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Effect of Cycloheximide on Development of Methotrexate Resistance of Chinese Hamster Ovary Cells Treated with Inhibitors of DNA Synthesis

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We examined the effects of 18 h of incubation of Chinese hamster ovary (CHO K1) cells with cycloheximide, hydroxyurea, and aphidicolin. Treatment of cells with cycloheximide alone at a concentration adequate to inhibit DNA synthesis to <10% of control was significantly less cytotoxic and clastogenic than treatment with hydroxyurea or aphidicolin, did not induce unbalanced cellular growth, and had no effect on the frequency of resistant cells in methotrexate selections compared with control cells. When combined with hydroxyurea or aphidicolin and compared with the effects of either drug alone, cycloheximide blocked the induction of unbalanced growth during drug treatment, reduced the frequency of chromosomal aberrations in recovering cell populations, and decreased cell killing. In addition, the increased frequency of methotrexate-resistant cells observed after treatment with hydroxyurea or aphidicolin was eliminated when cycloheximide was present during drug treatment.

The frequency with which cultured mammalian cells become resistant to the antifolate methotrexate (MTX) can be increased if cells are treated with any of a number of agents before drug selection. Included in these treatments are hydroxyurea (HU) (5, 14, 21), MTX (24), UV irradiation (15, 33), hypoxia (5), tumor promoters (2, 35), and carcinogens (17, 18, 35). Several features are common to most of these treatments including transient inhibition of DNA synthesis (S-phase block) and the induction of unbalanced cellular growth (increase in cell size without increase in DNA content), chromosomal damage, and cytotoxicity.

Transient inhibition of DNA synthesis induced by these treatments has been suggested to be the crucial event leading to the facilitation of MTX resistance during subsequent selections (28). While the mechanism of this effect is not understood, dihydrofolate reductase (DHFR) and several other unidentified proteins accumulate during the cell cycle block induced by HU and aphidicolin (APC) (16). These proteins have been proposed to include proteins involved in DNA synthesis which contribute after release of the cell cycle block to the overreplication of DNA and facilitate the development of MTX resistance (16, 28). While the relative frequency of cells with amplified genes is increased by these treatments, this effect is not specific for gene amplification and presumably the frequency of cells with altered DHFR enzyme and transport alterations is also increased (5).

In this study, we explored the role of continued protein synthesis during inhibition of DNA synthesis by examining the effect of treating cells with cycloheximide alone or in combination with HU and APC. Our data showed that continued protein synthesis during inhibition of DNA synthesis is required for the induction of chromosomal aberrations and cytotoxicity by HU and APC and that the increased frequency of MTX-resistant cells observed after pretreatment with HU and APC is eliminated if cycloheximide is present during inhibition of DNA synthesis.

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MATERIALS AND METHODS

Cell culture. Chinese hamster ovary cells (CHO K1) were obtained from the American Type Culture Collection. The cells were maintained in Ham's F12 medium without glycine, hypoxanthine, or thymidine and supplemented with 10% dialyzed fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.) and gentamicin (50 μg/ml). Cells were passaged twice per week and tested to be free of mycoplasma by fluorescence microscopy after Hoechst staining.

Cytotoxicity determinations and drug treatments. Cytotoxicity was measured as the frequency of colonies surviving after drug treatment. Known numbers of cells were plated onto 100-mm dishes and allowed to attach for 6 to 8 h. Medium containing specified concentrations of drug was then added, and the cells were incubated with the drug(s) for 18 h. After drug treatment, cells were washed twice with large volumes of warm phosphate-buffered saline and fresh medium was added. After 1 to 3 weeks of incubation, cells were fixed and stained with crystal violet, and colonies (>50 cells per colony) were counted. To measure MTX resistance, we allowed drug-treated cells to recover for 18 h before adding fresh medium containing 100 nM MTX. MTX-resistant colonies were counted after 3 to 4 weeks of growth.

Chromosome analysis. Chromosome aberrations were measured as the frequency of metaphase cells with unequivocal chromatid breaks as described previously (31). At various times after drug treatment, cells were treated with colcemid (0.7 μg/ml) for 1 h and metaphase cells were harvested by standard procedures. Other types of aberrations were also observed (e.g., aberrant chromosome condensation, endoreduplication) but were not used to quantify the frequency of chromosomal aberrations because the frequency of such aberrations was low relative to that of chromatid breaks and they were difficult to quantify.

Cell cycle and cell growth measurements. The effects of drug treatment on cell cycle progression and cell growth were measured by flow cytometry. Drug-treated cells were trypsinized, washed in phosphate-buffered saline, and fixed in cold 70% ethanol. Cells were stained for DNA content with chromomycin A_3 (100 μg/ml in phosphate-buffered

saline plus 50 mM $MgCl_2$) or with propidium iodide (25 μg/ml) after RNase treatment (50 μg/ml for 1 to 3 h at 37°C). Cell size was measured by forward angle light scatter. Integrated fluorescence signals were measured on a Coulter Epics 753 flow cytometer with 488-nm excitation.

Total cellular protein content was measured with fluorescein isothiocyanate by the method of Crissman and Steinkamp (9). These data were corroborated by assay of soluble cellular protein (4). Samples of cells used for cytometric analyses were counted and subjected to four rounds of rapid freeze-thawing to disrupt cells. Membrane material was removed by centrifugation, and the protein concentration was measured with a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.).

RESULTS

Cytotoxicity. After 18 h of cycloheximide treatment (10 μg/ml), cell survival was reduced to 62% of control (Fig. 1, dotted line). While this represents a significant level of cell killing, it is less than that observed at all but the lowest concentrations of HU (0.3 mM) and APC (0.5 μg/ml) used in these experiments. This concentration of cycloheximide produced nearly complete inhibition of DNA synthesis ([^{3}H]thymidine incorporation, <10% of control) and protein synthesis ([^{35}S]methionine incorporation, <5% of control). Equivalent levels of inhibition of DNA synthesis were achieved with 0.5 mM HU and 1.0 μg of APC per ml.

Both APC and HU produced a concentration-dependent biphasic reduction in cell survival (Fig. 1). Over the range of drug concentrations examined, the rate of cell killing decreased at higher drug concentrations. At the highest concentration of inhibitors used, cell survival was 0.030% for APC-treated cells (5.0 μg/ml)-treated cells and 0.015% for HU (3 mM)-treated cells.

Cell survival increased when cycloheximide (10 μg/ml) was added simultaneously with either HU or APC (Fig. 1). For both drugs, survival declined initially to about 40% of control at low drug concentrations when cycloheximide was present and then remained at that level with increasing concentrations of HU or APC. Combined treatment thus resulted in a positive deviation (greater survival than expected) from the expectations for additive cell killing at all concentrations of HU and APC tested (Table 1). At the highest concentrations (5 μg of APC per ml, 3 mM HU), the deviations from additive expectation were 10-fold and 36-fold for APC and HU, respectively. These results are consistent with other reports showing that cycloheximide antagonizes the cell-killing effects of drugs that inhibit DNA synthesis (3, 20).

Chromosome aberrations. Cycloheximide was relatively nonclastogenic in CHO K1 cells under the conditions used. Resumption of cell cycle progression after 18 h of cycloheximide treatment was slow, and metaphase cells were not found in significant numbers before 6 to 8 h of recovery. The maximum proportion of cells with chromosomal aberrations appeared about 24 to 30 hours after release (Table 2). Typically, the maximum frequency of aberrant metaphases was about 15 to 20%, and in general, the number of aberrations per cell was low (Table 2). Cells were examined up to 72 h after removal of cycloheximide to ensure that there was not a delayed peak in the appearance of aberrant metaphases.

Both HU and APC induced a high frequency of cells with aberrations (up to 60% of metaphases scored), and a high proportion of such cells displayed many aberrations per cell

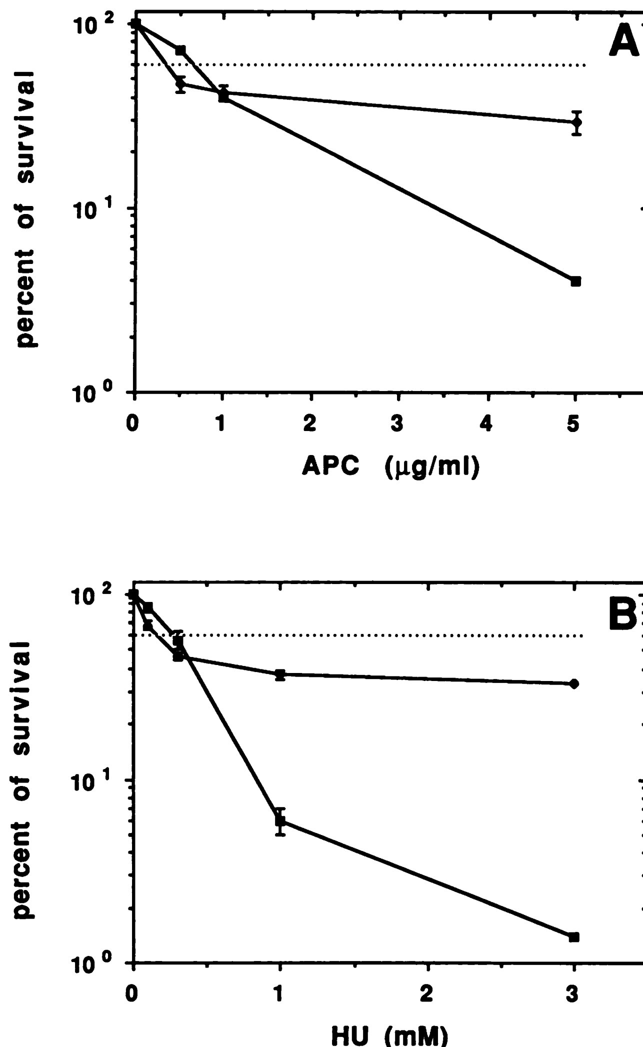

FIG. 1. Effect of cycloheximide treatment on the survival of cells exposed to HU or APC. Cells were treated with different concentrations of APC (A) or HU (B) in the presence (◆) or absence (□) of cycloheximide (10 μg/ml) for 18 h. Colonies were counted 1 to 3 weeks later. The dotted line indicates cell survival after 18 h of cycloheximide treatment. Bars show standard deviation.

(Table 2). The frequency of metaphase cells at the end of 18 h of treatment (before release from inhibition) was very low (0.1 to 0.5%); however, metaphase cells were abundant by 4 h after release. While the data are more complete for APC-treated cells than for HU-treated cells, the temporal pattern of appearance of aberrant metaphases in recovering populations was similar for both drugs. Typically, the cells observed during early recovery (4 h) contained fewer aberrations than those appearing later (12 to 24 h), and for both HU and APC there was a peak in the frequency of cells with aberrations and the number of aberrations per cell after 18 to 24 h of recovery (Table 2).

The presence of cycloheximide during APC or HU treatment greatly reduced the frequency of cells with chromosomal aberrations, as well as the number of aberrations per cell (Table 2). While the frequency of aberrations was not as low as in cells treated with cycloheximide alone, a significant nonadditive reduction in chromosomal aberrations was apparent in both HU- and APC-treated cells at all time points during recovery from combined treatment.

TABLE 1. Protective effect of cycloheximide on cells subject to prolonged inhibition of DNA synthesis[a]

Treatment	% Survival			
	A	B	C	D
APC (μg/ml)				
0.5	72	47	44	1
1.0	40	42	25	2
5.0	4	29	3	11
HU (mM)				
0.1	85	68	52	1
0.3	57	46	35	1
1.0	6	37	4	10
3.0	1	33	1	36

[a] Cells were treated for 18 h with different concentrations of APC and HU in the absence (A) or presence (B) of cycloheximide (10 μg/ml), and surviving colonies were counted after 1 to 3 weeks of growth. Expected additive survival (C) was calculated as the product of the survival of cells exposed to cycloheximide alone (62%) and of cells exposed to different concentrations of APC and HU in the absence of cycloheximide. The ratio of observed to expected survival is shown in column D.

Cell cycle and cell growth effects of inhibitors. The cell cycle and cell growth effects of drug treatments were examined by flow cytometry. Cells treated for 18 h with cycloheximide were blocked in the G1 and S phases. Cells in the M phase and possibly the late G2 phase were able to progress through the M phase in the presence of cycloheximide, resulting in a depletion of these cells from the population. This can be seen as a small reduction in the size of the G2-M peak for cells treated with either HU or APC and cycloheximide (Fig. 2). HU and APC induced a specific S-phase cell cycle arrest. In HU-treated cells, those cells in the S phase at the time of treatment were inhibited from further progression in the S phase, while cells in the G2-M and G1 phases progressed through the cell cycle and accumulated either in the early S phase or at the G1-S boundary (Fig. 2A). At the concentrations used here, APC produced a significantly more leaky S-phase block. APC-treated cells initially accumulated at the G1-S boundary and subsequently moved very slowly through the S phase (Fig. 2B). This process was both concentration and time dependent such that at relatively low APC concentrations (equal to or less than 1 μg/ml), cells moved into the S phase after approximately 14 h of treatment, while at higher concentrations, this process was delayed as a direct function of APC concentration (up to at least 10 μg/ml). The addition of cycloheximide blocked the S-phase accumulation of cells induced by both HU and APC. DNA histograms showed that cells were blocked more uniformly over the cell cycle and that the accumulation of cells in the early S phase was completely prevented by cycloheximide treatment (Fig. 2).

TABLE 2. Frequency of metaphase cells with chromosomal aberrations after different treatments

Treatment[a]	% Aberrant metaphases at the following time (h) after release[b]:					
	4	8	18	24	36	48
APC	64	57	80	76	77	20
HU	60	67	69	44	ND[c]	ND
CYX	2	2	14	14	14	2
APC + CYX	9	0	7	7	7	8
HU + CYX	15	ND	6	11	ND	ND

[a] Cells were treated with APC (2 μg/ml) or HU (1 mM) in the presence or absence of cycloheximide (CYX) (10 μg/ml) for 18 h, washed with phosphate-buffered saline, and incubated in normal medium for the times indicated. Metaphase cells were harvested after 1 h of colcemid treatment (0.7 μg/ml).

[b] Numbers are the percentage of metaphases with aberrations (chromatid or chromosomal breakage). A minimum of 100 cells was counted per time point, except for hours 4 and 8 after cycloheximide alone for which the minimum was 50 cells.

[c] ND, Not determined.

While HU and APC inhibited DNA synthesis and thereby prevented cell division, cell growth continued and consequently, after 18 h of treatment with these drugs, cell size was 140 to 160% of control, based on forward angle light scatter (Fig. 2). Cycloheximide inhibited the increase in cell size in cells treated with APC or HU alone, such that after combined treatment, cell size in these cells was 85 to 95% of control. After 18 h of cycloheximide treatment, cell size was reduced to 90% of control (Fig. 2).

While forward angle light scatter is linearly related to cell size (27), the relationship of scatter to cell size is complex and poorly understood. Therefore, we sought other measures of continued cell growth during treatment with metabolic inhibitors. Total protein per cell measured by fluorescein isothiocyanate staining increased about twofold relative to controls after 18 h of treatment with HU or APC. Based on three experiments, the mean protein content was 195% of control for HU-treated cells and 188% of control for APC-treated cells. Protein content after cycloheximide treatment decreased to 85% of control, and protein content after combined treatment was 94% of control for HU-cycloheximide-treated cells and 97% of control for APC-cycloheximide-treated cells (Table 3).

To confirm the cytometric data, we measured the total soluble cellular protein content of cells used for cytometric analysis (4). The cytometric data were corroborated by direct protein measurement (Table 3). Cells treated with APC and HU contained 2.1 and 1.9 times the total protein of control cells, respectively, and this was reduced to 75 to 90% of control when cycloheximide was present during treatment. If cells were left in either APC or HU for more prolonged periods, the protein content of these cells continued to increase such that after 48 h of continuous treatment, cells contained up to four times the protein of control cells (data not shown).

MTX resistance. We measured the frequency with which cells became resistant to a single concentration of MTX (100 nM, which is two to four times the 50% lethal dose for these cells) after 18 h of treatment with APC or HU in the presence or absence of cycloheximide. For these experiments, cells were treated with drug(s) for 18 h and allowed to recover for 18 h before the addition of MTX to the medium. When cells were treated with cycloheximide alone, the frequency of MTX-resistant cells did not increase relative to that of controls (untreated exponentially growing cells plated directly into MTX). Both APC and HU treatment resulted in an increase in the frequency of MTX-resistant cells which was directly related to the preselection concentration of the drug (Fig. 3). A similar concentration dependency of the frequency of resistant cells after pretreatment was observed for HU (14) but not for UV irradiation or treatment with *N*-acetoxyacetoaminofluorine (33). At the highest concentrations of HU (3 mM) and APC (5 μg/ml) used, the enhancement was about 80- and 7-fold, respectively. Pretreatment of cells with cycloheximide alone did not affect the frequency of resistant cells (Fig. 3), and when combined with HU or APC, cycloheximide blocked the increased frequency of resistant cells after both HU and APC treatment (Fig. 3A and B).

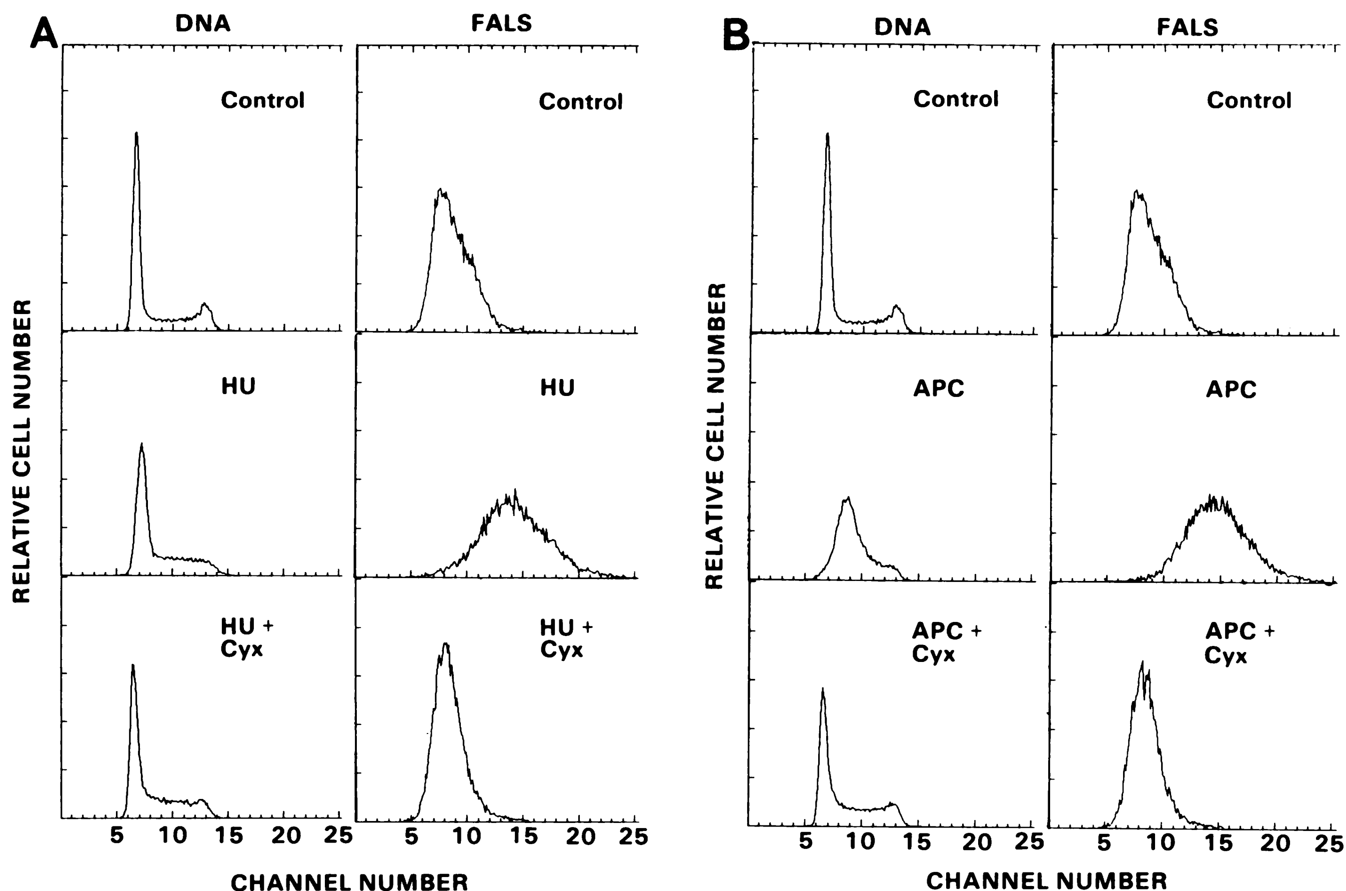

FIG. 2. Effect of APC and HU on cell cycle progression and cell size. Cells were treated for 18 h with 1 mM HU (A) or 1 μg of APC per ml (B) in the presence or absence of cycloheximide (Cyx) (10 μg/ml), fixed, and stained with propidium iodide. DNA histograms are shown on the left, and cell size (forward angle light scatter [FALS]) is shown on the right. A total of 10^4 cells were analyzed for each histogram.

DISCUSSION

Our data showed that the consequences of inhibiting DNA synthesis with cycloheximide differ markedly from those of specific inhibitors of DNA synthesis, HU and APC. Cycloheximide at a concentration adequate to inhibit DNA synthesis to <90% of control was relatively noncytotoxic and induced a low level of chromosome damage. Under the conditions used and at similar levels of DNA synthesis inhibition, both HU and APC induced high levels of chromosome damage and were very cytotoxic. Furthermore, cycloheximide when present during HU or APC treatment reduced the cytotoxic effects of HU and APC and acted to block the facilitation of MTX resistance seen when cells were treated with HU or APC before MTX selection. This effect was correlated with the inhibition of unbalanced cellular growth occurring during S-phase arrest induced by HU and APC.

TABLE 3. Relative protein content of drug-treated cells[a]

Treatment	Relative protein content	
	FITC	Bradford assay
Control	1.0	1.0
APC	2.1	2.1
HU	2.0	2.0
CYX	0.8	0.7
APC-CYX	0.9	0.9
HU-CYX	0.9	0.9

[a] Cells were treated for 18 h with APC (1.0 μg/ml), HU (1 mM), and cycloheximide (CYX) (10 μg/ml), harvested, and either fixed in 70% ethanol for flow cytometric measurement of cellular protein content with fluorescein isothiocyanate (FITC) or frozen for measurement of soluble protein content by the Bradford[4] assay. Protein content is expressed relative to asynchronously growing untreated cells.

Cycloheximide has been shown to reduce the cytotoxicity of drugs which inhibit DNA synthesis (3, 20). We extended these observations by showing that there is a similar reduction in the frequency of cells with chromosome aberrations in cell populations recovering from treatment with these drugs. DNA strand breaks have been shown to accumulate during prolonged inhibition of DNA synthesis with MTX, and this effect (as well as cytotoxicity) is strongly reduced in growth-arrested cells (19). Similar results were reported for HU treatment by Coyle and Strauss (7), who also found that, in contrast to HU, the mild cytotoxicity of cycloheximide (which was very similar to the level reported here) was not associated with extensive DNA strand breakage. Ayusawa et al. (1) showed enhanced cell survival and reduced DNA strand breakage in thymidine auxotroph mouse cells treated with cycloheximide during thymidine starvation, and these effects were attributed to the growth arrest induced by the drug. Our data are consistent with these observations, and taken together, these data provide evidence that continued protein synthesis (cell cycle progression) is necessary for

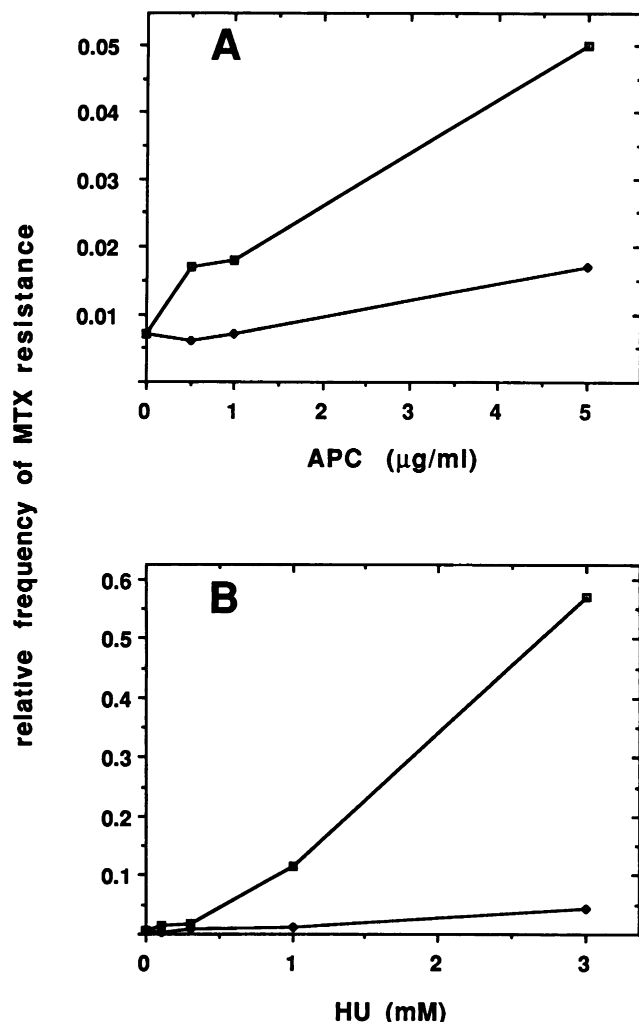

FIG. 3. Effect of cycloheximide on the frequency of MTX-resistant cells pretreated with HU or APC. Cells were treated for 18 h with different concentrations of APC (A) or HU (B) in the presence (◆) or absence (□) of cycloheximide (10 µg/ml) and were allowed to recover for 18 h before 100 nM MTX was added. Colonies were counted 3 weeks later. Frequency of resistance is calculated as the number of resistant colonies normalized to cell survival in the absence of selection (i.e., divided by the number of colonies surviving initial drug treatment).

both the induction of chromosome aberrations and cytotoxicity by treatments whose primary mechanism of action is the inhibition of DNA synthesis. More recently, it has been found that both DNA strand breakage and cytotoxicity of the epipodophyllotoxin etoposide is reduced by inhibition of protein synthesis with cycloheximide during drug treatment, indicating that continued protein synthesis is necessary for the cytotoxicity of at least some drugs which act directly at the level of DNA, rather than more indirectly (e.g., intermediary metabolism of DNA precursors) (6).

Cycloheximide inhibits DNA synthesis in direct proportion to the degree of protein synthesis inhibition induced (32). However, cycloheximide alone did not increase the frequency of MTX-resistant cells in subsequent selections and inhibited these effects in cells treated with HU and APC. This observation clearly indicates that while inhibition of DNA synthesis may be necessary, it is not sufficient for the facilitation of drug resistance observed in cells treated before MTX selection. We used MTX resistance as an assay for genetic effects and did not address the specific question of whether the DHFR gene is amplified in the resistant cells. We presume, as was first described by Brown et al. (5) and Tlsty et al. (33), that not all the resistant cells became resistant via DHFR gene amplification.

Cellular DHFR content has been shown to increase during HU and APC blocks, and this accumulation is inhibited by cycloheximide treatment (16). Based in part on this observation, it has been proposed that the accumulation of proteins occurring during S-phase block with HU is important for facilitating the emergence of MTX-resistant cells during subsequent selection (16, 28). The data shown here are consistent with the proposal, insofar as they show that HU and APC induce significant unbalanced growth in S-phase cells (increase in cell size and protein content without an increase in DNA content) and that this effect is inhibited or reduced by treatment with cycloheximide. However, because total cellular protein content increases significantly during these blocks, it is difficult to determine the exact contribution of S-phase-specific proteins to the total protein accumulation and whether subsequent cellular responses directly reflect specific protein imbalances (26). It is also possible that the accumulated proteins act to transiently protect cells from the cytotoxic effects of MTX (10). These data serve, in addition, to point out the potential problems in using cell synchronization methods based on inhibiting DNA synthesis for analysis of cell cycle-related processes.

Hill and Schimke (14) observed that after HU treatment of mouse lymphoma cells, cells with an increased frequency of MTX resistance were derived from a subset of cells which were large in size, had an elevated DHFR content, contained more than the G2-M DNA content before mitosis, and contained a variety of chromosomal aberrations. Similar results have been obtained by other treatments including hypoxia (25) and UV irradiation and APC (14). The findings that enhanced drug resistance frequencies are derived from cells with more than a G2-M DNA content has been interpreted to support the overreplication model of gene amplification (28). Interpretation of the data used to support this hypothesis is subject to debate (22, 23). However, Hill and Schimke (14) also suggested that overreplication could result from severe perturbation of cell cycle progression such that the first cell division after reinitiation of DNA synthesis was aborted and cells initiated a second S phase without an intervening mitosis. Various investigators have reported altered rates of cell cycle progression after HU (8, 13, 30), thymidine (12, 34), and cytochalasin (11) treatment. In CHO cells, Hoy et al. (15) have shown that for HU and APC, the consequences of drug treatment for the rate of cell cycle progression vary as a function of where the cells are in the cell cycle at the time drug treatment is initiated.

We observed premature chromatin condensation as well as the persistence of nucleoli in metaphase cells after prolonged inhibition of DNA synthesis, indicating that integration of cell cycle events can be disturbed under conditions of prolonged inhibition of DNA synthesis and the uncoupling of cell growth from DNA replication. The occurrence of mitotic events in S-phase cells has recently been reported for cells treated with HU and caffeine (29), indicating that inhibition of DNA synthesis does not necessarily induce a cell cycle block.

While it is difficult to establish causality in the complex effects of drug treatments as used in our studies, the data presented suggest that inhibition of DNA synthesis per se is

not responsible for the chromosomal aberrations, cell killing, and enhanced frequency of drug-resistant cells observed after treatment with HU and APC. We are examining further the effects of these agents on CHO K1 cells with particular reference to protein accumulation occurring during inhibition of DNA synthesis and the nature of the cell cycle disturbances induced by these treatments.

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