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# Effect of the Interval between High Dose 1-β-D-Arabinofuranosylcytosine Injections on Leukemic Cell Load, Intestinal Toxicity, and Normal Hematopoietic Stem Cells in a Rat Model for Acute Myelogenous Leukemia

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## ABSTRACT

**One injection of 1-β-D-arabinofuranosylcytosine (ara-C) in BN rats bearing myelocytic leukemia induces recruitment and synchronization of the leukemic cells. A second ara-C injection, given when the largest fraction of cells is in S phase, causes the largest reduction in leukemic clonogenic cells. The relevance of recruitment and synchronization of leukemic cells after high dose ara-C (200 mg/kg) by rapid i.v. injection (comparable with 1 g/m² in patients) has been tested in rats with respect to survival time and toxicity. Several groups of leukemic rats have been treated with seven injections of ara-C; the intervals between the injections per group were 4, 6, 9, 12, 15, 18, and 24 h, respectively. The longest mean survival time is observed in the group treated every 9 h which is 70.8 days compared to 22.6 days in nontreated leukemic controls. This 9-h interval of ara-C administration corresponds with the moment when DNA synthesis of the leukemic cells resumes after its inhibition by the ara-C. The most severe toxic side effects on the gastrointestinal system are observed in the group that received ara-C every 6 h; no toxic death has occurred in the animals treated with 15-h or longer intervals. The effect of the increasing interval between two ara-C injections on the normal hematopoietic stem cells has been measured with the colony forming unit spleen assay. This study showed that the reduction of normal stem cells due to ara-C is independent of the interval of administration. This differential effect of ara-C on leukemic and normal hematopoietic stem cell kinetics might in part explain the mechanisms of achieving a complete remission in acute leukemia.**

## INTRODUCTION

The BNML[1] model has in common with human acute myeloid leukemia that a considerable number of the leukemic cells are not actively proliferating (1). Whether these cells are "quiescent" $G_0$ cells which can be triggered in cell proliferation phase after an appropriate stimulus or are $G_1$ cells with a very long or variable cell cycle time (2) has not yet been elucidated. In clinical leukemia, much attention has been focused on the effect that ara-C exerts on the number of cells in S phase (3–6).[2]

Lampkin *et al.* (4) and Smets *et al.* (6) reported that the killing of the cells in S phase by ara-C is followed by recruitment and synchronization of the leukemic cells. In the BN myeloid leukemia, recruitment and synchronization of the leukemic cells after an injection of ara-C are observed (7). An increased influx of cells in proliferative phase takes place and the administration of a second ara-C injection at this moment results in a very effective tumor load reduction, as measured with the LCFU-S assay. This effect is most pronounced when the second ara-C injection is given at the time when the cells are accumulated in S phase (8). If normal hematopoietic stem cells are not or at least less involved in the process of recruitment and synchronization, a therapeutic gain can be achieved.

In this study which is performed in the BNML model, data are presented which show that ara-C has no recruiting and synchronizing effect on normal hematopoietic stem cells, in contrast to the effect on leukemic cells. In addition, it shows that repeated ara-C injections induce an intestinal toxicity syndrome the severity of which is dependent on the time interval between ara-C injections.

## MATERIALS AND METHODS

**Animals.** Female inbred brown Norway (BN) rats, 12–14 weeks old and ranging in weight from 150 to 190 g, were used. The CFU-S experiments were done in C57BL/L1 Ry × C3t/LW Ry $F_1$ mice. The animals were raised in the Rijswijk Colony of the Radiobiological Institute TNO, Rijswijk, the Netherlands.

**Rat Leukemia Model (BNML).** The BNML model has been described in detail elsewhere (1, 9). Briefly the leukemia was chemically induced in a female BN rat by 9,10-dimethyl-1,2-benzanthracene. It shows a reproducible growth pattern upon i.v. cellular transfer within BN rats. Cytologically and cytochemically it is similar to human acute promyelocytic leukemia, and a severe suppression of normal hematopoiesis occurs during the disease. In contrast to other leukemia models (L1210, L5222) the growth fraction is relatively low, 0.60 to 0.40. An inoculum of $10^7$ BNML cells i.v. kills the rats in 19–24 days.

**Bone Marrow Cells.** Femurs (of BN rats) were excised and adherent muscle tissue was removed. They were then broken into two parts and cells from each fragment were collected by repeated flushing with Hanks' solution and scraping with a bent needle placed on a syringe. The collected bone marrow cells were filtered through nylon gauze to obtain a monocellular suspension. The concentration of nucleated cells in suspension was determined by counting the cells in Türck's solution in a hemocytometer.

**CFU-S Assay for Normal Rat Hematopoietic Stem Cells.** This method of quantifying the number of pluripotent HSC is first described by Till and McCulloch (10) for mouse bone marrow and has been modified for the rat (11). The CFU-S are determined by pooling femoral bone marrow cells of three rats. A fraction of the suspension is injected in 15 C57 BL/L1 Ry × C3H/Lw Ry $F_1$ mice which have received 10.25 Gy total body γ-irradiation.

At day 9 after injection of the bone marrow cells, the mice are killed and after fixation of the spleens the macroscopically visible colonies are counted. A linear relationship has been found between the number of cells injected and the number of spleen colonies formed.

**Hematological Supportive Care.** In the experiments in which the survival time was evaluated after intensive treatment with ara-C, blood transfusions were given to all rats to prevent the animals from dying due to anemia and thrombocytopenia. Therefore 1.5–2 ml blood were given on days 4, 6, 9, and 11 after start of treatment. The donor blood was obtained from retired BN female and male breeders. To prevent coagulation 30 IU of heparin/ml were mixed with the blood in the syringe.

**Experimental Design.** The ara-C dose chosen for this study was based on dose-response data which showed a plateau curve for ara-C at dosages above 200 mg/kg (12). The effect of different intervals between ara-C injections on the leukemic cell load has been evaluated by means of survival time experiments. Groups of rats have been treated with 7 rapid i.v. injections of ara-C, each group with its own interval of 4, 6,

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[1] The abbreviations used are: BNML, brown Norway myelocytic leukemia; ara-C, 1-β-D-arabinofuranosylcytosine; HSC, hematopoietic stem cells; CFU-S, colony forming unit spleen; LCFU-S, leukemic colony forming unit spleen.

[2] L. P. Colly, R. Willemze, M. W. Honders, and W. G. Peters. Synchronization of leukemic cells after an i.v. injection of ara-C 1000 mg/m² in 9/10 patients with acute myelogenous leukemia (AML), submitted for publication.



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12, 15, 18, and 24 h, respectively. In these studies, treatment was started at day 14 after an inoculum of $10^7$ leukemic cells i.v. At this stage of the disease, the bone marrow contains 90–95% leukemic blast cells, which start to appear also in the peripheral blood.

Effects of the increase of the interval between two ara-C injections, (3, 6, 8, 10, 12, 14, and 17 h, respectively) on normal hematopoietic stem cells have been evaluated by means of the CFU-S assay. This study was performed in normal rats, because normal hematopoiesis including CFU-S is severely suppressed in leukemic rats (13).

## RESULTS

The survival time of groups of rats that have received either no treatment (leukemic control group) or 7 injections of ara-C (200 mg/kg rapid i.v.) is shown in Fig. 1. Each group of rats received 7 ara-C injections at its own interval. The longest survival time is observed for the group which received 7 injections of ara-C every 9 h. In contrast to this beneficial effect of the treatment resulting in a mean survival of 70 days, no therapeutic gain is achieved when the ara-C injections are given with a 24-h interval because the latter group of rats died at the same time as the leukemic controls (no treatment).

All dead animals have been subjected to autopsy in order to determine whether they died from relapse of the leukemia (large spleen and liver) or from other (macroscopically visible) pathologic conditions.

All the animals which died immediately after start of the treatment and during the first week thereafter have been regarded as having died due to toxicity. The predominant cause of death was of intestinal origin [severe diarrhea and extended intestines filled with (bloody) fluid material]. In Fig. 2 the percentage of rats in each group that died from leukemia or toxicity, respectively, is shown. The ara-C treatment given with a 6-h interval was extremely toxic; 15 of 19 rats died from intestinal toxicity, compared to only 3 of 22 in the group injected at 12-hourly intervals. In the groups treated with a 15-h interval or longer no rats died from intestinal problems.

In Fig. 3 the effect of increasing the interval between 2 ara-C injections is shown on normal stem cells as measured as CFU-S. No significant difference in the number of CFU-S in the untreated control group and the rats that received 1 injection of ara-C is seen. After variation of the interval between 2 ara-C injections from 3 to 17 h, no additional stem cell reduction is observed; this plateau level is not significantly different from the control group or from the rats that received only 1 injection of ara-C.

## DISCUSSION

The cell killing effect of ara-C is due to its S-phase specific cytotoxicity (14). The property to cause a transient block in the cell cycle traverse of cells from $G_1$ to S (15) might induce the effect of synchronization of leukemic cells. Flow cytometry studies in the BNML show the mentioned properties in succession (Fig. 4); shortly after i.v. injection of the drug, a small number of cells in S phase (killing + blocking in $G_1$-S) is detected and a sudden release of the blocking effect on the $G_1$-S boundary between 8 and 10 h after injection is followed by an accumulation of cells in S phase which is explained as recruitment and synchronization of leukemic cells (7, 8).

In survival experiments shown in Fig. 1, the most effective treatment consists of repeated ara-C injections given when the synchronized cells accumulate in early S phase; Fig. 1 also

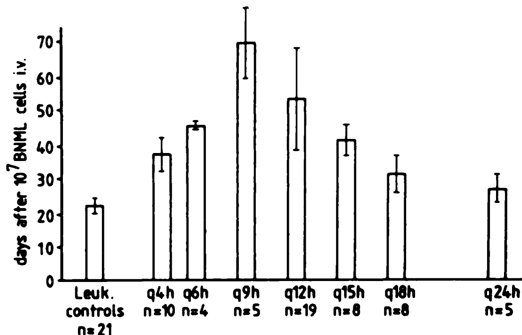


Fig. 1. Mean survival time ± SD (*bars*) of groups of rats which have obtained either no treatment (leukemic controls) or 7 ara-C injections (200 mg/kg rapid i.v.). Each group has been treated with its own interval.

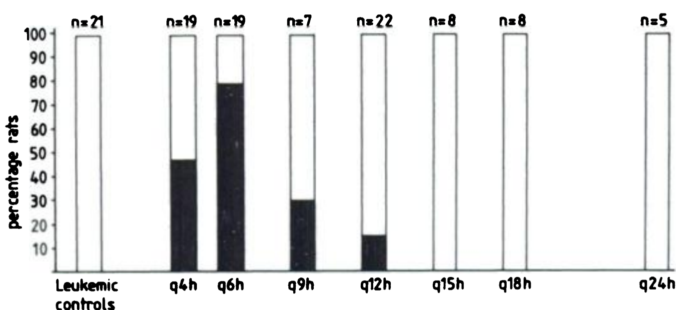


Fig. 2. Comparison of cause of death (■, toxicity; □, leukemia) of BNML rats; same study as in Fig. 1.

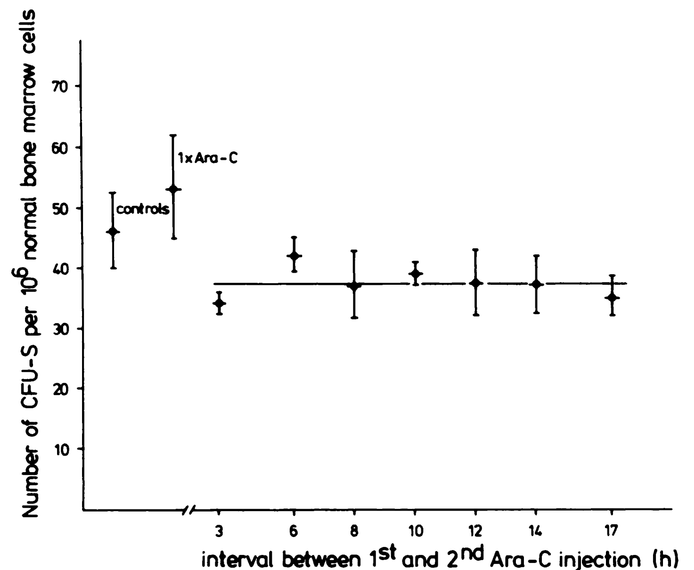


Fig. 3. Changes in the number of CFU-S ± SE when the second ara-C injection was given at various time intervals after the first one in normal rats. The animals were sacrificed at 3 h after the second ara-C injection.

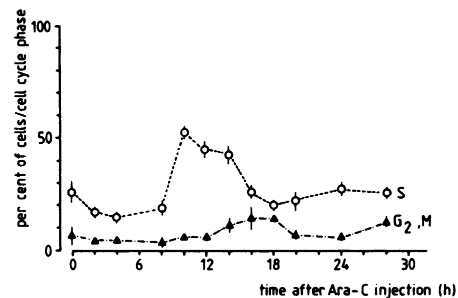


Fig. 4. Flow cytometric measurements of the distribution of leukemic bone marrow cells over S and $G_2$-M phases after one i.v. injection of ara-C (200 mg/kg). Each *point* represents means SD (*bars*) of 5–10 separate experiments.



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shows that treatment is less effective if the cells have almost finished DNA synthesis (the 15-h interval group). Hardly any increase in survival time is observed for the group of animals which have received ara-C every 24 h; at 16–30 h after one ara-C injection about 25% of cells are in S phase. This lack of effect in the latter group might be due to the fact that the cell cycle time of the BNML is 14 h (1), so that cells can continue the cell cycle phases in between the ara-C injections. The group treated every 9 h has shown a mean survival time of 70.8 ± 10.3 (SD) days. The tumor load at day 14, the starting day of the treatment, is $5 \times 10^9$ leukemic cells. It can be calculated from a dose-response curve (number of injected leukemic cells *versus* survival time) that the observed 70.8 days correspond with a reduction in tumor of 7–8 logs (8). These data also have shown that the animals can survive the aplastic period with hematological supportive care consisting only of blood transfusions, without bone marrow transplantation. The observed relationship between ara-C interval and effect on the tumor load, expressed as increase in survival time, is similar to that observed in a LCFU-S assay, performed after 2 doses of ara-C (Fig. 1; Ref. 8). The influence of time between the subsequent ara-C injections in both types of experiments (survival time and LCFU-S data) shows the same pattern. This effect is easily explainable regarding the curve of S-phase cells after ara-C administration in cell kinetic studies as described above. The survival and the LCFU-S curves reflect the cell kinetic measurements after one injection of ara-C. A comparable assay for the LCFU-S, but focusing on normal HSC, is the CFU-S assay. Flow cytometric determination of the cell cycle phases of the stem cells is not a sensitive assay because these cells are rare in normal bone marrow; less than 1% of the nucleated cells is a hematopoietic stem cell (11). Because the LCFU-S reflect the S-phase compartment in cell kinetic studies of leukemic cells after ara-C administration, so the CFU-S data are assumed to depict the effect of ara-C on the S-phase cells of the normal stem cells. In Fig. 3 it is shown that no ara-C interval dependency is observed; this is in sharp contrast with what is observed for the leukemic cells. In these kinds of biological assays the variation is considerable, which might explain the overlapping value for the controls and for the group which has obtained one injection of ara-C. In a study previously published (12) it has been shown that the killed fraction of cells of the CFU-S compartment increases after 7 repeated ara-C injections. This suggests that CFU-S in rapidly proliferating bone marrow is more vulnerable to the toxic effect of ara-C than CFU-S in normal proliferating bone marrow.

With respect to the toxic death after ara-C treatment as shown in Fig. 2, the explanation for the high mortality observed in the animals treated with the shorter interval might be that the intestinal crypt cells are synchronized and no cells can continue through S phase without being damaged by ara-C; this event might result in a loss of crypt and villous cells in the intestinal mucosa. In mice, Phelps (16) has described a drop in the labeling index of the intestinal crypt cells immediately after ara-C administration, followed by an increase of DNA synthesis 6 h later. The observation in our study suggests that a similar phenomenon occurs in the rat intestinal cells. Further research on this subject is in progress in our institute.

The data presented in this article show that ara-C can reduce the leukemic cell mass very effectively due to recruitment and synchronization of the leukemic cells when the optimal interval of treatment (9 h) is used. The intestinal cells probably synchronize too. The most pronounced toxicity is observed when the interval between subsequent ara-C injections is 6 h, whereas no ara-C interval dependency is observed for hematopoietic stem cells.

These observations suggest that leukemic cells can be recruited and synchronized much more easily than the normal stem cells, which might in part explain the differential sensitivity of these cells for ara-C.

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