Predictions of Response to Chemotherapy in Acute Leukemia by in Vitro Drug Sensitivity Testing on Leukemic Stem Cells

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ABSTRACT

Twenty-two patients with acute myeloid leukemia were studied for in vitro drug sensitivity of the leukemic clonogenic cells in agar. The cells were preincubated for 1 hr with 1-β-D-arabinofuranosylcytosine (ara-C; 0.15, 0.3, 0.6, 1.2, and 2.4 μg/ml) or daunorubicin (0.018, 0.037, 0.075, 0.15, and 0.30 μg/ml), washed, and plated in agar, and cluster/colonies were counted after 10 days of incubation. Survival curves were constructed and used for calculation of the surviving fraction of clonogenic cells. In 18 patients treated with thioguanine-daunorubicin-1-β-D-arabinofuranosylcytosine-prednisone, the in vitro drug sensitivities could be correlated to the in vivo response to therapy. Patients who entered remission (12 of 18) were significantly more sensitive to ara-C (p < 0.005) and to daunorubicin (p < 0.02) than patients who did not enter remission (six of 18). All patients who entered remission, except two, had normal or increased sensitivity to both drugs, and all patients who did not enter remission, with one exception, had decreased sensitivity to one or both drugs. Comparison of the cytostatic effect of [3H]thymidine and ara-C suggested that, in some cases, ara-C killed more clonogenic cells than those in S phase, and in some cases, the cells seemed to be metabolically resistant to ara-C.

We conclude that in vitro drug sensitivity tests on leukemic clonogenic cells reflect the patient’s in vivo response to the tested drugs and may be used to study the biological properties of leukemic stem cells that determine their drug sensitivity.

INTRODUCTION

Although a majority of patients with acute leukemia achieve remission with currently used chemotherapeutic regimens, cytostatic drugs produce considerable toxicity also in those patients where therapy fails to induce remission. Some of these patients will eventually achieve remission after a change to another chemotherapeutic regimen, but it is not known if the initial therapy alters the chances for subsequent remission. It would therefore be an improvement if the therapeutic response to chemotherapy could be predicted and if the most effective regimen were selected.

The leukemic cell population is dependent on an influx of cells from a leukemic stem cell pool, because of the limited proliferative capacity of the majority of leukemic blast cells (11). Normal and leukemic stem cells are clonogenic in vitro, and it is thus possible to study the effects of chemotherapeutic drugs on these cells in vitro. Such tests could possibly provide a means to predict the response to chemotherapy in acute leukemia and eventually be used to select the most efficient drug combination in individual patients. Another development would be to use such in vitro tests to establish the efficacy of new drugs before clinical trials are started. There are a few reports demonstrating the feasibility of this approach in leukemia. One report (16) showed that patients with acute myeloid leukemia whose clonogenic leukemic cells were sensitive to ara-C and daunorubicin in vitro entered remission, while patients resistant to either or both drugs failed to enter remission. Similar findings have been reported by others (15), while another study (13) demonstrated that the self-renewal capacity of blast progenitor cells in peripheral blood showed a stronger correlation to induction of remission and survival than the in vitro sensitivities to ara-C or doxorubicin. Large efforts have also been made to develop clonogenic tumor stem cell assays for quantitation of chemotherapy drug sensitivity in vitro of nonhematopoietic cells (18).

In this paper, we have studied the in vitro drug sensitivity of normal and leukemic clonogenic cells in agar using ara-C and daunorubicin over a wide concentration range and different incubation times to establish a standardized method for in vitro drug sensitivity testing. In 18 patients with acute myeloid leukemia, the in vitro results, with few exceptions, reflected the in vivo response to therapy in terms of achievement of remission.

MATERIALS AND METHODS

Patients. Twenty-six patients with acute myeloid leukemia were studied. Cells from 4 of them did not grow in vitro, and these patients were excluded from further analysis. For the remaining 22 patients, the studies were performed on bone marrow cells obtained at diagnosis in 21 cases and in one case (No. 14) on cells obtained after 5 courses of TRAP; in one case (No. 14), cells from diagnosis and after 6 courses of TRAP were studied. Eighteen of the patients were treated and analyzed for correlation between in vitro and in vivo response to chemotherapeutic drugs. The French-American-British (FAB) classification (3) was used for morphological diagnosis. The 22 patients studied for drug sensitivity are presented in Table 1.

Treatment. Induction therapy (TRAP) consisted of daunorubicin (60 mg/sq m) on Day 1, ara-C (70 mg/sq m) twice daily i.v. in bolus doses on Days 2 to 6, thioguanine (70 mg/sq m) twice daily on Days 2 to 6, and prednisone (30 mg/sq m) on Days 2 to 6. The treatment was repeated if possible on Day 10. Consolidation therapy consisted of TRAP, and maintenance therapy, when given, was TRAP alternating with cyclophosphamide-oncovin-1-β-D-arabinofuranosylcytosine-prednisone. Complete remission was defined as normal bone marrow cellularity with less than 5% blast cells, return of neutrophil counts to 1.5 × 10⁹/liter or greater, platelet counts of at least 100 × 10⁹/liter, and a hemoglobin value of at least 100 g/liter. Twelve patients achieved remission, and 6 patients did not. Of the 4 remaining patients, 2 had refractory anemia.

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2 To whom requests for reprints should be addressed.

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with excess of blasts for 3 and 20 months, respectively (Patients 21 and 22) prior to overt leukemia; one of these patients was treated with ara-C and thioguanine intermittently (Table 1).

Cell Preparation. Bone marrow cells, 3 to 5 ml, obtained by sternal puncture were collected in heparinized McCoy's medium, suspended by repeated suction through a needle (0.8 x 80 mm), and separated on Isopaque-Ficoll (Lymphoprep; Nyegaard & Co., Oslo, Norway). The mononuclear cell fraction was washed twice in McCoy's medium and adjusted to $3 \times 10^8$ cells/ml in McCoy's medium containing 1% FCS (Flow Laboratories, Solna, Sweden). Normal marrow cells were obtained from healthy volunteers.

Exposure to ara-C and Daunorubicin in Vitro. ara-C (Cytosar; Upjohn) and daunorubicin (Cerubidin; Leo Rhodia) were dissolved in 0.15 M NaCl, filter sterilized, and diluted in McCoy's medium. Fresh solutions were made for each experiment. Fifty µl of drug solution were added to 0.5 ml of bone marrow cells, 3 x 10^6/ml, to achieve final drug concentrations of ara-C of 4.8, 2.4, 1.2, 0.6, 0.3, and 0.15 µg/ml, and of daunorubicin of 0.6, 0.3, 0.15, 0.075, 0.037, and 0.018 µg/ml. The cells were then incubated at 37° for 1 hr in a CO² incubator and washed twice in McCoy's medium-1% PCS before culture in agar. Cells incubated in medium only served as controls. In some experiments, combinations of ara-C and daunorubicin, or [3H]thymidine were cultured in agar at a concentration of 2 to 5 hr of preincubation was studied.

To calculate the SF of clonogenic cells from the following equation.

$$SF = \frac{\text{area under curve (surviving cells)}}{\text{total area (surviving plus dead cells)}}$$

The concentration ranges for ara-C of 0 to 2.4 µg/ml and for daunorubicin of 0 to 0.3 µg/ml were used for calculation of SF in the standard assay.

**RESULTS**

Survival of Clonogenic Cells after Drug Exposure. Chart 1 shows typical survival curves of clonogenic cells after 1-hr exposure to increasing concentrations of ara-C and daunorubicin. Drug resistance is demonstrated by flat curves with minimal or no reduction of clonogenic cells. Drug-sensitive cells gave a single exponential curve for daunorubicin, whereas ara-C typically gave a biphasic curve.

We also investigated the effect of ara-C and daunorubicin as a function of time. Both drugs, ara-C (1.2 and 4.8 µg/ml) and daunorubicin (0.075 and 0.30 µg/ml), were effective within 1 hr, and there was no major additional effect at 2, 3, or 5 hr of incubation (8 experiments, data not shown). In 5 cases (one
normal and 4 patients with acute myeloid leukemia), 1-hr drug exposures were compared with continuous exposure. At the concentrations tested, continuous exposures to ara-C (0.037 to 2.4 µg/ml) resulted in almost total inhibition of colony/cluster formation except for the lowest concentrations. Continuous exposures to daunorubicin (0.018 to 0.30 ng/ml) resulted in the same or slightly more pronounced inhibition as observed with 1-hr pulse exposure (data not shown). Therefore, 1-hr incubations were chosen for standard assays.

**Combined Exposure to ara-C and Daunorubicin.** In 7 patients, combinations of ara-C and daunorubicin were tested, 4 of them are shown in Chart 2. Patient A was sensitive to both ara-C and daunorubicin, and the combination of ara-C and daunorubicin caused an increased killing effect. Patient B was sensitive to ara-C but insensitive to daunorubicin; however, the combination of the 2 drugs caused markedly increased killing at all concentrations (one additional patient showed a similar pattern). Patient C had low sensitivity to both ara-C and daunorubicin, but the combination resulted in 50% killing, even at the lowest concentrations. Patient D showed a pattern of insensitivity to both ara-C and daunorubicin, and the combination was also ineffective (2 additional patients were similar to Patient D).

**Comparison of LD₅₀ Values and the SF.** LD₅₀ values and the SF were calculated for ara-C and daunorubicin in all 22 patients. There was a strong correlation between the SF and log LD₅₀ values for both ara-C (r = 0.95) and daunorubicin (r = 0.92). Table 2 shows the SF values for ara-C and daunorubicin. Marrow cells from one patient (No. 14) were studied twice; at diagnosis, SF was 0.88 and 0.90 for ara-C and daunorubicin, respectively, and after 6 courses of TRAP, it was 0.86 and 0.88.

**Relationship between ara-C and Tritiated Thymidine Exposure.** Tritiated thymidine suiciding is an established method for determination of the fraction of clonogenic cells in S phase (14). ara-C kills the cells during DNA synthesis (10), and a correlation between the number of surviving cells after exposure to ara-C and [³H]thymidine would be expected. As shown by the data in Table 2, this is not always the case. In Cases 2, 6, 8, 11, 13, 17, and 18, ara-C kills fewer cells than would be expected from the fraction of cells in S phase, whereas in Case 21, and possibly Cases 3 and 4, ara-C kills more cells than expected.

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**Table 2**

<table>
<thead>
<tr>
<th>Patient</th>
<th>[³H]Thymidine</th>
<th>ara-C</th>
<th>Daunorubicin</th>
<th>CR/NR</th>
</tr>
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<tr>
<td>1</td>
<td>0.22</td>
<td>0.78</td>
<td>0.38</td>
<td>CR</td>
</tr>
<tr>
<td>2</td>
<td>0.60</td>
<td>0.68</td>
<td>0.59</td>
<td>CR</td>
</tr>
<tr>
<td>3</td>
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<td>0.44</td>
<td>0.48</td>
<td>CR</td>
</tr>
<tr>
<td>4</td>
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<td>0.59</td>
<td>0.40</td>
<td>CR</td>
</tr>
<tr>
<td>5</td>
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<td>0.61</td>
<td>CR</td>
</tr>
<tr>
<td>6</td>
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<td>0.68</td>
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<td>CR</td>
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<td>7</td>
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<td>0.21</td>
<td>0.92</td>
<td>CR</td>
</tr>
<tr>
<td>8</td>
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<td>CR</td>
</tr>
<tr>
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<td>0.54</td>
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<td>CR</td>
</tr>
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<td>CR</td>
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<td>0.54</td>
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</tr>
<tr>
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<td>0.97</td>
<td>0.52</td>
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<td>0.90</td>
<td>NR</td>
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<td>0.78</td>
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<td>0.93</td>
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<tr>
<td>22</td>
<td>0.39</td>
<td>0.31</td>
<td>0.62</td>
<td>NR</td>
</tr>
</tbody>
</table>

CR, complete remission; NR, nonremission.

<table>
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<tr>
<th>Normals</th>
<th>0.63 ± 0.04</th>
<th>0.89 ± 0.08</th>
<th>0.56 ± 0.10</th>
</tr>
</thead>
</table>

n = 14; n = 8; Mean ± S.D.
Correlation between in Vitro Drug Sensitivity and in Vivo Response to Therapy. Chart 3 shows the relationship between in vitro drug sensitivity and the clinical outcome in the 18 patients treated with TRAP. With regard to ara-C, patients who achieved remission showed significantly higher in vitro sensitivity [mean SF, 0.60 ± 0.15 (S.D.)] than did patients who did not achieve remission [mean SF, 0.86 ± 0.12 (S.D.)], p < 0.005. This difference was also found for daunorubicin; patients who entered remission were more sensitive to daunorubicin (mean SF, 0.58 ± 0.18) than were those who did not enter remission (mean SF, 0.78 ± 0.14), p < 0.02 (Student’s t test).

In Chart 4, the SF values for ara-C are plotted versus SF for daunorubicin together with the normal mean ± 2 S.D. With 2 exceptions, all patients who achieved remission (•) had normal or increased drug sensitivity for both ara-C and daunorubicin; the exceptions were Patients 6 and 7, who were insensitive to daunorubicin but had normal and high sensitivity to ara-C, respectively. With regard to Patient 6, it is noticeable that the in vitro combination of ara-C and daunorubicin resulted in markedly increased killing effect (Chart 2B). Among the patients who did not enter remission (○), all except one showed decreased in vitro sensitivity to one or both drugs. The exception was Patient 15, who had normal sensitivity to both ara-C and daunorubicin but died after 2 courses of TRAP from an intracerebral bleeding (at the time of death, he had rising WBC to 50 × 10^9/liter and predominantly blast cells). Patient 13 had normal sensitivity to daunorubicin but was insensitive to ara-C and died from intracerebral bleedings and with a severely hypoplastic marrow after 2 courses of TRAP.

DISCUSSION

This study describes a standardized method for in vitro drug sensitivity testing of clonogenic cells in acute myeloid leukemia and demonstrates a correlation between in vitro results and in vivo response to therapy.

The concentrations of ara-C and daunorubicin were chosen to fall within the range that can be achieved in plasma (1, 2, 8, 9, 19) and used in culture medium containing 1% FCS. However, it should be remembered that the pharmacokinetics, protein binding, and drug metabolism that modify the drug concentrations in vivo are not possible to obtain in vitro. Daunorubicin produced single negative exponential curves in agreement with earlier findings (4, 5), which allows calculation of LD_{50} values from the slope of the curve. ara-C, on the other hand, acts primarily on cells in S phase (10), which probably explains why ara-C produces biphasic curves with the initial slope suggesting corresponding to sensitive cells in DNA synthesis and the plateau phase corresponding to insensitive cells. Therefore, LD_{50} values can only be extrapolated from the first part of the curve. We found that a calculation of the SF from linear box diagrams reliably reflected the in vitro drug sensitivity for both ara-C and daunorubicin and strongly correlated to the LD_{50} values. The construction of a dose-response relationship is preferable to the use of single drug concentrations; e.g., sampling errors at one of the drug concentrations becomes less important when a dose-response curve is constructed, and furthermore, as illustrated in Chart 1, major differences between some patients would have passed unobserved if only single drug concentrations in the lower range had been studied. Maximal drug effects were seen after 1-hr incubation except for ara-C, which during continuous exposure produced severe suppression of cluster/colony formation even at the lowest concentrations tested. However, continuous exposure for 10 days may not be relevant, and we found it to be less informative than the 1-hr pulse exposure. Furthermore, short incubation times are preferable because they will not interfere with the cell cycle status.

We found a strong correlation between in vitro drug sensitivity and in vivo response to therapy. In general, only those patients sensitive to both ara-C and daunorubicin entered remission. However, exceptions to this general agreement between in vitro sensitivity and response to therapy emphasize the fact that other factors than drug sensitivity contribute to the clinical outcome.

These findings agree with previous reports (15, 16). In one study (16), the cells were incubated with ara-C (0.3 and 3.0 μg/ml) and daunorubicin (0.1 μg/ml) for 1 hr prior to culture in agar. By relating the fraction of surviving clonogenic cells exposed to ara-C (0.3 μg/ml) and daunorubicin (0.1 μg/ml) to the in vivo response, the author found that patients who entered remission had more sensitive cells than did those who failed to enter remission. Only those patients who were sensitive to both drugs responded favorably to treatment. It was also found that simultaneous exposure to ara-C and daunorubicin was the most sensitive test for recognition of patients who responded with
remission or no remission to therapy (16). We found that the combination of suboptimal doses of ara-C and daunorubicin in 4 of 7 patients resulted in increased killing. However, the result of simultaneous exposure to ara-C and daunorubicin is subject to individual variations and cannot be predicted from the response to each of the drugs tested separately.

In another study (15), leukemia cells from 9 patients with acute myeloid leukemia were exposed to single-dose doses of vincristine, ara-C, prednisone, rubidazone, 6-thioguanine, and doxorubicin, and the results were compared with those of a simultaneously studied normal control. Patients who had cells more sensitive than the control entered remission, whereas patients with less sensitive cells did not. However, since there was a considerable variation in sensitivity among the controls, the outcome of the test will depend very much on the sensitivity of the randomly chosen control.

In agreement with a previous report (16), a comparison of the suicidal effect of tritiated thymidine and ara-C suggested that, in some cases, ara-C killed more cells than those in S phase, and in other cases, clonogenic cells in S phase seemed to be metabolically resistant to ara-C. The mechanisms on a cellular level for these discrepancies may be related to the degree of retention of ara-C in the leukemic cells (17) and may be of critical importance for the in vivo response to ara-C.

In studies of cytostatic drug effects on blast progenitor cells in peripheral blood, sensitivity to ara-C but not to doxorubicin correlated to the clinical outcome (13). However, only self-renewal capacity of the blast progenitor cells was related to survival; a low self-renewal capacity was prognostically favorable (6, 13). It was also reported that the in vitro drug sensitivities to ara-C and doxorubicin and the self-renewal capacity are heritable properties within the blast progenitor cell population and change only slowly (12). We observed on patient (No. 14) who had identical sensitivity to both ara-C and daunorubicin at diagnosis and after 6 courses of TRAP. However, some patients may change their sensitivity during the course of the disease (12, 16).

Although it is possible to demonstrate a correlation between in vitro sensitivity to chemotherapeutic drugs and the response to therapy, it is too early to apply the tests for selection of the most effective therapy on an individual basis. As pointed out, the drug sensitivity is only one of several factors that determine the clinical response to therapy. At present, we regard it as more important to develop the in vitro tests for studies of the biological properties of the leukemic cells that determine their drug sensitivity. As a long-term goal, it should be possible to use the in vitro tests for development of new chemotherapeutic regimens and, perhaps, to design individualized chemotherapy.

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