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Cell Cycle Synchronization of Human Lymphoid Cells *in Vitro* by 2,3-Dihydro-1*H*-imidazo[1,2-*b*]pyrazole[1]

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SUMMARY

2,3-Dihydro-1*H*-imidazo[1,2-*b*]pyrazole (IMPY), a DNA synthesis-inhibitory drug, reversibly arrests growth of human lymphoblasts *in vitro*. DNA distribution histograms of cultures exposed to 0.5 to 2.0 mM IMPY show accumulation of cells with G_1-early S DNA content. On reincubation in fresh medium, cell cycle traverse is resumed by the blocked cells in a synchronized manner. Maximum incorporation of [H^3]thymidine into DNA (174 to 220% of control) and labeling indexes (72 to 86%) are seen after 4 hr of incubation, and a major increase in cell number is seen between the 9th and 13th hr. DNA distribution histograms of cells reincubated in fresh medium (after double block), show an initial increase in the number of cells with S-G_2-M DNA content and a corresponding decrease in the number of G_1-early S cells. After 4 hr of reincubation, a gradual increase in the number of G_1-early S cells was seen as the earlier blocked cells completed cell cycle traverse and mitosis. Cells exposed to 2.0 mM IMPY took approximately 2 hr longer to traverse than did cells exposed to 0.5 mM IMPY.

INTRODUCTION

A number of physical and chemical methods have been used for cell cycle synchronization of cultured mammalian cells (13, 16, 22). Physical procedures have included harvesting of mitotic cells by selective detachment from monolayer cultures (19), differential centrifugation (12), or the use of abnormal temperatures to block the cell cycle traverse (14, 18). A variety of chemical agents have also been used to achieve similar results either by blocking the cell cycle traverse in G_1-early S (TdR,[3] hydroxyurea) or in G_2-M (colchicine, *Vinca* alkaloids, or podophyllotoxin), or by a selective killing of cells in the S part of the cell cycle (methotrexate, high-specific-activity [^{3}H]TdR). A number of these techniques have been used alone or in combination (13, 16, 22) to obtain synchronized populations from mammalian cells growing in monolayer or suspension cultures. By use of the mitotic selection procedure alone or in combination with cell cycle-blocking agents like colchicine, or *Vinca* alkaloids, highly synchronized cell populations can be obtained from monolayer cultures (3, 15).

A number of human lymphoid cell lines have been recently established and well characterized both morphologically and biochemically (11). Due to their unique growth characteristics and increased sensitivity to a variety of chemical agents (6), some of the techniques normally used for synchronization of nonlymphoid cells are not directly applicable to these cell lines.

The recent availability of flow microfluorometry for rapid determination of DNA per cell (21) has made it relatively easy to follow the progression of a synchronized population through the cell cycle (20). We have used this technique in combination with [^{3}H]TdR incorporation to follow the cell cycle traverse of human lymphoid cells synchronized by a variety of methods.[4] In the present report we describe synchronization of human lymphoblasts of the CCRF-CEM cell line by exposure to the DNA synthesis-inhibitory drug IMPY (1, 5).

MATERIALS AND METHODS

Human lymphoblasts of the CCRF-CEM cell line initially isolated from the peripheral blood of a pediatric leukemic patient (11) were grown in suspension cultures and nourished with Eagle's minimal essential medium (for spinner cultures), supplemented with 10% fetal calf serum and antibiotics, penicillin, and streptomycin. Only log-phase cultures (1 to 2 × 10^6 cells/ml) were used in the present study.

IMPY was a generous gift from Dr. P. Schmidt, Dr. K. Eichenberger, and Dr. K. Scheibli of Ciba-Geigy, Ltd., Basel, Switzerland. Stock solutions of IMPY were made in Hanks' balanced salt solution and stored in a light-proof glass vial. Appropriate dilutions were made in serum-free medium before addition to the cultures.

For reversal of the IMPY-induced cell cycle block, aliquots of cells were washed twice with serum-free medium and reincubated in fresh medium containing 10% fetal calf serum and the usual antibiotics. Cell counts were made in a hemocytometer, and for mitotic indices, acetocarmine squashes were used.

Cells for cytofluorographic analysis were removed at various time intervals and stained in propidium iodide (50 μg/ml) dissolved in 0.1% sodium citrate solution (2, 8). Suspensions of stained cells were analyzed in a Cytofluorograf Model 4801 (Bio/Physics Systems, Inc., Mahopac, N. Y.). In this instrument, a single-cell suspension of the sample is exposed in a flow chamber to a focused argon ion laser beam (488 nm). Fluorescence resulting from the laser

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[3] The abbreviations used are: TdR, thymidine; IMPY, 2,3-dihydro-1*H*-imidazo[1,2-*b*] pyrazole; TCA, trichloroacetic acid.

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excitation of the propidium iodide-stained cells is collected and quantitated through a combination of appropriate mirrors, filters, and photomultipliers. The resulting electrical pulses (based on excitation of the DNA-propidium iodide fluorescence in each cell) are collected and stored in a pulse-height distribution analyzer. Histograms generated from analysis of 10,000 cells/sample are photographed from the cathode-ray tube of a storage oscilloscope. The abscissa of the histograms (generated by the pulse-height analyzer) is divided into 100 channels of increasing linear value from 0 to 100. The height of the bars indicates the number of cells recorded in each channel. These data were also simultaneously printed on paper tape.

DNA distribution patterns of some selected cell populations used to identify and correlate the position of human diploid cells in various parts of the cell cycle with their recorded channel position in the DNA frequency distribution histograms have been recently published (8, 10).

For [^{3}H]TdR incorporation into acid-insoluble DNA, 3 to 5 ml of cells were incubated with [^{3}H]TdR (1 μCi/ml; specific activity, 54 Ci/mmole) for 30 min. Isotope uptake was terminated by the addition of cold 0.9% NaCl solution. Triplicate samples were washed twice with cold 0.9% NaCl solution and centrifuged, and cell pellets were precipitated in cold 10% TCA for 30 min. Precipitates were collected on Millipore filters and washed twice with 5% TCA. Dry filters were placed in scintillation vials containing 10 ml scintillation fluid [4 g Omnifluor (New England Nuclear, Boston, Mass.) in 1 liter toluene]. Vials were counted in a Beckman Model LS-335 liquid scintillation system.

Cells incubated with [^{3}H]TdR (similar to those used for scintillation counting) were centrifuged, washed, and resuspended in fetal calf serum. Smears were fixed in acetic: alcohol (1:3). Rehydrated slides were coated with Kodak NTB-3 emulsion and exposed at 10° for 24 to 72 hr. Slides were developed in D-19 for 2.5 min, fixed in Kodak fixer, and stained in carbolfuchsin. Cells showing more than 5 grains/nucleus (background less than 5 grains/100× oil immersion field) were counted as labeled. A majority of the labeled cells (~90%) had more than 100 grains/nucleus.

RESULTS

In log-phase cultures of CCRF-CEM lymphoblasts, viable cell counts doubled in 24 hr. Pulse labeling with [^{3}H]TdR showed a labeling index of 50 to 60%, and cpm as determined by scintillation counting (after TCA precipitation) were between 13 and 16 × $10^3/10^6$ cells.

In cultures incubated with the various IMPY concentrations for 18 hr, increase in cell counts was either small (less than 10% in 0.5 mM IMPY) or none at all (2.0 mM). Incorporation of [^{3}H]TdR into acid-insoluble DNA was inhibited within 30 to 60 min of exposure to IMPY. The cpm incorporated after 60 min of exposure to IMPY concentrations of 0.5, 1.0, and 2.0 mM were 57, 50, and 7% of the control, respectively. In cultures exposed to 2 successive 18-hr blocks of 0.5, 1.0, and 2.0 mM IMPY (separated by a release of 10 hr) labeling indices were 80, 50, and less than 10%, respectively. Multipolar mitosis and heteropyknotic nuclei were rare in cultures exposed to a double block of 0.5 mM IMPY. In cultures exposed to 2.0 mM IMPY, approximately 4% of the mitotic figures were multipolar and 2% of the population had heteropyknotic nuclei.

In cultures removed from a 2nd block of 0.5 to 2.0 mM IMPY and resuspended in fresh medium, a major increase in cell counts was seen between the 9th and 13th hr of incubation. In cultures exposed to 2.5, 1.0, and 0.5 mM IMPY for 18 hr and subsequently washed and reincubated in fresh medium for 26 hr, cell counts were 84, 89 and 103% of the control, respectively. However, exposure to IMPY had no effect on the subsequent growth of these cultures from the 26th to 48th hr of reincubation in fresh medium.

Data in Chart 1 (scintillation counts and labeling index after [^{3}H]TdR incorporation) and DNA distribution histograms in Chart 2 are of cultures reincubated in fresh medium after exposure to double IMPY blocks. IMPY concentrations used in these experiments were 0.5, 1.0, and 2.0 mM and the two 18-hr blocks were separated by a release period of 10 hr. Scintillation counts recorded in Chart 1*a* show that incorporation of [^{3}H]TdR into acid-insoluble DNA reached a peak (174 to 220% of control) after 4 hr of incubation in fresh medium, and by the 9th hr counts were between 76 to 95% of the control.

Chart 1*b* shows a similar increase in the number of labeled cells (after [^{3}H]TdR incorporation) in autoradiographs. Thirty-six to 53% of the population was labeled after 1 hr of incubation and the percentage of labeled cells reached a peak of 72% (2.0 mM IMPY) to 86% (0.5 mM) after 4 hr. By the 9th hr, 33% of the population was labeled. Mitotic index reached a peak of 10% between the 8th and 12th hr of release and cell counts that showed no significant increase during the 1st 7 hr, increased approximately 60% between the 9th and 13th hr of incubation.

DNA distribution histogram of log-phase cells is shown in Chart 2*a*. Fifty-two % of this population had the G_1-early S DNA content (Channels 20 to 35) and 25 and 22% of the population was, respectively, recorded with the DNA content of S (Channels 36 to 50) and late S-G_2-M (Channels 51 to 70). Histograms of Chart 2, *b* to *d* are of cells exposed to double blocks of 0.5, 1.0, and 2.0 mM IMPY, respectively. A blockage of cells in G_1-early S is indicated by an accumulation of 70 to 75% of the population with the DNA content of G_1-early S. Twenty-one to 27% of the population in these cultures [compared to 47% in log-phase cultures (Chart 2a)] had the DNA content of S and G_2-M.

DNA distribution histograms *e* to *g* of Chart 2 are of cells removed from the double IMPY block (0.5 mM) and incubated in fresh medium for 1, 2, and 4 hr. Resumption of the cell cycle traverse is indicated by the gradual decrease in the number of G_1-early S cells from a high of 75% (Chart 2*b*) to a low of 26% (Chart 2*g*, after 4 hr of incubation). This decrease was accompanied by a corresponding increase in the percentage of cells with the DNA content of S, G_2-M. Histograms *h* to *k* in Chart 2 are of cells incubated for 6, 8, 9, and 12 hr after 0.5 mM IMPY block and show the gradual decrease in the number of cells with the DNA content of S, G_2-M accompanied by an increase in the percentage of cells with the DNA content of G_1-early S from a low of 25% (Chart 2*h*, 6 hr) to a high of 48% (in Chart 2*k*, 12 hr). DNA distribu-

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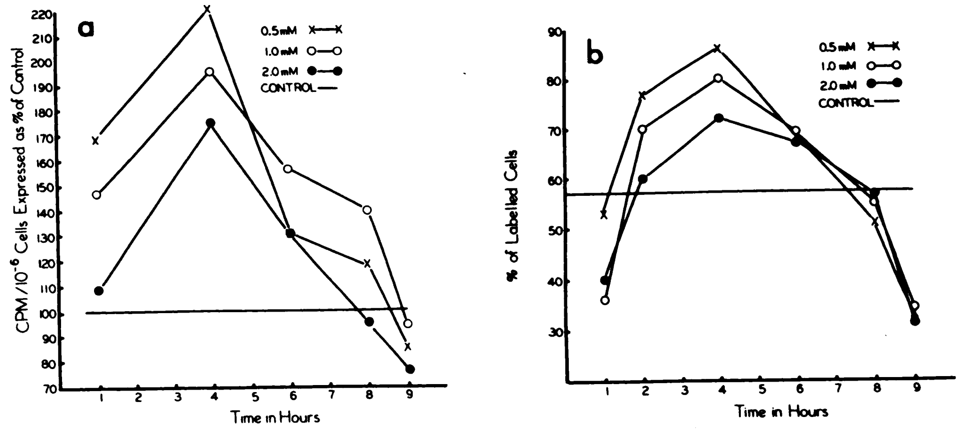

Chart 1. Log-phase cultures were blocked for 18 hr with IMPY (0.5 to 2.0 mM) and released by washing and reincubated for 10 hr; then IMPY was again added for 18 hr. Cells were removed from the 2nd block by centrifugation, and resuspended in fresh medium. *a*, incorporation of [^{3}H]TdR into acid-insoluble DNA of reincubated cells; *b*, percentage of labeled cells seen at various intervals after reincubation of blocked cells in fresh medium.

Chart 2. *a*, DNA distribution histogram of CCRF-CEM lymphoblasts from a log-phase culture. *Numbers* at the *base* (*20, 35, 50, 70*) indicate positions of the channels while *numbers* in *circles* record percentage of cells between Channels 20 and 35 (G_1-early S DNA content), 36 to 50 (S), and 51 to 70 (late S, G_2-M). *b* to *d*, cells after the 2nd (18-hr) block of IMPY. Concentrations used were 0.5, 1.0, and 2.0 mM, respectively. Note the accumulation of cells with DNA content of G_1-early S (Channels 20 to 35). *e* to *g*, cells released from the 2nd block of 0.5 mM IMPY for 1, 2, and 4 hr, respectively. *h* to *k*, cells released for 6, 8, 9, and 12 hr, respectively (after double 0.5 mM IMPY block). *l*, cells released for 12 hr (after double 2.0 mM IMPY block).

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tion histograms of cells released from the double blocks of higher IMPY concentrations (1.0 and 2.0 mM) were similar to those described above, although cell cycle traverse in these cultures was partially delayed. A comparison of Chart 2, *k* and *l*, which are of cells incubated in fresh medium for 12 hr (after 0.5 and 2.0 mM double IMPY blocks, respectively) shows that 48% of cells in the population exposed to 0.5 mM IMPY had the G_1-early S DNA content compared to 35% of such cells in the later population. On the basis of a comparison of DNA distribution histograms, cultures exposed to 2.0 mM IMPY took approximately 2 hr longer to complete cell cycle traverse than did cultures exposed to 0.5 mM IMPY.

DISCUSSION

Ennis *et al.* (5) were first to describe the inhibitory effect of IMPY on DNA synthesis in mammalian cells. In mouse L-cells, HeLa cells, and Sarcoma 180 mouse tumor cells exposed to IMPY, they reported complete inhibition of DNA synthesis without any significant inhibitory effect on RNA and protein synthesis. In L-cells incubated with 1 mM IMPY for 23 hr, inhibition of DNA synthesis was easily reversed by the washing and incubation of cells in fresh medium. High deoxyguanosine concentrations protected cells against the DNA-inhibitory effect of IMPY and, in contrast, deoxycytidine potentiated this effect. In a subsequent report Beer *et al.* (1) confirmed the initial observation of Ennis *et al.* (5) and reported cell cycle synchronization of HeLa cells by IMPY. These authors also confirmed the immediate resumption of DNA synthesis by cells removed from IMPY-containing medium. On the basis of *in vitro* studies with isolated nuclei, these authors have suggested that IMPY inhibits DNA synthesis by interfering with the supply of deoxyriboside triphosphates rather than by directly affecting the process of DNA replication. Unlike mouse L-cells, in HeLa cells, deoxyguanosine does not prevent the inhibitory effect of IMPY on DNA synthesis, presumably due to the reported low capacity of HeLa cells for phosphorylation and utilization of exogenous deoxyguanosine (1).

A number of physical and chemical methods have been used to obtain cell cycle-synchronized populations of mammalian cells. Excellent critical reviews of the various procedures are found in some of the recent articles on the subject (13, 16, 22). However, as mentioned before, many of these procedures are not directly applicable to the human lymphoid cells either due to the unique conditions of their growth or to their extreme sensitivity to some of the chemical agents. As a predominant number of lymphoid cell lines grow in suspension cultures rather than in monolayers (11), methods based on the selective detachment of mitotic cells and the subsequent selection of these or the remaining monolayer as a synchronized population cannot be applied to these cell lines. However, in an earlier report by Drewinko *et al.* synchronization by selective detachment of mitotic cells in a lymphoid cell line presumably capable of growing in monolayer cultures has been reported (4).

Excess TdR has been used extensively in cell cycle synchronization of mammalian cells (23). Exposure to TdR (1 to 2.5 mM) in single or double blocks, alone or in combination with other protocols, have been reported by a number of workers (see Refs. 13, 16, and 22 for review). Caution has also been sounded by workers who draw attention to the unbalanced growth and other biochemical anomalies that arise in cells synchronized by exposure to TdR or other similar chemical agents (7). In human and murine cells of leukemic origin, extremely toxic effects of TdR concentrations similar to those used for synchronization of other mammalian cells have been reported (17). For example, in CCRF-CEM cells exposure to 2.5 mM TdR, a concentration used routinely for the synchronization of L-929 and HeLa cells, leads to irreversible damage and cell loss. In L-929 and HeLa cells, 2.5 mM TdR is needed to block cells in the G_1-S boundary, whereas in CCRF-CEM cells TdR concentrations as low as 0.05 mM block cell growth. However, in contrast to cells synchronized by IMPY, CCRF-CEM cells released from an 18-hr block of 0.05 mM TdR have a large number of pyknotic nuclei (~10%) and a significant part of the population (~20%) fails to resume cell cycle traverse on reincubation in fresh medium.[4] A similar enhanced sensitivity is shown by the human leukemic lymphoid cells to a variety of other chemical agents (6). For example, colchicine and *Vinca* alkaloids that interact with microtubular proteins and thus arrest cells in mitosis have a pronounced cytolytic effect on human lymphoid cells of leukemic origin (9).

In our experience[4] and as shown in this study, human lymphoid cells of the CCRF-CEM cell line suffer the least morphologically visible damage or loss of viability on exposure to 0.5 to 2.0 mM IMPY. On reincubation in fresh medium, resumption of cell cycle traverse is apparent within 1 hr and no serious deleterious effects from exposure to IMPY are noted in the subsequent growth of cells exposed to 2 successive 18-hr blocks of IMPY. In this sense IMPY may be a better agent for cell cycle synchronization of human lymphoid cells that are otherwise extremely sensitive to the toxic effects of other compounds. A further improvement in cell synchrony is achieved by combination of IMPY with short exposure to C-mitotic agents like colchicine or vinblastine. In these experiments, C-mitotic agents can be added after 5 hr of release from the double IMPY block, and within the next 5 hr highly synchronized populations with as many as 80% mitotic cells are harvested. Replacement of colchicine or vincristine by podophyllotoxin (10) as a C-mitotic agent further yields highly synchronized mitotic populations that are also capable of cell division and of subsequent cell cycle traverse without excessive loss of viability.

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