Eur J Cancer Clin Oncol, Vol. 18, No. 8, pp. 763–769, 1982.
Printed in Great Britain.

0277-5379/82/080763-07$03.00/0

High-dose Cyclophosphamide Treatment of Acute Myelocytic Leukemia. Studies in the BNML Rat Model*

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Abstract—*In view of its application in patients with acute leukemia prior to bone marrow transplantation, the toxicity and efficacy of high-dose cyclophosphamide treatment were evaluated in a rat model for human acute myelocytic leukemia (BNML). The LD_{50} in leukemic rats proved to be lower than that in normal rats* (100 *vs* 164 mg/kg *respectively*). *With dosages above* 120 mg/kg, *bone marrow transplantation was required to overcome irreversible aplasia. Additional causes of death were lung-, bladder- and intestinal tract hemorrhages* (160–200 mg/kg) *or acute cardiopulmonary failure* (250–300 mg/kg). *In leukemic rats, excessive leukemic cell kill leading to tumor cell embolism was another contributing factor. In this respect, treatment of late-stage leukemia proved invariably fatal. In leukemic rats, the highest therapeutic index was achieved with* 100 mg/kg. *Depending on the stage of disease, a* 5–8.5 *log leukemic cell kill was achieved. An increased proliferation rate of residual leukemic cells after cyclophosphamide treatment appeared likely. Finally, the present data are extrapolated to the current treatment of human acute myelocytic leukemia in complete remission with high-dose cyclophosphamide in combination with supralethal total-body irradiation.*

INTRODUCTION

ALTHOUGH results of combination chemotherapy in acute myelocytic leukemia (AML) have improved significantly during the past ten years, relapse of leukemia still remains the main reason for ultimate treatment failure [1–4]. Obviously, residual drug-resistant leukemic cells are responsible in this respect. Attempts have been made to prevent the development of these clones by subjecting patients to high-dose chemo-radiotherapy at a stage of minimal residual disease, i.e. during the phase of complete remission. This treatment modality aims at eradicating the leukemia completely, but inevitably induces irreversible, lethal damage to the normal hemopoietic apparatus at the same time. Therefore, subsequent (allogeneic) bone marrow transplantation (BMT) is required. So far, results in terms of long-lasting leukemia-free survival look promising [5–7], although death due to toxicity, interstitial pneumonia, graft-vs-host disease and leukemia relapse is still a major problem. The commonly used conditioning regimen consists of cyclophosphamide (cyclo; 2×60 mg/kg) followed by total-body irradiation (TBI; 6.5–10.0 Gy). The advantage of using cyclo is three-fold. Firstly, in general, previous remission-induction chemotherapy does not include cyclo. Thus, drug resistance can be assumed to be minimal. Secondly, the alkylating action of the drug provides equal activity against proliferating and non-proliferating leukemic cells. In the third place, cyclo is a potent inducer of immunosuppression and will thereby prevent the host from rejecting the allogeneic bone marrow graft.

Since it is difficult, if not impossible, to determine tumor load reduction quantitatively in human patients, the efficacy of high-dose cyclo treatment was studied in a transplantable rat leukemia model (BNML), which shares many essential characteristics with human AML [8–10].

Accepted 2 March 1982.
*These studies were supported by the Koningin Wilhelmina Fonds of the Dutch National Cancer League (Grants No. RBI 76/4 and 80/1).
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MATERIALS AND METHODS

Experimental animals

The experiments were performed with the inbred Brown Norway (BN) rat strain produced in the Rijswijk colony. Male rats between 12 and 16 weeks of age were used (body weight, 190–230 g).

Rat leukemia model

The rat leukemia model (BNML) has been described in detail elsewhere (origin, classification, transplantation procedure, growth characteristics, etc. [8–10]). The leukemia was induced in a female BN rat by 9,10-dimethyl-1,2-benzanthracene. It shows a reproducible growth pattern upon intravenous cellular transfer within the BN rat strain. Cytologically and cytochemically, it is similar to human acute promyelocytic leukemia. Further analogies with the human disease are: (a) a slow growth rate (10^7 BNML cells killed after 18–23 days; growth fraction, 0.60–0.40); (b) a severe suppression of normal hematopoiesis due to an absolute numerical decrease in the number of normal hematopoietic stem cells; (c) diffuse intravascular coagulation; (d) prolonged blood transit time of leukemic cells (34–36 hr); (e) response to chemotherapy as in human AML; (f) presence of clonogenic leukemic cells (no *in vitro* colony formation:TD_{50}, 25 cells); (g) low antigenicity; and (h) no evidence for a virus as an etiologic agent.

Cyclophosphamide

Cyclophosphamide (ASTA, The Netherlands) was dissolved in 0.9% saline and injected i.p. or i.v. (tail vein) in a volume of 0.5 ml.

Experimental designs

1. *Toxicity studies.*

(a) Cyclo was administered i.p. in increasing dosages (100–300 mg/kg) to both normal rats and rats at days 13–15 after i.v. inoculation with 10^7 BNML cells. No subsequent BMT was performed. In both groups the LD_{50} was established.

(b) In a second series of experiments, rats at various stages of leukemia growth (i.e. at days 5–20 after 10^7 BNML cells i.v.) were treated with 200 mg cyclo per kg i.p., followed 24 hr later by isologous bone marrow transfer (10^8 cells i.v.). The fraction of animals dying from either drug-induced toxicity or leukemia as well as the number of long-term survivors were determined.

(c) Thirdly, rats at day 13 after 10^7 BNML cells i.v.—comparable with the stage of 'full-blown' relapse in human AML; approximate tumor load $1–5 \times 10^9$ cells [8, 9]—received varying dosages of cyclo (60–200 mg/kg) and subsequent BMT. Deaths from toxicity or leukemia were scored in each group. Non-treated leukemic rats served as controls. In addition, an experimental group receiving 4×50 mg cyclo per kg at 24-hr intervals (days 12–15) was included.

(d) Finally, at the optimal dose of 100 mg/kg, cyclo was injected either i.p. or i.v. in rats at day 13 after 10^7 BNML i.v. to investigate whether a different route of administration would result in a different leukemic cell kill. In the same experiment the possible beneficial effect of subsequent BMT was investigated.

2. *Efficacy against the BN leukemia.*

Groups of rats were injected with varying numbers of BNML cells (10^3 to 5×10^8). Cyclo in a dose of 100 mg/kg was given 48 hr later i.p. Direct quantitative information on the tumor load reducing capacity of this dose of cyclo was obtained by determining the fraction of long-term leukemia-free survivors.

RESULTS

1. *Toxicity Studies*

(a) In Table 1 the effects of increasing dosages of cyclo without subsequent BMT in both normal and leukemic rats are presented. By probit analysis the LD_{50} for normal rats was found to be 164 mg/kg, with 95% confidence limits ranging from 154 to 175 mg/kg. The LD_{50} for leukemic rats appeared to be much lower, i.e. somewhere between 100 and 120 mg/kg. The cause of death in the normal rats was bone marrow aplasia in combination with haemorr-

Table 1. Toxicity of cyclophosphamide treatment in normal and leukemic rats

Cyclophosphamide (mg/kg i.p.)	Deaths due to toxicity	
	Normal rats (A)	Leukemic rats (B)
100	0/8	0/8
120	0/8	8/8
150	—	8/8
160	7/12	—
200	10/12	8/8
250	8/8	—
300	8/8	—

In the leukemic rats, treatment was given at day 13 or 15 after inoculation with 10^7 leukemic cells. No subsequent bone marrow transplantation was performed.
Observation period: > 300 days.

hages in the gastro-intestinal tract, the lungs and the urinary bladder. The latter phenomenon is possibly due to a direct toxic effect of cyclo (or one of its metabolites) on the epithelia involved. With the higher doses (250 and 300 mg/kg), death almost invariably occurred within 48 hr. This appeared to be related to acute toxicity to the cardiopulmonary system. In the leukemic rats, excessive kill of leukemic cells leading to tumor cell embolism was an additional factor contributing to early death. The rats surviving the cyclo-related toxicity/aplasia either lived indefinitely (Group A) or died from recurrent leukemia (Group B). It should be emphasized that rats treated with 100 mg/kg apparently did not need a subsequent BMT to overcome the aplastic phase.

(b) Death from drug-induced toxicity appeared to be clearly related to the stage of leukemia growth as can be seen in Table 2. Aplasia as an ultimate cause of death was ruled out in these experiments because BMT was performed after cyclo treatment. When treatment was given at days 5, 10 or 11 after leukemia transfer, toxicity-related deaths were observed in 37.5–60% of the rats. Beyond day 13, virtually all rats died shortly after high-dose cyclo treatment was given. Among the early-treated animals, 11 out of 28 (39%) were leukemia-free long survivors. As the total tumor load at days 5–11 ranges from 6×10^7 to 5×10^8 cells, 200 mg cyclo per kg at least induced an 8.5 log cell kill in these rats. Only 3 out of 28 rats (11%), of which none were treated at day 5, one was treated at day 10 and two were treated at day 11, died of recurrent leukemia. In all but the day 11 treatment group the median survival time (MST) was shorter (14–21 days) than that of non-treated leukemic controls (MST: 23 days). MST calculated from the time of treatment was longest in early-treated rats (days 5 and 10: 9 and 7 days respectively) as compared to rats treated in a later stage of disease (days 13–20: only 1–3 days survival after cyclo). Apparently, in the latter group excessive tumor cell kill possibly in combination with impaired functions of vital organs infiltrated with leukemia are additional factors contributing to early death.

From these experiments it was concluded that in order to prevent lethal drug-induced toxicity, either (a) the dose of cyclo should be lowered, or (b) treatment should be given at an early stage of disease. Because one of the major aims of the study was to evaluate the efficacy of cyclo during the phase of overt disease, dose–effect experiments were performed at day 13 after inoculation with 10^7 BNML cells as described below.

(c) As can be seen in Table 3, doses up to 100 mg cyclo per kg did not induce lethal toxicity. In the higher dose range (120–200 mg/kg) an increasing fraction of toxicity-related deaths were observed. Apparently, these were due to reasons mentioned before and not to irreversible marrow aplasia because BMT was performed after cyclo treatment. In this respect the experiment differs from the one presented in Table 1. The rats dying from recurrent leukemia showed an increase of life span over

Table 2. Efficacy and toxicity of high-dose cyclophosphamide treatment given at various stages of leukemia growth

Cyclophosphamide (200 mg/kg i.p.) given at day:*	MST† (days)	Range (days)	Cause of death‡ T	Cause of death‡ L	Long-term disease-free survivors
5	14	11–>300	6/10	0/10	4/10
10	17	17–>300	5/10	1/10	4/10
11	35	19–>300	3/8	2/8	3/8
13	14	14–20	10/10	0/10	0/10
15	18	16–58	13/14	1/14	0/14
16	17	17–18	6/6	0/6	0/6
20	21	20–21	6/6	0/6	0/6

*After inoculation with 10^7 leukemic cells.
†MST: Median survival time, calculated from date of leukemia transfer.
‡T: Toxicity; L: leukemia.
All rats received 6×10^7–10^8 normal isologous bone marrow cells i.v. 24 hr after the injection of cyclophosphamide.
MST of non-treated leukemic controls: 23 days (range: 21–25 days).
Observation period: > 300 days.

Table 3. Efficacy and toxicity of varying dosages of cyclophosphamide followed by isologous bone marrow transplantation in leukemic rats

Cyclophosphamide (mg/kg i.p.)	Cause of death*		MST†—days (Range)		ILS‡—days
	T	L	T	L	L
60	0/8	8/8	—	35 (31–37)	12
80	0/8	8/8	—	38 (36–48)	15
100	0/8	8/8	—	44 (40–49)	21
120	2/8	6/8	20, 21	48 (45–51)	25
160	6/8	2/8	18 (16–21)	44, 69	21,46
200	10/10	0/10	14 (14–20)	—	—
4 × 50§	3/10	7/10	22 (21–22)	78 (52–99)	51
—	0/11	11/11	—	23 (21–26)	—

Treatment was given at day 13 after inoculation with 10^7 leukemic cells. All rats received 6×10^7 normal isologous bone marrow cells i.v. after the (last) injection of cyclophosphamide.

*T: Toxicity; L: leukemia.

†MST: Median survival time, calculated from date of leukemia transfer.

‡ILS: Increase in life span, as determined by: (MST treated rats) − (MST non-treated controls) − (duration of treatment).

§In the 4 × 50 mg/kg regimen, individual dosages were given 24 hr apart, from day 12 to day 15.

non-treated leukemia controls proportional to the cyclo dose administered. Based on MST the increase in life span (ILS) was 12–25 days after 60–120 mg cyclo per kg. In addition, in the group receiving 160 mg/kg, two rats died from leukemia at days 44 and 69 (ILS: 21 and 46 days respectively). The relation between these values for ILS and log leukemic cell kill will be discussed later.

If the total dose of 200 mg/kg was given in four fractions of 50 mg/kg each at 24-hr intervals, the fraction of rats dying from toxicity decreased from 100 to 30% (Table 3). The remaining 70% died from recurrent leukemia (MST: 78 days; ILS: 51 days).

(d) The optimal single dose of 100 mg cyclo per kg was chosen for further studies. As shown in Table 4, there appeared to be no difference in terms of prolongation of life span after (a) cyclo was administered either i.v. or i.p. and (b) a subsequent BMT was performed or not. Thus in the final experiments (see below), 100 mg cyclo per kg was given i.p. without marrow transplantation.

2. *Efficacy against the BN leukemia*

From Table 5 it is clear that 100 mg cyclo per kg could effectively kill up to 5×10^8 leukemic cells. However, when the total tumor burden exceeded 10^6 cells, the fraction of rats in which a leukemia relapse occurred steadily increased. With approximate tumor loads of 10^7, 10^8 and 5×10^8 cells at the time of cyclo treatment, recurrent leukemia was observed in 8, 20 and 50% of the rats respectively. None of the rats died from cyclo-related toxicity.

DISCUSSION

From the data presented it is clear that cyclo has a potent anti-leukemia effect in the BNML rat model. With single-dose treatment, the highest therapeutic index was obtained with 100 mg/kg. This dose induces a significant leukemic cell kill (Table 5). However, it did not cause irreversible fatal marrow aplasia (i.e., no subsequent BMT was required; Table 4), nor was lethal toxicity to other organ systems observed (Table 3). Increasing this dose led to an increasing fraction of rats dying from drug-induced toxicity (Table 1), and decreasing it resulted in a reduced anti-leukemia effect as can be judged from the shorter values for ILS (Table 3). However, splitting up a high dose of 200 mg/kg in four fractions of 50 mg/kg each

Table 4. The efficacy of cyclophosphamide injected along different routes in leukemic rats

Cyclophosphamide	MST† (days)	Range (days)	ILS‡ (days)
100 mg/kg i.v.	43	41–45	21
100 mg/kg i.p.	43	40–46	21
100 mg/kg i.p. + BMT*	44	40–49	22
—	22	20–25	—

Treatment was given at day 13 after inoculation with 10^7 leukemic cells; 8 rats per group.
*BMT: 6×10^7 normal isologous bone marrow cells were injected i.v. 24 hr after the cyclophosphamide injection.
†MST: Median survival time, calculated from date of leukemia transfer.
‡ILS: Increase in life span, as determined by: (MST treated rats) − (MST non-treated controls).

given at 24-hr intervals decreased the percentage of toxicity-induced deaths from 100 to 30% (Table 3). The anti-leukemia effect appeared to be maximally strong as judged from the 78-day median survival time. This finding supports the clinical application of high split doses of cyclo prior to bone marrow transplantation.

Furthermore, the route of administration (either i.v. or i.p.) did not make any difference as regards leukemic cell kill (Table 4).

There appeared to be a clearcut difference as regards the LD_{50} for cyclo between normal and leukemic rats (164 and approximately 110 mg/kg respectively). The LD_{50} in normal rats compares well with that reported in different rat strains, i.e. 150–180 mg/kg [11, 12]. As has been pointed out, toxicity-related deaths in normal rats were due to irreversible aplasia in combination with lung-, bladder- and intestinal tract haemorrhages (160–200 mg/kg) or to acute cardio-pulmonary failure (250–300 mg/kg). In leukemic rats, excessive leukemic cell kill leading to tumor cell embolism in combination with impaired functions of vital organs due to leukemia infiltration were additional factors contributing to early death. In this respect, treatment of late-state leukemia proved to be invariably fatal (Table 2).

As regards normal tissues, the rat seems to be far more sensitive to cyclo than man. Based on the equivalent surface area dosage conversion factor [13], 100–300 mg cyclo per kg in the rat compares to 15–40 mg/kg in man. In the conditioning regimen for BMT in human acute leukemia 2×60 mg cyclo per kg is admisistered [5–7]. This dose, which is equivalent to a total dose of 840 mg/kg in the rat, is tolerated without lethal toxicity in man.

Direct quantitative information on the efficacy of the optimal cyclo dose against the BN leukemia was derived from the study presented in Table 5. Up to a 8.5 log leukemic cell kill was observed. The survival time of rats dying from relapsed leukemia varied greatly (39–110 days). Theoretically, if 100 mg cyclo per kg would kill the same fraction of leukemic cells in all rats (first order kinetics), a decreasing MST would be expected with increasing initial tumor loads. The reason that this is not observed might be (a combination of) host variation in drug absorption, metabolism and excretion which occur even with inbred animals. Furthermore, biochemical drug-resistance of a small fraction of leukemic cells as well as anatomical resistance of cells hidden in sanctuaries which cannot be reached by the drug might have led to a varying log leukemic cell kill and thus to a variation in MST in these

Table 5. The efficacy of cyclophosphamide in leukemic rats carrying fixed numbers of leukemic cells

Number of BNML cells i.v. at day 0	Cyclophosphamide (mg/kg i.p.) at day 2	Deaths due to leukemia	MST* (days)
10^3	100	0/5	—
10^4	100	0/11	—
10^5	100	0/10	—
10^6	100	0/6	—
10^7	100	1/13	57
10^8	100	1/5	110
5×10^8	100	4/8	51 (range: 39–68)

*MST: Median survival time of rats dying from leukemia, calculated from date of leukemia transfer. No subsequent bone marrow transplantation was performed. Observation period: > 300 days.

rats. In addition, altered kinetics of proliferation of leukemic cells surving cyclo treatment might be involved, as illustrated by the data in Table 4. The tumor load at the time of treatment (day 13 after 10^7 BNML cells i.v.) is approximately $1–5 \times 10^9$ cells [14]. With 100 mg cyclo per kg maximally inducing an 8.5 log cell kill (Table 5), the minimal number of surviving leukemic cells will be 2–10. According to the linear relationship between the number of inoculated leukemic cells and the survival time [15], this small number of cells would cause death from leukemia only after 50–60 days. However, calculated from the time of treatment, the observed MST was only 30–31 days (deduced from Table 4). This might indicate that the residual leukemic cells proliferate much faster as compared to a similar number in non-treated leukemic controls. In earlier studies it was found that for inocula of $10^2–10^7$ leukemic cells, survival was prolonged by 4 days with every ten-fold decrease in the number of injected leukemic cells [15]. According to this rule, 100 mg cyclo per kg would yield a 5 log cell kill as deduced from the 21–22 days ILS (Table 4). Thus, either the proliferation rate of residual leukemia has increased after cyclo treatment or the log leukemic cell kill induced by 100 mg cyclo per kg is less in a stage of full-blown disease (a 5 log cell kill at day 13 after 10^7 BNML i.v.; Table 4) as compared to an early phase of leukemia growth (up to an 8.5 log cell kill at 48 hr after 10^7 BNML i.v.; Table 5). In favor of the latter hypothesis is the observation that cyclo followed by supralethal total-body irradiation (9.0 Gy), which by itself induces a 4 log cell kill in the BNML [16], is still not curative for rats at day 13 after 10^7 BNML i.v. bearing 5×10^9 leukemic cells [17]. Whatever the mechanism involved might be, there appears to be a correlation between the ILS and the cyclo dose given at an advanced stage of disease (Table 3).

In conclusion, cyclophosphamide administered in high dosages proved to be an effective drug in this rat model for human AML. Depending on the stage of disease, a 5–8.5 log leukemic cell kill was achieved. As this leukemia model showed similar sensitivity to various cytostatic agents as compared to human AML in previous studies [18–20], extrapolation of the present data to human AML seems to be justified. This statement is supported by the observation that a substantial proportion of patients treated with high-dose cyclo and supralethal total-body irradiation followed by bone marrow transplantation in first complete remission of AML (approximate tumor load equal to or less than $10^9–10^{10}$ cells) are presently long-term disease-free survivors [5–7].

Acknowledgement—The skillful technical assistance by Carla van Marrewijk is greatly appreciated.

REFERENCES

1. SPIERS ASD, GOLDMAN MJ, CATOVSKY D *et al.* Prolonged remission maintenance in acute myeloid leukaemia. *Br Med J* 1977, **2**, 544–547.
2. FREIREICH EH, KEATING MJ, GEHAN EA *et al.* Therapy of acute myelogenous leukemia. *Cancer* 1978, **42**, 874–882.
3. PREISLER HD, RUSTUM Y, HENDERSON ES *et al.* Treatment of acute nonlymphocytic leukemia: use of anthracycline–cytosine arabinoside induction therapy and comparison of two maintenance regimens. *Blood* 1979, **53**, 455–464.
4. BLOOMFIELD CD. Treatment of acute nonlymphocytic leukemia 1980. *Ann Intern Med* 1980, **93**, 133–135.
5. THOMAS ED, BUCKNER CD, CLIFT RA *et al.* Marrow transplantation for acute nonlymphoblastic leukemia in first remission. *N Engl J Med* 1979, **301**, 597–599.
6. POWLES RL, MORGENSTERN G, CLINK HM *et al.* Bone-marrow transplantation in acute myelogenous leukaemia. *Lancet* 1980, **i**, 1047–1049.
7. BLUME KG, BEUTLER E, BROSS KJ *et al.* Allogeneic marrow transplantation in acute leukemia. *N Engl J Med* 1980, **302**, 1041–1046.
8. HAGENBEEK A, VAN BEKKUM DW, eds. Proceedings of a workshop on comparative evaluation of the L5222 and the BNML rat leukaemia models and their relevance for human acute leukaemia. *Leuk Res* 1977, **1**, 75–255.
9. VAN BEKKUM DW, HAGENBEEK A. Relevance of the BN leukemia as a model for human acute myeloid leukemia. *Blood Cells* 1977, **3**, 565–579.
10. HAGENBEEK A, MARTENS ACM. The pathogenesis of a rat model for human acute myelocytic leukemia. *Haematologica* 1980, **65**, 293–308.
11. PHILIPS FS, STERNBERG SS, CRONIN AP, VIDAL PM. Cyclophosphamide and urinary bladder toxicity. *Cancer Res* 1961, **21**, 1577–1589.

12. SYRKIN AB, ZAITZEVA LA. The rate of cyclophosphamide activation. *Neoplasma* 1970, **17**, 377–381.
13. FREIREICH EJ, GEHAN EA, RALL DP *et al.* Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey and man. *Cancer Chemother Rep* 1966, **50**, 219–229.
14. HAGENBEEK A, MARTENS ACM. Functional cell compartments in a rat model for human acute myelocytic leukemia. *Cell Tissue Kinet* 1979, **12**, 361–372.
15. HAGENBEEK A. Introduction of the BN myelocytic leukemia. *Leuk Res* 1977, **1**, 85–87.
16. HAGENBEEK A, MARTENS ACM. The effect of fractionated versus unfractionated total body irradiation on the growth of the BN acute myelocytic leukemia. *Int J Radiat Oncol Biol Phys* 1981, **7**, 1075–1079.
17. HAGENBEEK A, MARTENS ACM. The efficacy of high-dose cyclophosphamide in combination with total body irradiation in the treatment of acute myelocytic leukemia. Studies in a relevant rat model (BNML). *Cancer Res* (In press).
18. COLLY LP, TÖNS A, HAGENBEEK A. Experimental chemotherapy in the BN myelocytic leukaemia. *Leuk Res* 1977, **1**, 247–250.
19. AGLIETTA M, SONNEVELD P. The relevance of cell kinetics for optimal scheduling of 1-β-D arabinofuranosyl cytosine and methotrexate in a slow growing acute myeloid leukemia (BNML). *Cancer Chemother Pharmacol* 1978, **1**, 219–225.
20. HAGENBEEK A, MARTENS ACM. Efficacy of piperazinedione prior to bone marrow transplantation: studies in a rat model for human acute myelocytic leukemia. *Cancer Treat Rep* 1981, **65**, 575–582.