Selection and Characterization of L1210 Sublines Resistant to Teniposide (VM-26)  
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ABSTRACT

Two spectra of L1210 sublines with gradations of resistance to teniposide (VM-26) were selected by stepwise exposure of cultures to increasing concentrations of the drug. Cultures representing the first spectrum were from 20 times to 1200 times more resistant to VM-26 than were cultures of parental cells. At 24 hr after addition of 22 nM VM-26 to the medium, the growth of cultures of parental cells was inhibited by 50%. Increases in resistance to VM-26 among the sublines coincided with increases in population doubling times. When cells were transferred to drug-free medium, there was a sharp decrease in resistance over the first 10 days; the subsequent decline in resistance, over 2 to 4 months, correlated with a decrease in population doubling times. The second spectrum of resistant sublines arose from the first spectrum after the latter had been maintained for about 1 year on various selective concentrations of VM-26. Resistance to VM-26 by this second group of sublines was from 400 times to over 2000 times greater than that of the parental cell line. Doubling times for these resistant cell populations were similar to the normal rate of the parental cell line. Eight sublines were characterized by two chromosomes with homogeneously staining regions, while the remaining subline had a single chromosome with this anomaly. One of the regions appeared on a submetacentric chromosome in seven of the nine sublines, while the other was on an acrocentric chromosome. These observations indicate that a longer doubling time facilitated selection of increasingly resistant sublines but was not essential for the resistance of sublines in the second spectrum.

INTRODUCTION

Teniposide (VM-26), a semisynthetic derivative of podophyllotoxin (33), is a mutagenic (31), cytostatic (11, 18, 23, 34), and cytotoxic (1, 10, 12, 13, 20, 24, 26, 29) antineoplastic agent with logical actions of VM-26 are associated with an inhibition of cellular respiration (7-10, 14), a depression of thymidine incorporation into DNA (23, 33), a block of the mitotic cycle (18, 23, 33), and the formation of single-strand and double-strand breaks in DNA, as well as DNA-protein cross-links (21, 28). The lesions in DNA have been attributed to an inhibition of topoisomerase (22).

In combination with certain other antineoplastic drugs, VM-26 is therapeutically effective against L1210 leukemia and in some patients with acute lymphocytic leukemia (3, 27), neuroblastoma (3, 15), brain tumors (32), or carcinoma of the lung (24); however, the majority of patients treated with VM-26 eventually succumb to drug-resistant disease.

The basis for the therapeutic action of VM-26 and for relapse after an initially favorable response has not been defined clinically. Nonetheless, the acquired resistance of Ehrlich ascites tumor cells to etoposide, a similar derivative of podophyllotoxin, was associated with decreased cellular accumulation of drug (30), and the resistance of a Chinese hamster ovary cell line to VM-26 was associated with cross-resistance to a variety of structurally and metabolically unrelated anticancer drugs, again suggesting an altered transport of epipodophyllotoxins by resistant cells (12). Similarly, the selection of CCRF-CEM human lymphoblasts with resistance to vinblastine produced sublines with cross-resistance to VM-26 (4, 5).

In this paper, we describe the stepwise selection and partial characterization of L1210 cells with acquired resistance to VM-26. Preliminary reports on certain of these data have been presented elsewhere (19, 20).

MATERIALS AND METHODS

Chemicals and Drugs. Crystalline VM-26 was a gift from Bristol Laboratories, Syracuse, NY, and Sandoz Limited, Basel, Switzerland. VM-26 was placed in aqueous solution immediately before each study by dissolving 5 mg of the drug in 0.5 ml of dimethylsulfoxide and subsequently diluting with cell culture medium. Colcemid and trypsin were obtained from the Grand Island Biological Co., Grand Island, NY. Giemsa, improved R66 solution, was purchased from Hopkin and Williams of Chadwell Heath, Essex, England.

Cell Culture. Both the parental and mutant L1210 sublines were grown in Auto-Pow Eagle's minimal essential medium with 10% horse serum and L-glutamine from Flow Laboratories, McLean, VA. Cells were maintained as suspension cultures at 37° in an atmosphere with 5% CO2 and subcultured every 4 or 5 days. Unless otherwise noted, the L1210/VM-26 sublines were routinely maintained in medium with selected concentrations of the drug.

Selection of Mutants. The resistant sublines were selected by growing the parental L1210 cell line sequentially in medium with 10, 100, and 1000 nM VM-26. The concentration of VM-26 in the medium was increased when the recovery of cultures provided a sufficient number of cells for continuation of the selection, for maintenance of cultures at the level of selection, and for storage in liquid nitrogen. Subsequently, the

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4 The abbreviations used are: VM-26, teniposide or 4'-demethyllepipodophyllumotoxin-9(4,6-O-2-thiénylide-β-D-glucopyranoside); HSR, homogeneously staining region; IC50, concentration of VM-26 required to inhibit the growth of cultures by 50% at the end of 24 hr.
5 The term "L1210/VM-26" is used to designate L1210 sublines with acquired resistance to VM-26.
surviving cells were cloned in medium with 0.12% agar and 1, 2, 5, or 7.5 μM VM-26 (16). The colonies appearing in soft agar were transferred to liquid medium with a corresponding concentration of the drug.

IC50 Value and Doubling Time. The sensitivity of L1210/VM-26 sublines is specified by an IC50 value. The proliferation rate, expressed as population doubling time, was determined from the increase in cell number during a 24-hr period (25). Cultures in the logarithmic phase of growth after 48 hr in drug-free medium were diluted to about 300,000 cells/ml and incubated with various concentrations of VM-26 for 24 hr. The cell number was determined with a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL). In each experiment, IC50 values were determined for 3 to 4 sublines, and L1210 cells were titrated as a control.

Chromosome Banding. G-banded chromosomes were prepared by a modified trypsin-Giemsa technique. Cell cultures were treated with Collcemid (0.036 μg/ml) for 20 min and then with 0.075 M KCl for 25 min before fixation in 3 changes of a solution with methanol and glacial acetic acid (3:1). Individual slides were processed by the following protocol: (a) 2 min in a 0.25% solution of trypsin in Hank’s balanced salt solution (pH 7.4); (b) 3 or 4 dips into 0.9% NaCl solution; (c) 4 min in 10% Giemsa stain (pH 6.8); and finally, (d) 4 to 8 rinses with water before drying.

RESULTS

Selection of L1210/VM-26 Sublines. The selection of L1210/VM-26 sublines was begun with 10 nm VM-26, and the concentration increased stepwise to 100 nm VM-26 and then to 1000 nm VM-26. Because of extensive lysis of cells at the higher concentrations of VM-26, it was necessary after each of these increments to wait for a repopulation of the cultures before proceeding with the selection of resistant cell lines.

After repopulation of the cultures with cells that could proliferate in 1 μM VM-26, 50,000 cells were added to 5 ml of medium containing 0.12% agar plus 1, 2, 5, or 7.5 μM VM-26. Numerous colonies were observed after 2 weeks in medium supplemented with either 1 or 2 μM VM-26; several colonies developed in 5 μM VM-26, and a single colony developed in soft agar with 7.5 μM VM-26. Individual colonies were transferred to liquid medium and maintained in a concentration of VM-26 equivalent to that in which the clone originated. Soon after the colonies were placed in liquid medium with the selective concentration of VM-26, portions of the cultures were transferred to a liquid nitrogen bank, while the remainder was maintained in the medium with drug.

There were 2 exceptions to this procedure. The HI subline, isolated and provided by Paula Franklin, was maintained for a period of several weeks in drug-free medium before we knew that the resistant sublines slowly reverted in the absence of VM-26. The LI 7.5 subline was established from a colony that developed in soft agar over 5 weeks in 7.5 μM VM-26. This clone continued to grow slowly after transfer to liquid medium with 7.5 μM VM-26 and therefore was transferred to drug-free medium for 3 months until the culture became established. Subsequently, both the HI and LI 7.5 sublines were maintained in medium with 100 nm VM-26; partial reversion of drug resistance prevented maintenance of these 2 sublines in medium with VM-26 at the same concentration used in the selection on soft agar.

Growth Characteristics. The sensitivity of the cell lines to VM-26 is expressed as the IC50 value (Table 1). The mean IC50 value for 22 assays of the parental L1210 cells was 22 ± 1 (S.E.) nm. For the 9 L1210/VM-26 sublines selected for study, it ranged from 0.4 ± 0.03 μM to 28 ± 0.6 μM when the sublines were assayed initially. When they were reassayed, after maintenance of the cultures for about 1 year in medium containing VM-26, the IC50 values of 7 sublines were 1.5 to 10 times greater than initially (Table 1).

Most cultures of L1210/VM-26 sublines proliferated at a slower rate than did the parental line (Table 1). The mean population doubling time for the L1210 cell line was 16 ± 0.6 hr, whereas that of the sublines ranged from 16 to 38 hr. When the sublines were assayed initially, the increase in population doubling times correlated with an increase in the respective IC50 values (Chart 1).

The correlation between doubling times and IC50 values (ρ < 0.01) did not persist in cultures reassayed after maintenance for 1 year in medium with VM-26 (Table 1). During this time, in which the IC50 value increased for 7 sublines, population doubling times returned to the normal, or near normal, rate of the parental L1210 line.

Reversion of Sublines. Three highly resistant sublines, designated as Lla5μM, Llb5μM, and Llc5μM, were transferred from medium containing 5 μM VM-26 to drug-free medium. Initially, the 3 sublines had IC50 values of about 25 μM and population doubling times of about 37 hr (Table 1). By Day 10, the Lla5μM and Llb5μM sublines had lost about 30% of their resistance to
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μM VM-26, and the Llc5/μM cells had lost about 75% (Chart 2). Thereafter, the IC50 value decreased more slowly, and for Llb5/μM and Llc5/μM, it continued to decrease linearly for the next 120 days. For Llb5/μM, the IC50 value decreased linearly for 60 days, after which the rate of decrease changed. For 2 to 4 months after the initial decrease in resistance, loss of resistance to VM-26 correlated significantly with the decrease in doubling times of the sublines (p < 0.01); however, resistance regressed at different rates for the 3 sublines. A 1 μM change in the IC50 value for Llb5/μM, Llb5/μM, and Llc5/μM sublines was associated with respective decreases of 1.8, 2.7, and 7.7 hr in population doubling times. Although we monitored the regression of sublines for only 6 months, they were regularly transferred to fresh drug-free medium for another 6 months before the study was terminated. At termination, the 3 sublines were as sensitive to VM-26 as was the parental cell line and proliferated as rapidly.

Karyotype of the Sublines. The second spectrum of L1210/VM-26 cell lines was karyotyped because VM-26 induces lesions in DNA and because gene amplification is associated with acquired resistance to certain drugs (2, 6, 17). The cultures selected for karyotypic analysis had been maintained with VM-26 for at least 7 months.

The modal chromosome number of the parental line was 40 with a mean of 41 ± 1.3 (Table 1). The mode for the L1210/VM-26 sublines ranged from 37 chromosomes for Llc2/μM cells to 44 chromosomes for Llc5/μM cells and Ll7.5 cells. The population of cells in the respective sublines was more karyotypically heterogeneous than in the parental line, as indicated by the greater S.E.s of the mean. Double minute chromosomes were not observed in any cell line.

Metaphase cells from each of the sublines also were characterized by acrocentric or submetacentric chromosomes that were not observed in spreads of metaphase cells from either the parental line or the fully revertant lines. Furthermore, in almost every metaphase L1210/VM-26 cell, one to 3 chromosomes displayed a HSR, and in about 40% of the spreads, one of the HSRs was associated with a submetacentric chromosome (Fig. 1). Presumably, the submetacentric chromosome was the product of a translocation. The Ll2/μM cell line was the only resistant subline in which 2 chromosomes with HSRs were not found routinely in metaphase cells.

DISCUSSION

Resistance to VM-26 developed gradually in L1210 cultures, and over a period of about 4 months, a spectrum of 9 L1210/VM-26 cell lines was selected by culturing cells in increasingly higher concentrations of VM-26. The IC50 values for these sublines originally ranged from about 20 times to over 1200 times that of the parental cells. Cells from this original spectrum were subsequently cultured in selective concentrations of VM-26 for 1 year, at which time the IC50 values ranged from 400 to over 2000 times that of the parental cells. The selection of resistant cell lines was terminated when the concentration of dimethyl sulfoxide, in which the drug was dissolved, began to interfere with the titration of resistance.

Resistance to VM-26 in the original spectrum of sublines was accompanied by a decrease in the rate of proliferation of the sublines. To reduce any residual effect of VM-26 on the movement of cells through the division cycle, we collected cells from the various sublines by centrifugation and resuspended them in drug-free medium, where they were cultured for 48 to 72 hr before dilution to an appropriate concentration and titration of drug sensitivity. With these precautions, the increase in population doubling times of the original sublines correlated with the observed increases in the respective IC50 value (p < 0.001).

When cells from the 3 most highly resistant sublines in the original spectrum were placed in drug-free medium, the cell lines gradually began to proliferate more rapidly over a period of several months as sensitivity to VM-26 returned (Chart 2). The gradual decrease in population doubling time correlated with a decrease in the respective IC50 values of the partially revertant cultures (p < 0.01), again indicating that a longer doubling time is associated with resistance. After 6 months of culturing in drug-free medium, the doubling times of these cell lines returned to that of the parental line, whereas about 1 year elapsed before...
the original sensitivity to VM-26 was restored.

These observations demonstrated a pronounced decrease in the proliferation rate of the original spectrum of L1210/VM-26 sublines. Furthermore, because of the length of time over which cell lines reverted, the decrease cannot be attributed to residual drug. On the other hand, maintenance of cultures in medium supplemented with selective concentrations of VM-26 produced a second spectrum of more resistant sublines and, at the same time, permitted selection of more rapidly dividing cultures. Apparently, an increase in population doubling time contributed to the initial selection of resistant sublines but was not essential for resistance to VM-26.

The presence of HSRs on chromosomes in the resistant sublines suggests that gene amplification was associated with the development of resistance (2, 6, 17). Generally, 2 HSRs were present in cells from sublines in the second spectrum, on both acrocentric and submetacentric chromosomes. The temporal relationship between the appearance of an HSR and the apparent translocations in the second spectrum of sublines is not known, nor do we know the contribution of drug-induced lesions in DNA to the development of these karyotypic variations. After 7 months in drug-free medium, the karyotypes of partially revertant derivatives of the L1210/VM-26 cell lines reverted, the decrease cannot be attributed to residual drug. Maintenance of cultures in medium supplemented with selective concentrations of VM-26 produced a second spectrum of more resistant sublines and, at the same time, permitted selection of more rapidly dividing cultures. Apparently, an increase in population doubling time contributed to the initial selection of resistant sublines but was not essential for resistance to VM-26.

In summary, the gradual development of resistance to VM-26 by L1210 cultures was apparently facilitated by a longer population doubling time, which was not essential for the maintenance of resistance. Rather, continued resistance was generally accompanied by chromosomal translocations and HSRs on 2 chromosomes, suggesting a link between genetic instability and cellular resistance to VM-26.

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REFERENCES

Fig. 1. Chromosomes with HSRs from L1210/VM-26 sublines. Representative chromosomes were selected from a single metaphase cell of each subline.
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