

Increased Life-span in AKR Leukemia Mice Treated with Prophylactic Chemotherapy

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SUMMARY

AKR mice were treated prophylactically (age 5 to 10 months) on single-drug schedules of Cytoxan, methotrexate, or 1,3-bis(2-chloroethyl)-1-nitrosourea. The schedules in which methotrexate or 1,3-bis(2-chloroethyl)-1-nitrosourea were used were ineffective. The mean survival time and 12-month survival rate for each of the 5 nontoxic Cytoxan-treated groups were significantly greater than the mean survival time and 12-month survival rate of untreated controls. The 12-month survival rates for the 5 Cytoxan groups were approximately 50% compared to 17% for controls.

In a second experiment in which mice were treated from age 4 to 9 months the increases in survival time were similar to the above. In addition a group that received Cytoxan and methotrexate in combination had a further increase in mean survival time.

The data indicate the therapeutic effectiveness of prophylactic chemotherapy of AKR mice. A number of hypotheses are provided to explain the increases in survival with emphasis on the possible existence of an early lag phase in tumor growth.

INTRODUCTION

From 80 to 90% of AKR mice die of spontaneous leukemia by 1 year of age. The Gross leukemia virus initiates the disease (1), primarily in the thymus (3, 5). Subsequently, there is enlargement of the thymus, lymph nodes, and spleen by lymphoma tissue (6). The spontaneous onset of the tumor has provided rationale for the increasing use of the AKR mouse as a model to develop improved therapy for human neoplasms (9, 10).

The time between the detectable appearance of lymphoma cells in the thymus and clinically diagnosable widely disseminated disease (10^9 cells) is about 1 month (9). The overall doubling time before diagnosis is about 1 day. Death at 10^{10} cells is noted about 15 days after diagnosis in untreated animals and occurs, on an average, at 9.5 months of age.

Skipper *et al.* (10) have comprehensively tested various cytotoxic agents, administered after diagnosis, for their

effect on survival time of the mice. Most drugs were without significant effect. However, Cyt² administered alone, in a 200 mg/kg dose and repeated every 10 days (to a total of 4 doses), resulted in 25% survivors 60 days after diagnosis. Similarly, Cyt and ara-C administered in very substantial doses resulted in 33 to 46% 60-day survivors.

The present study was conducted to determine whether prophylactic chemotherapy could increase the life-span of AKR mice. Pollard and Sharon (8) have reported that increased life-span of 15 germ-free AKR mice occurred after treatment with Cyt from age 6 months. We have reported the results of experiments comparing the effect of various schedules of Cyt on the life-span of AKR mice when administered to the mice from 5 to 10 months of age (11). This report includes the above mice followed to 18 months of age and includes a 2nd experiment in which the mice were treated from 4 to 9 months of age.

MATERIALS AND METHODS

Mice. AKR/Cr mice were received from Drug Research and Development, Division of Cancer Treatment, National Cancer Institute, at 8 weeks of age. They were housed in plastic cages in a constant temperature facility and provided with water and laboratory chow *ad libitum*.

Randomization. Approximately 5% of the mice died or evidenced runting and weight loss before treatment was initiated; they were discarded. Males and females were randomized separately, 4 to 5 mice/cage. Cages with males and cages with females were randomized separately to 10 groups. Therefore, each group contained approximately the same percentage (50%) of each sex. Two groups were randomly chosen as untreated control groups. The other 8 groups were assigned to the various treatment schedules.

Drug. Cyclophosphamide (Cyt; Mead Johnson Co., Evansville, Ind.; NSC 26271 and BCNU; NSC 409962) were dissolved in a sterile 0.85% NaCl solution immediately before use. Both drugs were administered i.p. Amethopterin (MTX; Lederle Laboratories, Pearl River, N. Y.; NSC 740) was dissolved in 2% sodium bicarbonate immediately before use and was administered i.p. All drugs were administered in a volume of 0.01 ml/g body weight. In treatment groups,

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² The abbreviations used are: Cyt, Cytoxan; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; MTX, methotrexate; q1w, weekly; q2w, every 2 weeks; q4w, every 4 weeks; 12 sr, 12-month survival rate; 15 sr, 15-month survival rate; MST, mean survival time.

Table 1

Prophylactic chemotherapy of AKR mice

Each treatment group of approximately 41 mice received the drugs i.p. from age 5 to 10 months. There were 86 control mice.

Drug	Dose schedule (mg/kg)	% survivors			MST ^a (mo.)
		10-mo.	12-mo. ^b	15-mo. ^b	
Cyt	120, q1w	22	2 ^c	0 ^c	8.6 ^c ± 0.24 ^d
Cyt	120, q2w	63	53 ^e	25 ^e	11.8 ^e ± 0.61
Cyt	120, q4w	64	50 ^e	17	11.6 ^e ± 0.48
Cyt	60, q1w	65	50 ^e	30 ^e	11.8 ^e ± 0.59
Cyt	60, q2w	69	48 ^e	21	12.0 ^e ± 0.52
Cyt	60, q4w	66	46 ^e	27 ^e	12.2 ^e ± 0.57
MTX	20, q1w	24	15	7	9.0 ± 0.48
BCNU	10, q1w	40	13	3	9.3 ± 0.37
Controls		41	17	9	10.0 ± 0.30

^a Mice that were alive at 18 months of age were counted as 18-month survivors for computation of MST. The true MST should not differ significantly from given values because there were few (or no) 18-month survivors in each group. The analysis is based on the least significant difference test using the log-transformed survival time.

^b The χ^2 test was used to compare the 12- and 15-month survivors.

^c Values are significantly smaller than control ($p < 0.05$).

^d Mean ± S.E.

^e Values are significantly greater than control ($p < 0.05$).

therapy was initiated at 5 months of age and continued until 10 months of age in the 1st experiment. In the 2nd experiment mice were treated from 4 to 9 months of age.

Mortality and Spleen Weights. Mice were checked for mortality daily until 1 year of age and then twice weekly. Spleens were removed and weighed twice weekly from mice that died within the previous 24 hr.

RESULTS

Experiment 1

The survival time of 9 different groups were compared (Table 1).

Six groups of mice received Cyt. Three groups of mice received 120 mg/kg q1w, q2w, or q4w, respectively. Three other groups received Cyt, 60 mg/kg, either q1w, q2w, or q4w. One group of mice received a weekly schedule of MTX and 1 group received BCNU weekly. The 12 sr, 15 sr, and MST of untreated controls were 17%, 9%, and 10.0 months, respectively.

Cytosan. Cyt, 120 mg/kg q1w, was toxic; and the 12 sr, 15 sr, and MST of the group on this schedule were significantly smaller than for untreated controls. In the other 5 Cyt-treated groups the 12 sr ranged from 46 to 53%, significantly greater than that of controls ($p < 0.01$). The 15 sr was increased in the 5 groups and significantly ($p < 0.05$) in 3 groups, to 25 to 30%. The MST of all 5 groups were increased significantly to 11.6 to 12.2 months; $p < 0.001$. The results of these 5 groups with respect to 12 sr, 15 sr, and MST were similar. Although the 3 values for the 2nd group (Cyt, 120 mg/kg q2w) were significantly increased, there were a few early toxic deaths, which accounts primarily for why the standard error of the group was the greatest.

MTX and BCNU. The schedules used for both MTX and BCNU were ineffective. Furthermore, some early toxic deaths occurred with MTX.

We have directed our attention particularly to Group 6, which received the least amount of Cyt (60 mg/kg q4w). The survival curve for this group is shown (Chart 1) and is compared to controls. The curves plotted on a semilog scale demonstrate an initial shoulder region to about 8 to 10 months, after which they become linear with divergent slopes.³

Experiment 2

The survival time of 11 groups were compared (Table 2). Treatment was initiated earlier (4 months) and terminated

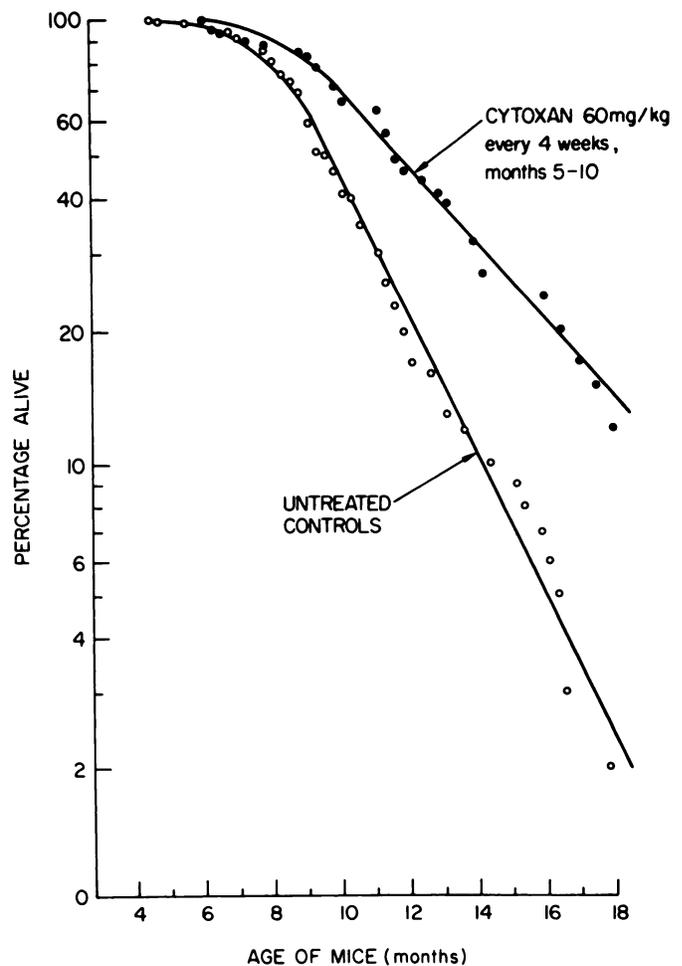


Chart 1. Comparison of survival curves of untreated AKR mice and AKR mice treated with Cyt, 60 mg/kg q4w, Months 5 to 10. Data were collected until the mice were 18 months of age.

³ An equation to estimate the proportion of the survivors in the 2 groups (Chart 1) based on a log normal assumption was computed. The estimated percentage of survivors was computed from $100 [1 - F(t)]$ with $F(t) = P[Z \leq (\log t - \hat{\mu})/\hat{\sigma}]$, where Z has a standard normal distribution, t denotes the month, and $\hat{\mu}$ and $\hat{\sigma}$ denote the estimated mean and standard deviation of Z , respectively. The estimated percentage of survivors is similar to the observed percentages in both groups.

Table 2
Prophylactic chemotherapy of AKR mice

Each treatment group of approximately 42 mice received the drugs i.p. from age 4 to 9 months. There were 68 control mice.

Drug	Dose schedule (mg/kg)	% survivors			MST ^a (mo.)
		9 mo.	12 mo. ^b	15 mo. ^b	
Cyt	120, q1w	0	0 ^c	0	6.4 ^c ± 0.20 ^d
Cyt	120, q2w	67	39	32 ^e	11.5 ^e ± 0.69
Cyt	120, q4w	74	62 ^e	29 ^e	12.1 ^e ± 0.62
Cyt	60, q1w	23	16	7	8.2 ^c ± 0.48
Cyt	60, q2w	68	44	28 ^e	11.8 ^e ± 0.63
Cyt	60, q4w	84	69 ^e	24	12.8 ^{e, #} ± 0.49
MTX	20, q1w	0	0 ^c	0	6.5 ^c ± 0.14
BCNU	10, q1w	32	8	3	8.8 ^c ± 0.31
Cyt and MTX	60, q2w	50	48	29 ^e	10.7 ± 0.75
Cyt and MTX	20, b1w, ^f q2w				
Cyt and MTX	60, q4w	83	62 ^e	29 ^e	13.2 ^{e, #} ± 0.51
Cyt and MTX	20, b1w, q4w				
Controls		66	32	12	10.6 ± 0.44

^aMice that were alive at 18 months of age were counted as 18-month survivors for computation of MST. The true MST should not differ significantly from given values because there were few (or no) 18-month survivors in each group. The analysis is based on the least significant difference test using the log-transformed survival time.

^bThe χ^2 test was used to compare the number of 12- and 15-month survivors.

^cValues are significantly smaller than control ($p < 0.01$).

^dMean ± S.E.

^eValues are significantly greater than control ($p < 0.05$).

^fb1w, twice a week.

[#]Values are significantly greater than of any other group ($p < 0.05$).

earlier (9 months) than in Experiment 1 (Table 1). Six groups of mice received Cyt as in Experiment 1. Cyt, 120 mg/kg q1w, was again toxic. In addition Cyt, 60 mg/kg q1w, was toxic in this experiment. The MST's of the 4 other Cyt groups were significantly greater than that of controls ($p < 0.05$). The MST of the group that received Cyt, 60 mg/kg q4w, was 12.8 months, 2.2 months longer than the MST of controls (10.6 months). A difference of 2.2 months in MST's was also noted for the corresponding groups in Experiment 1. Again, MTX and BCNU were not beneficial on the schedules tested. Two combination schedules in which Cyt and MTX were used were tested. Mice treated with Cyt, 60 mg/kg q2w, and MTX, 20 mg/kg twice weekly q2w, evidenced a bimodal death distribution. There were a number of early toxic deaths. However, the 15 sr was significantly increased. The group that received Cyt, 60 mg/kg q4w, and MTX, 20 mg/kg twice weekly q4w, survived the longest (MST, 13.2 months). Its MST was significantly greater than that of all other groups except the mice that had received Cyt, 60 mg/kg q4w.

Spleen weights of untreated controls at death and of treated groups, except where toxicity was clearly evident, were over 135 mg. This is greater than 3 S.D. above the mean spleen weight (90 mg) of nonleukemic mice (M. J. Straus, unpublished data), indicating that these animals died with leukemia. The mean spleen weight of untreated

controls at death was 411 mg, and the mean for the treated animals was 398 mg.

DISCUSSION

These data indicate that significant increases in survival time are achieved on a number of schedules of Cyt therapy when prophylactic treatment of AKR mice is initiated prior to the time of grossly detectable disease. The group with the longest MST in the 1st experiment was treated with Cyt, 60 mg/kg q4w; MST, 12.2 months. This represents a 66-day increase over the MST of untreated controls. This schedule was without demonstrable toxicity for the host. The results obtained in this group and in the other 4 "nontoxic" Cyt groups compare favorably with the reported median survival times (of 30 to 35 days after day of control death) in AKR mice treated after diagnosis (10).

In the 2nd experiment the group with the longest MST, treated with Cyt alone, also received Cyt, 60 mg/kg q4w; MST, 12.8 months. The addition of MTX to this schedule in another group resulted in a further increase in MST of 13.2 months. This represents an 80-day increase over the MST of untreated controls.

Using 1st generation AK leukemia cell transplants, Skipper *et al.* (10) suggested that following the appearance of 1 tumor cell the time to death is less than 2 months. If we were to assume that death invariably took place within 3 months after the appearance of the 1st tumor cells, then the present results would suggest that some animals were "cleared" of tumor at the time of termination of prophylactic treatment. In such case, for example, mice alive at 13 months would have had no tumor at 10 months. Fourteen % of control animals were alive at 13 months and 35% were alive in the group that received the least amount of Cyt. The difference is 21%, which would represent the additional percentage of mice free of tumor at 10 months in the treated group.

Similarly, if one then looks at the percentage alive in the 2 groups at 16 months, it is 5% for controls and 14% for treated mice. The difference is 9% which would represent the additional percentage of mice free of tumor at 13 months as the result of treatment. Nine % of controls died between 13 and 16 months, and therefore 9% apparently developed tumor spontaneously between 10 and 13 months. Similarly, 21% of treated mice died between 13 and 16 months and would have developed tumor between 10 and 13 months which is much higher than the expected 9%, suggesting the possibility of reinduction of tumor by the virus in approximately 12% of the mice. Such viral reinduction of disease in mice apparently "cured" of systemic AKR leukemia has been suggested by Skipper and Schabel (9, 10).

In the foregoing the long-term survival of some animals following termination of prophylactic treatment has been explained on the basis of complete clearance of tumor cells at that time. Alternatively, such long-term survival could be explained even if tumor cell clearance did not occur. Surviving tumor cells could have had reduced growth rates

whether through direct modification by prophylactic treatment or because of preferential survival of tumor cells with lower growth rates. In either case, since treatment with tolerated intermittent doses of Cyt exerted a prophylactic effect it is suggested that continuation of such treatment beyond the 10th month would be worthy of exploration. Continuation of therapy could result in further inhibition of tumor growth in instances in which the tumor cell population has not become extensive. Also, such treatment might prolong the period of inhibition of primary induction of tumor cells and tend to inhibit any reinduction in animals that may have been "cleared" of the disease.

Skipper and Schabel have reported that the time between when detectable lymphoma cells appear in the thymus to the time of diagnosis of systemic disease (10^9 cells) is approximately 1 month. Such rapid growth with a short doubling time (1 day) for the tumor cell population would suggest that, within the limits of cumulative toxicity, treatment be administered at relatively short time intervals in order to avoid "escape" of the tumor cell population during the interval between treatments.

The observation of a prophylactic effect with a low dose of Cyt given at monthly intervals is noteworthy. Following termination of therapy the number of mice in which prolongation of a "tumor-free" state is estimated to have occurred would appear to be higher than might have been expected in the presence of exponential leukemic growth of newly induced tumor cells (doubling time, 1 day). The marked increases in survival in groups in Experiment 2, in which treatment was terminated 1.6 months before the MST of controls, suggests that spontaneous AKR tumor growth may be slower than predicted from transplant assays (9, 10).

It is not clear to what extent the therapy exerts its effect on target cells in the thymus either before or during leukemic transformation, prior to the time when the tumor cells are actually detectable in the thymus. It is possible that the transition to cancer occurs at a relatively low rate in the thymus, constituting a form of lag phase, and that the cells at this time are highly susceptible to therapy. High susceptibility of leukemic target cells prior to induction in early leukemic transition stages could contribute to the effectiveness of low intermittent (monthly) doses of Cyt. Alternatively, the therapy may exert some effect on the metastatic process.

It has also not been determined whether the therapy has interfered with viral replication. The higher incidence of deaths in treatment groups as compared with controls, following termination of therapy, suggests the possibility of tumor cell reinduction by a higher titer of virus.

The action of drugs on target cells in the predetectable stages of spontaneous leukemia and on viral replication, as well as on viral-cellular interactions, is worthy of further investigation.

An additional basis for early delayed growth may be immune capacity for cell kill of small numbers of cells as has been suggested by Mathé *et al.* (4) and others. If the immune capacity were exceeded, tumor cell growth would then proceed exponentially. Depression of immune response

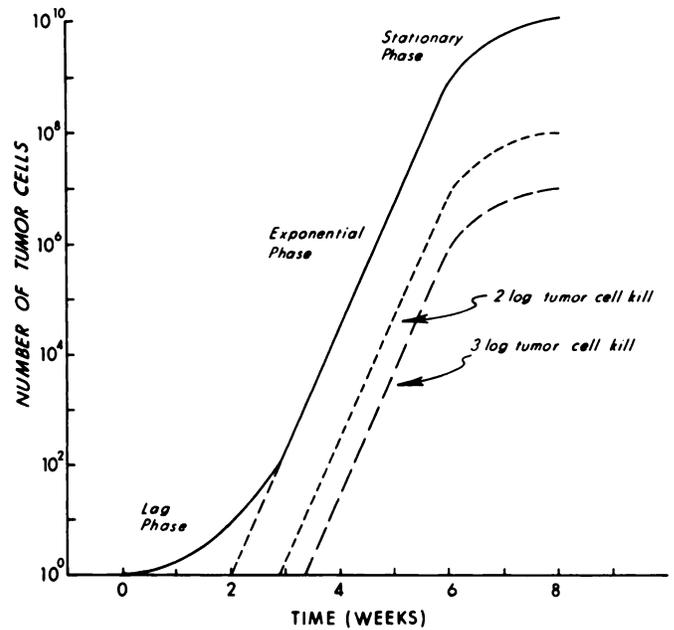


Chart 2. A schematic comparison of 2 possible growth curves for spontaneous AK leukemia. One curve indicates that initial tumor growth is exponential. Another curve includes an early lag phase from 10^0 to 10^2 cells followed by exponential growth with a doubling time of 1 day. Two additional curves indicate the reduction in tumor cell population that would occur following administration of a dose of drug that kills 3 logs or 2 logs of tumor cells. (It is assumed that a dose of drug kills the same percentage of cells irrespective of tumor size.)

in mice with AKR leukemia has been reported (7), although a recent report by Hargis and Malkiel (2) indicates that AKR leukemic mice exhibit normal immediate and delayed hypersensitivity reactions.

The constructed growth curve of spontaneous AKR leukemia, which includes an initial lag phase (11) from 10^0 to about 10^2 cells with exponential growth from 10^2 to 10^9 cells followed by slower growth to death, is shown in Chart 2. The curve is of a Gompertzian form. If a small initial lag phase exists that increases tumor growth by 2 weeks, then the potential for achieving cure with a dose of drug that kills 2 or 3 logs of cells⁴ is markedly enhanced.⁵

The potential cure rate is based on the assumption of a Poisson distribution for the number of surviving tumor cells following drug treatment. If the treatment kills 3 logs of cells it will reduce a cell population of 10 to an average of 0.01, and there is then a 99% probability that all cells will have been destroyed and thus that cure was achieved. The corresponding cure rates are 90.4% for 100 initial cells, 36.8% for 1000 initial cells, and 0.005% for 10,000 initial cells. The chance of cure is thus enhanced where there is a prolonged lag period during which the cell population is small, so that the probability of extinguishing all tumor cells is high. Where not all tumor cells are destroyed by a 1st prophylactic treatment, the existence of a lag period can

⁴ Cyt, 60 mg/kg, has not killed more than 3 decades of tumor cells in almost every *in vivo* mouse tumor system tested.

⁵ The calculation of the cure rate would depend on the distribution of the tumor cell population at the time of treatment.

serve to keep cell populations limited at the time of the 2nd prophylactic treatment so that again a high probability of cure may exist.

In order to achieve a comparable increase in cure rate by maintaining early exponential growth (10^9 to 10^8 cells) and increasing the doubling time (by altering slope), it would be necessary to increase the total time of exponential tumor growth by more than 6 weeks.

The early lag phase then facilitates cure with low doses of drug given at infrequent intervals and would not necessitate large increases to the estimated time of tumor growth of 1 cell to death. Furthermore, early-lag-phase tumor growth is consistent with growth patterns demonstrated in other biological systems, particularly bacterial growth.

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