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Cure of Advanced L1210 Leukemia After Correction of Abnormal Red Blood Cell Deformability

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Summary. *Chemotherapeutic efficacy is inversely related to pretreatment tumor burden. A possible contributory factor in chemotherapy resistance is the occurrence of decreased red blood cell deformability in mice with advanced tumors. Poorly deformable red blood cells may prevent adequate drug delivery to tumor cells. Two methods for improving red cell deformability were found in this study. The first involved treatment of L1210 leukemia-bearing mice with red cell metabolic substrates, including inosine, adenosine, glucose, sodium pyruvate, and ascorbic acid. The combination of inosine plus sodium pyruvate (3 mg of each drug in 0.5 cm^3 phosphate-buffered saline) was most effective in restoring deformability to normal. Administration of an active chemotherapeutic agent (BCNU or cyclophosphamide) also improved red cell deformability, with maximal restoration occurring 4–5 days after drug treatment. Standard and 50% of standard drug doses were equally effective in restoring deformability. The optimal therapy program for day 7 L1210 leukemia utilized inosine plus sodium pyruvate given 10–15 min before BCNU 15 mg/kg on day 7 and before BCNU 30 mg/kg on day 12. This treatment yielded 44% cures, whereas BCNU alone, in identical dose and schedule, gave no cures. Median survival was 50 days for the inosine-pyruvate-treated mice, as against 30 days for BCNU alone. Therefore, treatment with non-toxic doses of red blood cell metabolic substrates plus optimal timing of chemotherapy, two maneuvers that significantly increased red blood cell deformability, resulted in significant therapeutic benefit.*

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Introduction

Chemotherapy efficacy is inversely related to pretreatment tumor burden. Treatment of intravenously transplanted L1210 leukemia with optimal single doses of cyclophosphamide or BCNU resulted in greater than 50% cures when the tumor burden was 8×10^5 cells. When the tumor burden was 8×10^7 cells, however, the cure rate decreased to less than 5% for both drugs [15]. For Lewis lung carcinoma optimal single-dose BCNU or cyclophosphamide treatment on day 2 after intravenous inoculation of 10^6 tumor cells produced 50% and 80% cures, respectively. When treatment with these two drugs was delayed to 14 days after tumor transplantation no cures were achieved [10]. In human neoplasia it is difficult to precisely measure tumor burden. Estimations of tumor cell mass within the body can be made, however, by determining the number of organ systems involved by cancer. In patients with diverse neoplasms, including small cell lung cancer or female breast cancer, patients presenting with multiple organ system involvement by tumor have a significantly worse prognosis than do individuals with disease confined to one or two sites [7, 9].

Chemotherapy sensitivity decreases with progressive tumor growth, in part because of unfavorable cell kinetic changes [13] and because enlarging tumors outgrow their blood supply [16]. Another factor that may influence chemotherapy efficacy is circulatory stasis within tumor capillaries. Stasis, if present, would be expected to decrease oxygen, nutrient, and chemotherapy delivery to tumor cells. Capillary blood flow is principally determined by red blood cell deformability, since the diameter of a red blood cell often exceeds the diameter of a capillary [18]. Abnormal red blood cell deformability, in advanced cancer, has been demonstrated in mice bearing L1210 leukemia and Lewis lung carcinoma [3]

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and in patients with small cell anaplastic bronchogenic carcinoma [4]. In the former model red cell deformability was normal for the first 4 days after intraperitoneal transplantation of 10^5 viable cells. During days 5–9 after tumor transplantation, however, red cells became significantly more rigid. Decreasing deformability was associated with a rapid decline in hematocrit, presumably because poorly deformable red cells were cleared from the circulation [8].

While the red blood cell abnormality accounting for decreased deformability has not been identified, empirically it was observed that deformability could be improved by in vivo treatment with effective cytotoxic chemotherapy or by the use of various red cell metabolic substrates including inosine, adenosine, glucose, sodium pyruvate, or ascorbic acid. These drugs have been used in blood-banking to restore function to stored, outdated, human red blood cells [5, 17]. The function of sodium pyruvate or ascorbic acid is to oxidize intracellular NADH, thus maintaining red cell glycolysis [5].

The purpose of the present study was to determine whether treatment with red blood cell metabolic substrates plus proper timing of chemotherapy, based on red cell deformability results, would improve the survival of mice bearing advanced L1210 leukemia.

Materials and Methods

(BALB/c × DBA)F_1 male mice were obtained from the Charles River Breeding Laboratories, North Wilmington, Mass. Mice were 8–10 weeks old and weighed 26–30 g at the time of tumor transplantation. Mice received 10^5 hemocytometer-counted, trypan blue-excluding L1210 cells IP on day 0. Following this inoculum of tumor cells untreated mice survived 11–13 days.

Red blood cell deformability studies were performed after various experimental manipulations. For these studies mice received 15 U heparin IV via a tail vein, 5–10 min before bleeding. Bleeding, after ether anesthesia, was achieved by unilaterally cutting the jugular vein and carotid artery. Generally one, but occasionally two, animals were used for each blood sample.

Blood samples were centrifuged to remove the plasma. Red blood cells were washed three times with 3 ml calcium-free and magnesium-free phosphate-buffered saline (PBS), pH 7.2, containing 1% human serum albumin. Following the third wash a 0.5% red cell suspension was made in the above washing fluid. All washings and subsequent filtrations were performed at room temperature.

Red blood cell deformability was measured by the method of Reid et al. [12] with slight modification. The 0.5% red cell suspension, in a 12-ml graduated vessel, was filtered through a 13-mm Nuclepore filter (3 μm pore size) (Nuclepore Corp., Pleasanton, Calif.). The negative filtration pressure was 20 cm water. The total filtered volume was determined at 1, 2, 4, 6, 8, and 10 min and plotted on semilog paper. The slope of this line represented the deformability index (DI) (cm^3/10 min).

To determine the effect of metabolic substrates on red cell deformability, day 7 L1210-bearing mice were given a single IP injection containing 3 mg inosine, adenosine, or glucose together with 3 mg sodium pyruvate or ascorbic acid in 0.5 cm^3 calcium-free and magnesium-free PBS. Mice were bled 3–4 h later.

To study the effect of chemotherapy on red cell deformability, day 7 L1210-bearing mice received cyclophosphamide 125 or 250 mg/kg or BCNU 15 or 30 mg/kg IP in a volume of 0.01 cm^3/g body weight. Groups of mice were bled daily on days 9–14 after L1210 transplantation.

To determine a dose/response effect of chemotherapy on red cell deformability, day 7 L1210 mice received cyclophosphamide 62.5, 125, or 250 mg/kg or BCNU 15 or 30 mg/kg IP. Groups of mice were bled on day 12.

In therapy trials day 7 L1210 mice received inosine, adenosine, or glucose with sodium pyruvate or ascorbic acid. Drug doses were 3 mg/mouse IP in 0.5 cm^3 PBS, 15–30 min before IP BCNU 15 mg/kg. On day 12 after tumor transplantation mice received a second IP dose of the substrate combination that they received on day 7. BCNU 30 mg/kg IP was given 15–30 min later. Control mice received metabolic substrates alone or BCNU alone. Mice were then monitored for survival, with 56+ day survivors considered cured. Reported results were based on three or four separate experiments. There was only slight variation in survival results from one experiment to another. Animals dying within 5 days of chemotherapy were assumed to have died of drug toxicity.

Statistical comparisons were made according to Student's *t*-test. Survival results after chemotherapy were compared by the log rank method [11].

Results

The red blood cell DI for normal, non-tumor-bearing, mice is 4.6 ± 1.4 cm^3/10 min [3]. The mean DI for mice bearing day 7 L1210 leukemia is

Table 1. Effect of various red blood cell metabolic substrates on restoration of red blood cell deformability

Metabolic substrate	No. of studies	DI (cm^3/10 min) (Mean + SEM)
None	15	1.8 ± 0.7
Inosine + Na pyruvate	4	4.6 ± 0.6
Inosine + ascorbate	4	2.8 ± 1.3**
Adenosine + Na pyruvate	4	3.3 ± 0.6*
Adenosine + ascorbate	4	3.4 ± 0.8*
Glucose + Na pyruvate	4	2.4 ± 0.8***
Glucose + ascorbate	4	2.5 ± 1.0***

Day 7 L1210-bearing mice received either no treatment, or treatment with the above drugs in doses of 3 mg/mouse in 0.5 cm^3 PBS. The inosine dose used in these experiments approximates the dose used for restoration of outdated blood [5, 17]. Mice were bled 3 h after drug treatment and deformability was determined. Red blood cell deformability after inosine plus sodium pyruvate treatment was significantly better compared to the other substrate combinations under consideration.

* $P < 0.05$
** $P < 0.025$
*** $P < 0.005$

Table 2. Time-course of restoration of red blood cell deformability after chemotherapy

Days after L1210 transplant	No. of studies	DI (cm^3/10 min) (Mean ± SEM)
7	15	1.8 ± 0.7
9	10	2.0 ± 0.6
10	7	2.5 ± 1.6
11	11	3.4 ± 1.4
12	22	3.8 + 1.5
13	7	2.9 ± 1.0
14	7	2.3 ± 1.3

Day 7 L1210-bearing mice were treated with BCNU or cyclophosphamide. Groups of mice were bled daily starting 48 h after treatment and the deformability index determined. Improvement in deformability achieved on day 12 after L1210 transplantation (5 days after cytotoxic drug administration) was significantly better than that achieved on days 10, 13 or 14 ($P < 0.05$) and significantly better than that achieved on day 9 ($P < 0.005$). There was no significant difference in red cell deformability on days 11 or 12 after L1210 transplantation

Table 3. Effect of drug dose on restoration of red blood cell deformability

Drug	Dose mg/kg	No. of studies	DI (mm^3/10 min) (Mean ± SEM)
Cytoxan	250	4	3.9 ± 2.0
	125	15	4.1 ± 1.2
	62.5	4	1.0 ± 0.2
BCNU	30	4	2.5 ± 0.9
	15	4	2.5 ± 0.9

Day 7 L1210-bearing mice were treated with cyclophosphamide or BCNU, in the doses indicated. Red blood cell deformability was determined on day 12. Deformability after treatment with cyclophosphamide 62.5 mg/kg was significantly impaired compared with results with cyclophosphamide 250 mg/kg ($P < 0.001$)

1.8 ± 0.7 cm^3/10 min. Differences in deformability between these two mouse groups are significant at the $P < 0.0005$ level. The capacity of various red cell metabolic substrates to correct the DI abnormalities associated with tumor growth is indicated in Table 1. Inosine plus sodium pyruvate was the most effective pretreatment, giving significantly improved red cell deformability compared with any of the other drug combinations tested.

Results of studies to determine an optimal time to administer a second dose of BCNU or cyclophosphamide are shown in Table 2. Following treatment of day 7 L1210 mice with a single dose of BCNU 15 or 30 mg/kg, or cyclophosphamide 125 or 250 mg/kg, maximal improvement in red cell deformability was noted on days 11 and 12 (4 and 5 days after treatment). Red cell deformability on day 12, for example, was significantly improved relative to results obtained on days 9, 10, 13, or 14.

The results of work performed to determine the relationship between chemotherapy dose and red cell deformability improvement are indicated in Table 3. BCNU 30 mg/kg and cyclophosphamide 250 mg/kg are optimal single treatment doses producing maximal survival prolongation with less than 10% treatment-related mortality. A 50% dose reduction for either drug gave comparable deformability results. When 25% of the optimal cyclophosphamide dose was used, however, red cell deformability was significantly decreased ($P < 0.005$) compared with that obtained with higher cyclophosphamide doses.

Initial therapy trials in day 7 L1210-bearing mice evaluated either red cell metabolic substrates alone or single doses of BCNU. Evaluation of cyclophosphamide treatment was also planned. However, many cyclophosphamide-treated mice died with hind limb paralysis, presumably due to the failure of this drug to effectively control central nervous system metastases. Consequently cyclophosphamide was not evaluated further. Treatment with any of the red blood cell substrate combinations listed in Table 1 failed to increase survival over that in control, untreated, mice. Twelve L1210-bearing mice received BCNU 30 mg/kg on day 7. The median survival was 25 days and no mouse survived beyond 40 days. One mouse died on day 17, presumably from drug toxicity. Twenty mice received inosine and pyruvate prior to BCNU 30 mg/kg on day 7. Four mice died on or before day 17. There were no cures. Twelve mice received inosine and pyruvate prior to BCNU 15 mg/kg. There were no toxic deaths. Median survival, for these mice, was 27 days with no cures.

Subsequent experiments employed two doses of BCNU administered on days 7 and 12 after tumor transplantation. Twenty-one mice received BCNU 30 mg/kg on day 7 and 15 mg/kg on day 12, while 20 mice received BCNU 15 mg/kg on day 7 followed by 30 mg/kg on day 12. Four mice in the former group (19%) died on or before day 18 (apparent drug toxicity), while one mouse (5%) in the latter group died during the same time period. The median survival for BCNU 30 mg/kg followed by 15 mg/kg was 31 days, as against 38 days for mice receiving BCNU 15 mg/kg followed by 30 mg/kg. One mouse in the former group was cured. There was no statistically significant survival difference between these two treatments. Because of the relatively high percentage of toxic deaths in the BCNU 30 mg/kg followed by 15 mg/kg schedule, and because of the failure of

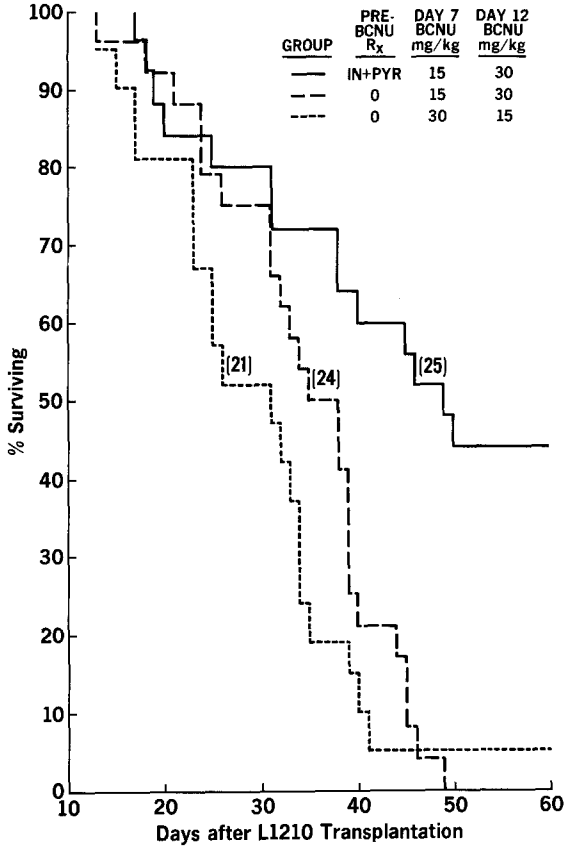

Fig. 1. Survival of L1210-bearing mice that received BCNU alone or with red blood cell metabolic substrates on days 7 and 12 after tumor transplantation. Survival of mice pretreated with inosine and sodium pyruvate was significantly prolonged compared with that in either group of mice receiving BCNU alone ($P < 0.001$)

inosine plus sodium pyruvate to decrease presumed drug-related mortality in the above-mentioned single-dose BCNU experiments, therapy trials with the BCNU 30 mg/kg followed by 15 mg/kg schedule were discontinued.

When inosine and sodium pyruvate were given prior to BCNU 15 mg/kg on day 7 and 30 mg/kg on day 12 to 25 mice (Fig. 1) the median survival was 50 days, with 44% cures. This survival was significantly better than either of the BCNU alone schedules ($P < 0.001$). Thirty mice received inosine plus ascorbic acid prior to BCNU, given on the above schedule. The median survival was 40 days, with 31% surviving beyond 56 days. These results were also significantly better than those achieved with BCNU alone ($P < 0.005$). Cure rates between 0 and 5% and median survivals between 35 and 40 days were achieved when 20 mice each received pretreatment with adenosine plus sodium pyruvate, adenosine plus ascorbic acid, glucose plus sodium pyruvate, or glucose plus ascorbic acid.

Discussion

Multiple factors, including those enumerated in the *Introduction,* account for decreasing chemotherapy efficacy with increasing tumor burden. The hypothesis tested in this study was that decreasing red blood cell deformability associated with increased tumor burden contributes to chemotherapy resistance. Testing this hypothesis required development of techniques to improve red cell deformability. Two reliable methods were found. Pretreatment of mice with with red blood cell metabolic substrates, similar to those used in blood-banking to restore outdated blood [5, 17], and treatment with an active chemotherapeutic agent (BCNU or cyclophosphamide) both significantly improved red cell deformability. Based on studies in which deformability was determined after treatment with various substrate combinations, it appeared that inosine plus sodium pyruvate was significantly better than any of the other combinations. When treatment experiments were done, however, inosine plus ascorbic acid gave comparable survival results to inosine-pyruvate. Thus it is possible that the inosine-ascorbate combination, or ascorbic acid alone, influences other cellular events in addition to having an effect on red cell deformability. Data indicating that ascorbic acid might have a variety of beneficial effects on cancer patients have been reported [2]. Red cell deformability experiments did, however, predict that adenosine- and glucose-containing combinations would be less effective therapeutically than inosine-containing combinations, and this was observed.

The second method for improving red cell deformability was to treat with an effective chemotherapeutic agent. Deformability significantly improved 4–5 days after drug administration. While the mechanism responsible for improved deformability is unknown, the timing and the transient nature of the improvement suggest that release of metabolic products from dying tumor cells might be responsible [6]. This hypothesis is in accord with the finding of improved red cell deformability of L1210-bearing mice shortly before death [3].

The observation that optimal and 50% of optimal chemotherapy doses yielded equivalent deformability improvement allowed testing of the question as to whether administration of full chemotherapeutic doses when deformability was subnormal was less effective than giving smaller chemotherapeutic doses initially, followed by a full dose when deformability returned to normal. While there was a suggestion that the former method of drug administration was better than the latter (Fig. 1), as measured by a decrease in early, presumably drug-related, mortality from 19%

to 5% and by a 1-week increase in median survival, the survival curves were not statistically different. The more important finding from these experiments was that only 1 of 45 mice (2%) treated with two doses of BCNU survived beyond 56 days. In contrast, 11 of 25 inosine plus sodium pyruvate-pretreated mice (44%) survived beyond that time period. While the tumor cell kill required for an increase from 2% to 44% cures is small (less than 50 cells [1, 14]), administration of BCNU alone did not kill these few remaining cells.

Therefore, treatment with red blood cell metabolic substrates plus proper timing of chemotherapy, based on effects on red blood cell deformability, resulted in significant survival benefit in mice bearing advanced L1210 leukemia. Since red cell substrates were non-toxic, full doses of cytotoxic chemotherapy could be administered. Further trials of this approach in other experimental models and in human tumors seem warranted.

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