Cytotoxic Thresholds of Vincristine in a Murine and a Human Leukemia Cell Line in Vitro

Don V. Jackson, Jr.,* and Richard A. Bender

Medicine Branch, National Cancer Institute, NIH, Bethesda, Maryland 20205. Division of Oncology/Hematology, Bowman Gray School of Medicine, Winston-Salem, North Carolina 27103. Received June 19, 1978; accepted July 25, 1979.

ABSTRACT

L1210 murine leukemia and CEM human lymphoblastoid leukemia cells were exposed to vincristine sulfate in vitro. The response of these cell lines to this agent was measured by the colony-forming ability of L1210 cells in soft agar and inhibition of growth of CEM in suspension culture. Incremental increases of vincristine concentrations in excess of $2 \times 10^{-9}$ M produced a progressive reduction of survival of L1210 cells and suppression of CEM growth under the condition of constant drug exposure. A maximum cytotoxic effect was reached with drug concentrations between $10^{-8}$ and $10^{-7}$ M. When L1210 cells were exposed to vincristine for a variable length of time ranging from 0.5 to 24 hr, $10^{-7}$ M produced a noticeable cytotoxic effect following an incubation of only 30 min. A 50% cell kill of L1210 cells and a 50% reduction of CEM cell growth were produced by $10^{-7}$ M following a 1- to 3-hr period of exposure; 6 to 12 hr were required to produce a similar effect at a vincristine concentration of $10^{-6}$ M. Therefore, the antitumor effect of vincristine is critically dependent on both concentration and duration of exposure. These data suggest the possibility that the effectiveness of vincristine as an antitumor agent could be enhanced if methods are developed to prolong exposure intervals.

INTRODUCTION

Of the Vinca alkaloids isolated from the periwinkle plant, Vinca rosea Linn. VCR and VLB have proved to be the most active antitumor agents. VCR has been widely used in the treatment of many neoplastic diseases, including the non-Hodgkin's and Hodgkin's lymphomas, acute lymphoblastic leukemia, breast carcinoma, Wilms' tumor, neuroblastoma, and embryonal rhabdomyosarcoma (4, 20). The major antitumor effect of this agent appears to be related to its high-affinity binding to the basic protein subunit of microtubules, tubulin, which results in disruption of the mitotic spindle apparatus and arrest of cells in metaphase (17, 18, 20). Many in vitro studies of both VCR and VLB in mammalian cell lines have illustrated this effect (3, 6, 11–13, 15, 21, 22), but a number of investigators have demonstrated reversibility of mitotic arrest in tissue culture (11, 12, 21). Therefore, mitotic arrest may not invariably be translated into loss of cell viability.

Several in vitro studies have demonstrated a dose-dependent antitumor effect of VCR (14, 16, 19, 23). However, due to the limited number of exposure intervals investigated, these studies have not clearly established the relationships between cytotoxicity, drug concentration, and duration of exposure. The present study examines the antitumor effect of VCR in relation to a wide range of exposure intervals in a murine and human leukemia cell line in vitro. The concentrations of VCR used in this study have been clinically attainable in plasma following i.v. administration (1, 9).

MATERIALS AND METHODS

Drugs. VCR sulfate was obtained from the Drug Development Branch, National Cancer Institute, Bethesda, Md. Stock solutions of VCR were prepared by its addition to RPMI Medium 1640 (Grand Island Biological Company, Grand Island, N. Y.) and filtering through a 0.20-μm membrane filter (Nalge Sybron Corp., Rochester, N. Y.). The resulting solution was stored at 4° and was used for up to 2 weeks after preparation. Suitably diluted aliquots of this stock solution were used in all the experiments described herein.

Cells and Media. L1210 murine leukemia cells were maintained in 75-cm² tissue culture flasks (Falcon Plastics, Oxnard, Calif.) in RPMI Medium 1640 supplemented with fetal calf serum (5%), penicillin (20 units/ml), and streptomycin (20 μg/ml). A human lymphoblastoid leukemia cell line originally isolated from a leukemic child, CEM (7), was obtained from H. E. M. Research, Inc., Rockville, Md. This cell line was grown in spinner bottles supplemented with serum and antibiotics as indicated above. L1210 and CEM cell lines were maintained in log-phase growth by periodic dilution and had mean doubling times of approximately 12 and 62 hr, respectively.

Cloning. The colony-forming ability of L1210 cells was determined by the soft-agar cloning technique modified from the methods of Chu and Fisher (5). Soft agar medium was prepared by autoclaving 1.3% Noble agar (Difco Laboratories, Inc., Detroit, Mich.) in double-distilled water. The agar was cooled to approximately 44°, and 10 ml were added to 90 ml RPMI Medium 1640 (supplemented as above) which had been prewarmed to 44°. Three ml of this agar preparation were dispensed into 12-× 75-mm plastic tubes (Falcon Plastics) and cooled to 37°. L1210 cells in log-phase growth were serially diluted to a concentration of 100 cells/ml, and 1 ml was dispensed into each tube to give a final agar concentration of 0.032%. In constant-exposure experiments, aliquots of stock VCR solution sufficient to give concentrations of VCR between $10^{-10}$ and $10^{-6}$ M were added directly to 3 replicate tubes following inoculation with cells. In variable-exposure experiments, cells were exposed to VCR ($10^{-9}$ to $10^{-7}$ M) for varying time intervals (0.5 to 24 hr). In these studies, the cells were initially grown in suspension culture at 4 to 8 $\times 10^5$ cells/ml in tightly capped 75-cm² flasks, incubated with VCR for varying intervals at 37°, washed twice with bicarbonate-buffered 0.85% NaCl solution (pH 7.4), and serially diluted to deliver...
100 cells into each of 3 replicate tubes. All tubes were capped, inverted 3 times, and placed in an ice bath for 15 min to facilitate gelation of the agar. After warming to room temperature for 45 min, the tubes were placed in a humidified incubator at 37° with 5% CO₂ to maintain media pH. The colonies were visually counted at 14 days and reported as their cloning efficiency [number of colonies of test cells/number of control colonies] × 100. The average cloning efficiency of control cultures was approximately 90%. CEM cells were not suitable for cloning studies due to a very low (<5%) plating efficiency.

Suspension Culture. CEM cells were initially grown in suspension culture at 1 × 10⁶ cells/ml in 20-ml volumes in 75-sq cm flasks to which were added various concentrations of VCR. The flasks were loosely capped and placed in a humidified incubator at 37° with 5% CO₂. In constant-exposure experiments, using 10⁻¹⁰ to 10⁻⁶ M VCR, aliquots of the cell suspension were removed from 3 replicate flasks daily for 4 days and counted using a Model B Coulter Counter (Coulter Electronics, Hialeah, Fla.). In variable-exposure experiments, cells were exposed to VCR (10⁻⁹ to 10⁻⁷ M) for selected time intervals (0.5 to 24 hr), centrifuged at 750 × g for 5 min, washed twice with bicarbonate-buffered 0.85% NaCl solution (pH 7.4), re-suspended in duplicate flasks containing fresh media, and incubated as detailed above. The cells were counted 72 hr after being inoculated into fresh media. The data are expressed as the relative cell number [(cell density of exposed cells/cell density of controls) × 100].

All experiments were performed 3 times on different experimental days.

RESULTS

Effect of Constant Exposure of Murine Leukemia Cells to VCR. The cloning efficiencies of L1210 cells constantly exposed for 14 days to increasing concentrations of VCR are presented in Chart 1. A progressive decrease of survival was produced by incremental increases of VCR concentrations in excess of 2 × 10⁻⁹ M, and no surviving cells were noted following exposure to VCR concentrations between 6 × 10⁻⁸ and 1 × 10⁻⁶ M. The concentration which produced a 50% cell kill in L1210 murine leukemia cells under the conditions of constant VCR exposure was approximately 1 × 10⁻⁸ M.

Effect of Variable Exposure of Murine Leukemia Cells to VCR. The cloning efficiencies of L1210 cells following exposure to 10⁻⁹, 10⁻⁸, and 10⁻⁷ M VCR for various time intervals are shown in Chart 2. Following exposure to 10⁻⁷ M VCR, a noticeable reduction of cell survival was seen beginning as early as 0.5 hr (the first observation period), whereas a 1 hr period of incubation was required to produce evidence of cytotoxicity with 10⁻⁸ M VCR. Following a 24-hr exposure to 10⁻⁷ and 10⁻⁸ M VCR, cell survival was reduced to 3.8 and 23.5% of control values, respectively. The intervals of exposure required to produce a 50% cell kill in L1210 murine leukemia cells under these in vitro conditions were approximately 3 hr for 10⁻⁷ M and more than 6 hr for 10⁻⁸ M VCR. A cloning efficiency of 42% was recorded following a 14-day period of constant exposure to 10⁻⁸ M VCR, whereas a 12-hr period of exposure resulted in 40% cloning efficiency and a 24-hr period of exposure resulted in 24% cloning efficiency. The greater effect of 10⁻⁸ M VCR under conditions of variable drug exposure may relate to the traumatic effect of washout procedures.

While such procedures allow exchangeable intracellular VCR to leave the cell, they may also damage the integrity of the cell and make comparisons between constant-exposure and variable-exposure experiments difficult.

Effect of Constant Exposure of Human Lymphoblastoid Cells to VCR. Daily cell counts of CEM cells constantly exposed to VCR for variable time intervals and cloned in soft agar. Experimental details are described in the text. Each point is the mean of 3 experiments. Bars, S.E.
been exposed to $1 \times 10^{-9}$ and $1 \times 10^{-10} \text{ M}$ VCR were capable of doubling their number. The mean doubling time of control cell cultures under experimental conditions was 60 to 64 hr. Increasing concentrations of drug between $1 \times 10^{-9}$ and $1 \times 10^{-8} \text{ M}$ produced an incremental suppression of cell growth. Cells grown at VCR concentrations in excess of $10^{-8} \text{ M}$ dropped from initial densities of $1 \times 10^6/\text{ml}$ to 8.5 to 8.8 $\times 10^5/\text{ml}$ by 24 hr and showed no evidence of subsequent growth. A similar reduction of cell density was noted initially in cells exposed to 6 and $8 \times 10^{-9} \text{ M}$ VCR, but partial recovery of growth was noted after the initial 24-hr period. Chart 4 demonstrates the reduction of cell number expressed as a percentage of untreated controls produced by increasing concentrations of VCR following a 72-hr period of constant exposure of CEM cells. Cell growth was increasingly diminished by VCR concentrations in excess of $2 \times 10^{-8} \text{ M}$ until a plateau of maximal effect was reached with concentrations greater than $10^{-8} \text{ M}$.

**Effect of Variable Exposure of Human Lymphoblastoid Cells to VCR.** The results for CEM cells which were exposed to $10^{-9}$, $10^{-8}$, and $10^{-7} \text{ M}$ VCR for selected time intervals, resuspended in fresh media, and cultured for 72 hr are shown in Chart 5. The data are expressed as ‘relative cell number’ as defined earlier. Growth retardation of CEM cells with $10^{-7} \text{ M}$ VCR was apparent following a 0.5-hr exposure. This concentration displayed a greater inhibition of growth with increasing exposure intervals until a plateau was reached between 12 and 24 hr. A similar diminution of cell growth was shown with $10^{-8} \text{ M}$ VCR although no appreciable effect was demonstrated until CEM cells had been incubated in this concentration for at least 3 hr. A 24-hr period of exposure to $10^{-8}$ and $10^{-7} \text{ M}$ VCR was comparably toxic as shown by a reduction of relative cell number to 20.9 and 17.9%, respectively. The response of cell growth to $10^{-9} \text{ M}$ VCR was variable.

**DISCUSSION**

L1210 murine and CEM human leukemia cells demonstrated sensitivity to a wide range of VCR concentrations from $10^{-9}$ to $10^{-6} \text{ M}$ in vitro. Under conditions of constant exposure at concentrations in excess of $2 \times 10^{-9} \text{ M}$, a brisk reduction of survival and growth of L1210 and CEM cells, respectively, resulted with a maximal effect noted between $10^{-8}$ and $10^{-7} \text{ M}$. The sensitivity of both cell lines to VCR was critically dependent on the length of drug exposure, as well. Whereas a 1- to 3-hr period of exposure to $10^{-7} \text{ M}$ VCR produced a 50% cell kill of L1210 cells and 50% reduction of CEM cell growth, exposure intervals of 6 to 12 hr were required to produce a 50% reduction of both L1210 cell survival and CEM cell growth with $10^{-8} \text{ M}$ VCR. Critical exposure times and concentrations appear to be similar in these 2 cell lines despite significant differences in their growth kinetics. However, since cells in log-phase growth were selected for study because of their uniformity it is unlikely that there would be a sizable difference in the fraction of cells at risk for VCR-induced cytotoxicity during the experimental period in either cell line, a fact which may account for these apparent similarities. Moreover, because CEM cells doubled in 60 to 64 hr under experimental conditions and cell number was measured at 72 hr and scored in assessing the effects of various concentrations of VCR and intervals of ex-
posure, a delay in mitosis and not cell death may account for some differences between control and experimental cultures.

The current study has demonstrated a rapid effect of VCR on survival and growth of 2 mammalian leukemia cell lines in vitro which appears dependent on both drug concentration and on the length of drug exposure. Furthermore, concentrations of VCR between $10^{-8}$ and $10^{-7}$ M which have been shown to produce maximum cytoidal or growth-suppressent effects on these cells lines in vitro are clinically achievable blood levels, although differences between drug metabolism and/or decomposition in vitro and in vivo remain undefined. Following an i.v. bolus of 2 mg of [3H]VCR in patients with non-Hodgkin's lymphomas, Bender et al. (1) noted an instantaneous mean peak blood level of $3.6 \times 10^{-5}$ M followed by a triphasic decay of blood radioactivity with half-lives of 0.85, 7.4, and 164 min, respectively. Despite this rapid decay, low concentrations of VCR and its metabolic and/or decomposition products ($\sim 10^{-8}$ M) may be detected for 1 to 3 hr or more following i.v. injection (1, 9), which may be a consequence of enterohelial recirculation and gradual release from tissue stores (8). The present study demonstrates that such low concentrations of VCR may be cytotoxic or cytostatic in some leukaemia cell lines in vitro when they are exposed for periods as short as 3 hr.

Studies using in vitro techniques also support the importance of duration of exposure as well as concentration of VCR in producing cytotoxicity. Bruce et al. (2) demonstrated progressive killing of murine lymphoma cells following fractionated i.p. doses of VCR. Using a murine bioassay technique to estimate survival of L1210 cells exposed to VCR in vitro for 0.5 to 24 hr, Wilkoff et al. noted a 24-hr period to be the most effective drug exposure time (24). These authors also calculated the minimum effective concentrations of VCR to be between $1.2 \times 10^{-8}$ and $1.2 \times 10^{-9}$ M (24), which is in agreement with the minimum effective concentrations of VCR in L1210 cells ($>2 \times 10^{-9}$ M) obtained by direct measurement in the present study.

The similarity between these data and those reported with VLB using in vitro cloning techniques is of interest (3). A cell survival of 50% was obtained 8 to 12 hr following in vitro exposure to approximately 0.5 to 1.0 $\times 10^{-7}$ M VLB in Earle's L-cells. After a 16-hr exposure period in the same cell line, Valeriote and Bruce (22) noted a cloning efficiency of 50% of control using 2 to $4 \times 10^{-8}$ M VLB.

The present in vitro investigation correlates well with early in vivo trials in pediatric leukemia which suggested an increased response rate with increasing VCR dosage (10), although optimal doses have not been established for most human neoplasms. However, the positive in vitro effect of increasing exposure time of neoplastic cells to this drug has not been possible clinically. Marked cytotoxicity and growth suppression effects of VCR concentrations between $10^{-8}$ and $10^{-7}$ M were consistently demonstrated in this study following exposure periods of 12 hr or more. Maintenance of these concentrations of VCR in vivo has not been possible with the current i.v. bolus method of administration. Furthermore, attempts to increase the frequency of administration of VCR have been fraught with significant host toxicity, principally in the form of dose-limiting neuropathy (10). In the future, if methods are developed to prevent or abrogate the neurotoxicity associated with the administration of VCR, it is likely that enhanced antitumor responses will be observed following the use of infusion therapy or other methods designed to prolong the exposure of neo-plastic tissues to effective concentrations of this agent.

ACKNOWLEDGMENTS

The authors would like to express their appreciation for the assistance of Carol Johnson in the preparation of this manuscript.

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