Biological Characterization of a Prolonged Antileukemic Effect of 5-Azacytidine

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SUMMARY

A prolonged cytotoxic effect of 5-azacytidine (aza-CR) on leukemic colony-forming units (LCFU) was observed in mice with transplanted L1210 leukemia. LCFU showed rapid reaccumulation in the marrow 12 hr after injection of 0.1 mg of aza-CR per mouse. However, after 0.5 mg of aza-CR, repopulation was delayed for at least 6 days. Experiments were performed to determine the mechanism of this prolonged antileukemic effect. Suspensions of leukemic marrow prepared from mice treated 4 days previously with 0.5 mg of aza-CR were exposed to [3H]thymidine in vitro in order to kill cells in S phase. Suspensions exhibited a 40% reduction in LCFU, indicating the prolonged effect was not due to cell cycle progression delay. Mice given whole-body irradiation prior to receiving L1210 demonstrated the same delayed repopulation following the high dose of aza-CR as nonirradiated mice, suggesting that the effect was likely not due to an immune reaction.

aza-CR, when given to normal mice as long as 2 days prior to leukemic transplantation, was able to prolong the survival of leukemic mice, but not when given at longer intervals. Administration of aza-CR to mice 1 day or 1 hr prior to leukemic transplantation resulted in decreased LCFU survival as well as delayed repopulation of LCFU; the rate of repopulation was not changed. This indicated a prolonged residual activity of the drug, but not sufficient to explain the total in vivo suppression.

In contrast, administration of aza-CR to leukemic mice suppressed repopulation of a subsequent leukemic transplant for 4 days, even when the cells were given 2 days after the aza-CR. Cytidine was partially able to reverse the delayed repopulation of LCFU when given 1 day after aza-CR, but it was unable to reverse the phenomenon 2 days after aza-CR. Therefore, a high dose of aza-CR produces a prolonged antileukemic effect which is probably mediated by continued availability of an aza-CR metabolite. Since this effect is more pronounced in leukemic mice than in nonleukemic mice, the pharmacokinetics of high doses of aza-CR probably differ in normal and leukemic mice.

INTRODUCTION

aza-CR, a pyrimidine analog, has been shown to have an important role in the therapy of acute leukemia in humans (6, 8, 14, 16). Determination of the kinetics of aza-CR cytotoxicity in vivo is important in order both to devise methods of administration of the drug that maximize the therapeutic effect and to suggest possible drug combinations with additive or synergistic antitumor effects.

Previous work from this laboratory using L1210 leukemia in vivo (11) indicated that aza-CR has 2 unusual properties. The 1st property was a biphasic dose-response curve. The 2nd unusual property was a prolonged antileukemic effect lasting many days after a single injection and not observed with any other drug previously examined. The purpose of these experiments was to define the mechanism of the prolonged antileukemic effect of aza-CR.

MATERIALS AND METHODS

Drugs. [3H]Thymidine (specific activity, 50 Ci/mmole) was purchased from New England Nuclear, Boston, Mass. Cytidine was purchased from Sigma Chemical Co., St. Louis, Mo. aza-CR (NSC 102816) was obtained from the Drug Development Branch of the National Cancer Institute, Bethesda, Md., and was dissolved in distilled water immediately prior to use and administered i.v. in a volume of 0.5 ml.

L1210 Leukemia. The L1210 leukemia model was used as previously described (11). Two- to 3-month-old DBA/2J mice (The Jackson Laboratory, Bar Harbor, Maine) were transplanted weekly with 4 x 104 L1210 leukemia cells, and a monodispersed cell suspension was prepared 7 days later from the spleens of these mice. Two million nucleated cells in 0.5 ml were injected i.v. into recipient 6- to 8-week-old BALB/c x DBA/2 F1 (hereafter called CD2F1) mice (obtained from the National Cancer Institute) weighing 20 to 25 g (0.006 sq m). Four days later, these leukemic mice were used in the experiments described below.

Assay for LCFU. LCFU were enumerated using the spleen colony assay (1, 2). Femoral marrow was obtained from...
groups of 4 leukemic CD2F1 mice, and 0.5 ml of an appropriately diluted monodispersed suspension (sufficient to give 2 to 4 colonies/spleen) was then injected i.v. into 8 recipient CD2F1 mice. The spleens were removed 8 days later, and macroscopic colonies were counted. The average number of colonies per spleen and the marrow dilution were used to estimate the number of LCFU present in the femoral marrow of the donors. The fractional survival of LCFU was determined by normalizing all results to an untreated control group assayed at 4 days after leukemic cell injection, except as noted.

**Assay for Survival.** Groups of 10 mice received a transplant of L1210 cells i.v. The number of mice surviving was determined daily. The median survival for the group was determined to the nearest 0.5 day by interpolation.

**Assay for [3H]Thymidine Reduction of LCFU (Thymidine Suicide).** Bone marrow suspensions were diluted in a volume of α-minimum essential medium that contained 20 mM 4-(N-morpholino)propane sulfonic acid buffer in place of bicarbonate sufficient to give a cell concentration of about \(10^6\) cells/ml. [3H]Thymidine was added to a final concentration of 100 μCi/ml, and cell suspensions were incubated at 37°C for 20 min with periodic agitation. Control suspensions lacked [3H]thymidine. Cell suspensions were then diluted 1:200 in α-minimum essential medium containing 20 mM 4-(N-morpholino)propane sulfonic acid buffer in place of bicarbonate and nonradioactive thymidine, 100 μg/ml, and 0.5 ml was injected into recipient mice for assay of LCFU as described above. The proportion of LCFU surviving after [3H]thymidine treatment was calculated from LCFU in cell suspensions incubated with [3H]thymidine, divided by LCFU in control cell suspensions incubated without [3H]thymidine.

**Statistical Analysis.** Differences between sample means were determined by Student's t test. Fitting of lines through data points and differences between lines were determined by linear regression analysis.

**RESULTS**

**Time Dependence of LCFU Survival.** In order to define the model to be examined here, we administered 0.5 mg of aza-CR to each mouse, and LCFU survival was measured at various time intervals thereafter (Chart 1, closed data points). At that dose, reduction in fractional survival of femoral marrow LCFU to \(5 \times 10^{-2}\) was apparent by 4 hr after aza-CR injection. This curve is similar to that shown previously for a higher dose (1 mg/mouse) of aza-CR (11). The delayed repopulation is in contrast to the rapid repopulation of LCFU following a lower dose, 0.1 mg aza-CR (Chart 1, solid line), taken from our previous publication (11). The following experiments were performed to determine the mechanism of the continued suppression of LCFU proliferation of femoral marrow LCFU following 0.5 mg of aza-CR per mouse.

**Effect of [3H]Thymidine on LCFU Survival.** One possibility was that the surviving LCFU after 0.5 mg aza-CR failed to repopulate the marrow because drug persisted in the host sufficient to inhibit cells in their progression through the cell cycle. If this were the case, such cells should not be killed by exposure to high-specific-activity [3H]thymidine, since they would not be proceeding through DNA synthesis (17). Thus, marrow suspensions were prepared from leukemic mice 4 days after injection of 0.5 mg aza-CR per mouse. The cell suspensions were incubated with [3H]thymidine immediately after their preparation. Nonradioactive thymidine was then added, and the suspension was injected into recipient mice for assay for LCFU. The fraction surviving was compared to that in similar suspensions incubated without [3H]thymidine (Table 1). LCFU content was significantly decreased after aza-CR treatment (p < 0.01). Of the LCFU remaining, the mean fraction surviving [3H]thymidine treatment was 0.6. In comparison, the mean

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**Table 1**

<table>
<thead>
<tr>
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<th>LCFU/femur</th>
<th>Proportion of LCFU surviving after [3H]thymidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aza-CR-treate</td>
<td>4.4 ± 0.83 (\times 10^4) &amp; 0.36 ± 0.12</td>
<td></td>
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<tr>
<td>d</td>
<td>6.7 ± 0.25 (\times 10^3) &amp; 0.60 ± 0.11</td>
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* Mean of 3 experiments.

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fraction of untreated LCFU surviving \( ^3 \text{H} \)thymidine treatment was 0.36. This difference is not significantly different (\( p > 0.2 \)). Since \( ^3 \text{H} \)thymidine was able to decrease aza-\( \text{CR} \) treated LCFU by 40%, at least 40% of LCFU were synthesizing DNA 4 days after aza-\( \text{CR} \) injection. Thus, there was minimal (if any) delay in progression through S phase, and drug-induced progression delay was not the explanation for the delay in LCFU repopulation following aza-\( \text{CR} \).

**LCFU Survival in Irradiated Mice.** From the above results it would appear that cell proliferation was balanced by cell loss. It has been demonstrated in experimental leukemia that anticancer agents, specifically, dimethyltriazenoimidazolecarboxamide, can result in residual cells being more immunogenic than the original cell population (4). Possibly, immunological rejection of tumor cells resulting from aza-\( \text{CR} \) treatment or simply from a tumor-host response between our particular cell and animal lines led to the lack of repopulation of LCFU following the high dose of aza-\( \text{CR} \). To test this theory, we measured the survival of LCFU at various times following a 0.5-mg dose of aza-\( \text{CR} \) in irradiated (400 rads whole-body \( \gamma \) radiation) leukemic mice. Mice were given leukemia cells immediately after the radiation and 4 days later received 0.5 mg aza-\( \text{CR} \). The change in LCFU thereafter is shown in Chart 1 (open data points). LCFU survival in 2 separate experiments showed moderate variability. However, in neither experiment was repopulation of LCFU observed. Further, in both experiments, LCFU survival was the same as that found in nonirradiated mice.

**Effect of aza-\( \text{CR} \) Pretreatment on Life-span and LCFU Survival in Leukemic Mice.** We next examined the possibility that cytotoxic levels of aza-\( \text{CR} \) or a metabolite remained in the mice for an extended period of time. Groups of 10 mice received injections of varying numbers of L1210 cells (10^3, 10^4, 10^5, and 10^6/mouse) 1 day after receiving 0.5 mg aza-\( \text{CR} \) per mouse, and the mean survival time was determined. In mice receiving aza-\( \text{CR} \), mean survival was prolonged 2.0 to 3.0 days compared to mice not receiving aza-\( \text{CR} \), which survived 7.6 days. This prolongation corresponded to a cell kill of approximately 99%. For comparison, injection of 0.5 mg aza-\( \text{CR} \) 2 days after 10^6 L1210 cells produced a prolongation of survival by 5.7 days (11).

Next, groups of 10 mice were given 0.5 mg aza-\( \text{CR} \) at different times prior to transplantation of 10^6 leukemia cells, and the mean survival time was determined (Table 2). There was a significant prolongation in life-span when aza-\( \text{CR} \) was injected within 2 days of leukemia transplantation (\( p < 0.001 \)).

Next, varying doses of aza-\( \text{CR} \) were given 1 day prior to transplantation of 10^6 leukemia cells. Following a low dose of aza-\( \text{CR} \), 0.1 mg/mouse, mean survival was 8.2 ± 0.2 (S.E.) days, or a prolongation of only 0.6 days (see Table 2). Following 0.5 mg/mouse, there was a prolongation of life-span of 2.2 days.

In order to determine whether the prolongation of life-span was due to a cytotoxic effect on LCFU, marrow was assayed after aza-\( \text{CR} \), 0.5 mg/mouse, was given at varying times prior to leukemia transplantation. LCFU survival was normalized to LCFU content in mice that had not received aza-\( \text{CR} \) and that were assayed 1 day after transplantation of 2 × 10^6 leukemia cells, which ranged from 110 to 240 LCFU/femur. Four separate experiments were performed, and the results are presented in Chart 2. Mice that had received no aza-\( \text{CR} \) (Chart 2, closed circles and solid line) or that received aza-\( \text{CR} \) 2, 3, or 4 days prior to transplantation (Chart 2, closed symbols), demonstrated a similar increase in LCFU survival 2 to 6 days after transplantation.

In contrast, in mice that had received aza-\( \text{CR} \) 1 day (Chart 2, open squares) or 1 hr (Chart 2, open triangles) prior to transplantation, the LCFU survival 1 day after transplantation...
tion was decreased by approximately 90% compared to untreated mice. The repopulation of LCFU was delayed by 1 day, and the rate of repopulation, once begun, was less than that of the control (p < 0.0001 compared to control mice and mice receiving aza-CR 2, 3, or 4 days prior to transplantation). These results in LCFU survival are thus in agreement with the prolongation in life-span in mice receiving aza-CR prior to transplantation (Table 2).

Lack of Cytidine Reversal of Delayed Repopulation of LCFU. In order to characterize more specifically the molecular species responsible for the prolonged suppression of LCFU following high-dose aza-CR, mice were given 0.5 mg aza-CR followed by cytidine at different times. If mice received a bolus injection of 12 mg cytidine per mouse i.v., 2 min after aza-CR, the fractional survival of LCFU per femur 24 hr later was 0.36 ± 0.13. If cytidine was not given, fractional LCFU survival was 0.008 ± 0.003. If cytidine was given as a 24-hr infusion of 200 mg beginning 2 min after the aza-CR injection by a technique previously described (3), the fractional survival obtained 15 min after the end of the infusion was 0.33 ± 0.13 (Table 3). Thus, cytidine was capable of reversing much of the aza-CR cytotoxicity if given immediately after aza-CR.

Cytidine administration was delayed until the phase of prolonged suppression of LCFU. Leukemic mice that had received 0.5 mg aza-CR were given 50 mg of cytidine i.p. 1 day after aza-CR, followed by a 24-, 48-, or 72-hr infusion of cytidine, 200 mg/mouse/day. The results (Table 3) show that LCFU increased from a fractional survival of 0.008 to 0.11 during these 3 days. This rate of proliferation was slightly less than that of untreated LCFU (Chart 2, solid line). If, however, aza-CR-treated mice received cytidine starting 2 days after aza-CR treatment, fractional LCFU survival 48 hr later, 4 days after transplantation, was 0.011. Thus, cytidine was relatively effective in reversing the prolonged suppression of LCFU survival if given 1 day after aza-CR but was unable to reverse the phenomenon if given 2 days after aza-CR. The factor responsible for the prolonged inhibition of LCFU proliferation 2 or more days after aza-CR (as shown in Chart 1) was not reversed by cytidine.

Proliferation of Leukemia Rechallenge in aza-CR-treated Leukemic Mice. There was a discrepancy between the observed proliferation of LCFU beginning 2 days after aza-CR in normal mice receiving aza-CR and, later, L1210 leukemia (Chart 2) and the continued suppression of LCFU repopulation for 6 days in mice receiving L1210 leukemia and, later, aza-CR (Chart 1). We therefore examined the rate of proliferation of a 2nd challenge of L1210 cells in aza-CR-treated mice. Leukemic mice that received aza-CR 4 days after transplantation of $2 \times 10^6$ L1210 cells, were given a 2nd injection of $2 \times 10^6$ leukemia cells 2 days after the aza-CR. The femoral marrow content of LCFU (Chart 3, closed data points) never rose above the background level (Chart 3, solid line). In contrast, an injection of $2 \times 10^6$ leukemia cells in normal mice not treated with aza-CR resulted in an exponential increase of LCFU (Chart 3, open data points and solid line). By Day 3 after rechallenge, LCFU survival in aza-CR-treated mice was 100 times lower than in untreated mice. Thus, the factor responsible for inhibition of repopulation of LCFU persists for a longer period of time in aza-CR-treated leukemic mice than in aza-CR-treated normal mice and remains at a concentration sufficient to inhibit a leukemia rechallenge even 2 days after 0.5 mg aza-CR.

![Chart 3. LCFU survival as a function of time after aza-CR injection.](chart3.png)

**Table 3**

<table>
<thead>
<tr>
<th>Initiation</th>
<th>Duration (hr)</th>
<th>Cessation (hr before LCFU assay)</th>
<th>Time of LCFU assay (days after aza-CR injection)</th>
<th>Fractional LCFU survival</th>
<th>Cytidine infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15 M NaCl infusion</td>
<td>0.008 ± 0.003</td>
<td>0.46 ± 0.14</td>
<td>2 min after aza-CR</td>
<td>24</td>
<td>0.25</td>
</tr>
<tr>
<td>0.008 ± 0.003</td>
<td>0.33 ± 0.13</td>
<td>1 day after aza-CR</td>
<td>24</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>0.023 ± 0.001</td>
<td>0.035 ± 0.015</td>
<td>1 day after aza-CR</td>
<td>48</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>0.026 ± 0.023</td>
<td>0.145 ± 0.065</td>
<td>1 day after aza-CR</td>
<td>72</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>0.11 ± 0.04</td>
<td>0.011 ± 0.003</td>
<td>2 days after aza-CR</td>
<td>48</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

* Survival of LCFU in treated mice is compared to that in untreated mice assayed 4 days after L1210 cells.

* Mean ± S.E.
DISCUSSION

The experiments presented here, plus the experiments previously described from this laboratory (11), indicate that high-doseaza-CR treatment of mice with L1210 leukemia results in a prolonged suppression of femoral marrow LCFU. Previously published results indicated that the prolonged suppression occurred at doses of aza-CR of 0.25 mg/mouse and above. The data presented here are consistent with those observations (Chart 1). Marrow suspensions from aza-CR-treated leukemic mice incubated with $[^3H]$thymidine showed decreased LCFU survival not significantly different from that found for control LCFU (Table I). Since the number of LCFU remain constant but are progressing through the cell cycle at a rate similar to that of untreated cells, about half of the progeny must be dying each generation time. Possible reasons for such cell killing are immune rejection or residual cytotoxic levels of drug or drug metabolites.

Mice that had been irradiated prior to leukemia cell transplantation were still capable of demonstrating the prolonged suppression of LCFU survival following aza-CR treatment (Chart 1). This indicates that radiation-sensitive immune responses are not required for the phenomenon.

Experiments were then performed to determine whether aza-CR cytotoxicity persisted in normal mice that only later received L1210 cells. aza-CR injected into normal mice 1 or 2 days prior to leukemia transplantation resulted in an increased life-span of leukemic mice by 2 to 3 days (Table 2). This phenomenon was not observed after 0.1 mg of aza-CR, but only after 0.5 mg aza-CR. This correlated with the dose dependence of the phenomenon of prolonged suppression of LCFU survival which was previously published (11). It should be emphasized that treatment of leukemic mice with 0.5 mg aza-CR results in more marked prolongation of survival, as demonstrated previously (11) and as expected from the data in Chart 1.

This persistent antileukemic effect was confirmed in studies of LCFU survival following aza-CR treatment of normal mice prior to leukemia transplantation (Chart 2). However, the failure of any cytotoxic effect of aza-CR to persist for more than 2 days after treatment of normal mice is in contrast to the 5- to 6-day prolongation of suppressed LCFU content following aza-CR treatment of leukemic mice (Chart 1 and our previous report (11)].

LCFU repopulation began 2 days after L1210 transplantation into aza-CR-treated normal mice (Chart 2) but did not commence even by 4 days after transplantation into aza-CR-treated leukemic mice (Chart 3). This may be due to altered drug metabolism in leukemic mice leading to increased amounts of cytotoxic drug or drug metabolites available at later times after aza-CR treatment. We have previously reported enhanced cytotoxic effect of aza-CR against hematopoietic colony-forming units in leukemic mice compared to hematopoietic colony-forming units in normal mice (11).

These studies demonstrate that the antileukemic effect of aza-CR persists for 1 to 2 days in normal mice. aza-CR has been measured by biological assay, and the half-life in normal mice was found to be less than 6 hr (9). The half-life of radioactivity of radioactive aza-CR is less than 12 hr in humans (15). It is therefore likely that this persistent antileukemic effect is not mediated by intact aza-CR.

Experiments performed with cytidine reversal confirmed this hypothesis. Cytidine immediately after aza-CR was able to reverse most of its cytotoxicity, confirming the observations of others (7). However, cytidine given 1 day after aza-CR treatment of leukemic mice was only partially able to reverse the prolonged suppression of LCFU survival, and at 2 days after treatment, aza-CR was unable to do so. This suggests that a metabolite of aza-CR is responsible for the phenomenon of prolonged suppression. It is possible that the defective transfer RNA which is produced after aza-CR exposure (5, 13) is related to this prolonged antileukemic effect. Since this cytotoxic factor prevents repopulation of a 2nd challenge of L1210 cells (Chart 3), it must either be released extracellularly or be directly transferred between cells. Further biochemical studies are needed to define the nature of the cytotoxic factor responsible for this phenomenon.

The documentation of prolonged suppression of LCFU survival following aza-CR and of prolonged antileukemic effect following aza-CR may be important in explaining the appearance of delayed myelosuppression observed in some patients receiving repeated doses of aza-CR in clinical trials (8). Furthermore, it is possible that tumor cells persisting in this “nongrowth” phase following aza-CR may be sensitive to antitumor drugs otherwise ineffective against those tumor cells. Further studies of drug-drug interaction are in progress to determine whether additive or synergistic antitumor effects can be produced by combination of other drugs with high doses of aza-Cr.

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REFERENCES

Prolonged aza-CR Cytotoxicity


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