

CYTOFLUOROMETRIC STUDIES ON THE ACTION OF PODOPHYLLOTOXIN AND EPIPODOPHYLLOTOXINS (VM-26, VP-16-213) ON THE CELL CYCLE TRAVERSE OF HUMAN LYMPHOBLASTS

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ABSTRACT

Flow microfluorometric analysis of human lymphoid cells exposed in vitro to cytostatic concentrations of podophyllotoxin (0.01–5 $\mu\text{g}/\text{ml}$ for 24 h) shows that a major part of this population (40–60%) has the DNA content of cells in the G_2 -M part of the cell cycle, and that approximately 60% of these cells are arrested in mitosis. Although a similar pattern of DNA distribution is seen in cultures exposed to cytostatic concentrations of VM-26 (0.01 $\mu\text{g}/\text{ml}$) and VP-16-213 (0.1 $\mu\text{g}/\text{ml}$), no mitotic cells are seen in these cultures. Exposure to higher concentrations of VM-26 (0.1 $\mu\text{g}/\text{ml}$) and VP-16-213 (1.0 $\mu\text{g}/\text{ml}$) inhibits cell cycle traverse, and after 24 h of exposure a major part of the population is arrested with the DNA content of cells in the S part of the cell cycle. Exposure to higher drug concentrations leads to a reduction in the number of cells with the late S- G_2 DNA content.

Whereas the cell cycle block induced by cytostatic concentrations of podophyllotoxin (0.01 $\mu\text{g}/\text{ml}$) is readily reversible by reincubation of cells in drug-free medium, cells blocked by VM-26 and VP-16-213 are unable to resume cell-cycle traverse under similar conditions.

Medicinal properties of root extracts from *Podophyllum* sp. have been known for more than a century. However, interest in the possible pharmacological and cancer chemotherapeutic properties of the various podophyllin derivatives has been currently revived by observations that topical application of podophyllin will cure condyloma acuminatum (9). A number of active ingredients have been isolated from podophyllin and include podophyllotoxin and 4-demethyl podophyllotoxin (10). An excellent review of the biochemical, pharmacological, and clinical literature on podophyllin published up to the year 1954 has been compiled by Kelly and Hartwell (10).

Some of the earlier cytological studies have shown the colchicine-like effect of podophyllotoxin on the mitotic and meiotic spindles of both plant and animal cells (22). In eggs, destruction of the mitotic and meiotic spindles by exposure to podophyllotoxin, picropodophyllotoxin, and podophyllinic acid has been reported (3). A number of subsequent reports have confirmed these observations and also described podophyllotoxin-induced nuclear fragmentation and necrosis (10).

Some of the recent studies have shown that podophyllotoxin binds to microtubular proteins (tubulin) and thereby prevents the polymerization of microtubules and formation of the mitotic spindle (25). The resulting failure of cells to divide leads to the accumulation of mitotic-arrested cells by a mechanism similar to that of colchicine and the vinca-alkaloids, vinblastine, and vincristine.

A number of new podophyllin derivatives have been prepared recently and include VM-26 (4'-demethyl-epipodophyllotoxin thenylidene glucoside [or 4'-demethyl-1-0-4, 6-0-thenylidene- β -D-glucopyranosyl] epidodophyllotoxin) and VP-16-213 (*N*-demethyl epipodophyllotoxin ethylidene glucoside). Both of these compounds are of possible chemotherapeutic value in cancer and have undergone phase I clinical trials (2). However, in contrast to the situation with podophyllotoxin, exposure of cells to cytostatic concentrations of podophyllotoxin derivatives, VM-26 and VP-16-213, leads neither to depolymerization of microtubules¹ nor to the accumulation of cells in mitosis (21).

In view of the possible differences in the mode of action of podophyllotoxin and its derivatives VM-26 and VP-16-213, we have in the present report analyzed the effect of various concentrations of these three related compounds on the cell-cycle traverse of cultured human leukemic lymphoblasts.

MATERIALS AND METHODS

Human lymphoblasts of cell line CCRF-CEM initially isolated from the peripheral blood of a pediatric leukemic patient by Foley et al. (6) were grown in suspension cultures and nourished with Eagle's minimal essential medium (for spinner cultures) supplemented with 10% fetal calf serum and antibiotics, penicillin, and streptomycin. Only log phase cultures ($0.5-2 \times 10^6$ cells/ml) were used for incubation with the various drug concentrations in the present study.

Podophyllotoxin (NSC-24818), 4'-demethyl-epipodophyllotoxin- β -D-thenylidene-glucoside (VM-26, NSC-122819), and *N*'-demethyl-epipodophyllotoxin-ethylidene glucoside (VP-16-213, NSC-141540) were obtained from the Cancer Therapy Evaluation Branch, National Cancer Institute. Stock solutions of podophyllotoxin were made in ethanol and diluted in fresh medium before addition to the cultures. Sealed glass ampules of VM-26 and VP-16-213 containing 10 and 20 mg/ml, respectively, in a mixture of benzyl and absolute alcohol were prepared by Sandoz Ltd., Basel, Switzerland.

Viable cell counts were taken after vital staining with

¹ A. Krishan, manuscript in preparation.

trypan blue, and acetocarmine squashes were used for mitotic indices.

For cytofluorographic analysis of DNA content per cell, aliquots of cells were removed at various time intervals and fixed in 50% methanol. Digestion in RNase (1 mg/ml) was followed by staining in propidium iodide according to the published method of Crissman and Steinkamp (4).

Suspensions of stained cells were analyzed in a Cytofluorograf model 4801 (Bio/Physics Systems Inc., Mahopac, N.Y.). In this instrument, fluorescence emanating from excitation of propidium iodide-stained cells by a focused argon laser beam is collected and quantitated through a combination of appropriate mirrors, filters, and photomultipliers. Data on the frequency distribution pattern of the DNA-based fluorescence per cell (after propidium iodide staining) were collected and analyzed in a pulse height analyzer (Bio/Physics model 2100). Histograms generated after analysis of 10,000 cells per sample were photographed from the cathode-ray tube of a storage oscilloscope. Cells per channel data for tabulation were also available on printout tape.

For reversibility studies, cultures exposed to 0.01 μ g/ml of podophyllotoxin, VM-26, and VP-16-213 (0.1 μ g/ml) for 18 h were centrifuged and the cell pellets were washed twice in Hanks' balanced salt solution before being reincubated in drug-free fresh medium. Aliquots from these cultures were analyzed at 2-, 4-, and 6-h intervals for cell counts, mitotic indices, and DNA per cell distribution analysis.

RESULTS

In DNA distribution histograms of Figs. 1-4, the DNA content per cell is recorded in arbitrary units of 100 channels on the abscissa while the height of the bars indicates the number of cells in each channel. The numbers recorded in circles on the histograms represent the percentage of total population recorded between channels 20 and 35 (G_1 -early S DNA content), 36 and 50 (S-DNA content), and 51 and 70 (late S, G_2 , and M DNA content).

Fig. 1 A-E shows DNA distribution histograms of some selected populations of human lymphoblasts (CCRF-CEM) *in vitro*. The DNA distribution of log phase CCRF-CEM cultures (Fig. 1 A) is recorded mainly between channels 20 and 70 of the histogram and contains two prominent peaks of cells with the G_1 DNA content (channels 20-35) and a smaller peak between channels 51 and 70 (late S- G_2 /M DNA content). In between channels 35 and 51 are cells with a DNA content intermediate between that of G_1 and that of G_2 (S-phase). Histogram 1 B shows the DNA distribution of a CCRF-CEM culture exposed to 0.5 mM of DNA

synthesis inhibitory drug IMPY² for 24 h. A major part of this population has the DNA content of the G₁-early S and is recorded between channels 20 and 35 of the histogram. In Fig. 1 C, D are shown DNA distribution histograms of cells released from IMPY-induced G₁-S block and reincubated for 3 and 10 h, respectively, in fresh medium. After 3 h of reincubation, a major part of the cell population is in mid-S and, as seen in Fig. 1 C, 51% of the population has the DNA content recorded between channels 36 and 50. By the 10th h of release and reincubation in fresh medium (Fig. 1 D), a major part of this population either is in G₂-M or has already completed cell division and entered G₁. Accordingly, histogram 1 D shows two major peaks of G₁ cells (channels 20–35) and of cells in the late G₂-M part of the cell cycle (channels 51–70). In Fig. 1 E, we show a DNA distribution histogram of lymphoblasts exposed to 0.01 μg/ml of vincristine sulfate for 18 h. Approximately 60% of the cells in this population were arrested in mitosis by the stathmokinetic effect of vincristine and, as seen in DNA distribution histogram 1 E, 60% of this population had the DNA content of channels 51–70.

On the basis of these representative histograms (Fig. 1) and some of our recent publications on propidium iodide staining (13) and cell cycle synchronization in mammalian lymphoid cells,³ we feel safe to assume that propidium iodide-DNA complex fluorescence as collected and quantitated through the Cytofluorograf represents a fair approximation of the DNA content per cell.

Podophyllotoxin

Table I shows the viable cell counts and mitotic indices of CCRF-CEM human lymphoblasts exposed to various concentrations of podophyllotoxin for 24 h. These cultures were started at an initial concentration of 0.6×10^6 cells/ml, and cell counts in the untreated cultures had doubled after 24 h of incubation. As is evident from this Table, exposure to 0.01–5.0 μg/ml of podophyllotoxin had a cytostatic as well as a mitosis-arresting effect on the cell population. In these cultures, 48–60% of the cells were arrested in mitosis after 24 h of exposure to the drug. The other half of the population contained an approximately equal

number of cells with heteropyknotic⁴ or interphase nuclei.

DNA distribution histograms of some representative cell populations from cultures exposed to various podophyllotoxin concentrations are shown in Fig. 2. Histogram 2 A represents an untreated control cell population while histograms 2 B and C represent cultures exposed to 0.01 μg/ml of podophyllotoxin for 4 and 7 h, respectively. Cell counts recorded on the histograms show that exposure to podophyllotoxin leads to a build-up in the number of cells with the DNA content of channels 51–70, accompanied by a corresponding decrease in the number of cells with the DNA content of channels 20–35. This is presumably due to the mitotic block induced by these concentrations of podophyllotoxin without any pronounced inhibitory effect on the movement of cells from G₁ to S and G₂. DNA distribution histograms of cultures exposed to 0.1 and 1.0 μg/ml of podophyllotoxin for 4–7 h were similar to those of Fig. 2 B, C.

Histograms 2 D and E show DNA distribution of cultures exposed to 0.01 and 1.0 μg/ml of podophyllotoxin, respectively, for 24 h. In both of these cultures, accumulation of cells with the DNA content of channels 51–70 is noted. This increase (40–56%) in the number of cells with the G₂-M DNA content was accompanied by an approximate mitotic index of 50% (40–60%).

In Fig. 2 D the number of cells with the G₁ DNA content (channels 20–35) is higher (26%) than that of cells in Fig. 2 E (15%) and could possibly be due to the escape of some cells from the podophyllotoxin-induced mitotic block at the lower drug concentration.

VM-26

Viable cell counts and mitotic index data in Table I show that incubation of cultures with low concentrations of VM-26 had a cytostatic effect on the cell population, while at higher drug concentrations cytotoxic effects were also seen. In contrast to podophyllotoxin-treated cultures, very few mitotic cells were seen in cultures exposed to this drug. In fact, no mitotic figures were recognizable in cultures exposed to 0.1 and 5.0 μg/ml of VM-26 for 24 h.

⁴ In acetocarmine squash preparations, heteropyknotic cells have dense, heavily stained nuclei. Some of these nuclei do show a faint outline of individual chromosomes and may in fact be mitotic cells with highly clumped chromosomes.

² IMPY is abbreviation for 2,3-dihydro-1-H-imidazo[1,2-b]pyrazole

³ A. Krishan, manuscript submitted for publication.

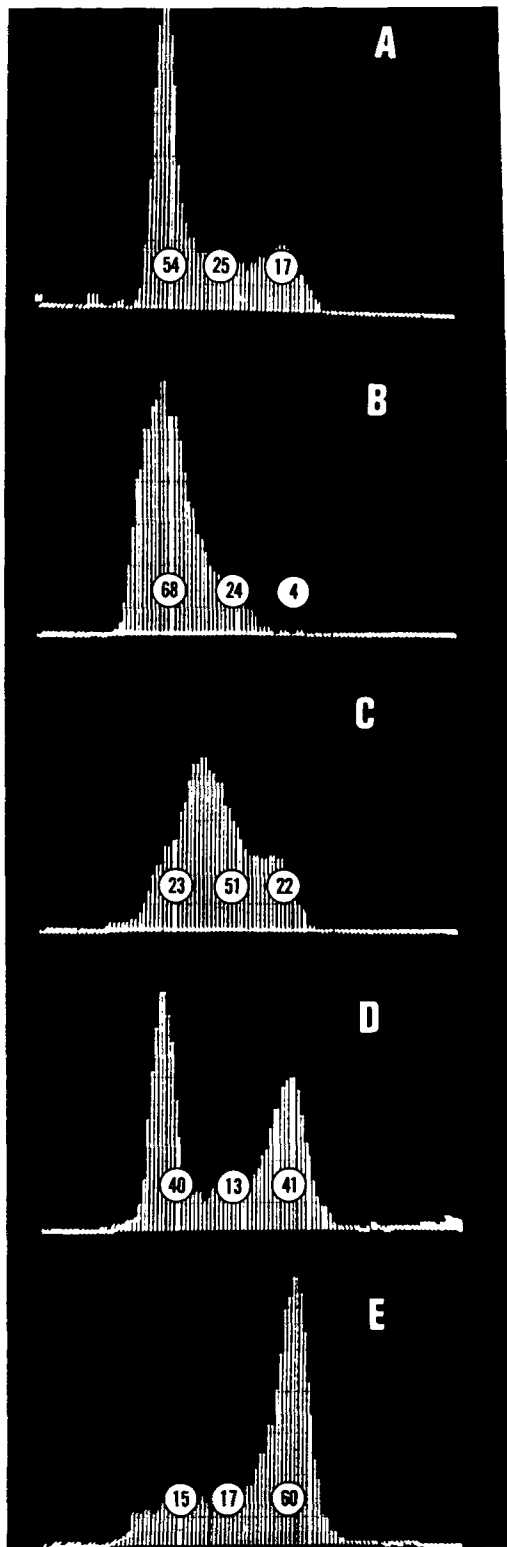


Fig. 3 A-E shows representative DNA distribution histograms from the VM-26-treated cultures. As seen in Fig. 3 A and B, which show DNA distribution after 4 and 7 h of exposure, respectively, to 0.01 $\mu\text{g}/\text{ml}$ of VM-26, a progressive accumulation of cells between channels 51 and 70 is accompanied by a corresponding decrease in the number of cells between the 20th and 35th channels.

A further depletion in the number of cells with the G_1 DNA content (channels 20-35) was seen after 24 h of incubation and, as seen in Fig. 3 C, only 20% of this population had the DNA content of channels 20-35. By this time, the proportion of cells between the 51st and 70th channels (G_2 -M DNA content) had also increased and was now 59% of the total population. However, no mitotic cells were seen in these cultures (Table I).

Exposure to higher concentrations of VM-26 had an adverse and inhibitory effect on the cell cycle traverse of CCRF-CEM cells. In histograms 3 D and E, from cultures exposed to 0.1 and 1.0 $\mu\text{g}/\text{ml}$, respectively, of VM-26 for 24 h, no accumulation of cells between channels 51 and 70 is seen. The DNA per cell distribution histogram of Fig. 3 D (0.1 $\mu\text{g}/\text{ml}$) shows an accumulation of cells in the S part of the cell cycle while in Fig. 3 E

FIGURE 1 A DNA distribution histogram of a log-phase CCRF-CEM culture. A major part of this cell population is recorded between channels 20 and 70. The first major peak is seen between channels 20 and 35 and represents cells with the approximate DNA content of G_1 cells. Inclusions of some cells from the early S part of the cell cycle in this peak is also possible. A second, smaller peak is seen between the 51st and 70th channels and presumably includes cells with double the DNA content of G_1 cells. Cells recorded between these channels would be in the G_2 -M phase, although some contamination with cells in the later part of the S phase cannot be ruled out. Cells recorded between channels 36 and 50 are presumably in various stages of their genome replication (S). **B** DNA distribution in a culture exposed to DNA synthesis inhibitory drug IMPY for 24 h. Note the accumulation of cells in G_1 -S. **C** and **D** DNA distribution histograms of cells removed from culture of Fig. 1 B and reincubated in fresh medium for 3 and 10 h, respectively. In Fig. 1 C, a large proportion of the cells (51%) have the DNA content of channels 36 to 50. By the 10th h of release, two major peaks each having ~40% of the population are seen between channels 20 and 35 and 51 and 70. **E** DNA distribution in a CCRF-CEM culture exposed to 0.01 $\mu\text{g}/\text{ml}$ of vincristine sulfate for 24 h (mitotic index, 60%).

TABLE I
Viable Cell Counts (after Trypan Blue Staining) and Mitotic Indices of CCRF-CEM Cultures Exposed to Various Concentrations of Podophyllotoxin, VM-26, and VP-16-213 for 24 h

	0.01	0.1	1.0	5.0
	μg	μg	μg	μg
Podophyllotoxin	60*/48†	54/57	51/60	50/60
VM-26	54/1.0	41/0	40/0	39/0
VP-16-213	89/2	65/1	51/0	40/0

* Cell counts from drug-treated cultures are expressed as percent of counts from untreated cultures. Thus, a score of 50 and 100 would represent no increase and a doubling of cell counts, respectively.

† This figure represents percent mitotic figures seen after acetocarmine staining. A minimum of 500 cells were counted in each preparation.

(1.0 $\mu\text{g}/\text{ml}$) a reduction in the number of cells with the late S-G₂/M DNA content is evident. No further movement of these cell populations was seen on continued incubation (30 h) in the drug-containing medium.

VP-16-213

Table I shows the viable cell counts of cultures exposed to various concentrations of VP-16-213. Whereas cell multiplication in cultures exposed to 0.1 $\mu\text{g}/\text{ml}$ was more or less inhibited, a reduction in the number of viable cells (after trypan blue staining) was seen in cultures exposed to higher concentrations of VP-16-213.

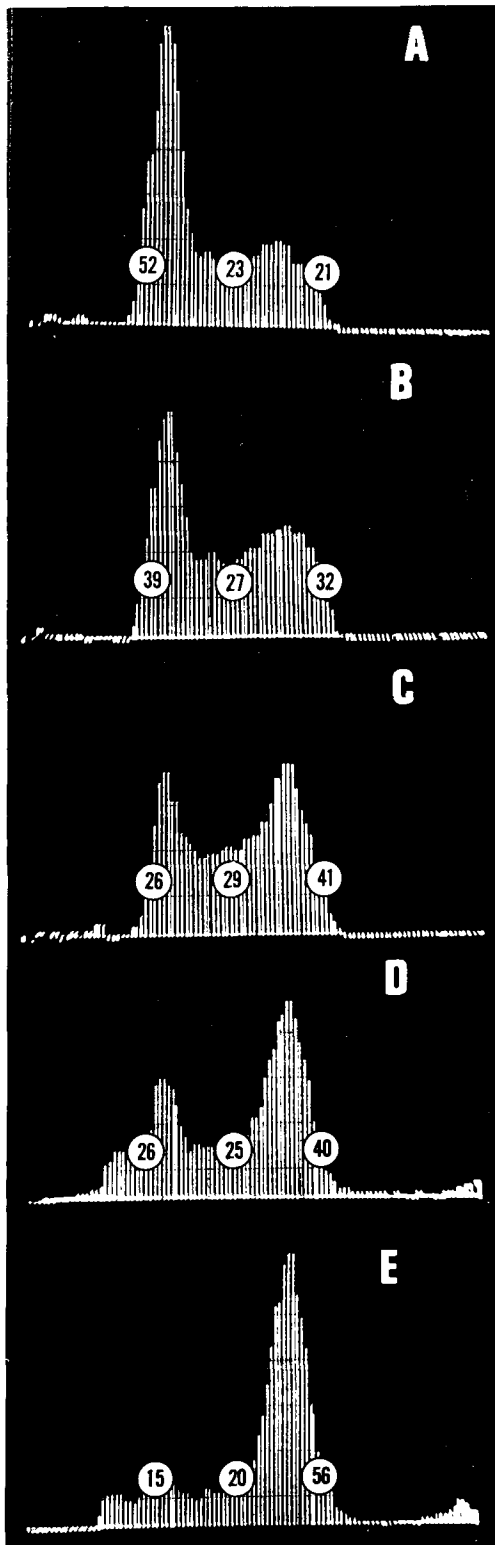
DNA distribution histograms of cultures exposed to 0.01 $\mu\text{g}/\text{ml}$ of VP-16-213 for 6 and 24 h (Fig. 4 A, B) are more or less similar to those of untreated log-phase cultures (Fig. 1 A). However, in both Fig. 4 A and B, a slight increase in the number of cells between channels 51 and 70 (from 17% of untreated cultures) is seen. In cultures exposed to 0.1 $\mu\text{g}/\text{ml}$ of VP-16-213 for 24 h (Fig. 4 C), accumulation of cells with the late S-G₂/M DNA content (channels 51–70) was evident and, although these cells constituted 56% of the total population, no mitotic stages were seen in these cultures. Exposure of cells to higher drug concentrations (1.0 $\mu\text{g}/\text{ml}$ for 24 h) inhibited cell cycle traverse and, as seen in Fig. 4 D, a large number of these cells were accumulated with the DNA content of cells in the S part of the cell cycle. In comparison to Fig. 3 D, which shows a similar pattern of DNA per cell distribution in cultures exposed to 0.1 $\mu\text{g}/\text{ml}$ of VM-26, a larger proportion of these cells were in the early S part of the histogram between channels 36 and 51. As in the case of VM-26 treated cultures (0.1 $\mu\text{g}/\text{ml}$), further incubation of these cultures (up to

30 h) did not cause any major change in the DNA per cell distribution of the population. Exposure of cells to higher concentrations of VP-16-213 (5–10 $\mu\text{g}/\text{ml}$) gave DNA per cell distribution histograms similar to those of Fig. 3 E, with depletion of cells in late S and G₂.

In contrast to cultures exposed to various concentrations of podophyllotoxin (and resembling cultures exposed to VM-26), no mitotic figures were seen in VP-16-213-treated cultures, even though in some of them, such as those in Fig. 4 C, 56% of the population had the late S-G₂/M DNA content.

Reversibility of the Podophyllotoxin-VM-26- and VP-16-213-Induced Blocks

Fig. 5 A shows the DNA distribution histograms of CCRF-CEM cells incubated with 0.01 $\mu\text{g}/\text{ml}$ of podophyllotoxin for 18 h. As seen in this figure, a large number of cells in this culture had the DNA content of late S-G₂/M cells and were recorded between channels 36 and 70 of the histogram. Approximately 36% of these cells were arrested in mitosis. Aliquots of cells were removed from this culture, washed twice in balanced salt solution, and reincubated in fresh medium. In Fig. 5 B, which shows DNA distribution histograms of cells reincubated in drug-free medium for 2 h, a shift in the DNA content per cell of the population is evident. This shift was accompanied by a decrease in the mitotic index from 36% at the end of the 18-h block to 24% after 2 h of reincubation in the drug-free medium. Whereas in podophyllotoxin-blocked cultures only arrested mitotic figures were seen, in the reincubated cultures anaphases and telophases were readily recognizable. On continued incubation in the drug-free medium, a further decrease in the mitotic index was seen (8% after 6 h



of reincubation) with a corresponding increase in cell counts from 0.9 to 1.2×10^6 /ml after 6 h of reincubation. Fig. 5 C, D, which are DNA distribution histograms of cells after 4 and 6 h of reincubation, respectively, in drug-free medium, show a gradual decrease in the number of cells between channels 20 and 35 (G_1) from a low of 20% (Fig. 5 A and B) to a high of 62% in Fig. 5 D. It is evident from these histograms that a large number of cells arrested in mitosis by exposure to podophyllotoxin can resume their cell cycle traverse once removed from the drug-containing medium.

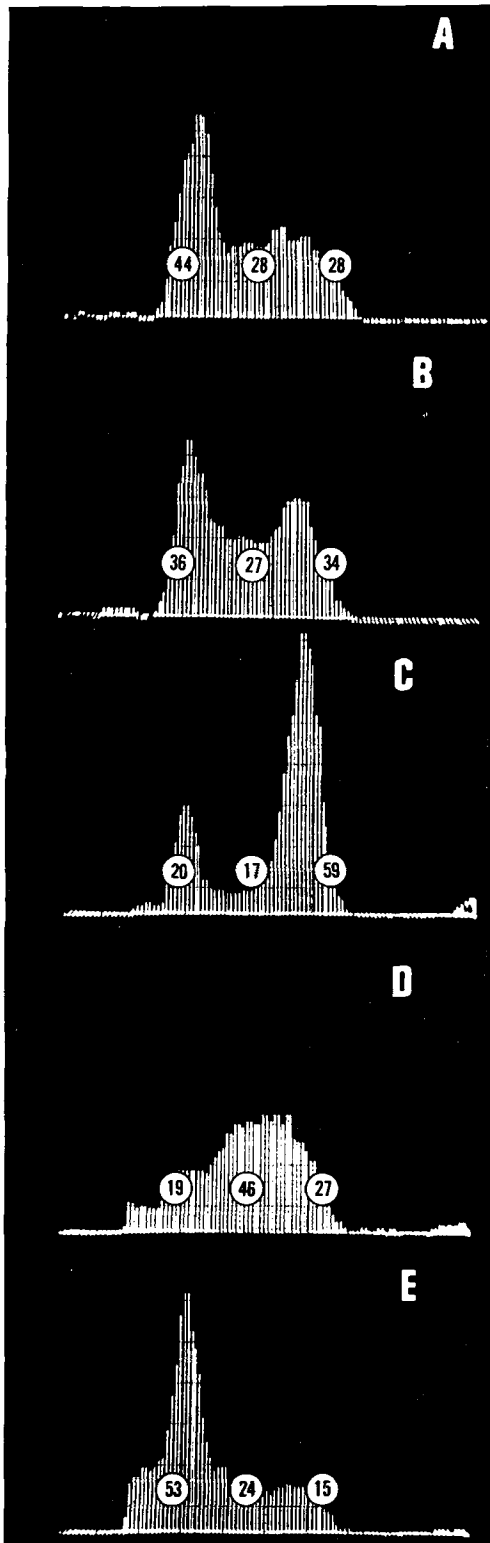
In contrast to podophyllotoxin, the cell cycle block induced by low concentrations of VM-26 ($0.01 \mu\text{g/ml}$) and VP-16-213 ($0.1 \mu\text{g/ml}$) was not reversed by washing and reincubation of the cells in fresh medium. In cultures incubated with $0.01 \mu\text{g/ml}$ of VM-26 for 18 h and subsequently washed and reincubated in fresh medium, no significant changes in DNA per cell distribution histograms, mitotic indices, or cell counts were seen after 6 h of reincubation. Although occasional mitotic figures were seen in the 2–6-h reincubated cultures, they constituted less than 1% of the population.

Similarly, in cultures incubated with $0.1 \mu\text{g/ml}$ of VP-16-213 for 18 h and subsequently incubated in fresh medium, drug-induced G_2 arrest could not be reversed by washing the cells in fresh medium. No significant increase in the number of cells with the G_1 DNA content was seen in these cultures even after 6 h of incubation.

DISCUSSION

After the discovery of cellular microtubules as the essential structural and functional elements of the mitotic spindle (14, 15, 20), a number of studies have demonstrated the ability of the so-called c-mitotic agents to bind to the microtubular proteins (tubulin) and thus prevent organization of the mitotic spindle (7, 11, 17). A number of subsequent studies have described the variety of morphologi-

FIGURE 2 A DNA distribution in a CCRF-CEM culture used as a control for the podophyllotoxin experiments. B, C, and D DNA distribution histograms of cultures exposed to $0.01 \mu\text{g/ml}$ of podophyllotoxin for 4, 7, and 24 h, respectively. Note the gradual increase in the number of cells between channels 51 and 71. E DNA distribution histogram of a culture exposed to $1.0 \mu\text{g/ml}$ of podophyllotoxin for 24 h. 56% of this population had the DNA content of channels 50 to 70.



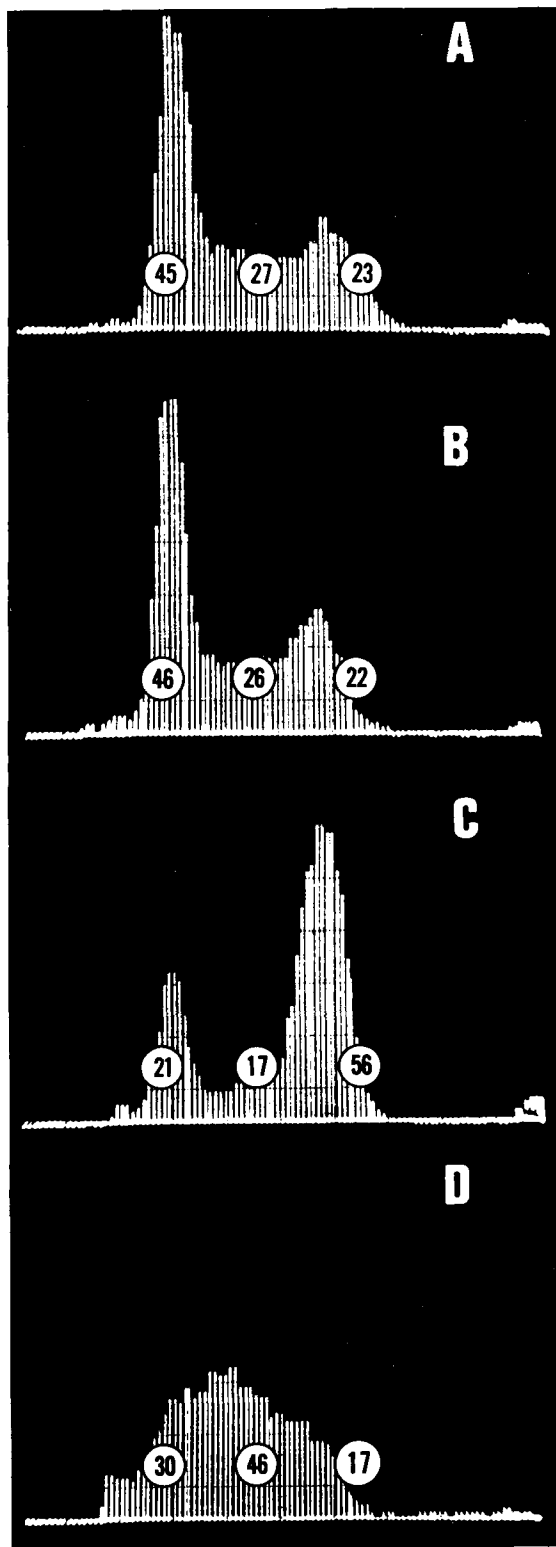
cal (1, 12, 16, 23), biochemical, and physiological changes (5, 17-19, 24) seen apparently as a direct consequence of this affinity between tubulin and the c-mitotic agents such as colchicine and the vinca alkaloids, vinblastine and vincristine. At the morphological level, a prominent manifestation of this affinity between some of the c-mitotic drugs and tubulin is seen in the destruction of the mitotic spindle and accumulation of cells in mitosis.

Although the spindle-destroying effect of podophyllin and some of its derivatives has been known for many years, it was only recently shown that podophyllotoxin shares with colchicine a common binding site on the microtubular proteins or tubulin (25). This binding affinity probably accounts for the colchicine-like mitosis-arresting action of podophyllin and some of its derivatives. Cells arrested in mitosis by exposure to podophyllotoxin show subcellular alterations similar to those of cells exposed to low mitosis-arresting concentrations of colchicine or the vinca alkaloids, vinblastine and vincristine. Besides showing the presence of large kinetochores, annulate lamellae, and micronuclei, these cells are characterized by the absence of microtubules normally seen in association with centromeres and centrioles of mitotic cells.¹

Microfluorometric observations in the present study show the gradual accumulation of cells in mitosis after exposure to various concentrations of podophyllotoxin. Progression of cells through the G_1 -S- G_2 part of the cell cycle is not inhibited by podophyllotoxin, and a large number of cells duplicate their DNA content and accumulate in the G_2 -M part of the cell cycle. The appearance of very high mitotic indices (60%) in cultures incubated for 24 h indicates that it is only at or near the mitotic stage (after chromosomal condensation) that further progress of the cells through the cell cycle is blocked, presumably due to the podophyllotoxin-induced destruction of the spindle microtubules.

Cultures exposed to low concentrations of

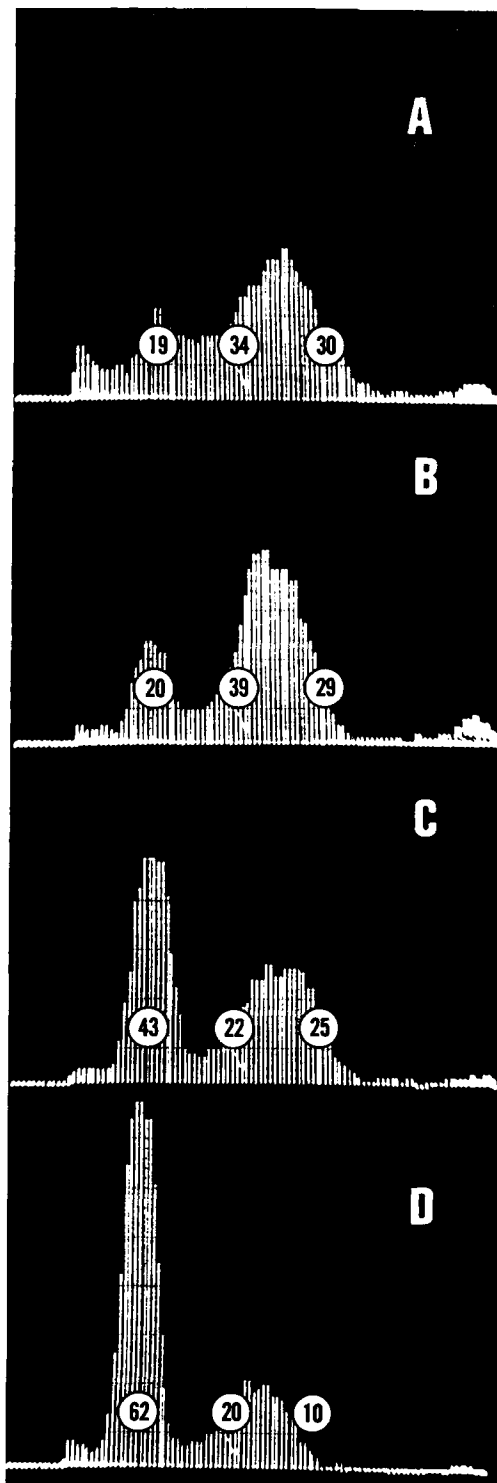
FIGURE 3 A-C Show DNA distribution in cultures exposed to 0.01 $\mu\text{g}/\text{ml}$ of VM-26 for 4, 7, and 24 h. Note the gradual accumulation of cells between channels 51 and 70. D and E DNA distribution in cultures exposed to 0.1 and 1.0 $\mu\text{g}/\text{ml}$ of VM-26 for 24 h. In histogram 3 D, cells are blocked with the DNA content of channels 36 to 70, while in 3 E a reduction in the number of cells between channels 36 and 70 is noted.



VM-26 and VP-16-213 show an accumulation of cells in G_2 (but before the condensation of chromatin into discrete chromosomes) while higher concentrations show inhibitory effects on cell cycle traverse. In cultures where a predominant part of the cell population had the DNA content per cell of G_2 -M, hardly any mitotic cells were seen, although examination of cells by electron microscopy revealed the presence of normal-appearing microtubules (especially in the pericentriolar area). These observations would indicate that even though VM-26 and VP-16-213 share with podophyllotoxin a cytostatic action, they apparently do not act by preventing the formation of microtubules but act at some site between completion of DNA synthesis and condensation of chromosomes. These observations confirm those of Stahelin (21) who had reported that, contrary to the effect of all previously known active podophyllotoxin derivatives, VM-26 did not cause accumulation of cells in mitosis. In fibroblasts exposed to various concentrations of VM-26, he reported an initial arrest of cells in mitosis followed by an inhibitory effect of the various drug concentrations on the entry of cells into mitosis. At high concentrations, cells preparing to enter mitosis were seen to disintegrate into small droplets. In contrast to the action of other c-mitotic agents, such as podophyllotoxin, colchicine, and vinblastine, the action of VM-26 on cells was irreversible. VM-26 also differed from other podophyllin-derived spindle poisons in having a pronounced inhibitory effect on DNA synthesis.

As seen in the present study, the effect of VP-16-213 on cell cycle traverse of CCRF-CEM cells is similar to that of VM-26. At high concentrations, VP-16-213 inhibits cell cycle traverse, while at $0.1 \mu\text{g/ml}$ it leads to an accumulation of cells with the G_2 -M DNA content but before their entry into the chromosome condensation stage. These observations support those of Grieder et al.

FIGURE 4 A and B DNA distribution in cultures exposed to $0.01 \mu\text{g/ml}$ of VP-16-213 for 24 h. Except for a slight increase in the number of cells between channels 51 and 70, these histograms are similar to those of log-phase cultures. C Accumulation of cells between channels 51 and 70 of cultures exposed to $0.1 \mu\text{g/ml}$ of VP-16-213 for 24 hr. D Accumulation of cells between channels 36 and 50 in cultures exposed to $1.0 \mu\text{g/ml}$ of VP-16-213 for 24 h.



(8) who reported that in murine mastocytoma cells exposed to VP-16-213, loss of mitotic activity was accompanied by an increase in the DNA content per cell. Cytophotometric analysis of Feulgen-stained cells showed that the number of cells with twice the DNA content of normal G_1 cells had increased twofold in cultures exposed to 1-10 $\mu\text{g}/\text{ml}$ of VP-16-213 for 6 h.

To conclude, the present study shows that various concentrations of podophyllotoxin allow cells to progress through the cell cycle until they are reversibly stopped in a c-mitotic stage. Compared to this result, podophyllotoxin derivatives VM-26 and VP-16-213 in low concentrations allow cells to traverse the cell cycle until a predominant number of them are irreversibly blocked with the DNA content of G_2 cells but cannot progress to the mitotic stage. Since apparently normal-looking microtubules are still seen in these cells, it is highly suggestive that the VM-26- and VP-16-213-induced lesion prevents the entry of cells that have duplicated their DNA content into the chromosome condensation stage and mitosis.

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FIGURE 5 A A cell population exposed to 0.01 $\mu\text{g}/\text{ml}$ of podophyllotoxin for 18 h. B-D DNA distribution in aliquots removed from the above culture and reincubated in fresh medium for 2, 4, and 6 h, respectively. Note the gradual reduction in the number of cells between channels 51 and 70, accompanied by a corresponding increase in cells between channels 20 and 35.

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