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Clinical and Cell Kinetic Studies of the Effects of the Epipodophyllotoxin VP16-213 During Therapy of Refractory Acute Nonlymphocytic Leukemia*

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Summary. *After induction failure or relapse on primary therapy, acute nonlymphocytic leukemia (ANLL) has historically been highly resistant to reinduction chemotherapy. In order to evaluate a new drug combination for this condition, 24 children and young adults with ANLL failing to attain first remission [5] or in relapse [19] were treated with VP16-213 plus 5-azacytidine (5AZ). Each patient had previously received combination chemotherapy with several antileukemic agents, including anthracyclines and cytosine arabinoside. These patients received 5-day courses of VP16-213 (200 mg/m^2) daily × 3 days, followed immediately by 5AZ (300 mg/m^2) daily × 2 days, repeated every 1–2 days until the bone marrow became hypoplastic. After 2–4 courses, 19 patients demonstrated an objective anti-leukemic effect by achieving marrow hypoplasia without evidence of blasts. Eleven of these (11/24 = 46%) achieved complete remission after a median time of 45 days from the initiation of therapy, making this combination of VP16-213 and 5AZ the most active regimen we have tested for ANLL in relapse. In order to determine the effects of VP16-213 on leukemic blast proliferation in vivo, serial bone marrow aspirates were studied in eight of these children. VP16-213 200 mg/m^2 IV was given daily × 3 days, and the mitotic index, labeling index and fractions of cells in G_0/G_1, S and G_2 + M by flow cytometric measurement of DNA content were determined. Bone marrow samples were obtained before, 4 h and 20–24 h after the first dose in all patients, and 20–24 h after the third dose in six of the patients. The cytokinetic studies 4 h after the first dose of VP16-213 consistently showed a G_2 phase arrest of the bone marrow myeloblasts, indicating uptake of the drug and production of the same cell kinetic lesion which has been shown for cells treated with VP16-213 in vitro. In each patient studied 24 h after the third drug dose, there was a decrease in the fraction of cells in S phase compared to the pretreatment study (median decrease 56%, range 24–67%). The blast count per ml of marrow decreased by a median of 84% (range 72–91%) over this same interval. The observed decrease of fraction of cells in S phase after three doses of the drug suggests selective kill of cells in S compared to G_0/G_1. We conclude that the activity of VP16-213 against ANLL blasts can be demonstrated in vivo by the production of G_2 phase arrest and reduction of marrow blast count with findings consistent with increased cytotoxicity for cells in S and G_2 phase of the cell cycle. In addition, this intensive combination of VP16-213 and 5AZ has shown clear evidence of effectiveness in patients with refractory ANLL, and thus may be useful during initial therapy.*

Introduction

Complete remission rates of 60–80% are now being reported for new patients with acute nonlymphocytic leukemia using intensive combinations of anthracyclines and ara-C in addition to other agents such as 6-thioguanine [8]. Despite intensive therapy during remission, the majority of patients will relapse with drug-resistant disease [18]. The purpose of this study was to test the efficacy of two new drugs, VP16-213 and 5-azacytidine (5AZ), based on the proven activity of each of these drugs as single agents for the therapy of refractory acute nonlymphocytic leukemia (ANLL) [3, 9, 15–17].

An additional goal of this study was to determine the effects of VP16-213 on leukemic blast proliferation in vivo, based on in vitro studies which have shown that this drug induces premitotic arrest of cycle

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progression and is most cytotoxic for cells in S and G_2 phase [6, 11]. By employing the new methology of DNA flow cytometry in addition to the classical techniques of mitotic and labeling indices and cell count determination, we could determine whether the cell kinetic effects of VP16-213 which had been observed in vitro could also be observed in patients during therapy with the drug.

Materials and Methods

Twenty-four patients with previously treated acute nonlymphocytic leukemia were treated on this study, and their characteristics and previous therapy are listed in Table 1. Twenty-two patients entered this study after failing therapy with the St. Jude AML-76 Protocol study for newly diagnosed patients with acute nonlymphocytic leukemia [4]. In accordance with this protocol, patients received vincristine, daunomycin, 6-azauridine and cytosine arabinoside for remission induction. Continuation therapy with vincristine, cyclophosphamide, adriamycin, 6-mercaptopurine and cytosine arabinoside was given in monthly cycles. Three of the patients treated with VP16-213 plus 5-azacytidine failed induction on the AML-76 Protocol and 19 were in first hematologic relapse which occurred while receiving maintenance chemotherapy. The two most recent patients entered on this study failed induction on the St. Jude AML-80 Protocol which employed intravenous daunomycin plus continuous cytosine arabinoside infusions.

Drug Schedule. An intensive schedule of VP16-213 plus 5AZ was employed. Patients were treated with VP16-213 200 mg/m^2 IV daily × 3 days followed by 5AZ 300 mg/m^2 IV daily × 2 days. These 5-day courses were repeated every 1–2 days until marrow hypoplasia or progressive leukemia was documented by bone marrow aspiration. When marrow hypoplasia was achieved, chemotherapy was held and weekly bone marrow aspirates were performed to monitor normal marrow recovery or a return of blasts. If leukemic blasts rather than normal marrow cells returned, two to three additional courses of chemotherapy were given.

Cell Kinetic Studies. Serial bone marrow aspirates were obtained during therapy with VP16-213 from eight of the children on this study. Bone marrow samples were obtained before, 4 h and 24 h after the first dose of VP16-213 in all eight patients and 24 h after the third dose in six of the patients. After the bone marrow needle was placed, the first 0.2 cm^3 of marrow drawn was used to determine the DNA histogram by flow cytometry and the mitotic index, in order to minimize contamination by cells from the blood. Then 1.5 cm^3 of marrow was drawn in a syringe containing sodium citrate and used to determine the ^{3}H-thymidine labeling index, an additional DNA histogram to correlate directly with the labeling index, a total nucleated cell count, and a differential cell count from a Wright stained smear. Samples were stained for flow cytometry by adding a small volume of the marrow suspension directly to a solution of propidium iodide (0.05 mg/ml) and 0.1 sodium citrate to achieve a final dilution of 10^6 white cells/ml [10]. The marrow samples in propidium iodide solution were allowed to stain for 10 min at 4° C and then stored at 4° C and analyzed within 24 h. The relative fluorescence intensities of cells stained with propidium iodide were analyzed by a Coulter model TPS-1 cell sorter (Coulter Electronics, Inc., Hialeah, Fla., USA). With this instrument, single cells in suspension were exposed to a focused argon-ion laser beam (488 nm), and fluorescence from laser excitation of the propidium iodide-DNA complex in each cell was quantitated after passing through a system of filters and photomultipliers [2]. Measurements of the relative fluorescence intensities of all cells were recorded in the form of a frequency histogram. These data were transferred directly to a Data General Eclipse computer and analyzed for the fraction of cells in G_0/G_1, S and G_2 + M phases of the cell cycle using a computer program which was generously supplied to us by Dr. Jerrold Fried [7]. In order to produce consistent results from the computer program analysis, the basic program was systematically rerun while adjusting the G_1 mean by 0.01 channel units until the height of the S region at the mean of the G_1 peak divided by the height of the S region in mid-S was greater than or equal to 0.1. The results of the computer program, including the predicted regions of the histogram containing cells in the phases of the cell cycle, were then plotted with a Tektronix 4025 graphics terminal and 4662 plotter. The DNA specific fluorescence of 50,000–100,000 cells was measured for each histogram, at a rate of 300–500 cells/s. The mitotic index was determined by direct enumeration of mitotic figures in a wet mount of an aliquot of cells stained with 2% acetocarmine. Ten thousand cells were counted from each sample and the results were expressed as mitotic figures per 1,000 cells. The labeling index was determined using standard autoradiographic methods. Cells were incubated at 37° C after adding 1 μC/ml of ^{3}H-thymidine (specific activity of 1.9 C/mM). Smears were then made fixed in acetic acid alcohol (1 : 3), dipped in Kodak NTB-2 emulsion and stored at 4° C for 11–13 days. Slides were developed in Kodak D19 developer, stained with Wright stain and 1,000 cells were examined and the percent of cells with more than 5 grains over the nucleus was determined. Statistical analysis of the difference between paired cell kinetic measurements at different time points for the same patient was performed using Wilcoxon's signed ranks test [12].

Table 1. Patient characteristics

Total no. of patients	24 (11 males, 13 females)
Median age in years (range)	10 (1–19)
ANLL type[a]:	
Myelocytic (M1, M2)	13
Progranulocytic (M3)	1
Myelomonocytic (M4)	5
Monocytic (M5)	5
Treatment history:	
Induction failure on primary therapy	5
First relapse	19

[a] Classified according to the FAB Cooperative Group [1]

Criteria for Response. Marrow hypoplasia was defined as hypocellularity of the marrow aspirate with less than 5% total blast cells and no identifiable malignant blasts on two Wright stained smears. The criteria for complete remission were findings of a cellular marrow with active normal hematopoiesis, less than 5% blast cells, with no definite leukemic blasts, and normal blood cell counts.

Results

Response to Therapy

The intensive schedule of VP16-213 plus 5AZ employed in this study was effective therapy since 19

of 24 patients had marrow hypoplasia and 11 of 24 patients achieved complete remission (Table 2). To achieve remission, five patients required two courses, four patients required three courses, and two patients required four courses of chemotherapy. Significantly, two of the patients who achieved complete remission were patients who had failed induction with an intensive schedule of daunomycin plus continuous ara-C infusions. VP16-213 plus 5AZ was not found to be more effective for any particular morphologic subgroup of acute nonlymphocytic leukemia as shown in Table 3. Of the 11 patients who achieved complete remission, five received no further therapy and remained in remission for 12–29 weeks (median, 19 weeks), and four received monthly courses of chemotherapy with a remission of 8–18 weeks (median, 11 weeks). The remaining two patients had bone marrow transplants during remission; one died of infection 7 weeks later and the other is still in remission over 2 years after transplant.

The major toxicity produced by VP16-213 and 5AZ in combination was prolonged pancytopenia accompanied by infectious and hemorrhagic complications. A detailed analysis of the length of pancytopenia, the incidence of infection and bleeding, and a comparison of this intensive schedule of VP16-213 plus 5AZ with a less intensive program are described in a separate report [13].

Table 2. Efficacy of therapy

Total no. of patients	24
No. with marrow hypoplasia and	
Complete remission	11
Leukemic regrowth	7
Death in hypoplasia	1
Subtotal	19
No. without response	3
No. who died during induction	2

Table 3. Response by ANLL blast morphology

Morphology	Proportion in complete remission
Myelocytic (M1, M2)	8/13
Promyelocytic (M3)	0/1
Myelomonocytic (M4)	1/5
Monocytic (M5)	2/5
Total	11/24

Cell Kinetic Studies During VP16-213 Administration

A typical DNA histogram from a patient before VP16-213 therapy which illustrates the result of the computer analysis is shown in Fig. 1. Due to the low percentages of cells in S and G_2 + M phase which are typical of acute nonlymphocytic leukemia blasts, the distributions of cells in these regions can be more easily visualized in a ten-fold enlargement of the histogram (Fig. 1, Panel B). The computer program fits the G_0/G_1 and G_2 + M regions with Gaussian distributions and when implemented as described, results in a relatively rectangular-shaped S-phase region. The percent of cells in DNA synthesis from the citrate bone marrow sample was measured by both the DNA histogram and the ^{3}H-thymidine labeling index on 22 samples from eight patients. The percent of cells in S-phase calculated from the DNA histogram was found to be highly correlated with the results of the labeling index (correlation coefficient = 0.89) but the slope obtained from least squares linear regression was 0.66, indicating that the percent of cells in S-phase from the DNA histogram consistently underestimated the percent of cells incorporating ^{3}H-thymidine as measured by the labeling index.

Figure 2 shows an example of serial blast cell kinetic studies from a patient receiving the 3 daily doses of VP16-213. DNA histograms are shown before treatment, 4 h and 24 h after a single dose and then 24 h after the third dose of VP16-213. G_2-phase arrest of cell cycle traverse was evident 4 h after treatment with the drug, with a fall in the mitotic index from 7.1/1,000 cells to 0.9/1,000 cells and an increase in the percent of cells in G_2 + M from 2.6%–3.5%. Twenty-four hours after the first dose and 24 h after the third dose of the drug, the mitotic index remained low, and there was a decrease in the percent of cells in S-phase on the DNA histogram compared to the pretreatment value.

Despite individual variations in cell kinetic perturbations induced by VP16 from patient to patient, two consistent patterns were identified. The first consistent finding was G_2-phase arrest of cycle progression which was evident 4 h after the first dose of VP16-213, as shown by a marked decrease in the mitotic index observed in all eight patients studied (Fig. 3, Panel A). Twenty-four hours after the drug was given there was partial recovery of the mitotic index to less than pretreatment values. Accumulation of cells in the G_2 + M region of the DNA histogram was not uniformly observed (Fig. 3, Panel B) even 24 h after the first dose of VP16-213, despite evidence from the mitotic index that the G_2 blockade was sustained over this time period.

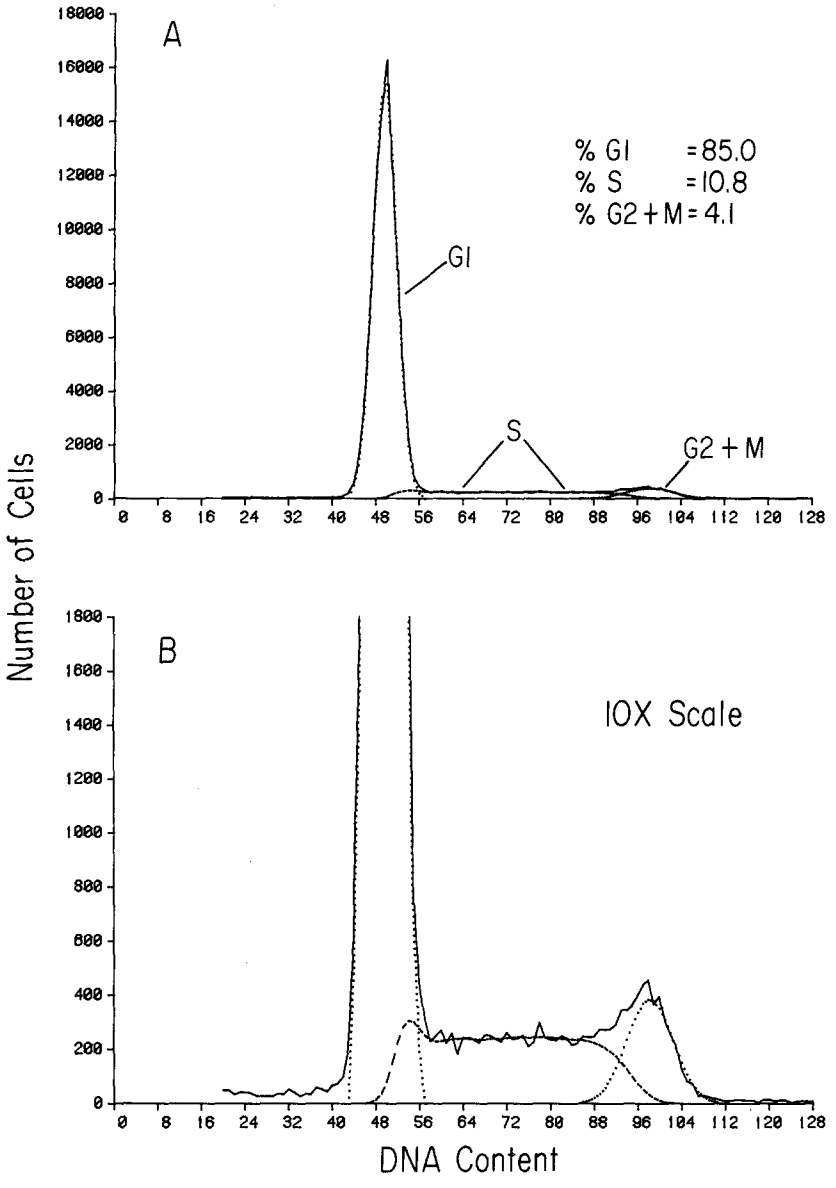

Fig. 1A and B. The DNA histogram of marrow myeloblasts from a patient before therapy with VP16-213. The G_1, S, and $G_2 + M$ regions are indicated on the full scale histogram (*panel* **A**). The 10-fold enlargement (*panel* **B**) gives greater detail of th S and $G_2 + M$ regions, outlined by dashed lines as determined from the computer analysis. The % of cells in G_1, S, and $G_2 + M$ (*upper right corner*) are based on computer integration of these regions

The second consistent cell kinetic finding was a decrease of the percent of cells in DNA synthesis observed after 3 daily doses of VP16 (Fig. 4). The percent of cells in S-phase on the DNA histogram decreased in all six patients studied 24 h after the third dose of VP16-213, with a median decrease of 56% and a range of 24%–67% (Fig. 4, Panel A). When the labeling index was used to assess the percent of cells synthesizing DNA, the value also decreased over the same time period, with a median decrease of 62%, and a range of 36%–82% (Fig. 4, Panel B).

In addition to studies of the perturbations of the cell cycle of the leukemia blasts during therapy, total nucleated cell counts and differential counts on the aspirated bone marrow were performed. When the leukemic blast count/ml of bone marrow 24 h after the third dose of the VP16 was compared to the pretreatment value, a marked decrease was observed in all six patients studied, with a median decrease of 84%, and a range of 72%–92% (Fig. 5).

Of the six patients with studies at all four time points, all six achieved marrow hypoplasia with three achieving complete remission and three demonstrating a return of leukemic blasts instead of normal cells after hypoplasia. No correlation was found between the cell kinetic response to VP16-213 and the clinical response of individual patients.

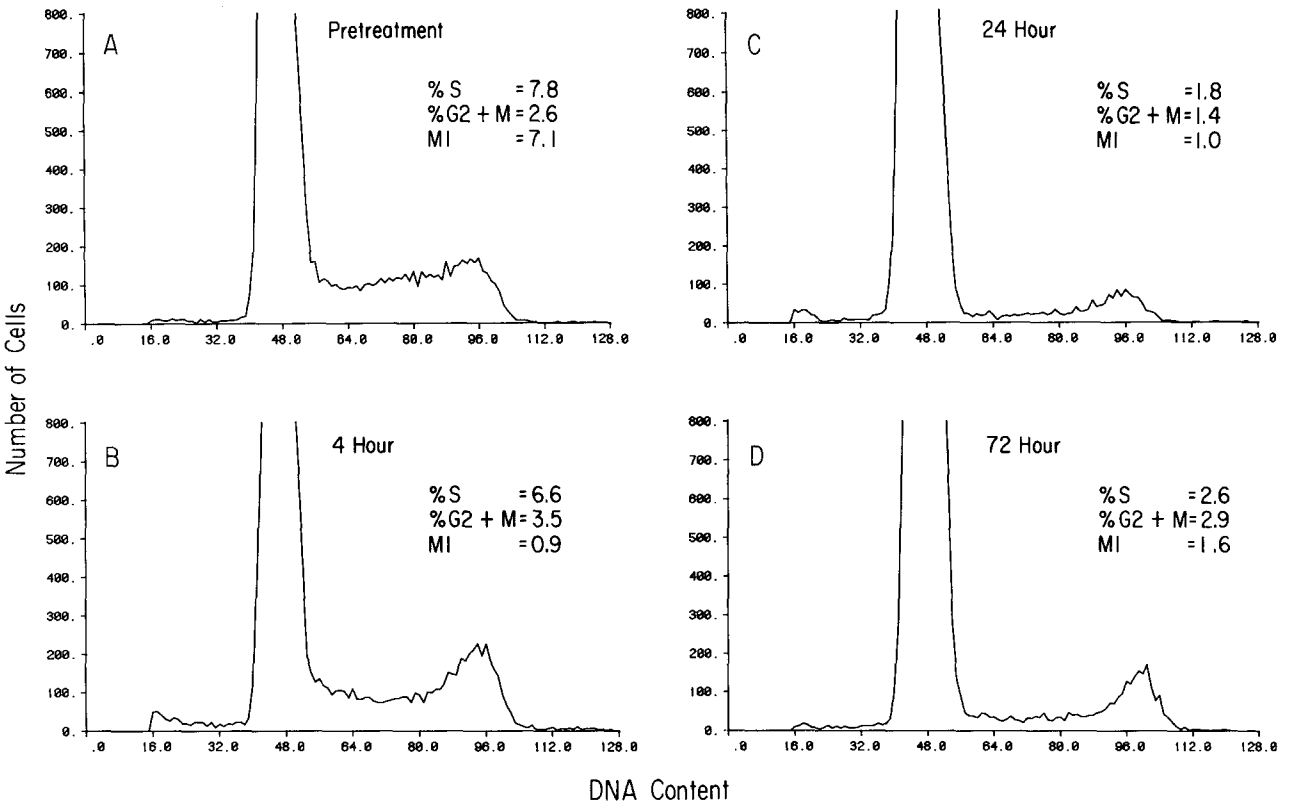

Fig. 2A–D. Serial DNA histograms (10× scale) and mitotic indices of bone marrow myeloblasts from a patient before and during therapy with VP16-213. G_2 phase arrest is evident 4 h after the first dose of VP16-213 (*panel* **B**) based on the decreased mitotic index and increased % G_2 + M compared to the pretreatment values (*panel* **A**). The mitotic index remains low 24 h after the first dose of the drug (*panel* **C**) and 24 h after the third dose of the drug (*panel* **D**) implying sustained G_2 phase interruption of cycle traverse. The % of cells in S phase is also decreased at these times compared to the pretreatment value

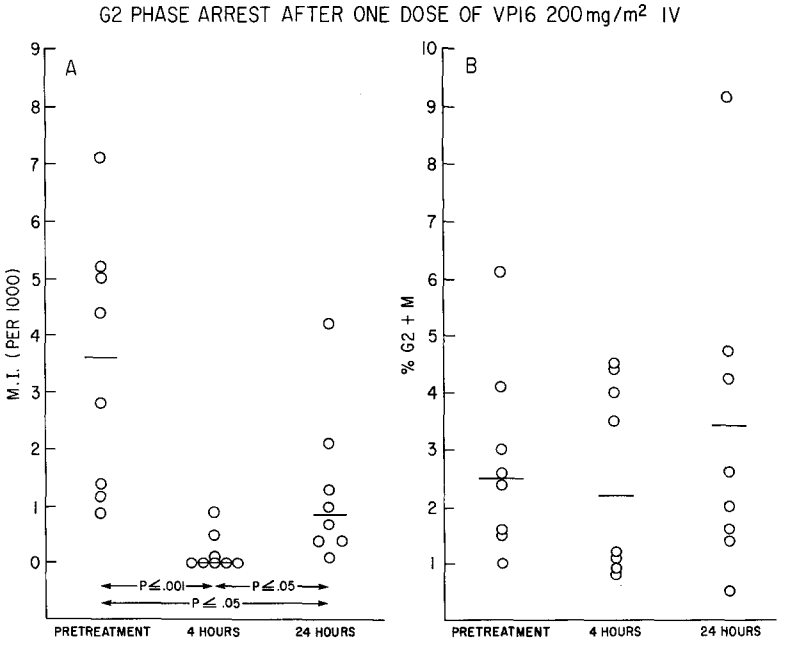

Fig. 3A and B. G_2 phase arrest of cell cycle traverse is evident in all eight patients studied 4 h after VP16-213, based on the markedly decreased mitotic index compared to pretreatment values (*panel* **A**). The mitotic index remains significantly lower 24 h after the drug, implying sustained G_2 blockade. The % G_2 + M from the DNA histograms do not uniformly increase even 24 h after the drug (*panel* **B**), which is consistent with cell death in G_2 phase in addition to the arrest of cycle progression

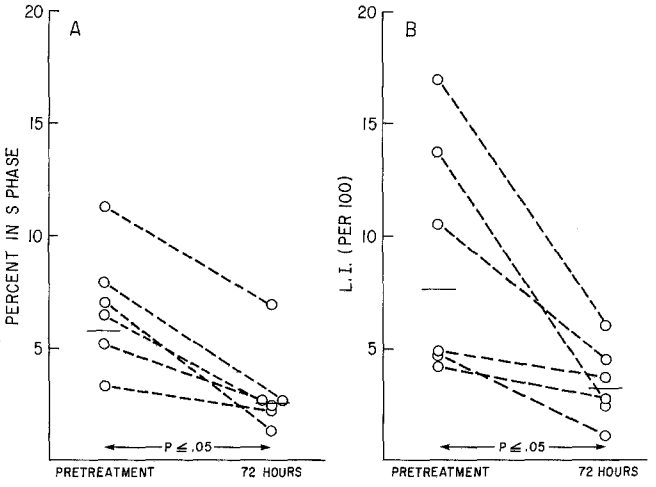

Fig. 4A and B. The percent of cells in DNA synthesis consistently decreased after 3 daily doses of VP16-213, as measured by the DNA histogram (*panel* **A**) and the labeling index (*panel* **B**). These results suggest selective cytotoxicity for cells in S phase compared to G_1 induced by the drug

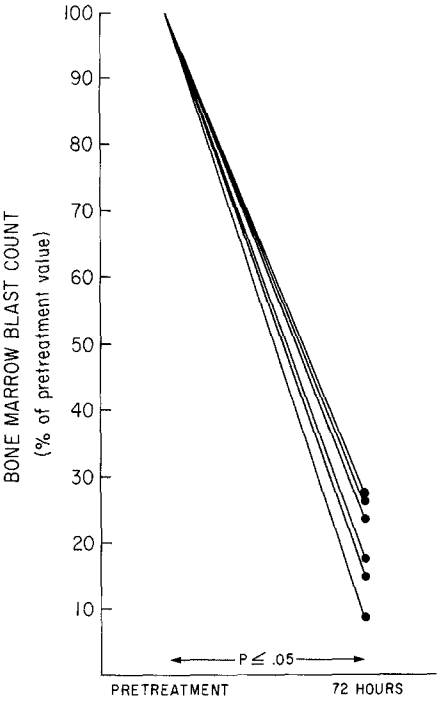

Fig. 5. The cytotoxicity of VP16-213 for bone marrow myeloblasts is demonstrated by the decrease in blast count per ml observed after 3 daily doses of the drug (median decrease 84%)

Discussion

VP16-213 and 5AZ in the intensive combination schedule employed in this study were effective therapy for patients with acute nonlymphocytic leukemia who failed induction therapy or relapsed during primary therapy, which included anthracyclines and cytosine arabinoside. The two drugs were combined because they showed promise as single agents in pediatric patients studied by Rivera et al. [16], Chard et al. [3], and Karon et al. [9]. Since the complete remission rate was nearly 50% (11 of 24 patients), this combination appears to be one of the best alternatives available for acute nonlymphocytic leukemia that is refractory to primary therapy. The selective activity of VP16-213 against monocytic and myelomonocytic variants of acute nonlymphocytic leukemia which has been reported in single-agent trials [3, 14] was not observed for this drug combination. The apparent increased activity of this combination for acute myelocytic leukemic compared to VP16-213 alone may be due to the contribution of 5-azacytidine or to the fact that VP16-213 was used in relatively high dosage compared to other studies.

VP16-213 was given first in this combination, so that serial bone marrow leukemic blast cell kinetic studies of the effect of this drug could be obtained. G_2-phase arrest of cell cycle progression was observed in the leukemic blasts of all eight patients studied 4 h and 24 h after the first dose of VP16-213, as manifested by a marked decrease in the mitotic index which did not recover to the pretreatment value before a second dose of the drug was given. It is interesting that accumulation of cells in the $G_2 + M$ region of the DNA histogram was not observed, which is probably due to selective death of cells in G_2 with elimination of these cells from the population. In addition, a decrease in the percent of cells in DNA synthesis was observed 24 h after the third dose of VP16-213 when compared to the pretreatment value in each patient studied. In view of the marked reduction in blast number/ml occurring over this same time period, the most likely explanation of the finding of decreased percentage of cells in DNA synthesis is selective kill of cells in S-phase compared to G_0/G_1-phase by the drug.

The cell kinetic changes observed in bone marrow leukemic blasts during therapy are consistent with the findings with cell lines incubated with VP16-213 in vitro as reported by Krishan et al. [11] and Drewinko et al. [6]. As previously noted, the marked accumulation of cells in G_2-phase of the DNA histogram which was pronounced in the in vitro studies, was not observed in the in vivo studies of leukemic blasts. This is probably due to the low growth fraction which is characteristic of human leukemia and results in a different relationship between the rate of cell accumulation and the rate of cell death in G_2 compared to the in vitro studies, which employ cells with a much higher proliferative fraction.

The finding that the percent of cells in S-phase from the computer interpretation of the DNA histogram was consistently less than the labeling index from the same sample may result from the computer program when implemented as described in this study to produce a rectangular shaped S phase region. Cell cycle phase analysis with other computer programs, for example the one described by Dean [5], produce greater overlap of early S with G_0/G_1 and late S with $G_2 + M$, which would increase the proportion of cells in S phase. Greater overlap than shown in Fig. 1 is reasonable, since S phase includes cells with slightly more DNA than G_0/G_1 and slightly less DNA than $G_2 + M$.

The findings that VP16-213 induces G_2-phase arrest and preferential kill of cells in S- and G_2-phase as well as striking marrow blast cytoreduction provide further support of the activity of this agent in acute nonlymphocytic leukemia. Since most patients with acute nonlymphocytic leukemia currently relapse during continuation therapy, it is important to develop new drug combinations that can be employed earlier in therapy to prevent relapse. Since VP16-213 plus 5AZ in the intensive schedule employed in this study produced complete remission in nearly 50% of patients with refractory acute nonlymphocytic leukemia, this combination may have potential earlier in therapy to prevent relapse.

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