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Enhanced cytotoxicity with methotrexate in conjunction with hypoxanthine in L1210 cells in culture*

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Summary. By inhibiting dihydrofolate reductase, methotrexate (MTX) depletes cellular stores of reduced folates, resulting in the inhibition of DNA and RNA synthesis. Inhibition of RNA synthesis arrests cells in the G_1 phase of the cell cycle, preventing these cells from entering S phase and rendering them insensitive to MTX. Because MTX cytotoxicity can be enhanced by concurrent administration of hypoxanthine (HX), we examined the hypothesis that this modulation can allow normal rates of RNA synthesis and cell cycle progression from G_1 to S phase. For L1210 cells exposed to MTX for 12 h or 24 h, the addition of HX enhanced the cytotoxicity of MTX; however, no enhancement was observed with a 6-h exposure. Inhibition of RNA synthesis by MTX was prevented by concurrent administration of HX. The effect of HX on cell cycle progression was first examined using flow cytometry, which indicated that MTX treatment alone or with concurrent HX caused a buildup of cells with a G_1 content of DNA. Because this technique may fail to distinguish between cells in late G_1 phase, the G_1/S border, or early S, the method of premature chromosome condensation was used to determine cell cycle position based on chromatin morphology. A shift to a higher degree of chromatin decondensation was observed when HX was coadministered with MTX during a 12-h exposure, suggesting progression from G_1 towards S. This correlated with the enhancement of MTX cytotoxicity by HX after 12 h exposure. The results of these studies suggest that HX potentiates MTX cytotoxicity by maintaining RNA synthesis, allowing cells that might ordinarily be arrested in G_1 to progress into the cytotoxic S phase.

Introduction

The anticancer agent methotrexate (MTX), through inhibition of dihydrofolate reductase (DHFR), causes depletion of cellular reduced folate pools [13, 18] resulting in inhibition of de novo thymidylate and purine synthesis. Decreased availability of thymidylate and purines contributes to inhibition of DNA synthesis, and the lack of purines causes inhibition of RNA synthesis [3]. Although inhibition of DNA synthesis is lethal, inhibition of RNA synthesis, which is required for progression through the G_1 phase of the cell cycle, can cause cells to become arrested in this phase [6]. Arrest in G_1 could provide protection from the lethal action of MTX in S phase. As a result, the action of MTX is self-limiting and less than maximal cytotoxicity may be achieved.

The combination of MTX plus hypoxanthine (HX) has been shown to have increased cytotoxicity in murine and human tumor models [2, 15]. In mouse L cells, adenosine potentiated cytotoxicity as assessed by cloning in soft agar [2]. In both L1210 cells and two human malignant cell lines, various preformed purines increased MTX cytotoxicity at concentrations greater than 0.06 μM MTX [15]. Because both tumor cells and normal cells can salvage purines for nucleic acid synthesis, the addition of preformed purines to MTX treatment could allow cells to use salvage pathways to make purine nucleotides for RNA synthesis [11]. We hypothesize that concurrent HX administration permits normal rates of RNA synthesis, resulting in progression through G_1 and increased cytotoxicity.

In the present study we have examined the ability of MTX plus HX to modulate the self-limiting cytotoxicity of MTX. For the MTX/purine combination, the data support the hypothesis that abrogation of the inhibition of RNA synthesis, along with DNA synthesis inhibition during MTX exposure, alleviates the arrest and protection of some cells in G_1 by allowing continued cell cycle progression into the more toxic S phase.

Materials and methods

Drugs and chemicals. MTX was kindly provided by Dr V. Narayanan, Chief, Drug Synthesis and Chemistry Branch, National Cancer Institutes, Bethesda, Md. Sendai virus (UV-inactivated, 2000 hemagglutinating units/0.5 ml) was a generous gift from Dr Walter Hittelman, Department of Developmental Therapeutics, The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston, Tex. RPMI 1640 medium, alpha minimum essential medium, dialyzed horse serum, calf serum, and Colcemid (10 μg/ml in Hanks' buffered saline solution (HBSS) with phenol red) were all obtained from Gibco Laboratories, Grand Island, NY. Fetal calf serum was

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obtained from Biofluids. Rockville, Md. (5-^{3}H)-Uridine (Urd) (20 Ci/mmol) was obtained from ICN Pharmaceuticals. Irvine, Calif.

HX (stock solution) was made just before each experiment by dissolving the compound in 10 m*M* NaOH, heating at 60° C for 20 min, then diluting to 20 times the required concentration with RPMI 1640 medium.

Cell culture. L1210 cells were maintained in continuous suspension culture in RPMI 1640 medium with 10% dialyzed horse serum as described previously [7]. The doubling time was 12 h. A CHO-KI subline of cells was grown in monolayer cultures in alpha minimum essential medium containing 10% calf serum and 5% fetal calf serum with a doubling time of 16 h. Both cell lines were grown in a humidified atmosphere of 5% CO_2 and 95% air at 37° C.

Determination of cell viability. The soft agar cloning technique of Chu and Fischer [4] was used to determine the viability of L1210 cells after drug treatment. Exponentially growing L1210 cells were exposed to MTX with or without HX. After 6, 12, or 24 h of exposure, the cells were washed twice with fresh medium and diluted into RPMI 1640 medium containing 20% dialyzed horse serum and agar (0.12%) to an appropriate number, depending on the amount of cell kill expected. MTX is polyglutamated to forms that are retained in the cell after the removal of extracellular MTX [8, 19] and therefore thymidine (dThd) and HX (final concentrations of 10 μ*M* and 100 μ*M*, respectively) were included in the agar mixture to terminate the effects of MTX polyglutamates during the colony-forming assay. Cells were plated in triplicate and incubated at 37° C in humidified 5% CO_2 and 95% air. After 10–14 days the colonies grew to about 1 mm in size and were counted visually. Control plating efficiency was between 80% and 90%, which was normalized to 100% cloning efficiency.

Measurement of RNA synthesis. Incorporation of ^{3}H-Urd into acid-insoluble material was used as a measure of RNA synthesis according to the procedure of Mandel et al. [12]. L1210 cells in logarithmic growth were diluted to 2.5×10^5 cells/ml and MTX was added. In some cases HX was included at the same time. To determine RNA synthesis during the first 3 h of drug incubation, ^{3}H-Urd was added at the same time as MTX and duplicate 1-ml samples were removed and processed to assess the amount of radioactivity incorporated into the RNA, as described below. After 12 h of MTX exposure, RNA synthesis was determined by the addition of ^{3}H-Urd and the removal of duplicate 1-ml aliquots of cell suspension at specified times. Each aliquot was processed as follows. The cell suspension was added to an equal volume of ice-cold 0.9% NaCl solution to stop ^{3}H-Urd incorporation and filtered on wetted Whatman GF/C glass fiber disks. The cells were washed with 5 volumes of ice-cold 0.9% NaCl solution, after which the nucleic acids were precipitated with 10 volumes of 0.2 *N* PCA solution and the precipitate was washed again. The dried filters (70° C for 30 min) were then counted for radioactivity.

Flow cytometry. L1210 cells were exposed to MTX for 12 h washed, and resuspended in cold 0.9% NaCl (2×10^6 cells/ml). The cells were fixed in ethanol by adding a 70% solution (2.5 vol.) dropwise. The fixed cells were stained for 20 min with mithramycin (100 μg/ml) and 15 m*M* $MgCl_2$ in 25% aqueous ethanol, then examined by means of a FACS 4 flow cytometer (Beckton-Dickenson, Sunnyvale, Calif) [1, 5]. A Spectra Physics Model 164-05 argon ion laser at 457 nm was used to excite fluorescence. About 1.5×10^4 cells were analyzed for each DNA histogram. Pulse height frequency distribution histograms were normalized so that they were visually comparable. The optical alignment of the FACS 4 was maintained with standard microspheres (Coulter Electronics, Hialeah, Fla), which gave an average coefficient of variation of 2.1%.

Preparation of prematurely condensed chromosomes. Premature chromosome condensation was performed using the method of Hittelman and Rao [9]. L1210 cells in logarithmic growth were diluted to 2.5×10^5 cells/ml and incubated with MTX with or without HX for 6 or 12 h. Exposure times greater than 12 h with 0.5 μ*M* MTX damaged the cells sufficiently to make it difficult to prepare prematurely condensed chromsomes (PCC). The cells were washed with medium and counted. Mitotic CHO cells were prepared by the mitotic detachment of cells at confluency that had been treated with 0.05 μg/ml Colcemid for 3 h. One million mitotic CHO cells were combined with 3 million L1210 cells in 5 m*M* piperazine-*N*-*N*-bis(ethanesulfonic acid) (PIPES) buffer at pH 7.4 and mixed thoroughly. The mixture was centrifuged and resuspended in 0.5 ml HBSS containing about 2000 hemagglutinating units of UV-inactivated Sendai virus, then left 0° C for 15 min. Next, 50 μl of an $MgCl_2$/Colcemid solution (100 m*M* $MgCl_2$ and 4 μg/ml Colcemid in HBSS) was added to the fusing cells, and the fusion mixture was placed without shaking in a 37° C water bath for 45 min. After addition of 7 ml 0.075 *M* KCl, the cells were vortexed and allowed to sit at room temperature for 12 min, at which time the cells were centrifuged, the KCl solution poured off, and the tubes drained thoroughly. The insides of the tubes were wiped dry, and the cells were fixed twice with approximately 5 ml methanol/glacial acetic acid (3:1, v/v), which was added dropwise. The fixed cells were dropped onto a wetted microscope slide. After air drying, the cells were stained for 5 min with aceto-orcein. The slides were coded and then scored in a blind fashion using a light microscope. At least 120 PCC spreads were scored for each treatment group.

Results

Cytotoxicity

In the clonogenic studies presented here, dThd and HX were included in the soft agar to limit the "cytotoxic" period to the time during which the drug was present. If cells were only washed to remove extracellular MTX before cloning without HX and dThd, the cytotoxicity was always greater than when HX and dThd were present (data not shown). This protracted cell killing is probably due to the buildup and retention of cellular MTX polyglutamates [8, 19].

When L1210 cells were exposed to MTX at concentrations ranging from 0.5 μ*M* to 100 μ*M* for 12 h, a period of time equal to one cell cycle, there was a dose-dependent decrease in survival that plateaued at 1 μ*M* (Fig. 1). At this MTX concentration 90% of the tumor cells were rendered non-clonogenic. The addition of 100 μ*M* HX to these

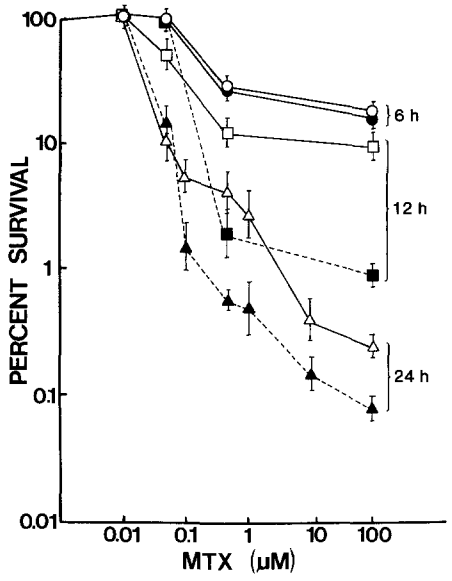

Fig. 1. Effect of MTX exposure on cytotoxicity and its modulation by HX. L1210 cells (2×10^5 cells/ml) were exposed to MTX (0.01 μ*M* to 100 μ*M*) for 6 h (○, ●), 12 h (□, ■) or 24 h (△, ▲) in the absence (*open symbols*) or presence (*closed symbols*) of 100 μ*M* HX, and then cloned in medium agar containing 10 μ*M* dThd plus 100 μ*M* HX. Colonies were counted 14 days later. The cloning efficiency for each treatment was determined and expressed as a percentage of control cloning efficiency. Each *point* represents the mean ± SE of at least four experiments

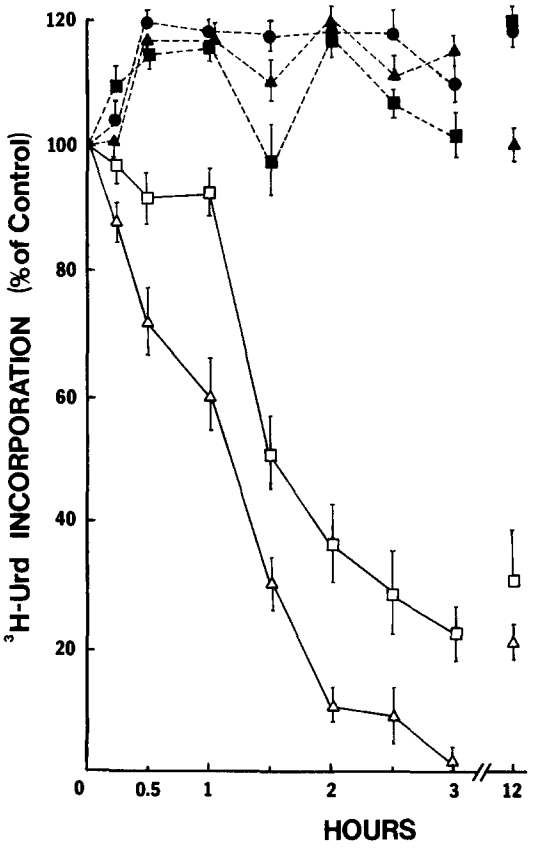

Fig. 2. Effect of HX on MTX inhibition of ^{3}H-Urd incorporation into RNA. L1210 cells (2.5×10^5 cells/ml) were incubated with 0 (●), 0.5 μ*M* (□, ■), or 100 μ*M* (△, ▲) MTX concurrently with 10 μ*M* ^{3}H-Urd in the absence (*open symbol*) or presence (*closed symbols*) of 100 μ*M* HX. At the times indicated, duplicate 1-ml aliquots of cells were removed and analyzed for the amount of tritium incorporated into RNA. Results are presented as the means ± SE for three experiments. Control RNA synthesis was 11.7×10^3 dpm/min per 10^6 cells

MTX exposures produced a one-log further reduction in clonogenic survival at 1 μ*M* and 100 μ*M* MTX (significantly different from MTX alone, $P < 0.001$, at all MTX concentrations ≥ 0.5 μ*M*). In contrast, a 6-h exposure to MTX alone was less cytotoxic at a given dose than a 12-h exposure. This probably occurs because fewer cells are exposed to MTX in the cytotoxic S phase during a 6-h than during a 12-h exposure. In addition, 100 μ*M* HX could not potentiate MTX cytotoxicity if the exposure was only for 6 h, probably because there was insufficient time for cells to become arrested in G_1. If the exposure period was increased to 24 h, the cell kill of MTX alone was greater than for the corresponding concentration after a 12-h exposure, especially at the higher MTX concentrations. However, the addition of 100 μ*M* HX to the 24-h MTX exposures potentiated MTX cytotoxicity at all MTX concentrations of 0.1 μ*M* or greater (significantly different than MTX alone; $P < 0.01$), although the potentiation was less than that seen for the same concentration of MTX after a 12-h exposure. Thus, it appears that L1210 cells must be exposed to MTX for at least one cell cycle (12 h) for HX to potentiate MTX cytotoxicity and that this effect occurs at MTX concentrations greater than 0.05 μ*M*, as HX was slightly protective at this or lower concentrations of MTX.

RNA synthesis

Because an exogenous source of preformed purines could provide purine nucleotides for use in RNA synthesis by way of the salvage pathway, we examined the ability of HX to prevent the inhibition of RNA synthesis. Over the first 3 h of drug exposure, MTX produced a dose-dependent inhibition of incorporation of ^{3}H-Urd into RNA at concentrations of 0.5 μl and 100 μ*M* (Fig. 2). RNA synthesis was inhibited nearly completely (100 μ*M*) or to 20% of control (0.5 μ*M*) after 180 min. Inhibition of RNA synthesis was maintained after 12 h exposure to both MTX concentrations. HX at 100 μ*M* protected RNA synthesis from inhibition by 0.5 μ*M* MTX and 100 μ*M* MTX over a 12-h period (Fig. 2). Concurrent administration of HX and MTX maintained RNA synthesis at levels that were slightly above control synthesis, as did HX alone.

Cell cycle progression as measured by flow cytometry

Flow cytometry was used to examine the ability of MTX to perturb cell cycle progression and the way in which HX might alter this effect. Figure 3 shows the results obtained from flow cytometric analysis of cells exposed for 12 h to MTX or MTX plus HX. The DNA histogram obtained for cells that have been treated with 100 μ*M* HX alone differs little from the control DNA histogram. However, for cells treated with 0.5 μ*M* or 100 μ*M* MTX, the resulting flow cytograms suggest an accumulation of cells in G_1 and indicate loss from G_2 plus M. When either concentration of MTX was combined with HX, the DNA histograms produced were similar to those seen when cells were exposed to MTX alone.

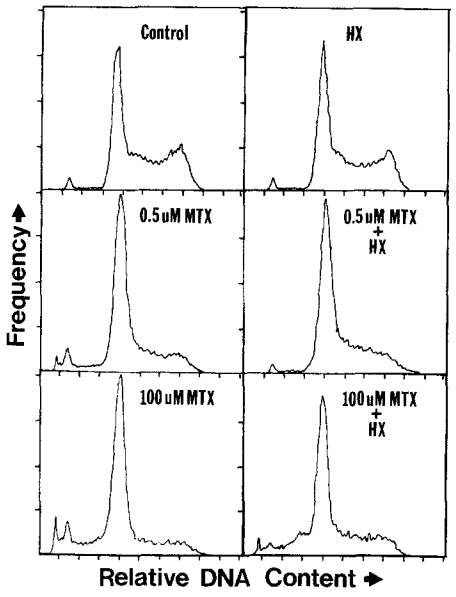

Fig. 3. DNA content histograms of L1210 cells treated with MTX plus HX or MTX alone. L1210 cells (2×10^5 cells/ml) were treated with 0.5 μM or 100 μM MTX alone or concurrently with 100 μM HX for 12 h. Cells were stained with mithramycin and analyzed for DNA content by flow cytometry

Cell cycle analysis by premature chromosome condensation

Premature chromosome condensation was used to determine the position in the cell cycle based on the decondensation state of chromatin in interphase cells. In unperturbed populations there is a typical distribution of PCC that parallels the cell cycle as defined by cellular DNA content [14], and as cells progress through G_1 and into S their PCC become more decondensed [9]. This deondensation is expressed in two ways. The first is macrodecondensation, in which the chromatids become extended and more narrow. The second is microdecondensation, which is manifest as apparent discontinuities in portions of the less condensed regions of the PCC. The apparent breaks in the PCC chromatin probably represent the unwinding of chromatin to such a degree that it is no longer visible under the light microscope.

The PCC spreads were scored as to the degree of chromosome decondensation according to the following scoring system which we have described in detail previously [7]. Figure 4 shows photomicrographs of each class. PCC in group 1 are a highly to moderately condensed form consisting of distinct unifilar structures that come from cells in the early to mid-G_1 phase. Group 2 PCC exhibit chromatin that is more extended than in group 1 and contain short regions of discontinuity. The chromatin in group 3 is mostly discontinuous, although regions in which the material is linearly organized can still be seen. Groups 2 and 3 show intermediate states of chromatin decondensation that are found in cells late in G_1 or early in S phase. The chromatin is most highly decondensed when in S phase, as seen in group 4, where the PCC are very diffuse and disorganized. Group 4 also includes PCC containing short, partially replicated regions. PCC spreads that were completely or nearly completely bifilar were classed as G_2, since spreads having this morphology come from cells that have finished replication and have initiated recondensation in preparation for mitosis [14]. G_2 PCC usually made up less than 10% of the total spreads and were not included in the scoring system.

Figure 5 shows the distribution of PCC after treatment of cells with 0.5 μM MTX and/or 100 μM HX for 12 h. PCC from control and HX-treated cells were distributed similarly. In either case, group 2 was the class containing the most spreads, whereas groups 3 and 4 contained somewhat fewer. Statistical analysis using the chi-square test indicated that these two distributions were not significantly different from one another ($P > 0.1$). For cells exposed to MTX, group 4 contained more spreads, and there were fewer present in groups 1 and 2 compared with controls. This represents a shift to more decondensed morphologies, as group 4 represents the maximal level of decondensation. The combination of MTX and HX resulted in a further increase in the number of spreads in group 4 and the loss of spreads from groups 1 and 2, producing a greater shift toward decondensation, and suggests progression through the cell cycle. After the combination of MTX and HX, over 80% of the spreads were found in groups 3 and 4. Chi-square analysis showed that the distributions from cells exposed to MTX or MTX plus HX were significantly different from each other as well as from controls ($P < 0.001$).

When cells were exposed to 0.5 μM MTX alone for 6 h or to MTX plus 100 μM HX and then PCC made, the resulting distributions were similar to those produced from control or HX-treated cells (data not shown). MTX alone produced a small increase in the percentage of cells with increased decondensation, but this effect was not enhanced by the simultaneous administration of HX.

Discussion

MTX is known to be a cell-cycle-specific yet self-limiting agent [20]. It has been reported that purines potentiate MTX cytotoxicity [5, 6]. Borsa and Whitmore [5] have suggested that the inhibition of de novo purine synthesis by MTX caused cells to accumulate in G_1, protecting them from the cytotoxic effects of MTX in S phase, and that the addition of purines circumvented the self-limiting effect. In this report, we show that the addition of HX to L1210 cells during MTX exposure abrogated the inhibitory effect of MTX on RNA synthesis and allowed cell cycle progression through G_1, producing enhanced cytotoxicity compared to MTX alone.

For cells exposed to MTX for 12 h or longer, HX increased the cytotoxicity of MTX (Fig. 1). Flow cytometric analysis of cells treated with 0.5 μM or 100 μM MTX showed a buildup of cells with a G_1 content of DNA and a corresponding loss of cells from G_2/M (Fig. 3). The addition of HX with MTX did not alter the flow cytotograms. This is in agreement with the results reported by Taylor et al. [16, 17] for exposure of CCRF-CEM cells to 100 μM MTX for one cell cycle. They observed that the addition of HX produced an increased cellular RNA content but did not prevent the accumulation of cells in G_1 as measured by DNA-content histograms. Because flow cytometry probably cannot distinguish between cells in late G_1 (at the border of G_1 and S) and in early S phase, we used the technique of premature chromosome condensation to determine cell cycle progression based on chromatin morpholo-

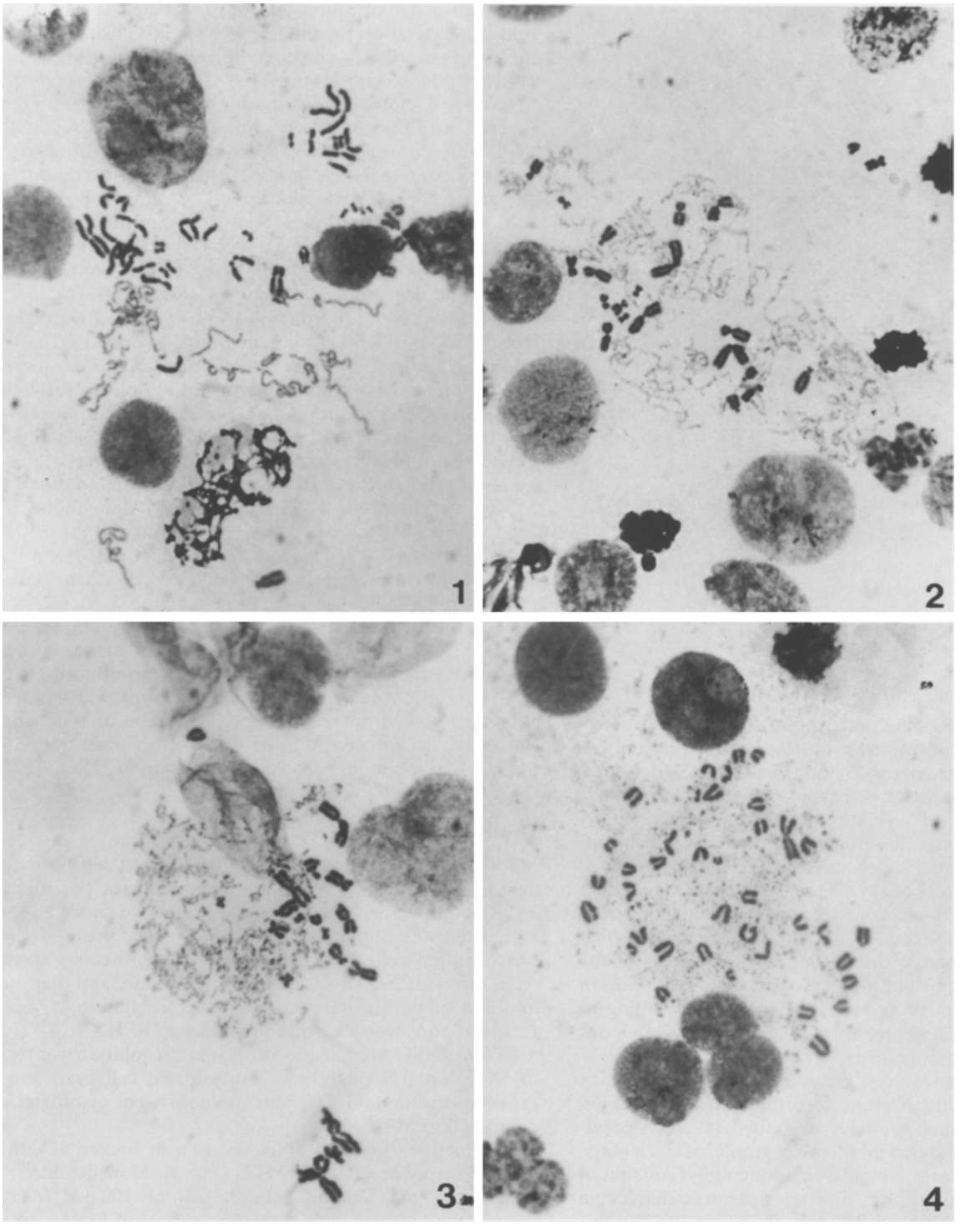

Fig. 4. Photomicrographs of PCC decondensation groups of L1210 cells showing various degrees of chromatin decondensation, which was used to assign PCC spreads to groups 1–4. Each group is described in the Results section. The darkly stained chromosomes seen in each panel are the mitotic CHO cell chromosomes.

gy. Our studies showed that in the presence of inhibition of DNA synthesis by MTX, chromatin decondensation continues (Fig. 5). Addition of HX during the 12-h (one cell cycle) exposure to MTX increased the proportion of cells with higher decondensation states. These results indicate that HX, by maintaining RNA synthesis in the presence of MTX, allows the progression of cells through G_1 toward S phase and results in enhanced toxicity as demonstrated here and by others [2, 15–17].

When cells were subjected to PCC analysis after a 6-h exposure to MTX or MTX plus HX, the distribution of PCC spreads was not altered by drug treatment (data not shown). Furthermore, HX did not enhance the toxicity of MTX observed after a 6-h exposure. Taylor et al. [15] and

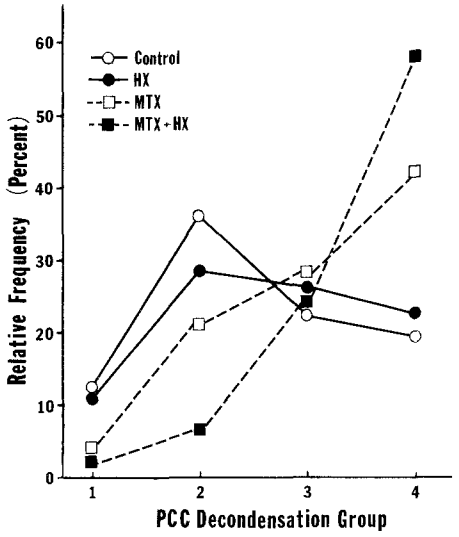

Fig. 5. Frequency distributions of PCC decondensation groups after exposure to 0.5 μ*M* MTX and/or 100 μ*M* HX for 12 h. After drug treatment, L1210 cells were fused with mitotic CHO cells to induce premature chromosome condensation. At least 120 spreads were scored for each treatment

Hryniuk [10] also could not demonstrate potentiation of MTX toxicity after a short exposure of CCRF-CEM cells to MTX. The lack of potentiation by HX and the failure of HX to promote the movement of cells toward lower states of chromatin condensation after 6 h of MTX exposure suggests that the subpopulation of cells (which become arrested in G_1 during a 12-h MTX treatment) could not reach S phase after a 6-h exposure. We propose that this subpopulation represents a portion of the cells that were initially in late S phase when purine synthesis was inhibited secondary to the depletion of reduced folates. In 6 h, these cells would not have sufficient time to traverse G_1 even if HX was provided. It is only when the duration of MTX exposure is increased to 12 h or more that the subpopulation becomes evident. Therefore, when these cells are provided with a source of preformed purines, they can traverse G_1 and enter S phase, where they undergo thymineless death.

When cells were exposed to either 0.5 μ*M* or 100 μ*M* MTX for 12 h about 10% of the cells remained clonogenic. The addition of HX reduced the number of clonogenic cells to about 1%, which indicates that as many as 9% of the cells were arrested in G_1 when treated with MTX alone. This is consistent with the results from the PCC studies, which showed a 13% decrease in the number of cells in G_1 (groups 1 and 2 in Fig. 5) when HX was added to the MTX treatment. However, after a 24-h exposure to 0.5 μ*M* and 100 μ*M* MTX, only 6% or 0.3% of the cells remained clonogenic, respectively. This level of survival is in the same range as that observed for 12 h of MTX plus HX, suggesting that cells arrested in G_1 retain their clonogenic potential for 12 h, but if exposed to MTX for an additional 12 h most of the cells are irreversibly damaged. Addition of HX to MTX during the 24 h treatment resulted in a further decrease in the clonogenicity, particularly at the higher MTX concentrations (0.5–100 μ*M*). This suggests that even after a 24-h exposure, some of the cells arrested in G_1 in the absence of HX remain clonogenic during this longer period of exposure. It is also possible that some of the highly resistant population could be made up of cells that are inherently resistant to MTX owing to increased DHFR or decreased MTX transport.

In summary, these studies show that the self-limiting aspect of MTX that occurs during 12-h (one cell cycle) exposure can be overcome by providing preformed pruines and allowing cell cycle progression. It is only through sparing RNA synthesis in the presence of inhibition of DNA synthesis that maximal MTX cytotoxicity is achieved.

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