In Vitro Combination Chemotherapy Demonstrating Potentiation of Vincristine Cytotoxicity by Prednisolone

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Summary

Human lymphoid cells grown in long-term tissue culture have been used to study the cytotoxic effects of the combination of vincristine and prednisolone. The potentiating effects of this drug combination in vitro cannot be consistently shown if the drugs are added without attention to the growth rate of the cultured population. If the cultured cells have achieved maximum density and have remained at this density for more than 24 hr, they are readily killed by vincristine alone and no further kill is achieved by adding prednisolone. Rapidly growing cell cultures, however, are relatively resistant to vincristine. The addition of prednisolone to such cultures restores their sensitivity to vincristine, but the combination is no more effective than is vincristine alone in stable cell populations.

These findings indicate that the effects of vincristine on the mitotic spindle, which produce metaphase arrest, do not account entirely for its ability to destroy cells. A second mechanism of action of vincristine at low concentrations is proposed.

Introduction

The use of combination drug therapy for cancer is gaining increasing acceptance. This has resulted from the demonstrated effectiveness of multiple-drug regimens in the treatment of acute leukemia (7, 9), Hodgkin's disease (4), and carcinoma of the breast (3 and tests (19)). Unfortunately, there are few guidelines that provide a rational basis for the selection of agents to be used in combination, since little is known of the factors responsible for the augmented cytotoxic effects observed when certain drugs are administered together.

Materials and Methods

Cell Line. The long-term human lymphoid cell line used in these studies, SK-LN, was kindly provided by Dr. Bayard Clarkson of the Sloan-Kettering Institute in New York. It was derived from a normal human lymph node obtained at surgery. Cells were grown in Eagle's minimum essential medium (Grand Island Biological Co., Grand Island, N. Y.), supplemented with 10% heat-inactivated, filtered fetal calf serum (Grand Island Biological Co.), glutamine, penicillin, and streptomycin. Cultures were maintained in an atmosphere containing 5% carbon dioxide at 37°C. Cells were diluted with fresh medium to a concentration of 2 x 10^6 cells/ml twice weekly.

Experimental Procedure. Each experiment was conducted in triplicate. Four ml of growth medium, 0.5 ml of drug in solution, and 0.5 ml of cell suspension were added to each culture bottle. On Day 4, cultures were "fed" with fresh medium.

Drugs were diluted in Dulbecco's phosphate-buffered saline (Grand Island Biological Co.) to a final concentration approximating in vivo levels achieved in normal adults given...
pharmacological doses of the same agents (10). When drugs were used in combination, they were prepared at double the standard strength and were added in 0.25-ml volumes to the test culture. Drugs of clinical grade, prepared for i.v. use and free of preservatives, were used in these investigations.

A portion of the vincristine used was supplied as a courtesy by Eli Lilly and Co., Indianapolis, Ind. (Oncovin). Fresh dilutions of drug from lyophilized powders were prepared for each experiment. Vincristine in powder form was stored at 4° as recommended by Lilly. Prednisolone (Meticortelone) was obtained from the Schering Corp., Union, N. J.

Cell counts were performed initially and on Day 7 with a Model F Coulter Counter (Coulter Electronics, Hialeah, Fla.). On Day 0, the cell concentration was usually $1 \times 10^6$ cells/ml. The results were expressed as $R_7$, that is, the ratio of cells counted on Day 7 to the number counted on Day 0. Total inhibition of cell growth for 7 days ($R_7 = 1$) in any culture is considered evidence of destruction of the culture population.

A typical dose (drug concentration)-cell kill curve is shown in Chart 1. $C_i$ is the lowest concentration capable of destroying the entire cultured cell population, while $C_m$ is the minimum concentration necessary to demonstrate any cytotoxic effect for a given drug. Each point on the curves shown in subsequent charts represents the average $R_7$ calculated for 3 cultures exposed to identical conditions. $R_7$ for samples in any triplicate set of cultures generally did not vary from each other by more than 0.5. The dose-cell kill curves are highly reproducible if cell age, drug concentration, and culture conditions are kept constant.

RESULTS

Initial efforts to demonstrate the synergistic cytotoxic effects of vincristine and prednisolone were hampered by variations in cell kill obtained with the use of vincristine alone. The SK-LN cell line was consistently sensitive to vincristine at concentrations above 20 ng/ml. However, on repeated experiments, $C_i$ varied between 1.2 and 20 ng/ml. This distressing lack of reproducibility could not be attributed to differences in medium or serum used or to variations in size of the initial cell inoculum. Eventually, a relationship was established between $C_i$ and the time elapsed since the test cells were last diluted with fresh medium. When cells subcultured 3 days earlier were used to measure vincristine cytotoxicity, they were generally found to be resistant to drug levels below 20 ng/ml. However, 5 days after subculture the cells became considerably more sensitive to vincristine. When concentration-kil determinations were made repeatedly on cells from 5-day-old cultures, a consistent $C_i$ of 5.6 ng/ml was found (Chart 2).

Once a consistent means for determining vincristine cytotoxicity had been established, the influence of concomitant cell exposure to vincristine and prednisolone could be studied. Chart 3 demonstrates the cell kill achieved when SK-LN cells, last provided with fresh medium 48, 72, 96, and 120 hr earlier, were exposed to vincristine alone, prednisolone alone, or the 2 agents together. Cells fed 48 to 72 hr earlier have considerably more resistance to vincristine than did 4- and 5-day-old cultures. Prednisolone itself reduced culture growth to approximately the same degree over a wide range of drug concentrations but did not destroy entire cultures, even when used at doses that, in vivo, would be equivalent to 500 mg/sq m. Prednisolone most clearly augments vincristine cytotoxicity when the 2 drugs are added in combination to recently subcultured cells. On Days 2 and 3 after subculture, SK-LN cell populations are not sensitive to vincristine alone at concentrations below 20 ng/ml (Chart 3). If vincristine is added together with prednisolone, the concentration-kil curve moves considerably to the right for cells of the same age. For example, in Chart 3, the $R_7$ for Day 2 cells treated with vincristine at 12.8 ng/ml is 2.1. When both vincristine and prednisolone are used for cells subcultured at the same time, $R_7$ is only 4. On Day 3, the respective figures for the same concentration of vincristine are $R_7$ of 24 versus $R_7$ of 2.5. Major

| Chart 1. Typical dose (drug concentration)-cell kill curve. $C_i$, lowest concentration capable of destroying the entire cultured cell population; $C_m$, minimum concentration necessary to demonstrate any cytotoxic effect for a given drug; $T$, cell exposure time. |
| Chart 2. Three separate concentration-kil determinations on 5-day-old cultures showing reproducibility of the method. MEM, minimum essential medium; CONC, concentration; FCS, fetal calf serum; $R_7$, the ratio of cells counted on Day 7 to the number counted on Day 0. |
slowing cell growth is demonstrated in Chart 9. When prednisolone is added, cells subcultured every 2 days have a growth curve similar to cells subcultured at 5-day intervals. Delayed growth under both conditions occurs primarily in the 1st 24 hr. Subsequently, the doubling time is the same regardless of frequency of feeding or previous addition of prednisolone to medium. Combination therapy, therefore, exerts its major influence on cell survival within the 1st day, perhaps during the 1st hours of cell exposure.

Chart 3. Cell kill achieved when cultured cells were provided with fresh medium 2, 3, 4, and 5 days prior to exposure to vincristine alone (O), prednisolone alone (●), or the 2 agents together (▲). MEM, minimum essential medium; FCS, fetal calf serum; R7, the ratio of cells counted on Day 7 to the number counted on Day 0.

Chart 4. Growth curve of SK-LN cell line. MEM, minimum essential medium; FCS, fetal calf serum.

Chart 5. Unexpected sensitivity of 3-day-old cells (●) to vincristine alone, behaving in a similar manner to 5-day-old cells (×) exposed to vincristine alone. MEM, minimum essential medium; FCS, fetal calf serum; CONC, concentration; DIL, dilution.

There were times when cultures began to grow with unexpected vigor and achieved twice the usual maximum cell density. During these periods no difference was noted between cells subcultured 3 and 5 days earlier (Chart 5). Ultimately, this was explained by the antecedent feeding history of the cell lines. Chart 6 shows that 3-day-old cells previously subcultured every 5 days were vincristine resistant; on the other hand, 3-day-old cells previously subcultured every 2 days were sensitive to vincristine. The influence of prednisolone on restoring susceptibility to vincristine-resistant cells is again apparent.

To explain the importance of antecedent frequency of subculture on resistance, Chart 7 shows the predicted growth curves for cells diluted with fresh medium every 2 days and every 5 days. If cultures are growing very rapidly, they may, when tested after 3 days for vincristine resistance, already be at maximum density and therefore in the stable phase of growth and sensitive to low doses of drug. When cultures grow more slowly, after 3 days they are in the midst of the logarithmic growth phase and are readily destroyed by vincristine. Actual growth curves at 2 feeding schedules are shown in Chart 8. The influence of prednisolone on differences are also seen between single and combination drug effects at lower concentrations. The drug combination has little advantage when used to destroy 5-day-old cultures, which are already very sensitive to vincristine alone.

When the growth curve for SK-LN cultures maintained in the laboratory as the standard source of test cells was determined (Chart 4), cells subcultured 3 days earlier were found to be in the logarithmic phase of growth. At 4.5 days, these cells reached maximum density and stopped growing, so that, 5 days after subculture, they were well into the plateau phase of the growth cycle. Vincristine resistance, therefore, appeared to be more characteristic of growing cells than of stable cells.

To explain the importance of antecedent frequency of subculture on resistance, Chart 7 shows the predicted growth curves for cells diluted with fresh medium every 2 days and every 5 days. If cultures are growing very rapidly, they may, when tested after 3 days for vincristine resistance, already be at maximum density and therefore in the stable phase of growth and sensitive to low doses of drug. When cultures grow more slowly, after 3 days they are in the midst of the logarithmic growth phase and are readily destroyed by vincristine. Actual growth curves at 2 feeding schedules are shown in Chart 8. The influence of prednisolone on
Potentiation of Vincristine Cytotoxicity by Prednisolone

Sk-LN (MEM + 10% FCS)

— 5–1 (VCR)
— 2–3 (VCR)
— 5–3 (VCR + PRED)

CONTROL

ble of producing mitotic arrest but have neither the therapeutic nor the toxic properties of vincristine. Johnson (12), in reviewing Vinca alkaloid research, summarizes conflicting evidence regarding vincristine action on DNA and RNA synthesis during interphase and concludes that further studies are needed to account for the oncolytic effects of this drug. Noble and Beer (17) have shown that Vinca alkaloids also inhibit the growth of nondividing tumor cells. While metaphase arrest is, therefore, one of the most distinctive results of vincristine treatment, it is by no means clear that this property of the drug can be used to explain all its effects.

The findings reported in the current paper favor the proposition that vincristine has more than 1 site of action and that, at low concentrations, vincristine cytotoxicity is not the result of metaphase arrest.

At concentrations equivalent to those produced by high clinical doses, vincristine kills growing and stable lymphoid cell culture populations equally well. At lower drug levels, however, growing cells are relatively resistant to vincristine (i.e., less than 20 ng/ml), while cells from stable (nongrowing) cultures retain their sensitivity. These observations cannot be accounted for either by assuming that vincristine cytotoxicity is dependent on a single phase of the cell cycle or is even independent of the cell cycle altogether. Drug activity, if it is limited to the G1, G2, or M phases of the cell cycle, should be reflected in greater sensitivity to the cytotoxic effects of vincristine in growing cells than in stable cells. They are not. If vincristine acts only in G1, why are growing cells readily killed at higher concentrations? As a cycle-independent drug, vincristine even at low levels should be as cytotoxic for growing cells as for stable cells. The data are best explained by assuming that there are 2 or more "mechanisms of action" of vincristine.

The cytotoxic effects of vincristine have been related to the mitotic arrest of treated cells (13). How this is achieved is unclear, but it appears most likely that vincristine acts by disrupting the microtubular substructure of cells (8), perhaps by interfering in the synthesis of a protein that binds microtubules together (1).

The cytotoxic effects of vincristine have been related to the mitotic arrest of treated cells (13) in a variety of tissue culture systems. The stathmokinetic influence of this agent has been observed in vivo in both animals (2) and man (6). These observations have led to the conclusion that the therapeutic effects of vincristine, when used in the treatment of human cancer, are dependent on the active growth of the neoplastic cells to be destroyed (14). It has been suggested, therefore, that the drug not be used together with any agent, such as a steroid, that slows the transition of growing cells through the cell cycle (5). Haghbin et al. (11) have taken cognizance of this admonition in planning the L-2 protocol for the treatment of childhood leukemia and deliberately separate the administration of vincristine and prednisone by 12 hr.

Marsden (16), in his extensive review of efforts to define the mechanism of action of vincristine, is much less certain that clinical activity can be correlated with the ability of the drug to arrest cells in metaphase. He cites other drugs, such as demecolcine, colchicine, and griseofulvin, that are capa-
population of cells in which many cells enter the early phases of mitosis. Vincristine at low levels did not affect the exposed lymphoid cells during logarithmic growth in long-term culture. Furthermore, prednisolone, which slows the transition of cells from G$_1$ to the S phase of the cell cycle, would, as predicted by Ernst and Killmann (5), be expected to interfere with cell destruction caused by a mitotic inhibitor. Instead, the addition of prednisolone to vincristine restores the ability of vincristine to kill growing cells, even though prednisolone itself is only minimally cytotoxic for the SK-LN cell line. Where does low-dose vincristine act? It may act in G$_1$, as suggested for vinblastine by Madoc-Jones and Mauro (15). This hypothesis would explain the sensitivity of stable cell cultures. By retaining more cells in G$_1$, prednisolone would then favor vincristine action.

One should take note of the observations of Wilkoff et al. (22) that actively dividing L1210 cells in culture are fairly sensitive to vincristine (0.1 to 25 µg/ml). After 24 hr of exposure to vincristine, the viable cells are considerably reduced. These observations are at variance with our data and may indicate that the experimental conditions were not the same or that all mammalian lymphoid cell lines in culture are not alike. Finally, Skipper et al. (20) have reported that spontaneous AKR lymphoma at diagnosis, when the tumor cells are doubling relatively slowly, responds to vincristine; prednisone increases this cell-kill considerably.

The in vitro findings reported here may have several important clinical implications. In planning multiple-drug regimens for the treatment of neoplastic disease, vincristine should be considered not only for the treatment of the actively cycling tumor cell population but also as 1 of the agents that may be capable of affecting the more stable neoplastic cells that are resistant to most cell cycle-dependent drugs, as has been proposed recently (21). There appears to be no justification for the practice of separating the time of administration of vincristine and prednisone or prednisolone, as in the L-2 protocol of Haghbin et al. (11). The present data strongly suggest that the synergistic activity of vincristine and prednisone, as empirically observed in the treatment of childhood leukemia, is dependent on the simultaneous use of the 2 drugs. More consistent therapeutic results may, therefore, be possible if attention is given to drug administration schedules to assure that they provide for the coincident exposure of tumor cells to both agents.

While cell cultures have been widely used to screen drugs for cytotoxicity or, more recently, to determine cell-cycle dependence of established chemotherapeutic agents, they have seldom been used in the direct study of clinical experience to determine the factors that underlie clinical success. The present study confirms our earlier conclusion (10) that in vitro investigations may be of value in the analysis of clinical problems relating to the chemotherapy of neoplastic disease if questions asked of the model system are simple and well defined. The ability to demonstrate reproducibly the additive cytotoxic effects of 2 antileukemic drugs against human lymphoid cells in culture provides a unique opportunity to study the conditions under which this synergism occurs. Ultimately, it is hoped that such studies will help to provide clinicians with a theoretical framework that will permit them to plan effective combination and sequential drug therapy regimens on a more rational basis.

ACKNOWLEDGMENTS

The authors are indebted to Rosa Pagan and Ann Trapani for technical assistance and to Sophie Falk for typing the manuscript.

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Chart 8. Growth curves of cells previously diluted with fresh medium every 2 days (A) or every 5 days (O). MEM, minimum essential medium; FCS, fetal calf serum.

Chart 9. Influence of prednisolone in slowing cell growth of cultures previously diluted with fresh medium every 2 days. MEM, minimum essential medium; FCS, fetal calf serum.
REFERENCES


MARCH 1975

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