Cytotoxic Effects of Cell Cycle Phase Specific Agents: Result of Cell Cycle Perturbation

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ABSTRACT

Although agents which act in a cell cycle phase specific manner are commonly used in the clinic and in basic research, it is as yet unclear why these agents are cytotoxic. In this paper, we examine the cellular events associated with the cytotoxicity of aphidicolin and vincristine in CHO strain AA8 cells. Cell killing resulting from aphidicolin treatment was found to require a period of inhibition-free growth following removal of the drug and was associated with characteristic aberrant mitotic processes. The cytotoxic effects of aphidicolin could be antagonized by the concomitant inhibition of protein synthesis with cycloheximide in the period of DNA synthesis inhibition. Cell killing resulting from treatment with vincristine was associated with the aberrant segregation of nuclear material and the formation of multiple nuclearized nuclei. Vincristine cytotoxicity was found to be antagonized by concomitant administration of cycloheximide or cytosine D. These data support a hypothesis that the cytotoxic effects of cell cycle phase specific agents do not derive directly from their biochemical action per se. We propose that cell death results from processes that are evoked by dissociation of normally integrated cell cycle events, and that dissociation is propelled by mitotic and karyokinetic events in the case of aphidicolin and karyokinetic/nuclear reformation events in the case of vincristine.

INTRODUCTION

Agents which inhibit specific cellular processes in proliferating cells are commonly used as chemotherapeutics as well as in basic cell research. The antimetabolites, e.g., methotrexate and nucleotide analogues (1, 2), as well as agents such as hydroxycytosine (1, 2) and aphidicolin (3) are inhibitors of DNA synthesis. The Vinca alkaloids (vincristine and vinblastine) and other plant alkaloids (colchicine and colcemid) are inhibitors of mitosis (1, 2). Although the biochemical actions of these agents are generally well understood, it remains unclear as to why these agents are cytotoxic.

Previous studies from this laboratory have shown that the transient inhibition of DNA synthesis by hydroxycytosine or aphidicolin results in 'unbalanced growth' (an increase in cell size/DNA ratio) which, subsequent to a period of recovery, leads to cell killing, extensive chromosomal aberrations, and enhanced methotrexate resistance. Further, if protein synthesis is concomitantly inhibited during the period of DNA synthesis inhibition, all of the above described consequences of drug treatment are greatly diminished (4). These results suggest that the cytotoxic effects of such agents are complex and require the continuation of certain cellular processes (e.g., protein synthesis) during the period in which the progression of other cellular processes (e.g., DNA synthesis) are inhibited.

In this paper we further examine the cellular events associated with the cytotoxicity of two classes of cell cycle phase specific agents: aphidicolin, which inhibits DNA synthesis, and vincristine, which disrupts assembly of the mitotic spindle apparatus. We examined the fate of individual cells by time lapse photomicrography for a period prior to, during, and subsequent to drug treatment. Cytotoxicity was measured by the exclusion of vital dyes within the period of time during and subsequent to drug treatment, as well as by clonogenic assays subsequent to treatment. Our results provide further support for the hypothesis that the cytotoxicity of these agents does not derive directly from the specific biochemical action of the drug per se, but rather results from the disparate inhibition of certain cell cycle processes relative to the progression of other processes, i.e., a dissociation of normally coupled cell cycle progression events.

MATERIALS AND METHODS

Cell Culture. Chinese hamster ovary cells strain AA8 were maintained as a monolayer in α-minimal essential medium, supplemented with 10% fetal bovine serum, 50 μg/ml gentamicin (GIBCO), buffered with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Sigma) at pH 7.3-7.4, and incubated at 37°C in 5% CO2. Cells were passaged with trypsin/EDTA (GIBCO) as necessary to maintain subconfluency.

Clonogenic Assays. Known numbers of exponentially growing cells were plated in duplicate into 100-mm dishes and allowed to attach for 2 h. Concentrated stock solutions of the drugs (1000×) were then added directly to the medium in the dishes and gently mixed to achieve the specified final concentrations. The cells were incubated 18 h with the drugs, then gently washed 3 times with large volumes (10 ml) of warmed PBS and replenished with fresh medium. After 6 to 8 days of incubation, the cultures were washed once with PBS, fixed with 70% ethanol, stained with crystal violet, and the colonies (>50 cells) were counted. Cell survival percentages were corrected for control plating efficiency (88%) or for survival in 10 μg/ml cycloheximide (63%) or cytosine D (48%) alone.

Cell Viability. Cells were seeded into 35-mm dishes and incubated 24-48 h. Concentrated stock solutions of drugs were then added directly to the medium as described above. At the specific time points during drug treatment and following removal of the drugs, dishes of cells were assayed for viability by exclusion of propidium iodide (5).

An aliquot of a concentrated stock solution (1000×) of PI was added directly to the medium to achieve a final concentration of 5 μg/ml and incubated for 5 min. The medium was then removed, centrifuged, and the nonadherent cells were resuspended in a small volume of PBS containing 5 μg/ml PI. The number of cells was determined microscopically in a hemacytometer, and the proportion of dead cells was determined by fluorescence microscopy. The adherent cells were assayed directly in the dishes by fluorescence microscopy, then detached by trypsinization and counted. The overall proportion of dead cells, both adherent and nonadherent, was then calculated.

Chromosome Preparation. Cells were seeded on 22- x 22-mm coverslips (No. 2, Clay Adams, Gold Seal) in 35-mm dishes and incubated overnight. Drugs were added as detailed above, and at specific times during and following drug treatment, metaphase spreads were prepared directly on the coverslips by standard procedures. Briefly, the medium was removed from the dishes, 2 ml of 75 mM potassium chloride were added, and the medium was then incubated 10 min at room temperature. An equal volume of 3:1 methanol-acetic acid was then added.

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2 To whom requests for reprints should be addressed.

The abbreviations used are: PBS, phosphate-buffered saline; PI, propidium iodide; CHO AA8, Chinese hamster ovary strain AA8.
After 10 min, the mixture was replaced with pure 3:1 methanol-acetic acid for 10 min. The coverslips were then air dried overnight and stained with Giemsa.

Time Lapse Photomicrography. Cells were photographed in sealed 25-cm² flasks during and following drug treatment at 15- or 30-min intervals by an automatic time-lapse system attached to an inverted microscope (Nikon). This interval was sufficient to accurately follow individual cells throughout the course of treatment. The temperature was stably maintained at 37°C by an incubator which surrounded the microscope stage, and the pH remained constant for the duration of each experiment. The microscope light was automatically turned off between exposures and the cells were further protected by a heat shielding filter.

RESULTS

Cytotoxicity of Aphidicolin. The treatment of CHO AA8 cells with aphidicolin for 18 h resulted in a concentration dependent reduction in clonal survival (Fig. 1A). To determine the time course of cell killing by aphidicolin, cell viability, as assayed by the exclusion of PI, was determined at various times during and following treatment with aphidicolin (Fig. 2A). The proportion of viable cells remained near control levels (>95%) throughout the 18-h course of aphidicolin treatment and for the first 6 h following the removal of aphidicolin. A modest decrease in viability in the subsequent 12 h was followed by a greatly increased rate of cell death beginning 18 h after the release from aphidicolin block. To address the possibility that cell lysis was artificially inflating the observed proportion of live cells (i.e., lysed cells, although dead, would not be stained with PI and would thus not be scored as such), the total number of adherent and nonadherent cells was determined for each time point. A decline in absolute cell numbers did not begin until 28 h after removal of drug (data not shown). Thus, within the time frame of the dye exclusion measurements, cell loss was not a complicating factor.

To preclude the possibility that the onset of cell death and the timing of drug removal was coincidental, the time course of cell killing was determined for cells which were exposed to aphidicolin for varying periods of time (Fig. 3A). Upon removal of aphidicolin, the time course of cell killing for cells treated 12 h with aphidicolin was nearly identical to cells blocked for 18 h such that with both treatment regimens there was a 6- to 12-h lag period between the removal of aphidicolin and the loss of membrane integrity (i.e., PI uptake). Additionally, cells exposed continuously to aphidicolin generally remained viable (~95%) throughout at least 48 h of inhibition.
added to the medium at time 0, and were removed at the designated times.

Inhibition with 5 ng/ml aphidicolin (A) or 100 nM vincristine (B) for 12 h (O), varying periods of time. The percentage of viable cells during and following treatment was determined by PI exclusion. The drugs were added to the medium at time 0, and were removed at the designated times.

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Time lapse photomicrography was used to examine the effects of aphidicolin on individual cells. By photographing one microscopic field for a period of time before addition of drug (15–20 h), throughout the duration of drug treatment (18 h), and for a period of time following the removal of drug (30–50 h), it was possible to follow the fate of individual cells in those periods of time. By determining the length of time it took for each cell to reach the interphase position in the period before drug treatment, it was possible to determine the age and estimate the cell cycle position of each cell at the time of drug addition, in addition to following the morphological changes resulting from drug treatment and subsequent to drug removal. Following 18 h of treatment with aphidicolin, all cells were significantly increased in size (Fig. 4B) relative to control cells (Fig. 4A). Previously reported flow cytometric data indicate the observed increase to be a true increase in cell volume and protein content relative to DNA content (4), and thus the increase in cell size described here does not represent merely an increase in surface area due to a flattened morphology.

After release from aphidicolin block, the cells that eventually died did so after progressing through three stereotypical processes involving aberrations of cell division, as detailed below.

First, the greatest number of cells (~50%) continued to increase in size over a period of time (30–50 h) equivalent to several cell cycles (~12 h; see below) and eventually lysed or detached from the flask. These cells never attempted to divide, and both the nucleus and cytoplasm retained an interphase morphology until cell death (lysis/detachment). This process of absence of mitosis is illustrated by cell 4 of Fig. 5.

A second typical response involved the cells that proceeded through multiple rounds of apparently aborted mitoses. These cells repeatedly assumed a refractile mitotic morphology, and then returned to a flat interphase morphology without successful division. Cytogenetic examination confirmed that these cells contained condensed chromosomes, many with aberrant (i.e., multipolar) metaphase arrays (not shown). Because these cells generally did not become multinucleated, this process appears to involve a failure of both karyokinesis and cytokinesis. Furthermore, there was often a disparity between the nuclear and cytoplasmic morphologies in these cells. For example, cell 3 of Fig. 5 displayed a typical flat interphase morphology immediately after release from aphidicolin block (Fig. 5B). After 8 h of recovery (Fig. 5C), the nucleus had resumed a refractile mitotic morphology, whereas the cytoplasm remained in an interphase morphology. By 10 h (Fig. 5E) the nucleus had resumed an interphase morphology but again rounded up 1 h later (Fig. 5F). The cell eventually lysed (Fig. 5G). This process of aberrant mitosis is also illustrated by cell 1 of Fig. 6.

A third group of cells attempted to undergo mitosis but were unable to successfully complete division to produce two daughter cells. For example, in Fig. 5, cell 1 assumed a mitotic morphology 8 h after release from aphidicolin block (Fig. 5C), but was unable to successfully complete mitosis and resumed an interphase morphology 2 h later (Fig. 5E). The presence of aberrant nuclear structures (micro- and multinucleation) suggests a failure of cytokinesis in addition to aberrant karyokinetic segregation. The cell eventually lysed after 28 h of recovery (Fig. 5I). This process of aberrant mitosis is also illustrated by cell 2 of Fig. 6.

Two other variants of this process were observed. One variant is illustrated by cell 2 of Fig. 5 and cell 3 of Fig. 6. In this group, division (karyo- and cytokinesis) proceeded to near completion, with seemingly complete separation of 2 daughter cells and resumption of an interphase morphology (Figs. 5G and 6F). However, either as a result of fusion, or an inability to complete the final stages of cytokinesis, a single multinucleated cell was subsequently formed (Figs. 5H and 6G). Such multinucleated cells were generally inviable in that they were unable to further divide and produce daughter cells, and ultimately lysed or detached from the flask. The other variant consisted of cells that either underwent multipolar divisions or unequal divisions (not shown). This variant of the aberrant mitosis group produced inviable daughter cells which rapidly lysed or detached.

It should be emphasized that the processes described above were not unique events observed in only one or two cells, but rather all cells that were killed by treatment with aphidicolin progressed through one of the above processes. Table 1 summarizes the aberrant mitotic processes associated with cell killing and the approximate proportion of cells in each category. The same processes were also observed after treatment with hydroxyurea (data not shown).

Analysis of time lapse photomicrographs (see Fig. 7) of cells in the period before aphidicolin addition revealed an average division to division cell cycle time of 12.1 h (for a detailed description of the CHO AA8 cell cycle, see Ref. 6). Only cells in the very last 2–2.5 h of the cell cycle (presumably G2-M
phase) at the time of drug addition were able to divide during aphidicolin treatment. Furthermore, in agreement with the PI exclusion data, cell death occurred neither during aphidicolin block, nor in the period immediately following removal of aphidicolin. Rather, cell death occurred only following a period of inhibition-free growth. No correlation between cell cycle position (estimated based on cell age) at the time of drug addition and specific “death processes” could be determined by analysis of time lapse photomicrographs.

Cycloheximide Antagonism of Aphidicolin Effects. By time lapse photomicrography, cells treated 18 h with both aphidicolin and cycloheximide (Fig. 4C) retained a morphology nearly identical to that of control cells (Fig. 4A), in contrast to the increased size and flattened morphology of cells treated with aphidicolin only (Fig. 4B). Additionally, the aberrant mitotic events leading to cell death observed with cells recovering from aphidicolin block were observed at a far lower frequency when protein synthesis was simultaneously inhibited with cycloheximide. These results are in agreement with the finding that the clonogenic cytotoxicity of aphidicolin was markedly antagonized by the concomitant administration of cycloheximide (Fig. 1A).

Cytotoxicity of Vincristine. Vincristine treatment of CHO cells produced a concentration dependent clonogenic cytotoxicity (Fig. 1B). Cell viability as measured by PI exclusion remained near control levels throughout the 18 h of vincristine treatment, followed by a consistent decrease in the proportion of viable cells beginning immediately following release from vincristine block (Fig. 2B). Following the removal of vincristine, the time course of killing of cells treated 12 h with vincristine was nearly identical to the time course of killing produced by treating the cells for 18 h (Fig. 3B). Continuous exposure to vincristine produced a considerable decrease in viability beginning approximately 24 h after the addition of vincristine.

The effect of vincristine on cells is illustrated in Fig. 8. Cells began accumulating in a refractile mitotic stage during the initial periods of vincristine block (Fig. 8B). However, as the duration of vincristine block continued, nearly all cells resumed an interphase morphology with the formation of multiple “partial” nuclei, with apparently fully formed nucleoli and condensed chromatin (Fig. 8, C–E), suggesting the cells had aberrantly traversed the metaphase block. This process is further illustrated in Fig. 9. After 4 h of vincristine treatment (Fig. 9B) there was an accumulation of cells which had condensed their chromatin and had arrested in metaphase, with a few multinucleated cells already apparent. As the vincristine block continued to 8 h (Fig. 9C) and 18 h (Fig. 9D), the number of cells blocked in metaphase decreased with a proportionate increase in the number of multinucleated cells.

Three processes leading to cell death were observed. The vast majority of the cells formed multinucleated cells which continued to increase in size following removal of vincristine, without undergoing subsequent mitosis (see Fig. 8, D–H, cell 1). These cells eventually lysed or detached (Fig. 8I, cell 1), usually between 12 and 24 h following removal of vincristine.
A smaller proportion of cells (<10%) formed multinucleated cells which eventually underwent an aberrant multipolar division, usually tripolar or tetrapolar (see Fig. 8, D–H, cell 2). The daughter cells were usually multinucleated, and eventually lysed or detached without further division.

A small number of cells (<10%) underwent division while in vincristine block, usually through a multipolar intermediate (Fig. 8C, cell 4 and Fig. 8D, cell 3). The resultant daughter cells generally lysed within 12 h of removal of vincristine (Fig. 8F, cells 3 and 4).

Cycloheximide Antagonism of Vincristine Effects. The concomitant administration of cycloheximide and vincristine resulted in a partial reduction in the clonogenic cytotoxicity of vincristine (Fig. 1B). By PI exclusion, the viability of cells remained near control levels during treatment with and following removal of vincristine (Fig. 2B). The cells treated with both vincristine and cycloheximide did not condense their chromatin throughout the 18 h of drug treatment (Fig. 9, E and F). The frequency of multinucleated cells was greatly reduced after 18 h of treatment (<5%), and these cells probably derived from cells in mitosis at the time of the addition of vincristine. After 18 h of treatment with vincristine and cycloheximide (Fig. 4E), the cells retained an interphase morphology that was nearly identical to control cells (Fig. 4A), in contrast to the cells...
treated with vincristine only (Fig. 4D).

Cytochalasin D Antagonism of Vincristine Effects. The concomitant administration of cytochalasin D, an inhibitor of actin polymerization, also antagonized the clonogenic cytotoxicity of vincristine (Fig. 1B) and maintained viability near control levels following removal of the drugs (Fig. 2B). Examination of metaphase spreads revealed that the cells proceeded to condense their chromatin, but the subsequent decondensation of the chromosomes and formation of multinuclei were inhibited. The majority of cells retained a refractile mitotic morphology (Fig.
Table 1 Aberrant mitotic processes associated with cell death

Summary of aberrant mitotic processes associated with aphidicolin cytotoxicity. Photomicrographic data from 5 time lapse aphidicolin experiments were analyzed. Classifications are based on the descriptions detailed in "Results."

<table>
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<tr>
<th>Classifications</th>
<th>Description</th>
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<tr>
<td>I. Absence of mitosis (47%)</td>
<td>A. Cell continues to increase in size, without ever attempting to divide; eventual lysis.</td>
</tr>
<tr>
<td>II. Multiple abortive mitosis (35%)</td>
<td>A. Whole cell assumes refractile mitotic morphology but flattens back down into an interphase morphology without division; one nucleus.</td>
</tr>
<tr>
<td></td>
<td>B. Only nucleus becomes refractile, with cytoplasm remaining flat. Eventually flattens back into interphase morphology without division; one nucleus.</td>
</tr>
<tr>
<td>III. Aberrant mitosis (18%)</td>
<td>A. Cell rounds up, undergoes mitotic contortions, then flattens back down without division. Multiple partial nuclei formed. No subsequent division.</td>
</tr>
<tr>
<td></td>
<td>B. Cell divides to near completion, then either is unable to complete cytokinesis or fuses, forming binucleate cell.</td>
</tr>
<tr>
<td></td>
<td>C. Cells undergo multipolar division or unequal division resulting in inviable daughter cells which soon die.</td>
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Fig. 7. Schematic analysis of time lapse photomicrography of aphidicolin-treated cells. A single micrographic field was followed by time lapse photomicrography for 20 h before drug addition (No Drugs), 18 h of inhibition with 5 μg/ml aphidicolin (APC Block), and 31 h following removal of aphidicolin (Release). Typically, an initial field of ~50 cells was selected for observation. The above data, representative of the progression of 6 cells, are presented only for the purposes of illustration and do not represent the actual proportion of cellular responses (see Table 1) or the actual detail and extent of analysis. Symbols: ———, division; ○, refractile morphology; ●, return to interphase morphology without division; †, cell death (i.e., lysis/detachment); ▲, abnormal morphology; △, normal morphology; ?, cell lost from field; ———, unsuccessful division/fusion.

4F), with their chromosomes remaining condensed throughout the 18-h block (Fig. 9, G and H). Cytochalasin D was as effective as cycloheximide in reducing vincristine cytotoxicity (Fig. 1B).

DISCUSSION

In the course of our continuing attempts to understand the mechanisms involved in the acquisition of resistance to various agents, we have begun to address the antecedent question of how such agents result in cell death. We have used three different criteria as end points of "cell death" in these studies: (a) the loss of membrane integrity as measured by propidium iodide uptake; (b) cell lysis or detachment as observed by time lapse photomicrography or measured as absolute counts of adherent cells; and (c) the classical clonogenic survival assay, in which cell death is measured as an inability to form colonies. We believe these criteria measure different events in the progression of cell death, such that loss of membrane integrity is followed by lysis/detachment and loss of reproductive potential.

The two agents studied herein, aphidicolin and vincristine, have very different biochemical actions. The former is a potent and specific inhibitor of DNA polymerase α (3), whereas the latter inhibits the assembly of the mitotic microtubule apparatus (1, 2). Although disparate in their mechanisms of action, we conclude that the cytotoxic effects of both agents require the active participation of cellular processes in producing cell death. Our hypothesis is that in each case, as specific events in cell cycle progression (i.e., DNA synthesis or karyokinesis) are inhibited relative to the continuation of other events in the normal progression of the cell cycle, there results a dissociation of normally integrated cell cycle events. We propose that it is the disruption of cell cycle events which triggers, by unknown cellular mechanisms, events resulting in cell death. Such a hypothesis is consistent with certain observations in fission yeast, as discussed below, where mutational events affecting cell cycle regulation have profound effects on cell viability (7, 8).

Perhaps the most striking finding with the DNA synthesis inhibitor aphidicolin (we also found similar results with hydroxyurea) was that cell death, as determined by time lapse photomicrography, PI exclusion, as well as cell detachment (decrease in absolute number of adherent cells), occurred only after restoration of DNA synthesis following removal of the inhibitors. These results are similar to the findings of Skog et al. (9), who reported that the peak of cell death in mouse T-lymphoma cells treated with hydroxyurea occurred within the first 12 h following removal of the drug. That cell death requires removal of inhibition is further demonstrated by our finding that if cells were continually exposed to inhibitory levels of aphidicolin, cell viability remained near control levels (>95%) for at least 48 h. In contrast, if DNA synthesis was transiently inhibited for 12 or 18 h, in each case there was an identical 6-h lag period between the removal of inhibition and the onset of cell death (i.e., PI uptake). Furthermore, in each case the kinetics of the loss of viability were identical following removal of inhibition. We have found that in the period following removal of inhibition, cell death was associated with several characteristic patterns of cell behavior. Approximately one-half of the cells underwent aberrant or aborted mitotic processes prior to death. The other half maintained a normal interphase morphology and eventually died without attempts to undergo mitosis. These processes resulting in death are similar to the morphologies associated with "apoptosis" and "necrosis," respectively (10), although we do not have corroborative biochemical evidence. We do not know if these different processes result from fundamentally different pathways, but our results indicate that there is no correlation between the cell cycle phase at the time of drug addition and the subsequent killing morphology.

Vincristine killing differs from that resulting from the inhibition of DNA synthesis in that cell death occurs irrespective of whether the cells are continually or transiently exposed to vincristine. However, the time course of cell killing that results from transient exposures to vincristine for 12 or 18 h is nearly identical. In both cases, there is a maintenance of viability near control levels throughout the course of inhibition, followed by a decrease in cell viability immediately following the removal of the drug. This observation is consistent with the concept that the absolute time of exposure to the drug is not the critical
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Fig. 8. Time lapse photomicrography of cells treated with vincristine. Cells were seeded into flasks, grown 24 h, and then treated 18 h with 100 nm vincristine. Photomicrographs (A)-(E) represent time points during vincristine treatment: (A) 0 h, (B) 6 h and 45 min, (C) 7 h and 24 min, (D) 10 h, (E) 18 h of vincristine treatment. The cells were then washed 3 times with warmed PBS and new medium was added. Photomicrographs (F)-(I) represent time points (F) 1 h and 30 min, (G) 2 h and 15 min, (H) 6 h, and (I) 12 h following removal of vincristine. The following processes are illustrated (see "Results"): cell 1 ( ), multinucleation with absence of mitosis, cell 2 ( ), and cell 3 ( ), multipolar division, and cell 4 ( ) mitosis during vincristine block.

determinant of cell death, and in this respect is similar to our results with aphidicolin.

The phenomenology associated with vincristine cytotoxicity has been previously described and differs somewhat according to cell type (11–13). We observed that CHO AA8 cells treated with vincristine underwent normal chromosomal condensation, but in the absence of normal mitotic microtubule apparatus assembly, remained blocked in metaphase for an extended period of time. The cells did not divide, but eventually aberrantly traversed the metaphase block with the formation of multiple partial nuclei, containing decondensed chromatin, fully formed nuclear membranes, fully formed nucleoli, and a flat interphase morphology by microscopic examination. These multinucleated cells all eventually lysed. These results are similar to the findings of Tennyson et al. (13), who reported that rat tracheal carcinoma cells treated 18 h with vincristine became multinucleated and that the multinucleated cells had no propagational potential. Additionally, the disruption of mitotic spindle formation in a CHO cell line which was temperature sensitive for tubulin assembly resulted in the same aberrations in
nuclear structure as reported herein, and also resulted in cell death (14).

To further study the phenomenology associated with cell killing, we have studied the effects of concomitantly administering certain other drugs with aphidicolin or vincristine. Previously, we have shown that the concomitant inhibition of protein synthesis with cycloheximide during inhibition of DNA synthesis results in a partial antagonism of many of the effects of transient DNA synthesis inhibition (i.e., cell killing, generation of chromosomal aberrations, enhanced methotrexate resistance) (4). Our current results confirm and further extend these observations to include: (a) cycloheximide prevents the aphidicolin induced loss of membrane integrity as assayed by vital dye exclusion, and (b) cells do not undergo aberrant/abortive mitoses when treated with both aphidicolin and cycloheximide. The major conclusion that can be drawn from these studies is that the events which occur subsequent to transient aphidicolin treatment, including loss of membrane integrity, generation of mitotic aberrations, and cell death, require continued protein synthesis during the period in which DNA synthesis is inhibited. Cycloheximide also prevents cell death resulting from vincristine treatment. Time lapse photomicrography of cells treated with vincristine and cycloheximide show them to maintain an interphase morphology. This is not surprising inasmuch as the inhibition of protein synthesis also inhibits progression through the cell cycle. These results do not distinguish among a large number of possible explanations, including suppression of the "induction" of proteins resulting in cell death or the prevention of cell cycle progression events that "provoke" cell death. However, these results do support the more general concept that cell death requires the participation of active cellular processes.

The concomitant administration of cytochalasin D, an inhibitor of microfilament assembly, also partially antagonized the cytotoxic effects of vincristine as measured by PI exclusion and clonogenicity. Microscopically, cytochalasin D does not prevent cells from condensing their chromatin but rather appears to prevent subsequent aberrant karyokinetic events. A large proportion of cells treated with both cytochalasin D and vincristine remained blocked in metaphase, with their chromosomes condensed, throughout the course of treatment, and the subsequent aberrant formation of multiple partial nuclei was proportionately reduced. Inasmuch as cytochalasin D was equally effective as cycloheximide in preventing cell death, we propose that these results are consistent with the concept that cell death results from the dissociation of normally integrated cell cycle events (i.e., karyokinetic/nuclear reformation events).

It has been shown in other systems that normally integrated cell cycle events can be dissociated. Schlegal and Pardee (15, 16) have reported that mitosis can be uncoupled from the completion of DNA synthesis in cells treated with caffeine, and Zetterberg et al. (17, 18) found that DNA synthesis and mitosis can be uncoupled from protein synthesis. There are several findings in yeast that point to the important role of the control of integrated cell cycle progression in maintaining cell viability. Hirano et al. (7) have reported in Schizosaccharomyces pombe...
that the lethality of a topoisomerase II mutant, which fails to allow normal chromosomal segregation at mitosis, is prevented by the presence of a mutation in the cdc11 gene which controls septation (cytokinesis). The double mutants remain viable and become polyploid. Additionally, Enoch and Nurse (8) have reported in S. pombe that certain cdc25 mutants, which are defective in the control of mitosis, are highly susceptible to the lethal effects of DNA synthesis inhibition by hydroxyurea in that they undergo mitosis without completing replication. In contrast, wild type cells do not undergo mitosis when DNA synthesis is inhibited and thus do not die. However, if such cdc25 mutants also contain a mutation in the cdc10 gene (thus preventing cells from entering S phase, i.e., START), then only those cells which have entered S phase prior to the addition of hydroxyurea are killed. These results clearly support the concept of the importance of an ordered progression of cell cycle events in maintaining cellular viability, and further support that regulation events involved in mitosis are critical. Further support for the major role of mitotic control comes from studies of the Rad9 gene in Saccharomyces cerevisiae (see Ref. 19 for review). This gene identifies a mechanism which "senses" DNA damage and is critical for preventing mitosis from occurring prior to the repair of damaged DNA.

Our results do not address the fundamental question of why cells die (i.e., lose membrane integrity/lyse) when treated with these and other agents. Although various studies concerned with cell death, as primarily studied in lymphocyte derived cell lines, have centered on the hypothesis that death results from the rapid digestion of chromatin by newly induced endonucleases as part of a "death program" (20, 21), more recent studies suggest that it is alterations in chromatin/nuclear structure that render DNA susceptible to the action of constitutively present endonucleases (22). Based on our results with vincristine and aphidicolin, and taking into account the studies in yeast as discussed above, we suggest that it is the disordered state of nuclear structure that results in a susceptibility to either constitutively expressed or induced enzymes which ultimately result in cell death, and that this occurs subsequent to aberrant karyokinetic processes. This hypothesis is consistent with our results that: (a) one-half of the cells that die with aphidicolin undergo aberrant mitotic processes before losing membrane integrity; (b) all cells that die from vincristine treatment undergo an aberrant traversal of metaphase with the disordered reformation of multiple partial nuclei; and (c) with all treatment modalities, cell membrane integrity is maintained as long as aberrantly segregated nuclear structures are not generated (e.g., continual S-phase block with aphidicolin and concomitant administration of vincristine and cytochalasin D). However, any suggestion that nuclear structure abnormalities are necessary for cell death is clearly too simplistic in light of the fact that one-half of the cells killed by aphidicolin do not undergo any gross changes in nuclear structure.

The finding that cell killing requires the continuation of certain cellular processes may have potential implications for various types of chemotherapy. Our results, most clearly evident with aphidicolin, demonstrate that in certain cases cell killing requires a period of drug-free growth following a period of inhibition. This suggests that the pharmacokinetics and dosing schedule of drugs may be important in maximizing cell killing. Maintenance of continual long-term inhibitory levels of drugs may be less effective than regimens alternating periods of inhibition followed by periods of drug-free growth.

The concept that cell killing requires active participation of cellular processes may also assist in understanding some types of drug resistance phenomena. Current understanding of acquired resistances, as studied in cell culture systems, has been centered on processes whereby action of the drug is circumvented either by preventing intracellular accumulation of the drug (23, 24) or by alterations in the structure (25) or concentration (26) of the target protein. We suggest that an additional mechanism of resistance may involve a lack of response to inhibitory processes that would otherwise evoke a cellular suicide process in more susceptible cell types. Indeed, we have found a remarkable difference in the susceptibility of various human and rodent cell lines to the cytotoxic effects of aphidicolin. Although DNA synthesis is equally inhibited in all of the cell lines studied, there exists a dramatic difference in cell killing.4 Thus, inherent resistance in this case does not derive from differences in the biochemical action of the agent but rather results from differences in the response of the different cell types to such inhibition. Our results are consistent with the concept that cell types that are not killed have an inherently more "stringent" control of cell cycle progression events in G2-M phases. This type of "inherent" resistance may partially underlie the striking differences in the success of therapeutic modalities among different clinical cancers.

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