Table 2.** Treatment of AML in CR1 – Program outline

Induction:	AMSA-OAP
	↓
Pre-BMT intensification:	HD Ara-C + AMSA
	↓
Maintenance:	AD-OAP
	↓
Harvest	AD-OAP
	↓
	AD-OAP
	↓
High-dose therapy:	CBV + ABMT
	↓
Maintenance:	AD-OAP X3
	↓
	AMSA-OAP X3

was infused. After hematopoietic recovery, three to six courses of AD-OAP/AMSA-OAP were administered. The results from 18 patients are documented in Table 3. Ten out of 18 patients (56%) are alive and in CCR; the shortest remission is 3 years, the longest 5 years. Eight patients relapsed, with a median remission time of 19 months (range, 7–22 months). It appeared that of the 12 patients with favorable prognostic factors (PCR1 ≥ 0.6) 8 are still in CCR (67%) whereas only 2 out of 6 patients (33%) with unfavorable factors (PCR1 < 0.6) are still in CCR.

**Table 3.** Complete remission duration: relapse-free patients

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Number: 10/18 (56%)
Median duration: 44+ months
58+, 50+, 50+, 50+,
49+, 44+, 41+, 40+,
38+, 37+

---

Our data are comparable to the European data published by Gorin [2], reporting a 56% DFS in patients treated with Cy + TBI and purged marrow. Although we have not used any in vitro manipulation of the marrow, the intensification with Ara-C and amsacrine prior to harvest may have acted as an in vivo purge, rendering the leukemic cell burden to a minimal level. We do not expect CBV alone to be more antileukemic than Cy + TBI; however, with the addition of CBV 2 months after treatment with HD Ara-C and Amsacrine, CBV might be strongly antileukemic. The Amsacrine, Ara-C combination might be very effective as observed in the BAVC regimen (BCNU, Ara-C, VP-16, Ara-C). A 75% DFS (median follow-up, 2 years) has been reported with this regimen in CR2 by the Rome Group [5].

Like the European data, the interpretation of our results is limited due to possible selection of patients. Promising as they may be, a randomized study to prove the biological significance is necessary.

#### *Auto-BMT in AML CR2*

The interpretation of transplantation results in second remission, as in first remission, is open to question. The biological effect of a procedure can be measured by comparing the length of remission after ABMT with the duration of remission preceding those remissions in which BMT occurred. When the transplantation remission is longer than the previous CR, this procedure may have changed the natural history of the disease. The inversion rate with conventional-dose chemotherapy is 10%–20% as published by Keating et al. [1]. Wiernick et al. (personal

communication) reported a 30% DFS and inversion rate in second remission with multiple pulses of HD Ara-C and mitoxantrone. The transplantation data reported by us, Gorin, and Santos are no different. Santos reported a 25%–30% inversion rate and contributed this to in vitro purging with 4-hydroperoxycyclophosphamide (4-HC). However, in the light of the data published by Keating and Wiernick, those data are not significantly better so that no conclusions concerning the efficacy of purging can be drawn. In addition, the transplantation data in CR2 reported by our group are not significantly better than those with conventional chemotherapy. The only transplantation data more favorable than the conventional chemotherapy data are those reported by the Rome group with the BAVC regimen, with an inversion rate of 50%.

The question of whether or not purging has a biological effect still remains unanswered. It is likely that a biological effect can only be expected when the leukemic cell population escaping the conditioning regimen is low. Primarily, this occurs in CR1 where the leukemic cell population is least resistant to cytoreductive therapy. Systematic studies to solve this question have not been performed.

In conclusion, in AML the question of ABMT still remains unresolved. Studies in CR1 are underway to prove its efficacy. The role of purging is controversial; especially in CR2 with potentially high leukemic burden after cytoreductive therapy, the significance of purging may remain questionable. We recommend a randomized study in CR1 with in vivo purged marrow in the good and intermediate prognostic patients, and a one-arm study with in vitro and in vivo purged marrow in bad prognostic patients.

#### **Acute Lymphocytic Leukemia**

The European ABMT data in adult ALL in CR1 reported by Gorin [2] are equivalent to the conventional chemotherapy data of the German Cooperative Group published by Hoelzer et al., reporting close to a 40%, 5-year DFS [6]. As in AML, prognostic factors need to be taken into account before any interpretation of data can be made. In



our studies the ALL data look less favorable from which to draw definite conclusions than the AML CR1 data although the median follow-up of 31 months is relatively short. Fifteen of the 28 patients are still in CCR; however, it looks as if a plateau of the curve has not been reached. A continuous relapse rate is noted in contrast with AML.

It may well be that in ALL more than one intensification is necessary and longer post-transplant chemotherapy. Potential elimination of ALL cells from the graft without loss of hematopoietic stem cells might be possible based on antibody separation technology, since the ALL cells differ in phenotype from early progenitor cells. Due to minimal loss with this technique a combination with chemopurge is possible without significantly compromising the hematopoietic restoration potential. Whereas good prognosis in AML may only need *in vivo* purging, our recommendations in ALL are a double *in vitro* purge. Effective intensification consolidation programs for *in vivo* purge do not yet exist. It is likely that a different mode of treatment is necessary for this disease. Not only are residual cells in the harvest marrow biologically significant, but also leukemic cells escaping the conditioning regimen. Escape may not be due to lack of sensitivity but to the sanctuary sites of ALL cells.

Results in CR2, CR3 are poor. The best data are those published by the Minnesota group in 22 children treated with Cy + TBI and monoclonal antibody purged marrow [7]. The inversion rate was 31%. Since this has not been compared with conventional chemotherapy results, the interpretation is limited.

In conclusion the role of ABMT in ALL is unclear. The results seem less favorable than in AML. A continuing relapse rate is noted after BMT so that more intensive and longer therapy might be necessary. *In vitro* purging with a combination of antibody purge and chemopurge might be effective. Studies for conclusive evidence need to be undertaken.

### **Efficacy of Purging**

As mentioned earlier it is unclear if the purging of bone marrow from leukemic cells is

beneficial to the long-term survival of AML patients undergoing ABMT. The biological significance of such procedures will depend in part on the role these reinfused leukemic cells play in the recurrence of disease after transplantation. The fewer leukemic cells to escape the transplantation conditioning regimen, the more significant the leukemic cell population in the graft will be. It is likely, then, that the biological significance of purging will be greater in first rather than in second or subsequent remissions. A significant drawback in the answering of this question has been the inability to monitor the assumed removal of these residual leukemic cells. There are two major constraints in the detection of minimal numbers (<1%) of tumor cells:

1. the lack of technology available with sufficient sensitivity and
2. identification of specific tumor markers.

When testing leukemic cell separation techniques on untreated or relapsed leukemic patients, it is possible to monitor the separations with little difficulty using standard techniques such as morphology, cytochemistry, cytogenetics, and the *in vitro* colony formation assays. None of these assays are specific and sensitive enough to detect the small numbers of leukemic cells which may be present in remission marrow. Since the purpose of the above-mentioned separation techniques is use for remission marrow, such detection methods leave us to do these separations blindly. For the past decade considerable effort has been made in the use of molecular technology to resolve these problems.

Since these tumor cells are part of a heterogeneous population of cells, the use of Southern and/or Northern blotting techniques have not enabled us to detect fewer than 1% contaminating tumor cells unless preselection is used through which our sensitivity increases to 0.1% at best. From estimates of the leukemic cell population in remission marrow, the frequency of the leukemic cell population is one leukemic cell in 20 000 cells, or 0.005%, which is far below the detection limits of the techniques discussed above. Therefore these assays cannot be used to monitor tumor cell separation techniques in remission marrow.

## Aberrant Gene Expression in Leukemia

There are numerous reports of aberrant proto-oncogene expression in tumor cells and more specifically in AML [8–16]. The importance of these genes in normal cellular proliferation and differentiation has become well accepted; many of these genes appear to be related to either growth factors or their receptors, or are involved in the signal pathway [17]. We have found that several of the proto-oncogenes analyzed by Northern blot hybridization were expressed in hematologically normal bone marrow [14], supporting the concept that these genes do play a role in adult hematopoietic metabolism. There is growing evidence that qualitative and quantitative changes in proto-oncogene expression may be associated with neoplasia [18].

The hematopoietic system has proven attractive for these studies since several proto-oncogenes are located at the breakpoint of chromosomal translocations or inversions specifically associated with various leukemias [19]. Perhaps this leads to the deregulated expression of these genes. Furthermore, the enhanced expression of cellular oncogenes has been consistently observed in neoplastic hematopoietic cells. Several groups in addition to ourselves have identified two genes, *MYC* and *SIS*, which are present at abnormally high levels in the peripheral blood and bone marrow cells of untreated and/or relapsed AML patients [20–25]. By Northern and dot blotting analyses we were unable to detect this abnormality in the bone marrow cells of any AML patient clinically classified as in remission. Since the limitations of these techniques are in the range of 1%–5% contamination of leukemic cells in the samples tested, any abnormality present at lower levels would remain undetectable.

### *Detection of Leukemic Cells by RNA In Situ Hybridization*

Our approach to this problem has been the identification of an abnormality which is detectable at the single cell level. We have reported the development of an extremely sensitive and rapid RNA-in situ technology which permits the detection of specific mRNAs within individual cells [26]. This

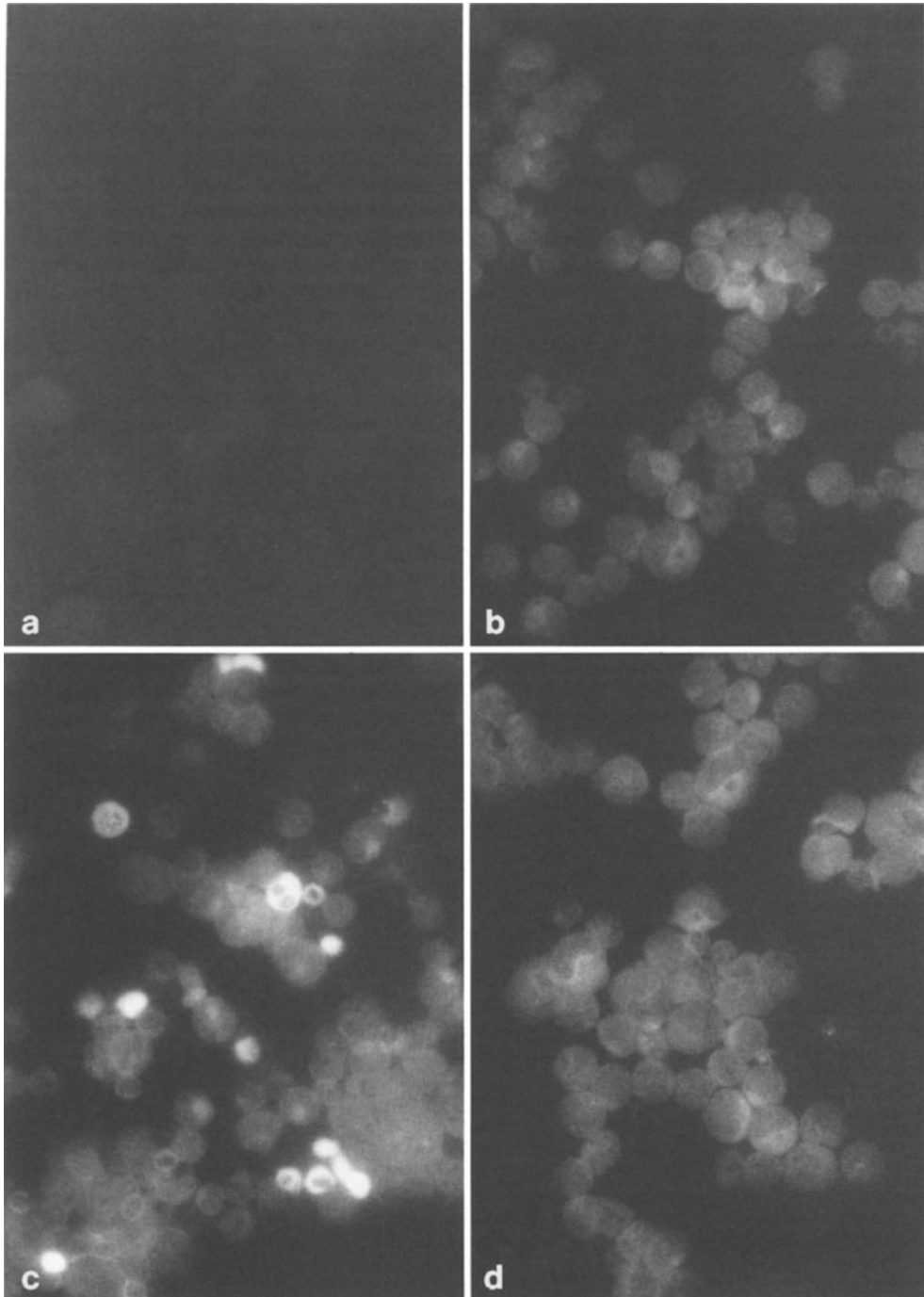
technology enables us to identify cells with the abnormal expression of any marker gene at a level of 1/50000 cells. Using this RNA-in situ hybridization methodology, we have identified the abnormally high expression of two genes, *MYC* and *SIS*, which occur in >90% of untreated and relapsed AML patients [25]. The high levels of *MYC* and *SIS* mRNA found in these cells cannot simply be attributed to the proliferative capacity of these cells or to the presence of normal, immature hematopoietic cells. Bone marrow cells from over 15 solid tumor patients have now been examined in a longitudinal study choosing several time points before transplantation and at two time points (1 month and 2.5 months) after BMT when the hematopoietic cells are in a highly proliferative state. Figure 1 is representative of the levels of *MYC* expression detectable in bone marrow cells obtained from

1. normal marrow,
2. AML remission marrow, and
3. solid tumor marrow, 1 month after ABMT.

With the exception of two patients who appeared to have a subpopulation of cells with high *MYC* expression before ABMT, in no case did Dr. Spencer in our group find any cells which express either *MYC* or *SIS* at the high levels found in the leukemic cell population (personal communication).

As one line of evidence that the cells we were examining actually belonged to the leukemic cell compartment, comparisons were made with the percentage of blast cells determined morphologically in these AML patients. The presence of such abnormal cells, as defined by gene expression, in these AML patients correlated well with the percentage of blast cells determined morphologically. As shown in Table 4, the percentage of cells overexpressing either *MYC* or *SIS* at least equals, and often exceeds, the number of blast cells present in the marrow.

In addition, we have identified the presence of such abnormal cells in AML patients studied shortly after remission induction, but these cells are often present at a much lower frequency than that found in either untreated or relapse AML (Table 5). Several, but not all, patients in which we found this abnormal group of cells have relapsed;



**Fig. 1 a–d.** MYC expression in hematopoietic cells. The expression level of the proto-oncogene MYC in hematopoietic cells was detected by RNA-in situ hybridization as described earlier [26] and visualized using a fluorescent label. Bone marrow samples were obtained from individuals under the following conditions: normal individuals, AML patients in remission, and solid tumor patients 1 month after ABMT; **a** represents a control for nonspecific fluorescence; **b** normal bone marrow; **c** AML remission marrow; **d** marrow from a solid tumor patient, 1 month after ABMT. The level of MYC expression in rapidly proliferating hematopoietic cells examined 1 month after ABMT was no higher than that present in the bone marrow cells examined from normal individuals

**Table 4.** Comparison of the percentage of blast cells with the percentage of cells with abnormal gene expression in AML bone marrow

Patient	% blast	% MYC	% SIS
1	7.2	70	70
2	24	25	25
3	40	90	90
4	49	30	50
5	51	50	50
6	60	75	75
7	63	85	90
8	65	65	90
9	76	70	75
10	78	75	80

**Table 5.** Overexpression of MYC and SIS in AML short-term remission patients

Patient	% MYC	% SIS	Remission duration
1	75	80	1 month
2	8.0	2.0	6 months
3	5.0	2.0	2 months
4	0.2	0.2	5 months
5	0.1	1.0	6 months
6	2.5	2.5	CR (4 months)
7	0.05	0.0	CR (1+ year)
8	0.0	0.0	CR (3 months)
9	0.0	0.0	CR (1+ year)
10	0.0	0.0	CR (1+ year)

in contrast, none of the patients whose bone marrow cells were found to be normal in their expression of *MYC* and/or *SIS* have relapsed since this study was completed. These results led us to question whether the presence of such an abnormal population of cells could be predictive of early relapse in acute leukemia.

To help us determine the significance of this abnormal cell population in the eventual clinical stability of the AML patient, we have also examined bone marrow cells of ten AML patients who are long-term survivors after BMT. The median CR duration at the time of examination for this group was 38 months, with the individual remissions ranging from 14 to 78 months.

The presence of an abnormal cell population expressing *MYC* at high levels similar to that found in AML short-term remission patients does not occur in this patient group. However, in three out of ten patients a high level of *SIS* expression alone was present in a variable percentage of cells, occasionally as high as 80%. At this time, none of the three patients identified with this abnormality at the RNA level have been classified as having a recurrence of leukemic cells in the bone marrow by conventional morphological criteria.

#### *Monitoring of Purging Procedures*

It is unclear at this time if the purging of bone marrow of leukemic cells is beneficial to the long-term survival of AML patients undergoing ABMT. As discussed earlier, a significant drawback in the answering of this question has been the inability to monitor the assumed removal of these residual leukemic cells. We have begun to apply our RNA-in situ hybridization technique to this problem. Since we find such high levels of *MYC* to be present in AML patient bone marrow, we were interested to see if these cells are removed during a purging procedure using monoclonal antibodies directed against the leukemic cell population. Very early results indicate that there is indeed a decrease, although not a complete elimination, of this *MYC*-overexpressing subpopulation of cells after purging. Although we have not yet attempted to quantify the efficiency with which these abnormal cells are removed, this test does appear to be a promising means by which to assess leukemic cell removal.

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## Long-Term Disease-Free Survival Following Autologous Bone Marrow/Blood Stem Cell Transplantation in 89 Patients with Acute Leukemia

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### Introduction

Myeloablative consolidation therapy followed by transplantation of the patient's prior harvested and cryopreserved bone marrow has been shown to be successful for the treatment of acute leukemia. Yeager et al. [1] recently reported data on autologous marrow graft harvested in second or subsequent complete remission (CR) of acute myelogenous leukemia (AML), treated *ex vivo* with the active cyclophosphamide (CY) derivative 4-HC, and eventually retransfused into patients with "high-risk" AML after myeloablative treatment with busulfan (BU) and CY. The 43% actuarial disease-free survival (DFS) in these patients was clearly superior to that with conventional treatment, which is 5% under such "high-risk" conditions [2].

These data (46% leukemic relapse) compare favorably with syngeneic bone marrow transplantations in patients autografted in first remission AML [3]. From this, it may be concluded that the *ex vivo* purged marrow does not significantly contribute to leukemic relapse in the autologous transplant situation. Therefore, the lack of graft-versus leukemia effect is probably the major cause of the higher leukemic relapse following ABMT.

In the autologous transplant situation, clonogenic tumor cell contamination of the

autograft not only depends on the efficacy of the "purging" technique, but also on the source of hemopoietic stem cells and cell composition of the autograft, and eventually on growth factors used to mobilize hemopoietic stem cells. The circulating stem cell pool is believed to be a stem cell source containing less tumor cells than the marrow when in complete remission and under steady-state conditions.

In 1985, we first reported on a patient whose hemopoietic system reconstituted completely and permanently after transfusion for blood-derived hemopoietic stem cells [4]. We then started a series of autologous blood stem cell transplantations preferentially in patients with low-risk AML. The reason for doing so was a higher prospective yield of blood-derived stem cells in patients whose prior chemotherapeutic treatment was not as heavy as it is under high-risk conditions [5].

In the following, we report on our experience with 89 autologous stem cell transplantations in 66 cases of AML and 23 cases of acute lymphoblastic leukemia (ALL).

### Patients

Patients were eligible for this study if the diagnosis of AML or ALL/AUL was made by examination of bone marrow aspirates, histological typing according to the French-American-British (FAB) classification, or immunophenotyping. All transplants were performed either in CR1 or in subsequent CR following intensive conventional chem-

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otherapy. The decision as to whether a given patient should receive a transplant derived from marrow or from the circulating blood was not made on the basis of a randomized prospective trial. All but one patient had no HLA-matched sibling donor. One patient preferred autologous bone marrow transplantation over allogeneic bone marrow transplantation despite having an HLA-identical sibling.

The study protocol was approved by the medical faculty, and informed consent was obtained from patients before entering the study.

### **“Purging” the Autograft**

#### *Chemoseparation*

In all AML patients and in most ALL patients, the marrow autograft was purged *ex vivo* using the cyclophosphamide derivative mafosfamide. The dose ranged from 60 to 80  $\mu\text{g}/2 \times 10^7$  white blood cells, resulting in a median CFU-GM reduction of 84% (range, 46%–97.5%). The marrow was processed by a two-step gradient procedure using the blood-cell processor IBM 2991: a buffy coat centrifugation followed by a Ficoll-Metrizoate (FM) gradient centrifugation. The blood stem cell autograft was not manipulated at all, to avoid the risk of major loss of hemopoietic progenitor cells due to the purging procedure.

#### *Immunoseparation*

In three patients with high-risk ALL, the marrow autograft was purged using the immunomagnetobead separation technique. The panel of monoclonal antibodies was chosen according to the immunophenotyping at the time of first diagnosis. The immunomagnetobead separation technique is described in detail by Kvalheim et al. [6].

### **Pretransplant Regimen**

In all but one patient, the pretransplant regimen consisted of highly fractionated total body irradiation (1200–1560 cGy) and CY

(200 mg/kg). One patient was treated with busulfan (16 mg/kg) and CY (200 mg/kg).

### **Results**

There were a total of 48 autologous bone marrow transplantations (ABMTs) in patients with AML, among them 21 in first CR and 27 in second and subsequent CR. Eighteen autologous blood stem cell transplantations (ABSCTs) were performed in first-remission AML. The actuarial DFS following ABMT in first CR AML was 62% compared with 28% in second or subsequent CR (CR2+) at a median follow-up of 28 and 27 months, respectively. The disease-free survival after ABSCT was 40% at 16 months posttransplant and not significantly different from CR1 patients following ABMT ( $P=0.75$ ). In first-remission ABMT patients, no late relapse after 1 year was seen, whereas in high-risk AML patients five late relapses occurred at 13, 13, 19, 20, and 30 months posttransplant. We have performed a total of 19 ABMTs in patients with high-risk ALL, among them 7 in first CR and 12 in second or subsequent CR. Five out of seven high-risk CR1 patients are in continuous CR at a median follow-up of 35 months (32–48 months), and 5 out of 12 CR2+ patients are in continuous CR at a median follow-up of 10 months (1–36 months). Three of those CR2+ patients had their autograft purged using a panel of monoclonal antibodies (immunomagnetobead separation) (Table 1). Four patients were given transplants using blood-derived stem cells, among them three in first CR and one in second CR. All three patients given transplants in first remission are in continuous CR at 21, 18, and 9 months. The patient given a transplant in second CR died of CNS relapse 5 months after ABSCT. The overall early transplant-related death rate (up to 100 days posttransplant) was low, being 8%.

### **Data Reported on ABMT in Acute Leukemia**

The latest survey of the European Cooperative Group for Bone Marrow Transplantation analyzed 1322 cases of acute leukemia

**Table 1.** Immunomagnetobead separation in patients with ALL in second complete remission (CR) DFS=disease free survival

ID No.	Age	Diagnosis	Status at transplantation	Ex vivo treatment of autograft	Pretransplant therapy	Status post-transplant DFS/survival
700913	18	ALL	CR2	McAb (CD10, CD19, CD20)	TBI/CY	+ 18 months
690419	19	ALL	CR2	McAb (CD10, CD19, CD20)	TBI/CY	+ 16 months
711205	17	ALL	CR2	McAb (CD20, HLA-DR)	TBI/CY	+ 9 months

Date of evaluation: 15. 8. 1989

**Table 2.** Disease-free survival after ABMT in patients with AML

	CR	Purged	Un-purged	Percentage patients disease-free	(Median) disease-free follow-up (months)
Stewart et al. [9]	1		×	3/13 (23%)	26–50
Burnett et al. [8]	1		×	7/12 (58%)	6.5–35
Gorin et al. [10]	1	×		9/13 (69%)	22
Santos et al. (personal communication)	1	×		9/16 (56%)	18.8
Gorin et al. [10]	2+	×		2/11 (18%)	9, 10
Santos et al. (personal communication)	2+	×		27/71 (38%)	17.0

autografted in remission [7]. The disease-free probability of AML patients autografted in first CR under standard-risk conditions with purged marrow was up to 60%, or 30% for AML patients autografted in the second remission of their disease. Table 2 shows data from single-institution studies reporting on AML patients, where the autograft was either unpurged or purged with cyclophosphamide derivatives (G.W. Santos, personal communication [8–10]). From data of the 1989 EBMT survey on ABMT, the DFS of high-risk ALL patients autografted in first CR after fractionated total body irradiation was up to 65%, or 20% in second remission, respectively [7].

### Conclusions

1. In AML following relapse long-term remissions can be attained following ABMT (1,11).

2. In first-remission AML there is no significant difference in the probability of DFS depending on whether autologous transplantation was performed with marrow or with blood-derived hemopoietic stem cells.
3. In ALL following relapse and ABMT, the probability of DFS is low; extensive purging of the marrow autograft by means of the immunomagnetobead-separation technique seems promising.
4. The transplant-related death rate is low (8%).
5. The therapeutic benefit of autologous bone marrow or blood stem cell transplantation in low-risk AML or high-risk ALL patients in first remission has to be evaluated by randomized prospective trials.



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## Allogeneic and Autologous Bone Marrow Transplantation for Acute Lymphoblastic Leukemia

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### Introduction

Acute lymphoblastic leukemia (ALL) includes heterogeneous clinical leukemic syndromes which differ in clinical presentation, immunophenotype, cytogenetics, and clinical outcome. Recognition of this heterogeneity has allowed definition of curative therapy for more than 50% of patients with ALL, especially in children. Despite these scientific and clinical advances, certain patient subgroups at presentation and most after clinical relapse have a poor prognosis and thus have been considered candidates for therapy with bone marrow transplantation (BMT). Since 1982, at the University of Minnesota, we have undertaken a series of consecutive parallel trials of bone marrow transplantation for patients with high-risk acute lymphoblastic leukemia using histocompatible sibling donor marrow when available and ex vivo purged autologous remission bone marrow for patients lacking an available matched donor [3]. Their clinical outcomes and analysis of potentially important clinical prognostic factors are presented.

### Patients and Methods

One hundred and seventy-eight patients undergoing bone marrow transplantation for

ALL in remission at the University of Minnesota have been included. Patients were eligible for transplantation in  $\geq$  second complete remission (CR) or in first CR if  $\geq$  21 years of age, or if 16–20 years with either WBC at diagnosis  $\geq 1 \times 10^4/\mu\text{l}$  or high-risk karyotype (e.g.,  $\text{Ph}^1+$  or  $t(4;11)$ ) or immunophenotype (T-cell or B-cell). Sixty-four patients with HLA-identical/mixed lymphocyte culture-compatible sibling donors received allogeneic bone marrow and patients lacking an available matched donor ( $n=105$ ) received autologous purged remission marrow, as described [6]. Allogeneic bone marrow recipients received graft-versus-host disease prophylaxis primarily with methotrexate, ATG, and prednisone [5] and no patients received ex vivo T-depleted allogeneic marrow.

From 1982 to 1984, patients were conditioned for transplantation using cyclophosphamide 60 mg/kg (day -7, -6) and fractionated total body irradiation (TBI) (165 cGy  $\times$  4 days = 1320 cGy) [8]. From 1984 to 1986, patients were prepared with single-dose TBI (850 cGy) and high-dose cytosine arabinoside (3 g/m<sup>2</sup> bid  $\times$  6 days). In 1987–1989, patients were conditioned with hyperfractionated TBI (120 cGy  $\times$  11 with lung shielding and electron beam chest wall boosting) followed by cyclophosphamide 60 mg/kg  $\times$  2 as described from Memorial Sloan-Kettering [1].

Autologous remission bone marrow in B-lineage ALL was purged with monoclonal antibodies BA1 (CD24), BA2 (CD9), BA3 (CD10) + complement ( $n=51$ ) and additionally since 1986, with 4-hydroperoxy-

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cyclophosphamide (4-HC) ( $n=39$ ) [4, 6]. T-lineage autologous marrow was purged with intact ricin-conjugated immunotoxins (anti-CD5, anti-CD7) ( $n=4$ ) and with immunotoxins + 4-HC ( $n=11$ ) [7].

## Results

The patients included in these trials and their pretransplant characteristics are detailed in Table 1. The recipients of allogeneic marrow were somewhat older than those undergoing autologous transplantation and tended to be transplanted in an earlier remission. Of the 22 patients transplanted in first complete remission, only 4 received transplantation as therapy solely because of age >21 years. The other 18 patients had Ph+ or t(4;11) ALL ( $n=7$ ), WBC > 50 000/ $\mu$ l ( $n=12$ ), CNS leukemia ( $n=5$ ), delay in achieving first CR ( $n=1$ ), or T-cell disease ( $n=6$ ). Almost 90% of autograft patients had B-lineage disease while a smaller percentage of allogeneic recipients with known immunophenotypes at transplantation had B-lineage disease. T-cell ALL was uncommon in both groups. The entire patient group had leukocyte counts at diagnosis ranging from 0.8 to  $1290 \times 10^3/\mu$ l, with a median of  $20.5 \times 10^3/\mu$ l, and underwent transplant at a median of 26 months following the original diagnosis of ALL. Those undergoing transplant following at least one

relapse ( $n=149$ ) had a median first remission duration of 20 months.

Recipients of autologous marrow showed evidence of bone marrow recovery and granulocyte production earlier than allograft patients. The nearly 1-week shorter duration of severe neutropenia in the autologous patients was accompanied by lesser morbidity and mortality manifest as a shorter time to hospital discharge alive. Autologous transplant recipients were discharged at a median of 43 days while allogeneic recipients were discharged at a median of 54 days following BMT ( $P=0.0003$ ). Fewer autologous marrow recipients died during their initial hospitalization; 6% (7 of 112 patients) as compared with 24% (16 of 66) of patients receiving allogeneic marrow.

Leukemic recurrence following transplantation, however, was both earlier and more frequent in recipients of autologous bone marrow. Autologous transplantation is projected to be followed by leukemic recurrence in 80% of patients (70%–90%; 95% confidence interval) while only 57% (41%–73%; 95% CI) of allograft recipients have relapsed following transplantation ( $P=0.003$ ). Among those who relapsed, in autografts the leukemia recurred between 1 and 24 months (median, 5 months) following BMT while in allogeneic recipients leukemia recurred between 1 and 27 months (median, 18 months) posttransplantation.

**Table 1.** Patient characteristics

	Allogeneic	Autologous
Number	66	112
Age (range; median in years)	4–48; 15	2–42; 9
Remission number 1st	13	9
2nd	31	51
3rd +	22	52
B-lineage	24 <sup>a</sup>	97
T-lineage	11 <sup>a</sup>	15
Pretransplant conditioning		
1982–1984 Cyclophosphamide + fractionated TBI	40	32
1984–1986 TBI + Ara-C	16	51
1987–1989 Hyperfractionated TBI + cyclophosphamide	10	29

<sup>a</sup> Immunophenotyping unknown for 31 allogeneic recipients

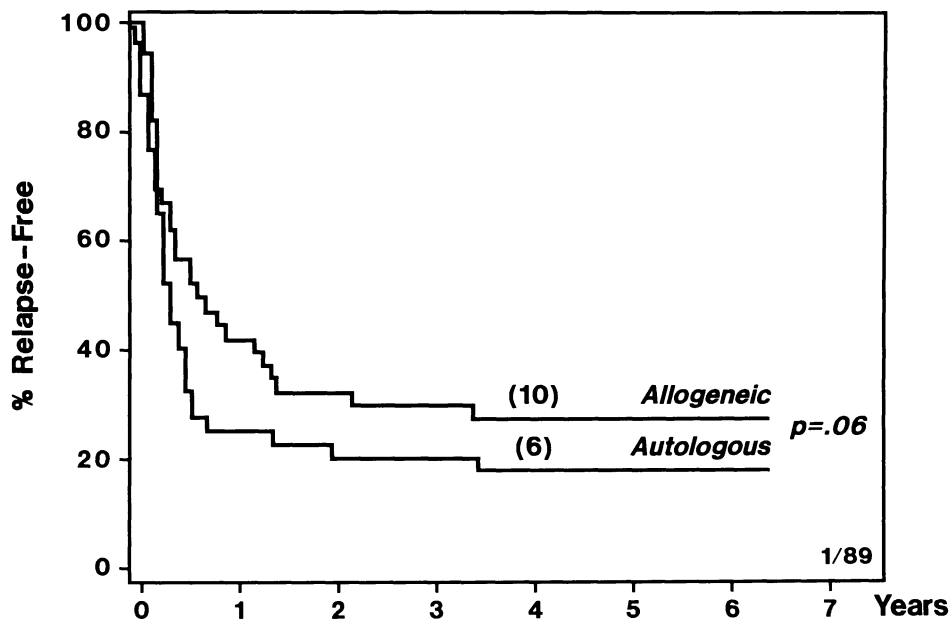


Fig. 1. Percentage of patients surviving disease free in years posttransplant. The number in parentheses represents patients alive and disease free beyond 4 years posttransplant

Despite this difference in the risks of leukemia recurrence, the disease-free survival in allogeneic and autologous recipients was similar, though there was a strong trend favoring allotransplantation. Shown in Fig. 1 is the Kaplan-Meier projection that 27% (15%–39%; 95% confidence interval) of patients will be alive and leukemia free at 3 years following allogeneic transplantation while 18% of autograft recipients (9%–27%; 95% CI) will be surviving ( $P=0.06$ ). The greater in hospital morbidity and mortality accompanying allogeneic transplantation, mostly due to complications of graft-versus-host disease and infection is contrasted with the significantly more frequent leukemic recurrence in recipients of autologous marrow.

Various pretransplant prognostic factors were assessed for their ability to predict leukemic relapse and disease-free survival following transplantation with either autologous or allogeneic bone marrow. None of the factors patient age, diagnostic WBC, remission number at transplantation, prior extramedullary leukemia was significantly associated with lesser risks of leukemic re-

lapse or improved disease-free survival following transplantation. However, a prolonged duration of initial remission following diagnosis (longer than the median of 20 months) was associated with a lesser likelihood of leukemic relapse after BMT ( $P=0.01$ ), though not with improved relapse-free survival.

The successively more intense conditioning regimens employed in this study using progressively more intense radiation schemes and patterned after conditioning regimens previously reported to be more effective in allogeneic transplantation [1, 2] have not produced better overall results. As shown in Table 2, rates of leukemic recurrence and disease-free survival (considering shorter follow-up in the more recent series) have not improved notably with these progressively more intense conditioning regimens.

## Discussion

This series of prospective, parallel trials of bone marrow transplantation for patients

**Table 2.** Clinical outcome following transplantation for ALL

	Relapse	Disease-free survival	Follow-up interval (years)
1982–1984			
Cyclophosphamide + Fr TBI	68.5% ± 12%	23.6% ± 10%	4
1984–1986			
Ara-C + TBI	80% ± 14.5%	14.8% ± 11.2%	2.5
1987–1989			
Hyper Fr TBI + cyclophosphamide	47% ± 21%	49.4% ± 21%	<1

Shown are Kaplan-Meier projections ± 95% confidence limits for post-BMT (allogeneic and autologous) leukemic relapse and disease-free survival. The minimum follow-up of patients in each successive protocol is shown

with remission ALL demonstrates a consistent proportion of patients alive and free of leukemia several years following transplantation. Using both histocompatible sibling marrow for allogeneic transplantation and immunologically purged autologous remission marrow for those lacking a sibling donor, one in five patients undergoing transplantation can achieve extended leukemia-free survival observed thus far to 7 years or more. Differing complications attend allogeneic and autologous transplantation with greater peritransplant morbidity and mortality accompanying allogeneic BMT, but significantly poorer control of leukemia in autologous BMT. These distinct problems suggest specific areas for the focus of ongoing clinical and laboratory research. In allogeneic transplantation, further attempts to improve pre-BMT conditioning continues through this sequence of trials. Unfortunately in the most recent trial, the promising results using the conditioning regimen reported from Memorial [1] appear not to be reproduced. Most importantly needed in allogeneic grafting, however, is reduction in the substantial peritransplant mortality due to graft-versus-host disease and opportunistic infection. Additionally, better methods for patient selection and establishing optimal timing of transplantation are required. Just as recognition of the heterogeneity of

ALL has allowed tailoring of more intense conventional chemotherapy regimens for those with higher-risk disease, perhaps the particularly high-risk marrow transplant candidate will benefit from more refined techniques in conditioning and in supportive care.

In autologous transplantation, however, leukemic relapse remains the major obstacle to improved success. The three successive regimens described here, all using remission marrow purged with monoclonal antibodies and/or 4-HC, have failed to contain leukemic recurrence. Notably, the role of purging has not yet been defined and will require improved methods of evaluating the significance of detectable minimal residual disease in the patient and in the cryopreserved marrow inoculum. Perhaps in autologous transplantation, the application of posttransplant intensification or maintenance chemotherapy or even immunotherapy may be particularly promising areas for future clinical investigation.

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## Treatment Strategies for Acute Lymphoblastic Leukemia

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Analysis of treatment strategies for acute lymphoblastic leukemia (ALL) is complex. Variables to be considered include age, risk group, timing, and therapeutic approach. Age is an important determinant of outcome; treatment must be analyzed separately in adults, adolescents, and children. Analysis of other risk factors is similarly complex; different risk factors predict outcome at diagnosis and at relapse. Another consideration is the time in which therapy is planned – first remission, second remission, or following subsequent relapse. In this review we consider five potential approaches to treating ALL: chemotherapy, HLA-identical transplants, autotransplants, a strategy of chemotherapy followed by transplantation in persons who relapse, and a strategy of transplantation followed by chemotherapy in those who relapse. Data are summarized from the literature [1–4].

### Adult ALL

Results of chemotherapy, HLA-identical transplants, and autotransplants in adult ALL in first and second remission are shown in Fig. 1. These data indicate equivalent leukemia-free survival (LFS) in persons

in first remission using all three approaches when results are adjusted for risk factors and for *time censoring* of persons with early relapse. These data also indicate that HLA-identical transplants result in superior LFS in persons in second remission compared with alternative therapies. Another approach to analyzing treatment results is in the context of controlled prospective trials. These are not reported in adults with ALL in first or second remission. A third approach to comparing treatment strategies is to use transplants in persons with a high risk of treatment failure with conventional therapy. This strategy assumes these adverse risk factors do not operate with transplants. However, recent analysis of risk factors in adults with ALL treated with chemotherapy or HLA-identical transplants indicates similar risk factors – age greater than 25 years, WBC at diagnosis greater than  $50 \times 10^9$ /liter, non-T immune phenotype, and slow response to induction chemotherapy.

Two other strategies to consider are use of chemotherapy followed by transplants in persons who relapse or the alternative, or transplants followed by chemotherapy in those who relapse. Comparison of these alternative strategies in adults reveals equivalent LFS of approximately 50%.

In conclusion, LFS in adult ALL in first remission is similar for the three therapies in adjusted cohorts of subjects. In second remission, results of HLA-identical transplants appear superior to alternative therapies. Overall, a strategy of chemotherapy followed by transplantation in persons who relapse may result in the highest LFS.

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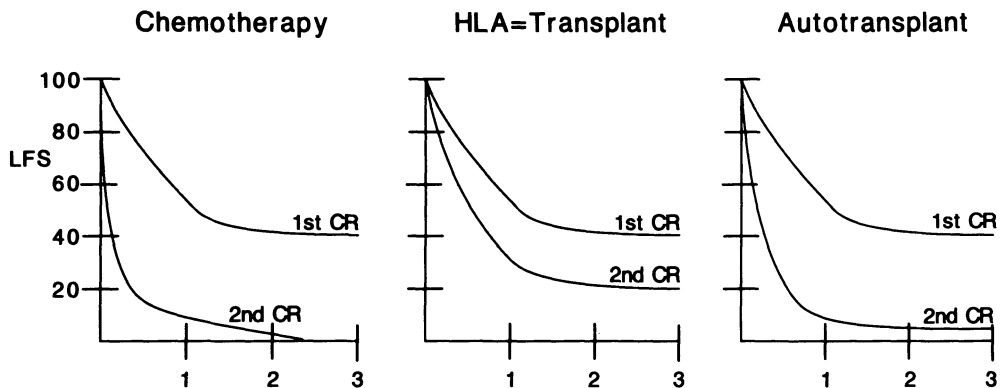


Fig. 1. Comparison of chemotherapy, HLA-identical sibling transplants, and autotransplants in adults with ALL. CR, complete remission

### Adolescent ALL

Optimal therapy of adolescents (16–22 years) with ALL is likewise controversial. Comparison of chemotherapy and HLA-identical transplants is shown in Fig. 2. These data indicate that results of chemotherapy are equivalent or superior to those achieved with HLA-identical transplants. Interestingly, results of chemotherapy in seemingly similar patients are different depending on the center in which treatment occurs; adolescents treated at pediatric cen-

ters have superior LFS than those treated at adult centers. The reason for this difference is unclear. There are insufficient data to analyze results of autotransplants in adolescents. Likewise there are no controlled trials evaluating alternative treatments nor are there sufficient data to analyze the combined use of chemotherapy and transplantation in adolescents.

In summary, results of chemotherapy at either pediatric or adult centers seems superior or equivalent to results of HLA-identical transplants. In second remission, HLA-

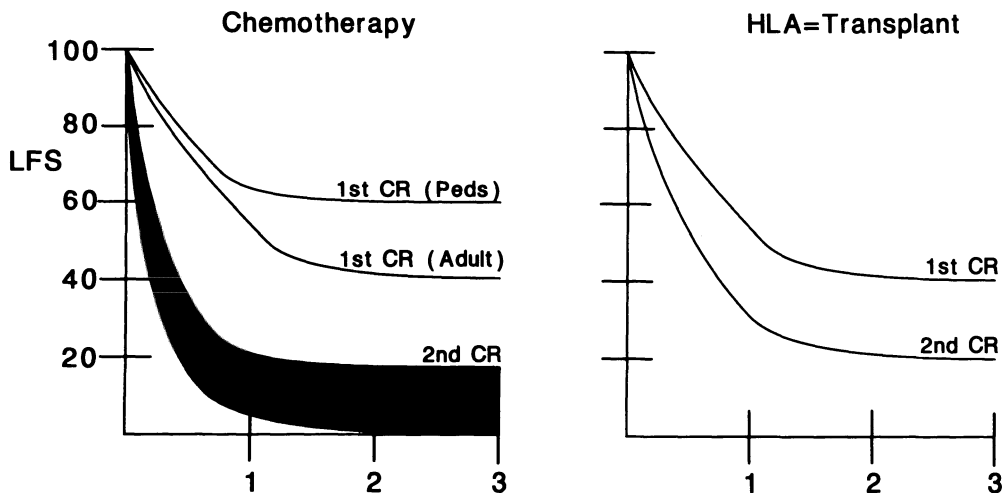


Fig. 2. Comparison of chemotherapy and HLA-identical sibling transplants in adolescents with ALL. CR, complete remission. Peds and Adult, outcome of adolescents at pediatric or adult centers. Shaded area is range of outcomes



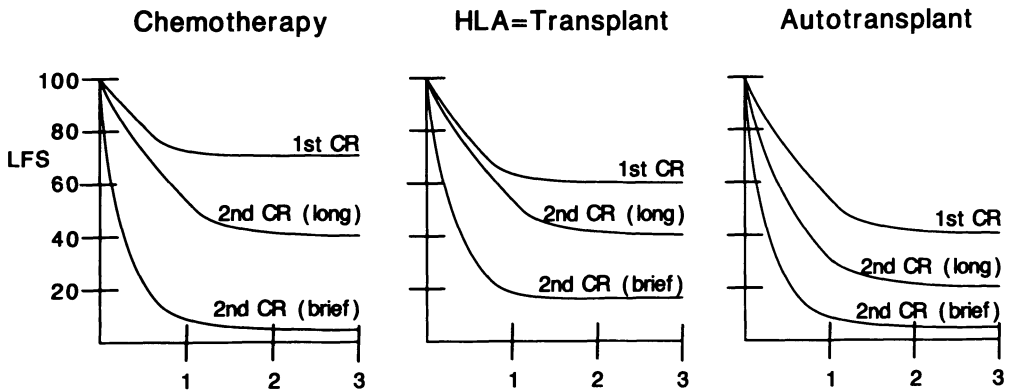


Fig. 3. Comparison of chemotherapy, HLA-identical sibling transplants, and autotransplants in children with ALL. CR, complete remission, *Long* and *brief*, length of first remission (>18 months or <18 months)

identical transplants and possibly autotransplants result in superior LFS. Overall, it seems that a strategy of chemotherapy followed by transplantation in adolescents who relapse may represent the best therapeutic approach.

### Childhood ALL

Results of alternative treatment strategies in children with ALL are shown in Fig. 3. Data on children in first remission refer to those with high-risk features. These data indicate equivalent results with chemotherapy and HLA-identical transplants; results of autotransplants seem inferior. Results of alternative treatments in second remission depend on the duration of first remission. Children with a brief first remission or who relapse on chemotherapy have superior LFS with HLA-identical transplants when compared with either chemotherapy or autotransplants. In children in whom the initial remission was long or who relapsed off chemotherapy, chemotherapy and HLA-identical transplants result in equivalent LFS; results of autotransplants appear slightly inferior. There are no reports of randomized trials comparing chemotherapy and transplantation in children with high-risk ALL in first remission nor in children with second remission in whom the first remission was brief.

Design or therapeutic strategies based on risk factor analysis in children are complex. Risk factors in children with ALL in second remission include the duration of first remission and whether relapse occurred on or off chemotherapy. Risk factors at diagnosis no longer operate in children in second remission. Similar risk factors also predict the outcome of transplants in this setting. Consequently, it is not possible to devise a preferred therapy based on risk factor analysis in children with ALL in second remission.

A comparison of chemotherapy followed by transplants in children who relapse or transplants followed by chemotherapy in those who relapse was also performed in children with high-risk ALL in first remission and in children in second remission in whom the first remission was brief. In both situations, a strategy of chemotherapy followed by transplantation in children who relapse resulted in equivalent or superior LFS.

In summary, chemotherapy is the preferred treatment of children with ALL in first remission including those with high-risk features. In children with ALL in second remission in whom the first remission is brief, or who relapsed on chemotherapy, HLA-identical transplants result in superior LFS to chemotherapy. There may be a role for autotransplants in this setting but this is not convincingly shown. In children with ALL in second remission in whom the first

remission was long, or who relapsed off chemotherapy, results of chemotherapy are superior to those of transplants. Initial treatment of these children should be chemotherapy followed by transplantation at the time of relapse or in third remission in those who fail. Children with advanced ALL (> second remission) have a poor outcome with chemotherapy and are reasonable immediate candidates for transplantation.

### Summary

We analyzed LFS in adults, adolescents, and children with ALL treated by using different therapies and therapeutic strategies. In most instances, the strategy of initial chemotherapy followed by transplantation in those who relapse produced the highest LFS. Results of HLA-identical transplants were generally superior to those of autotransplants. However, many persons do not have donors and should be considered for autotransplants or possibly for transplants from partially or fully HLA-matched related or unrelated donors. These persons are also potential candidates for evolving therapeutic strategies such as immune therapy or lymphoid growth factors.

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## Influence of Treatment Modality, Patient/Donor Characteristics, and Disease Stage on the Risk of Relapse After Allogeneic Marrow Transplantation for Acute Leukemia\*

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For patients with acute leukemia, allogeneic marrow transplantation (BMT) following myeloablative radiochemotherapy currently represents the most effective treatment to reduce the risk of leukemic recurrence. Approximately 50% of patients receiving allografts in first remission (CR) of acute myeloid leukemia (AML) or acute lymphoblastic leukemia (ALL) have become long-term survivors [1]. Substantial evidence for the high antileukemic efficacy of BMT comes from experiences in adult patients treated after first remission, who seldom achieve lasting remissions by conventional chemotherapy. Patients undergoing BMT in first early relapse or second remission still have a 25%–30% chance of becoming long-term disease-free survivors. A significant, albeit low, rate of long-term remissions and possibly cures has also been demonstrated in patients receiving allografts in end-stage relapse. Nevertheless, leukemic relapse still remains a prominent cause of treatment failure. The probability of leukemic recurrence is clearly correlated to the disease stage at the time of BMT, with increasing relapse frequencies occurring in more advanced stages of leukemia. Besides the apparent interrelationship between disease stage and relapse probability, the question has to be raised whether other factors might contribute to the risk of relapse after BMT. To determine the influence of different myeloablative regimens, posttransplant treatment

protocols, as well as patient and donor characteristics on the risk of leukemic recurrence after BMT, we retrospectively analyzed our experience in patients with acute leukemia, who had received HLA-identical sibling marrow transplants.

### Patients and Methods

This retrospective analysis included 125 consecutive patients (60 females, 65 males; median age 27 years, range 7–49 years), who received genotypically HLA-identical sibling marrow transplants for treatment of AML or ALL between December 1975 and 1988 at the University Hospital Essen. Ninety-two patients had AML (first CR  $n=55$ , second CR  $n=16$ , after second CR  $n=21$ ) and 33 patients had ALL (first CR  $n=14$ , second CR  $n=12$ , after second CR  $n=7$ ). Four different schedules of total body irradiation (TBI) were employed over the period covered by this analysis: 8.6 Gy single-dose TBI delivered from a linear accelerator (Linac) ( $n=35$ ) (dose rate, 12 cGy/min); 8.6 Gy single-dose TBI delivered from a cobalt-60 source (dose rate, 18 cGy/min) ( $n=6$ );  $4 \times 2.5$  Gy fractionated TBI (Linac) over 4 days (dose rate, 12 cGy/min) ( $n=14$ ); and  $4 \times 2.5$  Gy fractionated TBI (cobalt-60) over 4 days (dose rate, 4 cGy/min) ( $n=56$ ) [2]. Fourteen AML patients received busulfan 4 mg/kg body wt. on each of four consecutive days. TBI and busulfan were preceded or followed by cyclophosphamide 120 mg/kg body wt. administered over two consecutive days in 123 patients. The remaining two patients received etoposide

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60 mg/kg body wt. in conjunction with cobalt-60 fractionated TBI. Two different protective isolation systems were employed: 31 patients were treated in laminar down flow units and 94 patients were nursed in ultra-clean reverse isolation rooms [3]. For prophylaxis of acute graft-versus-host disease (GvHD), patients were either treated with methotrexate (MTX) intermittently ( $n=78$ ) [4] or given a short course of MTX in combination with cyclosporin (CSP) ( $n=47$ ) [5]. Diagnosis of relapse was based on bone marrow examination as well as peripheral blood smears. Whenever possible, informative markers (generally sex chromosome differences) were used to distinguish between host-type recurrence or the origin of leukemia from donor cells. In none of the relapsing patients could leukemia of donor cells be detected.

### *Statistical Analysis*

The event-free survival estimate and the probability of leukemic recurrence were calculated by the product-limit estimates of Kaplan and Meier [6]. For estimation of the probability of leukemic recurrence, patients who died in continuous remission were excluded from analysis from the time of their death. Tests of equality over stratified groups were performed using the log-rank test or Wilcoxon test. Categorical variables were compared by Fisher's two-tailed exact test. A time-dependent stepwise proportional hazards general linear (PHGLM) model was used to look for associations between leukemic recurrence and different covariables [7]: These included the preparative regimen employed, postgrafting prophylaxis of acute GvHD, isolation procedures, occurrence of acute or chronic GvHD, patient/donor age, sex-match and blood groups, diagnosis of AML vs. ALL, and disease stage at transplant. With regard to disease stage, two types of analyses were performed: patients were either divided into two disease-stage categories (first CR vs non-first CR) or were entered with a three-level category of disease stage. The latter analysis was based on a hazard of 1 for first CR patients, of 2 for second CR patients, and of 3 for patients after second CR, respectively. All analyses

were performed on an IBM 4361 computer using a statistical software package (LIFE-TEST, NPAR1WAY, UNIVARIATE and PHGLM procedures, SAS statistics, SAS Institute Inc., Cary, NC, United States). Date of analysis was 2 February 1989.

### **Results**

In 21 of 125 patients (17%), leukemic relapse occurred between 1 and 78 months after BMT. Only 3 of these 21 relapse events (14%) were observed beyond 2 years from transplantation. Four of 69 patients (6%) receiving marrow grafts in first CR of AML or ALL relapsed. This contrasts significantly to the 30% relapse incidence of 56 patients, who were grafted in more advanced disease stages ( $P<0.001$ , Fisher's two-tailed exact test). The 5-year relapse probability was 7% ( $SE \pm 4\%$ ) for patients grafted in first CR compared with 38% ( $SE \pm 13\%$ ) in second CR and 80% ( $SE \pm 12\%$ ) after second CR or in relapse, respectively ( $P=0.0001$ , Wilcoxon test) (Fig. 1). No significant influence of underlying disease (AML vs. ALL), different preparative regimens, isolation procedures, posttransplant immunoprophylaxis of acute GvHD, or patient/donor characteristics on leukemic recurrence could be detected by univariate analysis based upon the whole patient population. In the PHGLM model disease stage was also the sole covariable, which was significantly associated with an increased relapse risk (Table 1). After adjustment for disease stage, a significantly higher relapse rate was found in patients grafted after first CR who did not develop acute GvHD as compared with those with acute GvHD by univariate analysis ( $P=0.04$ ,  $\chi^2$ -test;  $P=0.04$ , Fisher's exact test). The 3-year relapse probability for patients without acute GvHD was 64% in contrast to 0% in those with acute GvHD ( $P=0.08$ , log-rank test). Again, underlying disease as well as treatment-related covariables and patient/donor characteristics were not associated with an increased risk of leukemic recurrence in different disease stages. After subdividing into disease stages, none of the above-mentioned covariables reached significance to enter the PHGLM model. In terms of overall event-

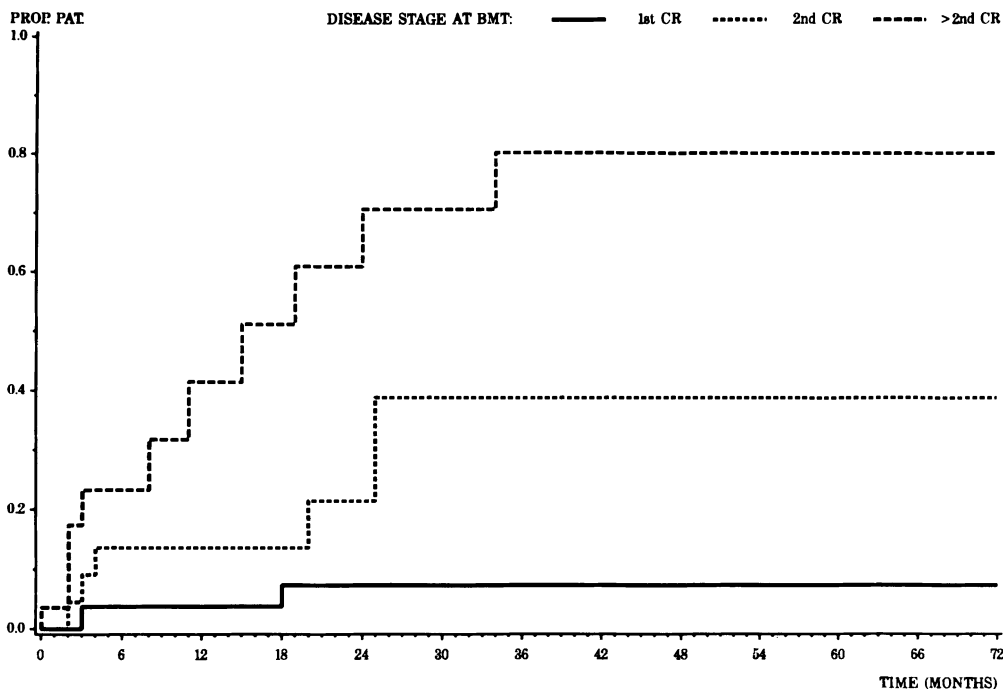


Fig. 1. Product limit relapse estimate after allogeneic BMT for acute leukemia: influence of disease stage at BMT ( $n=125$  February 1989)

Table 1. Influence of disease stage on risk of leukemic relapse

Disease stage at BMT	Relapse frequencies	Five-year relapse probabilities	Relative risk estimate <sup>d</sup>
First CR	4/69 <sup>a</sup>	7% ± 4%	1
Second CR	6/28 $P < 0.0001$ <sup>b</sup>	38% ± 13% $P = 0.0001$ <sup>c</sup>	3.7
>Second CR + relapse	11/28	80% ± 12%	13.7

<sup>a</sup> Proportion of patients

<sup>b</sup> By  $\chi^2$  test

<sup>c</sup> By Wilcoxon test

<sup>d</sup> Derived from time-dependent proportional hazards general linear model (after adjusting for disease stage, the following covariables did not reach statistical significance: underlying disease, preparative regimen, immunoprophylaxis for acute graft-versus-host disease, protective isolation system, patient/donor age, sex, or sex match)

free survival, disease stage was likewise the sole covariable significantly affecting the outcome after BMT in the PHGLM model. The 5-year overall event-free survival estimate of patients receiving BMT in first CR was 51% (SE ± 7%) as compared with 27% (SE ± 7%) in those after first CR.

## Discussion

The present analysis of 125 patients receiving allogeneic sibling marrow transplants as treatment of acute leukemia underlines the major importance of disease stage on the curative potential of BMT. It has to be em-

phasized that BMT in first CR of acute leukemia was the sole factor which concomitantly diminished relapse risk and favorably affected overall event-free survival. In contrast, we were unable to demonstrate any association between procedural factors and relapse probability, and this was especially true for the different myeloablative regimens employed. The 5-year relapse probabilities of 38%–80% in patients treated after first CR elucidates the incomplete efficacy of BMT in advanced disease stages and demonstrates that more effective preparative regimens are urgently needed to overcome the increased resistance and/or greater tumor burden in more advanced disease stages. Although some uncontrolled trials of new radiochemotherapeutic preparative regimens have suggested impressive antileukemic efficacy, there is currently no proof that these regimens would substantially reduce the relapse risk and improve survival of patients in advanced disease stages as compared with the broadly employed “standard” regimen of cyclophosphamide in conjunction with TBI. As long as preparative regimens with proven increased antileukemic efficacy are not available, it appears as a reasonable option to subject patients to BMT in first CR, who are prone to relapse under conventional postinduction chemotherapy. This provides that generally accepted prognostic factors for “high-risk” (in terms of relapse) leukemia enable patients to be identified who might benefit from either early or delayed BMT.

In considering that a very low number of relapse events occurred more than 2 years after BMT in our patients, it appears justified to assume that the pattern of relapse is different from that under conventional chemotherapy. Although leukemic recurrence has sporadically been reported up to 6.5 years after BMT, it is well established that patients beyond 2 years after BMT have a verified low risk of relapse [8] and most of these patients will eventually be cured.

This analysis demonstrates that modifications of the preparative regimens or the posttransplant treatment protocols employed in our patients had no influence on the relapse frequency after BMT. The 5-year

relapse probability of 7% provides further evidence to support the suggestion that performing BMT in first CR of acute leukemia currently represents the best option to reduce the risk of leukemic recurrence. To improve the chance of cure for patients in more advanced disease stages, more effective preparative regimens for BMT have to be developed.

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## Comparison of Allogeneic and Autologous Bone Marrow Transplantation for Treatment of Acute Lymphocytic Leukemia in Childhood

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Currently, chemoradiotherapy with bone marrow transplantation from an allogeneic HLA-matched sibling donor provides the best prospect to treat patients with leukemia who relapsed or who are at very high risk for relapse after primary induction therapy. However, it is only a minority of patients with acute leukemia who have a matched allogeneic marrow donor [1, 2]. In this report we compare the results of allogeneic and autologous marrow transplantation in patients with high-risk acute lymphoblastic leukemia (ALL).

### Materials and Methods

#### *Patients*

Fifteen children who had HLA-identical and MLC-negative sibling donors received allogeneic marrow transplants. Twelve patients were in second remission, two in first remission, and one in third remission. Two children with first remission were very high risk patients (BFM risk factors: 2.04 and 2.05, [3]). The relevant clinical data at presentation of the disease are given in Table 1.

Fifteen patients without a matched sibling donor received autologous marrow (14 unpurged, 1 purged marrow with a mixture of the three antibodies VIL-A1, VIB-C5, VIB-E3 plus human complement, W. Knapp, Vienna). Six patients out of 15 had initially

very high risk features concerning the BFM risk grouping, and two were prednisone non-responders and were autografted in first complete remission. Seven patients were transplanted in second and two in third remission.

About 30% of patients in both groups had white cell counts over 50000/mm<sup>3</sup> at time of diagnosis. Previous extramedullary relapses are shown in Tables 2 and 3. Initial marrow relapse occurred in the allogeneic group while receiving chemotherapy in four patients and after chemotherapy had been discontinued in nine patients.

#### *Conditioning Therapy*

*Allogeneic BMT.* All patients received cyclophosphamide (60 mg/kg) on two consecutive days and total body irradiation (TBI). Eight patients received 10 Gy TBI in a single dose, and seven patients received 12 Gy given as 2 × 2 Gy for three consecutive days. Patients were given two prophylactic doses of intrathecal methotrexate (MTX 12 mg/m<sup>2</sup>). Prophylaxis for graft-versus-host disease (GvHD) consisted of MTX and prednisone in 11 patients, MTX, cyclosporin A, and prednisone in 3 patients, and cyclosporin A in 1 patient.

*Autologous BMT.* Thirteen patients received cyclophosphamide 60 mg/kg on two consecutive days and two patients received 50 mg/kg on four consecutive days. Five patients received 10 Gy TBI in a single dose, six patients 12 Gy (2 × 2 Gy for three consecutive days), and two patients 9.9 Gy administered

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**Table 1.** Patient characteristics

Transplantation group	Allogeneic BMT		Autologous BMT	
	<i>n</i>	%	<i>n</i>	%
Patients, total	15	100	15	100
Median age (years/months)	9/0		7/6	
Age, range (years/months)	3/0–14/7		1/5–11/1	
Boys	12	80	8	53
Girls	3	20	7	47
Months from diagnosis to BMT	14–67		5–72	
Months from last relapse to BMT	3–32		1–21	
Median	4		9	
White blood cell count diagnosis				
10 GPT/liter	6	40	5	36
10–50 GPT/liter	4	27	5	36
50–100 GPT/liter	2	13	1	7
100 GPT/liter	3	20	3	21
Primary CNS involvement	–	–	3	21
Primary mediastinal mass	2	13	2	13
Primary lymph node enlargement	8	53	12	86
Enlargement of liver 5 cm	3	20	7	50
Enlargement of spleen 5 cm	4	27	8	57
Initial therapy: study V (Memphis)	3	20	2	13
VI (LSA <sub>2</sub> L <sub>2</sub> )	1	7	–	–
VII (BFM)	11	73	11	73
VIII (BFM)	–	–	2	13
BFM–RF: range	0.04–2.05		0.40–1.96	
median	0.94		1.29	
Risk group: SR	10	67	6	40
MR	3	20	4	27
HR	2	13	5	33

as 3.3 Gy for 3 days. Two patients were conditioned without TBI. They received cyclophosphamide (4 × 50 mg/kg) and busulfan 16 mg/kg on four consecutive days. Preparation for engraftment included two doses of intrathecal methotrexate (12 mg/m<sup>2</sup>). Lungs were shielded in the allogeneic and autologous BMT in the single-dose procedure at 8 Gy and in the fractionated TBI group at 10 Gy.

All patients were treated in laminar air flow rooms with total decontamination (neomycin, colimycin, nystatin). Prophylaxis against *Pneumocystis carinii* pneumonia consisted of trimethoprim sulfamethoxazole administered from day –14 to day +180.

### Statistical Analyses

Disease-free survival was calculated by the Kaplan-Meier plots [4].

## Results

### Engraftment

As shown in Table 4, the median time to engraftment, and thus the length of the neutropenic risk period, was similar in the allogeneic and autologous group. Patients with autologous BMT had a longer period of thrombocytopenia (42 vs. 28 days). The 100-day survival was the same in both groups.



**Table 2.** Summary of clinical data of patients with allogeneic transplantation

Patient No.	Patient name	Sex	Age at BMT (years/months)	Initial risk BFM	Site of relapse before BMT	Remission status at BMT	Conditioning	Interval		GVHD		Result of BMT	
								First diagnosis (months)	Relapse - BMT (months)	A	C	Survival in CCR (months)	Complications/cause of death
6	H. B.	M	9/0	0.62	BM	2nd CR		61	6	-	-	64 <sup>a</sup>	-
7	D. P.	M	8/0	0.94	BM	2nd CR		41	4	-	L	16	Septicemia
8	M. H.	M	9/0	0.61	BM	2nd CR		51	3	II	E	59 <sup>a</sup>	-
14	P. B.	M	4/8	0.82	BM	2nd CR	TBI	25	3	I	-	47 <sup>a</sup>	-
15	T. K.	M	13/8	2.04	-	1st CR	1 × 10.0 Gy	32		I	E	46 <sup>a</sup>	-
17	S. K.	M	10/4	2.05	-	1st CR		14		II	-	44 <sup>a</sup>	-
20	S. K.	M	7/4	1.24	BM	2nd CR		12	4	I	-	3	Leukemia
21	M. S.	M	12/10	0.04	BM + testis	2nd CR		60	4	III	-	1	AGVHD/cerebral bleeding
37	S. T.	F	9/1	1.12	BM	2nd CR	2 × 60 mg/kg cyclophosphamide	28	4	-	-	18	Relapse/remission
38	U. S.	M	10/9	1.28	BM	2nd CR		32	5	II	E	22 <sup>a</sup>	-
44	A. A.	M	3/0	0.84	BM	2nd CR		17	4	-	E	11 <sup>a</sup>	-
46	A. P.	F	7/0	0.76	BM/CNS	3rd CR	TBI	38	9	-	-	10 <sup>a</sup>	-
51	M. R.	M	4/2	1.38	BM + CNS	2nd CR	6 × 2.0 Gy	17	4	I	-	7	Relapse/-
59	M. K.	M	9/10	0.76	BM + CNS	2nd CR		53	9	-	-	4 <sup>a</sup>	-
67	A. M.	F	14/7	1.02	2nd CR	2nd		56	3	-	-	1 <sup>a</sup>	-

<sup>a</sup> Alive and well

**Table 3.** Summary of clinical data of patients with autologous transplantation

Patient No.	Patient name	Sex	Age at BMT (years/ months)	Initial risk RF BFM	Site of relapse before BMT	Remission status at BMT	Conditioning		Interval		Result of BMT	
							CP (mg/kg)	TBI (Gy)	BU (mg/kg)	First/ diagnosis - relapse (months)	Relapse - BMT (months)	Survival in CCR (months)
26	A. F.	M	8/0	?	BM	2nd CR	2 × 60	1 × 10	55	17	26	Relapse/leukemia
28	T. B.	M	9/4	1.24	BM/testis	3rd CR	2 × 60	1 × 10	71	1	30 <sup>a</sup>	-
29	S. P.	M	5/6	1.10	BM	2nd CR	2 × 60	1 × 10	30	6	29 <sup>a</sup>	-
31	S. B.	F	7/2	1.22	BM/BM	3rd CR	2 × 60	1 × 10	56	4	7	Relapse/leukemia
33	H. B.	F	8/4	1.80	-	1st CR	2 × 60	1 × 10	-	11	2 days	Congenital heart failure
34	A. D.	M	1/11	1.70	-	1st CR	2 × 60	6 × 2	-	6	6	Relapse/interstitial pneumonitis in remission
36	M. K.	F	7/6	1.90	-	1st CR	2 × 60	6 × 2	-	5	14 <sup>a</sup>	-
39	M. P.	M	1/5	1.78	-	1st CR	2 × 60	6 × 2	-	17	3	Relapse/leukemia
40	A. S.	F	9/6	0.78	BM	2nd CR	2 × 60	3 × 3.3	42	5	20 <sup>a</sup>	-
41	K. K.	F	6/10	1.35	BM	2nd CR	2 × 60	3 × 3.3	49	10	20 <sup>a</sup>	-
53	S. R.	M	8/4	0.82	BM	2nd CR	4 × 50	-	5	21	5 <sup>a</sup>	-
55	S. R.	F	3/2	1.96	-	1st CR	2 × 60	6 × 2	-	8	5 <sup>a</sup>	-
57	M. M.	M	6/6	0.86	BM	2nd CR	2 × 60	6 × 2	29	9	5 <sup>a</sup>	-
63	M. K.	M	9/3	1.42	-	1st CR	4 × 50	-	10	10	2 <sup>a</sup>	-
69	M. G.	F	11/1	0.59	BM + CNS	2nd CR	2 × 60	6 × 2	40	13	6 days <sup>a</sup>	-

<sup>a</sup> Alive and well

**Table 4.** Time to engraftment

Engraftment	Time of engraftment	
	Allogeneic (n = 15)	Auto- logous (n = 15)
Leukocytes (> 1000/mm <sup>3</sup> )	17 days	19 days
Platelets (> 40000/mm <sup>3</sup> )	28 days	42 days
Reticulocytes (> 10%)	23 days	32 days
100 day survival	11/14 <sup>a</sup>	11/13 <sup>a</sup>

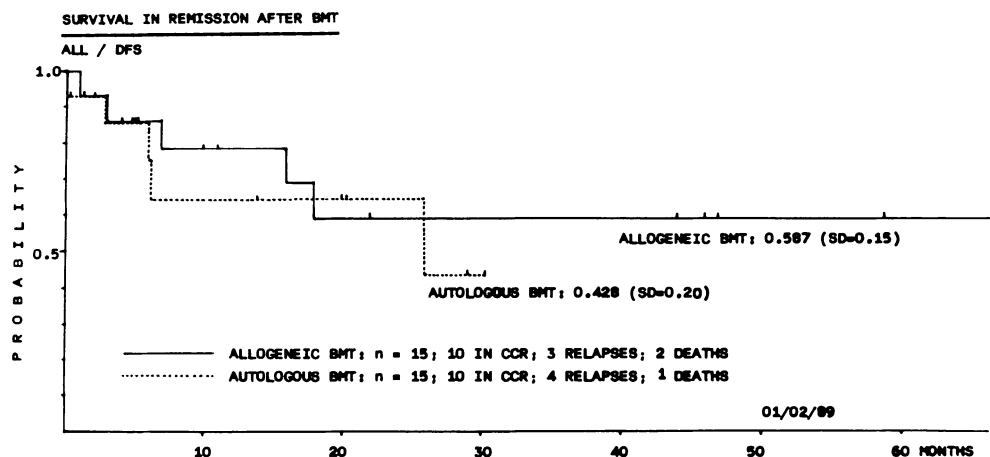
<sup>a</sup> The observation time of one patient in the allogeneic and two patients in the autologous group was shorter than 100 days

### Mortality and Cause of Death

The clinical causes of death are shown in Tables 2 and 3. After allogeneic grafting three patients relapsed. One patient died of *Streptococcus pneumoniae* septicemia, one patient died of cerebral bleeding during acute graft-versus-host disease grade III. In the autografting group four patients relapsed after 3, 6, 7, and 26 months. One patient died on day +2 from congestive heart failure. No case of interstitial pneumonitis after allo- and autografting was observed.

### Disease-Free Survival

Overall, 10 of 15 children after allogeneic BMT and 10 of 15 children after autografting survive relapse-free (Tables 2, 3). The Kaplan-Meier product-limit estimates show a disease-free survival at 5 years of 58% in patients with allogeneic grafts. As shown in Fig. 1, 43% children with autologous grafts survive disease-free at 2½ years. The estimates of the chance of relapse in the 5 years after allogeneic and in the 2½ years after autologous transplantation were 28% ± 14% and 54% ± 25%, respectively (Fig. 2). Among the patients who received allogeneic transplants for ALL in second remission, we investigated the effect of the duration of the initial remission on the rate of relapse after BMT. Children whose first remissions had a duration of more than 24 months, tended to have a higher probability of long-term disease-free survival. In the three relapsed patients the initial remission duration was 12, 17, and 28 months. The interval between relapse and BMT in these patients was 4 months (Table 2). In the autologous-graft subgroup, two of four patients with relapse had a duration of first remission of less than 18 months. In the allogeneic-graft subgroup relapses occurred 3, 7, and 18 months post-grafting. No relapses occurred in patients with chronic GvHD. The relapses in the autologous-graft subgroup occurred 3, 6, 7, and 26 months after BMT.



**Fig. 1.** Kaplan-Meier product limit estimates for probability of disease-free survival after allogeneic and autologous marrow transplantation

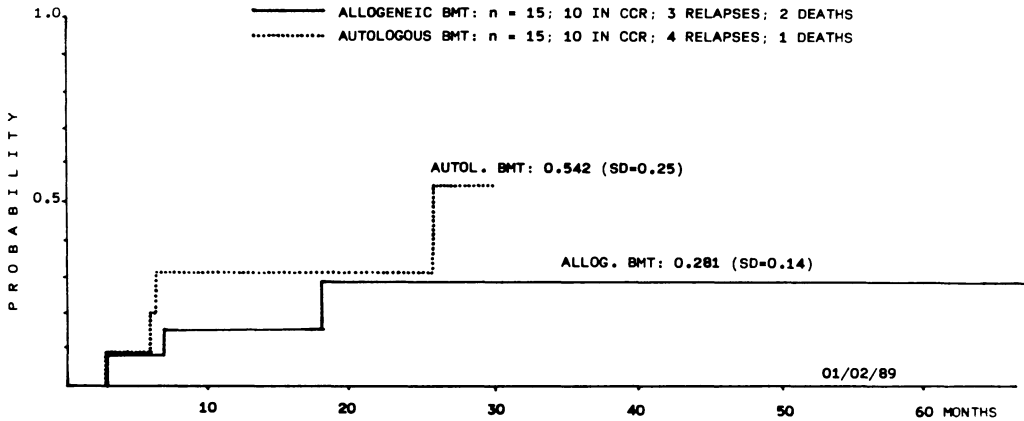


Fig. 2. Cumulative probability of leukemic relapse after allogeneic and autologous transplantation

**Discussion**

This report represents our results with allogeneic and autologous bone marrow transplantation in high-risk children with acute lymphoblastic leukemia in remission. The conditioning regimen for both types of transplantation was similar. Only two patients in the autologous-graft subgroup were conditioned without TBI with busulfan and cyclophosphamide. Previous single-institution prospective studies of children with ALL in second remission suggested that marrow transplantation was superior to chemotherapy [7–10]. However, the therapy of childhood ALL is improving both with chemotherapy and with marrow transplantation [5, 6].

Nevertheless, on the basis of the data of recent reports, children with ALL in second or third remission who have HLA-identical siblings are candidates for marrow transplantation [6, 11–13]. On the other hand, the indication for autologous marrow transplantation in children with ALL is not exactly defined. Therefore we report a single-institution study of 30 patients, comparing autologous and allogeneic BMT.

The marrow was purged only in one case. A probability of event-free survival of only  $29\% \pm 12\%$  in the high-risk group (32 patients of 524) in a modified BFM study, ALL VII-81, stimulated us to transplant

these very high risk patients in first complete remission [14].

The two children with allogeneic transplantations in first remission are disease free at 46 and 44 months. The event-free survival for six patients autografted in first remission is  $31\% \pm 25\%$  at 15 months. As Tables 2 and 3 show, there were no differences in peri-transplantation mortality between autologous and allogeneic transplantation. It is of particular note that the probability of relapse was 28% at 5 years after transplantation for the allogeneic-graft subgroup including patients with first, second, and third remission whereas the probability of relapse for the autologous-graft subgroup was 54% at 2½ years (patients in first–third remission). Kersey et al. [1] reported that the outcome in patients with autologous grafts may generally be determined by the end of 1 year.

Our findings concerning the overall incidence of acute and chronic GVHD are not appreciably different from those of other groups [6]. Two patients in our series with grade I acute GVHD relapsed. We found, as in other recently published reports, an association between the presence of chronic GVHD and a low relapse rate [6].

The results in patients with autologous and allogeneic marrow transplantation must be seen in comparison with those in patients receiving chemotherapy. Preliminary results of the BFM group are encouraging in that

they show chemotherapy alone can result in long-term survival [5]. With regard to the need for more effective pretransplantation cytoreductive regimens in allogeneic and autologous BMT, the BFM relapse therapy strategy for preparation could improve the transplantation results.

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## High-dose Chemotherapy with Noncryopreserved Autologous Bone Marrow Transplantation for Acute Myeloid Leukemia in First Complete Remission

H. Koepler, K. H. Pflueger, M. Wolf, R. Weide, and K. Havemann

### Introduction

Conventional chemotherapy as the TAD (thioguanine, Ara-C, daunorubicin) regime is able to induce a high number of complete remissions in the range of 60%–70% in patients with AML. Long-term survival, however, is poor due to a high relapse rate [1, 2]. Strategies to stabilize remission include maintenance therapy, intensification therapy or ablative forms of therapy with allogeneic or autologous bone marrow transplantation. While maintenance therapy gives a statistically significant but only small advantage in disease-free survival, the role of intensification with conventional chemotherapy as double induction or late intensification has not been clearly established. Initial results show an increased number of disease-free survivors [3, 4]. Increased long-term survival has been achieved with ablative therapies and allogeneic bone marrow transplantation resulting in 5-year survival rates between 40% and 50% [5, 6]. This approach, however, is limited to patients < 40 years with an HLA-identical-related donor. Recently, similar results in terms of disease-free survival have been reported by groups using an ablative therapy with an autologous bone marrow transplantation (ABMT) [7–9]. We report preliminary results of a phase I–II study with high-dose chemotherapy using escalating doses of

cyclophosphamide, etoposide, and Ara-C combined with an autologous noncryopreserved bone marrow transplantation for patients with AML in first complete remission.

### Patients and Methods

#### *Patients*

Seven patients with AML in first complete remission (CR) were treated with high-dose chemotherapy and ABMT 2–4 months after achieving CR. Age, sex, French-American-British (FAB) classification, and prior chemotherapy are shown in Table 1.

#### *Bone Marrow Procurement*

The methods of obtaining bone marrow and liquid storage have been described elsewhere [10]. Briefly: 750 ml/m<sup>2</sup> bone marrow was removed from the posterior iliac crest and transferred into CPDA 1 blood bags. The blood bags were stored in a refrigerator for 48–72 h at 4°C. Before reinfusion the marrow was filtered and then reinfused over a central line. Previous studies have shown that liquid storage in this way results in survival of stem cells as determined by survival or CFU-GM of 70%–80% at 48 h and 50%–60% at 72 h.

#### *Chemotherapy*

Patients L1–3 received cyclophosphamide 60 mg/kg day –2, etoposide 800 mg/m<sup>2</sup>

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**Table 1.** Patient characteristics and outcome

Patient no.	Age/sex	Diagnosis/ FAB classification	Prior chemotherapy	Disease-free survival after ABMT (months)
L1	25 ♂	AML M4	2 × TAD 1 × AD	17+
L2	43 ♀	AML M4	2 × TAD	8-
L3	48 ♀	AML M5	2 × TAD	9+
L4	40 ♂	AML M4	2 × TAD	5+
L5	62 ♀	AML M5	2 × TAD 1 × AD	5+
L6	35 ♂	AML M2	3 × TAD	4+
L7	45 ♂	AML M3	2 × TAD 1 × AD	1+

days -2, -1, and Ara-C 1000 mg/m<sup>2</sup> q12 h (five doses). Patients L4-6 received cyclophosphamide 60 mg/kg day -3, etoposide 700 mg/m<sup>2</sup> days -3, -2, -1, and Ara-C 1000 mg/m<sup>2</sup> q12 h (six doses). Patient L7 received cyclophosphamide 60 mg/kg days -3, -2, etoposide 700 mg/m<sup>2</sup> days -3, -2, -1, and Ara-C 1000 mg/m<sup>2</sup> q12 h (six doses).

### Results

All patients had a full hematological recovery. The mean time of neutropenia (neutrophils <500/μl) was 14 days (range 9-24 days), and the mean time of thrombocytopenia (platelets <20 000/μl) was 9 days (range 7-11 days). The acute toxicity of chemotherapy was tolerable and included mild to moderate nausea/vomiting, mucositis, and diarrhea, as shown in Table 2. The outcome in terms of disease-free survival is shown in Table 1. One patient relapsed 8 months after ABMT. Six patients remain in CR 17+, 9+, 5+, 5+, 4+, and 1+ months after ABMT.

**Table 2.** Nonhematological side effects

		No. patients/ No. treated patients
Nausea/ vomiting	(WHO grade 2/3)	7/7
Mucositis	(WHO grade 2/3)	6/7
Diarrhea	(WHO grade 1/2)	2/7
Fever	(WHO grade 3/4)	5/7

### Discussion

Recently, a promising number of disease-free survivors have been reported by groups using ablative therapies with ABMT as consolidation for patients with AML in first CR [6-8]. Though randomized trials are lacking, this approach seems to be superior to conventional chemotherapy and equal to allogeneic bone marrow transplantation. However, there are several issues under discussion as the optimal type of ablative chemotherapy, the role of total body irradiation, and approaches to purge the autologous marrow in vitro. In the study reported here, our aim was to give maximal antileukemic therapy with a safe and minimal risk involving easy procedure in terms of ablative therapy and bone marrow preservation. In a three-step escalating schedule for combination chemotherapy with cyclophosphamide, etoposide, and Ara-C, the nonhematological toxicity limit has not been reached and side effects were tolerable. The liquid storage of bone marrow was safe with a full hematological recovery in all patients. Due to the short observation time, the efficacy of this approach is not yet evaluable. One patient relapsed 8 months after ABMT. As most of the patients in this study had the FAB classification M4 or M5, subgroups with a poorer prognosis, and six out of seven patients are >35 years of age, a factor also correlated with a poorer prognosis, a greater number of patients with "standard" risk will be needed to answer this question.

In conclusion, high-dose chemotherapy with noncryopreserved ABMT is a safe pro-

cedure and may be useful as consolidation therapy for patients with AML in first complete remission.

### Summary

Seven patients with acute myeloid leukemia (AML) in first complete remission were treated with escalating high doses of cyclophosphamide, etoposide, and cytosine arabinoside (Ara-C). In all patients autologous bone marrow preservation was performed prior to therapy. Bone marrow was stored in blood bags in a refrigerator for 48–72 h at 4°C and then reinfused over a central line. All patients had a full hematological recovery. The mean time of neutropenia (neutrophils < 500/μl) was 14 days (range 9–24 days), and the mean time of thrombocytopenia (platelets < 20 000/μl) was 9 days (range 7–11 days). The nonhematological toxicity was tolerable with mild to moderate nausea/vomiting, mucositis and diarrhea, and so far not dose-limiting. Six patients remain in complete remission 17+, 9+, 5+, 5+, 4+, and 1+ months after autotransplantation. One patient relapsed 8 months after autotransplantation.

High-dose chemotherapy with noncryopreserved bone marrow autotransplantation may be useful as intensified consolidation for patients with AML in first complete remission.

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## Myelopoietic Reconstitution Following Autologous Bone Marrow Transplantation \*

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### Introduction

Autologous bone marrow transplantation (ABMT) is increasingly being considered as a therapy for patients with hematological malignancies. One of its major obstacles is the prolonged aplasia following the procedure that can be shortened by the administration of hematopoietic growth factors [1, 2]. However, little is known about the *in vivo* significance of these substances. Further, hematopoietic recovery may be impaired by cryopreservation damage to the stem cells. We therefore studied the colony-stimulating activity (GM-CSA) in sera of patients undergoing ABMT and the influence of different cryopreservation procedures on stem-cell proliferation.

### Material and Methods

#### *Clinical Methods*

One child (ANLL/M6/CR1) and five adults (ALL/CR2; ANLL/M2/CR2; ANLL/M5/CR2; 2 × ANLL/M5/CR1) were transplanted 3 months (2–7) after achieving complete remission with a conventional chemotherapy regimen. Nuclear bone marrow cells were enriched by centrifugation and frozen according to the procedures specified below (PN1–5). For technical reasons the PN6

graft was processed differently [3]. Patients were treated with fractionated total body irradiation (total 6 × 2 Gy; lungs 9 Gy; testes 16 Gy) and etoposide (PN1–5: 50 mg/kg; 1 mg/ml in 0.9% NaCl over 4 h) or cyclophosphamide (PN6: 2 × 60 mg/kg on two consecutive days). Two days later the frozen marrow cells were quickly thawed at 37°C and reinfused intravenously. Nursing was carried out with conventional reversed isolation. Oral amphotericin B and cotrimoxazol were given for selective intestinal decontamination. Febrile episodes were treated by cef-tazidime and netilmycin. Additional vancomycin was given when necessary. Blood counts and differentials were performed daily. Sera from all patients were collected twice weekly. All serum samples were sterilized by radiation and stored at –80°C before further processing.

#### *Stem Cell Assay*

*In vitro* stem cell proliferation was determined using a CFU-C test [4]. Briefly, 10<sup>5</sup> mononuclear bone marrow cells of healthy volunteer donors were suspended in 1 ml semisolid medium (30% test serum, 5% GCT medium, 0.9% methylcellulose in Dulbecco's Minimal Essential Medium (DMEM)). Test sera were either the sequentially collected probes or serially diluted sera (0.3%–5%) of a particular day after ABMT used together with a constant amount of growth-supporting fetal calf serum (FCS) (20%). All tests included controls with FCS, which was shown to support CFU-C growth

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in former assays. Cell suspensions were dispersed into 35-mm Lux Petri dishes and incubated at 5% CO<sub>2</sub>, 100% humidity, and 37°C for 14 days. Thereafter colonies of more than 40 cells were counted. All tests were performed in triplicate and mean values were calculated.

### *Cryopreservation*

Buffy-coat-enriched nuclear bone marrow cells were adjusted from  $2 \times 10^7$  to  $10^8$ /ml in freezing medium [60% autologous plasma, 10% dimethyl sulfoxide (DMSO) and 30% TC-199 medium], filled into Gambro-DF-700 bags, and molded to a defined volume ( $120 \times 145 \times 4$  mm) by two metal plates. Additional 2-ml probes at  $2 \times 10^7$  cells/ml were transferred into cryopreservation vials. Freezing of probes and bags was performed in a liquid nitrogen controlled-rate freezer (Planer BF-R-201) following three different procedures: (1) discontinuous cooling of the freezing chamber from 0°C to -80°C, (2) continuous (-1°C/min) temperature reduction to -80°C, and (3) continuous cooling with supercooling during the transition phase (graph 1). All probes were then cooled from -80°C to -150°C at a rate of -3°C/min and thereafter transferred to the liquid phase of a nitrogen storage container. Temperatures in bags and chamber were charted separately. Cell samples were tested for CFU-C growth before freezing and after they had been cryopreserved for 1 day. The proportion of CFU-Cs recovered after thawing was calculated.

## **Results and Discussion**

### *Clinical Course*

We observed no toxic deaths following ABMT and our patients were discharged from hospital on day 32 (25–65). However, hematotoxicity was more pronounced than after any other myelosuppressive therapy (including high-dose cytarabine and allogeneic bone marrow transplantation): five patients needed platelet support for 50 days (8–65 days) and one patient did not recover from thrombocytopenia. Leukocytes ex-

ceeded 1000/ $\mu$ l only at day 31 (days 20–55) and all patients had fever (1–6 days) and needed intravenous antibiotic treatment for 15 days (12–37 days). Four patients were additionally treated with vancomycin. Similar observations have been published [5–11]. This severe, prolonged cytopenia may be partially explained by stem cell toxicity of antibiotics or by the residual disease [7, 11], leading to relapse (on days 130–323) in four of our cytopenic patients. However, insufficient stem cell repopulation capacity may have further impaired the recovery of our patients.

### *Serum Colony-Stimulating Activity*

Serum hematopoietins, though used in several clinical trials to advance the reconstitution [1, 2], have not been studied so far following myeloablative procedures. We investigated here the serum GM-CSA levels in patients undergoing ABMT. It was of interest that GM-CSA was elevated in all patients at times when engraftment was not detectable (Table 1). This was most impressively seen in PN4, who had prolonged aplasia. We therefore concluded that GM-CSA was produced by cells resistant to the conditioning regimen. Controversially, two reports described serum megakaryocyte-stimulating activity (Meg-CSA) only after engraftment had occurred [12, 13]. Meg-CSA-producing cells were thus destroyed by the aplasia-inducing therapy, explaining longlasting thrombocytopenias and favoring Meg-CSA substitution following ABMT. Sera collected at a later phase did not stimulate stem cell proliferation. The decrease of GM-CSA was due to an inhibiting serum activity, as proven when increasing serum concentrations were added to CFU-C tests containing a constant amount of FCS (Table 2). This inhibiting activity may have been of granulocytic origin [14] because it occurred only after granulopoiesis was documented. Similar experiments with sera of patients after high-dose cytarabine treatment showed variable GM-CSA levels (data not shown), suggesting a less myelosuppressive action of this treatment.

**Table 1.** GM-CSA serum activity. The number of colonies that grew from healthy normal bone marrow samples with the serum of a patient following ABMT (PNn) collected on a particular day (week) is shown. If more than one serum was tested/week, mean values were given. On day 0 the bone marrow was reinfused

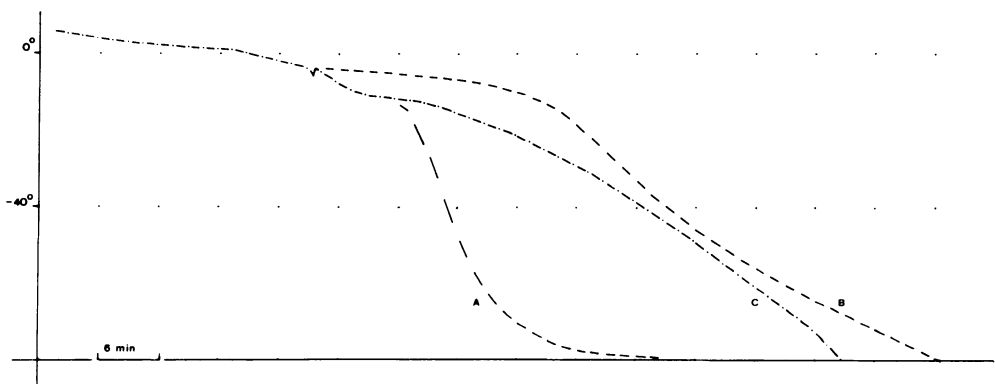
Day	PN1	PN2	PN3	PN4	PN5	PN6
-32			28			33
-10				44		
-9		0				
-5	21					54
-3					0	60
0	11		2		27	
2		38	16			35
3	73				41	
4			0			
5		99				88
7			53	50	81	
8	32	0				
10			32			60
11	43			69	14	
12		0				63
14	16		0		7	71
15		0		36		
17	16		1			
18				54		
19		0				
21	4			44	11	91
22-28	0	3		41	3	35
29-35	15	14		40	50	
36-42		19		44		
43-49	27	0	29	28	31	
50-56	31	30	29	20	16	
63-70				18	14	
71-77					25	
78-84			17		27	
85-91	0			30		

### Cryopreservation

Although the clinical significance of transfused CFU-Cs for the hematopoietic recovery following ABMT was controversially debated [15-17], we, like others, used the CFU-C assay to demonstrate stem cell integrity. We confirmed that high cooling rates following the crystallization [18, 19] and long transition times [19, 20] destroyed CFU-C proliferation: In program A (Fig. 1) the temperature in the freezing chamber was cooled discontinuously from 0°C to -80°C. Following a transition time of only 8 min, a steep temperature decline in the sample could be observed. CFU-C recovery of cells frozen according to this program was only 16% (Table 3). When the freezing chamber was cooled continuously (1°C/min; program B) the transition time was prolonged to 25 min and 42% CFU-C could be recovered. Using supercooling (program C) we were able to reduce the transition time to 16 min and to increase the CFU-C recovery to 71%. It is of note that at least 30% of

**Table 3.** Recovery of CFU-C. The amount of CFU-C (range) after cryopreservation according to programs A, B, or C is shown in comparison to nontreated samples

Program	n	CFU-C %
A	5	16 (0-81)
B	6	42 (0-83)
C	9	71 (31-91)



**Fig. 1.** Freezing procedure. The temperature reduction in the sample is depicted under different cooling conditions of the freezing chamber. A, discontinuous cooling from 0°C to -80°C; B, continuous cooling (-1°C/min); C, supercooling (-80°C for 5 min) during the transition time

**Table 2.** Inhibition of CFU-C. The concentration-dependent reduction of CFU-C proliferation by sera of selected patients (PNn) collected on particular days (nn) is shown

Serum %	PN1.28	PN2.10	PN2.17	PN3.16	PN3.50
30	0	0	0	0	0
5.0	0	0	0	8	0
2.5	0	0	5	11	1
1.2	0	6	15	16	8
0.6	8	14	24	27	14
0.3	36	17	27	38	25
0	87	75	71	70	70

stem cells retained proliferative capacity after freezing with program C, whereas procedures A and B completely destroyed CFU-C growth from some individual patients.

### Conclusions

Our results suggest:

1. The conditioning regimen for transplantation does not abrogate GM-CSA production; prolonged granulocytopenia therefore is not a GM-CSA deficiency effect. However, quantitative data on GM-CSA are necessary and are currently being obtained,
2. granulopoiesis is most likely under negative feedback control, and
3. obviously hematopoietic reconstitution following ABMT depends on the integrity of stem cells in the graft. They can be preserved when adequate freezing procedures are used.

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## Bone Marrow Transplantation with a Fixed Low Number of T-Cells in the Graft

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### Introduction

Ex vivo T-cell depletion of the marrow graft has decreased incidence and severity of graft-versus-host disease (GVHD), but has resulted in a higher incidence of graft failure and of relapse of the disease. In order to find an optimal T-cell number which avoids the extreme risks on both sides, we performed bone marrow transplants (BMTs) with  $1 \times 10^5$  T-cells/kg in the graft.

### Materials and Methods

#### Patients

Twenty-seven patients with malignancies or severe aplastic anemia received marrow from an HLA-identical sibling donor (Table 1). Transplant conditioning consisted of cyclophosphamide and total body irradiation (one fraction, regimen I; two fractions, regimen II), cyclophosphamide and busulfan (regimen III), or cyclophosphamide and melphalan (regimen IV). Except for one patient, all received cyclosporin A (3 mg/kg per day i.v.).

#### Treatment of the Donor Marrow

1. Isolation of mononuclear bone marrow cells (MBMCs) by centrifugation over Ficoll-Isopaque. From this fraction 1%–2% was set apart (fraction 1).

2. Differential agglutination with soybean agglutinin (SBA) followed by depletion of cells forming rosettes with sheep red blood cells (SRBCs) (fraction II).
3. T-cells were enumerated in both fractions by E-rosettes and by indirect immunofluorescence with anti-pan-T-cell monoclonal antibodies.
4. To obtain  $1 \times 10^5$  T-cells/kg, the shortage of T-cells in fraction II was adjusted by adding T-cells from fraction I.

### Results

#### Marrow Treatment

After SBA and SRBC treatment (fraction II) a median number of 0.2 (0.03–0.7)  $\times 10^5$  T-cells/kg was left in the graft. To obtain  $1 \times 10^5$  T-cells/kg a median number of 5.3 (2.4–11.9)  $\times 10^6$  T-cells was added from fraction I to fraction II.

#### Engraftment

Engraftment was achieved in all patients. Recovery of granulocytes  $>0.5 \times 10^9$ /liter occurred after a median of 21 (12–38) days and of thrombocytes  $>50 \times 10^9$ /liter after a median of 33 (20–291) days.

#### Graft-Versus-Host Disease (Table 1)

Fifteen of 26 evaluable patients (58%) had acute GVHD (grade I or II, only of the skin) and 7 of 23 evaluable patients (30%) had chronic GVHD. GVHD resolved completely with steroids in all patients.

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## Relapse Rate (Table 1)

Thirteen leukemic patients were transplanted with acute leukemias in first CR or with CML in first CP (standard-risk patients) and nine of these have a follow-up of at least 6 months (median 10, range 6–47 months), and, so far, one of these nine has relapsed.

## Discussion

Although this study is limited by the number of patients and by the heterogeneity of diseases and conditioning regimens, the results show that with  $1 \times 10^5$  T-cells/kg severe GVHD and graft failure can be avoided, whereas the risk of leukemic relapse may be low.

**Table 1.** Patients characteristics

Patient			Donor Sex/age (years)	Conditioning regimen	GVHD Acute/chronic	Survival after BMT (months)	Outcome
Unique patient	Sex/age (years)	Diagnosis					
054	F/20	CML-CP1	F/22	I	-/-	47+	A + W
071	M/36	CML-CP3	M/24	I	-/-	4	Relapse (2 months after BMT)
073	M/32	ANLL-CR1	F/40	I	-/-	38+	A + W
074	M/32	CML-CP2	M/31	III	II/+	16	Relapse (14 months after BMT)
077	F/42	ANLL-CR1	F/45	I	I/-	6	Septic shock
083	M/31	Melanoma	F/37	IV	I/NE	2	Progression
086	F/23*	ALL-CR1	F/26	I	II/NE	3	CMV-IP
088	M/32	Hodgkin's	M/31	IV	II/+	4	Relapse
090	F/35	Multiple myeloma	M/32	I	II/+	29+	Alive, relapsed 27 months after BMT
095	M/41	CML-CP1	M/39	III	NE/NE	1	VOD
096	F/17	ANLL-CR1	M/14	I	-/-	26+	A + W
097	M/35	Melanoma	M/36	IV	-/-	5	Progression
100	F/14	SAA	M/9	II	-/-	25+	A + W
103	M/19	ALL-Relapse 2	F/24	II	-/+	10	Relapse (9 months after BMT)
105	F/28	MDS	F/30	II	-/-	4	CMV-IP
116	F/41	CML-CP1	F/46	II	I/NE	3	Idiopathic pneumonia
126	F/18	ANLL-CR1	M/20	III	I/-	16	Relapse (10 months after BMT)
133	M/36	Melanoma	M/39	IV	I/-	15+	A + W
140	M/43	Multiple myeloma	F/44	II	II/+	11+	A + W
143	F/46	ANLL-Relapse I	F/36	III	I/-	9+	Alive, relapsed 5 months after BMT
145	M/39	ALL-CR1	M/24	II	II/+	10+	A + W
146	M/20	SAA	M/27	II	I/+	9+	A + W
150	M/35	ANLL-CR1	F/25	III	-/-	8+	A + W
152	F/18	ALL-CR1	M/17	II	-/-	6+	A + W
153	F/27	ALL-CR1	F/29	II	I/-	6+	A + W
156	M/36	CML-CP2	M/25	II	-/-	4+	A + W
157	M/29	ALL-CR1	F/26	II	I/-	3+	A + W

ALL, acute lymphoblastic leukemia; ANLL, acute nonlymphoblastic leukemia; CML, chronic myeloid leukemia; SAA, severe aplastic anemia; MDS, myelodysplastic syndrome; CR, complete remission; CP, chronic phase; I, cyclophosphamide/TBI (one fraction); II, cyclophosphamide/TBI (two fractions); III, busulfan/cyclophosphamide; IV, melphalan/cyclophosphamide; A + W, alive and well; CMV-IP, cytomegalovirus interstitial pneumonitis; VOD, venoocclusive disease of the liver; NE, not evaluable

## Complotyping and Subtyping of MHC Class I Gene Products in Haplotype Determination for Bone Marrow Transplantation\*

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### Introduction

In order to prevent major histocompatibility complex (MHC)-induced graft-versus-host disease (GvHD) after allogeneic bone marrow transplantation (BMT), the best possible match between donor and recipient is achieved between monozygotic twins [1]. It is well known that this is only possible in very few patients. In cases where parents share an HLA haplotype, or are phenotypically homozygous, or even appear to be identical, problems in the inheritance of HLA haplotypes among the offspring do occur. These uncertainties cannot be solved by the serological HLA typing or cellular typing of HLA-D-encoded gene products. For that reason we introduced two following additional biochemical typing methods:

1. Complotyping, i.e., definition of the MHC-encoded polymorphic complement components Bf, C2, C4A, and C4B. The C4A and C4B allotypes are as polymorphic as the HLA-A and HLA-B antigens, with 12 C4A and 18 C4B variants. In addition, a duplication of one of the C4 genes is observed in 1% of the cases [2].
2. Subtyping of class I gene products by one-dimensional isoelectric focusing (1D-IEF). As we showed recently, the applica-

tion of 1D-IEF followed by an immunoblotting raises the number of serologically defined antigens by nearly 60% [3, 4].

### Material and Methods

Complotyping was done on ethylene diamine tetra acetic acid (EDTA) plasma collected, and stored at  $-80^{\circ}\text{C}$  until use. C4 allotyping was performed as described by Koelble et al. [5] with modifications [6]. The analysis of the factor B (Bf) variants was performed as given by Martin and Ziegler [7], while C2 allotyping was carried out according to Doxiadis et al. [8].

The concentration of class I gene products by detergent-phase separation from mononuclear cells, their separation by isoelectric focusing, and visualization by immunoblotting were originally described by Neefjes et al. [3] and were used in the present study with minor modifications [4].

### Results and Discussion

To identify histocompatible donor/recipient pairs for allogeneic BMT, more than 1000 leukemic patients and their family members were HLA typed using the typical serological NIH technique. In addition, the mixed lymphocyte cultures (MLCs) were performed mostly between HLA identical/compatible pairs to prove MHC class II identity. However, in nearly 20% of the cases a clear-cut HLA haplotype ascertainment was not

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possible. HLA haplotype sharing, or homozygosity, identity between the parents, inter-MHC recombination, or extrapaternity of one or more offspring, and the inability to HLA type a patient unequivocally due to concomitant blast cells were the main obstacles. Complotyping was performed in 204 patients and their relatives. Of these, 134 cases represented the difficulties in HLA haplotype determination as given above, whereas the remaining 70 families were typed for other purposes, such as the unrelated donor marrow search program. Table 1 depicts the results of complotyping the 134 patients and their families. The individual complications in HLA haplotype ascertainment were grouped and counted separately. Parents sharing one HLA haplotype represented the main group (33 cases), followed by the group of partially mismatched donor/recipient pairs (32 cases). The efficacy of complotyping was best (7/11 cases solved) in HLA-B/HLA-DR recombinations while the haplotype definition in cases of a missing parent still represents a problem (1/21 cases solved). In summary, in 37 out of 134 cases (27%) complotyping enabled a clear-cut haplotype determination to be made.

**Table 1.** Efficacy of complotyping (Bf, C2, C4A, C4B) for the ascertainment of HLA haplotypes

Problem	Per- formed N	Solved	
		N	%
Haplotype sharing	33	7	21.2
Partial match	32	7	21.9
Homozygosity	25	12	48.0
Missing parent	21	1	4.8
HLA-B/DR recombination	11	7	63.6
D/DR typing problems	9	2	22.2
Extrapaternity	3	1	33.3
Total	134	37	27.0

The 1D-IEF of MHC class I antigens was performed in 58 patients and their families. The relevant results are given in Table 2. Biochemical subtyping of MHC class I gene products was found to be very efficient when serological typing problems or HLA ho-

**Table 2.** Efficacy of biochemical class I typing by 1D-IEF for the ascertainment of HLA haplotypes

Problem	Per- formed N	Solved	
		N	%
Haplotype sharing	21	5	23.8
Homozygosity	14	4	28.6
Missing parent	12	2	16.7
Typing problems	6	3	50.0
Extrapaternity	5	1	20.0
Total	58	15	25.9

mozygosity occurred. In total, 15 out of 58 cases with equivocal serological segregation analysis could be solved by 1D-IEF.

The results presented here clearly demonstrate the relevance of both methodologies for better clarification of segregating MHC haplotypes and consequently the improvement of matching donor/recipient pairs. These techniques will have further importance if unrelated donor/recipient pairs are to be analyzed.

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## Complications of Bone Marrow Transplantation in Chinese \*

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### Introduction

Over the past 2 decades BMT has evolved into an increasingly successful and widely applied treatment for a vast range of hematological disorders. Some of the complications restricting success have been overcome; such is not the case with acute GVHD and IP, which remain major obstacles encountered during the period of immunodeficiency following the BMT procedure. Since Taiwan is an HBV-endemic area [1, 2], 80% of patients test positive for HBV markers before BMT, but liver function tests are usually normal; elevation of enzymes often occurs, however, after the high-dose chemoradiotherapy preconditioning for BMT. To assess the extent and significance of these abnormalities, we reviewed the incidence of acute GVHD, IP, and VOD along with the LF test results of BMT patients treated with preconditioning regimens for BMT.

### Patients and Materials

In this study a total of 43 cases were admitted to the BMT unit at the Taiwan Veterans General Hospital, Taipei, including 18 cases of ANLL, 5 cases of ALL, 6 cases of CML,

8 cases of SAA, 1 case of thalassemia in allogenic BMT (10 had one or two loci mismatches), 3 cases of ANLL, and 2 cases of HD in autologous BMT. Of the 43 patients, 22 were male and 21 were female, with ages ranging from 3 to 46 years (mean age, 24 years). These patients were observed from May 1984 to October 1988, the majority being evaluated from 3 months to 1 year.

The preconditioning regimens for BMT were followed as described by Thomas et al. [3, 4]. The sera of all donor blood component transfusions were HBsAg negative. Ten patients received methotrexate (MTX) as immunosuppressive therapy while the other 28 patients received MTX and cyclosporin A (CSA) combination immunosuppressive therapy. The remaining five patients with autologous BMT did not receive immunosuppressive drugs.

### Serological Tests

The hepatitis B surface antigen (HBsAg), hepatitis B core antibody (HBcAb), hepatitis B surface antibody (HBsAb), hepatitis Be antigen (HBeAg), and hepatitis Be antibody (HBeAb) of sera were all measured by radioimmunoassay.

### Liver Function Test (LFT)

Alanine aminotransferase and aspartate aminotransferase were measured by a 24-factor automated chemical analysis using standard reagents. The normal range is below 40 IU/liter for both ALT and AST.

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## Results

### Acute GVHD

Thirty-three out of 38 allogeneic BMT cases were followed up for more than 3 months, only 3 patients (9.1%) suffering from moderate to severe acute GVHD, two of whom died. Two of 43 patients suffered from idiopathic IP, both of whom died of this complication. No VOD occurred in our series.

### Impaired Liver Function

Twenty-seven out of 35 patients (77%) developed one or more LF test abnormalities.

### Hepatitis B Virus Markers

The positive rates of HBsAg, HBsAb, HBeAg, HBeAb, and HBcAb were 12/37 (32%), 20/32 (63%), 3/27 (11%), 12/25 (48%), and 24/27 (89%), respectively. Following up HBV markers from 3 to 12 months after BMT, 6 of 20 patients (30%) were found to have changed HBV markers

in their sera. Figure 1 shows a typical case of changed HBV markers after BMT.

## Discussion

During the past 4 years, it has been noted that the most common symptomatic complications produced by BMT in Chinese patients differ significantly from those found in Westerners.

In the Western hemisphere, acute GVHD and IP are considered to be the leading complications of BMT [3, 5]; incidence varies between 30% and 70%, with death resulting in almost 50% of patients. In our hospital, while 10 of 37 patients (27%) receiving BMT received marrow from haploidentical donors mismatched by one or two loci and/or donors with positive mixed lymphocyte reactions (MLRs), the occurrence of acute GVHD was less than 9.1%. No patient received marrow that was treated *ex vivo* to decrease or eliminate donor T-lymphocytes in our series.

Could it be possible that Chinese people have unknown antigens in common that are different from the HLA group but are also

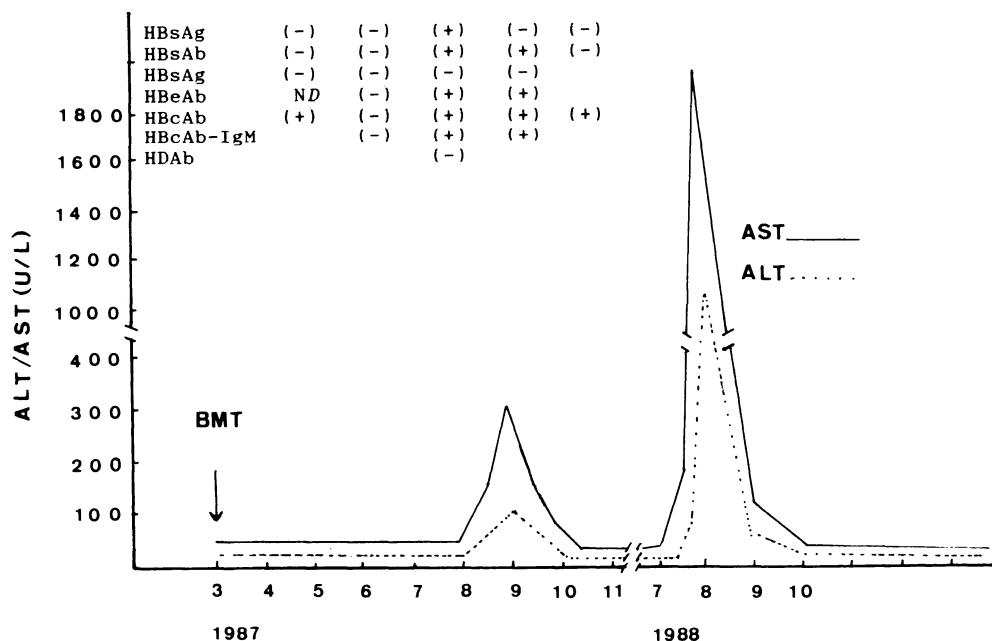


Fig. 1. A 23-year-old female with acute myelocytic leukemia developed acute hepatitis 5 months after bone marrow transplantation

significant determinants of tissue compatibility? The incidence of IP is also very low in our patients; consequently, mortality from these complications is very low.

Taiwan, being an area where hepatitis B is endemic, has a higher proportion of patients with impaired liver function tests than does the West.

Twenty-seven out of 35 patients (77%) developed one or more LF test abnormalities; however, 90% of these normalized within 1 year after BMT, with only one patient dying of hepatitis-related complications (in this case, subacute massive hepatitis). In conclusion, previous hepatic damage from HBV infection before BMT does not appear to increase the risk of posttransplant morbidity and mortality.

## Summary

Forty-three patients with hematopoietic disease were treated with intensive chemotherapy and radiotherapy, followed by allogeneic bone marrow transplantation (BMT) from 28 HLA-identical and 10 one to two antigen haploidentical sibling donors and autologous BMT (5 cases). Of these cases, there were 21 with acute nonlymphocytic leukemia (ANLL), 5 with acute lymphocytic leukemia (ALL), 6 with chronic myelocytic leukemia (CML), 2 with Hodgkin's disease (HD), 8 with severe-form aplastic anemia (SAA) and 1 with thalassemia. Complications of BMT were evaluated including acute graft-versus-host disease (GVHD), in-

terstitial pneumonia (IP), veno-occlusive liver disease (VOD), abnormalities of liver function (LF), and alteration of hepatitis B virus (HBV) markers. In thirty-three patients who were followed up for more than 3 months, we found that the incidence of moderate to severe acute GVHD (9.1%) and IP (two cases, 4.7%) were low. No VOD occurred in our series. During the follow-up period, 27 out of 35 patients (77%) had high alanine aminotransferase (ALT)/aspartate aminotransferase (AST) levels, even up to 1000 U/liter; however, only one patient succumbed to a hepatitis-related complication. Previous hepatic damage from HBV infection before BMT does not appear to increase the risk of posttransplant morbidity and mortality.

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## **Hematopoietic Growth Factors**

## Effect of Granulocyte-Macrophage Colony-Stimulating Factor on Neutropenia and Related Morbidity Induced by Myelotoxic Chemotherapy

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### Introduction

Myelosuppression-related neutropenia is the major side effect of most anticancer chemotherapy. Despite considerable improvements in supportive care due to the advent of a variety of new antibiotic combinations, infection remains the main risk arising during the neutropenic period that follows intensive chemotherapy for cancer [1]. In addition, neutropenia is the major obstacle to dose escalation, frequency of cytoreductive treatment, and thus to improved cancer control. Regarding reduction of the period of neutropenia and increase of the maximum tolerated dose of effective anticancer agents, autologous bone marrow transplantation (ABMT) has recently offered new promise. However, as many as 5% of patients having undergone ABMT die prior to engraftment with neutropenia-related infections as the major contributing cause [2]. The augmentation of number and function of circulating neutrophils by in vivo administration of hematopoietic growth factors, including granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), to cancer patients has been successfully shown in several recent phase I clinical trials [3–10], thus suggesting

the feasibility of spurring hematopoietic cell recovery after cytoreductive chemotherapy. Therefore, we have now intraindividually compared *Escherichia coli*-derived recombinant human (rh) GM-CSF to supportive care only in cancer patients having received myelosuppressive chemotherapy, to study prevention of neutropenia and related morbidity.

### Patients and Methods

#### Patient Selection

Patients with various types of solid tumor and lymphoid neoplasias with a Karnofsky performance score of >50%, including pancreatic cancer ( $n=2$ ), nonseminomatous testicular cancer (NSTC) ( $n=7$ ), bladder cancer ( $n=3$ ), ovarian cancer ( $n=1$ ), chondrosarcoma ( $n=1$ ), multiple myeloma ( $n=1$ ), follicular non-Hodgkin's lymphoma (NHL) ( $n=3$ ), and Burkitt-like non-Hodgkin's lymphoma ( $n=4$ ) who were undergoing myelotoxic chemotherapy were eligible for study. In three of the above patients (NSTC, ovarian cancer), myelotoxic chemotherapy was followed after 2 days by double ABMT. The first ABMT was followed by supportive care only, and, 2 days following the second ABMT, patients received GM-CSF. Signed informed consent conforming to institutional review guidelines was required. No exclusions were made for disease state or stage or prior therapy.

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## Clinical and Laboratory Monitoring

A complete history and physical examination were undertaken and the indices measured daily included complete blood cell count including differentials, hemoglobin, platelets, chemistries, prothrombin time, urine analyses, performance status, weight, temperature, and clinical evaluation of disease. Weekly chest radiographs and electrocardiograms were also obtained during the time of hospitalization and 2 weeks thereafter.

## Study Design

The study was a phase Ib/II study in which patients were consecutively enrolled according to the myelotoxic regimen they were assigned to. When exhibiting a neutropenic episode following the first cycle of chemotherapy, standard supportive care was instituted while the subsequent cycle also contained GM-CSF. In each chemotherapy group at least three patients had to be studied. The chemotherapy protocols used are as follows:

ADIP-FU: doxorubicin, 50 mg/m<sup>2</sup> day 1; ifosfamide, 4 g/m<sup>2</sup> day 1; cisplatin, 80 mg/m<sup>2</sup> per day days 1 and 2; 5-fluorouracil, 600 mg/m<sup>2</sup> day 2, for pancreatic cancer and chondrosarcoma [11]. NOSTE: mitoxantrone, 15 mg/m<sup>2</sup> per day days 1 and 2; prednimustine, 100 mg/m<sup>2</sup> days 1–5, for multiple myeloma and follicular NHL [12]. NHL BMF/81-protocol: cyclophosphamide, 200 mg/m<sup>2</sup> per day days 1–5; methotrexate, 500 mg/m<sup>2</sup> day 1; VM-26, 165 mg/m<sup>2</sup> day 5; cytarabine, 300 mg/m<sup>2</sup> day 5, for Burkitt-like NHL [13]. PEI: ifosfamide, 1.5 g/m<sup>2</sup> per day days 1–5; VP-16, 120 mg/m<sup>2</sup> days 1–5; cisplatin, 30 mg/m<sup>2</sup> days 1–5, for NSTC [14]. M-VAC: methotrexate, 30 mg/m<sup>2</sup> per day days 1, 15, 22; vinblastine, 3 mg/m<sup>2</sup> per

day days 2, 15, 22; doxorubicin, 30 mg/m<sup>2</sup> day 2; cisplatin 70 mg/m<sup>2</sup> day 2, for bladder cancer [15]. VP-16/CBDCA plus ABMT: VP-16, 1200 mg/m<sup>2</sup> day 1; carboplatin 1400 mg/m<sup>2</sup> day 1, for refractory NSTC and ovarian cancer [16].

All patients received two cycles of chemotherapy or ABMT. The first cycle consisted of administration of the respective chemotherapy protocol with or without autologous bone marrow support and standard supportive care. In the second cycle, patients received the identical protocol, but standard supportive care was supplemented by rh GM-CSF at a single dose (250 µg/m<sup>2</sup> per day as an s.c. injection; Fig. 1) for a total of 10 days, based on data obtained from a previous phase I safety and tolerance study (manuscript submitted). Patients experiencing fever ( $T > 38.5^{\circ}\text{C}$ ) were treated uniformly according to protocols with empirical antibody therapy. In patients having undergone ABMT, GM-CSF was administered until neutrophils rose above  $0.5 \times 10^3/\text{mm}^3$ .

## Patients

Twenty-two consecutive patients were studied on six different myelotoxic chemotherapy protocols. One of the protocols, to whom three patients were subjected to, consisted of double ABMT that followed high-dose chemotherapy. The mean age of all patients was 49 years (range, 16–77 years). There were 4 females and 18 males. Diagnosis, disease state, chemotherapy regimen, and other patient characteristics including age, sex, and previous anticancer therapy are listed by patient in Table 1.

## Preparation of rh GM-CSF

The GM-CSF used in this study was produced with *E. coli*, according to recombi-

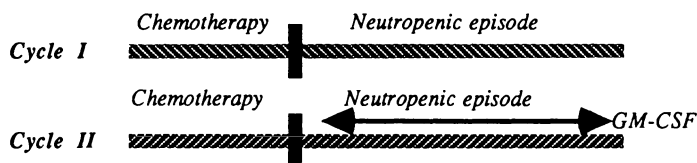


Fig. 1. Schema of study design



**Table 1.** Patient characteristics

Patient number	Age	Sex	Diagnosis	Prior therapy			Treatment protocol
				Surgery	Radiation	Chemotherapy	
<b>Chemotherapy</b>							
1	45	M	Chondrosarcoma	+	-	-	ADIP-FU
2	53	M	Pancreatic cancer	+	-	-	ADIP-FU
3	48	M	Pancreatic cancer	-	-	-	ADIP-FU
4	56	M	Multiple myeloma	-	-	+	NOSTE
5	77	M	Follicular NHL	-	-	+	NOSTE
6	59	M	Follicular NHL	-	-	+	NOSTE
7	49	M	Follicular NHL	-	-	+	NOSTE
8	52	M	NSTC	+	+	+	PEI
9	34	M	NSTC	+	-	+	PEI
10	25	M	NSTC	+	-	+	PEI
11	34	M	NSTC	+	-	+	PEI
12	33	M	NSTC	+	-	+	PEI
13	59	M	Bladder cancer	+	-	-	M-VAC
14	58	M	Bladder cancer	+	-	-	M-VAC
15	65	F	Bladder cancer	+	-	-	M-VAC
16	68	F	Burkitt-like NHL	-	-	+	NHL B1
17	58	M	Burkitt-like NHL	-	-	-	NHL B1
18	58	M	Burkitt-like NHL	-	-	+	NHL B1
19	67	F	Burkitt-like NHL	-	-	+	NHL B1
<b>Chemotherapy plus ABMT</b>							
20	34	M	NSTC	+	-	+	VP-16/CBDCA + ABMT
21	30	M	NSTC	+	-	+	VP-16/CBDCA + ABMT
22	16	F	Ovarian cancer	+	-	+	VP-16/CBDCA + ABMT

See text for explanation of abbreviations

nant-DNA techniques [17]. The specific activity of this material was  $5 \times 10^7$  units/mg protein. The agent was >95% pure and had no measurable endotoxin by limulus amoebocyte assay.

### Statistical Analysis

For each patient who completed the study, the differences in the variables between cycles 1 and 2 were determined. Two-sided *P* values were calculated by use of the Wilcoxon signed-rank test [18].

### Results

Twenty-two patients entered this study – at least three patients each in six different chemotherapy protocols. All patients com-

pleted the study and could therefore be evaluated for the following variables by comparing intraindividual data obtained in both segments of the study: days with temperature > 38.5°C, days in hospital, days on parenteral antibiotics, nadir of neutrophils, days with neutrophils <  $10^3/\text{mm}^3$ , nadir of hemoglobin, nadir of platelets, days with hemoglobin < 9 g/dl, days with platelets <  $20 \times 10^3/\text{mm}^3$ , number of red blood cell transfusions, and number of platelets transfused. None of the patients had received any anticancer therapy within 10 weeks prior to entry into this study. Three patients (patients Nos. 4, 6, and 7) had neoplastic bone marrow involvement with the underlying disease, but showed no significant difference in terms of bone marrow response to rh GM-CSF and are therefore included in the analysis.

**Table 2.** Effect of CM-CSF on neutropenia and associated morbidity due to chemotherapy

Treatment protocol	Number of cycles	Days Temp. > 38.5° C	Total days in hospital	Total days on parenteral antibiotics	Neutrophils	
					Nadir ( $\times 10^3/\text{mm}^3$ )	Total days < $10^3/\text{mm}^3$
ADIP-FU	6					
–GM-CSF	3	15	53	13	0.2	22
+GM-CSF	3	12	48	13	0.5	9
NOSTE	8					
–GM-CSF	4	2	27	7	0.3	60
+GM-CSF	4	2	25	0	0.2	26
PEI	10					
–GM-CSF	5	9	94	26	0.1	62
+GM-CSF	5	14	59	5	0.8	36
M-VAC	6					
–GM-CSF	3	8	53	13	0.1	22
+GM-CSF	3	1	40	0	0.3	9
NHL BFM/81	8					
–GM-CSF	4	3	98	0	0.1	13
+GM-CSF	4	3	98	0	1.0	13
VP-16/CBDCA + ABMT	6					
–GM-CSF	3	23	93	84	0.0	90
+GM-CSF	3	12	64	60	0.0	52
Mean						
–GM-CSF		11.0 $\pm$ 4.9	37.6 $\pm$ 12.3	14.5 $\pm$ 12.0	0.1 $\pm$ 0.09	26.2 $\pm$ 11.0
+GM-CSF		4.1 $\pm$ 4.5 <sup>b</sup>	30.8 $\pm$ 16.4 <sup>a</sup>	6.8 $\pm$ 8.3 <sup>b</sup>	0.84 $\pm$ 0.48	13.0 $\pm$ 10.9 <sup>c</sup>

<sup>a</sup> Not significant<sup>b</sup>  $P \leq 0.01$ <sup>c</sup>  $P < 0.001$ 

### First Course

The mean absolute neutrophil count of all patients entering the first part of the study was  $2.57 \pm 0.56 \times 10^3/\text{mm}^3$ . As shown in Table 2, after myelotoxic chemotherapy all patients experienced severe neutropenia with a neutrophil nadir below  $0.3 \times 10^3/\text{mm}^3$  (mean,  $0.1 \pm 0.09 \times 10^3/\text{mm}^3$ ). Mean duration of neutropenia (days with neutrophils  $< 10^3/\text{mm}^3$ ) was  $26.2 \pm 11.0$  days. Overall, the patients spent 416 days (mean,  $37.6 \pm 12.3$  days) in hospital and had temperatures of  $> 38.5^\circ\text{C}$  over a total of 58 days (mean,  $11.0 \pm 4.9$  days) requiring parenteral administration of antibiotics for a total of 159 days (mean,  $14.5 \pm 12.0$  days) until reso-

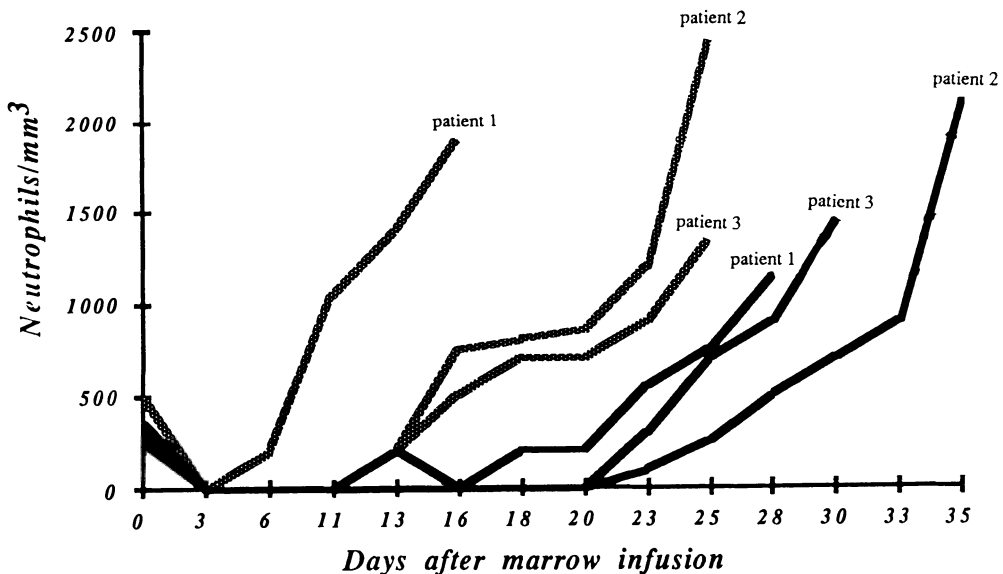
lution of clinical signs of infection. The mean hemoglobin value and platelet counts prior to study were  $9.7 \pm 1.2$  g/dl and  $162.3 \pm 27.1 \times 10^3/\text{mm}^3$ , respectively. After completion of the first part of the study hemoglobin decreased to  $8.9 \pm 1.3$  g/dl, requiring a total of 52 red blood cell transfusions to maintain hemoglobin levels above 8 g/dl. Platelet counts decreased to  $46.8 \pm 32.5$  and required a total of 48 units of transfused platelets to maintain platelet levels above  $20 \times 10^3/\text{mm}^3$ . By using this protocol, patients went through a total of  $11.0 \pm 4.9$  days with hemoglobin values  $< 9$  g/dl and a total of  $3.5 \pm 4.7$  days with platelet counts  $< 20 \times 10^3/\text{mm}^3$ .

*Second Course and Comparison with the First Course*

Myelotoxic chemotherapy administered in the presence of rh GM-CSF elevated the neutrophil nadir to  $0.84 \pm 0.48 \times 10^3/\text{mm}^3$  ( $P < 0.01$ ). The nadir of hemoglobin was  $8.1 \pm 1.1$  g/dl and of platelet counts  $41.2 \pm 37.9 \times 10^3/\text{mm}^3$  (not significant). The number of days passed with neutrophils  $< 10^3/\text{mm}^3$ , hemoglobin  $< 9$  g/dl, and platelets  $< 20 \times 10^3/\text{mm}^3$  was  $13.0 \pm 10.9$  ( $P < 0.001$ ),  $5.2 \pm 6.5$ , and  $4.7 \pm 5.2$  (not significant), respectively. Patients receiving GM-CSF spent a total of 334 days (mean,  $30.8 \pm 16.4$  days; not significant) in the hospital and had temperatures of  $> 38.5^\circ\text{C}$  for a total period of 45 days (mean,  $4.1 \pm 4.5$  days;  $P < 0.01$ ). Parenteral administration of antibiotics was required for a total period of 78 days (mean,  $6.8 \pm 8.3$  days;  $P < 0.01$ ). Analysis of neutrophil response in the patient group receiving ABMT failed to show elevation of the nadir count, but demonstrated highly significant ( $P < 0.001$ ) shortening of the neutropenic episode (Fig. 2).

*Toxicity of GM-CSF*

Granulocyte-macrophage-CSF was well tolerated by all patients. No patient failed to complete courses of rh GM-CSF because of suspected toxicity. The following clinical observations appeared related to GM-CSF administration: Three patients had slight bone pain, in two of whom associated only with the first two injections. Two patients had abdominal cramps, observed only after the first dose. Two patients had a constant low-grade temperature without evidence of infection. Respiratory symptoms noted in a previous phase I trial using continuously infused GM-CSF were not encountered. No renal, hepatic, neurological, cardiac symptoms, or fluid retention occurred. Mucosal toxicity (grade III, according to WHO criteria) was seen in three patients during the first phase of the study. Incidence and severity of mucositis was, however, significantly reduced when GM-CSF was administered.



**Fig. 2.** Mean neutrophil counts in three patients receiving two cycles of high-dose chemotherapy plus ABMT. The first cycle (solid line) was performed in the absence of GM-CSF. In the second cycle patients received a single daily s.c. injection of GM-CSF over a 10-day period starting 48 h after bone marrow infusion (dotted line)

## Discussion

Shortening the period and degree of neutropenia should reduce the incidence and severity of infections. Infection due to neutropenia following myelotoxic anticancer treatment is the most important factor contributing to morbidity and mortality of chemotherapy-treated patients. Therefore, reduction of neutropenia after administration of cytotoxic agents may prove highly beneficial in terms of morbidity and mortality of chemotherapy but may also be of value in terms of hospital costs by abbreviating stay in the hospital and reducing the amount of expensive antibiotics to be administered. Increase of dose of cytotoxic chemotherapy may also improve disease outcome [19]. For a variety of cytotoxic regimens, myelosuppression has emerged as the limiting toxicity of chemotherapy. In this study, we have administered rh GM-CSF after high-dose myelotoxic chemotherapy to cancer patients and have intraindividually compared its effect on neutropenia and related morbidity with neutropenia-related variables obtained during a preceding chemotherapy cycle delivered in the absence of GM-CSF. On GM-CSF, neutrophil nadir was significantly elevated as was the time patients experienced a neutrophil count below  $10^3/\text{mm}^3$ , a threshold known to be critical for acquiring infective complications [20] and thus resulting in a significant reduction of febrile episodes and need for antibiotics. No significant effect was seen concerning hemoglobin/platelet nadirs or duration of chemotherapy-related anaemia/thrombopenia. GM-CSF was well tolerated in all patients. Some deleterious effects of chemotherapy on rapidly dividing cells other than hematopoietic cells proved also to be ameliorated by GM-CSF. Foremost, severe mucositis that occurred in 14% of our patients off GM-CSF was not seen on GM-CSF. While we have shown that chemotherapy-associated morbidity can be significantly reduced in patients receiving myelotoxic cancer chemotherapy, additional studies will, however, be needed to assess whether the use of GM-CSF in anticancer chemotherapy may allow us to increase dose intensity leading to improved response rates and survival among cancer patients.

## Summary

A phase Ib/II clinical study was undertaken to assess the efficacy of recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) to attenuate neutropenia and associated morbidity caused by high-dose anticancer chemotherapy administered in the presence or absence of autologous bone marrow support. We treated 22 patients with various solid tumors and lymphoid neoplasias with a single daily subcutaneous dose of GM-CSF ( $250 \mu\text{g}/\text{m}^2$ ) 48 h after a second cycle of highly myelotoxic chemotherapy for a period of 10 days and compared intraindividually neutropenia-related clinical and laboratory variables with data obtained from the same patients having previously received a first neutropenia-inducing cycle of identical chemotherapy in the absence of GM-CSF. We show that GM-CSF is active in neutropenic patients by significantly increasing the neutrophil nadir, reducing the time of relevant neutropenia, and reducing the duration of the patient's hospital stay and necessity for parenteral antibiotics. No significant toxicity was encountered with subcutaneous GM-CSF treatment.

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## Recombinant Human Granulocyte-Macrophage Colony-Stimulating Factor After Chemotherapy for Acute Leukemias at Higher Age or After Relapse

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### Introduction

Despite great efforts in supportive care early death during the phase of induction treatment still remains an unsolved therapeutic problem in patients with acute leukemias. For acute myelogenous leukemia (AML) in adults the data of six multicenter trials [1–6] comprising 3438 patients show early deaths in 17%–32% of patients overall and in 27%–52% of patients 60 years of age or older. In addition, intensive second-line chemotherapy for relapsed and refractory AML and acute lymphoblastic leukemia (ALL) produced complete remission (CR) in 56% of cases, but also 29% early deaths [7, 8]. Markedly lower response rates in most other studies (overview in [9]) suggest still higher early death rates in second-line treatment. Furthermore, 5%–20% of patients receiving first-line postinduction intensification chemotherapy for AML died from toxicity (overview in [6]). Infections have been identified in multicenter trials as the cause of at least one-half of the early deaths during induction therapy for AML [1–4], and they may in addition account for many early deaths from unidentified causes.

Although at some institutions early lethality may have decreased today to levels below those reported in multicenter trials, myelosuppression and infections due to neutropenia still represent the major dose-limiting toxicity. Thus, reducing the phase of critical cytopenia will allow more inten-

sive and effective antileukemic chemotherapy.

Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF), not applied as yet in patients with acute leukemias, has been proven effectively to stimulate granulopoiesis under various conditions. In primates a dramatic rise in the number of neutrophils was induced [10, 11]; this also occurred in an animal with virus-induced pancytopenia [10]. After irradiation [12] or combined irradiation and cytostatic treatment and autologous bone marrow transplantation [13, 14] the recovery of neutrophils was accelerated by GM-CSF. Given to humans, GM-CSF induced an increase in neutrophils in patients with acquired immunodeficiency syndrome [15] and in patients with aplastic anemia [17]. The phase of therapy-induced critical neutropenia could be reduced after chemotherapy for sarcomas [18] and after chemotherapy followed by autologous bone marrow transplantation for breast cancers and melanomas [19]. As preleukemic disorders, myelodysplastic syndromes have now been treated with GM-CSF [20, 21] and have shown an effective increase in neutrophil counts.

When considering GM-CSF as a part of treatment for acute leukemias, its stimulatory effect on leukemic blasts in AML must be taken into account. Thus, in myelodysplastic syndromes some patients responded with an increase of blasts and even a transformation into AML [21]. In vitro leukemic blast growth in both colony assays and suspension cultures was found stimulated in the presence of GM-CSF in the majority [22–28]

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and up to 97% [25] of AML cases. Thus, as a first step the present study on GM-CSF in acute leukemias (begun in September 1987) was restricted to patients at high risk of early death due to early or multiple relapse or higher age. The design and entry criteria aimed at avoiding an interference of GM-CSF with chemotherapy, an unnecessarily high dose, and an unnecessarily high risk for patients with favorable prognosis. The evaluation of the hematologic effects of GM-CSF was provided by comparison to related control groups treated in phase II and III studies at the same institutions.

### Patients and Methods

Those entered into the study included

- a) adult patients at all ages with early relapse occurring in the first 6 months of remission or with multiple relapse, and
- b) patients 65 years of age or older with relapsed AML or ALL or with newly diagnosed AML.

Median age was 65 years (range, 17–84). Patients having had bone marrow transplantation prior to a relapse and secondary leukemias were included. The historical control groups treated at the same institutions and receiving the same chemotherapy corresponded to the GM-CSF group in terms of age and therapeutic situations but did not include patients with transplantions or secondary leukemias.

Recombinant human GM-CSF has been provided by Behringwerke (Marburg, FRG). The recombinant protein was expressed in yeast and purified by Immunex (Seattle, Washington, USA), as described [29]. Sterility, general safety, and purity studies met United States Food and Drug Administration standards.

For chemotherapy early or multiple relapses were treated with high-dose cytosine arabinoside (ara-C) randomly either 1 or 3 g/m<sup>2</sup> q12 h by intravenous infusion on days 1, 2, 8, and 9 and mitoxantrone 10 mg/m<sup>2</sup> intravenously on days 3, 4, 10, and 11 (HAM) [30]. Only one course of sequential HAM was given to each patient. Newly diagnosed AML and later AML relapses in the higher age group were treated with 6-

thioguanine 100 mg/m<sup>2</sup> q12 h orally on days 3–9, ara-C 100 mg/m<sup>2</sup> daily by continuous intravenous infusion on days 1 and 2 and q12 h intravenously on days 3–8, and daunorubicin randomly either 30 or 60 mg/m<sup>2</sup> by intravenous injection on days 3–5 (TAD9) [4]. Depending on adequate blast clearance from the bone marrow, patients received one or two courses of TAD9.

Laboratory tests included daily complete blood counts including those of white and red cells and reticulocyte, platelet, and differential counts. Bone marrow aspirates and biopsies were obtained before and after chemotherapy and after GM-CSF when CR criteria in blood counts were achieved, or a persistent cytopenia had to be cleared up. Bone marrow microscopy included quantification of cellularity (scoring from 0 to 4) and percentage of blasts and normal hematopoietic cells. DNA histograms from bone marrow cells were obtained by flow cytometry using methods described [31]. DNA aneuploidy was defined by a significant clonal deviation of the cellular DNA content from admixed normal blood reference cells. Colony-forming progenitor cells in the bone marrow growing on methyl cellulose were monitored using methods described [32]. Leukemic colonies were identified morphologically after Pappenheim staining and by their ability to grow in secondary plates.

If the bone marrow 3 days after the end of chemotherapy was aplastic with no blasts, GM-CSF 250 µg/m<sup>2</sup> was given by continuous intravenous infusion beginning on day 4. When a neutrophil count of 2000/mm<sup>3</sup> was achieved and maintained for 4 days, the dose was reduced to 125 µg/m<sup>2</sup> for 4 days and to 50 µg/m<sup>2</sup> for another 4 days until the infusion was discontinued.

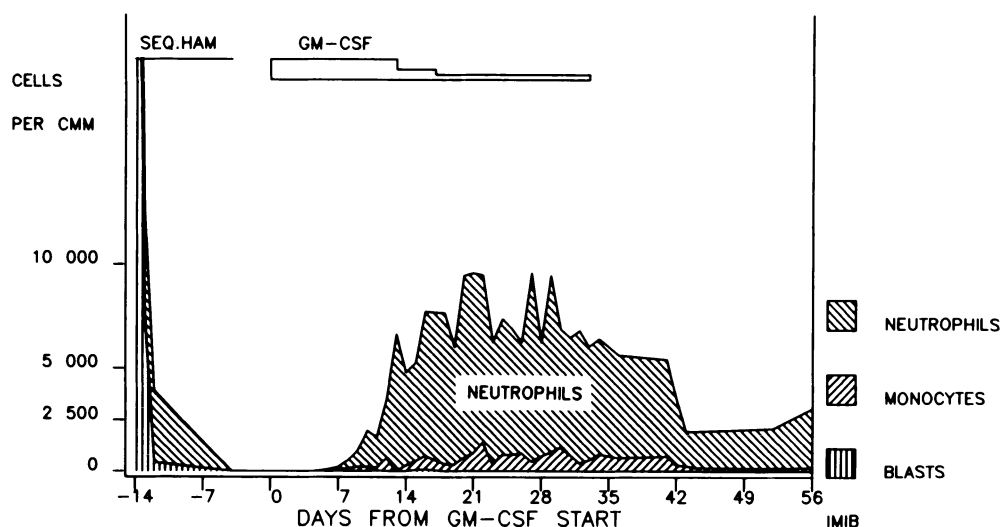
### Results

Data on the 23 patients in the study are presented in Table 1. Among 13 patients with relapses five had ALL and eight AML. Relapses were early first relapses in five patients, one late first relapse in a 77-year-old patient and seven second relapses. CR was achieved in 12 out of 23 patients: in four out of five with relapsed ALL, three out of eight with relapsed AML, and five out of ten with

**Table 1.** Patient characteristics, treatment, and outcome

Patient	Age	Diagnosis	Special risk	Chemo-therapy	Outcome
1 AB	30	ALL 1st relapse		S-HAM	CR
2 HO	21	ALL 1st relapse		S-HAM	CR
3 LA	17	ALL 1st relapse		S-HAM	CR
4 SC	26	ALL 2nd relapse		S-HAM	CR
5 TO	35	ALL 2nd relapse		S-HAM	ED
6 RI	65	AML 1st relapse		S-HAM	ED
7 KI	44	AML 1st relapse		S-HAM	ED
8 EI	61	AML 2nd relapse		S-HAM	CR
9 TE	34	AML 2nd relapse	auto-BMT	S-HAM	CR
10 FI	35	AML 2nd relapse	allo-BMT	S-HAM	NR (hypoplasia)
11 KO	27	AML 2nd relapse	auto-BMT	S-HAM	ED
12 HE	61	AML 2nd relapse	Tumor chemotherapy	S-HAM	AML regrowth, NR
13 KL	77	AML 1st relapse		TAD9	CR
14 KI	65	AML de novo		TAD9	ED
15 BR	77	AML de novo		TAD9	CR
16 LA	75	AML de novo		TAD9	CR
17 ME	70	AML de novo		TAD9	AML regrowth, NR
18 SC	84	AML de novo		TAD9	CR
19 GI	75	AML de novo		TAD9	CR
20 AM	70	AML de novo		TAD9	NR (hypoplasia)
21 BO	65	AML secondary	Tumor chemotherapy	TAD9	NR (hypoplasia)
22 DU	78	AML secondary	MDS	TAD9	AML regrowth, CR
23 BO	75	AML secondary	MDS	TAD9	ED

S-HAM, Sequential course of high-dose cytosine arabinoside and mitoxantrone; TAD9, 9-day course of 6-thioquanine, cytosine arabinoside, and daunorubicin; CR, complete remission; ED, early death; NR, non-response.



**Fig. 1.** Typical time course of nucleated white blood cell counts under GM-CSF. *Seq. HAM*, Sequential high-dose Ara-C and mitoxantrone. GM-CSF intravenous infusion is shown with dose deescalation in a 20-year-old patient with second relapse of ALL



newly diagnosed AML at 65 years or older. The median age of responders was 61 years (range, 17–84). Special additional risks were present in seven patients, including three cases of previous bone marrow transplantations (one allogeneic and two autologous), two AML cases secondary to tumor chemotherapy, and two AML cases with underlying myelodysplastic syndromes. In the remaining 16 patients 10 achieved CR. Early death occurred in 6 out of 23 patients and in

3 out of 12 patients aged 65 years or older. In the nontransplanted, nonsecondary leukemia group there were 5 early deaths out of 16 and in patients aged 65 years or older 2 out of 7.

Figure 1 shows a typical time course of white blood cell counts under GM-CSF. Figs. 2 and 3 compare recovery time of neutrophils between the nontransplanted leukemias in the GM-CSF groups with that in the control groups. No differences in neu-

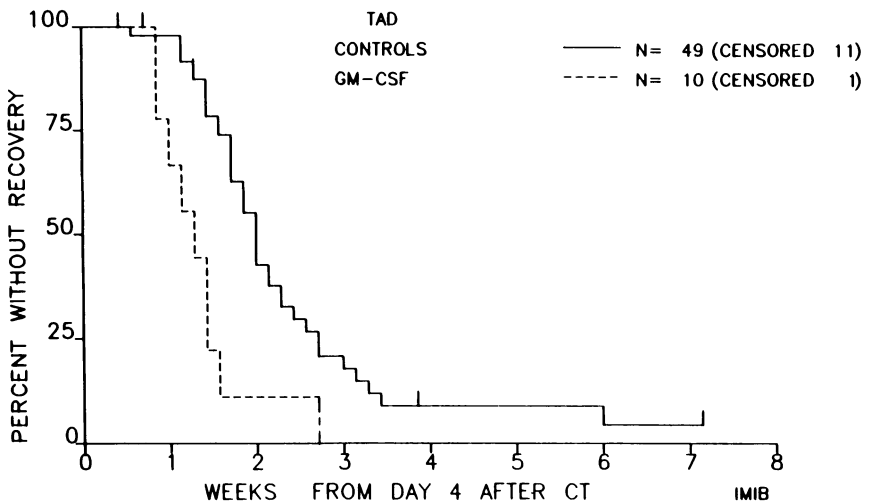


Fig. 2. Kaplan-Meier test of recovery time to 500 neutrophils from day 4 after the end of chemotherapy (CT) comparing patients with and without GM-CSF after chemotherapy with TAD9 ( $p=0.02$ )

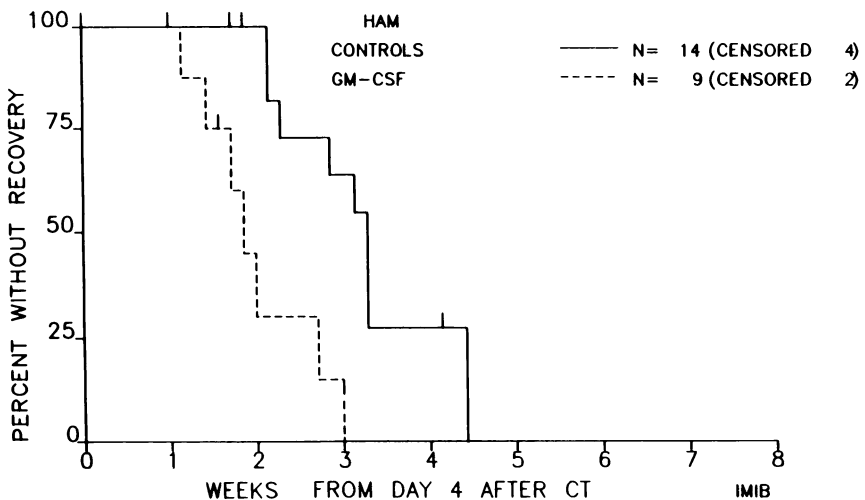


Fig. 3. Kaplan-Meier test of recovery time to 500 neutrophils from day 4 after the end of chemotherapy (CT) comparing patients with and without GM-CSF after chemotherapy with HAM ( $p=0.02$ )

trophil recovery time in terms of different doses of ara-C and daunorubicin were found between the groups compared. No significant differences in the recovery time between patients with and without GM-CSF were found for platelet and reticulocyte counts.

A regrowth of leukemic blasts in blood and bone marrow under GM-CSF was observed in 3 out of 18 patients with AML, including a second relapse of AML secondary to tumor chemotherapy and an underlying myelodysplastic syndrome. The leukemic regrowth was reversible after discontinuation in a 78-year-old patient (Fig. 4) who is in remission since 14 months without further chemotherapy. A different pattern was found in two other patients in whom the discontinuation of GM-CSF had no influence on the kinetics of leukemic regrowth, however a monocytosis in one patient was rapidly reversible (Fig. 5).

Monitoring of bone marrow cells for DNA aneuploidy revealed a presence of this leukemic cell marker in three patients. Aneuploidy disappeared after chemotherapy in two patients but was still detectable in one patient in whom it disappeared only after GM-CSF. Monitoring of leukemic colony growth in vitro did not show a

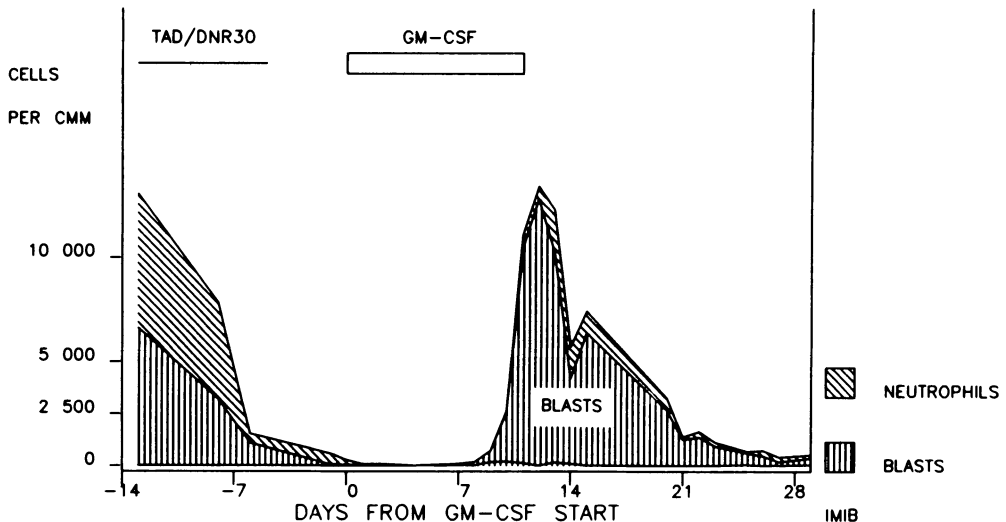
leukemic regrowth in addition to the three cases described.

Since September 1987 three of the four responders with ALL have again relapsed. Of the eight responders with AML two relapsed after 6 and 12 months in remission.

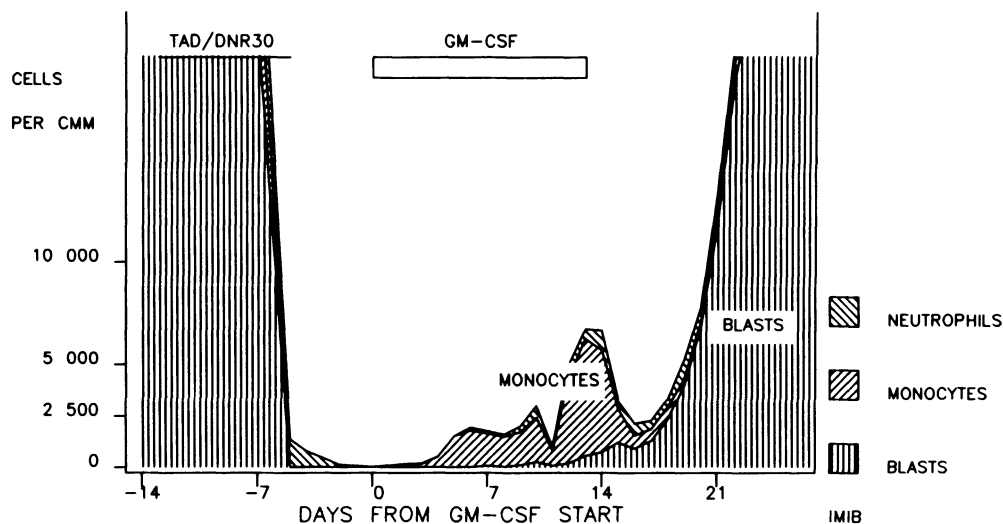
GM-CSF side effects were generally mild and consisted of fever, malaise, or fluid retention.

## Discussion

In this clinical study we explored the use of GM-CSF for acute leukemias by treating a group of patients at high risk of early death. We could demonstrate that GM-CSF given to relapsed or older patients in aplasia after chemotherapy reduces the median recovery time of blood neutrophils by 6 and 10 days, respectively, when compared to related control groups given the same chemotherapy alone. This effect was not seen in three patients having had prior bone marrow transplantation, a procedure which did not occur in the control groups. The use of GM-CSF resulted in 52% CR in this group of high-risk patients. In the group of patients over the age of 65 years 50% achieved a CR after GM-CSF. The early death rate of de novo



**Fig. 4.** Time course of blood cell counts for the different white blood cells in a patient with AML. *TAD/DNR30*, Chemotherapy with 9-day 6-thioguanine, Ara-C, and daunorubicin at 30 mg/m<sup>2</sup> single dose. For GM-CSF see Fig. 1. Under GM-CSF a rapid regrowth of leukemic blasts was observed. After discontinuation of GM-CSF the regrowth, like a spleen enlargement, was reversible and the 78-year-old patient is in stable remission since 14 months without further chemotherapy



**Fig. 5.** Time course of peripheral blood counts for the different white blood cells in a patient with AML. For TAD/DNR30 see Fig. 4, and for GM-CSF see Fig. 1. The patient showed a monocytosis and a regrowth of leukemic blasts under GM-CSF. After discontinuation of GM-CSF the monocytosis, similarly to that observed in some other patients, was rapidly reversible whereas the blast counts continued to increase with no influence on their kinetics. This pattern suggests that the regrowth in this patient occurred spontaneously rather than being induced by GM-CSF

AML in this age group was 20% at the three centers participating in this study versus previous 39% in patients aged over 65 years in the entire study conducted by the AML Cooperative Group [4].

Of major concern is the risk of promoting leukemic cell growth by GM-CSF. Thus, some myelodysplastic syndromes showed a leukemic transformation under GM-CSF [21], and leukemic cell growth in vitro was stimulated in up to 97% of AML cases [25]. However, in vivo a corresponding promotion of AML does not seem to occur at the same incidence. In addition, as also shown in myelodysplastic syndromes [20], a regrowth of blasts may be reversible after removing GM-CSF, as we could demonstrate in one out of three patients. Furthermore, a regrowth of blasts may be spontaneous and not require GM-CSF, since its discontinuation did not affect the kinetics of blast regrowth in two patients. It could be argued that GM-CSF in these cases was required only for the recruitment of quiescent blasts into the cell cycle and not for their subsequent proliferation. This exclusively recruiting effect, however, has no evidence in the in vitro experiments which – as far as described

– show an increase in the number of leukemic colonies by growth factors [22–28] regularly occurring together with a stimulation of proliferation as expressed in an increase in colony size [22, 24, 28]. Special risk factors may contribute to the regrowth of blasts. Thus, two out of three patients with regrowth had secondary AML. Except for the three cases of clinically detectable blast regrowth no further evidence of persisting leukemia was obtained from concurrent monitoring for DNA aneuploidy and leukemic colony growth. In contrast, a residual DNA aneuploidy disappeared even under GM-CSF without any further chemotherapy. Since the duration of remissions does not seem to be short, GM-CSF may not reduce remission duration in AML.

The stimulatory effects of GM-CSF on recovery of neutrophils with no promotion of the disease in most patients, as demonstrated here, may reduce the therapeutic risk in this high-risk group. The use of GM-CSF may allow further intensification of chemotherapy for acute leukemias and thereby increase the cure rate in these diseases. Our findings deserve further investigation by a controlled trial.

## Summary

Chemotherapy (CT) induced critical cytopenia remains as the major dose limiting problem in the treatment of acute leukemias. This is especially true in patients at high risk of early death due to high intensity chemotherapy for relapse or to higher age. In an attempt to reduce the phase of risk we administered human recombinant GM-CSF to 23 patients at a median age of 65 (range 17–84) years including 8 acute myelogenous (AML) and 5 lymphoblastic (ALL) leukemias after early or second relapses and 10 patients of 65 years and older in primary induction treatment for AML. 3 patients with AML had prior bone marrow transplantation. 4 AML's were secondary to tumor chemotherapy (2) or myelodysplastic syndrome (2). The study was part of chemotherapeutic phase II and III studies using TAD9 for primary induction chemotherapy and S-HAM for relapses. Starting on day 4 after the end of intensive CT GM-CSF 250 µg/m<sup>2</sup>/day was given by continuous i.v. infusion and after recovery of neutrophils was deescalated in two 4 day steps and discontinued. 12/23 patients achieved a complete remission (CR) and median age of responders is 61 (range 17–84) years. In the group of the non-transplanted patients the median recovery time of neutrophils is reduced by one week when compared to controls receiving the same chemotherapy ( $p=0.002$ ). We observed a leukemic regrowth in 3 patients of 61, 70, and 78 years, 2 of whom having secondary AML. After discontinuation of GM-CSF the regrowth of blasts was reversible in 1 patient and continued unaffectedly in its kinetics in 2 patients. Since the study started in September 1987 among the total of 12 responders relapses occurred in 2 out of 4 patients with ALL and 2 out of 8 patients with AML. Our results show that GM-CSF effectively stimulates neutrophil recovery after chemotherapy of acute leukemias and seems to improve therapeutic outcome in a high risk group. A leukemic regrowth may be reversible or spontaneous and appears less frequent than expected from in vitro effects. A longer observation time and a controlled study will be necessary to further substantiate the effect and benefit from GM-CSF in acute leukemias.

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## Treatment of Poor-Prognosis, Newly Diagnosed Acute Myelogenous Leukemia with High-Dose Cytosine Arabinoside (Ara-C) and rHUGM-CSF

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### Introduction

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is one of several growth factors that affect the survival, proliferation, and functional activation of myeloid cells in culture [1]. Clinical trials have indicated that GM-CSF can increase neutrophil counts in patients with solid tumors [2], aplastic anemia [3], and myelodysplastic syndromes [4]. Other trials have demonstrated that GM-CSF can shorten the duration of chemotherapy-induced neutropenia in patients with solid tumors [5].

Death from infection occurs in 10%–50% of patients following initial chemotherapy of acute myelogenous leukemia (AML). Infection almost always occurs in a setting of chemotherapy-aggravated neutropenia with the risk of infection increasing as the neutrophil count falls below 1000/ $\mu$ l. Hence techniques that could shorten the duration of neutropenia might reduce the frequency of infection. One such technique is the use of GM-CSF.

However, the use of GM-CSF in patients with AML is not without risk of its own. Myeloid leukemia cells in culture can use GM-CSF as a growth factor [6, 7], and its use in patients with myelodysplastic syndromes and relatively high pretreatment blast cell counts has been associated with conversion to AML [8]. Under these circumstances we decided that eligibility for the

clinical trial reported here would be limited to those patients who, because of the risk of fatal infection, were relatively unlikely to attain a remission following chemotherapy given without GM-CSF. We also administered GM-CSF only after completion of chemotherapy, the objective being to shorten the duration of chemotherapy-associated neutropenia.

### Patients and Methods

All patients had less than a 0.9 probability of surviving the initial 28 days following chemotherapy in the absence of GM-CSF. The predictions were based on a logistic regression model developed in 569 consecutive patients with newly diagnosed AML treated at MD Anderson Cancer Center between 1975 and 1985 [9]. The model had previously been found accurately to predict the probability of 28-day survival in an additional 198 patients with newly diagnosed AML who had not contributed to the derivation of the model and who were treated between 1985 and 1988 [9]. The median probability of 28-day survival among our patients with newly diagnosed AML is 0.9. Hence the patients treated during this study were that half of our patients least likely to survive the initial 28 days following chemotherapy if chemotherapy were to be given without GM-CSF. As a group, these patients have in the past had a 30% mortality rate during these 28 days, a 40% mortality rate during induction therapy, and only a 45% CR rate. The principal cause of death has been infection.

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Twelve such poor prognosis patients were consecutively entered onto this study. All had greater than 30% blasts in the pretreatment marrow. The median age was 63 years, and 5 of the 12 patients had a Zubrod performance status of 2 or worse. Seven patients had a demonstrated cytogenetic abnormality. Two patients had trisomy 8, and one patient each had an 8:21 translocation, a deletion of the long arm of chromosome 5, a 15:17 translocation, an isochromosome 17q, and monosomy 7. Circulating blast cell counts ranged between 0 and 135 000, and circulating neutrophil counts ranged between <100 and 20 000. Consistent with the relatively low likelihood of surviving induction therapy and of entering CR, three of the patients had either septicemia or pneumonia prior to therapy, and an additional patient was febrile.

Chemotherapy consisted of "high-dose" Ara-C (HDAC): 6 g/m<sup>2</sup> by continuous i.v. infusion over 96 h with a 3 g/m<sup>2</sup> dose given 12 h before the start of the infusion. We chose this regimen because, in patients with newly diagnosed AML, it has produced comparable CR and 28-day survival rates as combinations of conventional dose Ara-C and Adriamycin, but with shorter durations of neutropenia [10], and because we had considerable recent experience with HDAC, facilitating comparisons between this therapy with and without GM-CSF. Recombinant human GM-CSF (rHUGM-CSF) was supplied by Schering Corporation, Kenilworth, New Jersey. This GM-CSF is synthesized in *Escherichia coli* and is not glycosylated. rHUGM-CSF was administered at a dose of 120 µg/m<sup>2</sup> per day as a continuous i.v. infusion beginning 2 days after completion of HDAC (day 6 of therapy). The infusion was to continue until the neutrophil count was at least 1000/µl. Patients achieving CR (<5% blasts in the marrow, >1000 neutrophils, and >100 000 platelets/µl) received postremission therapy with Ara-C but without further rHUGM-CSF.

Blood counts were obtained daily. Bone marrow was examined 2 weeks after beginning HDAC and weekly thereafter. In patients with abnormal pretreatment karyotypes, cytogenetic analysis was repeated in remission.

## Results

Complete remissions were observed in 6 of the 12 patients. These occurred following one course of HDAC + rHUGM-CSF in all but one patient. Pretreatment cytogenetic abnormalities were present in four of the six patients who entered CR. These disappeared in remission in a patient whose initial karyotype contained trisomy 8, a patient who initially had an 8:21 translocation, and a patient who initially had monosomy 7. However, trisomy 8 persisted in one patient despite the presence of hematologic CR. This patient relapsed 2 months after entering CR, while relapses have not been documented to date in any other responding patient.

In three of the patients who entered CR the neutrophil count rose abruptly over 2–4 days from <50 to over 10 000/µl. This rapid rise occurred after 6–9 days of rHU-GM-CSF. All three patients had a neutrophil count of at least 1000 within 19 days of beginning HDAC. This pattern and rate of recovery is highly unusual in our experience. Although the two populations may not be strictly comparable, only 6 of 61 patients who entered CR in one course following HDAC without rHUGM-CSF during a previous study had a neutrophil count of at least 1000 within 19 days of chemotherapy versus three of five patients treated during this study. The median time to reach a neutrophil count of at least 1000 in patients entering CR in one course was 25 days when HDAC was given without GM-CSF compared with 19 days in this study. In those patients in whom the neutrophil count rose above 10 000/µl following administration of rHUGM-CSF, neutrophilia generally persisted for 1–2 weeks after discontinuation of rHUGM-CSF. In none of the patients who entered CR did maintenance of a normal neutrophil count depend on continued administration of rHUGM-CSF. However, in one other patient the neutrophil count did fall after discontinuation of GM-CSF despite the presence of a normal marrow differential, and promptly rose following reinstatement of rHUGM-CSF. The patient also had persistent thrombocytopenia requiring platelet transfusions and the marrow showed only rare megakaryocytes. With this

exception thrombocytopenia was not a clinical problem during this study. All patients who entered CR had at least 25 000 platelets/ $\mu$ l in the absence of transfusions within 4 weeks of the start of chemotherapy, and none had hemorrhagic complications.

In only one patient did the percentage of blasts cells in the marrow increase after initiation of rHU-GM-CSF following chemotherapy (11 patients survived long enough to have at least one marrow examined at least 1 week after beginning rHUGM-CSF). This patient, the only one treated who had promyelocytic leukemia, had a hypocellular marrow (5% cellularity) containing 9% promyelocytes after completing HDAC, i.e., immediately before beginning rHUGM-CSF. One week later the marrow was hypercellular (90% cellularity) and contained 80% promyelocytes. Disseminated intravascular coagulation was present, therapy was changed to daunorubicin (without rHU GM-CSF), and CR occurred.

As expected in these poor prognosis patients, major infection (pneumonia or sepsis, proven or presumed) occurred frequently. Such infections occurred in seven patients, resolved in two when the neutrophil count rose, but were the likely cause of death in the five patients who died during the study. Four of the five died while still neutropenic within 20 days of beginning HDAC, i.e., before the anticipated rHUGM-CSF-associated rise in neutrophil count as observed in patients who achieved CR (days 18–19), or, at the latest, only at this time. The fifth patient died despite being in hematologic (and cytogenetic CR) with  $>1000$  neutrophils/ $\mu$ l for 3½ weeks. The cause of death in this patient was pneumonia which developed 10 days after discontinuation of rHUGM-CSF (permission for an autopsy was not granted). The causes of death in the patients who died while still neutropenic were: Ara-C-induced colitis and resulting sepsis, disseminated aspergillosis, disseminated candidiasis, and presumed sepsis and pneumonia. There was nothing to indicate that rHUGM-CSF was responsible for death in any of these poor prognosis patients.

## Discussion

Because GM-CSF can serve as a growth factor for myeloid leukemia cells [6, 7], as well as for normal myeloid progenitors [1], we were concerned that administration of rHUGM-CSF might aggravate leukemia in our patients. However, following study completion we observed normal marrow differentials in seven patients. In only one patient was there evidence of growth of leukemia following rHUGM-CSF. This contrasts with the frequency with which rHUGM-CSF stimulates the *in vitro* proliferation of blast cells obtained from patients with AML [6, 7]. The contrast may reflect the use of chemotherapy immediately prior to rHUGM-CSF in our patients or the more complicated nature of the *in vivo* environment, e.g., the presence of normal as well as leukemic progenitors.

Our results suggest that rHUGM-CSF may be able to accelerate neutrophil recovery following chemotherapy in some but not all patients with newly diagnosed AML, although a definitive conclusion must await more formal study. In some patients accelerated neutrophil recovery appears to result from preferential stimulation of normal myelopoiesis. Thus in two patients in whom neutrophil recovery occurred within 19 days of the beginning of therapy with HDAC, the remission marrow contained only diploid metaphases, suggesting that the neutrophils were derived from normal precursors. Proof that myelopoiesis in some patients is truly normal must await demonstration that the neutrophils are functionally normal.

Other responses to rHUGM-CSF following chemotherapy were observed. In one patient in whom a rapid neutrophil recovery occurred following administration of rHUGM-CSF, cytogenetic analysis demonstrated persistent aneuploidy in remission, suggesting that myelopoiesis contained to be derived from the original leukemic clone and that rHUGM-CSF may have accelerated proliferation of this clone after it had been induced to terminally differentiate by HDAC. In other patients, time to neutrophil recovery was comparable to that usually seen following HDAC without rHUGM-CSF. The variability in response to rHUGM-CSF following chemotherapy, the



infrequently observed stimulation of leukemia observed to date, and the possibility of preferential stimulation of normal myelopoiesis leads us to believe that further study of rHUGM-CSF in conjunction with chemotherapy in patients with AML is warranted to define which patients may benefit from this therapy.

### Summary

We gave 4 days of high-dose Ara-C followed 2 days later by rHUGM-CSF (which continued until the neutrophil count was  $>1000/\mu\text{l}$ ) to 12 patients with newly diagnosed AML and a relatively poor prognosis. Six CRs occurred, there were four deaths during induction, and in only one case was there an rHUGM-CSF-associated growth of leukemia. The pattern of hematologic recovery was variable but in some patients rHUGM-CSF seemed to accelerate normal myelopoiesis following chemotherapy. Continued investigation of rHUGM-CSF and chemotherapy in AML is warranted.

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## Use of Recombinant Human Granulocyte Macrophage Colony-Stimulating Factor To Speed Engraftment and Treat Graft Failure Following Marrow Transplantation in Man\*

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### Introduction

Recombinant human granulocyte macrophage colony-stimulating factor (rhGM-CSF) is a regulatory glycoprotein which stimulates proliferation, differentiation, and maturation of myeloid hematopoietic precursors [5]. Administration of rhGM-CSF to normal nonhuman primates and patients with acquired immunodeficiency syndrome, myelodysplasia, and aplastic anemia appears capable of elevating peripheral leukocyte and granulocyte counts [1, 6, 7, 14]. We have been exploring the use of rhGM-CSF following marrow transplantation in man. Two uses of rhGM-CSF which are described here are the use of rhGM-CSF immediately following autologous marrow transplantation for lymphoid malignancies and the use of rhGM-CSF for marrow graft failure.

### Use of RhGM-CSF Immediately Following Autologous Marrow Transplantation

Marrow transplantation is being used with increasing frequency to treat patients for a

variety of diseases. Following transplantation, there is usually a period of severe pancytopenia lasting 3–4 weeks before the transplanted marrow is able to replenish peripheral blood counts. During this period, despite the use of intensive and expensive infection prophylactic measures including prophylactic antibiotics, immunoglobulin, granulocyte transfusions, and laminar airflow isolation, the vast majority of patients become febrile, at least 30% develop documented septicemia, and as many as 5% die prior to engraftment either directly due to infection or with infection as a major contributing cause.

We were interested in studying the use of rhGM-CSF immediately posttransplant to see if it had important toxicities and to see if it might be capable of speeding engraftment. Although rhGM-CSF could have a role following transplantation in many different settings, we chose first to investigate its use following autologous transplantation for lymphoid malignancies since in this setting there is little concern that rhGM-CSF might aggravate graft-versus-host disease (GVHD), cause graft failure, or stimulate tumor regrowth, concerns more reasonably applied to the settings of allogeneic transplantation and transplantation for myeloid diseases.

Our initial study involved the use of rhGM-CSF given by 2-h intravenous infusion beginning on the day of transplant and continuing for 14 days [2, 9, 11]. The starting dose was 15  $\mu\text{g}/\text{m}^2$  per day with the plan to give that dose to three patients and to double the dose in subsequent groups of three patients until either intolerable toxicity was

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**Table 1.** Outcome of a phase I–II trial of rhGM-CSF following autologous marrow transplantation for patients with lymphoid malignancies

rhGM-CSF ( $\mu\text{g}/\text{m}^2$ )	Patients	Day ANC > 500	Day PLT > 20 000	Days febrile	Day of discharge
0	86	25 $\pm$ 10	38 $\pm$ 20	12 $\pm$ 7	41 $\pm$ 25
< 60	6	22 $\pm$ 10	30 $\pm$ 22	11 $\pm$ 6	30 $\pm$ 9
60+	9	14 $\pm$ 2	29 $\pm$ 5	6 $\pm$ 3	29 $\pm$ 9

ANC, absolute neutrophil count; PLT, platelet count

seen or biologic activity observed. If activity without toxicity was seen, we planned two more dose escalations before stopping. For this study, biologic activity was defined as recovery to > 500 granulocytes/mm<sup>3</sup> before day 14 in two of three patients at a given dose. This was based on our previous experience with 86 patients treated similarly but without rhGM-CSF, where only four patients (< 5%) achieved this level by day 14.

At the first two dose levels (15 and 30  $\mu\text{g}$ ), no patient reached 500 granulocytes by day 14, but at 60  $\mu\text{g}$  the three patients reached that level by days 12, 13, and 15. Since no apparent toxicities were seen, two more dose escalations were performed. The results of the initial phase I–II study are summarized in Table 1. As shown, patients treated at 60  $\mu\text{g}/\text{m}^2$  and above engrafted faster, had fewer febrile days, and were discharged sooner. RhGM-CSF was well tolerated by all patients. No patient failed to complete the course because of toxicity. Mild toxicities included abdominal cramps in three patients, low-grade fever thought to be due to rhGM-CSF in one, and skeletal pain in one patient.

Following completion of this first study, it was our view that these results, although encouraging, required confirmation in a prospective double-blind study. However, before proceeding with such a study, a number of questions were addressed. These included the duration of rhGM-CSF administration, the day of initiation of administration, and whether to give the drug by 2-h or continuous infusion. Interest in the duration of infusion was raised by the observation that, with a 14-day infusion, some patients who had recovered to greater than 500 gran-

ulocytes/mm<sup>3</sup> would, with discontinuation of rhGM-CSF, drop back to granulocytopenic levels. In the first series of patients, the average drop was 35% (4%–63%) and four patients who had recovered again became temporarily granulocytopenic. Accordingly, the duration of rhGM-CSF infusion was prolonged to 21 days in four subsequent patients. All four tolerated the longer treatment without any apparent increased toxicity and with avoidance of the early drop. When these four patients discontinued rhGM-CSF, their baseline counts were sufficiently high that granulocytopenia did not recur [11].

Some experiments in animal models suggest that continuous infusion of GM-CSF may be superior to bolus infusion. There are no data which address the comparative utility of a 2-h infusion. A 2-h infusion has some practical benefit in freeing up a central line for other uses and in more easily allowing outpatient administration. We treated three patients with continuous infusion rhGM-CSF at 240  $\mu\text{g}/\text{m}^2$  per day for 21 days and compared their outcome with that previously seen. This very brief experience suggested no benefit for continuous infusion. Finally, we treated four patients with rhGM-CSF starting at day 7 rather than day 0 given the possibility that delaying administration might allow early stem cell self-renewal rather than differentiation, hopefully resulting in a more striking response to rhGM-CSF later. No evidence for this hoped for response was seen and so this approach was abandoned [11].

Given these results, we have initiated a prospective randomized double-blind study of rhGM-CSF versus placebo in patients

with lymphoid malignancies undergoing autologous marrow transplantation in which the drug is given by 2-h infusion daily for 21 days starting on day 0 at a dose of 240  $\mu\text{g}/\text{m}^2$  per day. We hope this study will help provide definitive results concerning the role of rhGM-CSF following autologous transplantation.

### **Use of RhGM-CSF as Treatment for Marrow Graft Failure**

Although rapid sustained engraftment is the general rule after autologous or allogeneic marrow transplant, it does not always occur. Some patients fail to engraft, others do so but only at a very slow pace, and others will, after a period of successful engraftment, lose their grafts. Graft failure can be divided into two broad categories. One category is graft rejection, a syndrome in which there is clear cytogenetic evidence of regrowth of host hematopoiesis. Graft rejection is almost certainly mediated by host immunocompetent cells which survive the preparative regimen and are not eliminated by transplanted donor cells. The incidence of graft rejection is significant, particularly with the use of mismatched donors and with the use of marrow T-depletion techniques. In the second broad category of graft failure, no evidence for regrowth of host cells can be found but the transplanted marrow still does not function normally. There are probably many causes for this sort of graft failure, including viral infections, adverse drug reactions, intrinsic or acquired damage to the transplanted stem cell, and unrecognized autoimmune or alloimmune reactions.

Patients who fail to engraft, engraft slowly, or reject their grafts do poorly. The chances of surviving a transplant are reduced roughly by half in those patients who do not recover to 100 granulocytes/ $\text{mm}^3$  by day 28 after transplant. This difference in survival is attributable wholly to non-leukemic, that is, transplant-related deaths. In the past, the only treatments for graft failure were continued support or a second marrow transplant. Such transplants may consist simply of a second marrow infusion if continued donor hematopoiesis is documented, or an intense and potentially toxic

preparative regimen followed by marrow transplant if return of host immunocompetent cells is found. Second transplants for graft failure are sometimes useful but overall are usually unsuccessful. Survival 1 year following an attempted second transplant, whether autologous or allogeneic, is roughly 10% in our experience.

Since rhGM-CSF seemed to work so well in our phase I–II trial with little toxicity, we began a similar phase I–II trial in patients with graft failure. In this study, eligible patients were those who did not achieve a granulocyte count of 100/ $\text{mm}^3$  by day 28 or those who initially engrafted but later lost their grafts as defined by a granulocyte count of less than 500/ $\text{mm}^3$  for 2 weeks. Patients were treated with up to three cycles of rhGM-CSF at doses of 60–500  $\mu\text{g}/\text{m}^2$ . This study is still ongoing, but preliminary results have been published in abstract form [10]. With 17 patients entered, 7 of 10 autologous transplant recipients, and 4 of 8 allogeneic recipients appear to have responded to rhGM-CSF by increasing granulocytes to above 500/ $\text{mm}^3$  within 2 weeks of beginning treatment. All responses were sustained after stopping rhGM-CSF and 10 of the 11 responders (as opposed to 3 of the 8 nonresponders) remain alive and well at this time. RhGM-CSF was well tolerated at doses up to 250  $\mu\text{g}/\text{m}^2$ , but at 500  $\mu\text{g}/\text{m}^2$  mild to moderate toxicity was seen in all four treated patients.

This phase II study (240  $\mu\text{g}/\text{m}^2$  is our established dose) will continue to accrue patients in order to learn more about the use of rhGM-CSF in this setting. If subsequent results continue to appear as promising as those so far seen, an interesting question will emerge. Given the past inadequacy of our treatment of graft failure, and given the results and lack of toxicity seen with rhGM-CSF, is participation in a prospective randomized trial with one arm being a placebo really in the patients' best interest?

### **Discussion**

The results we saw in our phase I–II trial of rhGM-CSF given immediately post-transplant were in large part predicted by studies performed in nonhuman primates by Mon-

roy et al. and Nienhuis et al. [8, 12]. They are also similar to those reported by Brandt et al. in a group of patients given high-dose combination chemotherapy followed by autologous transplantation as treatment for a variety of solid tumors, with the exception of apparently increased toxicity in the study by Brandt et al. [4]. The increased toxicity seen in that study might, in part, have been due to the use of higher doses of rhGM-CSF in their study, the use of a different preparation of rhGM-CSF, and the use of a different preparative regimen with different toxicities of its own. An additional "toxicity" mentioned by the Duke group is the observation that migration of granulocytes to skin chambers is diminished in the presence of rhGM-CSF [13]. Whether this observation has any clinical significance is unclear. We did not witness progressive infections in the face of adequate granulocyte counts in our study and the overall number of days with fever and infection appeared to be dramatically reduced with rhGM-CSF. Thus, at present, it is difficult to know the meaning of this laboratory finding.

In both the Duke study and ours, there was a strong suggestion of accelerated engraftment with rhGM-CSF. However, a study published by Blazar et al. did not show as strong a tendency toward accelerated engraftment with rhGM-CSF [3]. In that study, all marrows were incubated with 4-hydroperoxycyclophosphamide (4-HC) *in vitro*, a form of *in vitro* treatment not used in our study or Brandt's. Since 4-HC kills many committed myeloid progenitors, it is possible that *in vitro* use of 4-HC eliminates the class of cells most responsive to rhGM-CSF and therefore diminishes any apparent effect of the agent. In this circumstance, use of a growth factor which reacts with an earlier progenitor might be required. A final definition of the beneficial effects and toxicities of rhGM-CSF used immediately post-transplant will require a prospective, randomized trial. Several such trials are currently underway.

Use of GM-CSF or other hematopoietic growth factors to treat graft failure has not previously been described in man, although Nienhuis et al. did describe such use in two rhesus monkeys [12]. Our preliminary results suggest a benefit of rhGM-CSF in this

setting as well, and again we saw very little toxicity. Specifically, rhGM-CSF did not seem to exacerbate GVHD or lead to graft rejection. Nor did we witness rapid regrowth of myeloid tumors in those patients treated for graft failure after transplantation for AML. However, our results are quite preliminary and this study is still actively accruing patients.

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## Regeneration of Granulopoiesis with Recombinant Human Granulocyte-Macrophage Colony-Stimulating Factor After Bone Marrow Transplantation

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### Introduction

In the period immediately following bone marrow transplantation, most patients contract infections which contribute significantly to morbidity and mortality. These infections during the granulocytopenic phase following pretreatment for BMT are more severe than during the granulocytopenia caused by conventional cytotoxic treatment. The higher doses of cytostatic drugs damage the defense mechanisms of the mechanical barriers such as skin and mucous membranes. The mucositis in the mouth and the entire gastrointestinal tract caused by cytostatic drugs and radiation can be superinfected by colonizing microflora, producing an initial focus for severe septic infections during granulocytopenia.

It has long been known that the severity and duration of granulocytopenia are correlated with an increased risk of infection [3]. This is also true for patients after BMT [6]. It would be a great therapeutic step forward if it were possible to significantly reduce the extent and duration of severe granulocytopenia after bone marrow transplantation.

### Human Recombinant Granulocyte Macrophage Colony-Stimulating Factor (rhGM-CSF)

Normal hematopoiesis cell proliferation and differentiation is controlled by a group of growth factors known as colony-stimulating factor (CSF) [9, 10]. They support the proliferation and differentiation of hematopoietic precursor cells in semisolid culture systems and activate the function of the granulocytes and macrophages in the peripheral blood. GM-CSF is produced by recombinant DNA technology in yeast or *Escherichia coli* expression vectors [5].

### Clinical Phase I Studies with rhGM-CSF

Phase I dosage escalation studies of yeast derived material showed that a dose of 500 µg/m<sup>2</sup> a day in the form of a continuous infusion could be administered to humans without problems [14]. A biological effect was found with doses as low as 30 µg/m<sup>2</sup> per day. A continuous infusion caused a much more effective rise in granulocyte levels than did a short infusion of the same total amount [7].

It could be concluded from the preclinical and clinical phase I studies that the intravenous administration of rhGM-CSF after bone marrow transplantation can shorten the duration of granulocytopenia [2, 10]. Based on this a phase I/II therapy study with rhGM-CSF after bone marrow transplantation was designed.

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**Table 1.** Autologous and allogeneic bone marrow transplantation

No.	Age/sex (years)	Diagnosis, status
<b>Autologous</b>		
39-L-02	6 F	Neuroblastoma IV, CR
46-L-05	10 M	Neuroblastoma IV, PR
47-L-06	6 F	Neuroblastoma IV, CR
55-L-09	15 F	Alveolar rhabdomyosarcoma
72-L-10	15 M	Ewing's sarcoma
-B-01	5 M	Neuroblastoma
-B-02	10 F	Neuroblastoma
-B-03	10 M	Ewing's sarcoma
-B-04	30 M	Ewing's sarcoma
44-L-04	15 F	ALL, second CR
<b>Allogeneic</b>		
37-L-01	19 F	T-ALL, second CR
49-L-07	30 M	Lymphoblastic NHL i.v., PR
50-L-08	29 F	PRE-B-ALL, second CR

**Goals of This Study**

1. To investigate the effect of rhGM-CSF on hematological reconstitution after bone marrow transplantation.
2. To test the toxicity and safety of rhGM-CSF.

**Patients and rhGM-CSF Therapy Protocols (Tables 1,2)**

Patients with the following diseases were included in this study:

1. Neuroblastoma, Ewing's sarcoma, alveolar rhabdomyosarcoma
2. Acute lymphoblastic leukemia after autologous or allogeneic bone marrow transplantation
3. Patients with bone marrow failure after autologous BMT.

**Table 2.** Conditioning regimens for bone marrow transplantation

<b>Neuroblastoma</b>		
Cisplatin	120 mg/m <sup>2</sup>	day -19
Irradiation to former sites of bulky disease 2 fractions/day	2100 cGy	days -18 to -10
BCNU	200 mg/m <sup>2</sup>	day -9
Melphalan	2 × 30 mg/m <sup>2</sup>	days -8, -7, -6
VP-16	300 mg/m <sup>2</sup>	days -5, -4, -3
<i>Or</i>		
Melphalan	25-30 mg/m <sup>2</sup>	day -7, -6, -5, -4
VP-16	1800 mg/m <sup>2</sup>	day -3
Fractionated total body irradiation	2 × 150 cGy	day -7, -6, -5, -4
<b>Leukemia and NHL</b>		
1. Fractionated total		
Body irradiation on days		total dose: 12 Gy -10, -9, -8, -7
Daily fractions lung dose		2 10 Gy
VP-16 (etoposide) on days		15 mg/kg per day -6, -5, -4, -3;
<i>Or</i>		
2. Fractionated total		
Body irradiation on days		total dose: 12 Gy -9, -8, -7, -6
Daily fractions lung dose		2 10 Gy
Cyclophosphamide on days		50 mg/kg per day -5, -4, -3, -2



**Treatment Schedule with rhGM-CSF**

Patients were given 500  $\mu\text{g}/\text{m}^2$  rhGM-CSF a day by intravenous infusion over 24 h in 0.9% normal saline with 1% albumin. The first dose was administered within 24 h of the completion of bone marrow transfusion. This dose was maintained until the absolute

neutrophil count reached 1000/ $\mu\text{l}$  or more for three consecutive days. Then the rhGM-CSF dose was reduced to 250  $\mu\text{g}/\text{m}^2$  a day. If the neutrophil count increased further or remained stable at greater than 1000/ $\mu\text{l}$  for at least three consecutive days the dose was reduced again to 125  $\mu\text{g}/\text{m}^2$ . After 3 more days at this dose level and, again, neu-

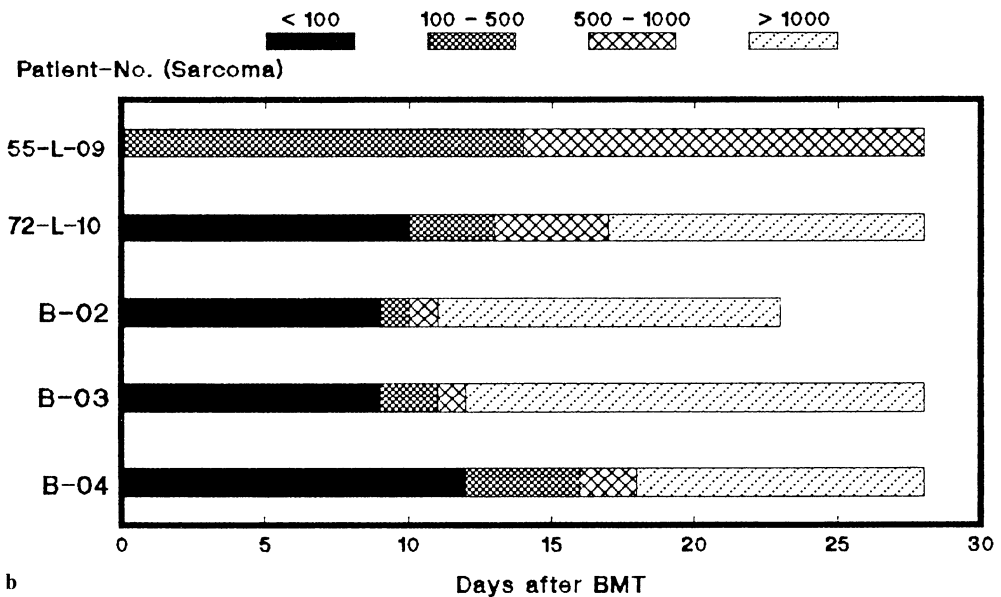
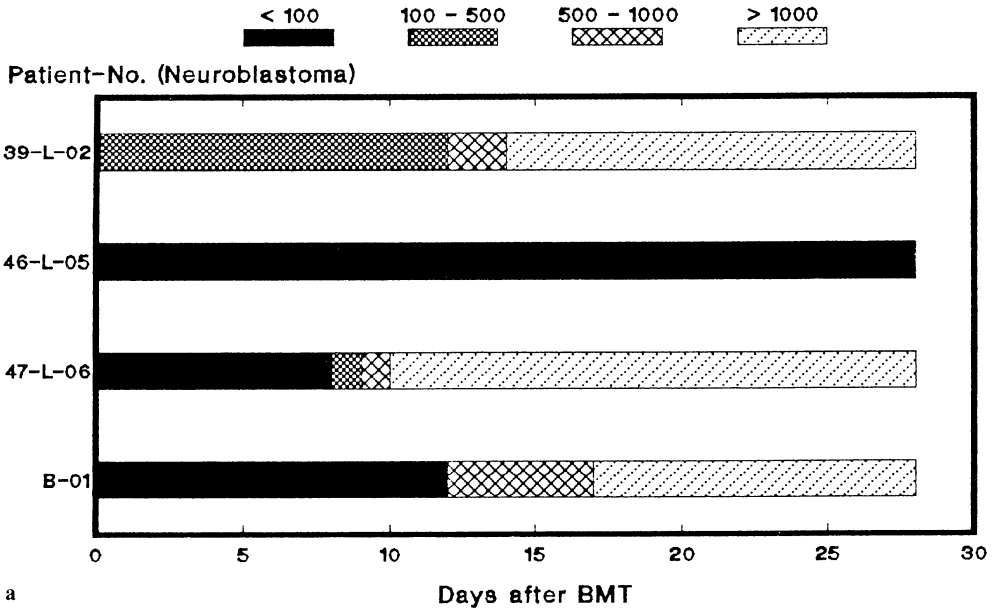


Fig. 1 a, b. Time of absolute neutrophil count after autologous bone marrow transplantation

trophils above 1000/ $\mu$ l, a reduced dose of 50  $\mu$ g/m<sup>2</sup> was administered for 3 days, which completed rhGM-CSF treatment. Therapy should not extend beyond 28 days after BMT.

**Results**

The duration of granulocytopenia after autologous BMT for neuroblastoma and sarcoma is shown in Fig. 1. The kinetics of leukocyte regeneration after autologous BMT in a patient with ALL are shown in Fig. 2, and Fig. 3 shows the duration of neutropenia after allogeneic BMT in ALL.

*Toxicity*

In general, side effects were mild to moderate. The most frequent ones were fever, erythema, abdominal complaints, tachycardia, and slight weight gain. Only one patient after allogeneic BMT suffered from a capillary leak syndrome, which was aggravated by rhGM-CSF.

**Discussion**

The most important aim of this study was to test the biological effect, toxicity, and feasibility of a continuous rhGM-CSF infusion after bone marrow transplantation. The rhGM-CSF dosage took the results of phase I studies into account, in which a maximum tolerable dose of 500  $\mu$ g/m<sup>2</sup> per day was established for material produced in yeast (G. Schulz, A.G. Behringwerke, personal communication [8]). There was no severe toxicity clearly attributable to rhGM-CSF administration.

After autologous BMT in solid tumors eight out of nine patients responded to the rhGM-CSF therapy. The continuous infusion with rhGM-CSF led to a rapid increase in leukocytes, in most cases from a proliferation of the neutrophils, i.e., granulocytes and stab cells. In several patients the eosinophil and monocyte counts also rose excessively. A significant increase in leukocytes could not be determined in any of the patients before day +8. This differs from results obtained from patients with bone marrow dysfunction [14], aplastic anemia

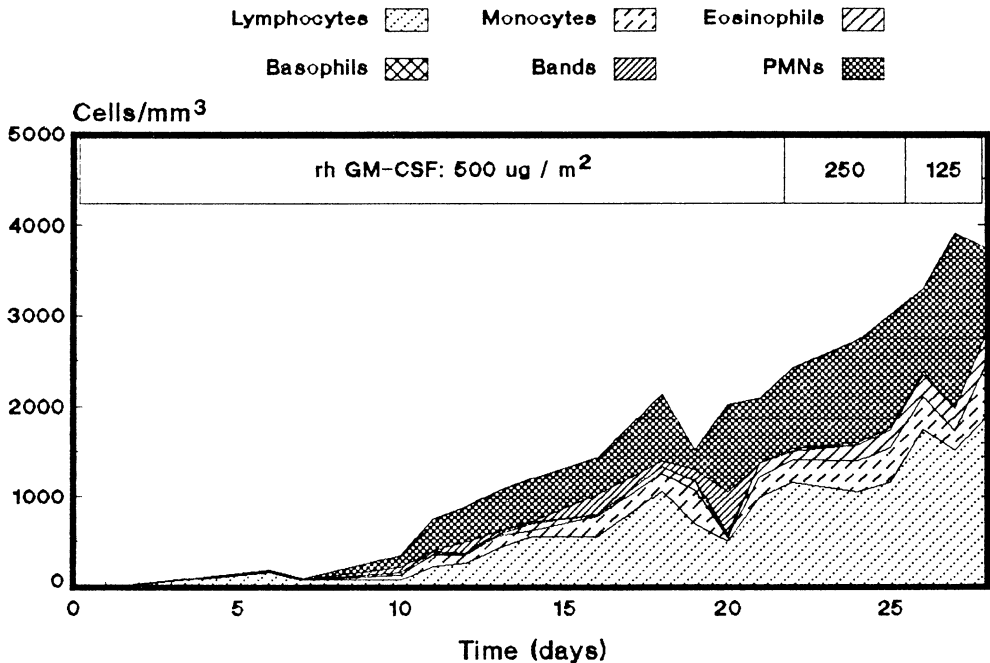


Fig. 2. Patient 44-L-04, female, 16 years old, with ALL after autologous bone marrow transplantation

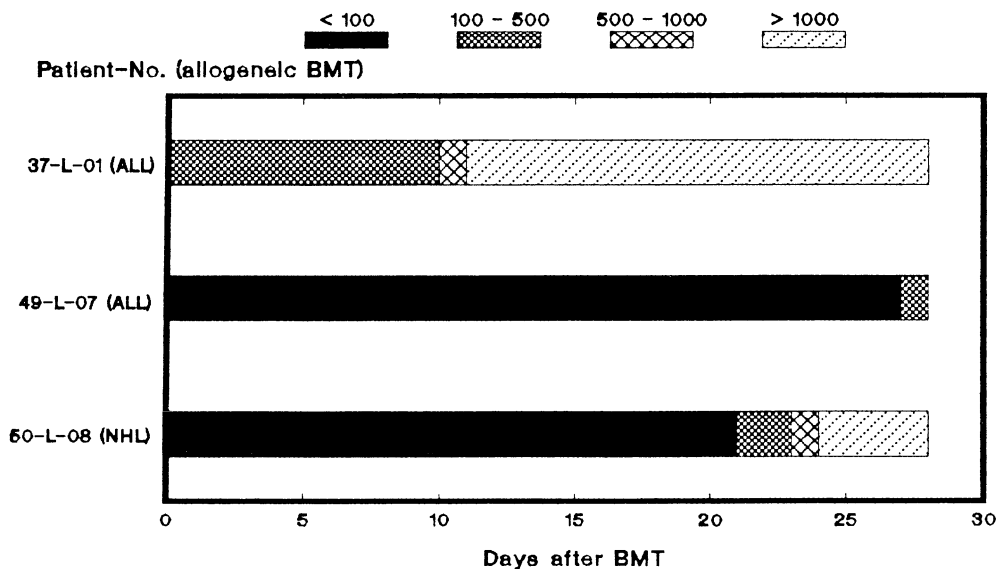


Fig. 3. Time of absolute neutrophil count after allogeneic bone marrow transplantation

[13], and myelodysplasia [1]. In these patients the leukocyte count rose markedly within 1–5 days [15].

Besides the additional effect on marrow release, margination, and extravascular distribution of leukocytes [12, 13], it can be assumed that the stimulative progenitor cells do not yet react after autologous BMT or are only present in small numbers and must first grow again from the pluripotent stem cells.

In acute lymphocytic leukemia (ALL) after autologous BMT the bone marrow recovered more rapidly than one would have been expected, within about 14–40 days, median 24 days.

In allogeneic BMT it is not yet clear if rhGM-CSF can accelerate the regeneration of hemopoiesis, as the time to reach 500 neutrophils/ $\mu$ l ranged from 8 to 41 days. Further studies are necessary.

Neutropenia under 500/ $\mu$ l lasted 10–16 days after autologous BMT for neuroblastoma and sarcoma when patients responded to rhGM-CSF. Thus, the phase of life-threatening neutropenia lay under the time generally observed with this chemotherapy protocol. In our protocol, rhGM-CSF was discontinued after a gradual reduction as

soon as the neutrophil count remained above 1000/ $\mu$ l, and at the latest after 28 days of therapy. The granulocyte level fell again when rhGM-CSF was discontinued, but never under the critical limit of 500/ $\mu$ l. In another study it was shown that the mean ( $\pm$ SD) granulocyte level rose significantly higher after a 14-day continuous infusion ( $1160 \pm 1030/\mu$ l) than in a control group. A level of 500 granulocytes/ $\mu$ l was reached after a mean of  $15.8 \pm 6.3$  days in patients with rhGM-CSF, earlier than in the historical control group, where it was reached after  $19 \pm 4.6$  days. When rhGM-CSF was discontinued, the leukocyte and granulocyte levels fell within 2–3 days to those of the other patients [4]. It can be concluded from these data and from the discontinuation effect that rhGM-CSF stimulates and accelerates granulocytogenesis after autologous BMT. It is still not clear, however, by how many days granulocytopenia can be shortened.

Our results need confirming in a larger study with equivalent conditioning regimens. We have initiated a multicenter trial in Europe with 15 transplantation centers. Only patients with ALL and non-Hodgkin's lymphoma (NHL) who receive an au-

tologous bone marrow graft after total body irradiation with at least 10 Gy in combination with high-dose chemotherapy will be included in this randomized double-blind study with rhGM-CSF.

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## Colony-Stimulating Factors (rhG-CSF, rhGM-CSF, rhIL-3, and BCFG) Recruit Myeloblastic and Lymphoblastic Leukemic Cells and Enhance the Cytotoxic Effects of Cytosine-Arabinoside\*

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### Introduction

The treatment of adult and pediatric acute lymphoblastic leukemia (ALL) and acute myeloblastic leukemia (AML) has resulted in long-term survival and cures in a substantial number of patients [1–3]. Best results have been achieved in pediatric ALL with approximately 70% of patients in ongoing long-term remission [4]. Results in patients with adult leukemias are much worse: though 60%–80% of patients with AML achieved complete remission, only 15% are alive after 5 years [1]. In adult ALL, the incidence of remission is ca. 80%–90%, but only 35% of these remain in long-term remission [1, 3, 5]. These results have not improved significantly over the last 15 years, despite efforts in many centers and in multicentric trials to test new drug schedules, drug combinations, and treatment modalities, including bone marrow transplantation [6]. Clearly, new approaches to improve survival of these potentially curable patients are needed.

Numerous prognostic models have been developed and a multitude of clinical and laboratory factors have been identified as having prognostic relevance [7–9]. These prognostic models have been useful in identifying patients with high, intermediate, and poor risk of achieving complete remission and remaining in remission for extended periods of time, and they have been useful in selecting patient groups for new chemotherapeutic approaches. Unfortunately, however, few if any of these factors have provided guidance for therapy based on the biology of the acute leukemias.

At Memorial Hospital studies in AML [3, 10] have investigated cell cycle kinetics of leukemia cells and identified cell kinetic quiescence as unfavorable for:

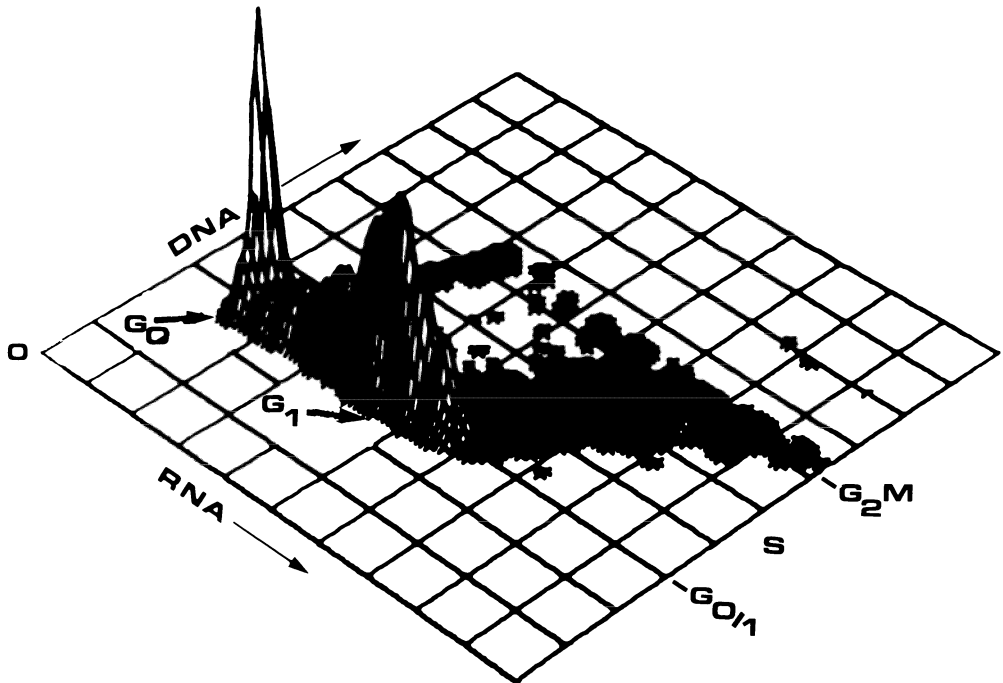
1. achievement of complete remission,
2. remission duration, and
3. survival.

Earlier cell kinetic studies focused primarily on the potential prognostic importance of cells in DNA synthesis, as assayed by thymidine-labeling autoradiography or DNA flow cytometry. Results of these studies have been recently reviewed [11]. DNA/RNA flow cytometry allowed us to further analyze cell populations within the cell cycle, in particular, to discriminate quiescent G<sub>0</sub> cells from proliferating G<sub>1</sub> cells [12]. Subsequent application of this technique to acute leukemia allowed the development of the above-mentioned prognostic models [3, 10, 13]. Figure 1 shows a typical DNA/RNA histogram obtained from a bone marrow sample of acute nonlymphocytic leukemia:

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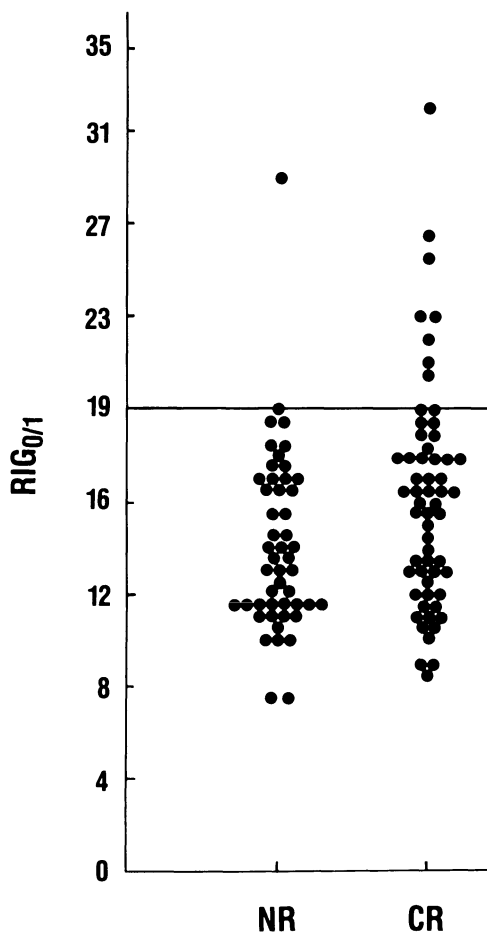
**Fig. 1.** DNA/RNA histogram of a leukemic cell population as determined by acridine orange flow cytometry. Cells in  $G_0$  can be distinguished from cells in  $G_1$  by differences in RNA content and cells in S and  $G_2M$  by increased DNA content. Some 10000 cells were measured in a flow cytometer and analyzed according to [12]

cells in  $G_0$  can be distinguished from those in  $G_1$ , S, and  $G_2M$ . The RNA content of these subpopulations can be normalized by relating them to the RNA content of normal control lymphocytes (RNA index) [10, 12].

Some 89% of adult patients with AML with RNA index  $G_{0/1}$  greater than 19 and 65% of patients with RNA index  $G_{0/1}$  greater than 16 achieved complete remission (CR). In contrast, only 49% of patients with RNA index  $G_{0/1}$  less than 16 achieved CR. This association is shown in Fig. 2. A complete analysis of this data and multivariate analysis of clinical and laboratory variables was previously reported [10]. Of patients with RNA index above the mean 64.9% had CR and of those with RNA index below mean 47% had CR ( $P=0.059$ ). No significant correlation between remission incidence and the number of cells in S phase was noted. Stepwise Cox regression analysis identified the log RNA index  $G_1$  as the most significant predictor for remission duration

( $P=0.026$ ). Age was another significant variable; young patients with high RNA index did significantly better than those with low RNA index or than older patients. When survival was analyzed, it was found that patients with RNA index  $G_1$  less than 16 did not survive more than 12 months. All patients surviving longer than 12 months were in the group with higher RNA index. Stepwise Cox regression analysis again demonstrated RNA index to be independently significant ( $P=0.026$ ; Fig. 3).

Analysis of ALL in adults resulted in development of a prognostic model consisting of white blood cell count (WBC;  $<$  or  $>$  20 000 or percentage of circulating blasts  $>$  80%,  $P=0.0$ ), immunophenotype (null or B cell,  $P=0.014$ ), RNA index  $G_{0/1}$  ( $<$  or  $>$  14,  $P=0.017$ ), age at diagnosis (younger or older than 60 years,  $P=0.049$ ), time to less than 5% marrow blasts in more than 28 days ( $P=0.066$ ;  $P$  values derived from final Cox model and score test) [3]. For



**Fig. 2.** Distribution of RNA index  $G_{0/1}$  ( $RIG_{0/1}$ ) in patients with acute myeloblastic leukemia at diagnosis: *NR*, no response; *CR*, complete remission (treated with Memorial Hospital L16/M protocols). Patients with  $RIG_{0/1}$  greater than 19 have a higher likelihood of achieving CR than patients with  $RIG_{0/1}$  less than 19

achievement of complete remission, unfavorable prognostic factors were identified as follows: L3 or acute undifferentiated leukemia (AUL) morphology ( $P=0.0008$ ), WBC over  $10000/\text{mm}^3$  ( $P=0.0009$ ), weight loss over 5% ( $P=0.010$ ), low log RNA index  $G_{0/1}$  ( $P=0.011$ ), old age at diagnosis ( $P=0.012$ ). Low RNA index, i.e., a quiescent cell kinetic state at diagnosis, was again associated with low likelihood of achievement of remission [3]. For remission duration, poor prognostic predictors were WBC greater than 20000 or percentage of circulating blasts over 80%, non-T phenotype, high

RNA index, old age, and slow achievement of CR [3].

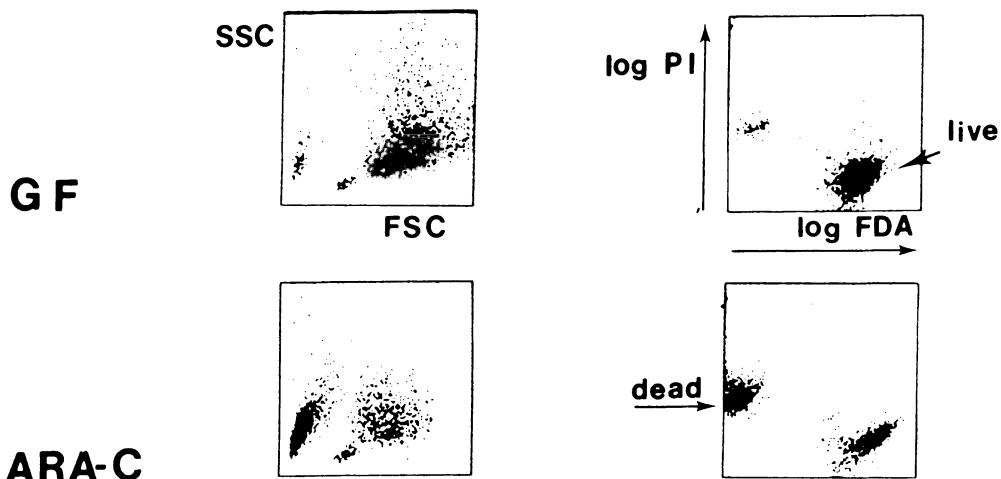
Analysis of prognostic factors in ALL in children resulted in the identification of time to remission as the most important prognostic factor: Patients with early remission had longer remission durations [13]. Early CR (within 14 days) was influenced by:

1. age,
2. WBC,
3. platelet count,
4. RNA index  $G_0$ , and
5. DNA index.

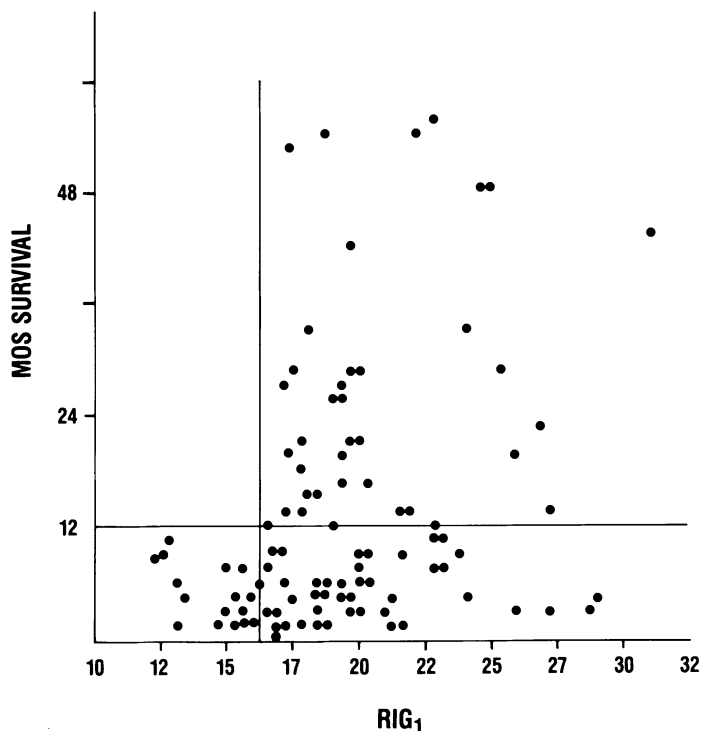
Very young (<2 years) and adolescent patients, low platelet count, WBC over  $10000/\text{mm}^3$ , and low RNA index as well as low DNA index were all associated with low likelihood of achievement of complete remission (Fig. 4).

All three prognostic models indicate that cell kinetic quiescence of leukemic cells at the time of diagnosis may confer poor prognosis in AML and ALL. We have previously reported on the recruitment of ALL cells in vitro into the cell cycle by a combination of interleukin-2 (IL-2), phorbol 12-myristate 13-acetate (PMA) and monoclonal antibody OKT3 (OKT3) [14]. Recruitment was observed in 16 of 18 patients with ALL, and DNA aneuploidy served as a marker for the identification of leukemic cells. Normal cells in the same samples were recruited into  $G_1$ , S, and  $G_2M$ , while leukemic cells were only recruited into  $G_1$ . The conditions required for recruitment varied greatly from case to case. In some samples individual components were sufficient for maximum recruitment into  $G_1$ , in others, combinations of factors were most effective. It is likely that recruitment into the cell cycle under these conditions was mediated by normal hematopoietic cells, which were present in all cultures.

The advent of recombinant human colony stimulating factors provides new opportunities for the recruitment of leukemic cells into the cell cycle. In this paper, we report on the effects of rhG-CSF, rhGM-CSF, rhIL-3, and low molecular weight B-cell growth factor (BCGF) on blast cells from AML and ALL. We then report improved cytotoxicity of cell cycle-specific chemotherapy, i.e., Ara-C, against the recruited leukemic cells.



**Fig. 3.** Correlation between RNA index of G<sub>1</sub> cells (*RIG*<sub>1</sub>) and survival (months) in patients with acute myelogenous leukemia. Patients with *RIG*<sub>1</sub> less than 16 do not survive longer than 12 months ( $P=0.026$ )



**Fig. 4.** Prognostic factor analysis of pediatric acute lymphoblastic leukemia. Achievement of complete remission (CR) in 14 days after initiation of induction therapy was identified as the most important prognostic factor with regard to remission duration. The variables shown here (white blood cell count, WBC, age, platelet count, DNA index, and RNA index G<sub>0</sub>) were analyzed with locally weighed logistic regression analysis. Patients with very low *RIG*<sub>0</sub> have a significantly lower likelihood of achieving early CR than patients with higher *RIG*<sub>0</sub>. Low and older age (>7 years), high WBC, low platelet count, and low DNA index are likewise correlated with low likelihood of achieving early CR

## Material and Methods

### Cells and Cell Cultures

Peripheral blood or bone marrow samples were obtained, after written informed consent, from 13 patients with AML and 3 pa-

tients with CML in blastic phase (2 myeloid and 1 lymphoid). AML patients were classified according to the FAB criteria (Table 1). In all samples the percentage of blasts was more than >90.

Cells were separated by centrifugation on Ficoll-Hypaque density gradient. Mono-



**Table 1.** FAB classification of the leukemia samples studied

Diagnosis	No.	FAB
AML (new leukemia)	5	M1, M2, M3 (2), M5
AML (2nd leukemia)	5	M1, M2 (3), M4
AML (relapsed)	3	M1, M2, M5
CML (blastic phase)	3	1 lymphoid, 2 myeloid
Total	16	

Newly diagnosed "de novo" acute leukemias are listed separately from newly diagnosed secondary leukemias, relapsed leukemias and cases of blastic transformation of CML.

cytes were removed by adherence to plastic. In some instances leukemic cells were further purified by eliminating T lymphocytes (E rosetting).

AML cells were resuspended in RPMI-1640 supplemented with 10% FCS, 1% L-glutamin and 1% penicillin-streptomycin (all by GIBCO, Grand Island, NY).

Cells were cultured at a starting concentration of  $0.5 \times 10^6$ /ml for 4 days at 37°C in 5% CO<sub>2</sub> with or without (control culture) the following recombinant human growth factors: G-CSF 500 U/ml (AMGEN), GM-CSF 500 U/ml (AMGEN), and IL-3 20 ngr/ml (IMMUNEX).

ALL blast cells were likewise separated on a Ficoll-Hypaque gradient, monocyte depleted, and incubated with rhG-CSF, rhGM-CSF, rhIL-3, and low molecular weight (15 kDa) BCGF (kindly provided by Dr. F. Uckun, University of Minnesota, Minneapolis, MN).

#### *Drug Treatment*

Ara-C (1 mM; Upjohn Kalamazoo MI) was added after 48 h of preincubation with cytokines and in control cultures. The time of exposure to the drug was from 12–48 h. The drug exposure was stopped by washing the cells twice with cold 4°C phosphate-buffered solution (PBS).

#### *Viability/Clonogenicity*

In modification of a procedure reported by Ellwart et al. [15], an aliquot (1–2 ml) of cell

suspension was taken from the liquid culture at 12-h intervals and incubated at room temperature for 30 min with 0.5 µg/ml final concentration of fluorescein diacetate (FDA; Sigma, St. Louis, MO) dissolved in acetone. Propidium iodide (PI; Sigma, St. Louis, MO) in PBS was added to achieve a final concentration of 50 µg/ml. The tubes were then placed on ice and measured by flow cytometry (FACscan, Becton and Dickinson, Mountainview, CA) for 1 min at the set flow rate of 12 µl/min. Viable cells are those which are able to convert (by unspecific esterases) nonfluorescent FDA to fluorescein which has bright green fluorescence. Nonviable cells display red fluorescence because PI can penetrate the membrane and stain the nuclei (Fig. 5).

#### *Proliferation and Recruitment*

Assessment of proliferation was performed at 24 and 48 h of suspension culture by counting cells in a chamber and assessing cell viability by trypan blue. Determinations of cellular DNA and RNA content and cell cycle distribution (i.e., G<sub>0</sub>%, G<sub>1</sub>%, S%, G<sub>2</sub>M% and RNA index) were obtained using the acridine orange (AO) technique as previously described [10, 12]. For the identification of cells in active DNA synthesis, simultaneous cytometric staining for BrdU and cellular DNA content was used as described by Dolbeare [16]. Growth fraction was determined by the simultaneous flow cytometric measurement of Ki67 [17] and cellular DNA/Ki67 content as described by Larsen [18].

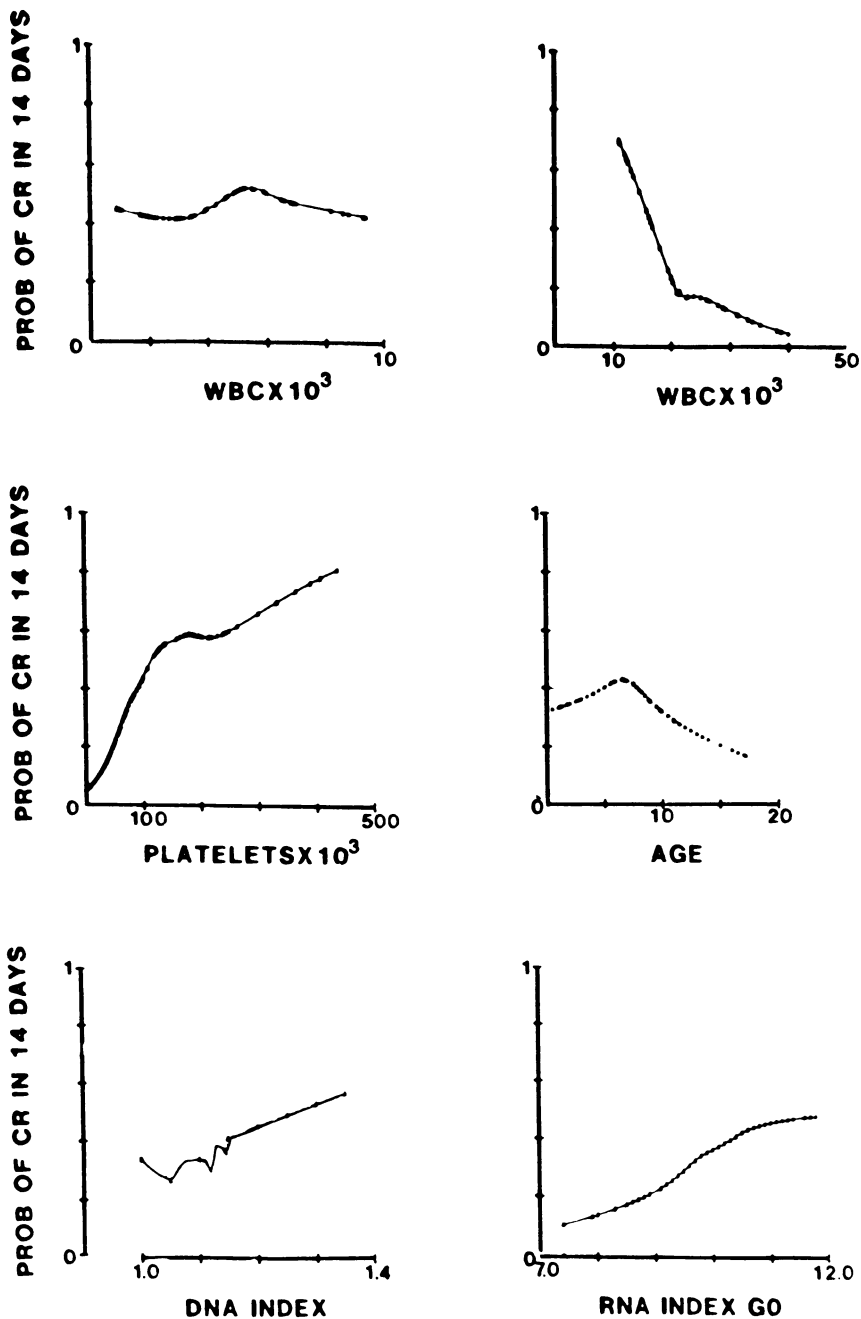


Fig. 5. Cell viability/clonogenicity in AML. Cells were stained with fluorescein diacetate and propidium iodide. Live cells convert FDA into fluorescein (green fluorescence) and dead cells do not. Dead cells allow penetration of PI into the cell nucleus (red fluorescence). *Upper panels* demonstrate viability of AML cells in the presence of the cytokines rhG-CSF + rhGM-CSF + rhIL-3: few cells are identified as dead. *Lower panels* show the effect of Ara-C (1 mM) on cell viability: a substantially larger number of cells are dead and light scatter properties are also changed (*FSC*, side scatter; *FFC*, forward scatter)

### Flow Cytometry Measurements and Analysis

For the AO samples, a computer-interfaced research cytofluorograph (modified model FC 201, Ortho Instruments, Westwood, MA) was used to measure the fluorescence of individual cells in two separate wavelength bands (F530 and  $F > 600$ ). Fluorescence signals generated by each cell at the time of crossing the focus of the argon-ion laser beam were optically separated and measured by photomultipliers. The pulse width, i.e., the time the cell or nucleus takes to pass through the illuminating beam, was also recorded and used to distinguish single cells from cell doublets and other aggregates. Data were stored in a Nova 1220 minicomputer, and software developed by Sharpless [12] was used to analyze each sample.

For measurement of cells stained simultaneously with FITC-MoAb (anti-BrdU, anti-Ki67) or fluorescein (FDA) and PI (DNA content) a FACScan was used (Becton Dickinson, Mountainview, CA). The samples were analyzed using a Hewlett-Packard computer and Consort 30, FACScan Research and Paint-a-gate software (Becton Dickinson, Mountainview, CA).

### Results

A total of 16 samples from 16 patients with AML and blastic transformation of CML were analyzed. The different diagnostic subgroups are shown in Table 1. Table 2 summarizes the results of cytokine treatment in all samples studied.

The numbers represent mean percentage of cells in  $G_0$ ,  $G_1$ , S, and  $G_2M$  as well as RNA index of cells in  $G_1$ , all determined by acridine orange flow cytometry. Significant ( $P=0.04$ ) reduction of the number of cells in  $G_0$  was accompanied by significant increase in the number of cells in S-phase ( $P=0.001$ ) and in  $G_2M$  ( $P=0.06$ ). The RNA content of cells in  $G_1$  increased significantly as well ( $P=0.001$ ), indicative of an increased cellular RNA content of these cells. The rate of DNA synthesis, as measured by the relative amount of incorporated bromodeoxyuridine (BrdU) did not change significantly. The growth fraction of the leukemic population, as measured by the expression of the cell cycle related nuclear antigen Ki67, increased significantly ( $P=0.05$ ). Only minimal recruitment was observed in the three patients with relapsed AML and in all patients with blastic transformation of CML.

No recruitment of any kind was observed in samples from ALL (data not shown). Of the 13 patients with AML, six patients had a significant (three fold) increase in the number of S phase cells. Their data is shown in Table 3, a significant depletion of  $G_0$  cells from 50.7% to 17.2% was accompanied by an increase in the number of S phase cells ( $P=0.008$ ), in cells in  $G_2M$  ( $P=0.05$ ), and in the RNA index of cells in  $G_1$  ( $P=0.001$ ). There was great heterogeneity from case to case with regard to response to rhG-CSF, rhGM-CSF, and IL-3. Figure 6 demonstrates the increase of cells in active DNA

**Table 2.** Cell cycle effects and recruitment by cytokines of all sixteen samples studied.

	Cell cycle					Growth fraction (Ki67)	DNA synthesis rate <sup>a</sup> (BrdU)
	$G_0$	$G_1$	S	$G_2M$	RNA-I $G_1$		
Controls	44.0	45.4	8.8	1.6	18.3	6.2	123.6
G+GM+IL-3	26.8	43.1	26.3	3.7	24.4	41.2	140.6
<i>P</i>	0.04	0.6	0.001	0.06	0.001	0.05	0.3

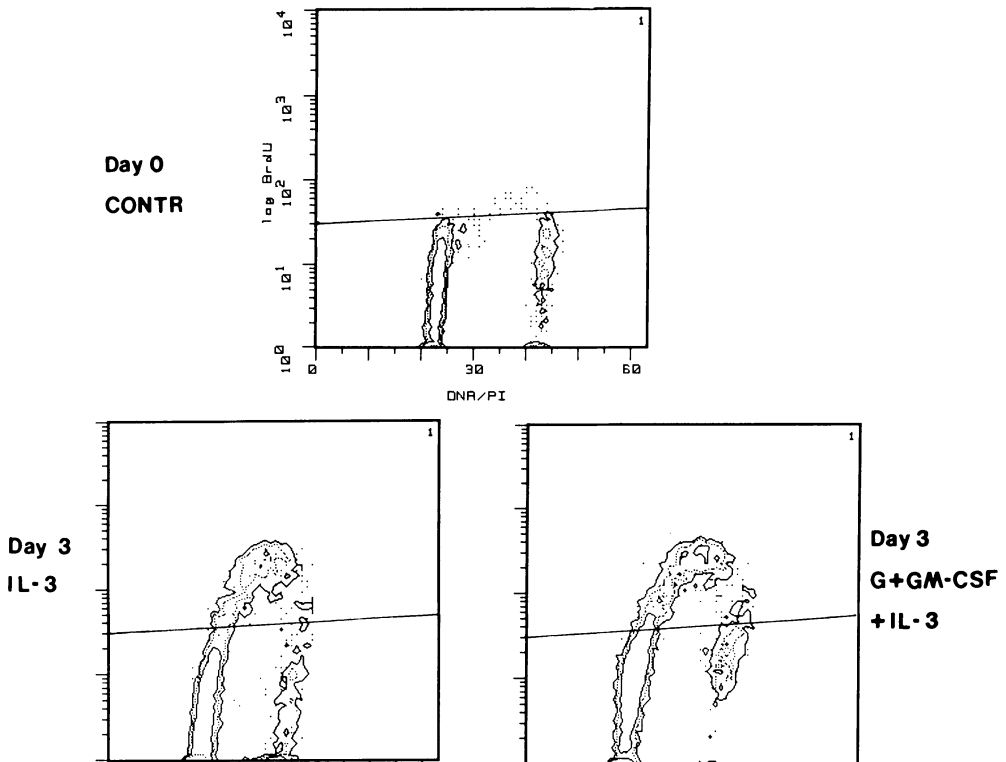
<sup>a</sup> As determined by the mean amount of incorporated BrdU.

Cell cycle distribution (Percentage of  $G_0$ ,  $G_1$ , S,  $G_2M$ , and RNA-Index  $G_1$ ) are given for controls studied at 0 time and for rhG+rhGM+rhIL-3 in combination. The *P* value denotes confidence levels for differences between controls and cytokine-treated samples. Growth fraction was determined as percentage of Ki67 positive cells. DNA synthesis rate was measured as the mean amount of incorporated BrdU. A decrease of cells in  $G_0$  is accompanied by an increase of cells in S and  $G_2M$ . RNA-index  $G_1$  and growth fraction are also significantly increased.

**Table 3.** Recruitment by cytokines

%	AML ( <i>n</i> =6) more than threefold ↑ S					AML ( <i>n</i> =7) less than threefold ↑ S				
	G <sub>0</sub>	G <sub>1</sub>	S	G <sub>2</sub> M	RNA index	G <sub>0</sub>	G <sub>1</sub>	S	G <sub>2</sub> M	RNA index
Controls	50.7	43.1	5.1	0.9	17.1	46.4	41.9	10.1	1.4	19.4
G GM IL-3	17.2	49.0	28.3	5.4	25.8	35.2	31.6	30.7	2.7	25.3
<i>P</i>	0.004	0.4	0.008	0.04	0.001	0.5	0.4	0.05	0.1	0.06

Cell kinetic changes and recruitment of AML are divided into two groups: one with less than threefold increase in the number of S-phase cells and one with more than threefold increase in percent S. Of note is a significant decrease in the percentage of G<sub>0</sub> cells in sample with more than threefold increase in S-phase and the absence of significant recruitment from G<sub>0</sub> in samples with a less than threefold increased percentage of S-cells.



**Fig. 6.** DNA/bromodeoxyuridine histograms of AML cells incubated with rhIL-3 and rhG-CSF + rhGM-CSF + rhIL-3 for 3 days in suspension culture. *X-axis*, DNA content (linear); *Y-axis*, BrdU (log scale). *Line* defines background fluorescence as determined by isotypic control antibody. Cells with positive BrdU fluorescence (higher than isotypic control antibody fluorescence) are in DNA synthesis (incorporating BrdU). *Upper panel* shows control sample with 2.6% cells in S-phase (mean fluorescence 59.2). *Left lower panel* shows the effect of rhIL-3 after three days of incubation: percentage of cells in S=25.4, mean fluorescence 225.9. Combination of cytokines on day 3 (*right lower panel*): S-phase = 28.9%, mean fluorescence 171.0. A tenfold increase in the number of DNA synthesizing cells and increased DNA synthesis rate are apparent

synthesis from 2.6% (control) to 25.4% (IL-3) and 28.9% (G + GM + IL-3), respectively. The DNA synthesis rate in this sample was also increased (see legend).

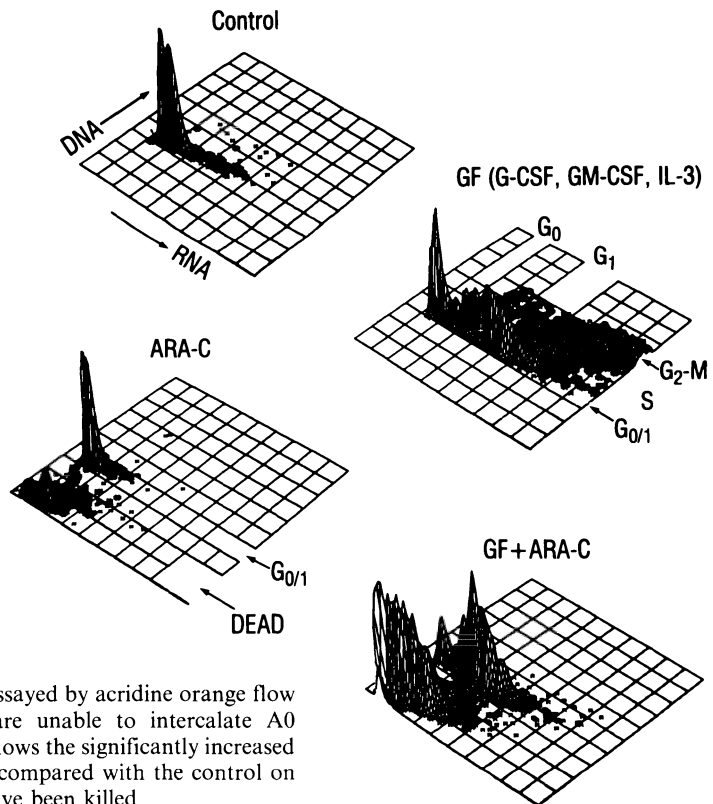
Typical examples of successful recruitment from  $G_0$  are shown in Figs. 7 and 8 (upper right panels). The combination of rhG-CSF and rhIL-3 was most effective. In both cases, the relative number of cells in  $G_0$  decreased, while a significant increase in the number of cells in G and  $G_2M$  was observed. The increased RNA content of  $G_1$  cells (RNA index  $G_1$ ) is also apparent from these figures.

The time course of recruitment, in general, was as follows: recruitment from  $G_0$  into  $G_1$  occurred after 24 h, recruitment into S phase after 48 h, and increase in cell number after 72 h. Again, we observed variability from case to case and between different cytokines.

Because maximum increase in S phase cells was observed after 48 h, Ara-C (1 mM) was added to cultures at this time point. Cytotoxicity was assessed at multiple subse-

quent time points. As shown in Fig. 7, increased recruitment into  $G_1$  and S in stimulated cultures was accompanied by increased cell killing by Ara-C (lower panels). Figure 5 verifies this result independently in the FDA/PI assay. In some cases, however, increased recruitment was not paralleled by increased cytotoxic activity of Ara-C. As can be seen in Fig. 8, numerous cells are still present in S phase, following recruitment with rhG-CSF, rhGM-CSF, and rhIL-3 and treatment with Ara-C. Cell kill is only moderately increased as compared to controls.

Table 4 correlates recruitment and cytotoxicity of Ara-C: recruitment was again defined as an increase in the percentage of S phase cells by a factor of 3 or greater. Five samples showed such recruitment and three of five had an increase in sensitivity to Ara-C. In three cases, cytotoxicity increased by 92% (1.92-fold) with a range from 1.3- to 3.1-fold. Two samples did not show such increased Ara-C cytotoxicity. In none of the four cases that failed to demonstrate signifi-



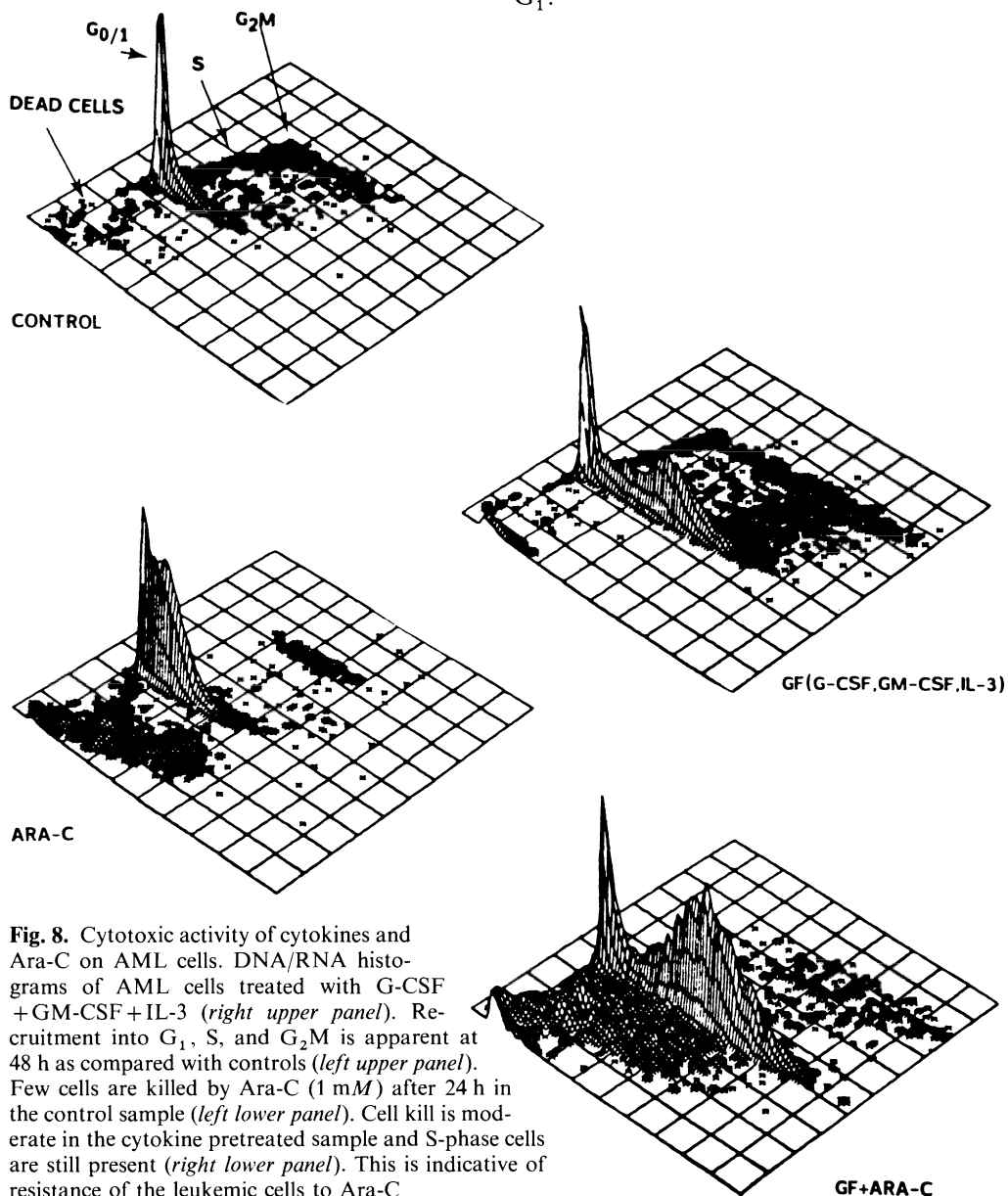
**Fig. 7.** Recruitment and enhanced sensitivity of AML cells by cytokines and Ara-C. DNA/RNA histograms of AML cells treated with G-CSF + GM-CSF + IL-3 (right upper panel), as compared with control cells (left upper panel). A significant increase of cells in  $G_1$ , S,  $G_2M$  is apparent. Stimulation for 48 h was followed by incubation with Ara-C (1 mM) for 24 h. Left lower panel demonstrates cell kill by Ara-C as assayed by acridine orange flow cytometry: only few cells are unable to intercalate A0 (=dead). Right lower panel shows the significantly increased cytotoxic effect of Ara-C as compared with the control on day 0. All cells in S-phase have been killed

cant recruitment was increased cytotoxicity of Ara-C observed. The median difference in cytotoxicity was 0.95 (range 0.74–1.0).

### Effects of BCGF on ALL

Cell kinetic effects of low molecular weight BCGF (15 kDa) are shown in Fig. 9. This case of ALL was characterized by a hyper-

diploid DNA stemline (DNA index: 1.2). DNA aneuploidy allows the discrimination of leukemic cells (hyperdiploid) from non-leukemic cells (diploid). As can be seen, recruitment is achieved with a highly significant increase in the number of cells in  $G_1$ . However, a decrease of cells in S phase was observed under these conditions. Table 5 lists the associated cell kinetic changes with decrease of cells in  $G_0$ , increase of cells in  $G_1$ , and increase of RNA index of cells in  $G_1$ .



**Fig. 8.** Cytotoxic activity of cytokines and Ara-C on AML cells. DNA/RNA histograms of AML cells treated with G-CSF + GM-CSF + IL-3 (*right upper panel*). Recruitment into  $G_1$ , S, and  $G_2M$  is apparent at 48 h as compared with controls (*left upper panel*). Few cells are killed by Ara-C (1 mM) after 24 h in the control sample (*left lower panel*). Cell kill is moderate in the cytokine pretreated sample and S-phase cells are still present (*right lower panel*). This is indicative of resistance of the leukemic cells to Ara-C

**Table 4.** Correlation between recruitment and increased sensitivity to Ara-C in AML

	Cytotoxicity		
	increase	no change	total
+ <sup>a</sup>	3	2	5
- <sup>b</sup>	0	4	4
total	3	6	9

<sup>a</sup> More than threefold increase S; significant decrease  $G_0$ .

<sup>b</sup> Less than threefold increase S; no decrease  $G_0$ . Five of nine AML samples had significant recruitment. Increased Ara-C cytotoxicity was observed in 3 of 5 of these samples: recruitment was associated with an increase in the sensitivity to Ara-C at 24 h. Increase in cytotoxicity was 1.9-fold (range 1.3–3.1). No increase in cytotoxicity to Ara-C was observed in the absence of recruitment (0 of 4 samples). The median difference in cytotoxicity was 0.95 (0.74–1.0) for this group.

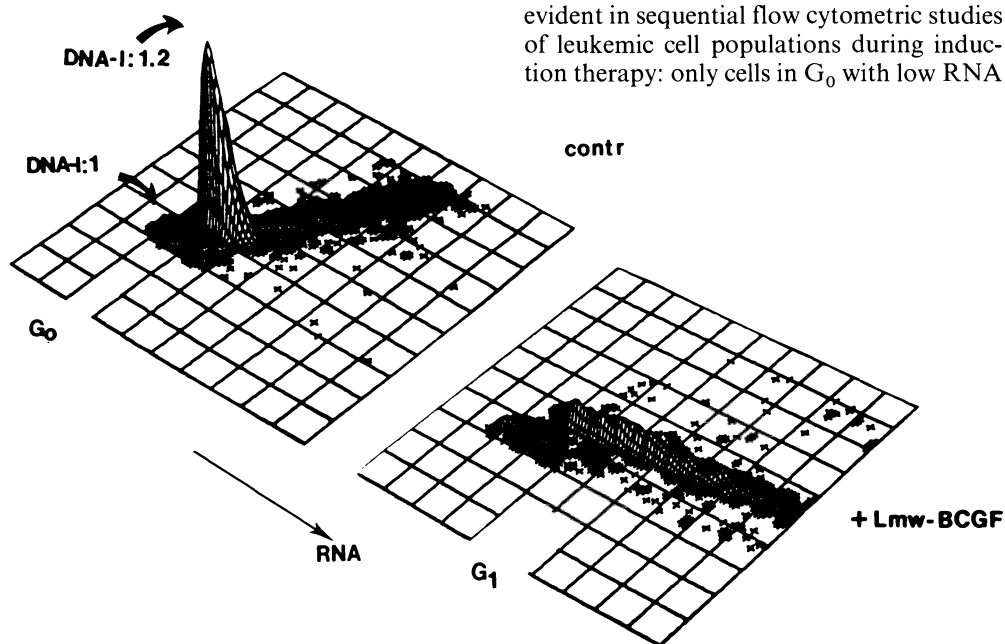
**Table 5.** Recruitment of ALL by L-BCGF

	$G_0$ (%)	$G_1$ (%)	RNA index $G_1$
Controls	39	50	15.5
L-BCGF	15	63	20.2

Cells kinetic effects of low molecular weight BCGF (15 kDa) on ALL cells in suspension culture ( $n=3$ ). A decrease of  $G_0$  cells was accompanied by an increase of  $G_1$  cells. The RNA content of cells in  $G_1$  (RNA- $G_1$ ) is also increased.

## Discussion

Prognostic models for ALL and AML in adults and children have identified cell cycle parameters as important predictors for achievement of CR (adult and pediatric ALL, adult AML), for remission duration (adult ALL and AML) and for survival (adult AML). The prognostic importance of quiescent (“ $G_0$ ”) leukemic cells can not only be deduced from in these models, but is also evident in sequential flow cytometric studies of leukemic cell populations during induction therapy: only cells in  $G_0$  with low RNA



**Fig. 9.** DNA/RNA histogram of a pre-B cell ALL sample at day 0. Diploid (DNA index: 1.0) and hyperdiploid (DNA index: 1.2) leukemic cells can be separated based on differences in their DNA content. A high number of cells in S-phase is apparent. After stimulation with low molecular weight BCGF (15 kDa) for 48 h, a significant increase in hyperdiploid leukemic cells in  $G_1$ -phase is apparent. There is a decrease of cells in S-phase. A low degree of stimulation can be seen of diploid cells with entry into  $G_1$ .

content are detectable after reduction of the leukemic cell mass to residual leukemia at the end of induction therapy [19]. The efficacy of cell cycle-specific drugs such as Ara-C and daunorubicin implies that quiescent cells enter the cell cycle and become susceptible to these drugs. Otherwise Ara-C would kill only the approximately 10% of cells engaged in DNA synthesis at any given time, or the cells entering S phase while cytotoxic levels of Ara-C are present. The normal flow of quiescent cells into the cycle is enhanced by Ara-C, as previously demonstrated [20]. Nevertheless, cell killing is inadequate in most acute leukemias, since only a small proportion of patients will remain in long-term remission. The role of consolidation and maintenance therapy has recently been questioned, and present evidence points to the importance of maximal initial cytoreduction for long-term remissions [21].

In vitro and in vivo investigations with cytokines have suggested recruiting and differentiating effects of rhG-CSF, rhGM-CSF, and IL-3 on normal hematopoietic cells, and we have previously demonstrated recruitment from  $G_0$  to  $G_1$  for G-CSF in normal bone marrow blasts [11].

Effects of recombinant cytokines on leukemic cells have been investigated by a number of groups [22–29]. The recruitment of leukemic cells by rhG-CSF, rhGM-CSF, and rhIL-3 observed in this study was significant in most cases, however, it was not detectable in the lymphoblastic transformation of CML and in samples from patients with ALL. This could be related to the absence of specific cytokine receptors on the surface of these leukemic blast cells or to an already maximal autocrine stimulation of blasts by cytokines. The latter hypothesis, however, has been questioned by a report that the expression of m-RNA for G-CSF and GM-CSF in fresh leukemic blasts may be related to in vitro manipulations of these leukemic blast cells and that unprocessed samples have considerably lower, or absent, mRNA expressions [30]. Receptor studies on leukemic blast cells are presently underway.

In a proportion of samples, recruitment was unequivocally demonstrated by depletion of  $G_0$  cells and transition of cells to  $G_1$ , S, and  $G_2$  as well as by an increase in the

growth fraction of leukemic cells, as determined by the nuclear antigen Ki67 [17]. The cell number in stimulated cultures increased approximately twofold. DNA synthesis rate did not seem to be affected, on average, as shown by quantitation of the amount of incorporated BUdR into S phase cells (Table 2). The example shown in Fig. 6 which shows a tenfold increase in the number of cells in S phase also demonstrated higher DNA synthesis rate. Labeling studies of leukemic cells in vivo with BrdU will probably determine if cytokines also affect the DNA synthesis rate.

Recruitment should render leukemic cells more sensitive to Ara-C, which is known to be specific for cells in S phase, and to topoisomerase II-inhibitors such as daunomycin, adriamycin, idarubicin, VP-16, and VM-26. Levels of topoisomerase II were shown to be very low in quiescent cells and increased significantly after stimulation and recruitment into the cell cycle [31]. Hence, recruitment from  $G_0$  to  $G_1$  should greatly enhance the cytostatic action of this class of drugs.

The increased cytotoxic effect of Ara-C in our experiments was demonstrated by a decrease in cell number, an increase in the number of trypan-blue positive cells, and an increase in the number of cells unable to intercalate acridine orange (Fig. 7). Decreased intercalation can be explained by partial denaturation of DNA: double-stranded DNA becomes single stranded and loses the ability to intercalate acridine orange. Dead cells are therefore found below the level of  $G_{0/1}$  cells in DNA histograms. A similar increase in the number of dead cells was observed in the independent FDA/PI flow cytometry test. This novel test assays the ability of cells to convert FDA to fluorescein, a process for which esterases are needed. PI can penetrate the cell membrane only in dead cells and live cells are therefore characterized by the absence of PI fluorescence and the presence of FDA fluorescence (Fig. 5). Conversely, dead cells have characteristically increased PI fluorescence and decreased FDA fluorescence. This assay was shown to be correlated to the clonogenicity of leukemic cells [15].

These results and previous reports from our group [32–34] and other investigators



[35–37] provide increasing support of a “recruitment” therapy of leukemia. However, as shown in Table 4 and Fig. 8, successful recruitment into the cell cycle does not always result in increased cytotoxicity of Ara-C. Pharmacological resistance *in vitro* to Ara-C in a given cell population cannot be overcome by recruitment, and future clinical trials will have to test Ara-C sensitivity or resistance in order to avoid misinterpretations of clinical trials. A recent report by Bhalla [38] provided additional rationale for “priming” of AML with rhGM-CSF: leukemic cells had increased levels of Ara-CTP as compared to normal cells after treatment with GM-CSF, and it was concluded that this might result in preferential killing of leukemic cells.

This leads to the crucial question of whether cytokine-induced recruitment of leukemic cells could also result in increased recruitment of nonleukemic hematopoietic progenitor cells. Although we demonstrated previously that G-CSF recruits normal myeloblasts into the cell cycle [11], the presence of inhibitory factors produced by leukemic cells such as leukemia-inhibitory activity (LIA) [39] and others may result in a relative protection of normal progenitors from the recruiting effects of hematopoietic cytokines. In cases with DNA aneuploidy, the effects on leukemic versus normal cells can be discriminated by determination of the expression of cell cycle-related antigens such as Ki67 on diploid and aneuploid cells. In addition, techniques such as premature chromatin condensation (PCC) [40, 41] and fluorescence *in situ* hybridization using chromosome specific DNA probes [42] will facilitate the discrimination of cytokine effects on normal and leukemic cells.

How many leukemic cells can be recruited by cytokines? Data reported here indicate that, at 24 and 48 h, significant recruitment of myeloid cells into the cell cycle can be achieved. Continuous or repetitive labeling with BrdU and subsequent staining with anti-BrdU-antibody *in vitro* and *in vivo* will shed light on the number of recruited and nonrecruited cells. Again, different cytokines may act differently, and the time periods required for optimal recruitment remain to be determined. These studies will be of major importance for clinical trials and will

hopefully aid in the design of optimal treatment schedules.

Finally, the induction of differentiation [43] by cytokines is a distinct possibility and will have to be addressed in clinical trials. If differentiation is induced by cytokines, these cells may be less sensitive to the cytotoxic effects of chemotherapeutic agents. Careful monitoring of differentiation markers and the simultaneous determination of clonality in differentiated cells will be important for the interpretation of results of clinical trials.

Our preliminary observations do not provide evidence of recruitment of ALL cells or of blast cells in the lymphoblastic transformation of chronic myelogenous leukemia by rhG-CSF, rhGM-CSF, and rhIL-3. It will be of interest to compare the *in vitro* and *in vivo* effects of recombinant colony stimulating factors. This approach may lead to the development of optimized cytokine-chemotherapy combination protocols for the treatment of leukemia.

Previous attempts of recruiting ALL cells have met with only limited success: incubation of ALL with OKT3, PMA, and IL-2 resulted in recruitment from  $G_0$  into  $G_1$  but not into the S phase [14]. Likewise, our preliminary data with low molecular weight BCGF in ALL failed to demonstrate recruitment into S phase. However, significant recruitment into  $G_1$  and depletion of  $G_0$  was observed, and it may be necessary to add additional factors for more complete recruitment of ALL cells.

After a long period of stagnation in the treatment of acute leukemias, recruitment of leukemic cells into the cell cycle followed by chemotherapy with cell cycle-specific agents may result in faster cytoreduction, a higher incidence of complete remission, and a better quality of these remissions. It is hoped that this strategy will ultimately result in longer remission durations and in improved survival of our patients with acute leukemia.

## Summary

Prognostic models for acute myeloid and lymphoid leukemias are presented that demonstrate that cell kinetic quiescence in acute leukemia is associated with poor response to chemotherapy, short remission

duration, and survival. Recruitment of cells into the cell cycle should therefore enhance cytotoxic effects of cell cycle – specific chemotherapeutic agents. We previously demonstrated recruitment of myeloid leukemic cells by cytokines. We have now investigated whether recruitment can be used to increase cell killing by cytosine arabinoside (Ara-C). Blast cells from 16 acute leukemias were stimulated with cytokines as follows: 13 acute myeloid leukemias (AML) and 3 chronic myeloid leukemia (CML) in blastic phase (1 lymphoid, 2 myeloid) were treated with recombinant human granulocyte colony stimulating factor (rhG-CSF), recombinant human granulocyte-macrophage colony stimulating factor (rhGM-CSF, AMGEN, 500 U/ml each), and recombinant human interleukin-3 (rhIL-3, IMMUNEX, 20 ng/ml), alone and in combination. After 48 h, at the time of maximal DNA synthesis, Ara-C ( $10^{-3}$  M) was added and cell counts, cytokinetics (DNA/RNA, DNA/bromodeoxyuridine and DNA/Ki67 flow cytometry), and cell viability/clonogenicity (fluorescein diacetate/propidium iodide exclusion flow cytometry) were investigated. In all 13 cases of AML recruitment was found; in 6 of these cases over a three fold increase in S phase ( $P=0.008$ ) and a significant ( $P=0.004$ ) depletion of  $G_0$  was demonstrated. In 9 of 13 patients with AML, the effect of Ara-C was investigated, and in 3 of 5 patients with over three fold increase in S phase, Ara-C toxicity was enhanced.

None of the patients with less than a three fold increase in S phase and no demonstrable recruitment from  $G_0$  had increased Ara-C cytotoxicity. Ara-C cytoreduction was paralleled by reduction in clonogenicity as demonstrated by fluorescein diacetate/propidium iodide (FDA/PI) flow cytometry. Four samples of acute lymphoblastic leukemia (ALL) were treated with low molecular weight B-cell growth factor (15 kDa) and recruitment of aneuploid cells from  $G_0$  to  $G_1$  was found in all patients (from 19.3% to 84.9%). These results indicate that recruitment of leukemic cells is inducible by cytokines and that the cytotoxicity of cell cycle-specific drugs such as Ara-C can be increased. This concept is presently being tested in vivo.

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## Effect of Treatment with rhGM-CSF and Low-Dose Cytosine Arabinoside on Leukemic Blast Cells in Patients with Myelodysplastic Syndromes

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### Introduction

Treatment of patients having myelodysplastic syndromes (MDS) with approaches such as differentiation induction, single cytostatic agents or supportive care only has, up to now, been rather unsuccessful. Aggressive chemotherapy followed by bone marrow transplantation is only suitable for a very small proportion of patients. Thus, there is a need for new therapeutic alternatives.

Recombinant hemopoietic growth factors such as erythropoietin, granulocyte and granulocyte-macrophage colony stimulating factor (G-CSF, GM-CSF), or interleukin-3 may offer such therapeutic possibilities by:

1. Inducing differentiation of the leukemic cell clone
2. Stimulating leukemic blast cell proliferation, thus increasing susceptibility to cytotoxic therapy
3. Stimulating other cell lineages to increase erythrocytes, thrombocytes and neutrophils
4. Shortening the regeneration time after chemotherapy

### *In Vitro and In Vivo Effects of GM-CSF on Proliferation and Differentiation of Leukemic Blast Cells*

GM-CSF stimulates in most but not all patients the in vitro proliferation of acute myeloblastic leukemia (AML) blast cells [1–5], including the clonogenic stem cell. GM-CSF stimulates in vitro colony formation of HL 60 cells and induces differentiation along the monocytic and eosinophilic lineages. An in vitro differentiation effect on blast cells of patients with AML is, however, only rarely observed.

The few clinical phase I/II studies using GM-CSF in MDS patients have shown contradictory results with regard to proliferation as well as differentiation [6–10]. In some studies no increase in the leukemic blast cell population in the peripheral blood and/or bone marrow was observed or the increase was only minor or transient [6–8]. It was assumed that normal and leukemic hemopoiesis are stimulated in a similar way but that the blast cell proliferation did not exceed that of normal precursors [6]. In

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contrast, of the 11 MDS patients treated with GM-CSF in our own study 5 patients experienced an increase in blast cells in the bone marrow and 4 in the peripheral blood [9]. Whether this blast cell increase might have been transient cannot be judged since the patients were later treated with low-dose cytosine arabinoside (LD-AraC) to avoid a further progression into overt leukemia [11].

Thus, at present it seems that GM-CSF can stimulate leukemic blast cells in MDS patients transiently but progressive leukemia can also be induced. Presumably the stimulation of leukemic blast cells in MDS patients is correlated to the initial blast cell content of the bone marrow.

With regard to the differentiation-inducing effect of GM-CSF in MDS the data published so far are scarce. There is, however, evidence in at least two studies that the neutrophils which emerge in MDS patients after GM-CSF treatment are partly derived from the leukemic cell clone. By cytogenetic analysis before and after GM-CSF treatment Ganser et al. [9] showed a stimulation and increase of the abnormal clone in two out of six patients. Since these patients had a substantial rise in mature granulocytes, it must be assumed that they derived at least partly from the malignant clone; this was most evident in one patient who had a dramatic increase in neutrophils, but only leukemic metaphases. Further, using the premature chromosome condensation method Hittelmann et al. [12] demonstrated terminal differentiation of leukemic cells in most MDS patients treated with GM-CSF. Since in both studies in some cases normal metaphases remained or even increased after GM-CSF treatment, one has to assume a mixed pattern with mature neutrophils being derived from leukemic cells and from the normal clone.

#### *Phase II Study of Recombinant Human GM-CSF Plus LD-AraC in Patients with MDS*

After the observation that GM-CSF can induce differentiation and proliferation of leukemic blast cells in MDS patients, a prospective multicenter trial was initiated in March 1988 to study the effect of GM-CSF

on hematopoiesis in patients with refractory anemia (RA) and the combined effect of GM-CSF and LD-AraC on hemopoiesis and on leukemic blast cells in patients with refractory anemia with excess blasts (RAEB), refractory anemia with excess blasts in transition (RAEB-T) or chronic myelomonocytic leukemia (CMML) [9]. The study had the following aims:

1. To test whether a reversal of granulocytopenia can be achieved in patients with RA and whether thrombocytopenia and anemia are alleviated as well
2. To study whether leukemic blast cells are eliminated and hemopoiesis normalized when GM-CSF is used in combination with LD-AraC in patients with RAEB, RAEB-T or CMML

#### **Material and Methods**

##### *Preparation of Recombinant Human GM-CSF*

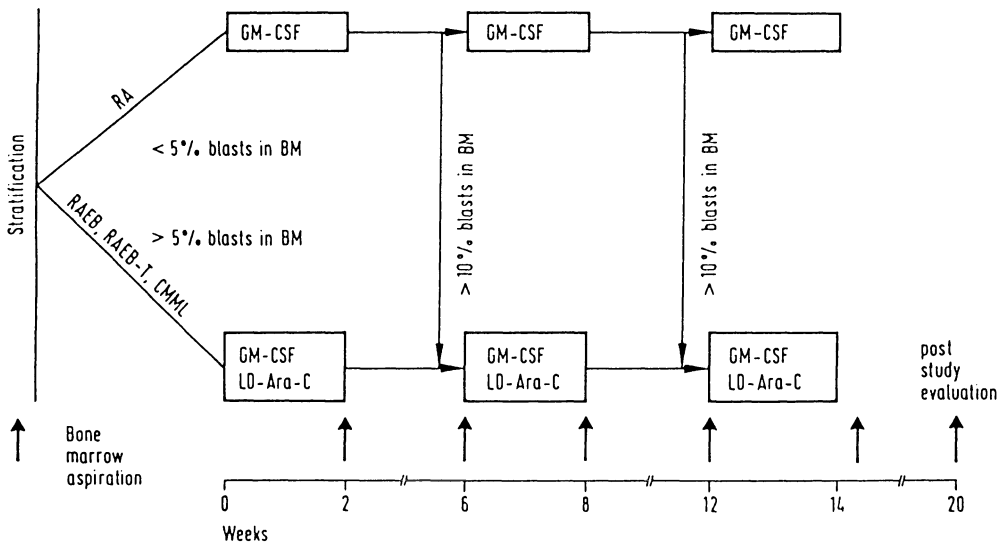
The recombinant human GM-CSF (rhGM-CSF) used in the study was expressed in *E. coli* and purified by Immunex Inc./Behringwerke AG (Seattle, USA/Marburg, FRG) as described previously [13]. The rhGM-CSF is not glycosylated and has a molecular weight of 14 kDa.

##### *Patient Selection and Study Design*

Only patients with clinical and hematological confirmation of MDS [14] either with RA or with RAEB, RAEB-T or CMML who had not been treated with antileukemic drugs for at least 4 weeks were included. Patients under the age of 50 years eligible for bone marrow transplantation or conventional high-dose chemotherapy were excluded.

##### *Treatment Plan (Fig. 1)*

The treatment schedule for patients with RA consisted of three sequential cycles of 14 days with daily subcutaneous injections of 250  $\mu\text{g}/\text{m}^2$  rhGM-CSF. Patients with RAEB, RAEB-T or CMML received the



**Fig. 1.** Treatment schedule for patients with myelodysplastic syndromes. Refractory anemia, *RA*; *RAEB*, refractory anemia with excess blasts; *RAEB-T*, refractory anemia with excess blasts in transformation; *CMML*, chronic myelomonocytic leukemia; *BM*, bone marrow; *GM-CSF*, granulocyte-macrophage colony stimulating factor; *LD-Ara-C* low-dose cytosine arabinoside

same three cycles of GM-CSF as s.c. injection at 8 a.m. and, in addition, s.c. injection of LD-AraC 20 mg/m<sup>2</sup> at 8 p.m. Patients were monitored daily and all constitutional symptoms were recorded. A complete blood count, including differential and reticulocyte counts, was done prior to therapy, at 2-day intervals during the treatment and weekly during the treatment-free intervals. Bone marrow aspirations were performed before and after the end of each treatment cycle for cytological examination. Dose-limiting toxicity was generally defined as toxicity of grade 3 or higher by WHO criteria, except hematological toxicity (thrombocytopenia, anemia, neutropenia). If the blast cells in the peripheral blood increased by 50% above baseline, the administration of GM-CSF was stopped while LD-AraC continued.

## Results

A total of 58 patients with MDS entered the study. The median age was 58 years (range 32–80 years). The distribution of the different subtypes of MDS is given in Table 1. Twenty patients had RA, 17 RAEB, 17

**Table 1.** Characterization of patients in the prospective GM-CSF/LD-Ara-C study

Total no.		58
Evaluable <sup>a</sup>		32
Age (years)	Median	54
	Range	30–80
Diagnosis	RA	20
	RAEB	17
	RAEB-T	17
	CMML	4

<sup>a</sup> One cycle GM-CSF/LD-Ara-C completed. GM-CSF, granulocyte-macrophage colony stimulating factor; LD-Ara-C, low-dose cytosine arabinoside; RA, refractory anemia; RAEB, refractory anemia with excess blasts; RAEB-T, refractory anemia with excess blasts in transformation; CMML, chronic myelomonocytic leukemia

RAEB-T and 4 had CMML. So far 32 patients have finished the first cycle of therapy; 9 RA patients had one cycle of GM-CSF; 9 patients with RAEB, 10 patients with RAEB-T and 4 patients with CMML had one cycle of combined treatment with GM-CSF and LD-AraC.

**Table 2.** Change in peripheral blood counts in patients with MDS after one cycle of GM-CSF/LD-Ara-C

Diagnosis	n	Blast cells			Neutrophils		
		Before (median values/nl)	After	After	Before (median values/nl)	After	After
RA	9	0	0		1.6±0.4	11 ±4.7	1.7±0.8
RAEB	9	0.8±0.5	7 ±5	0.3±0.2	1.4±0.5	21 ±13	1.7±1.1
RAEB-T	10	1.2±0.8	4.1±2.9		1.4±0.6	7.3±4.4	
CMML	4	1.6	3.4		14	42	

For abbreviations, see Table 1

The change in peripheral blood counts after one cycle of therapy is shown in Table 2. In nine patients with RA there was no increase of blast cells after one cycle of GM-CSF. In seven of nine patients with RAEB the blast cells in the peripheral blood remained constant, whereas in two patients a slight increase was observed, causing the rise in the median value (Table 2). Even in these two patients the increase was transient and the blast cell content returned to the pre-treatment level within 4 weeks. The increase in the median blast cell count for patients with RAEB-T was caused by a rise of blast cells in three out of these ten patients. In one patient the blast cell increase was transient, whereas the remaining two progressed to overt leukemia. For the four CMML pa-

tients the blast cell content of the peripheral blood did not change significantly.

In all patient groups there was a substantial increase in the neutrophils (Table 2). The median value for the RA patients rose from 1.6 to 11 cells/nl, for the RAEB patients from 1.4 to 21 cells/nl, for the RAEB-T patients from 1.4 to 7.3 cells/nl and from 1.6 to 3.4 cells/nl for the patients with CMML. Here, in addition to neutrophils, a substantial rise in monocytes and immature myelomonocytic cells was observed.

Parallel to these peripheral blood values, the changes in bone marrow blast cells and nondividing myelopoietic cells for MDS patients who completed one cycle of combined treatment are given in Table 3. There was no increase in the blast cell content in the nine

**Table 3.** Change in bone marrow composition in MDS patients after one cycle of GM-CSF/LD-Ara-C

Diagnosis	n	Blast cells		M <sub>5</sub> -M <sub>8</sub>	
		Before %	After %	Before %	After %
RA <sup>a</sup>	10	1.4 (0-3)	0.7 (0-2)	35 (10-68)	34 (13-56)
RAEB	7	8.4 (5-15)	6.8 (1-17.5)	29 (6-56)	26 (3-45)
RAEB-T	5	20 (10-29)	11 (6-19)	19 (6-39)	23 (6.5-40)

<sup>a</sup> GM-CSF only

Results expressed as mean percentages (range).

MDS, myelodysplastic syndrome; M<sub>5</sub>-M<sub>8</sub>, nondividing myelopoietic cells; for other abbreviations, see Table 1



RA patients in whom the bone marrow was evaluable or in the seven RAEB patients or the five RAEB-T patients. In the two CMML patients with bone marrow evaluation before and after treatment the blast cell content decreased from 30% and 40% to 20% and 0% but later showed a blast cell increase to 46% and 70%, respectively. Altogether, in three of four patients with CMML the disease progressed.

The observation time was too short to evaluate the effect of GM-CSF or the combined treatment with GM-CSF/LD-AraC on erythropoiesis and megakaryopoiesis.

## Discussion

There are several arguments which may justify the combined modality of stimulation and cytotoxic treatment in patients with MDS. As first clinical studies have shown, GM-CSF is capable of recruiting leukemic blast cells *in vivo* in patients with RAEB, RAEB-T or CMML. GM-CSF was therefore used in this treatment approach for blast cell stimulation combined with a cytostatic agent. For the latter, LD-AraC was chosen for two reasons. Firstly, there is ample experience that LD-AraC alone can achieve responses in MDS [15, 16], and secondly, there is evidence for a synergistic effect with GM-CSF.

*In vitro*, the combination of GM-CSF with low-dose AraC appears to be more potent in the induction of differentiation in leukemic cells [17, 18]. In addition, leukemic progenitor cells are more sensitive to long-term LD-AraC in the presence of GM-CSF as compared with more mature nonself-renewing primary clonogenic cells, while normal secondary clonogenic cells are spared [19]. Furthermore, leukemic blast cells express more binding sites for AraC after exposure to GM-CSF, thereby facilitating the uptake of AraC which is otherwise the limiting process to treatment with LD-AraC. These *in vitro* results provide the basis for combination therapy of LD-AraC and GM-CSF in patients with MDS.

When combined treatment with hemopoietic growth factors and AraC is considered for MDS patients there are several theoretical arguments on how to schedule these

drugs [20]. There is the possibility of pre-stimulating the blast cells with GM-CSF and then continuing with a combined cytotoxic treatment with LD-AraC and further stimulation by GM-CSF, assuming a continuous recruitment of leukemic blast cells into cycle. Alternatively, there are arguments to support subsequent cytotoxic treatment with LD-AraC to eliminate pre-stimulated blast cells. At present, there are no convincing *in vitro* experiments to favor one or the other possibility. Therefore in our ongoing study a simultaneous application of GM-CSF and LD-AraC was chosen. Clearly such an approach is focused on stimulating leukemic blast cells to proliferate and not on improving hemopoietic regeneration, as in the ongoing EORTC study in MDS where GM-CSF is administered after LD-AraC therapy.

One of the main interests in our study was to see whether in patients with RA blast cells might appear in the peripheral blood or bone marrow after GM-CSF treatment. This was apparently not the case in the first nine patients, who completed one cycle of GM-CSF alone. Thus, in patients with RA the administration of GM-CSF in the schedule used here does not, apparently, risk the recruitment and stimulation of a leukemic cell clone. For the majority of patients with RAEB (7/9) the blast cells remained constant or decreased. In the two patients with an increase of blast cells in the peripheral blood it was transient. Some patients receiving the combined treatment modality even showed a decrease in blast cell content of the bone marrow. These results contrast with our earlier findings where after GM-CSF only, an increase of blast cells in peripheral blood or bone marrow was found in 7 of 11 patients. Similar observations have been made by Estey et al. [10], who found an increase in 4 of 17 patients with RAEB/RAEB-T treated with GM-CSF alone. In the present study it seems that the stimulation of leukemic blast cells caused by GM-CSF in RAEB patients can be overcome by the simultaneous cytotoxic treatment with LD-AraC. For patients with RAEB-T the situation is somewhat different. Only three of ten patients showed an increase of blast cells in the peripheral blood and in all four patients with sequential bone marrow evalu-

ation the blast cell content decreased. However, in two patients with RAEB-T the blast cell increase in the peripheral blood continued and the patients developed overt leukemia. LD-AraC is therefore not sufficient to eliminate the GM-CSF stimulated blast cells in all patients with RAEB-T. The situation was different again in four patients with CMML. There was in two patients an initial decrease in the blast cell content of bone marrow but a later increase. Overall there was a progression of the disease, with an increase in neutrophils, monocytes, and myelomonocytic cells in the peripheral blood in three of four CMML patients. GM-CSF apparently results in a sufficient stimulation of the malignant clone in CMML but LD-AraC alone as a cytotoxic agent is inadequate to eliminate these cells. Therefore in this study the combined treatment with GM-CSF and LD-AraC for patients with CMML was discontinued. If GM-CSF is used in further patients with CMML, it should be combined with a more effective cytotoxic therapy.

In conclusion, in patients with RA, GM-CSF does not cause blast cell stimulation. We have to wait until more cycles with GM-CSF have been completed to find out whether, in addition to neutrophils, erythropoiesis and megakaryopoiesis are also stimulated. In patients with RAEB and RAEB-T it seems, at least after one cycle of GM-CSF/LD-AraC, that most of the time blast cells can be sufficiently controlled and that even a decrease is observed in some cases. The question of whether the completion of three cycles, as planned in this study, or more are necessary to eliminate leukemic blast cells completely is still open, as well as whether and to what extent normal hemopoiesis can be stimulated.

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