Cytokinetic and Molecular Pharmacology Studies of Arabinosylcytosine in Metastatic Melanoma

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SUMMARY

Eighteen patients with metastatic melanoma involving the skin were treated with 5-day courses of arabinosylcytosine (ara-C) every 3 weeks. Of the 13 adequately treated patients, there was 1 excellent objective response which lasted for 8 months and 2 transient partial responses. The ara-C kinase [activating enzyme (K)] and ara-C deaminase [inactivating enzyme (D)] were measured prior to treatment. The K values were higher in the responding patients, and the D values were higher in the nonresponding patients. The K/D ratio was significantly higher in the former patients, compared with that in the latter.

Similarly, the proportion of tumor cells in DNA synthesis as determined from labeling indices was highest in the response group. Also, the labeling index percentage was depressed to a greater degree during therapy and remained depressed for a longer period of time after therapy in the patients who responded to treatment, when compared with those who failed to respond. For the nonresponders, recovery of DNA synthesis was complete or nearly complete by Day 21 posttherapy.

It is concluded from this study that ara-C is inactive in the majority of patients with melanoma, that it has greater activity in females with melanoma compared to males, and that enzyme and cytokinetic studies can be used to predict clinical response to ara-C.

INTRODUCTION

ara-C is a pyrimidine nucleoside analog with substantial antitumor activity in experimental systems (4). In clinical trials, it has proved to be one of the most effective agents for the treatment of acute myelogenous leukemia. It is concluded from this study that ara-C is inactive in the majority of patients with melanoma, that it has greater activity in females with melanoma compared to males, and that enzyme and cytokinetic studies can be used to predict clinical response to ara-C.

ara-C is a pyrimidine nucleoside analog with substantial antitumor activity in experimental systems (4). In clinical trials, it has proved to be one of the most effective agents for the treatment of acute myelogenous leukemia. (1, 3). There is some evidence for activity in acute lymphocytic leukemia and in lymphoma (15).

In experimental in vivo tumor systems, it has been demonstrated that the schedule of ara-C administration may markedly influence the tumor response to ara-C (12). Thus, in both L1210 and spontaneous AKR mouse leukemia systems, the continuous administration of ara-C in courses that are at least twice the generation time of the neoplastic cells (with intervals between courses sufficient to allow for host recovery) results in the best therapeutic index (12). These observations have been extended to adults with acute myelogenous leukemia. With these patients, 5-day courses of continuous i.v. administration of ara-C (given every 2 to 3 weeks) provide complete remission rates of 30 to 40% as compared with daily treatment programs which result in complete remission rates of 15 to 20% (2, 3, 17). Cytokinetic studies in human melanoma indicate a modal generation time (Tc) of 3 days, but show a growth fraction of only 20%. We elected, therefore, to administer 5-day courses of ara-C by continuous i.v. infusion at 3-week intervals to patients with melanoma.

In a spectrum of experimental tumors, it has been demonstrated that the conversion of ara-C to ara-C nucleotide correlates directly with the in vivo antitumor activity of ara-C (10). The ara-C is activated by deoxycytidine and deoxycytidylylate kinase and is inactivated by deoxycytidine deaminase, which converts ara-C to the inactive metabolic product, arabinosyluracil. The activated product, ara-C triphospate, is a competitive inhibitor of DNA polymerase and thus inhibits DNA synthesis (6, 8). It was postulated that the ratio of these 2 enzyme activities (K/D) should determine the rate of activation of ara-C and, thus, the selective tissue toxicity. Additionally, since ara-C is a cell cycle-specific agent and inhibits cells only during DNA synthesis, the tritiated thymidine labeling index (LI%) was determined prior to treatment and repetitively during and after ara-C treatment.

This study was designed (a) to determine the activity of ara-C when given in the above-described dose schedule to patients with melanoma; (b) to determine the activities of ara-C kinase and ara-C deaminase in the tumor and their relationship to response; and (c) to determine the number of tumor cells actively synthesizing DNA (LI%) and to correlate these results with the enzyme determinations and clinical response.

Since the major objectives of this study were as indicated above, histological and immunological data were not included in this analysis.

MATERIALS AND METHODS

Clinical Material. Eighteen consecutive patients with metastatic melanoma and s.c. metastases that were suitable for
biopsy, who were admitted to the M. D. Anderson Hospital between October 20, 1970, and October 15, 1971, were entered into this study (Tables 1 and 2). Seventeen patients had extensive metastatic disease to the skin, lungs, liver, lymph nodes, and/or bone marrow; 1 patient had only extensive infiltration of the skin. Thirteen (72%) of the 18 patients had received prior therapy. Ten of the 13 patients had been treated with combination chemotherapy that included alkylating agents alone or in combination; 2 patients had received prior radiotherapy; 1 patient had received prior hydroxyurea. The minimum interval between the end of prior therapy and the start of this study was 30 days.

Of the 18 patients, 13 (72%) received an adequate trial, in that they received 2 or more courses of therapy or showed increasing disease after 1 course of therapy. Complete response was defined as the disappearance of all clinically evident melanoma at both the s.c. and other metastatic sites. Partial response was defined as a 50% (or greater) decrease in the product of the diameters of the measured tumor lesions.

ara-C was given by continuous i.v. infusion over a 5-day period and repeated at 2- to 3-week intervals. The dose and interval were adjusted in accordance with the degree of myelosuppression. The starting dose of ara-C for all patients was between 100 and 150 mg/sq m/day.

**Cytokinetic Studies.** Excisional biopsies of 1 to 2 discrete s.c. melanoma nodules were obtained from patients prior to therapy, and repetitively during therapy, as indicated under "Results." In the majority of cases, the entire nodule was surgically removed for analysis. Tumors of small and comparable size were chosen. The average tumor size was 0.42 ± 0.09 g.

The melanoma tissues were minced in Eagle's essential media, and the suspension was centrifuged at 500 X g (1500 rpm) for 10 min. The supernatant was discarded, and the cell pellet was resuspended in Eagle's media and adjusted to a cell count of 3 X 10^6 cells/ml. One ml of cell suspension was placed into each of 2 separate 25-ml Erlenmeyer flasks for duplicate determinations. Five μCi of thymidine—H (specific activity, 6 Ci/mmole) were added to each flask and incubated in a shaking, 37° water bath for 1 hr. Following incubation, we terminated isotope incorporation by setting the samples in an ice bucket for 20 min. The cells were washed twice with cold 0.9% NaCl solution. The supernatant was discarded, the cell pellet was resuspended in 1 ml of 0.9% NaCl solution, and tumor cell smears were made from the cell suspension for...
autoradiography. The slides from the duplicate determinations were dipped in Kodak NTB₂ emulsion, exposed for 24 hr, developed in D¹⁹ developer at room temperature, fixed in general-purpose Kodak fixer, and stained with May-Grünewald-Giemsa. Background count obtained from the average of 3 background fields was 1 grain per cell nucleus, and a labeling criterion of 3 grains per cell was adopted. One hundred cells were counted on each slide, and the number of labeled cells per 100 counted cells was defined as the LI%. To check for reproducibility of results, we determined the LI% on the slides made from the duplicate fractions of the pretherapy tumor samples obtained from 11 of the 18 patients. The average difference in reading between slides made from the split tissue samples was 3%.

Enzyme Studies. Enzyme studies were performed on biopsy material obtained from 17 patients prior to treatment. ara-C (NSC 63868) [that is, tritiated ara-C (specific activity, 19 Ci/mmmole)] and tetrahydrouridine (NSC 112907) were used in the determination of the ara-C kinase and ara-C deaminase activities and were supplied by the drug development branch of the Cancer Chemotherapy National Service Center at the National Cancer Institute. The radiochemical purity was > 97%, as determined by chromatographic and radiochemical techniques. Other chemicals that were used were commercially available.

The melanoma tissue that we obtained from the patients was processed immediately, rinsed with 0.9% NaCl solution, homogenized in 0.25 M sucrose, and centrifuged at 100,000 X g for 60 min. The supernatant was recovered for the assays of the ara-C kinase and deaminase activities. The separation and identification of the product from its substrate have previously been described (8). (The assay procedure is to be published elsewhere.)

Hematology. Complete blood and platelet counts were obtained on all patients prior to, during, and at 2- to 4-day intervals following therapy and were plotted for the 13 patients with adequate trials.

Changes in the peripheral blood for the patients with adequate trials were correlated with changes in the various marrow compartments, as determined from smear differentials and clot sections of aspirates obtained at 5- to 7-day intervals prior to, during, and after the 1st course of chemotherapy.

Statistical Methods. The Fisher exact test (13) and the Wilcoxon rank test (13) were used in the analysis, as indicated in “Results.” An observed difference is referred to as “significant” if the p value is less than 0.05 and as “highly significant” if the p value is less than 0.01.

RESULTS

Response

Clinical. Of the 18 patients studied, 13 (72%) had an adequate trial (Table 1). Of the 13 patients who received adequate trials, 3 (23%) had an adequate response.

The results obtained with individual patients are presented in detail in Table 2. The patients are divided into the categories of adequate and inadequate trial (Table 2, Column 1). Within the adequate trial category, patients are ranked in order of tumor response, with the pretreatment tumor size expressed as 100% (Column 6). For responding patients, the tumor size recorded was taken at the time of maximum response. For nonresponding patients, the tumor size is that recorded at the end of the study. Thus, for patients who failed to respond, the recording of 120 to 200% indicates increasing tumor size during treatment.

Of the 5 patients with inadequate trials, 4 had early deaths and 1 refused more than 1 course of therapy.

The 1st patient, V. M. (Column 2), had an excellent, almost complete response which lasted for 8 months. The next 2 patients, D. H. and E. D., had marginal partial remissions, each of which lasted only 1 month.

The patients with adequate trials were examined for the effect of certain clinical variables on response. It was found that age (Table 2, Column 3), time from diagnosis to metastases (median, 2 months), and time from metastases to treatment (median, 7 months) did not significantly affect response. However, as can be seen from Table 3, the response in women was suggestively superior to that of men (p < 0.07).

Enzyme Assays

The pretreatment enzyme assays for the individual patients are presented in Table 2. The median ara-C kinase activity (Column 7) was 329 in the 3 responding patients, compared to a median of 34 (p = 0.07) for the entire “nonresponse” group and a median of 41 (p = 0.07) for the patients who failed to respond with an adequate trial of therapy (p = 0.07). The patient with the maximum tumor response (V. M.) had the highest K value (506). Among the nonresponding patients, there was a tendency for the lower K values to be found in patients with more progressive disease.

The difference between responders and nonresponders with respect to ara-C deaminase (Table 2, Column 8) was the converse of that for ara-C kinase, although the difference was not significant. Thus, the median D value for the responding patients was 278 compared to a median of 349 for the entire nonresponse group and a median of 410 for patients who failed to respond with an adequate trial of therapy.

The difference between the response and nonresponse groups was more striking when the K/D ratios were compared (Table 2, Column 9). There was a high negative rank correlation (−0.68) between the K/D ratio and tumor response (9). The median K/D ratio for the responding patients was 51, compared to a median of 10 for the entire nonresponse group (p < 0.05) and a median of 7.5 for the patients who failed to respond.

### Table 3

<table>
<thead>
<tr>
<th>Sex</th>
<th>No. of patients</th>
<th>No. of patients responding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>8</td>
<td>0 (0)³</td>
</tr>
<tr>
<td>Female</td>
<td>5</td>
<td>3 (60)</td>
</tr>
</tbody>
</table>

³ Adequate trials only.

| Numbers in parentheses, percentage of patients responding. |
respond with an adequate trial of therapy ($p \leq 0.05$). The highest K/D ratio was found in the only patient (V. M.) who had an excellent response to therapy.

Among the nonresponding patients with an adequate trial of therapy, there was evidence that stabilization of disease tended to be associated with higher K/D ratios than occurred in those patients with progressive disease (patients with a 50% or greater increase in tumor size during treatment). The K value appeared to have a greater influence on the correlation of the K/D ratio to response than did the D value, as determined from the comparison of the rank correlation between tumor response and the K value ($-0.66$) and the rank correlation between tumor response and the D value ($+0.12$). The correlation between tumor response and K was statistically significant ($p = 0.05$).

LI%

The pretreatment LI% also correlated with response and with the K/D ratio (Table 2, Column 10). Thus, the highest mean LI% (14%) occurred in the responding patients, compared to a median of 4% for the entire nonresponse group ($p = 0.05$) and a median of 3% for patients who failed to respond with an adequate trial of therapy ($p = 0.05$). LI%'s were obtained on Days 5 (Table 2, Column 11) and 21 (Column 12) following the start of the 1st course of treatment. For the patient (V. M.) who achieved maximum response, there was a marked decrease in the LI%, which was maintained through Day 21 posttherapy. For the nonresponders, a 50 to 100% decrease in the LI% occurred on Day 5 of therapy, but there was a complete or nearly complete recovery in DNA synthesis by Day 21 posttherapy. The dashes (Table 2, Columns 10, 11, and 12) indicate that samples were not obtained; thus, it was not possible to perform repetitive biopsies on all patients.

Hematology

The effects of the 1st 5-day course of ara-C administration on the peripheral blood and bone marrow is presented in Charts 1 and 2. The reproducibility of the effects were impressive. Thrombocytopenia occurred in all patients with a mean nadir of 30,000. The nadir occurred on the 13th day following the beginning of the 1st 5-day course. Leukopenia due almost entirely to granulocytopenia occurred at 17 days, with a mean nadir of 400 granulocytes (PMN’s). Rapid recovery for both the platelet and granulocyte count followed the nadir. In this and in previous studies (5), it has been demonstrated that the lymphocyte count is not significantly affected by ara-C.

The bone marrow changes are indicated in Chart 2. The marrow differential was determined from the smear, and the cellularity was determined from particles in the clot section. The vertical scale represents the absolute cell concentration for each morphological cell type expressed as the product of the cellularity (as determined from clot sections) and the percentage of cells (as determined from smear differential) (7). As we anticipated, the cells with the most rapid turnover time and highest LI%, that is, the blasts and nucleated red cells of the bone marrow, decreased sharply during the 5 days of ara-C administration, followed by nearly complete recovery at Day 14. The promyelocytes also decreased, as did the myelocytes,
but there was a progressive and more extended delay for the nadir of the more mature elements (metamyelocytes and PMN's).

DISCUSSION

To date, ara-C has had limited and questionable activity in human solid tumors (5, 15). Of 51 ara-C-treated melanoma patients, there was only 1 partial response. However, all of these patients were treated with daily courses of therapy. In the present study, only 3 of the 13 patients with melanoma and an adequate trial of therapy had an objective response to ara-C. It has been demonstrated in L1210 mouse leukemia and in the spontaneous AKR lymphoma that courses of ara-C, given for twice the generation time of the neoplastic cells with intervals between courses sufficient to allow for host recovery, produce the best tumor response (12). Since the median generation time (T_50) of human melanoma cells is 3 days and the normal bone marrow recovery time is 9 or more days, 5-day courses of ara-C repeated every 2 to 3 weeks were used (5, 11). This has proved to be the most effective schedule in patients with acute leukemia (1). A limitation in melanoma, however, is the fact that the growth fraction is only 20% for relatively small s.c. metastases, and it is probable that for larger tumors it is substantially lower than this. Nevertheless, it is reasonable to hypothesize that ara-C might destroy the cells in cycle and thus “recruit” nonproliferating cells into cycle so that repetitive administration would result in a slow response. However, there was an increase in tumor size in 9 of the 10 nonresponding patients, in spite of ara-C administration. This indicates that ara-C was ineffective against cycling cells for these patients. Only 1 patient showed a nearly complete response, which lasted for 8 months, and 2 patients had brief partial responses.

In experimental in vivo systems, it has been demonstrated that the response of tumors to ara-C correlates with the conversion of ara-C to ara-C nucleotide by the tumor (10). In studies in patients with acute leukemia, Steuart and Burke (14) have demonstrated that ara-C deaminase correlates inversely with response. Thus, the lower the D value (the inactivating enzyme) in the leukemic cells, the greater is the responsiveness to ara-C. Moreover, they observed that, with continued treatment and the development of resistance to ara-C, there was an increasing D value in the leukemic cells (14). Our studies clearly indicate a positive correlation between the enzyme activity ratio (K/D) and responsiveness to ara-C. The only patient with a nearly complete and long-lasting response had a K/D ratio almost 2-fold higher than the next highest value and 2- to 6-fold higher than that of the patients who failed to respond. In contrast to acute myelogenous leukemia, however (14), the higher K values in the responding patients with melanoma contributed more to the high K/D ratio than did the higher D values in the nonresponding patients. The absence of response and, indeed, the progressive increase in size of the lesions in the nonresponding patients presumably resulted from the low K/D ratio and the ineffectual activation of ara-C, as well as from the very rapid selection of drug-resistant lines. In cytogenetic studies, it has been demonstrated that metastatic melanoma cells have marked aneuploidy, multiple stem lines, and cytogenetic instability (16). These are ideal circumstances for the development of resistant lines.

As with the K/D ratio, there was a correlation between the pretreatment LI% (proportion of tumor cells in DNA synthesis) and response. It would be anticipated that patients with a high LI% would have a more rapid response to ara-C. The major correlation in the responding patients was between the LI% and the K value. Thus, the 2 patients with the highest LI% had the highest ara-C kinase values. Since ara-C deoxycytidine kinase is involved in DNA synthesis, this correlation is not surprising.

The patient with the major tumor response (Table 2, V. M.) showed a marked decrease in the LI% which was sustained into the 2nd and 3rd week posttherapy. Although a decrease in the LI% occurred in the nonresponding patients on the 5th day of treatment, it was relatively slight and showed complete to nearly complete recovery by 2 to 3 weeks posttherapy.

The serial quantitative hematological effects of ara-C on the bone marrow which were concurrent with those on the peripheral blood have not previously been described. The effect of ara-C on both the bone marrow and peripheral blood were reproducible in this study.

The immature, rapidly proliferating cells (nucleated red blood cells, myeloblasts, and promyelocytes) decreased rapidly, as would be expected with a cell cycle-specific DNA synthesis inhibitor. The mature marrow myeloid compartments (myelocytes, metamyelocytes, and PMN's) also decreased, but at a slower rate, as a result of the inhibition of the more immature myeloid precursors. The decrease in the mature myeloid elements in the bone marrow correlated with the decrease in the PMN's in the peripheral blood.

REFERENCES


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