Correlation of Drug Sensitivity In Vitro With Clinical Responses in Childhood Acute Myeloid Leukemia

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Clonogenic cells from 41 children with newly diagnosed acute myeloid leukemia (AML) were tested in vitro for their sensitivity to cytarabine (Ara-C) and daunorubicin (DNR). The findings were then compared with the patients' responses to induction chemotherapy that uniformly included Ara-C and DNR. Light-density marrow cells were incubated with either or both drugs for one hour and cultured over leukocyte feeder layers; clusters and colonies were scored on days 7, 10, and 14. Only the percentage of cell kill in the presence of 1.8 μmol/L DNR was significantly associated with responses to induction therapy: median of 45% (range, 0% to 98%) for patients achieving complete remission and 16% (range, 4% to 23%) for nonresponders (P = .007). The relationship between clonogenic cell kill ≥23% and clinical responses was striking. Of the 11 evaluable patients with in vitro findings in this category, ten either failed induction therapy or relapsed within 1 year after attaining remission. Kaplan-Meier analysis of relapse-free survival times indicated longer durations of remission for patients whose blast cells showed increased sensitivity in vitro to Ara-C alone, DNR alone, or a combination of the two agents. Seven of 11 patients with cell kills of ≥49% in the presence of 1.25 μmol/L Ara-C remain free of leukemia, compared with only one of 12 whose cells were less sensitive to the drug (P = .006). We conclude that the in vitro sensitivity of clonogenic leukemic progenitors to DNR and Ara-C correlates with treatment outcome in children with newly diagnosed AML.

CLONOCGENIC ASSAYS of malignant stem cells have been proposed as a method for obtaining reliable information about the chemosensitivity of a patient's tumor cells and evaluating the probable clinical efficacy of anticancer therapy. In the human leukemias, normal progenitor cells are replaced by abnormal progenitors with a spectrum of proliferative potentials. Such cells from patients with acute myeloid leukemia (AML) usually form clusters or colonies in vitro when stimulated by leukocyte feeder layers or conditioned media. Preisler and others have reported that the in vitro drug sensitivity of clonogenic progenitors from different groups of adult patients with AML studied at diagnosis and relapse correlates with clinical responses to various induction regimens. Interpretation of these findings has been complicated by the lack of uniform treatment of blast cells in vitro and in vivo and by inclusion of patients who were studied after relapse as well as at diagnosis. To estimate the predictive value of clonogenic assays in childhood AML, we compared the in vitro sensitivity of cells to cytarabine and daunorubicin with induction responses to the same drugs. Additional comparisons were done to find whether sensitivity to these agents would reliably predict the long-term outcome of therapy.

PATIENTS AND METHODS

The study comprised 41 patients with newly diagnosed AML from whom sufficient cells for cultures were obtained during the period from April 1980 through Oct 1983. The distribution of ages, races, initial leukocyte and platelet counts, blast cell morphologic types and cytogenetic features among the study group did not differ significantly from that among 43 other patients who were treated according to the same therapeutic protocol. Responses to induction therapy and relapse-free survival times in the two groups were also similar (median times to failure, 293 and 341 days, excluding patients who received bone marrow transplants).

There were 27 boys and 14 girls, with ages ranging from 4 months to 19.3 years (median, 6 years). Thirty-six patients were white, and five were nonwhite. Leukocyte counts at diagnosis ranged from 1.6 to 400 × 10^9/L (median, 27.6 × 10^9/L). By the French-American-British (FAB) classification system, 8 patients had M₁ disease, 14 M₂, 3 M₃, 12 M₄, and 4 M₅. Cells from 17 children had Auer rods. Analysis of the patients' marrow cell karyotypes revealed normal ploidy in 16 cases, a combination of normal and abnormal ploidy in nine, and abnormal ploidy only in 13. Three children had inadequate chromosome preparations. Chromosomal translocations, including the (8;21), were identified in 13 patients. One child had monosomy 7, and two had an inversion of chromosome 16 (13q22). These investigations were performed with the informed consent of the patients or their parents and with the approval of the Clinical Trials Committee of St. Jude Children's Research Hospital.

Outline of therapy. The treatment protocol is summarized in Fig 1. Each child initially received three daily doses of daunorubicin (DNR) followed by a seven-day continuous infusion of cytarabine (Ara-C). If blasts were present in a marrow aspirate obtained on day 14, a second course of DNR/ara-C was given immediately. Patients with marrow hypoplasia and <5% blasts received a second course of DNR/Ara-C after marrow recovery. For this analysis, patients who entered hematologic remission after the first or second course of therapy were considered responsive to induction therapy. Those with residual leukemia or bone marrow recovery with leukemic blasts after two courses of induction therapy were classified as nonresponders. Patients who died during induction therapy without marrow recovery or failed to complete the protocol-specified therapy were excluded from this analysis. Six patients who achieved a complete remission and had a suitable marrow donor were given a bone marrow transplant. Two patients achieved hematologic but not complete remission; all others entered complete remission and received monthly cycles of chemotherapy as outlined in Fig 1 and were evaluated for duration of complete remission.

Cell culture methods and drug sensitivity testing. Aspirated bone marrow cells obtained at diagnosis were collected in preservative-free heparin. Light-density cells (<1.077 g/mL) were separated by density centrifugation with Ficoll-metrizate, yielding 58% to 100% blasts (median 96%). These were incubated for 60 minutes.
Fig 1. Schema of therapy. All patients received at least one course of DNR/Ara-C. If the marrow aspirate obtained on day 14 contained greater than 5% blasts, a second course of the same agents was given immediately. For children with hypoplastic marrows (≤5% blasts), the second course was delayed until marrow recovery occurred. Patients who attained remission and had an HLA-mixed lymphocyte culture (MLC)-compatible marrow donor received a bone marrow transplant. Those without a suitable donor received monthly cycles of continuation therapy, as shown in the figure. All postinduction treatment was stopped after 1 year.

(1 × 10^6 cells/mL) with 1.8 μmol/L DNR, 1.25 μmol/L Ara-C, and 12.5 μmol/L Ara-C or 1.25 μmol/L Ara-C plus 1.8 μmol/L DNR as reported by Preisler.\(^1\) When sufficient cells were available, additional incubations with drug for 18 hours were performed. The cells were then washed and plated in quadruplicate in 35-mm Petri dishes (Miles Scientific, Naperville, Ill) at 2 × 10^5 cells/mL in 0.3% agar with a leukocyte feeder layer.\(^5\) Cultures were incubated in a 7.5% CO₂ atmosphere at 37 °C, and clusters of more than three cells and colonies of >50 cells were scored on days 7, 10, and 14. The percentage of cell kill was determined by dividing the number of clusters and colonies of drug-exposed clonogenic cells (L-CFU) by that for cultures of untreated L-CFU and subtracting the result from 100%. Only studies for which control plates had 20 or more clusters and colonies were included in the analysis. In four of 144 drug assays, greater growth was noted for drug-exposed cells than for controls, resulting in negative cell kill; these values appear as zero in Fig 2.

Cells obtained from 46 of 72 patients, or 64%, met the criteria for drug sensitivity testing. However, five of the 46 presented prior to the beginning of in vitro drug sensitivity studies, leaving 41 eligible for testing. The numbers of clusters and colonies formed by clonogenic cells from 41 patients in this study ranged from 23 to 3,088, with a median of 538 per 2 × 10^5 cells plated. Cells from 17 of these patients formed more than 19 large clusters and colonies, and cells from 24 formed predominately small clusters.

The 43 patients not studied for drug sensitivity in vitro included the five patients cited in the preceding paragraph, three with insufficient cell numbers, 12 from whom samples were not collected, and 23 having fewer than 20 clusters or colonies on day 7. A comparison of the latter 23 patients to the 41 whose cells produced clusters and colonies in vitro revealed no significant difference in either the proportion achieving remission or the duration of remission. Growth patterns (cluster/colony ratios) were not related to response.

When clonogenic progenitors were scored on days 10 and 14, cell growth for the majority of patients had decreased (L. W. Dow, unpublished observation). The data are therefore reported for day 7.

Statistical methods. Mann-Whitney tests and chi-square tests were used to compare clinical characteristics between patients with and without cell culture results. The percentages of cells killed by Ara-C and DNR for responding and nonresponding patients were compared with use of Mann-Whitney tests. In the statistical analysis, patients were considered to have resistant disease if they failed to achieve remission after two cycles of DNR and Ara-C; additional analysis extended the definition of clinical drug resistance to cases in which relapse occurred within one year from the date of remission. Relapse-free survival curves were constructed by the Kaplan-Meier procedure, and significant differences between results were determined by the log rank test.\(^16\) Two patients in hematologic but not complete remission and six who received a bone marrow transplant were excluded from the Kaplan-Meier analysis. A method based on the log rank test was used to identify percentages of cell kill that yielded maximal differences in remission durations. The potential influence of various clinical and laboratory variables on duration of continuous complete remission was evaluated in a stepwise multivariate analysis using the model of Cox.\(^14\)

RESULTS

Thirty-one of the 41 patients whose blast cells were studied for chemosensitivity in vitro achieved a complete or hematologic remission after one or two courses of DNR and Ara-C, six had resistant disease requiring alternative therapy, and four could not be evaluated for clinical responses because of early death. Of these four, one died early in induction therapy (day 4); two others died on days 18 and 36 of intracranial hemorrhages without evidence of leukemia; and the fourth, who had monosomy 7, died with prolonged marrow hypoplasia and disseminated aspergillosis after two cycles of induction therapy.

Figure 2 shows the percentages of cell kill for clonogenic progenitors after one hour of incubation with either DNR or Ara-C, or both drugs, as determined from cluster and colony formation on day 7. The results obtained with 1.8 μmol/L DNR alone (panel A) were significantly related to clinical responses: 45% median cell kill (range, 0% to 98%) for patients entering remission vs 16% (range, 4% to 23%) for
induction failures resulting from persistent leukemia ($P = .007$). Close examination of our data revealed that low levels of chemosensitivity, as measured by the clonogenic assay, predicted clinical drug resistance with greater than 90% accuracy. Of 11 patients with in vitro cell kills of ≤23%, ten either failed induction therapy or relapsed within 1 year after attaining remission. At higher levels of cell kill, only three of 18 patients failed to achieve at least 1 year of remission.

The chemosensitivity of cells exposed to 1.25 or 12.5 μmol/L Ara-C alone or to Ara-C and DNR in combination did not achieve statistical significance as a predictor of induction responses. However, 11 of 19 patients with cell kills ≤49% with 1.25 μmol/L Ara-C failed therapy within 1 year, compared with two of 11 with higher percentages. With a tenfold higher concentration of Ara-C, nine of 17 patients with cell kills of ≤54% failed early, compared with three of nine whose blasts showed greater chemosensitivity. Combining Ara-C (1.25 μmol/L) with DNR (1.8 μmol/L) did not improve the results obtained with either Ara-C or DNR alone.

Twenty-three of the 31 patients who achieved remission were eligible for assessment of in vitro chemosensitivity as a predictor of remission duration. Kaplan-Meier analysis was performed for groups of patients defined by percentages of cell kill yielding maximal differences in remission durations (Fig 3). With a median follow-up time of 35 months, only three of nine patients with an in vitro cell kill of ≥53% in the presence of DNR have relapsed, compared with 12 of 14 with lower percentages ($P = 0.010$). Similarly, only four of 11 patients with ≥49% kill of progenitors exposed to 1.25 μmol/L Ara-C have failed therapy, compared with 11 of 12 with lower percentages ($P = .006$). Incubation of cells with 12.5 μmol/L Ara-C or a combination of DNR and Ara-C also yielded groups with significantly different relapse rates ($P$ values = .037 and .034, respectively). Importantly, median lengths of remission for patients with higher cell kill in the presence of 1.8 μmol/L DNR, 1.25 μmol/L Ara-C, 12.5 μmol/L Ara-C (data not shown), or a combination of DNR and Ara-C exceed 693 days and cannot be estimated, whereas those for the corresponding groups defined by lower levels of cell kill do not exceed 495 days.

To further assess the relationship of in vitro kill and other possible prognostic factors to remission duration, we per-
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formulated a Cox regression analysis. Variables included age, leukocyte count ($<25 \times 10^9/L$), presence or absence of CNS leukemia at diagnosis, FAB morphologic classification (M1 to M7, with M0 to M4), and percentage of cell kill in vitro with each drug or the combination of Ara-C and DNR. Only the cell kill by DNR in vitro was related to remission duration ($P = .04$).

To test the specificity of cell kill in vitro by DNR and Ara-C, we divided the patients into groups based on the same percentages listed in Fig 3. Twenty of 25 patients with low cell kill by DNR in vitro had a low cell kill with 1.25 mmol/L Ara-C, whereas ten of 12 with a high DNR-induced cell kill also had a high cell kill with Ara-C. Similar relationships were seen when DNR was compared with the tenfold higher concentration of Ara-C and when either drug as a single agent was compared with a combination of the two drugs.

We also examined the relationship of ploidy in vitro drug sensitivity to each of these agents, outcome of therapy, and to growth patterns in vitro. There were no demonstrable correlations (analysis not shown).

Twenty-three patients had sufficient cells for in vitro assessment of cell kill after incubation with Ara-C for 18 hours prior to plating, and 22 could be evaluated for response to DNR after a similar time of drug exposure. Cell kill in the 18-hour assays did not correlate with response to induction therapy; however, only four patients with resistant disease had sufficient cells for study (Fig 4). Among our smaller number of evaluable patients, remission durations were related to in vitro cell kill after an 18-hour incubation with DNR but not Ara-C. Only two of seven patients with cell kills greater than 41% in the presence of DNR have relapsed, compared with six of nine patients having lower percentages ($P = .03$).

**DISCUSSION**

Because clinical features have generally failed to predict responses to therapy in childhood AML, we sought in vitro measures of the chemosensitivity of the patients' leukemia cells. The stem cell model of human cancer predicts that clinical responses to treatment should relate only to the killing of cells with clonogenic potential. Recent observations of Preissler, Park et al, Browman et al, and others indicate that in vitro assays of cluster and colony formation by malignant progenitor cells will predict clinical drug resistance in a high proportion of cases; however, for children with newly diagnosed AML, it has not been demonstrated that the chemosensitivity of clonogenic progenitors correlates with treatment outcome.

Our study is the first in which clonogenic progenitor cells obtained only at diagnosis were exposed to the same agents used for remission induction. Results of the assay indicate that the in vitro sensitivity of leukemic progenitors to DNR, but not Ara-C, is highly correlated with subsequent responses to induction therapy. This suggests a greater therapeutic contribution from the anthracycline, consistent with the higher remission induction rates reported for DNR as a single agent in AML in doses similar to this regimen.

That the duration of complete remission correlates with percent DNR cell kill in vitro also suggests the relationship between in vitro and in vivo cell kill.

Typically, an assay that predicts clinical drug resistance is correct in 90% of cases, whereas the prediction of drug sensitivity is correct in 40% to 70% of cases. When induction responses were used as the end point, drug resistance in the assay (ie, cell kill of $<23\%$) was associated with clinical resistance in 55% of the patients. However, if both failure to achieve remission and early development of recurrent disease, within 1 year from the date of remission, were considered evidence of drug resistance, cell kill by DNR in vitro met the 90% criterion for a clinically useful predictor of drug efficacy.

The lack of a statistically significant relationship between cell kill by Ara-C, with or without addition of DNR, and clinical responsiveness to induction therapy conflicts with previous studies of clonogenic assays in AML. Preissler, for example, has reported that the sensitivity or resistance to either Ara-C or DNR in vitro correlated with the induction responses of 25 older patients with newly diagnosed or relapsed AML, some with leukemia following cytotoxic treatment for other malignancies. Similar findings were
reported by Browman et al\textsuperscript{8} for 38 adults treated with Ara-C and an anthracycline. Using different methods, Gustavsson and Olofsson,\textsuperscript{11} Lihou and Smith,\textsuperscript{9} and Park et al\textsuperscript{7} also found a positive correlation between the sensitivity of blast cells to Ara-C and anthracyclines in vitro and induction responses in mixed groups of newly diagnosed and relapsed patients. Finally, Marie et al\textsuperscript{16} demonstrated a correlation with the outcome of induction therapy for Ara-C but not doxorubicin in studies in which clonogenic cells were exposed continuously to drugs. These discrepancies may reflect different assay methods, different criteria for the inclusion of patients in the studies, or perhaps inherently different leukemic stem cells in childhood vs adult AML.\textsuperscript{22}

That in vitro kill with DNR alone differs from the kill with simultaneous exposure to Ara-C and DNR may be related to the cytotoxic effects of each drug on a patient's cells in vitro. Fried et al\textsuperscript{23,24} have shown that the cytotoxic effects of a one-hour simultaneous exposure of Chinese hamster ovary cells to Ara-C and DNR are related to the relative effects of each drug in the combination. When a moderately cytotoxic dose of DNR and a less cytotoxic dose of Ara-C were used, the observed cell kill with the combination was less than expected. When a higher, more cytotoxic concentration of Ara-C was coupled with a less cytotoxic dose of DNR, the results equaled the expected cytotoxicity. In their experiments, relatively low Ara-C concentrations inhibited cell cycle progression and provided partial protection from DNR cytotoxicity.

Methods of studying the sensitivity of leukemic cells to phase-specific antimetabolites such as Ara-C are controversial. Such studies have been complicated by the rapid deamination of the drug and its Ara-CMP (Ara-C monophosphate) metabolite. Preisler\textsuperscript{3} has recommended relating the sensitivity of clonogenic progenitors in S-phase to Ara-C by use of a thymidine suicide index to distinguish kinetically resistant from biochemically resistant cells. However, our data (not shown) do not indicate a relationship between the sensitivity of S-phase cells to Ara-C and the results of induction therapy or duration of remission. Lihou and Smith\textsuperscript{9} have recommended continuous exposure of cultures to Ara-C to obtain results more representative of the clinical situation, whereas Park et al\textsuperscript{7} has refed cultures daily, adding Ara-C as well as nutrients. We exposed clonogenic progenitors from 22 patients to Ara-C for 18 hours before plating and, despite greater cell kill, found no correlation with induction responses.

Weisenthal and Lippmann\textsuperscript{25} have suggested that the results of clonogenic assays reflect the general chemosensitivity of the proliferating leukemic cells in vivo rather than their responsiveness to particular agents. This hypothesis has been difficult to test in AML since most studies with clonogenic assays have not been performed in conjunction
with a prospective clinical trial. That the sensitivity of clonogenic progenitors to DNR or Ara-C alone, or to a combination of both drugs, showed a relationship to remission durations among our patients would support the idea of Weisenthal and Lippman. However, the failure of cell survival in the presence of Ara-C, even with added DNR, to predict early development of drug resistance suggests that the early responses of patients to chemotherapy may be related to the activity of specific drugs such as DNR. This is supported by the correlation of in vitro kill by DNR and remission duration. Alternatively, the separation of patients into favorable and unfavorable groups based on maximal differences in relapse-free survival could have increased the probability of false-positive results so that Ara-C might appear to have prognostic value. Further research comparing dose-response curves for AML cells exposed to different classes of antileukemic agents is needed to resolve this question.

The ability of the clonogenic assay to select patients who are most likely to fail anthracycline-based induction therapy or to relapse early should be confirmed in a prospective trial and exploited in treatment planning. Alternative methods may extend the prediction of clinical drug sensitivity to agents such as Ara-C and lead to clinically relevant models of drug combinations.

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REFERENCES

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