Analysis of Treatment Failure in Patients With Minimally Differentiated Acute Myeloid Leukemia (AML-M0)

By Roberto Stasi, Giovanni Del Poeta, Adriano Venditti, Mario Masi, Elisa Stipa, Teresa Dentamaro, Cristina Cox, Bruno Dallapiccola, and Giuseppe Papa

Reports of treatment of patients with minimally differentiated acute myeloid leukemia (AML-M0) are limited, heterogeneous, and controversial. We verified the prognosis of this subtype by analyzing the results of 189 consecutive patients with de novo AML. Fifteen cases fitting the criteria of AML-M0 were identified. No clinical features distinguished them from other patients with AML. The median age was 61 years (range 27 to 70), with a leukocyte count ranging from 0.6 to 185 × 10^9/L. In all cases the leukemic cells expressed CD34 and reacted with at least one of the antibodies to early myeloid antigens, i.e., CD13, CD33, or myeloperoxidase. Immunophenotypic analysis also showed positivity for CD7 in seven samples and the multi-drug-resistance P-glycoprotein (P-170) in six. Cytogenetic analysis was abnormal in 12 of the 13 patients in whom an adequate number of mitoses could be evaluated. No single abnormality prevailed, the most common findings being trisomy 8 (three cases) and aberrations of chromosome 7 (two cases). Antileukemic treatment differed according to age, but for remission induction, all patients received a combination of cytosine arabinoside and an anthracycline or mitoxantrone. The prognosis of patients with AML-M0 was remarkably poor as compared with the other French-American-British subtypes. Whereas the overall rate of complete remission (CR) was 58% with a median survival of 63 weeks, only 6 of the 15 patients with AML-M0 achieved a CR, and the median survival of this group was 16 weeks (range 3 to 39). The major determinant of treatment failure was unresponsiveness to chemotherapy, as only one patient died of infection during the hypoplastic phase. The CR duration of responders was short, ranging from 3 to 22 weeks, and no second remissions were observed. We conclude that conventional combination chemotherapy yields disappointing results in AML-M0. The reason for this may be the convergence of various unfavorable prognostic factors, such as (1) the high incidence of cytogenetic abnormalities; (2) the lack of differentiation features and the expression of immaturity markers such as CD34 and CD7; and (3) the frequent expression of P-170. Nonconventional therapeutic approaches should be developed to alter the prognosis of this form of leukemia.

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PATIENTS AND METHODS

Patients. The cases for this study were identified among 189 consecutive patients with de novo AML treated by intensive chemotherapy between 1987 and 1991 (Table 1). One-hundred and one men and 88 women with a median age of 58 years (range 15 to 81) and with a World Health Organization performance status ≤2 were studied. No patient had a history of prior treatment with mutagenic agents, a prior documented myelodysplastic syndrome dating more than 6 months, or history of other preexisting hematologic abnormalities. Antileukemic treatments were differentiated according to age. For 82 patients younger than 60 years old, induction therapy consisted of cytosine arabinoside (ARA-C) administered by continuous infusion at a dose of 200 mg/m²/d for 7 days, plus daunorubicin 45 mg/m²/d administered by intravenous (IV) push in the first 3 days of ARA-C administration. Eighty-six patients aged above 60 received, in induction, 1 g/m²/d ARA-C for 6 days by a 6-hour infusion, and 6 mg/m² mitoxantrone, 3 hours after the end of each ARA-C infusion. Twenty-one patients with acute promyelocytic leukemia (APL), regardless of age, received an induction course of idarubicin alone, 10 mg/m²/d IV push for 6 days, or associated with 200 mg/m²/d ARA-C by continuous IV infusion. All patients who attained remission received ARA-C-based consolida-

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tology laboratory of our institution. Bone marrow (BM) smears of patients less than 60 AML of the elderly, GIMEMA protocol was done independently by two morphologists (G.D.P. and A.V.). The immunophenotype was assessed by immunofluorescence with an Epics Profile flow cytometer (Coulter; Hialeah, FL). Double-color immunofluorescence studies were performed using combinations of phycoerythrin (PE) and fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (MoAbs). These included anti-CD33 (MY9, PE) and anti-CD13 (MY7, PE), purchased from Coulter; anti-CD15 (Leu-M1, FITC), anti-CD34 (HPCA-1, not conjugated), anti-CD2 (Leu-5b, FITC), anti-CD7 (Leu-9, FITC), anti-CD5 (Leu-1, FITC), anti-CD3 (Leu-4, FITC), anti-CD10 (anti-common acute lymphoblastic leukemia antigen, FITC), anti-CD19 (Leu-12, FITC), anti-CD20 (Leu-16, FITC), anti-CD22 (Leu-14, FITC) and anti-HLA-DR (IA PE), all obtained from Becton Dickinson (Mountain View, CA); anti-CD14 (FMC-17, FITC) supplied by Sera-Lab (Sussex, UK); and anti-CD41 (IOP-41, FITC) from Immunotech (Marseille, France). Mononuclear cell fractions of BM samples were separated after Ficoll-Hypaque gradient centrifugation (Sigma, St Louis, MO). All samples contained at least 80% of blasts. Cells were suspended in 0.1 mL of minimal essential medium containing 2.5% human AB serum to minimize Fc-receptor binding and washed twice with phosphate-buffered saline (PBS) afterward. Cells, 1 × 10⁶, were then incubated at 4°C for 30 minutes with the MoAbs at saturation concentrations and washed twice with PBS. For the unconjugated antibody HPCA-1, cells were further incubated for 30 minutes at 4°C with an FITC-conjugated F(ab); fragment of goat antimouse (Technogenetics, Milan, Italy). Nonspecific isotypic mouse MoAbs served as negative control for the primary reagents. A minimum of 8,000 events for each specimen was acquired. Blast cells were selected on the basis of forward-light scatter gating and a pan-myeloid marker, either CD13 or CD33. To eliminate possible contaminations of residual nonleukemic myeloid cells, a positive reaction was defined as 20% of gated cells being more fluorescent than the control. For CD3 a threshold of 10% was considered more appropriate to examine positivity, because in a normal BM only 1% to 2% of cells express CD34 and even a small increase above this background is likely abnormal. However, we observed that all samples expressing this antigen had a positivity greater than 20%, whereas negative cases had a fluorescence pattern comparable with normal marrows.

One-hundred thirty-one samples were also tested for the multidrug-resistance P-glycoprotein expression (P-170). As the MoAb against this protein recognizes an epitope on the inner surface of the cytoplasmic membrane, the cells were fixed and permeabilized in 3% paraformaldehyde/PBS and 50% cold acetone/PBS. Samples were then incubated at 4°C for 30 minutes with 1 μL of FITC-conjugated C219 MoAb (Centoocor, Malvern, PA) solution. Analysis was performed by flow cytometry as above. Given the heterogeneous expression of P-170 in terms of number of cells stained or fluorescence intensity, in accord with Campos et al., the threshold of positivity was set to a conventional 20%. Because the results obtained by multiple linear-regression analysis were very similar in terms of discriminating cases with relatively good from those with unfavorable prognosis, data are presented in this paper as positive or negative.

Intracytoplasmic and nuclear stains included: nuclear terminal deoxynucleotidyl transferase (TdT), cCD3, anti-MPO, cCD22, and cCD13. An indirect immunofluorescent assay using a polyclonal rabbit serum anti-TdT (SuperTeches, Bethesda, MD) was performed on cytosin smears, and in positive cases, a double-marker analysis was performed with MY7, MY9, and Leu-9 as previously described. In TdT/cCD7 cases, cytoplasmic CD3 was also investigated. Cytosin preparations were fixed in acetone for 10 minutes and air dried for 15 minutes. After incubation with 15 μL of FITC-conjugated Leu-4 MoAb (30 minutes in a moist chamber) cytosins were washed three times in PBS. Immunofluorescence was evaluated visually, using a Zeiss microscope (Zeiss, New York, NY) equipped with an epifluorescence set, a barrier filter set for blue fluorescence, and a 100× phase contrast objective. At least 200 cells per preparation were examined. Anti-MPO (Dakopatts AS, Roskilde, Denmark) was studied in most

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<th>Table 1. Patient Characteristics</th>
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<tr>
<td>Total no.</td>
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<tr>
<td>Women</td>
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<td>No. of patients</td>
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<td>WBC (×10⁶/L)</td>
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<td>CR rate</td>
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<td>Early deaths</td>
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<td>Median duration of survival (wk)</td>
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<td>Median duration of CR (wk)</td>
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Abbreviation: WBC, white blood cell.
cases with negative cytochemistry. cCD13 and cCD22 were investigated when surface myeloid markers were absent. The reactivity of these latter antibodies and of anti-MPO were evaluated either by immunofluorescence as above, or by an alkaline phosphatase antialkaline phosphatase method, or by both.

Cytogenetics. Procedures for the cytogenetic analysis of leukemia patients have been described in detail elsewhere.16 Briefly, both a methotrexate cell synchronization technique and a direct preparation were performed. Chromosomes were examined with conventional Giemsa stain. Whenever possible, at least 20 mitoses were analyzed. Karyotypes were assigned according to the recommendations of the International System for Human Cytogenetic nomenclature.17 The observation of a minimum of two mitoses with an identical rearrangement or extra chromosome was regarded as evidence for the existence of an abnormal clone.

Statistical analysis. The relationships of FAB subtypes to quantitative parameters at presentation (age, leucocyte and platelet counts, percentage of blasts in the BM, lactate dehydrogenase levels) were studied by analysis of variance. The relationships to qualitative parameters (sex, organomegaly, the achievement of CR) were analyzed by the chi-square test. A $P$ value of .05 or less was considered statistically significant. Because age is by far the most important prognostic factor in AML, $P$ values were computed with age strata to correct for any dependence on age of the prognostic factor. The Kaplan-Meier procedure was used for survival and remission duration curves. Survival was measured from the date of diagnosis to the date of death or last follow-up. Remission duration was measured from the date of CR until relapse. Six patients who underwent autologous or allogeneic BM transplantation were censored at the time of BM infusion. For comparison of remission duration or survival patterns of two or more groups, the log-rank test was applied. All calculations were performed with the WinSTAT 2.0 (Kalmia Co, Cambridge, MA) statistical program on an IBM computer (Greenock, UK).

RESULTS

The diagnosis of AML-M0 was made in 15 cases (7.9%). There were no clinical characteristics, abnormalities on physical examination or routine laboratory parameters that distinguished these patients (Table 2). Their median age was 61 (range, 27 to 70), only four being under age 50. The presenting leucocyte count ranged from 0.6 to 185 X 10^9/L (median, 27.5 X 10^9/L). Marrows were hypercellular in all but one patient (case 2). The light microscopy appearance of leukemic cells was that of type I blasts, ie, round cells with loose, open chromatin, distinct nucleoli, lightly basophilic cytoplasm without granules. In one patient (case 6) blasts showed a hand mirror-like morphology. No Auer rods were observed and cytochemistry was unremarkable in all cases. The immunophenotypic analysis showed the presence of myeloid-associated surface antigens, either CD13 or CD33, on the blasts of 12 patients. In the remaining three cases (nos. 1, 9, and 11), the leukemic cells reacted with anti-MPO; two of them (nos. 1 and 9) were also positive for cCD13. The expression of immaturity markers occurred frequently: CD34 was always expressed, HLA-DR was found in 13 samples, CD7 in seven and TdT in six. The concurrent expression of TdT and CD7 was observed in two patients; in two other patients, leukemic cells tested positive both for CD2 and CD7. No case showed cCD3 or cCD22. The P-170 glycoprotein was found in 6 of the 12 cases investigated. With regard to the expression of surface epitopes

![Table 2. Clinical and Laboratory Details of AML-M0 Cases](attachment:image.png)
in the other AML groups, significant differences were observed. The CD14 antigen, which tested positive in only one M0, was found in 76 cases of the other FAB classes, with preferential expression in those with monocytic differentiation (M4 and M5; P < .001). CD34 positivity was found in 94 of 153 non-AML-M0 patients (61.4%), with an intermediate tapering value in the M1 (32/42 cases), M5 (13/17), M4 (19/32) and M2 (23/41) subtypes; only one M3 was positive (P < .001). Leu-M1 (CD15), present in two AML-M0 cases, was generally weakly expressed in M1 AML (18/45), and positive in M2 (40/47), M4 (32/39), and M5 (17/22); reactions in APL were less consistent (9/21). CD7 was positive in 51 cases, mainly in the M4 and M5 groups (P = .005), and significantly correlated with CD34 (P < .001). Fifty-five of 119 samples (46.2%) were considered positive for P-170 expression. This phenotype was observed especially in the poorly differentiated M5 (13/15 cases) and M1 (20/31 cases) FAB classes, whereas all M3 patients were negative (P < .001). The distribution of immunologic markers did not significantly correlate with age.

Abnormal karyotypes were found in 12 of the 13 patients in whom an adequate number of mitoses was obtained. Anomalies were generally complex, and did not involve preferential chromosomes, the most common findings being trisomy of chromosome 8 (3 cases), and aberrations of chromosome 7 (2 cases). No Ph chromosome was found.

The CR rate for all patients of this series was 58% (110/189), with a median survival of 49 ± 6 weeks. The survival curves according to the FAB classification can be seen in Fig 1. AML-M0 patients had consistently poorer remission and survival rates as compared with the other FAB subtypes, and also relapsed more rapidly. A CR was achieved in five patients after one course of chemotherapy. The patient in case 15 did not respond to a first-line schedule with daunorubicin and ARA-C ("3 + 7" regimen); he was then treated with a combination of ARA-C, etoposide and mitoxantrone (MEC regimen) and achieved a CR. Induction treatment failed primarily because of refractoriness to chemotherapy, because only one case (4) died of infection during the hypoplastic phase. Five unresponsive patients achieved marrow aplasia during induction, but repopulation of the marrow with leukemic cells occurred. Marrow cellularity below 5% was not achieved in the other four cases with resistant disease. There were no apparent clinical or biologic differences between responders and nonresponders, excepting that no patient who entered CR after the first-line treatment expressed the multidrug-resistance phenotype at the time of diagnosis. All nonresponders died of therapy-related toxicity during subsequent salvage regimens with resistant disease. The median survival of AML-M0 patients was 16 ± 4 weeks (range 3 to 39), with a CR duration ranging from 5 to 22 weeks. No patient of this group underwent BM transplantation. All patients were retreated after first relapse, but no second remissions were observed.

**DISCUSSION**

Nearly 8% of the AML patients of our series was classified as AML-M0. This percentage averages the findings of Lee et al.\(^18\) and Yokose et al.,\(^19\) whereas Buccheri et al.\(^20\) seem to have a somewhat lower incidence (about 3%). In the 15 cases described here, the diagnosis of AML-M0 relied solely on immunologic studies. Noteworthy, anti-MPO was positive in all samples in which it was tested, thus confirming its usefulness as a sensitive diagnostic reagent in AML.\(^20,21\)

We have shown that the presenting clinical features of these patients were not distinctive, whereas their outcome was remarkably poor. They had a low remission rate with associated short remission duration and poor survival, disease resistance to chemotherapy being the primary cause of treatment failure. Comparisons with the data of the literature are difficult to assess, for reports of treatment of AML-M0 are limited and quite heterogeneous. Lee et al.\(^18\) have presented a relatively extensive series in which they described 10 cases classified as AML on the basis of cell marker studies or ultrastructural MPO staining. Treatment in eight of these patients with standard AML chemotherapy
resulted in only one CR lasting 5 months, with six patients showing resistant disease. Two other patients were treated with a regimen developed for acute lymphoblastic leukemia (ALL), and one of them achieved a CR. Mertelsmann et al. classified nine patients as AML-M0. Although response to therapy and survival parameters in this group tended to be worse, there was not a significant difference with the other subtypes. However, the diagnosis of these M0 cases may be questioned because it was based only on the morphologic impression. Yokose et al. have recently reviewed the results of the other 29 assessable cases in the literature where leukemic blasts were MPO negative at a light microscopy level, myeloid marker positive and B- and T-lineage marker negative. A CR was attained in 6 of 11 patients (54.5%) treated with an anti-AML regimen containing ARA-C or its derivative BHAC plus an anthracycline, and in 8 of 18 patients (44.4%) who received an anti-ALL regimen containing vincristine, corticosteroids and 6-mercaptopurine. The duration of CR and survival of these cases are not clearly reported. They also analyzed five cases of their series who were classified as AML-M0. Using a myeloid-oriented chemotherapy a successful induction was obtained in three patients, but only one remained alive at 15 months.

The laboratory investigations performed in this study have shown that the leukemic cells of this subtype present an accumulation of various biologic characteristics that may account for such adverse prognoses.

(1) An abnormal karyotype was found in all cases with adequate mitoses but one. Cytogenetic findings have a major prognostic impact in AML. We and others have previously shown that cases with normal karyotypes do considerably better, and that complex karyotypes are significantly associated with resistance to antileukemic treatment. In reviewing the previous mentioned series, only one examined chromosomes with banding techniques, obtaining a comparable frequency and pattern of abnormal karyotypes. No consistent abnormality was identified in our series, and some of these chromosome changes (eg, aberrations of chromosomes 8 and 7, minute chromosomes) may indeed be present in other myeloid subtypes, as well as in lymphoid forms.

One patient (no. 11) had t(6;11)(q15;q23), which is strongly associated with monoblastic/monocytic leukemia. In this case, leukemic cells might be monoblasts at a very early stage of maturation that did not yet express differentiation antigens and nonspecific esterase activity. One other patient (no. 2) had trisomy of chromosomes 13 and 19. Trisomy 13 is commonly found in association with other chromosome abnormalities, but is much rarer when it occurs as a sole cytogenetic abnormality. Recent investigations have delineated the clinical and laboratory features of leukemia patients with this cytogenetic finding. Trisomy 13 occurs both in secondary and in de novo acute leukemia, more frequently in an older male population, and is associated with a low CR rate and brief remission duration. Immunophenotypic analysis has shown an undifferentiated phenotype or biphenotypic markers in most cases. These results suggest the malignant transformation of an early stem cell, which retains the potential for myeloid and lymphoid differentiation. Baer and Bloomfield have speculated that an additional copy of chromosome 13 can result in an overexpression of a growth factor or of the receptor for a growth factor that acts on an early stem cell.

(2) Leukemic cells exhibited morphologic and phenotypic characteristics of cell immaturity, suggesting the involvement of an early hematopoietic precursor. The success of intensive cytoreductive therapy is dependent on the sensitivity of the neoplastic cell and on the immortality of the stem cell, which renders it resistant to conventional chemotherapy. Accordingly, the more the leukemic cell shows features that resemble the stem cell, the less likely it will be ablated by treatment. The prognostic importance of differentiation aspects in blasts cells, particularly SBB positive, has been recently shown after an extensive study by Hoyle et al. It is noteworthy that all patients with AML-M0 in our series expressed the CD34 antigen, a glycoprotein present on multipotent progenitors, and probably on the normal hematopoietic stem cell. Although CD34 leukemic cells do not seem to give rise to a higher proportion of clonogenic cells in an unconditioned CFU-L assay, clinical investigations have indicated that CD34 expression is an independent prognostic factor in AML and is associated with poor response to therapy. The TdT enzyme, which also is a marker of hematopoietic precursor cells of various lineages associated with adverse prognosis, was positive in six cases. Seven cases also presented CD7. The expression of this antigen in AML has been regarded for a long time as evidence of lineage promiscuity. However, Chabannon et al. have recently identified a minor subgroup of early hematopoietic precursors with the CD34+, CD7+ phenotype. Coexpression of CD7 and CD34 on leukemia cells may thus represent an amplification of a subpopulation of pluripotent cells. This view is supported by molecular studies (because CD7+ AML frequently have rearranged T-cell receptor and Ig heavy chain genes) and corroborated by the results of cell cultures, which show that the leukemic cells expressing this phenotype are capable of multilineage differentiation and which show the greatest growth response to IL-3. Even more than CD34 and TdT, CD7 expression appears to compromise treatment outcome.

(3) A substantial proportion of cases were positive for P-170. An increased expression of this glycoprotein is frequently observed in the blasts of refractory or relapsed patients, as well as in newly diagnosed leukemias. In the latter cases, P-170 positivity may be predictive of poor outcome of intensive chemotherapy. Our series has confirmed the prognostic implications of this finding because five of six patients who expressed this marker failed to achieve a CR. Interestingly, we found that P-170 is also more expressed in the M1 and M5 groups, other forms of AML with poor differentiation and the frequent coexpression of the CD34 antigen. This is consistent with the findings of Campos et al., who have described a correlation between the P-170 phenotype and the presence of CD34. A subgroup of AML patients with very poor prognosis was defined by the combined expression of these two markers.

In summary, this study shows the challenge of treating minimally differentiated AML. Poor results are obtained...
when patients with AML-M0 are treated with the conventional combination chemotherapies that have been successfully applied to other subtypes of AML. It is apparent that more intensive chemotherapy is needed to overcome drug resistance, but toxicity may be a limiting factor, especially considering that most of these patients belong to older age groups. Identification of factors that selectively stimulate or inhibit normal versus leukemia cells could increase the effectiveness of chemotherapy.43 Many data suggest that at least some leukemia stem cells respond to growth and regulatory factors, such as granulocyte-macrophage colony-stimulating factor.44 Use of these factors before chemotherapy might increase the proportion of proliferating leukemia stem cells killed, because most drugs are active against proliferating cells. Factors that favor differentiation of leukemia cells, such as tumor necrosis factor-α, may also be useful.45 Perhaps early cytoabative therapy followed by allogeneic BM transplantation might be proposed for eligible patients. For noneligible cases, consideration should be given to the use of innovative drugs, or new therapeutic regimens incorporating biomodulation or differentiating agents.

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REFERENCES

13. Catovsky D, Matutes E: The classification of acute leukemia. Leukemia 6:1, 1992 (suppl 2)
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R Stasi, G Del Poeta, A Venditti, M Masi, E Stipa, T Dentamaro, C Cox, B Dallapiccola and G Papa