Prediction of Chemotherapy Response in Human Leukemia Using an In Vitro Chemotherapy Sensitivity Test on the Leukemic Colony-Forming Cells

By C. H. Park, M. Amare, M. A. Savin, J. W. Goodwin, M. M. Newcomb, and B. Hoogstraten

An in vitro test system to quantitatively assess the chemotherapy sensitivity of human acute leukemic colony-forming cells (L-CFU) in relation to normal granulocytic precursor cells (CFU-C) has been developed. After simultaneous exposure of leukemic and normal bone marrow cells to individual drugs in vitro, cells were grown using an improved agar culture method with daily feeding. A sensitivity index (SI) was determined as the ratio of survival fraction of CFU-C to that of L-CFU. L-CFU being more (or less) sensitive than CFU-C if the SI were higher (or lower) than unity. Thirty SI were determined for 6 single drugs actually given in various combinations to a total of 9 patients (8 with acute nonlymphocytic leukemia and 1 with chronic myelomonocytic leukemia). A highly significant correlation was observed between high (or low) SI and achievement of (or failure to achieve) complete remission, with only 6 false correlations (p = 0.0013). Also, the mean of these SI (MSI) for the multiple single drugs given to each patient as components of a combination chemotherapy was used to indicate an overall sensitivity for each trial of the chemotherapy. Among the 10 chemotherapy trials (1 trial each for 8 patients and 2 trials for 1 patient), 4 trials resulting in complete remission had MSI higher than 1.0, and 6 trials not resulting in complete remission had MSI lower than 1.0 (p = 0.0048). This assay system appears useful in predicting the response of patients to chemotherapy and in the selection of the most effective drugs for use in individual patients.

A NUMBER of clinical1 and laboratory1-4 parameters are helpful in estimating the prognosis in individual patients with acute leukemia. However, none of these parameters can be directly translated into predictors of the sensitivity of leukemic cells to individual chemotherapeutic agents. It would be highly desirable to have a reliable test system comparable to that used to assay sensitivities of bacteria to antibiotics. Such a system could be used to test the response of an individual patient's leukemic cells to various drugs prior to institution of chemotherapy.

Previous studies have demonstrated that there is a specific subpopulation of tumor cells that is primarily responsible for the perpetual growth and survival of tumors: these are known as tumor stem cells and can be assayed by their ability to form colonies in vivo5-7 or in vitro.6-8 Using an in vitro colony-forming assay for tumor stem cells, it has been further shown that in vitro chemotherapy sensitivity tests can predict the response to chemotherapy of tumors in mice,9 rats,10 and man.11

Agar cultures have been used for more than a decade to assay normal granulocytic precursor cells (CFU-C).12,13 However, standard agar culture techniques do not satisfactorily support the growth of colonies from human leukemic marrows.3-4,14-18 We have recently developed a modified agar culture method that supports the growth of leukemic stem cell colonies (L-CFU) from the bone marrows of patients with acute nonlymphocytic leukemia (ANLL).19 Using this assay, we have examined the in vitro response to chemotherapeutic agents of L-CFU in comparison with that of CFU-C. Our preliminary results suggested that this assay might be of value as a predictive test for the clinical response to chemotherapeutic agents.

MATERIALS AND METHODS

Patients

Table 1 summarizes the clinical data and response to therapy of the initial nine leukemic patients in whom both clinical response and agar culture data could be evaluated. No patient had received prior treatment at the time of bone marrow aspiration. Eight patients had ANLL, and one patient (case 6) was in an accelerated phase of chronic myelomonocytic leukemia20 with a typical cell culture pattern of ANLL (vide infra) and was therefore included in this study. Normal bone marrow was obtained from hematologically normal patients with solid tumors undergoing bone marrow examination as part of their pretreatment staging workup. Informed consent was obtained from all patients in accordance with guidelines established by the University of Kansas Human Subject Committee prior to marrow aspiration. Eight patients received an intensive combination chemotherapy regimen consisting of vincristine (V), cytosine arabinoside (C), and prednisone (P) with or without adriamycin (A) or rubidazone, benzoylhydrazone daunorubicin (R). The administration schedule and drug dosages for VCP (also called OAP) and AVCP (also called AdOAP) are published elsewhere.22 The RVCP regimen (also called ROAP) is the same as AVCP except that A is replaced by 200 mg/sq m of R. One patient received 6-thioguanine (T) and C.23 All the drugs given to the patients were obtained from commercial sources, except R, which was obtained from the National Cancer Institute, Bethesda, Md.

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Materials and Methods for more details.

Colonies were counted in the control culture. In the remaining cultures, colony growth was observed in the control culture. The remaining datum point presented in Table 1 represents only 5.8% of the total colonies counted in the matched feeding culture. Thus, even if some of these colonies were CFU-C and also grew in feeding culture, they were shown to be as effective in vitro as fresh solutions. Refrigerated solutions of the drugs were used instead, since they were shown to be as effective in vitro as fresh solutions.

Cell Culture Assay

A detailed description of the cell culture method used in this study has been reported previously. Briefly, the culture system consists of 2 layers of 0.3% agar in a 35-mm plastic Petri dish perforated at the bottom by 6 small holes. Cells obtained by bone marrow aspiration are incorporated into the top agar layer. Both layers contain Alpha medium (GIBCO, Grand Island, N.Y.) containing 15% fetal calf serum (Flow Laboratories, Rockville, Md.) and 15% leukocyte-conditioned medium. The latter is prepared by incubation of normal human peripheral leukocytes with phytohemagglutinin (Wellcome Research Labs., Beckenham, England). Cultures were incubated for 2–3 wk at 37°C in an atmosphere continuously flushed with 7% CO2. Throughout the incubation period, each culture dish was removed from the incubator once daily and fed from the top with 0.5 ml of Alpha medium containing 15% each of fetal calf serum and leukocyte-conditioned medium. The fed medium diffuses through the agar layers and drains out of the dish through the holes on the bottom. Colonies of 50 or more cells were counted using an inverted microscope. Each datum point presented in this report represents the mean of quadruplicate cultures.

We have previously shown that this feeding culture technique does not significantly increase the growth of CFU-C from normal bone marrow. In contrast, when bone marrow from patients with ANLL is cultured by this technique, there is a marked increase in the number of colonies indicating growth of a functionally unique population of cells in 70% of the patients. This is the typical cell culture pattern of ANLL, and these colonies have been shown to be composed of CFU-C. It was reasoned that this dose of drug would determine the drug concentration permitting most effective in detecting differences in effect on survival of CFU-C and L-CFU, when L-CFU is more sensitive than CFU-C. If lower concentrations were used, the number of colonies indicating growth of a functional subpopulation of CFU-C, they represent a negligibly small fraction of the total number and would not materially alter the results of this study.

Because of the difficulty in having both fresh leukemic and fresh normal marrow available simultaneously for study, fresh marrows were stored frozen in liquid nitrogen until use as previously described.

In Vitro Exposure of Cells to Chemotherapeutic Drugs

Four drugs (V, C, A, and T), were obtained in pure powder form without preservatives from the respective manufacturers (Lilly, Indianapolis, Ind.; Upjohn, Kalamazoo, Mich.; Adria, Wilmington, Del.; and Burroughs and Wellcome Research, Triangle Park, N.C.). Clinical preparations were used for R and P (prednisolone sodium succinate, a hydroxylated compound that is also active in vitro) obtained from the National Cancer Institute, Bethesda, Md. Drug solutions were either prepared fresh immediately prior to study or frozen stocks were used. Solutions of C and T were not frozen, because freezing occasionally resulted in precipitation in the solution. Refrigerated solutions of the drugs were used instead, since they were shown to be as effective in vitro as fresh solutions. Cells were incubated at 10 times the final cell level for 1 hr at 37°C in Alpha medium containing 10% dialyzed fetal calf serum (GIBCO, Grand Island, N.Y.) with or without the addition of the selected concentration (vide infra) of test drug. Immediately following incubation, cells were transferred to an ice bath and washed twice prior to plating.

Statistical Analysis

A programmable calculator (Hewlett-Packard 65) was used for Fisher’s exact tests. The library program, 01694A, was used.

RESULTS

Survival of CFU-C After In Vitro Exposure to Chemotherapeutic Drugs

Figure 1 shows the results of a number of dose survival experiments performed on CFU-C with the six drugs used in treatment of our leukemic patients. The purpose of these experiments was to empirically determine the drug concentrations that represented the threshold of survival of normal granulocytic precursor cells, arbitrarily defined as the drug concentration permitting average survival of 60%–90% of N-CFU. It was reasoned that this dose of drug would be most effective in detecting differences in effect on survival of CFU-C and L-CFU, when L-CFU is more sensitive than CFU-C. If lower concentrations were used, the number of colonies indicating growth of a functional subpopulation of CFU-C, they represent a negligibly small fraction of the total number and would not materially alter the results of this study.

Because of the difficulty in having both fresh leukemic and fresh normal marrow available simultaneously for study, fresh marrows were stored frozen in liquid nitrogen until use as previously described.

Table 1. Clinical Data of Nine Leukemic Patients

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age/sex</th>
<th>Diagnosis*</th>
<th>Treatment†</th>
<th>CR‡</th>
<th>Other Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>39/F</td>
<td>AML</td>
<td>AVCp</td>
<td>Yes</td>
<td>CR achieved in 4 wk and lasted 15 mo, survival 20 mo</td>
</tr>
<tr>
<td>2</td>
<td>69/M</td>
<td>AMML</td>
<td>AVCp</td>
<td>Yes</td>
<td>CR achieved in 6 wk and lasted 5 mo, survival 9 mo</td>
</tr>
<tr>
<td>3</td>
<td>51/F</td>
<td>AMML</td>
<td>RVcp</td>
<td>Yes</td>
<td>CR achieved in 4 wk and lasted 7 + mo</td>
</tr>
<tr>
<td>4</td>
<td>56/F</td>
<td>AMML</td>
<td>RVcp</td>
<td>Yes</td>
<td>CR achieved in 9 wk and lasted 3 + mo</td>
</tr>
<tr>
<td>5</td>
<td>56/M</td>
<td>AMML</td>
<td>RVcp</td>
<td>No</td>
<td>Transient reduction of peripheral blood blasts noted</td>
</tr>
<tr>
<td>6</td>
<td>69/M</td>
<td>CMML</td>
<td>CT</td>
<td>No</td>
<td>In accelerated state at the time of the study</td>
</tr>
<tr>
<td>7</td>
<td>73/M</td>
<td>AMML</td>
<td>Vcp</td>
<td>No</td>
<td>Partial remission achieved</td>
</tr>
<tr>
<td>8</td>
<td>59/M</td>
<td>AML</td>
<td>AVCP/RVcp</td>
<td>No</td>
<td>Failed on both AVCP and later RVCP</td>
</tr>
<tr>
<td>9</td>
<td>69/F</td>
<td>AML</td>
<td>AVCP</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>

*AML, acute myelocytic leukemia; AMML, acute myelomonocytic leukemia; CMML, chronic myelomonocytic leukemia.
†A, adriamycin; V, vincristine; C, cytosine arabinoside; P, prednisone (or prednisolone for in vitro studies); R, rubidazone; T, 6-thioguanine. See Materials and Methods for more details.
‡CR, complete remission, i.e., disappearance of all evidence of disease with normal marrow (blast <5%) and normal peripheral smear.

The purpose of these experiments was to empirically determine the drug concentrations that represented the threshold of survival of normal granulocytic precursor cells, arbitrarily defined as the drug concentration permitting average survival of 60%–90% of N-CFU. It was reasoned that this dose of drug would be most effective in detecting differences in effect on survival of CFU-C and L-CFU, when L-CFU is more sensitive than CFU-C. If lower concentrations were used, the number of colonies indicating growth of a functional subpopulation of CFU-C, they represent a negligibly small fraction of the total number and would not materially alter the results of this study.

Because of the difficulty in having both fresh leukemic and fresh normal marrow available simultaneously for study, fresh marrows were stored frozen in liquid nitrogen until use as previously described.
Fig. 1. Dose survival of normal granulocytic progenitor cells (CFU-C) with 6 chemotherapeutic drugs. For the abbreviations of drugs (C, V, P, A, R, T), see the legend for Table 1 and the text. Drug concentrations are expressed as exponential with base of 10 (e.g., $2 \times 10^{-6}$). The arrows indicate the concentrations selected for simultaneous studies on CFU-C and leukemic colony-forming cells (L-CFU). In order to construct reliable dose survival curves, 10–20 experiments were done for each drug. The number of nucleated marrow cells plated per dish was $5 \times 10^6$ cells, and the typical number of colonies per dish in the control cultures without drug exposure were, in C for example, 54 (median) and 23–111 (range).

In Vitro Survival of L-CFU in Relation to That of CFU-C in Nine Leukemic Patients

Using the appropriate drug concentrations selected as described above, survival fractions of L-CFU were determined on cultures of the nine leukemic patients using the drugs actually employed in the treatment. Simultaneous control survival fractions of CFU-C were determined in each experiment. A sensitivity index of L-CFU in relation to CFU-C for each drug was determined as follows:

$$\text{Sensitivity index (SI)} = \frac{\text{Survival fraction of CFU-C}}{\text{Survival fraction of L-CFU}}$$

In this system, an SI of 1.0 means that there is no difference in the drug sensitivity between CFU-C and L-CFU. If L-CFU is more sensitive to the drug than CFU-C, the SI is higher than 1.0, and if L-CFU is less sensitive than CFU-C, the SI is less than 1.0. Actual examples of how SI were derived are shown in Table 2. In most cases, we did not have enough bone marrow cells to repeat the same studies, but these 2 patients were exceptional and allowed us to do the second experiment with a limited number of drugs. The survival fractions of CFU-C and L-CFU can fluctuate from one experiment to another. However, this fluctuation tends to occur in the same direction and by a comparable magnitude for a given set of experiments. Therefore, the ratio of these 2 fractions (i.e., SI) remains relatively constant, and on no occasion SI crosses the point 1.0 that divides the sensitive and resistant ranges. It is noteworthy that in case 4, who

<table>
<thead>
<tr>
<th>Leukemia Case Number</th>
<th>Experiment Number</th>
<th>Drugs Used for In Vitro Exposure*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>0.75</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0.74</td>
</tr>
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<td>9</td>
<td>1</td>
<td>0.64</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0.93</td>
</tr>
</tbody>
</table>

*For the abbreviations of drugs (A, R, C), see the legend for Table 1.
†Figures in parenthesis are fraction survival of CFU-C (numerator) and that of L-CFU (denominator) to calculate the SI (the figure in front of parenthesis).
achieved complete remission with a combination chemotherapy including R, the SI for R was consistently high while that for A was low. Excluding the data from the second experiments in cases where there were repeat studies, we have obtained 30 SI for single drugs used in the treatment of 9 leukemic patients, as shown in Fig. 2. The maximal possible number of SI that could have been obtained were 34, but in 3 cases (cases 1, 2, and 7) we did not have enough bone marrow cells to do all the studies.

Correlation of In Vitro SI and Clinical Response to Chemotherapy

The in vitro–clinical correlations can be made in two different ways. First, the values of each SI deter-

<table>
<thead>
<tr>
<th>Sensitivity index</th>
<th>Complete Remission</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>High (&gt;1)</td>
<td>11</td>
</tr>
<tr>
<td>Low (&lt;1)</td>
<td>2</td>
</tr>
</tbody>
</table>

Fisher’s exact test: \( p = 0.0013 \).

DISCUSSION

The findings presented here indicate that the in vitro sensitivity of L-CFU to chemotherapeutic agents correlates well with the clinical response to chemotherapy. We have made the in vitro–clinical correlations in two different ways: one is from the standpoint of individual SI for single drugs and the other from the standpoint of each treatment trial. The correlations appear extremely good for both methods, but the full and precise implication of these correlations is not as yet completely clarified. Analyzing the correlation based on the individual SI, 6 of 30 SI failed to correlate with clinical response. There are several possible explanations for these failures. The method by which the cells are exposed to drugs in vitro may not be optimal for testing a particular agent as it is used in treating acute leukemia. For instance, half the false correlations occurred with C, which is more effective...
in vivo when given by continuous infusion than by bolus injection. In vitro exposure for 1 hr may be too brief to reflect the sensitivity of cells to the drug when they are exposed over a much longer period. This will be tested by prolonging exposure time in future studies. More importantly, all patients were treated with combinations of two or more drugs, while the in vitro sensitivity tests in this report were performed on the individual drugs singly. Clinical response may reflect the combined effect, such as synergism, of these multiple agents, and any one of these drugs alone may not be effective. In future studies, multiple drugs can be used for simultaneous exposure to simulate the clinical situations more closely. Our preliminary study indicates the technical feasibility of in vitro exposure of bone marrow cells to a combination of drugs; however, the results are too premature to permit a conclusion as to their value. Another possible explanation for the lack of correlation is that although complete remission is achieved with a combination chemotherapy, one or more of the component drugs might not have contributed to the complete remission. Then the evaluation of SI for single drugs becomes extremely important in the selection of single drugs to be used for combination chemotherapy. Also, it will be reasonable to obtain the mean of these SI (MSI) for all the single component drugs used in the combination chemotherapy and to use this MSI as an overall indicator of sensitivity to the combination chemotherapy. This was our second method of correlation, and the result of this correlation was also extremely good. However, a larger number of studies is needed before the full implication of this correlation can be defined. In order to have more appropriate MSI, a differential "weighting" may have to be applied to the SI of different single drugs depending on their relative contribution to the combination chemotherapy. This "weight" can be determined only after a large number of correlations have been studied by a multiple regression analysis similar to the one used to assess clinical parameters for their prognostic values.

The potential value of this test system, which we would like to reemphasize, is the possibility that the most effective drugs or combination of drugs can be selected for use in treatment of individual patients. Thus, the test system differs radically from other well established prognostic indicators, such as age, chromosomal abnormalities, or growth patterns in cell culture, none of which enable the selection of effective drugs. There have been a number of previous attempts at developing test systems for drug selection using either biochemical or cytokinetic parameters. However, these are based on examination of the entire population of leukemic cells rather than on leukemic stem cells, the key compartment responsible for perpetuation of the leukemic cell clones. It has been shown previously that the results of colony-forming assays differ from those of other assays in the determination of cell survival following exposure to cytotoxic drugs.

One of the most important aspects of our test system is the use of simultaneous control cultures for CFU-C and the expression of tumor cell sensitivity as an index in relation to that of CFU-C. Since toxicity to hematopoietic tissue is the major limiting factor in chemotherapy, we believe that it is important to compare the effects of chemotherapy on tumor cells to the effects on normal hematopoietic cells. There is some fluctuation in survival data for CFU-C (Fig. 1), although its intrinsic chemotherapy sensitivity should remain the same, presumably resulting from day-to-day variations in culture conditions. However, by using relative survival data in the form of a sensitivity index, which is the ratio between two culture data and does not depend on a single absolute determination, this problem appears to be reduced and we have been able to demonstrate definite clinical correlations. The data of Table 2 directly demonstrate that SI remains relatively constant in spite of fluctuation of CFU-C and L-CFU survivals. This is another reason why the simultaneous survival study of CFU-C is important. In the in vitro experiment, cells are exposed to a constant concentration of drugs for an hour, while the drug concentration declines rapidly in most in vivo situations. It is therefore difficult to find the appropriate drug concentration to be used in vitro simply from the in vivo pharmacokinetic data. In addition, there are a number of other humoral, cellular, and tissue factors that differ between in vivo and in vitro situations and that might modify the effects of chemotherapeutic drugs. Examples are nutritional factors, nucleosides, and metabolites of drugs. The drug concentration in serum is not necessarily the one to which the malignant cells are exposed in vivo; rather, drug concentrations depend on tissue and organ arrangements and variation of blood supply. Therefore, it is difficult to derive an appropriate in vitro drug concentration from the in vivo drug concentration.

It was apparent from the outset that we would not have enough bone marrow cells to do multiple-dose experiments for each of the multiple component drugs of combination chemotherapy given to patients. We decided to test one "appropriate" dose for each of these multiple drugs rather than multiple doses for one of these drugs. We reasoned that an "appropriate" and relatively low dose should be effective in detecting a chemotherapy-sensitive leukemic cell population. Our initial plan was to test later, if bone marrow cells are
available, at least one other dose of relatively high concentration that should be more effective in detecting resistant cell population. However, our results with only one dose level are already good for detecting the resistant populations, and it may not be necessary to test the additional dose as originally planned.

Recently, an Arizona group has developed a promising colony-forming assay for tumor stem cells of various human malignancies. Their data on the in vitro response to chemotherapy of multiple myeloma and ovarian cancer appear to correlate very well with the clinical response to chemotherapy. Their approach is similar to ours, but there are a number of important differences. First, our target disease is acute leukemia, but this disease has not been included in their study as of their recent update. There are also significant differences in culture systems, e.g., the daily feeding unique in our system washes out any residual drug in culture that might remain after washing or might be released from dying cells. Therefore, the survival data after 1 hr incubation with drugs may not be directly translatable between these two systems. The ways of expressing the chemotherapy sensitivity of malignant cells are also different; ours involves CFU-C as a control, while theirs is based on dose survival curves. Because of these differences, caution is required in making a comparison between these two systems. Although the principle is similar, technically what applies to one system may not necessarily be good for the other.

An important potential problem with the use of our assay as a clinical tool is the turnaround time of 2 wk. If further studies establish its use in the selection of effective agents for individual patients, the initial choice of drugs will be made empirically with appropriate modifications being made when the culture results become available. This situation is analogous to that of the treatment of life-threatening infections, in which antibiotic treatment must be initiated prior to the availability of culture and sensitivity data. Future studies will be directed at shortening the turnaround time to improve the feasibility of using such a culture system in a clinical setting. Quite often we did not have sufficient marrow cells to complete all of the in vitro chemotherapy study. There were problems of dry tapping and poor procurement of marrow due to packed marrow as well as the need for large number of cells due quite often to low plating efficiency. These problems may be overcome in the future by multiple aspirations or bone marrow biopsy if the culture study results are potentially beneficial to the particular patients under study.

Overall, we are convinced that this system is very promising in the individualization of chemotherapy in ANLL. However, we believe that the study on a large number of patients is needed and the refinement of technique is desirable before the full and precise value of this system can be established.

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Prediction of chemotherapy response in human leukemia using an in vitro chemotherapy sensitivity test on the leukemic colony-forming cells

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