Cancer Chemother Pharmacol (1992) 30: 286 – 290

Cancer Chemotherapy and Pharmacology

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Vincristine potentiates cytochalasin B-induced DNA fragmentation in vitro*

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Received 8 November 1991/Accepted 2 March 1992

Summary. The cytochalasins are fungal metabolites that have previously been shown to have some chemotherapeutic potential. When various cell types are treated in vitro with both cytochalasin B and vincristine, the resultant DNA fragmentation is greater than the sum of that caused by each agent alone. The levels necessary to achieve this potentiation are obtainable in vivo. DNA fragmentation induced by cytochalasin E, an actin-specific agent, is potentiated by vincristine. Pretreatment of the mastocytoma line P815 with vincristine results in an enhancement of the ability of cytochalasin B to fragment DNA. These results indicate that cytochalasin B might be effective as a chemotherapeutic agent in the presence of vincristine.

Introduction

Current chemotherapeutic modalities have had little impact on the mortality associated with the more common cancers [3]. However, the ability to prolong the survival and enhance the quality of life of patients has been accomplished to a significant degree using various chemotherapeutic regimens for different tumors. The success of a given therapy may not be revealed by the solitary actions of a particular drug but may manifest itself in a multidrug regimen.

The cytochalasins are a family of fungal metabolites that have been evaluated as potential chemotherapeutic agents. Cytochalasin D has been shown to enhance in vivo survival in a number of rat tumors grown intraperitoneally [10]. More recently, cytochalasin B has been used to enhance survival in a number of tumors grown via the subcutaneous route but not in the same tumors established intraperitoneally [4]. The mechanism of cytochalasin cytotoxicity in vivo is not known, but recent in vitro work indicates that the cytochalasins can induce DNA fragmentation in a number of different cell lines [13]. These data suggest that the cytochalasins have chemotherapeutic potential, albeit of undetermined efficacy.

Vincristine is a chemotherapeutic agent that has been shown to potentiate the chemotherapeutic effects of other agents [18, 20]. It is a vinca alkaloid that binds specifically to tubulin, disrupting the microtubule system. Since this agent acts on a different component of the cyto-architecture than do the cytochalasins, we wished to evaluate whether the combination of these agents would enhance DNA fragmentation and cytotoxicity to an extent exceeding the sum of those induced by each agent alone. We found that the combination of these agents resulted in a potentiation of DNA fragmentation, which was followed by a concomitant increase in cytotoxicity. The DNA fragmentation was observed in a number of cell lines exhibiting varying degrees of sensitivity to either drug. Pretreatment of sensitive tumor lines with vincristine resulted in an enhancement of the ability of cytochalasin B to induce DNA fragmentation. We discuss the possibility that cytochalasin B could effectively be used as an adjuvant agent against tumors that are weakly sensitive to either drug given as a sole agent.

Materials and methods

Materials. Cytochalasin B and E were obtained from Sigma Chemical Co. (St. Louis, Mo.), and a 2-mg/ml stock solution was prepared in ethanol. Vincristine was obtained from LyphoMed (Rosemont, Ill.) and used as formulated (1 mg/ml). DNA fragmentation assays were performed in medium consisting of Hanks' balanced salt solution containing 0.02 M HEPES and 3% fetal calf serum (FCS) adjusted to a final pH of 7.4 with NaOH (referred to as HBSS). All assays were carried out at 37° C in room air. All media were adjusted to pH 7.4 with NaOH unless otherwise specified. All tissue-culture media consisted of RPMI 1640 containing 10% fetal calf serum (FCS), 6×10^{-5} M 2-mercaptoethanol, L-glutamine, penicillin, and streptomycin.

Cells. The cell lines EL4 ($H2^b$, T-cell lymphoma line), P815 ($H2^d$, mastocytoma), Z2B (human Epstein-Barr virus-transformed cell line),

* This work was supported in part by the Women's Cancer Association and by the Sylvester Comprehensive Cancer Center

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CH33 ($H2^k$, B-cell lymphoma), and LS102.9 ($H2^s$, B-cell lymphoma) were propagated by in vitro passage in the tissue-culture medium described above.

DNA fragmentation assayed by labeled thymidine release. It has been shown that cytochalasin B [13] and vinka alkaloids [16] degrade DNA as evidenced by gel electrophoresis. Since our prior work had shown that thymidine release reflected the DNA fragmentation induced by the cytochalasins [13], we used thymidine release as an indicator of DNA fragmentation. Target-cell DNA fragmentation was assayed by initially labeling target cells in the growth phase with [^{3}H]-thymidine (100 μCi) for approximately 18 h in standard tissue-culture medium. After the labeling period, the target cells were washed and resuspended in unlabeled tissue-culture medium for 1 – 2 h. Following the resting period, the target cells were washed and added to the microtiter-plate wells (15,000/well). Plates containing the targets and pharmacologic agent (final well volume, 150 μl) were incubated at 37° C in a room-air oven. After an appropriate incubation period as dictated by the individual experiment, the amounts of thymidine released from the experimental samples as well as the spontaneous release were determined by adding Triton X-100 to each sample (final concentration, 0.04%), mixing, centrifuging the plate for 5 min at 700 *g*, and recovering 100 μl supernatant for counting in a β-scintillation counter. The total release of [^{3}H]-thymidine was determined by adding 150 μl 0.5% sodium dodecylsulfate (SDS) aqueous solution along with 3 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM TRIS (pH adjusted to 7.4) to 50 μl target cells, followed by mixing, harvesting of 200 μl target cells, and counting radioactivity. It was found that the total radioactivity determined by this technique was identical to that obtained when the triton-releasable radioactivity in the supernatant was added to the radioactivity of the remnant pellet and supernatant solubilized with the SDS solution. The corrected percentage of DNA fragmentation was determined by the equation:

Corrected % DNA fragmentation = $100 \times (C_{exp} - C_{med})/(C_{det} - C_{med})$,

where *C* represents the amount of [^{3}H]-thymidine (expressed in counts per minute) released in the triton-soluble supernatants for the samples specified by the subscripts *exp* (experimental), *med* (targets incubated in medium alone), and *det* (targets solubilized with detergent).

The preincubation experiments were performed as follows: [^{3}H]-thymidine-labeled P815 cells were incubated for 6 h at 37° C in a room-air oven at 3×10^5 cells/ml in a 35-mm-diameter well (6-well plate) containing either HBSS, HBSS plus vincristine (10 μg/ml), or HBSS plus cytochalasin B (5 μg/ml). After the incubation period, the cells were harvested from each well and washed three times with HBSS prior to plating with the various pharmacologic agents as described above in microtiter wells for an 18-h assay.

Cytotoxicity assayed by release of ^{51}Cr from labeled cells. Cells were labeled with ^{51}Cr for 1 h (100 μCi/4 × 10^6 cells in 1 ml medium). Labeled cells were washed, allowed to rest for 30 min, and then washed with the medium appropriate for the individual experiment prior to their addition to the microtiter-plate wells. Labeled cells were incubated with the pharmacologic agents (final well volume, 200 μl) as deemed appropriate for each experiment. Plates containing cells and agents were incubated for 4 h at 37° C in a room-air oven. After the incubation period, supernatants were harvested (100 μl) following centrifugation for 5 min at 700 g, and the radioactivity was quantitated using a gamma scintillation counter. The maximal ^{51}Cr release was estimated by adding Triton X-100 to a final concentration of 0.1% to the target cells alone, whereas the spontaneous release was determined by incubating the targets with media alone. The specific lysis of cells was calculated using the above equation for the corrected percentage of lysis (specific lysis) with *C* representing the amount of ^{51}Cr (expressed in counts per minute) released in the supernatant for the samples specified by the subscripts *exp* (experimental), *med* (targets incubated in medium alone), and *det* (targets solubilized with detergent). Spontaneous-release values amounted to <10% unless otherwise specified.

Statistical analysis. Standard deviations are given for all points unless they are smaller than the symbol. Significance was determined using Student's *t*-test.

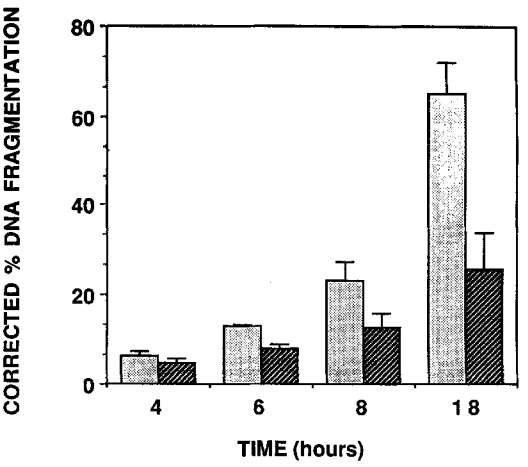

Fig. 1. Ability of vincristine to potentiate cytochalasin B-induced DNA fragmentation, expressed as the corrected percentage of DNA fragmentation plotted as a function of time. [^{3}H]-Thymidine-labeled P815 cells (15,000/well) were incubated at 37° C in vincristine (10 μg/ml) plus cytochalasin B (5 μg/ml) and in each agent alone for the times shown. *Open bars*, DNA fragmentation induced by combination treatment; *hatched bars*, sum of the DNA fragmentation caused by each agent alone. The spontaneous of [^{3}-H]-thymidine amounted to <35% for the 18-h incubation

Results

Vincristine potentiates DNA fragmentation and cytotoxicity induced by cytochalasin

Vincristine has been used to potentiate the effect of various chemotherapeutic agents [18, 20]. We addressed the question as to whether vincristine could enhance the effect of cytochalasin B on DNA fragmentation in vitro. We initially studied the ability of cytochalasin and vincristine to induce DNA fragmentation in P815 cells at different time points. Figure 1 shows the corrected percentage of DNA fragmentation plotted as a function of time. The sum of the DNA fragmentation of P815 cells treated with each agent alone and the fragmentation values observed following incubation of the cells with both cytochalasin B and vincristine are shown for different incubation periods. With prolongation of the incubation period, the potentiation was increasingly enhanced, being most pronounced at 18 h.

The effect of vincristine on DNA fragmentation as a function of the cytochalasin concentration is shown in Fig. 2. Cytochalasin B induced DNA fragmentation in P815 cells after an 18-h period of incubation in the absence of vincristine. When the cells were coincubated with vincristine and cytochalasin B, the amount of tritiated thymidine released was significantly greater than the sum of the amounts released following treatment with agent alone. The effect of incubation of the cells with various concentrations of vincristine in the presence or absence of cytochalasin B is illustrated in Fig. 3 as the corrected percentage of DNA fragmentation. At vincristine concentrations of ⩾1 μg/ml the treatment of P815 cells with both agents resulted in a corrected percentage of DNA fragmentation that was significantly greater than the sum of the values

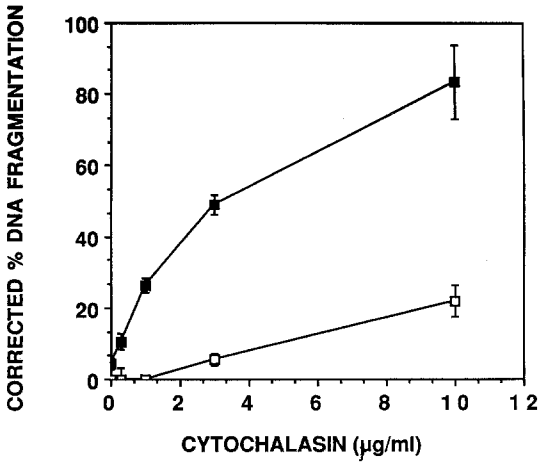

Fig. 2. Enhancement of cytochalasin B-induced DNA fragmentation by vincristine. The corrected percentage of DNA fragmentation is plotted as a function of the cytochalasin B concentration. [^{3}H]-Thymidine-labeled P815 cells (15,000/well) were incubated for 18 h at 37° C in the presence (■) or absence (□) of vincristine (10 μg/ml) at the cytochalasin B concentrations shown. The spontaneous release of [^{3}H]-thymidine amounted to 20%

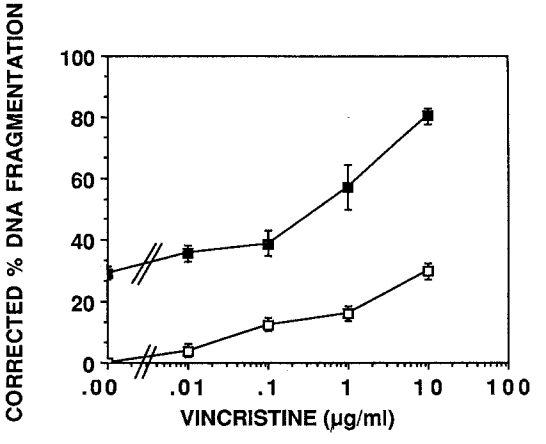

Fig. 3. Enhancement of vincristine-induced DNA fragmentation by cytochalasin B. The corrected percentage of DNA fragmentation is plotted as a function of the vincristine concentration. [^{3}H]-Thymidine-labeled P815 cells (15,000/well) were incubated for 18 h at 37° C in the presence (■) or absence (□) of cytochalasin B (5 μg/ml) at the vincristine concentrations shown. The spontaneous release of [^{3}H]-thymidine amounted to 22%

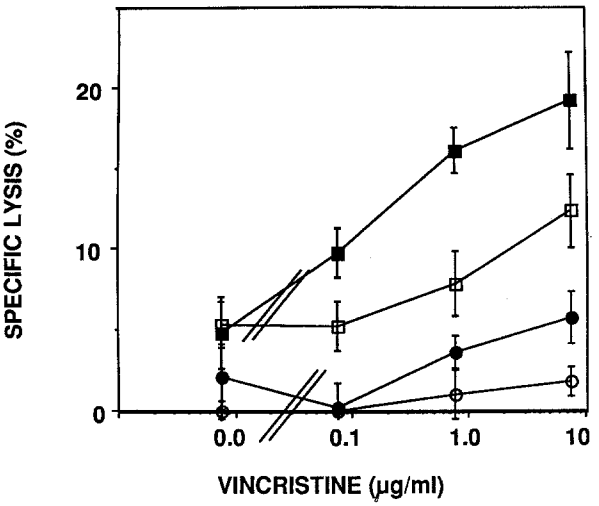

Fig. 4. Potentiation of the lysis of EL4 cells by vincristine and cytochalasin B. The specific lysis (%) is plotted as a function of the vincristine concentration for various fixed concentrations of cytochalasin B (■, 5 μg/ml; □ 2.5 μg/ml; ● 1.25 μg/ml; ○, no cytochalasin B). ^{51}Cr-labeled EL4 cells (15,000/well) were incubated for 4 h at 37° C in cytochalasin B and vincristine at the concentrations shown

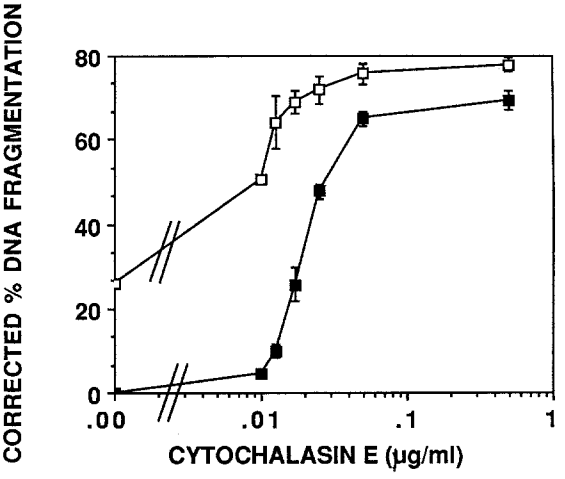

Fig. 5. Potentiation by vincristine of the ability of cytochalasin E to fragment DNA. The corrected percentage of DNA fragmentation is plotted as a function of the cytochalasin E concentration. [^{3}H]-Thymidine-labeled P815 cells (15,000/well) were incubated for 18 h at 37° C in the presence (□) or absence (■) of vincristine (10 μg/ml) at the cytochalasin E concentrations shown. The spontaneous release of [^{3}H]-thymidine was 15%

obtained in cells treated separately with each agent ($P < 0.05$).

In general, cells whose DNA underwent fragmentation proceeded to eventual death as evidenced by membrane lysis. To ensure that the cells examined were undergoing lysis, we looked at the release of ^{51}Cr from EL4 cells that had been treated with various combinations of cytochalasin B and vincristine. Figure 4 shows the specific lysis measured according to the release of ^{51}Cr as a function of the vincristine concentration for different cytochalasin concentrations. It can clearly be seen that the combination of vincristine and cytochalasin B enhanced cell lysis to a degree exceeding the sum of the specific lysis caused by each agent alone when the cytochalasin concentration was >1.25 μg/ml and the vincristine concentration was ⩾1 μg/ml. Similar results were obtained in P815 cells after an 18-h incubation period (data not shown).

In a previous study [13], we demonstrated that a maximal plateau of DNA fragmentation could be reached using concentrations of cytochalasin E that were low as compared with the cytochalasin B concentrations required to achieve similar levels of fragmentation. This finding enables the determination as to whether vincristine treatment results in an enhancement of the maximal DNA fragmentation induced by cytochalasin E alone. Figure 5 illustrates the corrected percentage of DNA fragmentation plotted on a logarithmic scale as a function of the cytochalasin E concentration in the presence or absence of vincristine. A plateau value of approximately 70% was reached using cytochalasin E alone, whereas a level of only 78% was

Table 1. Effect of cytochalasin B and vincristine on DNA fragmentation in different cells

Cell	Corrected % DNA fragmentation (SEM)[a]		
	Cyto B (5 μg/ml)	Vinc (10 μg/ml)	Cyto+Vinc
P815	18.7 (1.5)	16.1 (4.0)	58.3 (2.3)*
EL4	18.8 (4.8)	1.9 (1.2)	35.3 (6.3)*
Z2B	-2.1 (3.0)	15.4 (7.5)	12.9 (5.8)
LS102.9	2.2 (4.4)	2.8 (3.1)	7.1 (0.7)
CH33	8.4 (2.3)	8.0 (2.4)	31.4 (3.9)*

[a] All assays were performed using 15,000 cells/well for 18 h at 37°C except the EL4 assay, which was carried out for 4 h
* Significantly different ($P < 0.005$) from the sum of the DNA fragmentation caused by cytochalasin B and vincristine alone
Cyto B, Cytochalasin B; Vinc, vincristine

attained using both agents. This level was lower than the combined values obtained for each agent alone (ca. 93%). These data demonstrate that vincristine potentiates the ability of cytochalasin E to induce DNA fragmentation as evidenced by the shift in the curve to the left.

Various cell types were evaluated to determine whether vincristine could potentiate DNA fragmentation in the presence of cytochalasin B. Table 1 demonstrates that in many but not all of the cell lines examined, treatment with both cytochalasin B and vincristine resulted in a significant potentiation of DNA fragmentation as compared with the sum of the DNA fragmentation induced by each agent alone.

Pretreatment of P815 cells with vincristine enhances the DNA fragmentation induced by cytochalasin and vincristine

Vincristine is accumulated in cells and is bound unless displaced [2]. We wondered whether pretreatment of P815 cells with vincristine or cytochalasin would enhance the DNA fragmentation induced by these agents alone or in combination. Figure 6 shows the corrected percentage of DNA fragmentation plotted as a function of the pretreatment regimen. Pretreatment of the cells with vincristine resulted in potentiation of the cytochalasin effect as compared with pretreatment with HBSS. Pretreatment with cytochalasin B resulted in a slight increase in DNA fragmentation as compared with the negative control values. However, this finding is not surprising, since cytochalasin B induced only a small increase in DNA fragmentation following 6 h incubation (see Fig. 1) and enhanced fragmentation by only 14% after 18 h in this experiment. Thus, cytochalasin pretreatment did not enhance vincristine-induced DNA fragmentation. Cytochalasin B does not bind irreversibly to cells, and is removed during the washing steps following the pretreatment period.

The corrected percentage of DNA fragmentation for each group of cells was determined using the spontaneous DNA-fragmentation value found for cells that had been incubated in HBSS following preincubation with the appropriate agent. After the 18-h incubation period, the spontaneous release observed was 36%, 36%, and 24% for pretreatment with HBSS, cytochalasin B, and vincristine,

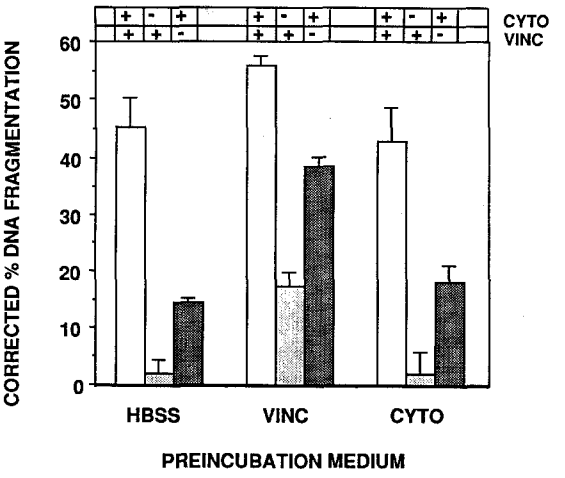

Fig. 6. Enhancement of cytochalasin B-induced DNA fragmentation by preincubation of P815 cells with vincristine. The corrected percentage of DNA fragmentation is plotted as a function of the preincubation medium under the experimental conditions described in Materials and methods. Following 6 h preincubation, [^{3}H]-thymidine-labeled P815 cells (15,000/well) were incubated for 18 h at 37°C in either vincristine (*VINC*, 10 μg/ml), cytochalasin B (*CYTO*, 5 μg/ml), or both of the agents as indicated by the key above the graph

respectively. The enhancement of DNA fragmentation by vincristine in the vincristine-pretreatment group that exceeded the values obtained in the other pretreatment groups was most likely attributable to the exposure of the cells to vincristine for a total of 24 h (18 h+6 h). Trypan-blue staining after the preincubation period yielded approximately 10% positive cells for each group.

Discussion

The cytochalasins are fungal metabolites that induce actin depolymerization by binding to the barbed end of F-actin and affecting the kinetics of polymerization [15]. In 1971, cytochalasin D injected intraperitoneally was found to inhibit the growth of ascites hepatoma AH-130 and Murphy-Sturm lymphosarcoma in rats [10]. However, when the drug was given to mice bearing various intraperitoneal tumors, there was no effect on the tumor index. More recently, cytochalasin B has been shown to prolong survival in mice bearing the subcutaneous tumors B16F10 (melanoma) and M109 (lung carcinoma) [4]. Although cytochalasin B has been reported to affect the viability of Friend erythroleukemia cells cultured in vitro, the mechanism of this effect was not well defined [17]. A recent study has demonstrated that cytochalasin B has the potential to induce DNA fragmentation in a number of cell lines [13]; this DNA fragmentation was most likely secondary to the effect of cytochalasin on the actin filaments.

One of the concerns about using cytochalasin B as a chemotherapeutic agent stems from is ability to affect adherence (see [5, 9]). A number of studies have investigated the incidence of disseminated tumor after pretreatment of tumor cells with cytochalasin [7, 8]. The results of these studies indicated an enhanced incidence of tumor in sites that are not typically affected. The tumors studied were not sensitive to cytochalasin B treatment in vitro. In only one

study was cytochalasin B given to mice bearing intraperitoneal tumors [4]; these investigators also found an increased incidence of disseminated tumor.

The success of cytochalasin B as a chemotherapeutic agent could depend on a number of factors that have not been well controlled; either the drug levels needed to provide a prolongation in survival have not been achieved or maintained, or the tumors studied have not been sensitive to cytochalasin given as chemotherapy. The reasons for pursuing the use of cytochalasin B as a chemotherapeutic agent are (a) it has shown some efficacy in vivo (and in vitro) and (b) it can enhance the immune participation of effector cells against various tumors [11, 12]. Studies examining the latter aspect indicate that cytochalasin B has some immune-enhancing potential.

In the present study, we extended our previous findings that cytochalasin B can fragment the DNA of various cells [13]. We addressed the question as to whether the DNA fragmentation induced by cytochalasins B and E could be potentiated by vincristine. Vincristine has been shown to be a useful agent, as it potentiates the effects of numerous other drugs [18, 20] and has chemotherapeutic potential in itself. Since vincristine induces the disruption of microtubules, we were interested in determining the effect of a combination of two drugs that affect different components of the cyto-architecture on both DNA fragmentation and cytotoxicity. We demonstrated that vincristine could potentiate the effects of cytochalasin B in numerous cell lines (Table 1). Potentiation was observed in the study in which DNA fragmentation secondary to cytochalasin E shifted the titration curve to the left (Fig. 5) and in the investigation of specific lysis as a function of various drug combinations. The concentration dependence of this effect was evaluated for both vincristine and cytochalasin B (Figs. 2, 3). The general resistance of P815 cells to vincristine-induced DNA fragmentation (Fig. 3) makes it difficult to discuss whether or not these agents act cooperatively, since the maximal DNA fragmentation inducible by vincristine was not obtained at the concentrations evaluated [6]. The potentiation occurred at vincristine levels that are attainable in vivo [1, 19]. The pharmacokinetics of intraperitoneal cytochalasin B have recently been evaluated by Lipski et al. [14], who report that levels of 5 μg/ml can easily be obtained and tolerated for extended periods.

Unlike EL4 cells, P815 cells (Table 1, Fig. 1) were not particularly sensitive to cytochalasin B- and vincristine-induced DNA fragmentation after 4 h incubation but exhibited an enhancement of DNA fragmentation as the incubation period increased (Fig. 1). The ability of vincristine to potentiate the effect of cytochalasin B was demonstrated even following the preincubation of P815 cells with vincristine (Fig. 6).

These data suggest that cytochalasin B can potentiate the chemotherapeutic actions of vincristine. This potentiation could be useful in vivo for a number of reasons; in particular, the combination of these two agents may overcome various multidrug-resistant states (see Fig. 1), and since the combination is more potent than either drug alone, the duration of exposure of a tumor required to induce cell death is diminished. In addition, the present study demonstrates that pretreatment with vincristine effectively sensitizes cells to cytochalasin B-induced DNA fragmentation; therefore, the drugs need not be used in combination. One potential in vivo complication would involve the existence of a population of normal cells that are sensitive to the drug combination; this would not be unique in chemotherapeutic modalities using multidrug regimens. However, the current work provides a potentially new multidrug regimen. Whether this combination regimen can enhance antitumor activity in vivo needs to be examined next.

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