

# Kinetics of Both Leukemic and Normal Cell Population Reduction following 5-Azacytidine<sup>1</sup>

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## SUMMARY

The cytotoxic effect of 5-azacytidine (AzaCR) on normal hematopoietic colony-forming units (NCFU) and L1210 leukemic colony-forming units (LCFU) in the femoral marrow of BALB/c × DBA/2F<sub>1</sub> mice was studied using the spleen colony assay. Dose-survival curves for LCFU and NCFU were biphasic. Repopulation of LCFU was rapid at a low dose of AzaCR (0.1 mg/mouse) but was delayed for greater than 6 days at higher doses (0.25 mg/mouse and above). Of the agents tested in this system, only AzaCR exhibited these properties. Survival of mice with L1210 leukemia following AzaCR administration was prolonged beyond that predicted by the degree of LCFU reduction alone, and reflected the delay in LCFU repopulation. In contrast, repopulation of NCFU in normal mice was not delayed at a high dose of AzaCR (0.5 mg/mouse). AzaCR produced a nine-fold greater reduction of NCFU in leukemic mice than in normal mice, measured 5 days after AzaCR injection. While divided doses of AzaCR produced LCFU cytotoxicity equivalent to a single dose, 24-hr infusions of high doses were inferior to single injections.

## INTRODUCTION

AzaCR<sup>3</sup> is a pyrimidine analog that has shown marked antineoplastic activity in experimental tumor systems (14, 19) and has demonstrated clinical antileukemic efficacy (5, 10, 15, 20). Following phosphorylation, AzaCR is incorporated into both DNA and RNA, and inhibits DNA, RNA, and protein synthesis (7, 16, 21). Studies at the cellular level have indicated that AzaCR is a cell cycle-specific agent (9) with its predominant cytotoxic effect against cells in S phase (8). AzaCR also blocks cells in their progression from G<sub>1</sub> into S at relatively low drug concentrations (16).

Since the kinetics of killing of leukemic cells by AzaCR

has been evaluated only *in vitro* (8, 9), this study was initiated to examine the kinetics of reduction of leukemic and normal cells *in vivo* by AzaCR. Our results suggest that AzaCR cytotoxicity is unusual. The kinetics of AzaCR killing differs from that expected for either phase-specific or phase nonspecific agents, and the repopulation of leukemic cells is dose dependent, being markedly delayed at high drug doses.

## MATERIALS AND METHODS

**L1210 Leukemia.** The L1210 leukemia model was used as previously described (3). Two- to 3-month-old DBA/2J mice (The Jackson Laboratory, Bar Harbor, Maine) were transplanted weekly with  $4 \times 10^4$  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 × DBA/2F<sub>1</sub> (hereafter called CD2F<sub>1</sub>) mice (National Cancer Institute) weighing 20 to 25 g (0.006 sq m). Four days later these leukemic mice were used in the experiments described herein.

**AzaCR.** AzaCR was obtained from the Drug Development Branch of the National Cancer Institute. It was dissolved and diluted in distilled water immediately prior to use and administered i.v. in a volume of 0.5 ml.

**Assays for LCFU and NCFU.** LCFU were enumerated using the spleen colony assay (1). Femoral marrow was obtained from groups of 4 leukemic CD2F<sub>1</sub> mice. Five-tenths ml of an appropriately diluted monodispersed suspension (sufficient to give 2 to 4 colonies/spleen) was then injected i.v. into 8 recipient CD2F<sub>1</sub> 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 either 4 or 5 days following leukemic cell injection, depending on the experimental study.

The assay for NCFU differed from that for LCFU in that the femoral marrow was obtained from 5 nonleukemic CD2F<sub>1</sub> mice, and the 10 recipients received 900 rads of whole-body  $\gamma$ -radiation, as previously described (18), prior to the injection of a dilution of the normal femoral marrow.

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<sup>3</sup> The abbreviations used are: AzaCR, 5-azacytidine, NCS 102816; LCFU, leukemic colony-forming units; NCFU, normal hematopoietic colony-forming units; ILS, increase in life-span.

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Macroscopic colonies in the spleen were counted 10 days after injection. The average number of colonies per spleen and the marrow dilution were used to estimate the number of NCFU present in the femoral marrow of the donors. The fractional survival of NCFU following treatment was determined by normalizing the results to an untreated control group.

## RESULTS

**Dose Dependence of LCFU and NCFU Survival.** Mice were given different doses of AzaCR, and the survival of LCFU and NCFU was measured 24 hr later. As shown in Chart 1, survival of both LCFU and NCFU decreased with increasing dose. The curves fitted to the data for the 2 cell populations were biphasic, with the initial slopes being steeper than the terminal slopes. Even for a dose of 0.01 mg/mouse, LCFU survival was significantly decreased (0.32). At a dose of 0.1 mg/mouse, NCFU survival was not different from the control, while LCFU survival had decreased to about  $6 \times 10^{-2}$ . At doses in excess of 0.5 mg/mouse, LCFU were more sensitive than NCFU by a factor of 100 or more.

**Time Dependence of LCFU and NCFU Survival.** Mice were given either 0.1 mg or 1 mg of AzaCR per mouse, and LCFU survival was measured at various time intervals thereafter (Chart 2). At the low dose, LCFU survival reached a minimum of about  $8 \times 10^{-2}$  at 8 to 12 hr after drug administration, following which repopulation of femoral marrow by LCFU was observed. After the high dose of AzaCR, a minimum surviving fraction of LCFU of  $5 \times$

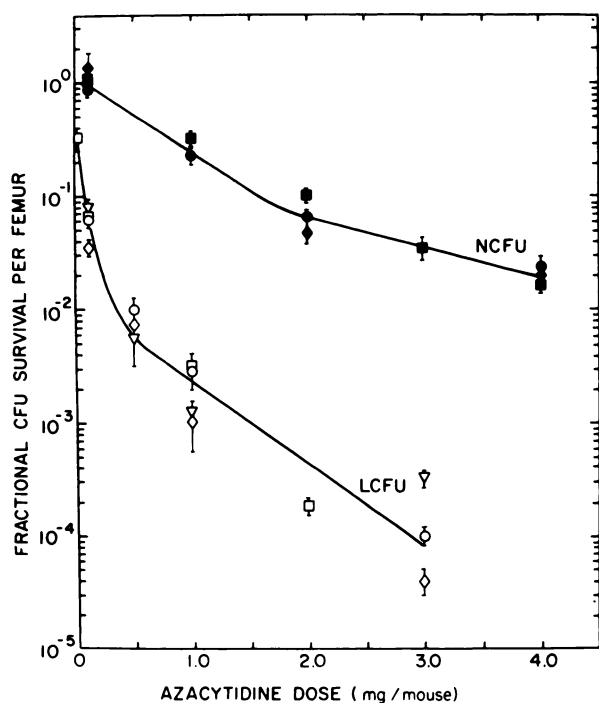


Chart 1. Fractional survival of femoral marrow NCFU and LCFU as a function of dose of AzaCR. Different symbols represent different experiments. Vertical bars,  $\pm 1$  S.E.

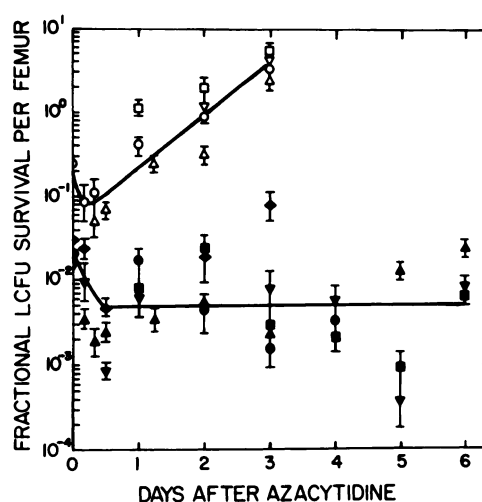


Chart 2. Fractional survival of LCFU as a function of time after 0.1 mg ( $\square, \nabla, \cup$ ) and 1 mg ( $\blacksquare, \blacklozenge, \bullet, \blacktriangle$ ) AzaCR per mouse. Different symbols represent different experiments. Vertical bars,  $\pm 1$  S.E.

$10^{-3}$  was reached by 12 hr, but repopulation had not commenced by 6 days after AzaCR injection. Mice receiving the high dose of AzaCR appeared not to be dying from leukemia but rather from drug toxicity, as expanded upon below in "AzaCR Toxicity."

A more accurate determination of the dose at which the transition occurred between prompt LCFU repopulation (as with 0.1 mg/mouse) and prolonged depression of LCFU content (as with 1.0 mg/mouse) was attempted. Leukemic mice received different doses of AzaCR, and the surviving fraction of LCFU was determined 1, 2, and 4 days later. LCFU survival increased over the observed period if the AzaCR dose was 0.1, 0.125, 0.15, or 0.2 mg/mouse. At 0.25, 0.3, 0.5, and 1.0 mg/mouse, significant repopulation had not occurred by Day 4.

Experiments were performed to determine whether this delay in repopulation occurred for NCFU as well. Each nonleukemic mouse was given 1.0 mg AzaCR, and survival of NCFU was measured. The minimum survival level (about  $8 \times 10^{-2}$ ) was reached by Day 2 and repopulation commenced thereafter (Chart 3). By Day 7, NCFU survival was 0.6 of the pretreatment level.

**Survival Studies.** Survival of leukemic mice following AzaCR administration should be proportional either to the decrease in LCFU, as occurs with all other agents studied against this tumor system, or to the decrease in LCFU plus an amount of time reflecting the delay in repopulation of LCFU. This factor was examined in mice that had received  $10^6$  leukemic cells i.v. and 2 days later received varying doses of AzaCR; survival was followed daily. Untreated leukemic mice survived 7 or 8 days. It was previously determined from the rate of leukemic proliferation that a 90% decrease in the number of LCFU should prolong survival by 1.1 days or yield a 15% ILS in this system (unpublished data). The predicted and observed values are shown in Chart 4 where ILS is plotted as a function of drug dose. After 0.1 mg AzaCR per mouse, the reduction of LCFU previously found (Chart 1) predicts an ILS of 22%,

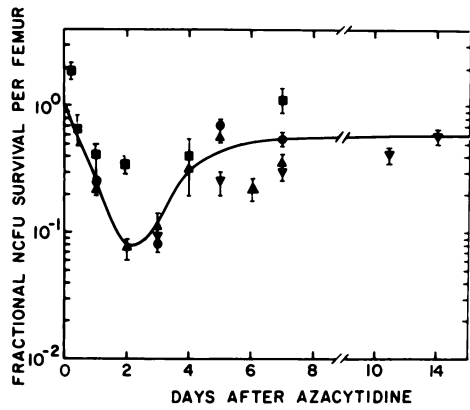


Chart 3. Fractional survival of NCFU as a function of time after administration of 1 mg of AzaCR per mouse. Different symbols represent different experiments. Vertical bars,  $\pm 1$  S. E.

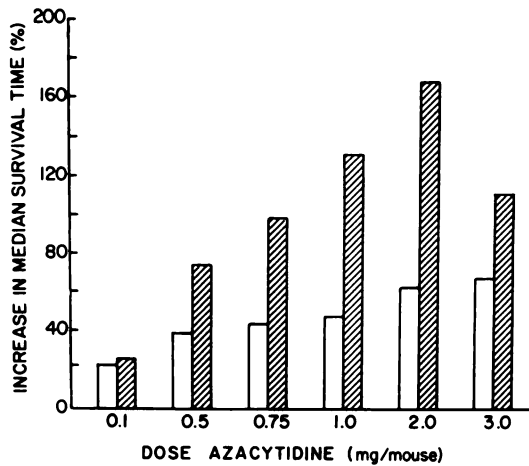


Chart 4. Percentage ILS of leukemic mice treated with AzaCR. The time at which 50% of the mice had died was determined to the nearest 0.5 day by interpolation. The experimental results (■) represent the mean of 1 to 3 experiments, each consisting of 10 mice. Mice receiving no AzaCR survived 7 or 8 days; □, ILS predicted from the decrease in LCFU alone, as determined from Chart 1.

in close agreement with the 24% ILS observed. For 0.5 mg and higher doses, disparities existed between the predicted and observed survival times. For example, the LCFU reduction observed after 1 mg AzaCR/mouse (Chart 1) would predict a 47% ILS, whereas 130% ILS was observed, a value more than 6 days beyond that predicted. Thus, the delayed repopulation of LCFU as measured by the spleen colony assay is reflected in the survival time assay.

**Effect of Repeated Doses or Infusion of AzaCR.** We next determined whether an optimum time interval existed for split doses of AzaCR. Leukemic mice received 0.1 mg/mouse of AzaCR and a 2nd 0.1-mg dose at either 4, 8, 12, 16, or 24 hr thereafter. The survival of LCFU was compared with that found for a single injection of 0.2 mg AzaCR alone ( $1.8 \times 10^{-2}$ ). The fractional survival for the 2 doses of 0.1 mg varied between  $1.3$  and  $2.8 \times 10^{-2}$  and is thus not significantly different from that for the single 0.2-mg dose.

We next attempted to determine whether drug adminis-

tration as an infusion would modify the extent of killing of LCFU. Mice were immobilized in a plastic chamber with free access to food, their tails were taped to tongue depressors, and 26-gauge needles were secured i.v. Twenty-four-hr infusions of AzaCR in 0.9% NaCl solution were administered and, 4 hr after the conclusion of the infusion, LCFU survival was assayed (Chart 5). There was no difference in survival of LCFU at doses up to 1 mg/mouse, compared with that found for single bolus injections (Chart 1). At doses above 1 mg/mouse, however, the acute injection produced a significantly greater decrease in LCFU than the infusion.

**Drug Stability Studies.** AzaCR was diluted in distilled water and stored at  $4^\circ$ . This solution was used for dose-survival studies (as in Chart 1) after various time intervals. The results are shown in Chart 6. For up to 2 weeks after preparation, little, if any, decrease in activity was observed. By 4 weeks, cytotoxic activity had decreased by approximately 50%.

**AzaCR Toxicity.** Groups of 10 normal CD2F<sub>1</sub> mice received different doses of AzaCR, and survival was measured at 30 days. Results of 5 separate experiments indicated that the LD<sub>50</sub><sup>30</sup> was approximately 3 mg/mouse.

Drug toxicity seemed to be increased in leukemic mice, since 80% of mice receiving AzaCR, 0.5 or 1 mg/mouse (4 days after receipt of  $2 \times 10^6$  leukemic cells), died between 6 and 8 days after drug injection, seemingly not from leukemia. Blood counts, films of peripheral blood, and bone marrow films were performed on these mice on Days 1, 2, 4, and 7, and these tests indicated the onset of profound pancytopenia 4 days after AzaCR injection. By 7 days after AzaCR treatment, white blood cell counts decreased to below 1,500/cu mm, platelet counts decreased to below

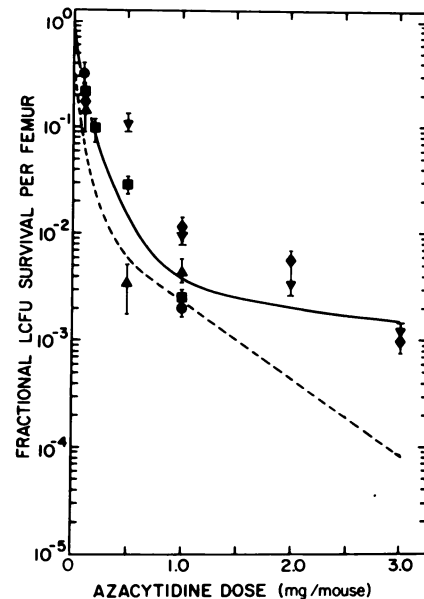


Chart 5. Fractional survival of LCFU as a function of dose of AzaCR administered as a 24-hr infusion. Different symbols represent different experiments; vertical bars,  $\pm 1$  S.E. ---, results obtained for a single injection of the same dose (shown in Chart 1). Fractional survival calculated as in Chart 1.

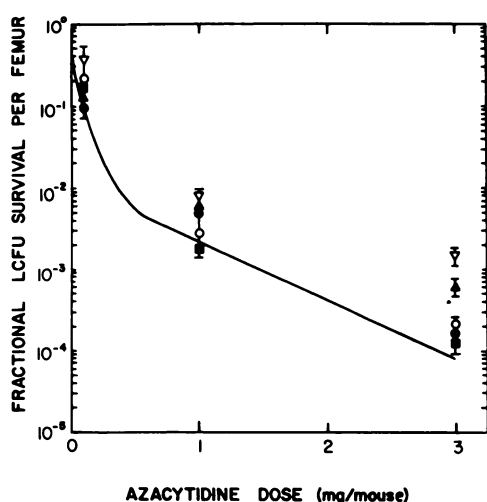


Chart 6. Stability of AzaCR in solution. The fractional survival of LCFU is plotted as a function of AzaCR dose. The periods of time after dilution are: immediate, taken from Chart 1 (—), 2 hr (●), 8 hr (▲), 24 hr (■), 2 weeks (○), and 4 weeks (▽). Fractional survival is calculated as in Chart 1.

25,000/cu mm, and hematocrit values, to below 25%. There was no increase in numbers of peripheral blood lymphoblasts at this time, indicating again the absence of leukemic proliferation. Deaths between Days 4 and 7 were determined at autopsy to be due to infection and hemorrhage. Gross and microscopic pathological examination of all tissues, including brain, demonstrated the persistence of mild leukemic infiltration, but there was no evidence of progressive leukemia that could account for death.

This suggested that marrow toxicity rather than leukemia caused the death of mice 6 to 8 days after doses of AzaCR much less than the  $LD_{50}$ <sup>30</sup>. Therefore, survival of NCFU in marrow of leukemic mice was measured 5 days following treatment with AzaCR, 0.5 mg/mouse. In order to reduce the LCFU in donor animals to undetectable levels, they were given cyclophosphamide, 2 mg/mouse i.v., 2 hr prior to sacrifice. This dose had previously been shown to reduce LCFU fractional survival to less than  $10^{-6}$  (12). The results were compared with the fractional survival of NCFU in normal mice that had also received AzaCR 5 days prior and cyclophosphamide 2 hr prior to sacrifice and assay. In leukemic mice, fractional NCFU survival was  $2.7 \times 10^{-2}$  while, in normal mice, it was 0.25. Thus, the survival of NCFU in leukemic mice was 11% that of normal mice 5 days after AzaCR treatment.

## DISCUSSION

Optimal chemotherapy requires knowledge of a number of factors relating to drug, tumor, and host. One such factor is the *in vivo* kinetics of tumor cell kill by clinically efficacious agents such as AzaCR.

*In vitro* studies have suggested that AzaCR is a cell cycle-specific agent which kills L1210 cells predominantly in S phase (7, 8, 16). However, the biphasic dose-survival curve obtained for both LCFU and NCFU (Chart 1) is inconsis-

ent with simple phase-specific cytotoxicity, since the more sensitive population seems to represent about 99% of the LCFU population. It is possible that, at low doses, AzaCR is S-phase specific, but at higher doses, its cytotoxicity becomes phase nonspecific, similar to that previously described for actinomycin D (18). In fact, the data of Li *et al.* (8) indicate cytotoxic effects in G<sub>1</sub>, G<sub>2</sub>, and M at a high drug concentration. A lack of phase specificity of AzaCR at higher doses is not surprising, since AzaCR not only inhibits DNA synthesis but also inhibits RNA and protein synthesis (7, 16), disrupts polyribosomes (2, 6), and produces a defective AzaCR-containing RNA (4).

Our time-survival studies indicate that survival of LCFU reaches a minimum 8 to 12 hr after AzaCR administration, which is consistent with the *in vitro* data of Lloyd *et al.* (9). However, repopulation was markedly dependent on the dose of AzaCR. At doses less than 0.2 mg/mouse, LCFU repopulation promptly occurred. At higher doses, little if any repopulation was apparent, a phenomenon unique to AzaCR. The phenomenon is not an artifact of the assay method, since white blood cell and differential counts of peripheral blood confirm the lack of progressive leukemic cell accumulation. In addition, survival was appropriately prolonged. The explanation for this phenomenon is not likely to be simple prolongation of generation time, since that would predict slower repopulation, which was not seen (Chart 2), as well as smaller spleen colonies, which were not observed. Persistent biological availability of AzaCR or an active metabolite is possible and might explain a lack of full NCFU repopulation after 14 days (Chart 3). An interesting, although presently unexplainable finding is that the dose at which the slope changed in the dose-survival curve of LCFU (Chart 1) corresponds to the dose at which the transition occurred between early repopulation and prolonged depression of femoral marrow LCFU found in the time-survival studies. Further studies to clarify the mechanism responsible for the prolonged depression of LCFU content are in progress.

This prolonged depression of LCFU content could produce spurious conclusions from mouse survival studies. It has been observed that cells surviving treatment with certain drugs proliferate at the same rate as before therapy, so that prolongation in survival is proportional to the number of cells killed (13). However, if repopulation is delayed for some period of time (as with AzaCR), then survival time would be prolonged in proportion to the length of time repopulation is delayed, even though there has been no additional killing of tumor cells. It is possible that this factor accounts for some of the observed prolongation in survival time of mice harboring L1210 leukemia treated with AzaCR in certain schedules (17, 19).

At low doses of AzaCR, equal cytotoxicity was observed, regardless of whether the drug was given as a single dose, split doses, or an infusion. Only with high doses in excess of 1 mg/mouse was a decreased cytotoxic effect of the infusion observed. This is consistent with the lack of significant schedule dependency demonstrated in survival time assays by Venditti (19). The decreased cytotoxic effect of infusion of high doses could be related to instability of the drug at room temperature.

It is significant that, despite the reported chemical instability of AzaCR in solution (9, 11), full cytotoxicity against LCFU was retained for 2 weeks with only a slight loss in effectiveness at 4 weeks. Although this cytotoxicity to LCFU may be due to metabolites, it indicates that infusions of AzaCR could be given without loss of therapeutic potential. The possibility of altered toxicity against NCFU or other tissues has not been studied.

The reduced NCFU survival in AzaCR-treated leukemic mice compared with AzaCR-treated nonleukemic mice is unexplained, but is consistent with the profound pancytopenia in leukemic mice 4 days after treatment with a high dose of AzaCR. It is possible that NCFU in leukemic mice are more sensitive to AzaCR due to an altered proliferative state, or it is possible that L1210 leukemia cells release an inhibitor of NCFU repopulation.

Therapeutically, the dose dependency of kinetics of AzaCR cytotoxicity suggests that the efficacy of regimens containing AzaCR may be dependent on whether high or low doses are used. At low doses of AzaCR, S-phase-specific cell kill might occur. The interaction of AzaCR at such doses, with other drugs, could be expected to differ from that at higher doses where phase-independent cell kill and delayed tumor repopulation are likely to occur.

#### ACKNOWLEDGMENTS

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