Methotrexate Cytotoxicity in Cultured Human Leukemic Cells Studied by Flow Cytometry

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ABSTRACT

Methotrexate (MTX) (2 x 10⁻⁸ M) inhibited DNA synthesis in CCRF-CEM cells, causing cells to accumulate in early S phase while cellular RNA content and cell size continued to increase. Two-parameter flow cytometric analysis of DNA and RNA showed these cells to be unbalanced with excessive RNA relative to DNA content. Fifty % of cells remained viable after a 96-hr exposure to 2 x 10⁻⁸ M MTX. In contrast, 10⁻⁴ M MTX inhibited cell cycle progression of cells in both G₁ and S phases and also prevented the development of unbalanced growth. In this instance, cell viability was reduced to 10% after 96 hr of drug exposure. The relative contribution of inhibition of thymidylate and purine biosynthesis to MTX cytotoxicity was investigated by addition of exogenous thymidine (10⁻⁵ M) or hypoxanthine (10⁻⁴ M). Thymidine reduced the cytotoxicity and inhibition of DNA synthesis caused by both doses of MTX and prevented classical unbalanced growth with 2 x 10⁻⁸ M MTX; treatment with 10⁻⁴ M MTX resulted in a form of unbalanced growth where cells had a relative excess of DNA compared with RNA content. The addition of hypoxanthine enhanced the classical unbalanced growth pattern seen with 2 x 10⁻⁸ M MTX but was accompanied by a partial reduction of both the MTX-induced cytotoxicity and the inhibition of DNA synthesis (to an extent similar to that seen with exogenous thymidine). Potentiation of cell killing was observed with the addition of hypoxanthine to cells treated with 10⁻⁴ M MTX. Complete rescue from MTX cytotoxicity at both concentrations was found only when both thymidine and hypoxanthine were present.

These findings suggest that MTX cytotoxicity is associated with inhibition of DNA synthesis resulting from the disturbance of both thymidylate and purine biosynthesis.

INTRODUCTION

MTX² is a potent inhibitor of mammalian dihydrofolate reductase (18, 19). The depletion of cellular pools of reduced folate cofactors following exposure to MTX disturbs thymidylate, purine, and amino acid metabolism. Reduction in cellular levels of 5,10-methylene-tetrahydrofolate inhibits the methylation of dUMP by thymidylate synthetase, thus blocking de novo thymidylate synthesis (6). Exposure of cells to MTX expands the dUMP pool and lowers markedly the dTTP pool unless exogenous dThd is added to the culture medium (26, 27), and cells have an active thymidine salvage pathway (2). Reduced folate cofactors are also utilized in the de novo synthesis of purines; although this pathway does not oxidize folate, disturbances of purine synthesis have been reported in cells exposed to MTX (8). Cells exposed to MTX may thus be perturbed by inhibition of de novo thymidylate biosynthesis, of de novo purine biosynthesis, or of both, depending upon the cell type and culture conditions. In the first case, cells would be expected to become unbalanced due to selective inhibition of DNA synthesis with continuation of RNA and protein synthesis; in the latter case, inhibition of both DNA and RNA synthesis might occur.

While flow cytometry has been widely used as a means of investigating the cell cycle perturbations caused by a variety of chemotherapeutic agents (30, 31, 35), most of these studies have been limited to single-parameter analysis of cellular DNA content, and little attention has been given to drug effects on other cell cycle-related parameters such as changes in RNA content and cell size. Because MTX may affect both DNA and RNA synthesis, we have studied cultured human leukemic cells exposed to MTX using flow cytometric single-parameter analysis of DNA, RNA, and cell size and correlated 2-parameter analysis of DNA and RNA. The effects of exogenous dThd and HX have also been studied to identify the relationship between MTX cytotoxicity and disturbance of thymidylate and purine biosynthesis.

MATERIALS AND METHODS

A long-term culture of human leukemic T-cells (CCRF-CEM) was used in this study (5). These cells grow as a suspension culture in Roswell Park Memorial Institute Medium 1640 supplemented with L-glutamine and 10% fetal calf serum (complete medium) with a cell-doubling time of about 24 hr. In all experiments to be described, 75-sq cm tissue culture flasks (Corning Glass Co., Corning, N. Y.) were inoculated with 90 to 150 ml of cell suspension at 10⁶ cells/ml. The cultures were then allowed to grow undisturbed for 24 hr before addition of drugs. All treatments were carried out on exponentially growing cell cultures. Cell counts were made by phase-contrast microscopy. This technique was also used to discriminate between live (phase-positive) and dead (phase-negative) cells.

Drug Treatment

dThd and HX were obtained from Calbiochem (Australia) Pty., Sydney, Australia. MTX was a gift from Dr A. Hellestrand, Cyanamid (Australia) Pty., Sydney, Australia. Razoxane was a gift from I.C.I. (Australia) Pty., Sydney, Australia.

Stock solutions of dThd and MTX were prepared at 30 times final concentration in Roswell Park Memorial Institute Medium 1640 without fetal calf serum. HX stock solutions were made by dissolving the drug in equimolar NaOH (10 mm), heating at 60° for 30 min, and then diluting the solution to 30 times the required final concentration with medium. Razoxane was made up at 50 times the final required concentration in 0.4 M HCl. All stock solutions were sterilized by Millipore filtration and kept

1 To whom requests for reprints should be addressed.
2 The abbreviations used are: MTX, methotrexate; dThd, thymidine; HX, hypoxanthine.

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frozen at −20°C until required.

In all experiments, the drugs were added as a small volume directly to cells in suspension 24 hr after culture initiation. Each drug was administered as a single dose and remained in the culture for the duration of the experiment. When drug combinations were tested, the drugs were added simultaneously. An appropriate volume of the drug vehicle was added to control flasks.

Flow Cytometry

DNA Analysis. Cellular DNA content was measured using an ICP22 flow cytometer (Ortho Instruments, Westwood, Mass.). The cell-staining procedure has been described previously (29). Briefly, 10^6 cells in complete medium were made permeable to stain by the addition of 1.5 volumes of ice-cold 0.1% (v/v) Triton X-100 containing 0.1 M HCl and 0.15 M NaCl. After 1 min, the cells were centrifuged, and the cell pellet resuspended in an ethidium bromide-mithramycin staining solution containing ethidium bromide (5 μg/ml), mithramycin (12.5 μg/ml), and 7.5 mM MgCl_2 in 0.1 M Tris-HCl buffer, pH 7.4. Samples of the stained cells were then excited at 360 to 460 nm, and the resulting fluorescence was measured at wavelengths >550 nm. Approximately 30,000 cells were analyzed for each DNA content histogram. Calculations of percentages of cells in various phases of the cell cycle and of coefficient of variation of the G_0 DNA peak were made using a curve-fitting method of analysis (17, 29). Unfixed chicken RBC (10^9) were added to each sample before staining as an internal standard which would account for variations in machine performance and staining procedures (29).

Dual-Parameter Analysis of DNA and RNA. Simultaneous measurement of cellular DNA and RNA content was obtained from cells stained with acridine orange by the 2-step procedure described by Traganos et al. (32). Cells (10^6/ml) were made permeable by the addition of a Triton X-100 solution containing acridine orange (10 μg/ml), 5 mM EDTA, and 0.15 M NaCl in 0.1 M citrate-phosphate buffer, pH 5.0. Acridine orange intercalates with double-stranded nucleic acids and dye stacks on single-stranded nucleic acids which when excited with blue light will cause green and red fluorescence, respectively. Experiments with cells treated with RNase or DNase (both at 1 mg/ml for 30 min) showed that approximately 75% of the red fluorescence and 85% of the green fluorescence could be attributed to RNA and DNA, respectively. The staining conditions used are therefore such that the green fluorescence provides a measure of cellular DNA content and the red fluorescence measures cellular RNA content.

The green and red fluoroses from acridine orange-stained cells were measured simultaneously with the ICP 22 flow cytometer in coincident mode using an excitation wavelength between 440 and 490 nm in conjunction with a 510-nm dichroic mirror. Green fluorescence was measured between 520 and 550 nm, and red fluorescence was measured at wavelengths greater than 620 nm. A 580-nm dichroic mirror was used in the fluorescent light path to separate green and red fluorescence. The correlated data were then plotted as a contour plot.

Single-parameter analysis of RNA was obtained in a similar fashion. Mean values of the RNA histograms were obtained as previously described (17).

Light Scatter Analysis. Measurements of narrow-angle laser light scatter (between 0.5° and 13°) were made using a fluorescence-activated cell sorter (FACS III; Becton Dickinson, Mountain View, Calif.). Narrow-angle light scatter of viable cells is closely correlated with cross-sectional cell area, and, because mammalian cells in suspension usually adopt a spherical shape, therefore reflects cell volume (33). In order to circumvent problems associated with cell debris and dead cells (which have low light-scattering properties), all cell samples were stained before analysis with fluorescein diacetate [50 μl of fluorescein diacetate (50 μg/ml) to 10^6 cells]. Fluorescein diacetate, itself nonfluorescent, is broken down by nonspecific esterase activity in cells to form fluorescein which when excited at 488 nm emits fluorescent light between 520 and 540 nm. Because fluorescein is retained only in viable cells with an intact cell membrane (22), the light scattering of debris and dead cells which have little associated fluorescence can be electronically gated out from the analysis. Values representing the histogram mean were then calculated from histograms of cellular light scattering as for the RNA histograms above. Proportions of live versus dead cells were on occasions estimated by this technique and found to be in good agreement with the results obtained by phase-contrast microscopy. For reasons of clarity, however, all estimates of viability referred to in the text were obtained by phase-contrast analysis.

RESULTS

In all experiments to be described, the addition of either 10^{-5} M dThd or 10^{-4} M HX as single agents to CCRF-CEM cells had no discernible effect on any of the measured parameters. For clarity, these results have been omitted from the data presented. Values shown for cell numbers, RNA content, and cell size represent the mean of 4 individual experiments unless so indicated. DNA content histograms and 2-parameter contour plots are from one experiment and were representative of results obtained in other experiments. An example of the DNA content analysis of control cell cultures is shown in Chart 1. No significant change in the proportion of cells in G_0 (38%), S

Chart 1. DNA distribution of untreated CCRF-CEM cells. Channel number, relative fluorescence intensity which is directly proportional to DNA content. The first peak corresponds to chicken RBC (CRBC) which acts as an internal biological standard. The largest population represents diploid CCRF-CEM cells (G_0). Cells with double the fluorescence of diploid cells are in G_1 or mitosis (G_1 + M). In all experiments to be described, the addition of either 10^{-5} M dThd or 10^{-4} M HX as single agents to CCRF-CEM cells had no discernible effect on any of the measured parameters. For clarity, these results have been omitted from the data presented. Values shown for cell numbers, RNA content, and cell size represent the mean of 4 individual experiments unless so indicated. DNA content histograms and 2-parameter contour plots are from one experiment and were representative of results obtained in other experiments. An example of the DNA content analysis of control cell cultures is shown in Chart 1. No significant change in the proportion of cells in G_0 (38%), S

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(49%), or G2 + M (13%) was found during a 96-hr period of cell growth. The ratio of the CCRF-CEM G1 peak position (Channel 50) to that of the chicken RBC (Channel 15) also remained constant with time.

Two dose levels of MTX were examined: 2 × 10⁻⁸ M (low-dose), which prevented any increase in viable cell numbers over a 96-hr period; and 10⁻⁴ M (high-dose), which resulted in a high level of cytotoxicity within 72 hr. The latter dose level approximates that which might be obtained clinically with high-dos MTX therapy.

Low-Dose MTX. 2 × 10⁻⁸ M MTX resulted in maintenance of a constant level of viable cells for a period of 96 hr (Chart 2). However, during that time, the proportion of dead cells in the culture increased to almost 50% (Table 1), indicating that some cell division was taking place. Analysis of the DNA content (Chart 3) of these cultures showed an initial accumulation of

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**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>24 hr</th>
<th>48 hr</th>
<th>72 hr</th>
<th>96 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2 (0.5)*</td>
<td>3 (0.8)</td>
<td>5 (0.5)</td>
<td>9 (0.5)</td>
</tr>
<tr>
<td>2 × 10⁻⁸ M MTX</td>
<td>15 (3.2)</td>
<td>15 (3.7)</td>
<td>33 (2.5)</td>
<td>48 (2.9)</td>
</tr>
<tr>
<td>+ 10⁻⁶ M dThd</td>
<td>7 (1.4)</td>
<td>15 (0.5)</td>
<td>17 (1.6)</td>
<td>21 (3.3)</td>
</tr>
<tr>
<td>+ 10⁻⁴ M dThd +</td>
<td>4 (0.8)</td>
<td>5 (1.8)</td>
<td>7 (2.1)</td>
<td>10 (1.5)</td>
</tr>
<tr>
<td>+ 10⁻⁴ M dThd +</td>
<td>16 (2.5)</td>
<td>57 (2.9)</td>
<td>90 (4.8)</td>
<td>100</td>
</tr>
<tr>
<td>+ 10⁻⁴ M dThd +</td>
<td>16 (2.9)</td>
<td>36 (6.7)</td>
<td>49 (5.7)</td>
<td>63 (3.0)</td>
</tr>
<tr>
<td>+ 10⁻⁴ M dThd +</td>
<td>35 (8.2)</td>
<td>86 (4.1)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>+ 10⁻⁴ M dThd +</td>
<td>3 (0.5)</td>
<td>8 (2.2)</td>
<td>67 (9.5)</td>
<td>91 (2.5)</td>
</tr>
<tr>
<td>+ 10⁻⁴ M dThd +</td>
<td>7 (0.8)*</td>
<td>10 (1.1)*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parentheses, 1 S.D. from the mean obtained from 4 individual experiments.

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With MTX exposure times up to 48 hr, the cellular RNA content and cell size increased compared to control values (Chart 5), and these increases, in conjunction with impaired DNA synthesis, indicate unbalanced cell growth. Unbalanced cell growth is readily demonstrated by the 2-parameter analysis of DNA and RNA contents at 48 hr where the cells can be seen to have an excess of RNA in relation to their DNA content when compared with controls (Chart 6). Between 48 and 96 hr, RNA levels return to normal, but cell size is still significantly increased even after 96 hr.
Addition of either dThd or HX to the low-dose-MTX-treated cells had similar effects on subsequent cell growth. Both agents allowed viable cell numbers to increase slowly in the presence of MTX (Chart 2) and reduced the degree of cytotoxicity (Table 1). Analysis of the DNA content of these cells suggests that both dThd and HX reduce the MTX-induced accumulation of cells in early S phase, but not completely, as shown by the perturbed DNA histograms between 24 and 96 hr (Chart 4). When, however, the effects of dThd or HX were measured in terms of RNA content or cell size, a marked difference between treatments was observed. While the addition of dThd largely prevented the MTX-induced increases in RNA content and cell size, the addition of HX exacerbated the effects previously described for MTX alone (Chart 5). Two-parameter analysis of DNA and RNA show that, while HX increases the degree of unbalanced cell growth over that for MTX alone, the addition of dThd completely prevents the appearance of this effect (Chart 6).

At first, it appeared that the addition of both dThd and HX to MTX treatment did not result in complete rescue of these cells. A delay in cell growth was observed between 24 and 48 hr (Chart 2) with minor perturbations in DNA content (Chart 3), RNA content, and cell size (Chart 5). The 2-parameter analysis also suggested some unbalanced growth at this time (Chart 6). However, it was found that these changes in the measured parameters could be directly attributed to the effects of the combination of dThd and HX, although no such effects were observed with either agent alone (Chart 6). The combination of dThd and HX would therefore appear to completely reverse the effects of low-dose MTX on CCRF-CEM cells.

In the absence of a suitable cloning assay for CCRF-CEM cells, long-term cell survival was assessed by the ability of cells to regrow in drug-free medium. Cells which had been exposed to each treatment for 96 hr were washed three times and resuspended in drug-free medium at a concentration of 2 x 10^5 viable cells/ml. Subsequent growth curves (Chart 7) showed that cells which had been exposed previously to low-dose MTX grew exponentially at a rate similar to that seen for control cultures after an initial lag period of 24 hr. Because this lag period could be due either to further cell death or to a delay in the return to exponential growth, differential counts of live versus dead cells were made 24 hr after drug-treated cells were resuspended in fresh medium. No significant increase in the number of dead cells over those present at culture initiation was found. This suggests that there is a delay in the return of MTX-treated cells to exponential growth and that most of the cells surviving 96 hr of MTX treatment are capable of further sustained growth. The lag period was reduced for cells previously exposed to MTX and HX or to MTX and dThd, and it disappeared completely for those cells previously treated with...
MTX Cytotoxicity

Chart 6. Correlated 2-parameter analysis of acridine orange-stained CCRF-CEM cells. Green and red fluorescences represent DNA and RNA, respectively. A, non-drug-treated controls; B, 2 x 10^{-8} M MTX; C, MTX plus 10^{-8} M HX; D, MTX plus 10^{-8} M dThd; E, MTX plus HX plus dThd; F, HX plus dThd. All measurements were made at 48 hr after drug addition. A, B, and C also show the contour plots obtained after RNase treatment (the left group of contours in each case). This shows that the increases in red fluorescence seen with treatment result from increases in cellular RNA content. Each contour plot was generated using 6 contour levels of 50, 100, 150, 300, 600, and 1000 cells. To facilitate comparisons between individual plots, a heavy solid line, representative of control values (A), has been superimposed on each contour plot.

the combination of all 3 agents.

High-Dose MTX. Exposure to 10^{-4} M MTX resulted in a rapid fall in cell viability between 24 and 96 hr (Chart 8). During this period, no increase in total cell numbers (live and dead) was observed; by 72 hr, the proportion of viable cells was reduced to 10% (Table 1). DNA analysis of these cells showed an increase in G_1 and a decrease in G_2 + M within 24 hr of treatment (Chart 9). No further changes in cell cycle distribution with time were then observed with the exception of the appearance of increasing cell debris. Constant cell cycle distributions as a function of time after MTX addition could mean either that MTX had no effect on cell cycle progression or that it prevented the progression of cells through all parts of the cell cycle. Because no increase in cell number was observed
with this dose of MTX (Chart 8; Table 1), the latter explanation seems more likely. This was confirmed by using the antimitotic agent razoxane (100 µg/ml) which causes cells to accumulate in G2 + M and prevents the return of cells to G1 (28). A large proportion of control cells treated with razoxane accumulated in G2 + M after 24 hr exposure, whereas only minor changes were observed in MTX cultures treated in a similar fashion (Chart 9). This concentration of MTX also had little effect on RNA content or with the exception of a small increase at 48 hr, on cell size (Chart 11). Two-parameter analysis of DNA and RNA at 48 hr show that no unbalanced growth occurred with this dose of MTX (Chart 12).

The addition of HX to MTX treatment had a profound effect on cell viability which decreased rapidly over a period of 72 hr (Chart 8; Table 1). Although DNA analysis of these cells at 24 hr was similar to that seen with MTX alone (Chart 9), increases in both RNA content and cell size were observed (Chart 11), and the 2-parameter analysis showed these cells to be unbalanced (Chart 12).

The addition of dThd partially reduced the cytotoxicity of MTX, with viable cell numbers decreasing more slowly than for MTX alone (Chart 8) and with approximately 40% of the cells remaining viable at 96 hr (Table 1). DNA analysis showed that the inhibitory effects of MTX on cell cycle progression were also reduced by dThd, and a large cohort of cells were seen to progress slowly through the cell cycle between 24 and 96 hr (Chart 10). Although no significant changes in cell size were observed with this treatment, marked changes in RNA content were found (Chart 11). The RNA content decreased continuously until 96 hr when it reached 40% of control levels. Two-parameter analysis showed that these cell populations had significantly less RNA per cell than did controls, although the DNA contents were within normal limits (Chart 12).

Apparent complete recovery from the effects of high-dose...
low-dose MTX were carried out for cells treated with $10^{-4}$ M MTX. $2 \times 10^5$ viable cells from either the MTX-alone group at 48 hr, MTX plus dThd at 96 hr, or MTX plus dThd plus HX at 96 hr were resuspended in fresh medium without added nucleosides. In each case, viable cell numbers were found to decrease to less than $10^5$ cells/ml within 48 hr. Similar results were obtained for the 3-agent combination even when further dThd was added to 48 hr.

**DISCUSSION**

MTX was observed to cause a dose-dependent effect on cell cycle progression. Low-dose MTX inhibited DNA synthesis and cells accumulated initially in early S phase and then progressed slowly through the remainder of the cell cycle. Following high-dose MTX, cell cycle progression through S phase ceased, and cells accumulated with a G1 DNA content. With the techniques used, it is not possible to know whether this latter group of cells is in G1, at the G1-S boundary, or even in very early S phase. With both MTX doses, the simultaneous addition of dThd partially prevented the disturbance of DNA synthesis and cell growth. However, complete rescue was seen only when both dThd and HX were present, suggesting that MTX at both doses was disturbing thymidylate and purine biosynthesis. An increase in cellular RNA content was seen following low-dose MTX, implying that purine biosynthesis was not completely inhibited; but the addition of HX increased both DNA synthesis and cellular RNA content, suggesting that some antipurine activity was present. In contrast, the lack of unbalanced growth with high-dose MTX while DNA synthesis is inhibited indicates a more pronounced inhibition of RNA synthesis presumably due to greater perturbation of purine metabolism. In view of this marked inhibition of RNA synthesis, it seems probable that high-dose MTX can inhibit the progression of cells through G1 (see above) when relatively high rates of RNA synthesis are known to occur (34).
Numerous studies have examined the biochemical effects of MTX in cultured cell lines and in vivo, but the precise mechanism by which MTX kills cells is still poorly understood despite a number of attempts to correlate MTX cytotoxicity with observed changes in a variety of biochemical parameters (7, 8, 11, 15, 21, 25, 27). Some authors have attributed disturbances of purine metabolism by MTX as a mode of cell death (8-11), while others have reported that inhibition of purine biosynthesis protects cells from subsequent MTX cytotoxicity (1, 23). In our studies with CCRF-CEM cells, the consequences of disturbances of RNA synthesis (indicative of purine metabolism) caused by MTX are dose dependent. With low-dose MTX, addition of HX partially rescued cells from toxicity; whereas with high-dose MTX, the addition of HX potentiated MTX cytotoxicity. Subsequent experiments have shown that HX potentiates MTX cytotoxicity with MTX concentrations in excess of $10^{-7} \text{M}$ (data not shown). This latter effect was not observed in an earlier study with CCRF-CEM cells (20), but in this instance...
no MTX concentrations greater than $10^{-7}$ M were used.

Several authors have reported that MTX may cause unbalanced cell growth due to selective inhibition of thymidylate synthesis (1, 3, 23). Unbalanced growth has also been reported following treatment with hydroxyurea (24), dThd (13), and 1-β-o-arabinofuranosylcytosine (12, 14). It has been suggested that the continuation of unbalanced cell growth for longer than one cell cycle time results in cell death (4, 12, 24). Some observations in this study appeared initially to be in agreement with these reports since dThd plus HX failed to maintain rescue from high-dose MTX for longer than 48 hr and resulted in unbalanced growth which was associated with a rapid increase in cell death unless further dThd was added at this time. Similarly, the addition of HX to cells treated with high-dose MTX caused unbalanced cell growth and significantly increased cytotoxicity. In contrast with these results were the studies with low-dose MTX which caused unbalanced cell growth between 24 and 72 hr (cell cycle time is 24 hr); when cells surviving 96 hr of this treatment were resuspended in fresh medium, the resulting growth curve suggested that the majority of the cell population was capable of normal growth. These results can be attributed neither to a population of cells which did not become unbalanced, since Chart 6 shows that the entire population is unbalanced at 48 hr, nor to MTX resistance, since these cells responded to further MTX treatment as before (data not shown). Furthermore, addition of HX to low-dose MTX resulted in a greater degree of unbalanced growth than did MTX alone, but this was accompanied by reduced cytotoxicity, similar to that accompanying dThd treatment which, however, also prevented unbalanced growth. Our results suggest that unbalanced cell growth is not the primary mode of cell death caused by MTX. Recovery from prolonged dThd-induced unbalanced growth has also been observed previously (16).

Unbalanced cell growth is generally held to occur when DNA synthesis is inhibited and cells continue to synthesize RNA and protein. We have observed that the converse pattern may also occur in cells exposed to high-dose MTX and dThd. In these cells, DNA synthesis continued while RNA synthesis was inhibited, resulting in unbalanced cell growth where cells contained excess DNA in relation to their RNA content. This effect was not due to the presence of dead cells (which frequently appear with a lower than normal RNA content), inasmuch as between 24 and 48 hr, where the phenomenon was observed initially, more than 60% of the cell population was viable. We are unaware of any such effect having been previously reported for MTX.

From the results obtained in this study, there would appear to be some correlation between MTX cytotoxicity and inhibition of DNA synthesis. The greatest degree of cytotoxicity was observed when DNA synthesis was almost completely inhibited as caused by high-dose MTX with and without added HX. Furthermore, when rescue with HX and dThd from high-dose MTX failed at 72 hr, the DNA histogram was similar to that for high-dose MTX alone, indicating that DNA synthesis was inhibited. This situation was prevented by the further addition of dThd which restored cell cycle progression and cell growth. With the other drug treatments examined, the degree of cytotoxicity diminished as the inhibition of cell cycle progression was reduced (as judged from the sequential DNA analysis) in order: high-dose MTX plus dThd; low-dose MTX plus HX; low-dose MTX plus dThd; low-dose MTX plus HX. In all instances where rescue from MTX cytotoxicity was effected, no substantial inhibition of cell cycle progression was observed.

In conclusion, this study suggests that the major effect of MTX on cell viability is mediated through the inhibition of DNA synthesis which results from interference with the de novo biosynthesis of both purines and thymidylate. The effects of MTX on RNA synthesis appear to be unrelated to cytotoxicity. We are presently engaged in further investigations to delineate the precise biochemical changes which correlate with MTX-induced cell death. The 2-parameter flow cytometric analysis used in this study clearly provided much more information pertaining to cell cycle-related drugs effects than did single-parameter analysis of DNA content alone, and we are extending this type of analysis to studies of cells treated with other antimetabolites including 5-fluorouracil and to deoxynucleosides.

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