A Rationale for Sequential High-Dose Chemotherapy of Leukemia Timed to Coincide With Induced Tumor Proliferation

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Studies in leukemic rats (LBN-ML) tested the hypothesis that an enhanced effect of drugs in sequence relates to temporally predictable induced tumor growth. As leukemia progresses, tumor labeling index (LI) declines in association with increasing tumor-associated inhibitory activity (TAIA) in serum. Graded initial doses of arabinofuranosyl cytosine (ara-C) (50–350 mg/kg every 8 hr × 6) produced relative increments of induced humoral stimulatory activity (HSA) and LI in vivo by day 6, while overcoming the antiproliferative effects of TAIA. Rats bearing late-stage tumor with high TAIA and low LI were treated with 50 or 100 mg/kg ara-C every 8 hr × 6 on days 0.1 and on other days in sequence (0.1–0.1 through 0.1–14.15). Serum, WBC and tumor LI were measured. With the 50 mg/kg initial dose, TAIA was not overcome, and the LI did not achieve levels above pretreatment. A second course of ara-C in sequence produced only additive survival, regardless of timing. In contrast, with the 100 mg/kg sequence, TAIA was overcome, the LI increased, and synergistic survival occurred in those treated with the second ara-C on days 6.7 (760% of controls) at the time of peak HSA and tumor LI. Survival times in other groups in sequence were only additive. Incremental doses of ara-C on day 6 produced linear prolongation of survival. These studies demonstrate that TAIA correlates with tumor LI and that the degree of resultant tumor growth relates to the initial drug dose, tumor kill, and TAIA reduction, and the amount of HSA induced. Survival relates directly to the dose of the second drug given at this predictable peak of maximal HSA and LI.

The value of sequential administration of antitumor agents and the increased activity of these drugs against a proliferating tumor have been demonstrated in vivo in rodents and humans. The goal of our clinical and laboratory studies is to develop principles to guide the use of drugs in sequence to increase antitumor effect. To this end, we have conducted trials in man based on laboratory models and in vitro results that suggest that the pharmacologic effect of cycle-active drugs is enhanced by the proper timing of the sequence, a time determined by detecting maximal induced tumor proliferation and alteration in humoral factors following initial drug perturbation.

Such serum factors influencing the growth of normal and malignant cells are detected before and during chemotherapy. In patients with tumor, inhibitors that suppress proliferation of hematopoietic cells are present in many with normal and tumor-infiltrated bone marrows. Similar activities have been found in media conditioned by normal and leukemic cells and may explain, in part, the often observed low tumor growth fraction and inhibited hematopoiesis at clinical presentation. In our experience, human leukemic and normal bone marrow hematopoietic cell growth is cytostatically suppressed in vitro by humoral tumor-associated inhibitory activity (TAIA). That leukemic cells retain appropriate responsiveness to humoral stimulatory and inhibitory factors in vitro has been noted by others as well. Our cell culture data also demonstrate that TAIA is not leukemogenic to normal marrow and enhances normal granulocyte maturation, and that its antiproliferative effects can be functionally overcome in vitro by drug-induced humoral stimulatory activity (HSA).

Our in vivo studies of timed sequential chemotherapy in human leukemia demonstrate that an increase in tumor labeling index (LI) temporally coincides with the detection of HSA following drug administration, suggesting that TAIA has been overcome. Maximal HSA occurs at a predictable time following initial aplasia-producing drug administration and is coincident with an increased LI of recovering normal marrow granulocytic elements and marrow tumor cells in vivo. In vitro, HSA effects "recruitment" of granulocytic precursors into cell cycle, but neither causes nor interferes with cellular maturation in that series. Malignant hematopoietic cells consistently proliferate without maturation in vitro in response to drug-induced HSA. In an in vitro model, the cytotoxic effect of cytosine arabinoside (ara-C) on human leukemic myeloblasts was enhanced in those cells initially cultured in serum containing HSA relative to cells initially cultured in autologous pretreatment serum containing TAIA.

Trials in human leukemia suggest that the positive effects of timed sequential chemotherapy, as measured by long unmaintained remissions, relate to a reduction of TAIA and the simultaneous induction of HSA; the net effect is increased tumor growth at a predict-
able time and sensitivity to accurately administered drug. This report details studies conducted in a rat model with progranulocytic leukemia to further substantiate this hypothesis.

MATERIALS AND METHODS

Rats

Female (Lew × BN) F_1 (LBN) rats, 9-12 wk of age, were obtained from Microbiological Associates, Bethesda, Md., and used within 6 wk of arrival. They were kept 4 to a cage and given Purina Rat Chow and water ad libitum. At the time the rats were inoculated with tumor, 20% streptomycin was added to their drinking water.

The BN myeloid leukemia was obtained from Dr. Saul Sharkis of our institution (with the permission of Dr. A. Hagenbeck and Dr. D. W. van Bekkum, Radiobiological Institute, Rijswijk, The Netherlands). For passage in LBN rats, spleens were taken from animals on the day prior to anticipated death, prepared as a single cell suspension, and injected i.v. For all subsequent passages, leukemia obtained from bone marrow was used.

In initial experiments, rats were treated with incremental doses of ara-C given every 8 hr × 6 s.c. beginning on day 0 and day 6 (the schedule predicted to be most toxic). Ara-C was kindly supplied by J. P. McGovern, the Upjohn Co., Kalamazoo, Mich. The maximal tolerated dose of this schedule was achieved at a total dose of 1800 mg/kg.

In subsequent experiments, rats that had received 10^6 LBN femur leukemic cells i.v. 14-17 days earlier received ara-C 50-200 mg/kg every 8 hr × 6 s.c. beginning on day 0 only when splenic weight was ≥1.5 g. Replicate animals (4 per point) were exsanguinated daily thereafter, sera collected, and WBC and femur tumor LI determined through day 14 posttherapy. Untreated control animals were handled similarly until death.

In sequential treatment studies, similar tumor-bearing animals received ara-C, 50-150 mg/kg every 8 hr × 6 s.c. beginning on day 0. One group received a double dose on day 0 (0,0). Other groups of 4-6 animals each received a second course of ara-C beginning on another day in sequence (0,2 through 0,14). Only the results of the initial experiment are reported. Each experiment was repeated three times and the results confirmed.

Sera Collection

Blood was obtained from replicate animals (4 per point) daily by exsanguination until WBC recovery after therapy. All sera were separated and stored immediately at −70°C. At the end of the collection period, all sera were assayed simultaneously to determine the effect of the sequential sera on 3H-TdR incorporation by varied marrow cells in the assay system.

Scintillation Assay

The effect of sera on 3H-TdR incorporation by normal and leukemic bone marrow cells was measured by scintillation-counting technique. Control studies in the laboratory have demonstrated that the sum of activities of distinct stimulatory and inhibitory humoral factors that influence cell proliferation is measurable by this method.11

In these assays, 1.0 µCi/ml of culture of 3H-TdR (specific activity, 1.9 Ci/m mole) was added to tubes in triplicate of Roswell Park Memorial Institute Medium (RPMI) 1640 and incubated with fresh femur cells (10^6/ml) at 37°C in a 7% CO_2-humidified atmosphere at a final volume of 2 ml. Sera to be evaluated were added to attain a concentration of 30%. After 18 hr of incubation, the cultures were terminated by immersion in ice, and the cellular contents of each tube were collected under vacuum on a glass-fiber filter. Each tube was washed with cold 0.9% NaCl solution, and the wash added to the filter. Following 3 washings with 0.9% NaCl solution, the acid-insoluble materials on the filter were precipitated by 3 washings with cold 5% trichloroacetic acid and were then washed 3 times with absolute ethanol. The dry filter was transferred to a screw-top vial to which 6 ml of scintillation fluid were added, cooled, and counted in a liquid scintillation spectrometer. Results are expressed as actual cpm for each experiment.

Preparation of 3H-TdR Microautoradiographs

Aliquots of rodent femur bone marrow tumor cells were added to 5 ml of heparinized RPMI 1640 culture media, with 30% autologous serum containing 3H-TdR at a concentration of 0.1 µCi/ml. After 1 hr and 15 min of incubation at 37°C in a 7% CO_2-humidified atmosphere, the cells were washed, resuspended in phosphate-buffered saline (containing per liter: CaCl_2, 100 mg; KH_2PO_4, 200 mg; KCl, 200 mg; MgCl_2, 6H_2O, 100 mg; NaCl, 8 g; and Na_2HPO_4, 2H_2O, 1.15 g, filter sterilized), and spun in a cytocentrifuge at 1500 rpm for 5 min onto slides coated with gelatin. Autoradiographs were prepared with Kodak NTB-2 photography emulsion and allowed to incubate for 21 days; then they were...
developed and stained with Giemsa. The \(^{3}\)H-TdR LI was determined by counting the number of cells per 1000 that contained 5 or more grains overlying the nucleus. Background labeling was estimated by the number of grains present in a cell-free area equivalent to the area of the myeloblast nucleus. Greater than 90% of all cells scored contained approximately 50 grains. Results are reported as percentage of labeled myeloblasts. The standard error of this method in our laboratory is ±1%.

RESULTS

Kinetics of Late-Stage LBN-ML (Fig. 1)

Animals given \(10^6\) femur leukemic cells i.v. die by day 22. During the 10-day period before death, the tumor cell count in the peripheral blood rises to >200,000/cumm, comprising 100% of the peripheral WBC for the last 3 days. Identifiable myeloblasts are detected in the bone marrow by day 12 and rapidly replace normal elements. The bone marrow tumor LI gradually falls from 45% to <15% as the cell density increases. TAIA, which inhibits DNA synthesis of cultured leukemic cells, parallels the decreasing LI. Serum obtained prior to death inhibits in vitro normal and leukemic rat bone marrow proliferation to 10% of values obtained in normal serum.

The Effect of the Initial Drug Dose

The Effect of 50 mg/kg ara-C Given Every 8 hr × 6 Doses (50) (Fig. 2)

LBN rats bearing late-stage leukemia (day 17 after \(10^6\) femur leukemic cells i.v.) received 50 mg/kg ara-C given every 8 hr × 6 doses. After an initial decrease, which persisted until day 6 following ara-C administration, the WBC began to rise. HSA reached maximal levels by days 4–6 (160% of pretreatment value), then declined rapidly as TAIA predominated with tumor regrowth. The LI of the femur leukemic myeloblasts fell from 40% to 27% by day 2, then increased to 37% on day 4. Subsequent LI reflected tumor regrowth, falling to 15% by day 15. The proliferative changes paralleled the TAIA and were the reciprocal of the WBC and spleen weight (not shown). At no time did the LI exceed pretreatment values.

The Effect of 100 mg/kg ara-C Given Every 8 hr × 6 Doses (100) (Fig. 3).

Rats bearing leukemia (day 17 after \(10^6\) femur leukemic cells i.v.) received ara-C, 100 mg/kg every 8 hr × 6 beginning on day 0. The effect of this treatment
on HSA induction, WBC, and tumor LI was monitored (6 rats per point). After an initial decrease, the WBC again began to rise by day 8. HSA, as measured by the influence of serum on DNA synthesis in leukemic cells in culture, reached maximal levels by day 6 (132% of pretreatment, 129% of normal serum control values). The LI of the femur leukemic myeloblasts increased from the pretreatment value of 37% to maximal levels of 55% by day 6, then gradually decreased to 19% as the leukemia progressed.

Remission Model—The Effect of High-Dose ara-C (200 mg/kg, Fig. 4)

Rats bearing late-stage tumor (day 17) were treated with ara-C, 200 mg/kg every 8 hr × 6. The tumor LI was 36% and steadily decreasing at the time of drug administration. Peak HSA was detected on day 6 at the time of initial early recovery of proliferative phase normal bone marrow elements, then fell to levels less stimulatory to DNA synthesis than normal serum after day 8, as marrow progressed to full granulocyte maturation by day 12. The presence of humoral inhibitory activity (HIA) at the time of normal bone marrow recovery and maturation after aplasia is a consistent finding.1,9,11,34 With leukemia relapse, there was progressive decrease in tumor proliferative activity paralleling the change in tumor LI of the untreated control group, with a steady increase in TAIA as the tumor load increased and the animals approached death.

The Induction of HSA and Reduction of TAIA by Incremental Doses of the Initial Drug (Fig. 5)

Normal and tumor-bearing rats were treated with incremental doses of ara-C (50-350 mg/kg every 8 hr × 6). The 3H-TdR incorporation induced by sera obtained from the normal animals at day 6 posttreatment gradually increased to 185% of control at the 350 dose level. The marked effect of TAIA (control tumor-bearing animals' sera inhibited 3H-TdR incorporation to 15% of normal) was not overcome on day 6 after a dose of 50 mg/kg every 8 hr × 6, but was normalized by the 100 mg/kg dose course. Thereafter, with increasing doses above 100, there was a gradual increase in measured stimulation, to equal that achieved in normal animals at the 350 dose level. These results are consistent with a combined effect of reduction of TAIA and the induction of HSA.

The Effect of Sequential Therapy

The Effect of Sequential Courses of ara-C Examined as a Function of the Interval Between Courses (Fig. 6)

Rats bearing leukemia (day 17) received ara-C, 50 mg/kg every 8 hr × 6 beginning on day 0. One group
received a double dose on day 0 (0,0). Other groups received a second course of ara-C beginning on another day in sequence (2 through 12). Untreated control animals died on days 3–8 (mean day 5). Those that had been given the single course (0-1) died on days 13–15 after drug (mean 14, 276% survival). All animals treated on days 0,4 died between days 4 and 7 of toxicity. The day 0,12 group died at approximately the same time as animals receiving the single course of chemotherapy (13–16, mean 14) with survival of 360% of controls. No beneficial effect of sequential chemotherapy was demonstrated in these animals in which the LI of tumor did not exceed the pretreatment values after the initial drug perturbation (Fig. 2).

**The Effect of High-Low Dose Sequence (Fig. 7)**

To determine any differential antitumor effect of the timing of the second drug given at a dose permitting survival in all sequence ranges, groups of rats with leukemia (day 17) were treated with an initial dose of ara-C known to effectively alter tumor growth kinetics (100 mg/kg ara-C every 8 hr × 6, Fig. 3). Control animals died on day 5 ± 2. Ara-C, in a dose of 50 mg/kg every 8 hr × 6 prolonged survival 5 ± 1 days over the controls. A dose of 100 mg/kg every 8 hr × 6 prolonged survival 8 ± 2 days. The predicted survival if these doses (100 + 50) were additive would be 13 days.
The majority of animals survived toxicity. The group given both doses beginning on day 0 survived the predicted 13 days beyond controls (360% increased life-span; ILS). Those treated on day 0.2 achieved ILS 520% of untreated controls and 162% of predicted additive effect, those on 0.4 (520%, 162%), and 0.6 (620%, 200%). On the day 0.8 schedule, ILS fell to 400% of untreated controls and 115% of predicted additive effect and by 0.10 to 380% and 108%. Three of 6 animals in the 0.12 groups died of recurrent leukemia before the second drug could be given, the remaining 3 having an ILS of 260% of untreated controls and 138% of predicted survival.

Enhanced survival with sequential therapy was achieved in all groups treated in sequence to day 8, the maximum (620%, 177%) in the group 0.6. When these data were compared using the Wilcoxon Rank Sum test, the survival of those treated on day 0.6 was significantly longer than those treated by longer sequences ($p < 0.001$) or by shorter sequences ($p < 0.05$). In addition, as a group, those treated early (0.2; 0.4; 0.6) survived longer than the late groups (0.8; 0.10; 0.12), $p < 0.001$. Only the survival of those treated in the early groups was better than the group that received the full dose over 2 days (0-0), $p < 0.001$. Those treated later (0.8; 0.10; 0.12) had no significantly greater response than those receiving the full dose on day 0 (0,0). The extent to which survival was increased by sequential therapy corresponded roughly to the increase in the LI at the time of beginning the second course of treatment (Fig. 3). The most marked effect of prolonged survival occurred in the 0.6 group, in which the second drug was administered at the time of peak HSA and tumor LI.

The Effect of the Second Drug

**Dose Response of the Second Drug in Sequence** (Fig. 8)

Rats bearing late-stage leukemia (day 17) received ara-C 100 mg/kg every 8 hr x 6 beginning on day 0. On day 6, groups of animals received either no drug (0), or increasing doses of ara-C (20-140 mg/kg every 8 hr x 6). Untreated animals died on day 10.5. Animals receiving only the initial 100 mg/kg dose died on day 21. Neither 20 mg/kg or 40 mg/kg every 8 hr x 6 beginning on day 6 prolonged survival over controls treated with only the 100 mg/kg dose on day 0. Between 60 and 120 mg/kg, a linear dose response for the second drug in sequence was seen. All animals treated at the 140 mg/kg dose died of toxicity.

**Sequential Therapy of LBN-ML With an Increased Second Dose** (Fig. 9)

Rats bearing leukemia (day 17) received ara-C, 100 mg/kg every 8 hr x 6 beginning on day 0. One group received a double dose on day 0 (0,0). Other groups received a second course of ara-C (100) beginning on another day in sequence (2 through 14). Control animals died on days 4-8 (mean 6). Those given the single course (0-1) died on days 18-21 (mean 19, 320% ILS). Those treated with the double dose died on days 16-18 (mean 17.5, 291% ILS). All animals treated on days 0.2 and 0.4 died between days 6 and 12 of toxicity with neutropenia and diarrhea. Those treated on days 0.6 died of leukemia between days 43 and 49 (mean 45.5, 758% ILS). Subsequent treatment groups had survivals of 425%-566% of controls. The data demonstrate that the optimal timing of a sequence of a cycle-active drug in the rat coincides with peak increase in LI and peak detectable HSA (Fig. 3) and that an increase of the second dose results in an increased survival. The window between toxicity, which seems to be primarily gastrointestinal mucosal breakdown with sepsis during neutropenia and enhanced antitumor effect, is narrow. Whereas maxi-
Dose-limiting toxicity does not relate to prolongation of myelosuppression, as recovery occurs at the same time regardless of the amount of drug given on the same schedule. In one sense, this resiliency of the bone marrow, and TAIA suppresses and thereby protects normal CFU-c, colony formation by normal bone marrow colony-forming units (CFU). Analysis of cell kinetic parameters with \textsuperscript{3}H-TdR pulse labeling techniques and autoradiography reveals that characteristics developing during the natural course of the disease are quite comparable to those observed in human AML. In the LBN rat, survival is a linear function of log inoculum down to at least $10^5$ cells with 100% mortality in close time span. Five days prior to death, the normal femoral bone marrow elements are completely replaced by a monotomy of cells with the morphology of progranulocytes and easily accessible for sequential kinetic determinations. Most relevant to our studies in late-stage disease and analogous to the human at clinical presentation is the correlation of increasing amounts of TAIA with a reduction in the proliferation of leukemic cells as the disease progresses to death.

**Effect of the First Drug**

These studies in the rat model confirmed assumptions made in our human trials. The data suggest that increased sensitivity to the second drug in sequence is the resultant of the effect of the initial drug on two opposing vectors, TAIA and HSA. The sequence studies and the dose--response curve support the postulate that adequate initial tumor kill must be achieved to reduce TAIA to levels sufficient for the expression of induced HSA, the quantity of which is also dose dependent (Fig. 5). The sum effect of drug on humoral influences is reflected in an increase in the proliferative index of residual leukemic cells at the time of peak induced HSA.

The time of maximal HSA and induced growth of residual leukemia and/or hematopoietic recovery is consistent in the species treated; the magnitude of each is dependent on the initial drug dose. In the rat, this growth curve peaks on day 6 after treatment, is the reciprocal of the WBC, and reflects the hematopoietic recovery time in that rodent. WBC recovery occurs by day 10--12 at the time of reciprocal HIA. In man, the peak HSA and LI in patients with AML, multiple myeloma, small cell carcinoma of the lung, and normal recovering bone marrow cells is reached on days 8--10 after aplasia induction, while in the mouse given comparable doses of drug, the zenith is attained on days 3--4 (unpublished observations). Dose-limiting toxicity does not relate to prolongation of myelosuppression, as recovery occurs at the same time regardless of the amount of drug given on the same schedule. In one sense, this resiliency of the hematopoietic precursor cell provides a therapeutic index. Because leukemia produces total cellular replacement of the bone marrow, and TAIA suppresses and thereby protects normal CFU-c,
successful ablation of tumor correlates with absolute bone marrow aplasia. Further increments of drug are limited only by toxicities other than myelosuppression, which is already complete. Concentrations of ara-C in excess of those producing granulocytopenia can be given to attack the tumor cell maximally without irreversibly affecting the stem cell. In our clinical experience, the limiting toxicity is damage to the gastrointestinal (GI) mucosa.47

Sequential Studies—Effect of the Second Drug

These studies demonstrate that peak HSA and tumor LI occur on day 6 in the rat, a time analogous to day 9 in man. When ara-C was given in doses that permitted survival in all sequences (100–50 mg/kg), maximal prolongation of life was achieved with the sequence given on days 0–6, confirming the temporal correlation of tumor growth, HSA, and response. As the dose of the second drug in sequence on day 6 was increased, the enhancement of survival time was linear (Fig. 8), supporting the clinical use of maximally tolerated doses of ara-C.48

However, no animals treated earlier than day 6 with the higher dose sequence (100–100 mg/kg) survived GI toxicity and associated sepsis. Regarding this toxicity, the repopulation kinetics of normal bone marrow cells and GI epithelial cells following aplasia-inducing drugs have been measured in the mouse by Young and colleagues.39,41 In their studies, growth recovery of ascitic L1210 subsequent to perturbation was unrelated to bone marrow DNA synthesis, but such third-space cells may be less responsive than bone marrow tumor cells to kinetic manipulations.43 However, the toxicity of therapy with ara-C in the mouse correlated with peak proliferation of the GI mucosa as it recovered from the initial drug. This preceded the zenith of bone marrow DNA synthesis by 12–24 hr.42 These data provide comparison with timing and toxicity, which can be applied to the rat and to man when recovery kinetics subsequent to initial drug perturbation are considered. Gastrointestinal and hematopoietic cell recovery, compared on the basis of 1:2:3 in mouse, rat, and man, respectively, predict that a second treatment beginning during GI proliferation (days 2, 4–5, 6–8 in mouse, rat, and man) would potentiate the toxicity of cycle-active drug to normal tissues, producing barrier breakdown during neutropenia. The devastating effect of ara-C given during this period was clearly demonstrated in this rat model and has been encountered in human trials.47 However, if the drug is given subsequent to gut recovery, but during reconstitution of hematopoietic (and leukemic) elements, the effect is salutary. Although maximal serum stimulation to proliferate hematopoietic (and leukemic) elements, the effect is salutary. Although maximal serum stimulation to proliferate hematopoietic (and leukemic) elements, the effect is salutary. Although maximal serum stimulation to proliferate hematopoietic (and leukemic) elements, the effect is salutary. Although maximal serum stimulation to proliferate hematopoietic (and leukemic) elements, the effect is salutary. Although maximal serum stimulation to proliferate hematopoietic (and leukemic) elements, the effect is salutary.

These results support the hypothesis that the increase in tumor growth relates to the dose of initial drug, its effect on tumor, the associated decrease in TAIA, and resultant magnitude of HSA induced. The optimal time of the second treatment correlates temporally with peak LI and HSA, and with minimal TAIA. A dose response to the second drug can be measured. While HSA and TAIA have not been demonstrated to be direct mediators of cell proliferation in vivo, this role seems likely. Studies to determine this association are underway.

ACKNOWLEDGMENT

We wish to thank Cynthia Garvin, Joyce Jung, and Patsy Sanford for their expert technical expertise and Barbara Chlan for her expert secretarial assistance.

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