Optimum Time Sequence for the Administration of Vincristine and Cyclophosphamide in Vivo

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Summary

The cytotoxic effect of the combination of vincristine sulfate and cyclophosphamide was quantitated by the spleen colony assay for both AKR- and L1210-transplantable leukemias as well as for normal hematopoietic stem cells. The lethal effect of this combination on the leukemic cell population was found to be schedule dependent. Subadditive cell killing occurred when the drugs were administered together or within a few hr of each other. A marked enhancement of the cytotoxicity was noted when cyclophosphamide was given after vincristine, with a nadir obtained when 12 hr had elapsed between the administration of the two drugs. The observed killing of normal hematopoietic stem cells by this combination was additive independent of the sequence or schedule of administration.

Introduction

VCR and CY are antineoplastic drugs with significant biological activity against a variety of experimental tumors and human cancers (1, 10, 16). VCR causes metaphase arrest, inhibits the synthesis of both DNA and RNA (5), and has been shown to kill cells selectively when the drug is added during the DNA-synthetic (S) phase of the cell cycle (11). CY is a cycle-specific drug because it preferentially kills proliferating cells (3, 20). It is thought to alkylate many cellular constituents but probably exerts its lethality by reacting with DNA (13).

Clinical experience (7, 8, 14, 15, 17) suggests that the combined use of these 2 drugs is superior to the use of either drug alone in terms of antitumor effect and prolonged duration of survival. However, the schedule for administration of both drugs is empirical and, in many instances, is based on convenience of administration.

In this report we examine the effect of sequence and time interval for administration of VCR and CY on transplantable AKR and L1210 leukemias. A quantitative cellular assay was used for these leukemic cell lines as well as for the normal hematopoietic stem cells. The drug sequence- and interval-dependent inhibition and synergism of cell killing is interpreted in terms of cell kinetics for both the leukemic and normal cell populations.

Materials and Methods

Drugs. VCR (Oncovin) (Lot 5JH82A) was supplied by Eli Lilly and Co., Indianapolis, Ind. in 1-mg vials. CY (Cytotoxan) (Lot MHM45A), in 100-mg vials, was obtained from Mead Johnson Laboratories, Evansville, Ind.

Both drugs were dissolved in sterile 0.15 M sodium chloride. The same diluent was used to prepare the required drug doses that were injected in a volume of 0.5 ml via the tail vein of the mouse.

Mice. AKR and BALB/c × DBA/2 (hereafter called CD2F1) mice were obtained from National Animal Laboratories, Crevé Coeur, Mo., and through the National Cancer Institute, Bethesda, Md., respectively. DBA/2J mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. Mice of either sex were used in these studies when they were 6 to 8 weeks old and weighed 18 to 25 g each.

Leukemic Cells. Two leukemic cell lines were utilized in these experiments: a transplanted AKR line, derived originally from a mouse with spontaneous AKR lymphoma and transplanted weekly into syngeneic mice as previously described (19); and an L1210 leukemia, obtained originally in the ascites formed in DBA/2J mice from the National Cancer Institute and passaged weekly into groups of DBA/2J mice by the i.v. injection of 10⁷ cells obtained from the spleens of leukemic mice. The leukemic mice used in the experiments described below received 10⁷ cells prepared from the spleen of a mouse with advanced leukemia in a volume of 0.5 ml i.v. 4 days prior to drug treatment.

Assay for LCFU. These assays were performed on groups of 4 to 5 leukemic mice at specified times following drug administration. The femurs of control and treated mice were removed and a monodispersed cell suspension of femoral marrow was prepared. After appropriate dilutions were made in α-minimum essential media (Flow Laboratories, Rockville, Md.), 0.5 ml/mouse, was given via the tail vein to groups of 8 to 10 recipients (AKR mice for the AKR leukemia and CD2F1 mice for the L1210 leukemia). Eight days later, the spleens of the recipient mice were removed, and placed in Bouin’s solution, and the number of macro-

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scopic colonies were counted. The number of LCFU from the original donor femur could then be determined (4). The fractional survival of LCFU was determined by normalizing all results to an untreated control group assayed at either 4 or 5 days, depending on the experimental study.

**Assay for NCFU.** This assay is similar to that for LCFU except that 5 normal donor and 15 recipient mice were used. After the recipients received 900 rads total-body irradiation they were given injections of the femoral marrow suspension. These mice were killed 9 to 10 days later, and the number of macroscopic colonies were determined (18). The fractional survival of NCFU following treatment was determined by normalizing the results to an untreated control group.

**RESULTS**

**Dose-Survival Curves for VCR and CY on LCFU.** Dose-survival curves for each drug alone were constructed to determine an appropriate dose of each to be used in the combination experiments. Single, increasing doses of each drug were given i.v. to groups of leukemic mice and the fractional survival of LCFU was assayed 24 hr later. The results were normalized to the 5-day control.

The dose-survival curves obtained for VCR are shown in Chart 1. The AKR leukemic cells were more sensitive than the L1210 cells. The curve for AKR leukemia was exponential throughout 6 decades of survival. The curve for L1210 leukemia was also exponential, but doses greater than 0.05 mg/mouse were lethal to the mice within 24 hr. A dose of 0.025 mg VCR per mouse was chosen for the combination experiments to obtain a surviving fraction of LCFU between 10⁻¹ and 10⁻³.

The dose-survival curves for CY are shown in Chart 2 and are exponential for both leukemic lines. A small shoulder region was observed for AKR leukemia. A dose of 0.5 mg CY per mouse was chosen for the combination studies to obtain a surviving fraction of LCFU between 10⁻¹ and 10⁻².

**Time-Survival Curves for VCR and CY on LCFU.** Before the effect of the combination of the 2 drugs could be studied it was necessary to determine the kinetics of the lethal effect of the selected doses of the 2 agents. This provided a correction factor for proliferation that occurred during the interval between drug combination. The kinetics also indicated the time at which the fractional survival reached its minimum so that the assays would not be done earlier than the optimum time. The assay of LCFU was done at specified times after the administration of 0.025 mg VCR per mouse. The results were normalized to the 4-day control. The fractional survival of LCFU with respect to time for both AKR and L1210 leukemia is shown in Chart 3. There was a gradual decrease in the surviving fraction of LCFU, reaching minimum levels of 10⁻¹ and 10⁻², respectively, at about 12 hr, following which repopulation predominated.

The kinetics of the reduction in the surviving fraction of LCFU after 0.5 mg CY per mouse is shown in Chart 4. There was a rapid decrease in survival for both cell lines, reaching a minimum within 2 hr after drug administration, followed by an increase of the LCFU with a doubling time of about 9 hr. The maximum reduction in fractional survival of LCFU was 10⁻¹ for AKR leukemia and 10⁻² for L1210 leukemia.

**Combined Effect of VCR and CY on LCFU.** Either VCR or CY was given at the dose levels described above, and the alternate drug was administered at time intervals varying from 1 min to 24 hr. One group was untreated and served as the control, another group received VCR only, and a 3rd group received CY only. Each group was assayed for LCFU/femur 24 hr after administration of the last drug. The results were normalized to the 5-day control. Proliferation of LCFU between the drug administrations was corrected by using the times in Charts 3 and 4 at which repopulation commenced. If the 2nd drug was administered during the time interval following the 1st drug when no net increase in LCFU occurred, no correction was made. For
Chart 3. Fractional survival of LCFU as a function of time following the administration of 0.025 mg of VCR per mouse. Different symbols represent different experiments; vertical bars, 1 S.E.

Chart 4. Fractional survival of LCFU as a function of time following the administration of 0.5 mg of CY per mouse. Different symbols represent different experiments; vertical bars, 1 S.E.

longer intervals, it was assumed that the residual cell population increased in number, with a doubling time of 9 hr, and an appropriate correction in LCFU surviving fraction was made.

The results for the AKR leukemia are shown in Chart 5. VCR given alone yielded a mean surviving fraction of $2.6 \times 10^{-3}$ LCFU/femur and CY alone resulted in a mean survival level of $8.1 \times 10^{-2}$ LCFU/femur. The predicted additive fractional survival for the 2 drugs when used in combination is the product of their cytocidal effect when administered independently ($2.1 \times 10^{-4}$). When VCR was given simultaneously with CY, the fractional survival was $3 \times 10^{-3}$, greater than the additive level and nearly equal to the level of VCR alone. As the interval between the administration of VCR and CY was increased, a gradual decrease in the surviving fraction occurred. When CY was given before VCR, the cytocidal effect increased and reached the additive level in approximately 12 hr, remaining at this level up to the 24-hr interval. However, when CY was given after VCR, the decrease in LCFU survival continued with the increase in the time interval between the 2 drugs, and a synergistic effect was found between the 6-hr and 24-hr intervals. The survival fraction returned to the range of the additive level after 24 hr.

The results obtained with L1210 leukemia are shown in Chart 6. The pattern was similar to that found with the
AKR leukemia. When VCR and CY were given at the same time or within a short interval, the cytotoxic effect was less than additive. When the 2 drugs were separated by a time interval ranging between 2 and 24 hr, the effect was synergistic. The minimum fraction survival level was reached when CY was given 8 hr after VCR and remained at this level up to the 24-hr interval. However, the effect was not as great as that observed for the AKR leukemia.

Combined Effect of VCR and CY on NCFU. VCR and CY, both singly and in combination, at the same dose levels described above and for the same intervals as in the previous experiments, were given to normal mice. After an appropriate interval of time, analogous to the LCFU studies, NCFU were assayed in the femoral marrow. The results are shown in Chart 7. VCR given alone resulted in a fractional survival of NCFU of \( 6 \times 10^{-4} \), and CY alone resulted in a survival level of \( 8 \times 10^{-4} \). The predicted additive level is thus \( 4.8 \times 10^{-4} \). All combinations used were found to be additive.

**DISCUSSION**

The results that we obtained can be divided into 2 categories, the effects observed with the drugs when used alone, or combined. The dose-survival curves for the transplanted AKR leukemia for both drugs were similar to those that have been obtained previously (2, 3). The differences found between the AKR and L1210 leukemias might be reasonably attributed to some biochemical differences between the cell lines. The time-survival curves indicate that the cell killing following CY seemed complete within 2 to 4 hr following drug administration, while the cell killing following VCR continued for 12 hr. This probably reflects a difference in the pharmacokinetics of the 2 drugs, in that the rate of disappearance of drug activity from the serum is rapid for the former agent (12) and slow for the latter agent (6).

For the combined drug studies, the results can be considered in terms of 3 distinct time intervals. First, when the agents were given together, the fractional survival of leukemic cells was less than additive and equal to the level achieved by VCR alone. This is possibly because both agents kill cells in the S phase of the cell cycle preferentially, and thus are not independent in their mode of action. This does not totally explain the results, because CY presumably is effective in other phases of the cell cycle and thus the killing should have been greater than that observed with VCR alone.

Second, as the time interval was increased for either sequence of drug, the fractional survival decreased to the additive effect. This possibly demonstrates that, if the drugs are not given at the same time, one might not interfere with the other's independent production of its full cytotoxic effect.

Third, when CY was given about 8 hr after VCR, a synergistic cell killing occurred. This could result from a partial synchronization of the leukemic cells so that the maximum number passes through the CY-sensitive phase at that time. Such a synchronizing effect has been demonstrated in vivo with Ehrlich ascites cells with combined VCR-CY treatment (9). Another possibility considered is that VCR may increase the permeability of the malignant cells, leading to increased incorporation of CY and a greater lethal effect.

Our results on the effect of the combination of VCR and CY on the normal hematopoietic stem cells did not show any schedule dependency. There was no potentiation of the cytotoxic effect of the 2 drugs when CY was administered before or after VCR, contrary to that noticed for the leukemic cell population.

Clinically, these results may be useful in terms of scheduling the use of the 2 drugs to obtain an optimum effect through an additional killing of the malignant cell population without an increased toxic effect on the normal cells. At the present time, we cannot be sure of the time interval with regard to human tumors because we do not understand the basis for the synergy in the experimental system. However, regardless of the cell-cycle-generation time parameter, it would seem advantageous to separate the administration of these 2 drugs by a time interval rather than giving them simultaneously.

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**REFERENCES**


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