Flow Cytometric Analysis by Bromodeoxyuridine/DNA Assay of Cell Cycle Perturbation of Methotrexate-treated Mouse L1210 Leukemia Cells

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ABSTRACT

The in vitro effects of methotrexate (MTX) on cell cycle progression and DNA synthesis of L1210 leukemia cells were studied by the bromodeoxyuridine (BrdUrd)/DNA analysis technique. Low dose (10^{-8} M) MTX, which slightly inhibits clonal replication of the cells, delays progression across the S phase, and treatment for 24 h results in a slight increase of the S-phase population. Much higher doses (10^{-7} M and 10^{-6} M) of MTX, which strongly reduce clonal replication, delay the progression of the cells at the G1-S boundary and across the S phase, but not in the other phases. The cells arrested at the G1-S boundary were able to incorporate BrdUrd in the medium for 6-12 h after the start of treatment and then lost the ability to incorporate BrdUrd. By determining the colony inhibitory activity of MTX, it could be shown that not only S-phase cells but non-S-phase cells are susceptible to cytotoxicity of MTX.

INTRODUCTION

Since the 1960s much information has accumulated on the effectiveness of MTX in killing cells in relation to the kinetic status of the exposed cells (1). However, many aspects of its action on cell cycle kinetics have not been clarified, except that the S-phase cells are the major targets of MTX. In 1970 Skipper et al. (2) classified MTX as an S-phase-specific drug with self-limitation in cell killing action, and this theory has been generally accepted. Some investigators (3, 4) have suggested, on the basis of autoradiography with [3H]dThd, that MTX inhibits cell transition at the G1-S boundary, and recent flow cytometric studies (4-6) have also shown G1-S block on DNA histograms of MTX-treated cells. If it is true that prolonged high MTX levels prevent G1-S transition with a subsequent decrease of cytotoxicity, there may be a paradoxical situation which would affect the timing of drug administration. However, the measurement techniques in the previous cytokinetic studies have some limitations in determining accurately G1-, S-, and G2 + M-phase fractions. For example, [3H]dThd-based autoradiography is suboptimal in the discrimination of weakly labeled cells from unlabeled cells (7). Univariate DNA distribution analysis by flow cytometry gives no information about the rate of DNA synthesis (8). Techniques with enough sensitivity to detect low levels of DNA replication on a cell-by-cell basis in vitro or in vivo have recently become available with the use of a monoclonal antibody to BrdUrd (9-11). We used this BrdUrd/DNA assay technique to study the cytokinetic effects of MTX on cultured L1210 leukemia cells. In this study, we show that the self-limiting action with MTX is different from that which has been commonly believed.

MATERIALS AND METHODS

Cells. L1210 leukemia cells were supplied by the Cancer Chemotherapy Center, Japan Foundation, Tokyo. The cells were grown in RPMI 1640 medium supplemented with L-glutamine and 5% FCS at 37°C in a humidified atmosphere of 95% air and 5% CO2. Cultures initiated at a density of 5 x 10^5 cells/ml grew exponentially to about 1 x 10^6 cells/ml in 3 days. In all the experiments described here 75-cm^2 tissue culture flasks were inoculated with 30 ml of cell suspension containing 1-3 x 10^5 cells/ml. All treatments were carried out on exponentially growing cells. Cell viability was assessed by trypan blue exclusion.

Cell Cloning Assay. The cytotoxicity of MTX was assessed by colony-forming activity. Cloning was performed with a two-layer agar system in 60-mm plastic tissue culture dishes (Corning, USA); a solid feeder layer (5 ml) containing 0.5% (w/v) Bacto agar, 20% FCS, and RPMI 1640 medium was overlaid with a semisolid layer (1.5 ml) containing 7,500 cells, 0.3% (w/v) Bacto agar, 20% FCS, and RPMI 1640 medium. Before plating, all cells were washed twice in warm RPMI-free RPMI 1640 medium to ensure drug removal. The dishes were then incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2 for 7 days. Colonies containing 50 or more cells were counted. The plating efficiency of untreated L1210 cells was more than 95%. Colony-forming activity was expressed as the percentage of plating efficiency of untreated cells.

Treatment. BrdUrd was obtained from Sigma Chemical Co., St. Louis, MO. MTX was supplied by Lederle Japan Co. Stock solutions of BrdUrd and MTX were prepared at 20 times the final concentration in RPMI 1640 medium. Two labeling schedules were used for the analysis of the cytokinetic response to MTX: (a) Cells were incubated for 20 min in medium containing 10 μM BrdUrd. The labeled cells were washed twice with warm complete medium and recultured in BrdUrd-free medium supplemented with MTX for designated intervals. MTX-treated cells were washed twice and fixed in 70% cold ethanol. The duration of the cell cycle (T) and of the S-phase (T_s) was determined in labeled cells incubated in MTX-free medium. (b) Cells were continuously incubated in MTX-containing medium and harvested after designated intervals; BrdUrd was added to the culture 20 min before harvest at a final concentration of 10 μM. The labeled cells were washed and fixed as described above. Fixed cells were stored at 4°C until use.

Staining. A modification of the method described by Dolbeare et al. (10) was used. Ethanol-fixed cells were rinsed with cold PBS and incubated in 4 N HCl for 20 min. Cells were washed twice with wash-buffer (pH 7.2, PBS containing 0.5% Tween 20), suspended in 20 μl of a monoclonal anti-BrdUrd antibody preparation (Becton Dickinson Co.) diluted with 80 μl of dilute-buffer (pH 7.2, PBS containing 0.5% Tween 20 and 0.5% bovine serum albumin) and incubated for 1 h. After two washings with wash-buffer, cells were exposed to 100 μg fluorescein isothiocyanate-conjugated goat antimouse IgG antibody (Cappel Lab.) diluted 1:200 in dilute-buffer for 30 min. After one rinsing in wash-buffer, cells stained with fluorescein isothiocyanate were resuspended in PBS containing 0.1% RNase (Sigma Chemical Co.). The staining of DNA was performed in 50 μg/ml of propidium iodide (Calbiochem-Berinko Co.). Clumps of cells were removed by passing the specimens through 43-μm nylon mesh and subjected to analysis by flow cytometry. Control cells without BrdUrd labeling were prepared by the same procedure.

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3 The abbreviations used are: MTX, methotrexate; dThd, thymidine; BrdUrd, bromodeoxyuridine.
Flow Cytometry. Fluorescence-stained cells were analyzed on a flow cytometer FACS IV (Becton Dickinson Co.). Cells were excited at 488 nm with an argon laser. Red fluorescence from propidium iodide was collected through a 590-nm-long pass filter and recorded as a measure of total DNA content. Green fluorescence from fluorescein was collected through a 530-nm band pass filter and recorded as a measure of the amount of incorporated BrdUrd. The green fluorescence was also recorded after logarithmic amplification for the three-dimensional display. Routinely, 30,000 cells were measured for each distribution at a flow rate of less than 300 cells/s. All data was stored on a floppy disc. A univariate 256-channel distribution and a bivariate 64 × 64 channel distribution showing the distribution of DNA on the X axis and BrdUrd on the Y axis were generated by software supplied by Fujisawa Pharmaceutica Co., Osaka, Japan. One half reduction of green fluorescence intensity in logarithmic amplification corresponded with four channels on the bivariate 64 × 64 channel distribution. Four windows were set on the bivariate BrdUrd/DNA distribution to analysis cell cycle progression: (a) G1, cells in the G1 phase; (b) G2M, cells in the G2M phase; (c) L, BrdUrd-labeled cells with a complete range of DNA; (d) LG1, BrdUrd-labeled cells with G1 DNA content in the region L (Fig. 1). The values for the cell cycle phases were shown as the ratio of the number of cells being in each window to the total number of cells in G1, G2M, and L regions. For comparisons of the rate of BrdUrd incorporation after treatment with different doses of MTX, the BrdUrd content of S-phase cells was quantified on the basis of the average channel number (fluorescence intensity). The average channel number was calculated by the integral value of the distribution divided by the number of labeled cells, and these values were plotted against the corresponding DNA contents.

RESULTS

Throughout the experiments described in this paper we shall arbitrarily define S phase from the biochemical point of view: the cells which incorporate DNA precursors (BrdUrd) and contain more DNA than G1 cells are considered to be S-phase cells. According to this definition cells in the LG1 region on the bivariate BrdUrd/DNA distribution correspond to cells at the G1+S boundary by conventional interpretation.

Cell kinetic parameters were determined by the procedure described by Sasaki et al. (12). Tc and T are calculated from the fraction of labeled cells in mid S-phase curve were 11.5 h and 6.3 h, respectively.

Cytotoxicity. Fig. 2 shows the effects of MTX on clonal replication of L1210 cells determined by colony assay. The clonogenicity was dose- and time-dependently inhibited at a range of MTX concentrations (10^-8-10^-4 M). The cytotoxicity of 10^-4 M MTX was slight: clonogenicity was 92.3% of untreated control cells after incubation for 24 h. 10^-7 M MTX caused a rapid fall in clonogenicity as the incubation time increased. At 10^-6 M or much higher concentrations (10^-5 and 10^-4 M), the clonogenicity after incubation for 12-24 h was strikingly reduced to less than 1% of the control value. Therefore, in all the experiments described here, 10^-8 M was used as the low dose and 10^-7 and 10^-6 M as high doses.

Cell Cycle Progression. During the first hour of incubation with MTX no significant change was observed at any concentration of MTX (10^-8-10^-4 M).

Considerable changes of the distribution of cellular DNA content were caused by 10^-8 M cells accumulated at the G1-S boundary at 12 h (Fig. 3B) and then from 12 to 24 h progressed slowly through the S phase (Fig. 3, C and D). During that period, there were alterations in the distributions of the BrdUrd-labeled cells which suggest a perturbation in the rate of BrdUrd incorporation of the S-phase cells (Fig. 3, B-D). S-Phase delay is also suggested by a gradual increase in the number of the labeled cells and decrease of G2M cells between 12 and 24 h (Fig. 4).

The effect of 10^-7 M MTX on cell cycle progression was marked in the bivariate BrdUrd/DNA distribution. The number of labeled cells (69.5% at the start of treatment) began to increase after incubation for 2 h, and by 6 h it had reached 91.5%; this rise was attributable exclusively to the increase of G1 population in the LG1 region (50.1% at 6 h), while at that period G1 and G2M populations were decreased to 7.2 and 1.3%, respectively (Figs. 3E and 4). This alteration is reflected as an increase of the G1 phase and a decrease of the G2M phase in the DNA histogram (data not shown). From 12 to 24 h no additional change was found in the DNA histograms, while in the bivariate distributions the labeled cell population except the LG1 region began to decrease and at 24 h there were almost none left (Fig. 3, F-G).

The effects of 10^-6 M MTX on the distribution of DNA content were very similar to those of 10^-7 M MTX (Fig. 3, H-J). However, in the bivariate distributions, the rise of the labeled cell population and the fall of G1 and G2M populations were more rapid than with 10^-7 M MTX. The percentages of cells in L, LG1, G1, and G2M at 6 h were 96.8, 68.7, 2.8 and 0.4%, respectively (Figs. 3H and 4). From 12 to 24 h there was also a marked decrease in the number of labeled cells; following the decrease of the population of labeled cells with DNA content of the S phase, the LG1 population began to decrease, and at
Fig. 3. Effects of MTX with time of exposure on bivariate BrdUrd/DNA distribution of L1210 cells. Green fluorescence emission (corresponding to relative BrdUrd content) is shown after logarithmic amplification. A, untreated cells; B–D, 10^{-8} M MTX; E–G, 10^{-7} M MTX; H–J, 10^{-6} M MTX. Time intervals of MTX treatment are shown. The bivariate distributions are representative examples of three individual experiments.

24 h very few labeled cells remained (Fig. 3, I–J). The decrease of the LG1 population between 6 and 24 h might conceivably be due to inhibition of the transition of cells from the G1 to the LG1 region. However, such a block is very unlikely, because during this period there were very few cells in G2M, and the mitotic indices were in the range of 0–0.3%.

The effects of 10^{-6} M MTX on cell cycle progression were also studied by the pulse-chase technique. In the pulse-chase experiments described here, pulse labeling with 10 \mu M of BrdUrd had no discernible effect on either the growth of L1210 cells or the cytotoxicity of 10^{-6} M MTX (data not shown). In the untreated control culture, 6 h after pulse labeling, cells which were in late S during the labeling period had divided and moved into G1, and a fraction of the cells that were in G1 during the labeling period had entered S (Fig. 5A). This chart also shows the effect of mitosis; the BrdUrd content of G1-phase cells is about half that of G2M-phase cells. 12 h after labeling, the distribution resembled that immediately after the labeling period, except that the BrdUrd content of labeled cells had halved (data not shown). In contrast with the control cells, in MTX-treated cultures the cells which were in G1 at the start of MTX treatment showed no progression across the S phase throughout the incubation period of 12 h (Fig. 5, B and C). The progression of cells in the S phase at that time differed between the early and mid S-phase cells and the late S-phase cells, the early and mid S-phase cells showing no progression,
Fig. 5. Bivariate BrdUrd/DNA distribution of L1210 cells prelabeled with BrdUrd. The labeled cells were grown continuously in MTX-free medium (A) or incubated with $10^{-4}$ M MTX (B and C) for 12 h. Time intervals after labeling are shown in the chart. A representative of three individual experiments is shown two- and three-dimensionally. Green fluorescence for relative BrdUrd content is expressed after logarithmic amplification.
while the late S-phase cells continued to divide and move into G\(_1\) during the first 6 h (Fig. 5B). After incubation for 12 h, progression through all phases seemed to stop (Fig. 5C).

These results with 10\(^{-7}\) and 10\(^{-6}\) M MTX show that high dose MTX inhibits the progression of cells through the S phase and the transition of cells from G\(_1\) to S. However, the progression of cells from late S or G\(_{2M}\) to G\(_1\) was not impaired. The cells which entered G\(_1\) were able to traverse the G\(_1\) phase and progress to the G\(_1\)/S boundary where they could incorporate BrdUrd but not initiate DNA synthesis.

DNA Synthesis. The effect of MTX on DNA synthesis in L1210 cells was studied by plotting the average rate of BrdUrd incorporation across the S phase. Fig. 6 shows the plotted data from untreated and treated L1210 cells. Little difference was observed in the shape of the curves for untreated cells in five different experiments, demonstrating the reproducibility of the method. The rate of incorporation accelerates in the early S phase, peaks in mid S, and decreases in late S. The differences in the effect of treatment with MTX at three different concentrations (10\(^{-8}\), 10\(^{-7}\), and 10\(^{-6}\) M) are apparent after incubation for 6 h: 10\(^{-7}\) and 10\(^{-6}\) M MTX caused alterations in the incorporation rate across the S phase but 10\(^{-8}\) M MTX did not (Fig. 6A). The incorporation rate was apparently lower in cells incubated for 12 h than in those incubated for 6 h, including cells incubated with 10\(^{-8}\) M MTX (Fig. 6B). These results suggest that the inhibition by MTX of DNA synthesis in L1210 cells varies with the dose of and duration of exposure to MTX.

DISCUSSION

The results of this study show that 10\(^{-8}\) M MTX, which has little effect on the clonogenicity of L1210 cells, delays progression of L1210 cells through the S phase and that much higher concentrations (10\(^{-7}\) and 10\(^{-6}\) M) of MTX, which strikingly reduce the clonogenicity of the cells, inhibit the progression at the G\(_1\)/S boundary and across the S phase, but not the other phases.

The results with 10\(^{-8}\) M MTX confirmed the S-phase delaying effect of low dose MTX reported by several investigators (13–15). Although the S phase arresting effect of high dose MTX has been mentioned in many other studies, no direct evidence for it has been presented so far (13–17). The results with the pulse-chase experiments of L1210 cells confirmed this effect.

There are some apparent contradictions in the effect of MTX on G\(_1\)/S transition. Lampkin et al. (16) and Ernst and Kilman (17), who studied the in vivo cell kinetic effects of MTX on human leukemia cells, concluded that MTX did not prevent the entry of cells from G\(_1\) to S. It is likely that they observed the effect of low dose MTX, because they used relatively low dose (1–2 mg/kg) of MTX. The [\(^{3}\)H]dThd-labeled cells in their experiments correspond with the BrdUrd-labeled cells in our experiments, so that our results also indicate that a rapid rise of [\(^{3}\)H]dThd-labeling index after injection of MTX is not confirmative evidence of the absence of G\(_1\)-S block.

Several in vivo and in vitro studies have suggested that MTX inhibits cell cycle progression at the G\(_1\)-S boundary. Their conclusions were based mainly on the following two findings: (a) a decrease in the percentage of cells labeled with [\(^{3}\)H]dThd after MTX treatment for 12–24 h (3, 4); (b) an accumulation of cells in G\(_1\) or at the G\(_1\)-S boundary on univariate DNA distribution measured by FCM in MTX-treated cells (4–6). The first finding is consistent with a decrease of the BrdUrd-labeled population during treatment with high dose MTX in our study. The interpretation of the DNA histograms illustrating cell accumulation near the G\(_1\)-S boundary varies among investigators, because of the inability to discriminate precisely the early S-phase cells from G\(_1\) cells with presently available analytical techniques. Accordingly, our results leave the possibility that some cells in the LG\(_1\) region are arrested in the early S phase. The effect of MTX, in the bivariate BrdUrd/DNA distribution, on the progression of cells at the G\(_1\)-S boundary is apparently different from that of cytosine arabinoside, a representative drug which allows G\(_1\) to traverse but prevents the G\(_1\)-S transition (18, 19), because this agent prevents BrdUrd incorporation from all S-phase cells, including cells located at the G\(_1\)-S boundary within a few hours of treatment; no BrdUrd-labeled cells are found in the bivariate BrdUrd/DNA distribution (11, 20).

Partial synchronization has also been reported as a cytokinetic effect of MTX. Lampkin et al. (16) observed synchronization in S in human marrow cells after a single injection of MTX, while Wheeler et al. (3) suggested that the rapid increase of the thymidine labeling index after incubation with MTX was due to a synchronization of H.Ep.No2 cells at the G\(_1\)-S transition stage. Our results show that the difference in the cell cycle phase of the synchronization may depend on the dose of MTX.

There is much evidence that the S-phase cells are the target of MTX (2, 17, 21), while some investigators have reported that MTX kills not only S-phase cells but also G\(_1\)-phase cells of L1210 ascites tumor (22). In our study of the cytotoxicity of MTX, the proportion of cells which lost their clonogenicity by 6-h treatment with 10\(^{-6}\) M MTX exceeded the percentage of S-phase cells at the beginning of treatment. This finding is probably explained by the loss of replicating activity of cells arrested at the G\(_1\)-S boundary and in the S phase. As many of the cells arrested at the G\(_1\)-S boundary were being in the G\(_1\) or G\(_{2M}\) phase during treatment with MTX, we infer that not only S-phase cells but non-S-phase cells are susceptible to cytotoxicity of MTX.

The biochemistry of the effect of MTX on the cell cycle has been studied extensively in various cell lines (1). Taylor et al. concluded, from the results of their studies on CCRF-CEM cells, that the major cytotoxic mechanism of MTX was inhibition of DNA synthesis resulting from deprivation of thymidilate and/or purines (13, 14). Bokkerink et al., in their recent studies with MOLT-4 cells, suggest that there is a close dose- and time-dependent correlation of the effects of MTX on cytotoxicity,
cell cycle perturbation and DNA synthesis (15). The present findings show that MTX-induced S-phase arrest is followed by a marked alteration in the distribution of BrdUrd-labeled cells, and that the rate of BrdUrd incorporation decreases as the drug dose and treatment period increase. These results suggest that the inhibitory effect of MTX on cell cycle progression through the S phase is closely associated with inhibition of DNA synthesis.

Cytokinetic concepts have not had as much of an impact on clinical cancer chemotherapy as originally hoped. One of the important reasons is that the experimental techniques used in cytokinetic studies often yield inaccurate or insufficient information (23). The present results show that cell synchronization near the G1-S boundary is not always precisely reflected in the level of [3H]dThd uptake and/or the DNA histograms and suggest that MTX prevents the G1-S transition, but is not self-limiting in cell killing action.

These cytokinetic findings will help in the evaluation of some synergistic effects of MTX and other cycle specific agents, for example combination of MTX and cytosine arabinoside (24), and may therefore be clinically important.

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