Prediction of in Vivo Cytotoxicity of Chemotherapeutic Agents by Their in Vitro Effect on Leukocytes from Patients with Acute Leukemia

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SUMMARY

In order to develop a test system for predicting cytotoxic response to chemotherapeutic agents, leukocytes from 36 adult patients and 2 teenagers with acute leukemia were cultured in vitro for 24 hr and the effects of drugs on the incorporation of uridine-$^3$H in RNA and of thymidine-$^{14}$C in DNA were measured. Cortisone, vincristine, and cytosine arabinoside produced inhibition of uridine-$^3$H incorporation in sensitive leukocytes. The effects of cortisol and vincristine on the incorporation of uridine-$^3$H in RNA and of thymidine-$^{14}$C were, in general, similar. In two studies, cytosine arabinoside produced a more profound depression of incorporation of radioactive thymidine than of radioactive uridine.

From 45 in vitro and clinical studies of leukocyte response to glucocorticoids and vincristine, it was concluded that the in vitro test system can predict the in vivo cytotoxicity of these compounds in acute leukemia in adults. From 8 in vitro and clinical studies of cytosine arabinoside, it appeared likely that the in vitro test system may have predictive value for the cytotoxic effect of this drug in vivo. Whereas the test system may have use in predicting in vivo cytotoxic effects, it cannot be used for predicting the likelihood of inducing a hematologic remission.

INTRODUCTION

In the past decade there have been a number of attempts to develop test systems which would predict the clinical effectiveness of various chemotherapeutic agents against specific human neoplasms. Such test systems have measured either an enzymatic function of the malignant cells or an alteration in metabolism induced by the chemotherapeutic agent in vitro (1, 2, 10, 11, 15, 18). The failure of widespread acceptance of these tests suggests either that they were not sufficiently reliable in predicting clinical response or that they were too cumbersome for routine use. In some instances the test systems had predictive value but the drug tested had only limited clinical usefulness (7).

We have recently described a test system for the prediction of in vivo cytotoxicity of chemotherapeutic agents by their effect on malignant human leukocytes in vitro (6). This test system was based on drug-induced inhibition of the incorporation of uridine-$^3$H into the cellular RNA of cultured malignant leukocytes. Three agents were selected for investigation: cortisol, vincristine, and cytosine arabinoside. It was clear from these initial studies that, although the in vitro test system might be used to predict cytotoxic effects of certain drugs against malignant cells, it could not predict the induction of a hematologic remission. The reasons for this are multiple and complex. Induction of a hematologic remission, defined as a return of the blood and bone marrow to morphologically normal status and an eradication of organ involvement by leukemic cells, depends on several factors, including (a) a differential cytotoxic effect of the drug on normal and malignant hematopoietic cells; (b) differential rates of regeneration of normal and abnormal cells after drug injury; (c) any existing host defense mechanisms against leukemic cells; and (d) the fortuitous occurrence of lethal complications during a period of potential normal marrow regeneration. It is obvious that no simple test can predict all of these factors. Is there any reason, therefore, to develop a test system which can predict whether a given drug will injure leukemic cells in vivo? We have found such a system to be useful in two clinical situations. When a patient with leukemia has experienced a relapse of his disease after treatment with multiple chemotherapeutic agents, it is usually difficult to select a drug for treatment. In such a situation there is often a feeling of urgency that time not be wasted on a trial with a compound that will not be effective; under such circumstances it is helpful to be able to eliminate those drugs that will have little or no antileukemic effect. The second situation in which a predictive test system may be useful is in treatment of patients with multiple chemotherapeutic agents simultaneously. Since the toxicity of a number of drugs may be additive, it is useful to eliminate those compounds which will have no antitumor effect. A third potential use of such an in vitro test system is the screening of new agents which may have antileukemic activity.

Using an in vitro test system, we have now completed approximately twice the number of clinical trials described in the...
original report (6) and the data are now available for a more rigorous evaluation of this test system. The present report describes our further experience.

**MATERIALS AND METHODS**

The study group consisted of 13 patients with acute lymphoblastic leukemia (ALL) and 25 patients with acute myelomonocytic leukemia (AML). Except for 2 patients, 16 and 17 years of age, all were adults. The morphologic criteria cited by Boggs et al. (4) were used in classifying acute leukemia. No attempt was made to discriminate acute myelocytic from acute monocytic leukemia, and these entities were considered together as acute myelomonocytic leukemia. All cases of leukemia which defied precise morphologic classification or which were classified as "undifferentiated acute leukemia" were included in the myelomonocytic group. Routine blood counts and bone marrow studies were performed by standard methods (5).

**In Vitro Test System**

The in vitro test system measured the incorporation of uridine-\(^3\)H into acid-precipitable RNA. The technic used was essentially the same as that described previously (6). Leukocytes were isolated from heparinized venous blood or bone marrow and were washed twice by centrifugation at 150 X g for 10 min at 22°C with Hanks’ balanced salt solution containing 0.10 volume fetal calf serum and 2 units of heparin/ml. The cells were suspended at a concentration of 1 X 10^6 cells/ml in Eagle’s minimal essential medium containing 0.20 volume fetal calf serum, 100 units of penicillin, and 50 μg/ml of streptomycin. Cell cultures in which at least 90% of the leukocytes were of a single morphologic type were prepared in triplicate. Saline solution, cortisol (hydrocortisone sodium succinate), vincristine sulfate, or cytosine arabinoside was added to the cell suspension. The final concentrations of the drugs employed were as follows: cortisol, 5 X 10^-6 , 1.5 X 10^-5 , or 5 X 10^-5 M; vincristine, 7 X 10^-6 , 2.2 X 10^-5 , or 7 X 10^-5 M; and cytosine arabinoside, 4 X 10^-7 , 4 X 10^-6 , or 4 X 10^-5 M. After incubation of the cell suspension for 4 to 5 hr in the presence of drug or saline solution, uridine-\(^3\)H (1 μc/ml, 3 c/mmole), or thymidine-2,\(^3\)H (0.05 μc/ml, 54 μc/mmole) was added, and the incubation was continued for 16 to 18 hr. The cells were washed twice by centrifugation with phosphate-buffered saline solution. Nucleic acids were precipitated with 5% trichloracetic acid and then washed twice with trichloracetic acid and once with methanol. The precipitate was dissolved in NCS (Nuclear Chicago, Des Plaines, Illinois), taken up in a phosphor, and the incorporated radioactivity was counted in a liquid scintillation spectrometer.

The effect of each drug on the incorporation of radioactive uridine or thymidine was expressed as the percentage of incorporation in control cultures to which the drug had not been added. The amount of uridine-\(^3\)H incorporated in different cell populations and the variation in replicate cultures were as previously described (6). Repeated studies of a single leukocyte population at a given time in a patient’s illness showed little variation (± 8% from the mean).

**Clinical Trials**

After the cells had been obtained for culture, the patients with acute leukemia were treated with 1 of 3 agents. These drugs were administered according to the following dosage schedule: prednisone, 60 to 80 mg/day, by mouth, for 14 days (4 trials) or 28 days (23 trials); vincristine, 0.05 to 0.075 mg/kg of body weight i.v., once weekly for 2 weeks (4 trials) or 4 weeks (15 trials); cytosine arabinoside, 1 to 3 mg/kg of body weight i.v., thrice weekly for 4 weeks (2 trials) or 8 weeks (6 trials) in the early studies, and 500 to 1000 mg/sq m once weekly in the later studies.

The decision to use one or another of the 3 drugs in treatment was made without knowledge of the results of the in vitro testing. The effects of the drugs in vitro and in vivo were compared at the end of the treatment period.

Since, in general, the period of trial with a single agent was not sufficiently long to allow application of the rigid criteria used to define remissions in leukemia (3), the following criteria were used to characterize a drug as cytotoxic in vivo: a reduction of 50% or more in the number of blast forms or immature cells in the peripheral blood; a decrease in the percentage of immature cells in the marrow, or the induction of hypoplasia of the bone marrow; and a decrease in lymphadenopathy and splenomegaly (when present) during the treatment period. It is apparent that clinical trials of 14 days with prednisone and vincristine can select only those patients in whom these drugs are rapidly cytotoxic. Patients who respond more slowly to these drugs would not be identified. This arbitrary time limit was imposed by the frequency with which therapy was altered. In those situations in which there was any doubt as to the cytotoxic effect of a drug in vivo, the data were eliminated from the study.

A drug was considered to be noncytotoxic in vivo if the patient had experienced a relapse of his disease while receiving adequate amounts of that agent. In 10 of 27 studies with cortisol and in one of 19 studies with vincristine, patients whose diseases were in relapse were receiving the drug at the time of the study.

**RESULTS**

**Correlation between In Vitro and In Vivo Effects of Glucocorticoids**

The results of 27 in vitro tests with cortisol on the incorporation of uridine-\(^3\)H in RNA of lymphoblasts and myeloblasts are shown in Chart 1. With 4 exceptions, the inhibition of incorporation of the radioactive uridine was a function of cortisol concentration, with higher concentrations producing greater inhibition. Although there was a wide range of responses in different blast cell populations, lymphoblasts were in general more sensitive to cortisol than were myeloblasts. In some lymphoblast populations, the incorporation of uridine-\(^3\)H was considerably depressed by cortisol (as low as 20% of the control value at 5 X 10^-6 M and as low as 10% of the control value at 5 X 10^-5 M). Incorporation of uridine-\(^3\)H by cells from patients with AML was never less than 70% of the control value at 5 X 10^-6 M cortisol, and only rarely less than...
an in vitro concentration of $5 \times 10^{-5}$ M cortisol. Incorporation of uridine-$^3$H, which was less than 50% of that in control cultures at this concentration, was correlated with a good clinical response to prednisone.

Four populations of lymphoblasts were stimulated in vitro by one of the concentrations of cortisol tested. In none of the patients from whom these cells were obtained was there clear evidence of acceleration of disease by the prednisone therapy.

The correlation between the results of in vitro testing with cortisol at two concentrations, $5 \times 10^{-5}$ and $5 \times 10^{-6}$ M, and the clinical cytotoxicity of prednisone for the 36 adults with acute leukemia are summarized in Chart 2. When all these cases are considered together, the in vitro test system appears to be useful for predicting clinical response. Expressed as percent of the control uridine-$^3$H incorporation, at a cortisol concentration of $5 \times 10^{-6}$ M, the mean ± 1 S.D. for the tests of leukocytes from patients nonresponsive to prednisone in vivo was $102 ± 36$; for leukocytes from patients in whom prednisone was considered to be cytotoxic in vivo, it was $54 ± 22$. The difference between these two groups was statistically significant ($P < 0.01$). At $5 \times 10^{-5}$ M cortisol, the results for "nonresponsive" and "responsive" leukocytes were $97 ± 42$ and $29 ± 11$ respectively. Again, the difference between the two groups was statistically significant ($P < 0.01$).

When the data were calculated separately for the 13 trials in patients with ALL, the results were as follows: at $5 \times 10^{-5}$ M cortisol, "responders" $29 ± 12$, "nonresponders" $110 ± 64$ ($P = 0.02$); at $5 \times 10^{-6}$ M cortisol, "responders" $54 ± 23$,

"nonresponders" $109 ± 47$ ($P < 0.05$).

As anticipated from the literature and previous clinical experience, prednisone administered without other drugs induced complete hematologic remissions only in acute leukemia morphologically characterized as lymphoblastic.

Prednisone was administered to 10 patients with AML in 14 clinical trials. In no case was prednisone cytotoxic in vivo by the criteria used in this study. In only one case was there a striking increase (threecfold) in the number of blasts in the peripheral blood and in organomegaly during prednisone therapy; this patient's white blood cells were stimulated by cortisol in vitro (uridine-$^3$H incorporation of 156% of control at $0.5 \times 10^{-5}$ M cortisol). Three other patients whose leukocytes were stimulated by cortisol in vitro (121% to 135% of control) did not show marked progression of disease during prednisone therapy. The correlation between the results of in vitro testing with cortisol and the clinical response to prednisone therapy by patients with AML is shown in Chart 1.

Prednisone was administered to 10 patients with ALL in 13 clinical trials. The drug was considered to be clearly cytotoxic in 5 trials and without effect during the period of observation in 8 trials. The correlation between the results of in vitro testing and the clinical response is shown in Chart 1. The in vitro test system discriminated those patients who responded to prednisone in vivo from those who did not respond. The separation of "responders" from "nonresponders" was best at 60% of the control value at $5 \times 10^{-5}$ M cortisol. Incorporation of uridine-$^3$H in RNA by some blast cell populations appeared to be stimulated by cortisol.

The results of in vitro tests with cortisol on the incorporation of uridine-$^3$H in RNA and thymidine-$^{14}$C in DNA of myeloblasts and lymphoblasts in general gave similar results in 8 studies. Since measurement of thymidine-$^{14}$C incorporation appears to offer no advantage over that of uridine-$^3$H incorporation, these data will not be reported in detail.

Chart 1. Correlation between the effect of cortisol on uridine-$^3$H incorporation by lymphoblasts and myeloblasts in vitro and the clinical response to prednisone. ALL, acute lymphoblastic leukemia; AML, acute myelomonocytic leukemia; •—•, prednisone noncytotoxic in vivo; △—△, prednisone cytotoxic in vivo; X—X, patients whose disease relapsed while receiving prednisone.

Chart 2. Correlation between the effect of cortisol in vitro on incorporation of uridine-$^3$H in vitro by lymphoblasts and myeloblasts and the clinical response to prednisone. •, noncytotoxic in vivo; X, noncytotoxic in vivo; disease relapsed while the patients were receiving prednisone; △, cytotoxic in vivo. The means are shown.
Correlation between *in Vivo* and *in Vitro* Effects of Vincristine

Individual populations of lymphoblasts and myeloblasts varied greatly in their sensitivity to vincristine. Considered as groups, these two types of leukocytes did not differ significantly in their *in vitro* responses to vincristine. The effect of vincristine on uridine-3H incorporation was, in general, a function of the drug concentration (6). Unlike cortisol, vincristine almost always inhibited the incorporation of tritiated uridine and, with one exception, never increased it above control levels.

The effects of vincristine on the incorporation of uridine-3H in RNA and of thymidine-14C in DNA were compared in 4 populations of myeloblasts and lymphoblasts. In general, there was good agreement between the dose-dependent inhibition of uridine and thymidine incorporation. Consequently, the remaining studies utilized uridine-3H alone.

Vincristine was administered to 18 patients with acute leukemia (AML 13 and ALL 5) in 19 clinical trials. In 13 trials in patients with AML, the drug was considered to be cytotoxic in 7 and without significant cytotoxicity in 6. In 6 trials in patients with ALL, the drug was considered to be clearly cytotoxic in 2, partially cytotoxic in 2, and without effect in 2. The correlation between *in vitro* and *in vivo* effects of vincristine is shown in Chart 3, where the data for cytotoxic and partially cytotoxic clinical responses have been grouped together.

At a concentration of 7 × 10^{-6} M vincristine, there was considerable overlap between the clinically responsive and nonresponsive leukocyte populations. At 7 × 10^{-5} M vincristine, there was reasonably good separation between these groups. Expressed as the percent of the control uridine-3H incorporation, the mean ± 1 S.D. for the responsive leukocyte populations was 9.6 ± 8, and for the nonresponsive populations, 36.0 ± 22. The difference between these groups was statistically significant (P < 0.01).

In the course of these studies, we made certain clinical observations on the use of vincristine in treating acute leukemia in adults. With the dosage and time schedule employed, vincristine administered without other drugs induced a complete hematologic remission in 2 of the 5 patients with ALL. Both patients had disease in relapse after the administration of other chemotherapeutic agents. Although vincristine was cytotoxic in 7 patients with AML, it never induced complete hematologic remission in patients with this disease. Reduction in the numbers of myeloblasts in the peripheral blood and in pathologic organomegaly were common with vincristine treatment, but significant reductions in abnormal cells in the bone marrow were rare.

**Correlation between *in Vitro* and *in Vivo* Effects of Cytosine Arabinoside**

There was wide variation in the sensitivity of different populations of leukocytes to cytosine arabinoside. In some populations, incorporation of uridine-3H was almost abolished at a concentration of 4 × 10^{-7} M cytosine arabinoside; in others, it was unaffected. In general, the suppression of uridine-3H incorporation was a function of the drug concentration in the range 4 × 10^{-7} M to 4 × 10^{-5} M (6). Enhanced incorporation of uridine-3H in the presence of the drug was not observed.

Only 8 clinical trials with cytosine arabinoside were completed. Two other trials were terminated by the deaths of the patients before sufficient time had elapsed for evaluation. In the 8 completed trials, the drug was considered to be highly cytotoxic in one patient, partially cytotoxic in 4, and without effect in 3. In the patient in whom the drug was highly cytotoxic, it rapidly produced a complete hematologic remission. This patient's disease relapsed several months later while on continuous maintenance therapy with this drug. In the 4 patients in whom the drug was considered to be partially cytotoxic, it produced partial but quite prolonged hematologic remissions which permitted the patients a good level of function. The *in vitro* test system appeared to separate the clinically responsive from the nonresponsive populations (Chart 4).

The effects of cytosine arabinoside on the incorporation of uridine-3H into RNA and thymidine-14C into DNA were compared in 2 populations of myeloblasts (Table 1). The incorporation of thymidine was depressed to a much greater extent than that of uridine.

**DISCUSSION**

An *in vitro* test system for predicting *in vivo* cytotoxic effects of chemotherapeutic agents should be technically simple and rapid; it should have a high degree of replicability and, above all, a high degree of accuracy in predicting clinical response. While occasional false positive results from such a test may be acceptable, even a few false negative results would be unacceptable, i.e., the test system should never exclude clinically useful drugs.
In an attempt to develop such a test system for predicting the response of malignant human leukocytes to chemotherapeutic agents, we surveyed the literature (1, 2, 7, 9—11, 14—19) and conducted a number of preliminary investigations of the effects of clinically useful drugs on several parameters of the metabolism of leukemic leukocytes in vitro: synthesis of RNA, DNA, and protein; respiration and glycolysis; and the ability to exclude supravital stains. From this survey, several conclusions seemed justified. The potential exists for developing an in vitro test system provided the site of cytotoxic action is a defined enzyme and the measurement of the effect of drug on that enzyme is technically feasible (7). It is also possible to develop an in vitro test system for drugs whose precise mode of action is not clear (e.g., cortisol and vincristine) provided certain conditions are met: (a) The drug must be active in the form in which it is added to the in vitro test system or must be converted to the pharmacologically active form by the constituents of the system. (b) A representative sample of the malignant tissue must be obtained for testing. (c) The metabolism of the leukocyte in vitro and in vivo must be sufficiently similar so that the drug effects under the two conditions are comparable. (d) There must be sufficient time for the drug to enter the cell, to reach the site of its activity and, if necessary, to be converted to the metabolically active form. (e) The drug must have measurable secondary effects on the metabolism of the isolated leukocyte which are related in some quantitative way to the primary—and as yet unknown—effect of the drug.

The requirement for the pharmacologically active form of the drug in vitro clearly excludes the testing of compounds such as cyclophosphamide, which must be converted to a metabolically active form in vivo (8). We have found that very high concentrations of this drug are inert when tested in vitro against malignant lymphoid cells which one might expect to be sensitive to alkylating agents. It is possible that a similar situation exists for certain purine analogs which must be converted to the riboside form before they are effective.

Obtaining a representative sample of malignant cells is not a major problem in acute leukemia, and our own studies have shown a good correlation between the response of peripheral blood and marrow cells provided the two populations are morphologically similar (6). In developing tests of in vitro cytotoxicity, consideration must be given to the duration of in vitro cultivation of malignant cells and to the duration of exposure to the chemotherapeutic agent. Paul (13) has called attention to the wide fluctuation in cell metabolism during the first few hours of their cultivation in vitro. Therefore, any test system utilizing very short periods of incubation of leukocytes may be doomed to failure because the in vitro metabolic activities of the cells are remote from their in vivo activities. Similarly, if insufficient time is allowed for complex chemotherapeutic agents to be taken up and (in some cases) metabolized by the cells, the outcome of the in vitro test may be prejudiced.

A quantitative relationship between secondary inhibition of RNA synthesis and the primary drug effect probably exists for certain chemotherapeutic agents. That such a relationship does, in fact, exist is suggested by the present study and by a preliminary report of the effects of L-asparaginase on human leukemic leukocytes in vivo and in vitro (12). While our own data must still be considered preliminary, they suggest that the following guide lines may be useful in predicting clinical cytotoxic effect of corticosteroids and of vincristine in acute leukemia in adults. Suppression of incorporation of uridine-3H in vitro of > 50% (compared with control cultures) by cortisol at concentrations of 5 × 10^{-5} M or 5 × 10^{-6} M and suppression of > 90% incorporation by vincristine at 7 × 10^{-5} M are correlates of in vivo cytotoxicity. Our data for cytosine arabinoside are still too scanty to determine similar guide lines for this drug, but our initial studies suggest that, with the accumulation of sufficient data, the test system may be clinically useful for this agent as well. With this compound, the inhibition of incorporation of radioactive thymidine may be a better indicator of cytotoxicity than that of uridine.

We have previously stressed the conclusion that, whereas an in vitro test system can predict the in vivo cytotoxicity of certain drugs for malignant cells, it cannot be used to predict the likelihood of induction of remissions with these drugs.
This conclusion is based on the observation that hematologic remissions depend on many factors in addition to injury of the neoplastic cells. If, however, an in vitro test system can eliminate those drugs which will not be cytotoxic, then it can be used in selecting among several potentially useful agents.

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