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Synchronization of cancer cell lines with methotrexate in vitro

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Abstract. Detailed procedure are described for inducing synchrony in cancer cell lines of human origin (Jurkat, K562, U937, SW626) and L1210 cell line of murine origin by using very low non-toxic concentrations (0.04–0.08 μM for 13–24 hours) of methotrexate under standard culture conditions. This protocol offers a method for synchronization of cells at the G_1/S boundary and through the S-G_2-M phases of the cell cycle. Kinetic behavior and biological properties of the synchronized cells are considered for characterisation of the system. Comparisons are made with MTX synchronization and that induced by aphidicolin alone or by a combination of serum deprivation and aphidicolin. Methotrexate appears to be the best choice for obtaining highly synchronous human cancer cell population without cytotoxicity or detectable physiological perturbations.

Key words: Cancer cell lines, Cell synchronization, Flow cytometry, MTX

1. Introduction

Synchronization remains an unique strategy to study the biological and biochemical events occurring at specific points of cell cycle progression. Perfect synchronization at a specific point of cell cycle is virtually impossible to achieve without affecting the metabolic processes of the cells. Many methods have been used so far but none of these methods appear completely satisfactory either because of the proportion of synchronised cell is not sufficiently high or because the cell manipulations perturb the cell physiology to an extent not acceptable for biochemical work. Most of the methods are not applicable to vivo. Thus, better methods need to be developed. Prolonged exposure to low doses of methotrexate (MTX) has been used by cytogeneticists for high resolution banding analysis. Recently we have successfully characterised the MTX synchronization system [1]. In the present paper we describe the synchronization of four cancer cells of human origin and one of animal origin in culture using very low non-toxic doses of MTX. The aim was to achieve satisfactory synchrony to study S phase cells. The kinetic behaviors and biological properties of the synchronised cells during reversal of the process are also considered.

2. Materials and methods

A. Chemicals and reagents
1. Methotrexate (MTX).[1]
2. RPMI 1640 medium, Cat. No. 22511-018.[2]
3. Foetal bovine serum (FBS), Cat. No. 40G3157F.[2]
4. L-glutamine 200 mM, Cat. No. 25030-024.[2]
5. Penicillin 5000 U-streptomycin 5000 μg/ml solution, Cat. No. 043-05070H.[2]
6. Phosphate buffered saline (PBS), Cat. No. 041-04040M.[2]
7. Trypsin solution 2.5%, Cat. No. 25090-028.[2]
8. Ethylenediamine-tetraacetic acid (EDTA), Cat. No. ED2SS.[3]
9. 2-Mercapto-ethanol, Cat. No. 63690.[4]
10. 5-bromo-2′-deoxyuridine (bromodeoxyuridine, BrdUrd), Cat. No. B-5002.[3]
11. Propidium iodide (PI), Cat. No. 537059.[5]
12. RNAse A, bovine pancreas, protease-free, DNAse I-free, highly purified Cat. No. 556746.[5]
13. Nonidet P40, Cat. No. 6507.[3]
14. Normal goat serum, Cat. No. X907.[6]
15. Sodium tetraborate, Cat. No. 478817.[7]
16. Anti-BrdUrd pure monoclonal antibody clone B44 (Mab), Cat. No. 347580.[8]
17. Tec-Gam Ig F (AB′)2-FITC conjugated Mab, Cat. No. TEC-3901-2.[9]
18. Tween 20, Cat. No. P 2690.[3]

B. Equipment and supplies
1. Round bottom cryogenic vial 2 ml, Cat. No. 4812 Falcon.[8]
2. Flasks for cell culture, Corning 25 cm,[2] Cat. No. 25100.[10]
3. Flasks for cell culture, Corning 75 cm,[2] Cat. No. 25110.[10]
4. Polystyrene conical tube 15 ml, Cat. No. 2095 Falcon.[8]
5. Polypropilene conical tube 15 ml, Cat. No. 2097 Falcon.[8]
6. Pipettes of 2, 5, 10 and 25 ml sterile pyrogen-free, Falcon.[8]

7. CO_2-auto-zero incubator.[8]
8. Varifuge 3.2S centrifuge.[11]
9. Microscope fluorescence BX60, Olympus.[12]
10. Inverted microscope for contrast phase, Nikon TMS.[13]
11. Orbital shaker.[14]
12. Vortex.[15]
13. Cell counter coulter model ZM equipped with coulter channelyzer 256.[16]
14. Immersion thermostatic circulator GTR 90.[17]
15. Stripettor with sterile filter, Cat. No. 4910.[10]
16. Micropipetter, P-20, P-200, P-1000.[10]
17. Cytofluorograph Facstar Plus.[8]

C. Preparation of culture medium

1. Jurkat, K562, U937 cell lines.
   To 100 ml of 1× RPMI 1640 add 1 ml of L-glutamine 200 mM and 2.8 ml of 7% sodium bicarbonate. The pH of the medium is adjusted to 7.4 by adding a few drops of 0.3 N HCl. Then add 10 ml heat-inactivated FBS.
2. L1210 cell line
   To 100 ml of 1× RPMI 1640 add 1 ml of L-glutamine 200 mM, 2.8 ml of 7% sodium bicarbonate and 10 μM 2-mercapto-ethanol. The pH of the medium is adjusted to 7.4 by adding a few drops of 0.3 N HCl. Then add 10 ml heat-inactivated FBS.
3. SW 626 cell line
   To 100 ml of 1× RPMI 1640 add 1 ml of L-glutamine 200 mM and 2.8 ml of 7% sodium bicarbonate. The pH of the medium is adjusted to 7.4 by adding a few drops of 0.3 N HCl. Then add 10 ml of FBS.
   All prepared medium were stored in sterile bottles at 4 °C and aliquoted asceptically as required.

D. Preparation of solutions

1. MTX for synchronization:
   Prepare fresh solution prior to use every time.
   Use 200 μl of 1 N NaOH to solubilize MTX.
   Dilution is done in complete culture medium with FBS.
2. Trypsin-EDTA solution:
   Dissolve 20 mg of EDTA in 100 ml 1× PBS and sterilize in autoclave at 120 °C for 15 min.
   Working solution: dilute 1 ml of 2.5% trypsin in 10 ml of EDTA solution.
3. Heat inactivation of FBS:
   Thaw serum, bring to room temperature.
   Incubate at 56 °C for 30 min in a pre-warmed bath. Store at 4 °C without freezing.
4. GM Saline solution for fixing cells:
   Prepare 1× GM saline solution by dissolving:
   1.1 g. glucose,
   8 g NaCl,
   0.4 g KCl,
   0.4 g $Na_2HPO_4.12\ H_2O$,
   0.15 g. KH_2PO_4
   0.2 g. EDTA
   Make up volume to 1.0 liter with sterile water.
   Sterilize the solution by filtration through sterile 0.02 μm filter and keep at 4 °C.
5. RNAse solution:
   Prepare 1 mg/ml solution in distilled water.
   Boil for 10 min to denature DNA.
   Bring to room temperature.
   Sterilize the solution by filtration through sterile 0.02 μm filter and keep at –20 °C for up to 4 months.
6. BrdUrd solution:
   Prepare 1 mg/ml solution in distilled water.
   Sterilize the solution by filtration through sterile 0.02 μm filter and keep at 4 °C in the dark for up to 6 months.

E. Cells and culture conditions

1. Revival cells from liquid nitrogen:
   The condition to preserve under liquid nitrogen and to thaw the cells are the same for all the cells used in this paper. We describe here the procedure to start a culture from frozen cells.
   Cells are stored in liquid nitrogen at the concentration of 3–4 cells/ml/vials in RPMI 1640 medium with 10% FBS and 10% DMSO.
   To culture cells from frozen stock, thaw a vial directly in a 37 °C water bath.
   Rapid thawing of cell suspension is essential for optimal recovery.
   The cells are transferred to a 15 ml polystyrene conical tube.
   Add drop by drop 10 ml of complete culture medium.
   Centrifuge at 1000 rpm for 10 min to remove the DMSO.
   Resuspend the pellet with 10 ml of culture medium and transfer the cells to a 25 cm^2 tissue culture flask at 37 °C in a 5% CO_2 incubator.
   Twenty-four hours later the culture medium is replaced with a new culture medium.
2. Propagation and culture of cell lines:
   a) Propagation of cells growing in suspension:
      - Mix cell suspension and disperse any cell aggregates by gentle pipetting.
      - Dilute 1 ml of cell suspension in 10 ml PBS and count the cells using a electronic particle counter.
      - Dilute to the appropriate seeding concentration by adding the appropriate volume of cells to a premeasured volume of medium in a culture flask.
      - Cap the flask and return to the incubator.
   b) Propagation of cells growing in attachment:
      - Discard medium.

- Add warm PBS to the side of the flask opposite the cells, to avoid dislodging cells.
- Put the flask on a orbital shaker with gentle agitation at 37 °C for 10–15 min.
- Remove PBS.
- Add 1 ml of prewarmed trysin-EDTA solution.
- Put the flask on a orbital shaker with gentle agitation at 37 °C till the monolayer starts to detach from the plastic surface.
- Stop the enzyme activity by adding 10 ml of complete (with serum) culture medium and disperse the cells by repeated pipetting over the plastic surface.
- Centrifuge the cell suspension for 10 min at 1000 rpm.
- Discard the supernatant, resuspend the cells in 10 ml of complete medium and count the cells by electronic particle counter.
- Dilute to the appropriate seeding concentration by adding the appropriate volume of cells to a premeasured volume of medium in a culture flask.
- Cap the flask and return to the incubator.
- Cell line are routinely cultured in a humidified incubator at 37 °C with 5% CO_2. Detailed culture conditions, seeding density and confluent density are reported below:

(1) Jurkat cell line:
Jurkat cell line, acute T cell human leukemia, is grown in suspension [2]. Exponentially growing Jurkat cells are resuspended in 10 ml of culture medium at a concentration of 5×10^4 cells/ml in a 25 cm^2 tissue culture flask. As shown in Figure 1 under these conditions the doubling time of Jurkat cells is 24 ± 3 hours and reach a confluent density of 1.5×10^6 cells/ml between 96 and 120 hours after seeding.

(2) K562 cell line:
K562 cell line, a chronic myelogenous human leukemia, is grown in suspension [3]. Exponentially growing K562 cells are resuspended in 10 ml of culture medium at the concentration of 1×10^5 cells/ml in a 25 cm^2 tissue culture flask. The doubling time of the K562 cells is 22.5 ± 3 hours and reach a confluent density of 1.5×10^6 cells/ml between 72 and 96 hours after seeding, as shown in Figure 1.

(3) U937 cell line:
U937 cell line, a histiocytic human lymphoma, is grown in suspension [4]. Exponentially growing U937 cells are resuspended in 10 ml of culture medium at the concentration of 8×10^4 cells/ml in a 25 cm^2 tissue culture flask. The doubling time of the U937 cells is 25.1 ± 3 hours and reach a confluent density of 1.7×10^6 cells/ml between 96

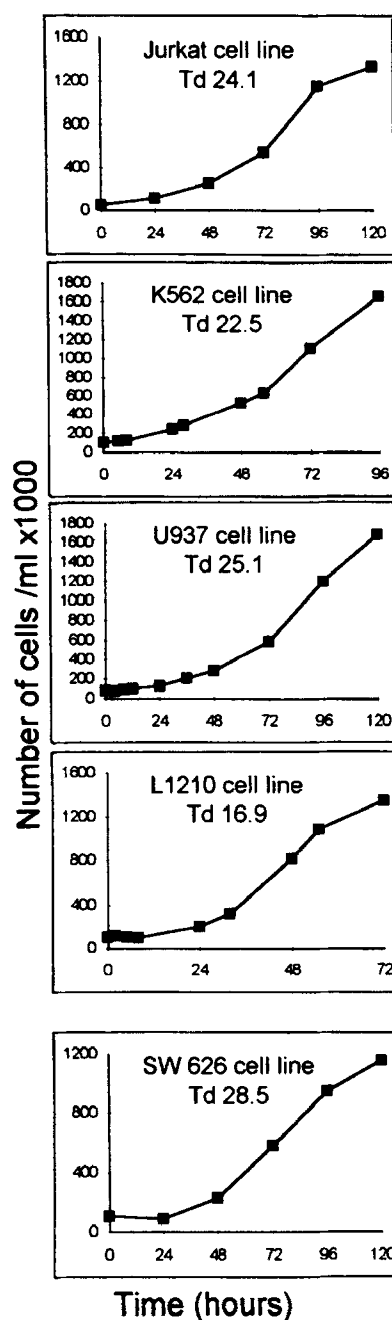

Figure 1. Growth curve of the cell lines used for MTX synchronization. The doubling time (Td) of each cell line is also indicated.

and 120 hours after seeding, as shown in Figure 1.

(4) L1210 cell line:
L1210 cell line, a mouse lymphoid leukemia, is grown in suspension [5]. L1210 are resuspended in 10 ml of culture medium at the concentration of 1×10^5 cell/ml in a 25 cm^2 tissue culture flask. 16.9 ± 4 hours is the doubling time of the L1210 cells seeded in this condition and reach a confluent density of 1.5×10^6 cells/ml 72 hours after seeding (Figure 1).

(5) SW 626 cell line:
SW 626 cell line, a human ovarian adenocarcinoma, is grown as monolayer [6]. To grow, exponentially growing cells are detached by trypsinization procedure described, counted and plated in 5 ml of culture medium at the concentration of 5×10^4 cell/ml in a 25 cm^2 tissue culture flask. The doubling time is approximately 28 ± 4 hours and they reach a confluent density of 1.2×10^6 cells/ml 120 hour after seeding, as shown in Figure 1.

(6) Synchronization with MTX
Exponentially growing cell populations are exposed to MTX at concentrations of 0.02 to 0.08 μM for periods of 16–24 hours. The optimum dose and period of incubation have been found to be:
- 0.04 μM for 24 h for Jurkat cell line
- 0.02 μM for 22 h for K562 cell line
- 0.04 μM for 16 h for U937 cell line
- 0.02 μM for 16 h for L1210 cell line
- 0.08 μM for 24 h for SW626 cell line.

The doses indicated above have been chosen based on the maximum effect on synchronization without detectable cytotoxicity.
Protocol:
- Seed the cells at the seeding concentration reported above for culture of each cell line.
- After 48 hours of seeding, incubate the cells with specific concentration of MTX, as reported.
- MTX is added to the culture medium to start the incubation.
- After lapse of indicated time of incubation, MTX is removed from the medium.
- For cells growing in suspension:
  (1) Remove the medium from the flasks and centrifuge in 15 ml polystyrene conical tube at 1200 rpm for 5 min.
  (2) Remove the supernatant, add 10 ml of warm PBS (37 °C) and centrifuge at 1200 rpm for 5 min.
  (3) Remove the PBS and add 10 ml of warm complete culture medium.

  Resuspend cells and return to new flasks. Cap the flask and return to the incubator.
- For cells growing in adhesion:
  (1) Remove the medium from the flasks
  (2) Wash the cells twice with 5 ml of warm PBS (37 °C) added to the side of the flasks opposite the cells to avoid dislodging cells.
  (3) Remove PBS and add 5 ml of warm complete medium. Cap the flask and return to the incubator.

To perform good synchronization, it is extremely important to work with solutions pre-warmed to 37 °C to avoid perturbations of cell physiology caused by stress due to cold PBS or cold medium. Another important point is related to the ability to remove completely traces of remaining MTX and to provide the cells with normal culture medium in a relatively short time. The temperature of the solutions and the time of recovery are two important steps for obtaining a satisfactory synchronization.
At different intervals, synchrony is checked by flow cytometry methods using monoparametric DNA and biparametric BrdUrd/DNA analysis.

(7) BrdUrd incorporation
Protocol A:
This protocol allows to study and compare the DNA synthesis rate and labeling index (percentage of cells in synthetic phase that are labeled with BrdUrd) at different

time of incubation and recovery in MTX-free medium. Comparison can be made with control and synchronized population as well as at different time during the synchronization

During the last 20 min of MTX incubation and during the last 20 min of different recovery time from MTX incubation, 20 μM or 30 μM of BrdUrd are added for leukemic or human ovarian cancer cells respectively. After BrdUrd incorporation the cells are washed with PBS and directly fixed in 70% ethanol and kept at 4 °C up to BrdUrd/DNA staining [7].

Protocol B:

This protocol allows to selectively label the S phase cells and to follow the movement of these cells through the cell cycle. When compared with the exponentially growing control cell population, the relative time taken by the synchronized population to cross the S phase can be worked out. Furthermore, it is possible to follow the fate of the BrdUrd positive and negative cell populations in the cell cycle (for details, see references [7, 8]).

Exponentially growing cells are treated with BrdUrd during the last 20 min of the MTX incubation. At the end of MTX incubation and during different time of recovery, control and treated cells are fixed in 70% ethanol and kept at 4 °C up to BrdUrd/DNA staining.

Extremely important is the dose of BrdUrd used in vitro. It is known that BrdUrd may modify the cell cycle phase distributions of the cells even if the time of BrdUrd incubation is very short as mentioned in this paper. High dose of BrdUrd *per se* causes a wave of synchronization of cells in S phase of the cell cycle and decreases the growth rate. As mentioned before, it is important to work with solutions pre-warmed to 37 °C to avoid perturbations of cell physiology during the recovery operations caused by stress due to cold PBS or medium. Another important point is that BrdUrd must be removed completely to avoid unwanted incorporation of BrdUrd by the present G_1 or G_2M phase cells when they will pass through their S phase in presence of traces of BrdUrd in future.

(8) Flow cytometry for cell cycle analysis

(a) Monoparametric cell cycle analysis:

Monoparametric DNA analysis using flow cytometry is performed with propidium iodide (PI) to evaluate the cell cycle phase distributions of the control and synchronized cell populations.

Protocol:

At the end of MTX incubation and 2, 4, 6, 8, 10, 12 and 24 hours after MTX-washout the cells are stained with the DNA specific stain, PI, or can be fixed in 70% ethanol solution for future processing with PI.

(b) Fixing cells for flow cytometry:

- Centrifuge the cells to remove culture medium at 1000 rpm for 10 min.
- The pellet of 5–10 × 10^6 cells is resuspended in 1 ml ice-cold GM saline solution using a vortex mixer.
- Add drop-by-drop 3 ml of 96% ethanol using a vortex mixer.
- The final cellular concentration should be 2–3 × 10^6 cells/ml.
- Keep the fixed cell on ice for 30 min and then store at 4 °C for at least overnight for later staining with PI.

(c) PI staining protocol for fixed cells:

- Centrifuge the fixed cells at 1200 rpm for 5 min to remove 70% ethanol.
- The pellet is washed with 5 ml PBS and centrifuged.
- Remove PBS.
- The pellet is stained with 2 ml of PI solution containing 50 μg/ml PI dissolved in 0.1% sodium citrate plus 25 μl RNAse 1mg/ml in water and 25 μl 0.1% Nonidet P40 in water.
- The time of PI staining is cell type dependent. Optimal staining is achieved in 30–60 min for leukemic cell lines

and in 120 min for human ovarian cancer cell line.

(d) Stain protocol for unfixed-(fresh) cells:
- Centrifuge the cells at 1200 rpm for 5 min to remove medium.
- The pellet is directly stained with PI using the same stain solution described for fixed cells.

(9) Biparametric BrdUrd/DNA analysis

(a) BrdUrd/DNA staining protocol:
- Centrifuge the fixed cells at 1200 rpm for 5 min to remove 70% ethanol.
- The pellet is washed with 5 ml PBS and centrifuged.
- Remove PBS.
- For detection of BrdUrd incorporation into DNA, double-stranded DNA is denaturated to allow the antiBrdU Mab to react with the BrdUrd incorporated in the DNA chain. To 3–4 × 10^6 cells add 1 ml of 2N HCl for 20 min for leukemic cells and 1 ml of 3N HCl for 30 min for human ovarian cancer cells at room temperature.

The denaturation step is critical for recognition of incorporated BrdUrd by the Mab in single standed DNA and for the stoichiometry of DNA staining with PI which requires double-stranded DNA.
- Stop the DNA denaturation by adding 4 ml (in excess) of 0.1 M sodium tetraborate, pH 8.5.
- Centrifuge at 1200 rpm for 5 min.
- The pellet is incubated with 1 ml of 0.5% Tween 20 in PBS and 1% normal goat serum for 15 min at room temperature.
- Centrifuge at 1200 rpm for 5 min.
- Incubate the pellet with 100 µl of anti-BrdUrd Mab diluted 1:10 in 0.5% Tween 20 in PBS for 1 hour at room temperature in the dark.
- Add 1 ml PBS and centrifuge at 1200 rpm for 5 min.
- The pellet is incubated with 1 ml of 0.5% Tween 20 in PBS and 1% normal goat serum for 15 min at room temperature.
- Incubate the pellet with 100 µl of FITC-conjugated goat anti-mouse IgG Mab diluted 1:50 in 0.5% Tween 20 in PBS for 1 hour at room temperature in the dark.
- Add 1 ml PBS and centrifuge at 1200 rpm for 5 min.
- Stain the cells with 2ml of a PI solution containing 2.5 µg/ml of PI in PBS and 25 µl RNAse 1 mg/ml overnight at 4 °C in the dark.
- The lower threshold of FITC fluorescence cut-off is determined based on analysis of a cell sample prepared without incorporation of BrdUrd in vitro.
- The specificity of the secondary FITC-conjugated Mab is determined on a cell sample prepared without the anti-BrdUrd Mab [7, 8].

(b) Instrument setting:

A FACStar Plus flow cytometry instrument using 488 nm excitation light was used to perform DNA cell cycle analysis on unsynchronized and synchronized cell populations. The fluorescence pulses were detected using a band pass filter 530 ± 30 nm and 620 ± 35 nm, for green and red fluorescence respectively, in combination with a dichroic mirror 570 nm. The coefficient of variation of the G_0/G_1 peak of the leukocytes used to set up the instrument was about 1.5–2.5% and must not be higher. The coefficient of variation of the G_1 peak of unsynchronized cells should not exceed 3–4%. For each sample, DNA analysis was performed on at least 10,000 cells and the percentage of the cell cycle phas distributions were calculated by the method of Krishan and Frei [9].

(c) Analysis of BrdUrd/DNA flow cytometry data: determination of synthetic phase time (TS)

Some theoretical considerations are necessary to calculate the TS of control and synchronized cells. We must assume that in the exponentially growing control population, at the time of BrdUrd incorporation the mean DNA content of the BrdUrd positive S phase cells is mid-way between G_1 and G_2 peaks and that the rate of cell progression through the S phase is constant. At this time the relative movement of the BrdUrd positive cells is assumed to be 0.5, that of the G_1 cells being 0 and that of the G_2M cells 1. The relative movement (RM) is calculated using the formula:

$$RM = (FS - FG_1)/(FG_2 - FG_1)$$

where F is the mean of PI red fluorescence of the corresponding phase of the cell cycle.

The TS is calculated from the mean DNA content of the BrdUrd-labeled cells. In fact, their position makes it possible to determine the rate at which these cells have progressed through the S to the G_2M phase in different time points after MTX washout. The TS is calculated as follows:

$$TS = 0.5/RM - 0.5/t$$

where t is the sampling time.

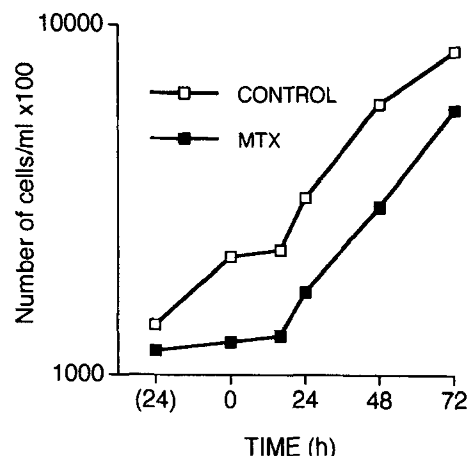

Figure 2. Time course of cell growth inhibition induced in SW626 incubated with 0.08 μM MTX for 24 hours evaluated during different time after MTX washout. (24): start of MTX incubation. 0: time of MTX washout. Cell number was counted at 24, 48 and 72 hours after MTX washout.

3. Results

A. Cell viability

Figure 2 shows the growth curve of control and MTX incubated SW 626 cells during the time of incubation and 24, 48 and 72 hours after MTX washout. MTX induced synchronization at the concentration of 0.08 μM caused only a reduction in the number of cells during the 24 hours of incubation, due to inhibition of DNA synthesis. No cytotoxic effect (as assessed by colony forming ability) was evident (data not shown, see [1]). When MTX was removed, the cells were able to recover in fresh culture medium and the synchronized cells were able to grow as control cells with the same doubling time. Absence of a lag period is a characteristic feature of this synchronization method.

Similar data have been obtained in all the other cell lines included in this paper regarding the non-toxic effect of MTX incubation.

B. Monoparametric cell cycle analysis

Figures 3, 4, 5, 6 and 7 show the degree of synchronization evaluated by monoparametric flow cytometric method at the end of MTX incubation and during different time after MTX washout on Jurkat, K562, U937, L1210 and SW 626 respectively. In the upper part of each figure we show the DNA histograms of the untreated and MTX treated cells and in the lower part the relative distribution in the different cell cycle phases.

In all cell lines, incubation with MTX caused accumulation of cells in the G_1/early S phase (SE). When MTX was removed and cells were allowed to grow in fresh medium, a wave of synchronization was evident.

In JURKAT cell line (Figure 3) just 2 hours after MTX washout very few cells were in the G_1 phase while majority of the cells were flowing through the S phase. At 6 hours, maximum accumulation of cells in S middle (SM) was evident. Between 6 and 10 hours of MTX washout, there were 70% cells in late S (SL) and G_2M phases compared to control cells. Between 24 and 48 hours the MTX synchronized cells resembled an exponentially growing control cell population.

In K562 cell line (Figure 4), 4 hours after MTX washout, a cohort of synchronized cells were in SE phase. Between 6 and 8 hours 40% of them were in SM. The cells became unsynchronized at 24 hours after MTX washout.

Figure 5 shows the flow cytometric analysis of control and MTX synchronized U937 cell line. After 2 hours of recovery in MTX-free medium, the majority of the cells (70%) were in G_1SE

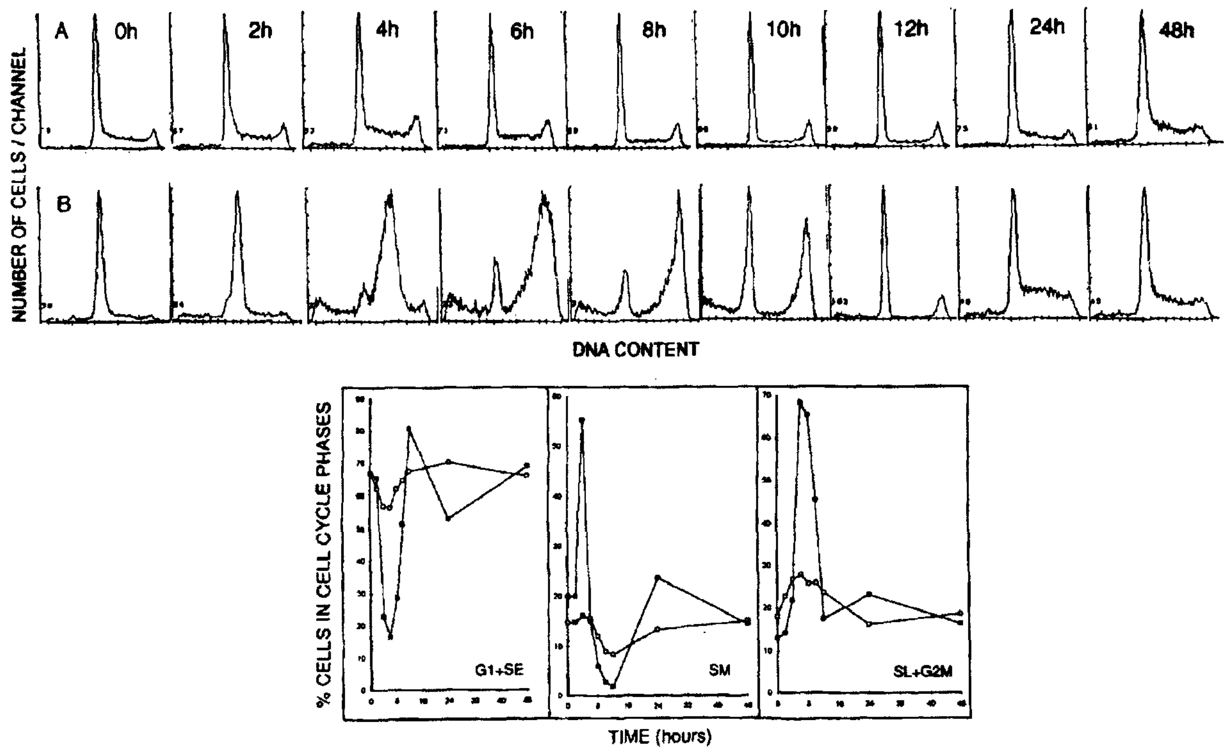

Figure 3. Cell cycle analysis of Jurkat cell line at various times of recovery after 24 hours of 0.04 μM MTX incubation. (A) control cells; (B) MTX synchronized cells. The percentage of control and MTX synchronized cells at various time of recovery after MTX incubation are also reported. □ : control cells; ■ : MTX synchronized cells.

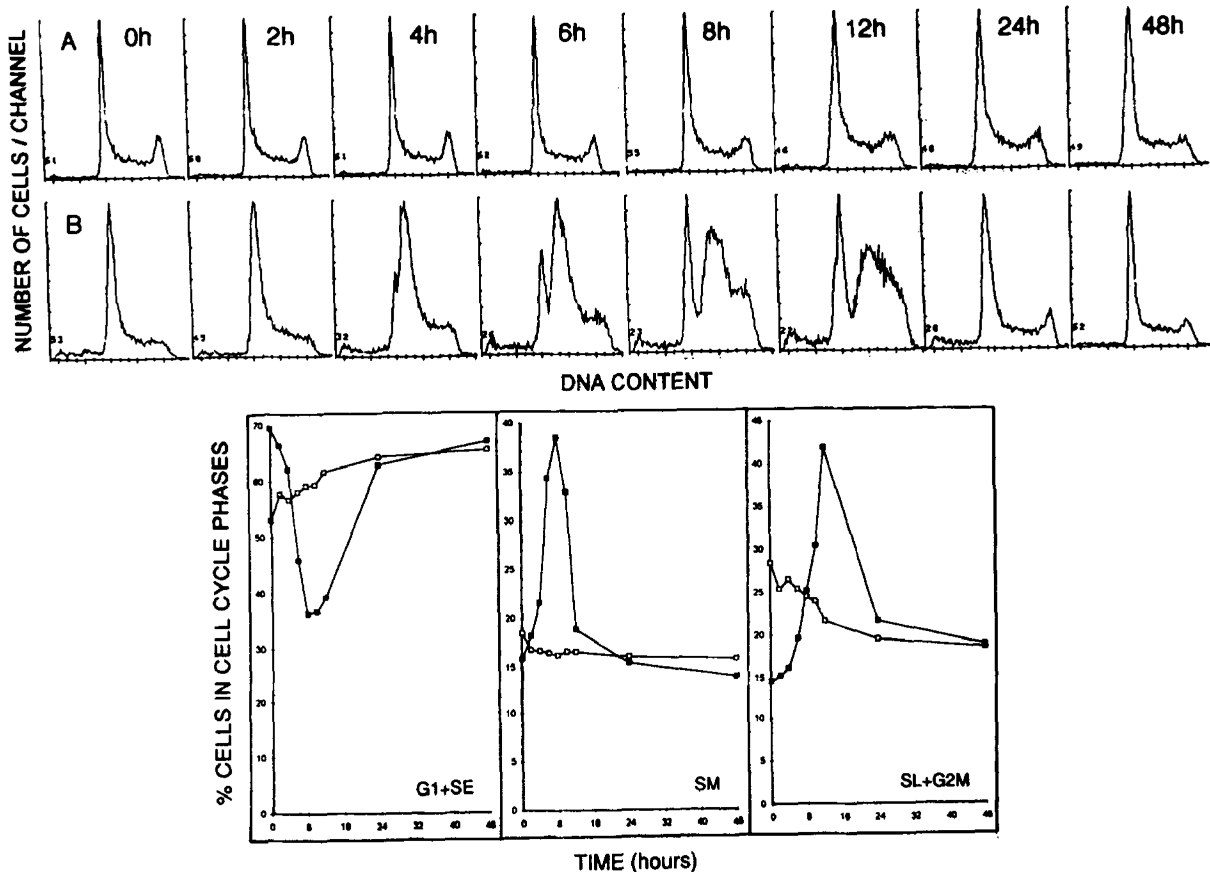

Figure 4. Cell cycle analysis of K562 cell line at various times of recovery after 22 hours of 0.02 μM MTX incubation. (A) control cells; (B) MTX synchronized cells. The percentage of control and MTX synchronized cells at various time of recovery after MTX incubation are also reported. □ : control cells; ■ : MTX synchronized cells.

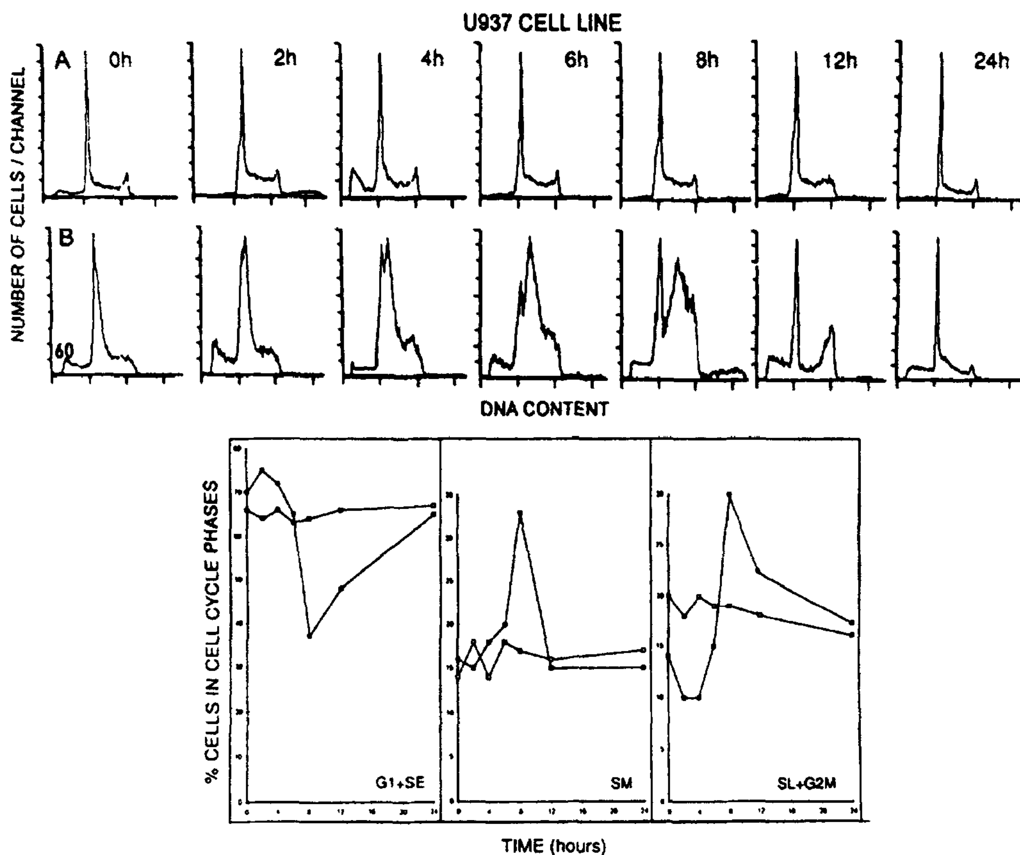

Figure 5. Cell cycle analysis of U937 cell line at various times of recovery after 16 hours of 0.04 μM MTX incubation. (A) control cells; (B) MTX synchronized cells. The percentage of control and MTX synchronized cells at various time of recovery after MTX incubation are also reported. □ : control cells; ■ : MTX synchronized cells.

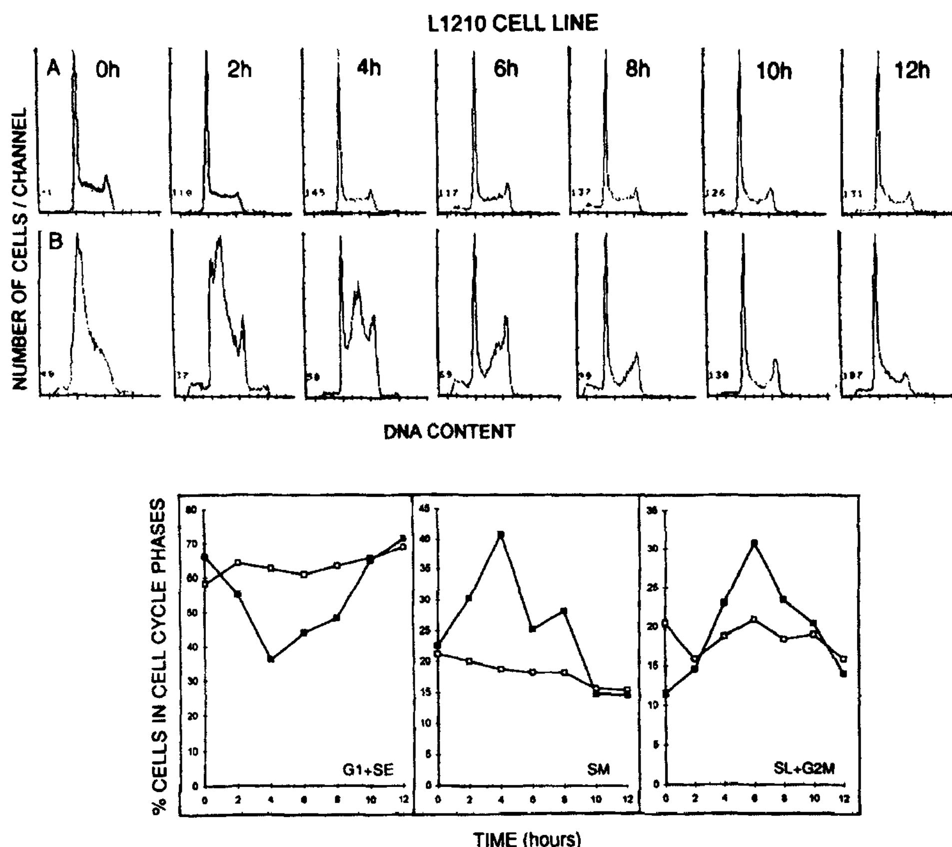

Figure 6. Cell cycle analysis of L1210 cell line at various times of recovery after 16 hours of 0.02 μM MTX incubation. (A) control cells; (B) MTX synchronized cells. The percentage of control and MTX synchronized cells at various time of recovery after MTX incubation are also reported. □ : control cells; ■ : MTX synchronized cells.

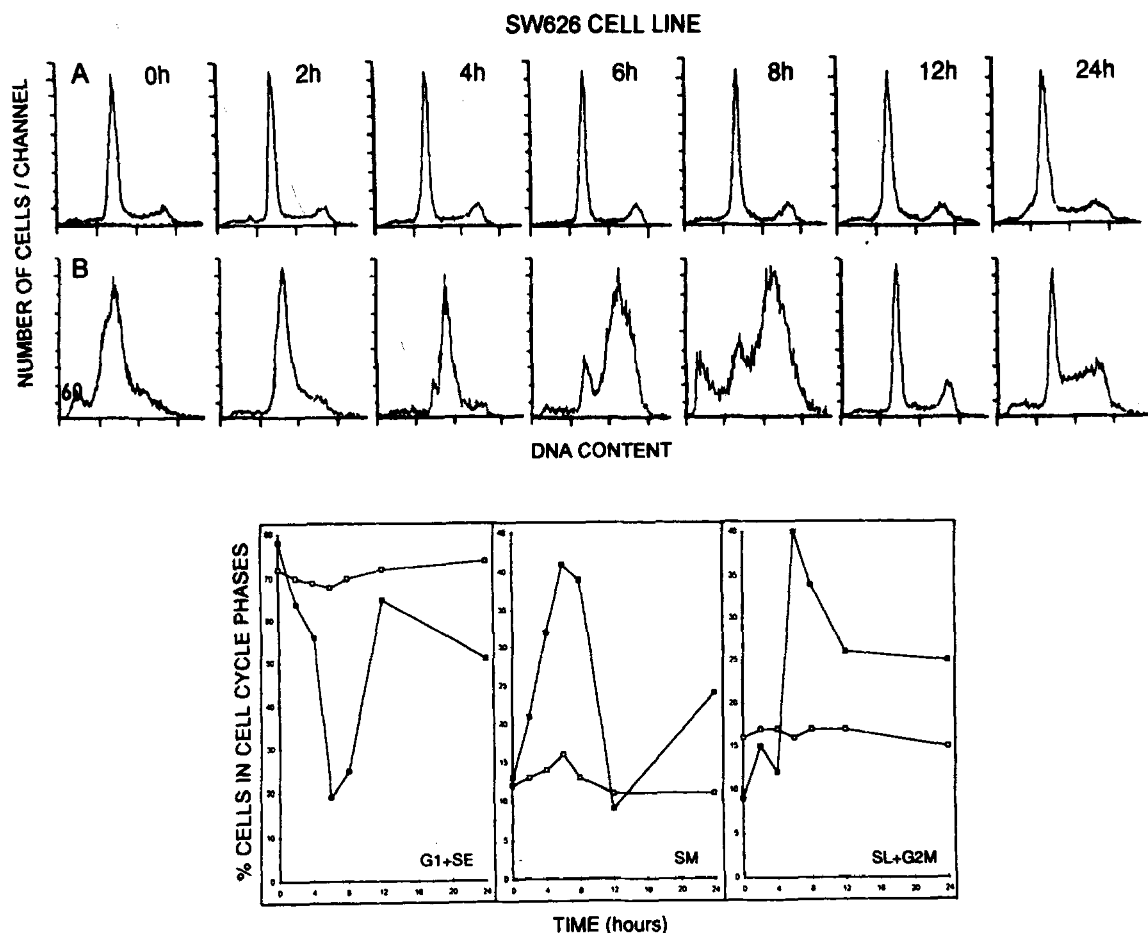

Figure 7. Cell cycle analysis of SW626 cell line at various times of recovery after 24 hours of 0.08 μM MTX incubation. (A) control cells; (B) MTX synchronized cells. The percentage of control and MTX synchronized cells at various time of recovery after MTX incubation are also reported. □ : control cells; ■ : MTX synchronized cells.

phase of the cell cycle. After 8 hours of recovery, a maximum accumulation of the cells in SM was evident. Between 12 and 24 hours in MTX-free medium there were more cells in SL and G_2M phases compared to control cells. At 24 hours after MTX washout the U937 cells were in a standard exponentially growing condition.

The synchronization caused by MTX on L1210 murine leukemic cell line is shown in Figure 6. Many experiments have been performed on L1210 to obtain a good synchronization using MTX, but the degree of synchronization was not satisfactory compared with the other cell line.

At 0 hour after MTX washout no cells were present in the G_2M phase of the cell cycle and the majority of the cells were in G_1SE phases. Between 2 and 4 hours, approximately 40% of the cells were in SM. After 6 hours in MTX-free medium maximum accumulation (30%) of the cells in G_2M phase of the cell cycle was evident. At 12 hours of recovery the cell cycle was completely desynchronized.

In human ovarian cancer SW 626 cells, immediately after MTX washout, 66% of the cells were in G_1SE phase of the cell cycle. 2 hours after majority of the cells were in SE-SM phases. At 6 hours of MTX washout maximum accumulation of the cells in SM and SLG_2M was evident while the cells became unsynchronized after 24 hours in MTX-free medium (Figure 7).

C. Biparametric BrdUrd/DNA analysis

Figure 8 shows the biparametric BrdUrd/DNA analysis on SW 626 cells. BrdUrd was added to the cells during the last 20 of MTX incubation (Oh) and as shown in *Protocol A*, BrdUrd incorporation, at different time after MTX washout samples were collected immediately after BrdUrd incorporation, washed and fixed in 70% ethanol. It appears very clear that the rate of DNA synthesis in MTX synchronized cells, evaluated as the mean of anti-BrdUrd green fluorescence, remained comparable to that of the control cells 24 hours after MTX incubation and also during the following 24 hours in MTX-free medium.

In separate experiments by exposing the cells to 30 μM BrdUrd during the last 20 min of MTX incubation it was possible to follow the progression of the cells through different cell cycle phases after MTX washout. Figure 9 shows the results of this 'pulse-chase' experiment (*Protocol B* BrdUrd incorporation). 24 hours after MTX incubation the BrdUrd positive cells were in SE

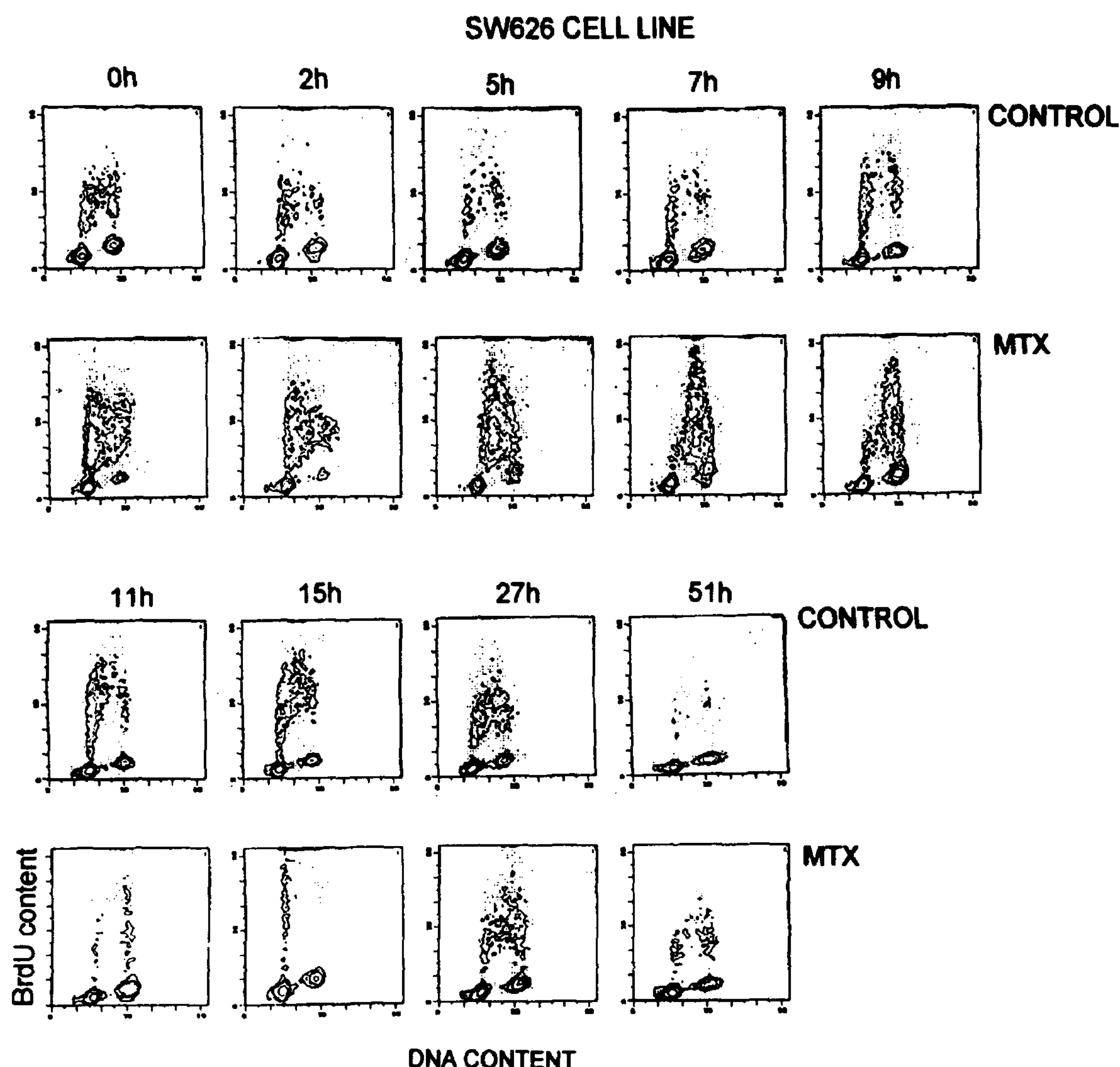

Figure 8. Biparametric BrdUrd/DNA analysis of SW626 control and MTX synchronized cells evaluated at different time after MTX washout. BrdUrd pulse labeling was done at the time indicated in the figure.

phase of the cell cycle. When the MTX was removed, the cohort of BrdUrd positive synchronized cells started moving into the S phase of the cell cycle at a rate similar to the control cells. The time taken to cross the S phase, determined by calculating the RM of the circulating cells during different time, was about 8 hours for control and MTX synchronized cells (Figure 10). The movement through the cell cycle of the cells which were in G_1 or in G_2M at the time of BrdUrd pulse (BrdUrd negative synchronized cells) were similar to the control cells.

4. Discussion

The present paper described synchronization procedure for five cancer cell lines such as Jurkat, K562, U937, L1210 and SW626 using MTX concentrations and exposure times that do not cause any detectable cytotoxicity. MTX is known to be a synchronizer causing a temporary arrest of cells at G_1/SE phase boundary [10] and cytogenetists have used low dose MTX to obtain high number of metaphase plates [11]. A comparative study by Fox et al. [12] investigated several methods of cell synchronization of Chinese hamster ovary (CHO) cells based on mitotic shake-off followed by hydroxyurea, aphidicolin (APC) or MTX for 12 hours. Approximately 20 to 40 time higher MTX, compared to our protocol, was used. This high concentration of MTX was somewhat cytotoxic.

The specific and reversible inhibition of DNA polymerase-α by APC [13; see this issue] without interference with RNA and protein synthesis and intracellular pool of nucleotides strongly recommends its use as a synchronizer [14]. Cordeiro-Stone & Kauffman [15] described a synchronization protocol for C_3H mouse T1/2 cell line consisting of a confluence arrest followed by replating in fresh medium containing APC for 24 hours. Four hours after APC was removed, 89% of nuclei were labeled

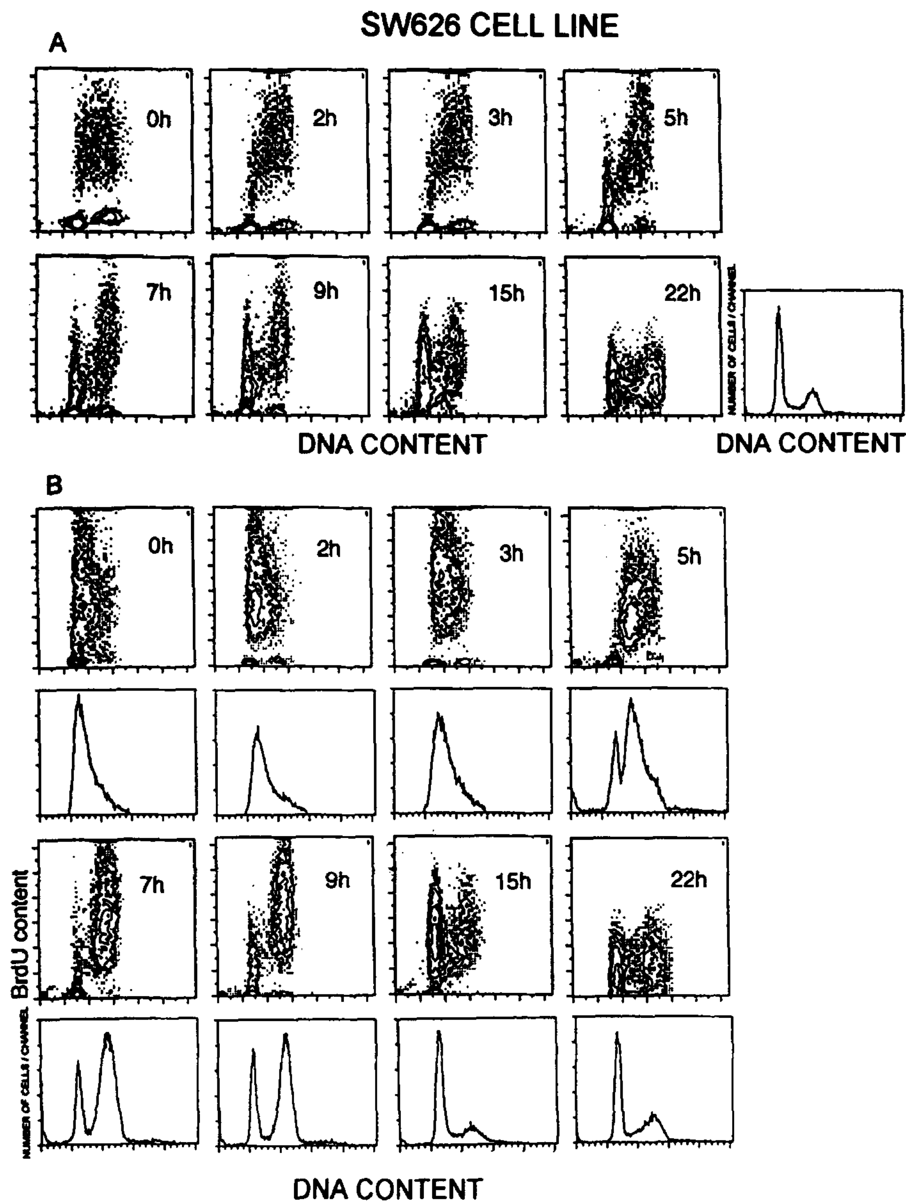

Figure 9. Monoparametric DNA and biparametric BrdUrd/DNA analysis of SW626 preselected with BrdUrd during the last 20 min of MTX incubation and evaluated at different time after MTX washout. (A) control cells; (B) MTX synchronized cells.

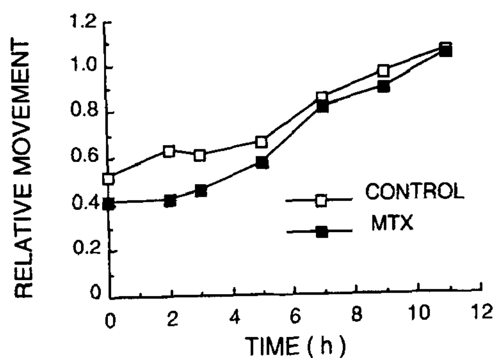

Figure 10. Relative movement of SW626 BrdUrd positive control and MTX synchronized cells evaluated at different time after MTX washout.

with tritiated thymidine within 30 min. Equally spectacular were the studies of Tobey et al. [16] on human foreskin-derived fibroblasts (HSF24, HSF55) using 5 μg/ml APC for 24 hours, after a 48 hour confluent growth in serum-deprived condition. Previously the same group reported optimal synchronization associated with high cytotoxitity in CHO cells [17].

Both the effects of cytotoxicity and DNA elongation and integrity are reported to be dependent very much on the cell lines investigated [15]. Our previous results [1] further supported the concept of differential sensitivity of various cell lines to APC [12, 17]. In the SW626 cell line, for example, 10 μg/ml APC produced a satisfactory wave of synchronization with 72% of the cells in SM, 4 hours after release in APC-free medium. Cytotoxicity was not apparent in this cell line. Instead, APC was extremely cytotoxic in

U937 human histiocytic lymphoma cell line [1]. High cytotoxic potential of APC against many human cancer cell lines have lead to clinical investigation of APC as a potential therapeutic agent [18]. Therefore, in some cancer cell lines, a synchronization protocol based on APC cannot be used. For those cell lines that cannot be satisfactorily synchronised by APC, MTX remains a potentially valid alternative.

In all the cell lines tested, MTX-induced synchrony was completely reversed within 24 hours in MTX-free medium and the cell cycle analysis resembled an exponentially growing population. MTX produced a good synchrony *through the entire S phase* of the cell cycle, but does not appear entirely satisfactory for obtaining cells in the G_2M phases (with the exception of Jurkat cell line) since the fraction of cells in this phase was almost always lower than in other phases and remained mixed with a higher proportion of cells in G_0/G_1 phase. For some inexplicable reasons, the level of synchrony in murine L1210 cell line was not satisfactory. Considering that a single murine line was tested, no generalised conclusion can be drawn on the role of MTX as a synchroniser of murine cells.

MTX at therapeutic dose is highly toxic to the S-phase cells. Exponentially growing cells treated with high concentration of MTX accumulate DNA breaks [19]. However, the concentration of MTX used as a synchronizer in our study caused neither growth inhibition, cell death, in terms of dye exclusion test, nor cytotoxicity, in terms of reduction of clonogenic potential, nor DNA damage (DNA single strand breaks assessed by alkaline elution methods; for details, see Sen et al. [1]). The doubling time and the duration of the S phase in the MTX synchronized cells appeared to be the same as that of the exponentially growing control cells.

The mechanism of the therapeutic effects of MTX is well known. MTX can inhibit DNA and RNA biosynthesis [20–24] and the effect is dependent on the intracellular accumulation of the polyglutamated form of the drug [25]. Our studies [1] in which thymidine was concomitantly applied with MTX showed that the low dose of MTX caused cell synchronization by inhibition of DNA biosynthesis [26–28]. Compounds that specifically inhibit DNA synthesis, such as CB3717 [29], migh produce better results than MTX for inducing synchronization. It is woth pointing out that the low dose of MTX as a synchronizer blocked DNA synthesis but RNA and protein synthesis continued in these cells as reflected by slightly higher RNA/DNA and protein/DNA values in the MTX synchronized cells and by a marked progressive increase in total stainable RNA and protein content during the first cell cycle as the cells traversed S phase [1]. The ratios remained altered in the first cell cycle only and then returned to control values in the subsequent cell cycle between 24 and 28 hours of MTX washout. Our results suggests that low dose MTX used as a synchronizer did induce slight perturbation of cell physiology similar to those reported on other DNA synthesis inhibitors [30–32].

Generally the polyglutamated form of MTX retained in the cell exerts its cytotoxicity even after MTX removal from the medium [33]. This is particularly evident when high concentrations of MTX are used. The fact that MTX synchronization did not change the duration of S phase is important. We have shown by preselecting BrdUrd positive cell population that the cells initially blocked in G_1/SE phase boundary start cycling immediately resembling a normal cell population in MTX-free medium. This further suggests that MTX or its polyglutamated form(s) are not retained in the cells at an active/cytotoxic concentration.

Notes on suppliers

1. Drug Synthesis and Chemistry Branch, Division of Cancer Investigation, National Cancer Institute, Bethesda, USA
2. Gibco, Grand Island, NY, USA; Gibco BRL UK-Life Technologies Ltd., P.O. Box 35, Trident House, Renfrew Road, Paisley, Renfrewshire, Scotland PA3 4EF, UK; Gibco BRL USA-Life Technologies Inc., 8400 Helgerman Court, P.O. Box 6009, Gaithersburg, MD 20884-9980, USA
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8. Becton-Dickinson (UK) Ltd., Betwee Towns Road, Cowley, Oxford OX43LY, UK; Becton-Dickinson, Microbiology Systems, P.O. Box 243, Cockeysville, MD 21030-0243, USA; Becton-Dickinson, Sunnyvale, CA, USA
9. Jackson Immuno Research Laboratories Inc., 872 West Baltimore Pike, P.O. Box 9, West Grove, PA 19390-0014, USA
10. Costar-Cornig-Nucleopore, 1 Alewife Center, Cambridge, MA 02140, USA; Costar/Nucleopore, 10 Valley Center, Gordon Road, High Wycombe, Buchs HP13 GEK, UK.
11. W.C. Heraeus gmbH, Pew, Germany
12. Olympus Optical Co., GmbH, Postfach 104908, Wendenstrasse 14–16, 2 Hamburg-1, Germany

13. Nikon (UK) Ltd., Instrument Division, Habrook, Halesfield 9, Teford, Shropshire TF7 4EW, UK
14. Bellco Glass Inc., Viveland, NJ, USA
15. Disa, Milan, Italy
16. Coulter Electronics, Luton, Beds, LU3 3RH, UK
17. I.S.Co s.r.l., Via Cesare Balbo 6, Milan, Italy

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