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SYNCHRONIZATION OF HeLa CELLS WITH 2,3-DIHYDRO-1-H-IMIDAZO[1,2-b]PYRAZOLE

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Abstract 2,3-Dihydro-1-H-imidazo[1,2-b] pyrazole (IMPY) has been shown to be a useful agent for synchronizing monolayer and suspension cultures of HeLa for cell cycle studies. Treatment of log cultures with 2·5 mM IMPY blocks DNA synthesis while permitting RNA and protein synthesis. After a 16-hr interval in which the cells are gathered at the entry to the S-period, removal of the agent allows the cells to proceed as a viable, highly synchronized population through S, G_2 and mitosis.

AGENTS that specifically inhibit biochemical or biophysical processes which occur in discrete phases of the cell cycle (e.g. DNA synthesis or mitosis) are potentially useful for synchronizing cell populations. Ennis *et al.*[1] have recently reported that 2,3-dihydro-1-H-imidazo [1,2-b]pyrazole (IMPY) can, at appropriate concentrations, rapidly inhibit DNA synthesis in cultured mammalian cells without significantly affecting RNA or protein synthesis. They also showed that when the IMPY-treated cells were restored to drug-free medium they rapidly resumed DNA synthesis. In the present communication, we show that both monolayer and suspension cultures of HeLa cells can readily be synchronized at the point of entry into the S-period by treatment with IMPY under appropriate conditions. On removal of the agent, these cells proceed synchronously through the replication cycle as a highly viable population.

METHODS AND MATERIALS

Cell cultures. HeLa cells were maintained under conditions which have been previously described.[2,3] Monolayer cultures were grown in 3-oz. prescription bottles by planting approximately 5×10^5 cells in 10 ml of Eagle's medium supplemented with 10% bovine serum, 0·1 mM glycine, 0·1 mM serine and 0·01 mM inositol (BEHM).[3] The cultures were incubated at 37° in air containing 5% CO_2. Suspension cultures were grown in shaker flasks in BEHM from which the calcium and magnesium were omitted but which contained 0·1% pluronic F-68† (spinner medium).[4] Cells were counted with a Coulter counter, monolayers after trypsinization,[3] and suspension cultures after gently pipetting to break up cell aggregates.

In certain experiments HeLa cell cultures were synchronized by reversibly blocking DNA synthesis for 16 hr with a mixture of amethopterin (final concentration, 10^{-6}M) and adenosine (final concentration, 5×10^{-5}M) following

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procedures already described.[3] The cells were released from the amethopterin block by adding thymidine (final concentration 4×10^{-6} M) to the cultures.

Incorporation of radioactivity into DNA, RNA and protein of HeLa cells. The synthesis of DNA, RNA and protein in the HeLa cells was usually measured by the incorporation of thymidine-methyl-^{3}H, uridine-5-^{3}H and L-leucine-^{14}C (U), respectively, into the acid-insoluble cellular material. The concentration and specific activities of the precursors and other incubation conditions are given in the figure legends. The labeled cells were washed (either directly as monolayers or after centrifuging aliquots of suspension cultures) successively with saline, 4% perchloric acid, 80% ethanol, absolute ethanol and ethyl ether and allowed to dry. The cell residues were dissolved in 2 ml of 88% formic acid and aliquots counted by a liquid scintillation spectrometer.

Radioactive precursors and other compounds. Thymidine-methyl-^{3}H (17 Ci/m-mole) and uridine-5-^{3}H (20·7 Ci/m-mole) were purchased from Schwarz-Mann, Orangeburg, N.Y.; L-leucine-^{14}C(U)(240 mCi/m-mole) was purchased from International Chemical and Nuclear Corp., Calif. 2,3-Dihydro-1H-imidazo[1,2-b]pyrazole (Ciba 21, 381Ba) was a gift from Dr. Prosper Loustalot, Ciba Ltd., Basle, Switzerland; it was dissolved in saline immediately before use and the solution sterilized by filtering through a 0·2 μm Millipore filter. Amethopterin was obtained from Lederle Laboratories.

RESULTS AND DISCUSSION

Effect of IMPY on growth of HeLa cells. The effect of IMPY concentration on the growth of HeLa cells in monolayer and suspension cultures is shown in Fig. 1. In both types of culture, the population growth was completely inhibited at drug concentrations of 1·0 mM. Cells which had been incubated for several hr in the presence of growth inhibitory concentrations of IMPY showed no evidence of gross cytological damage, although eventually many cells became substantially larger than those in control cultures.

The generation time of the HeLa cells used in the present study was 22–24 hr, of which the S-period occupies 6–7 hr.[5] It was therefore anticipated that, if DNA synthesis were blocked with IMPY for 16 hr, a high proportion of the cells would accumulate at or near the boundary between the G_1- and S-phases; when resuspended in drug-free medium these cells might be expected, in view of the reported reversibility of the inhibition,[1] to proceed as a synchronized population through the S-phase. Both the results in Fig. 1 and those of Ennis *et al.*[1] suggested that 1·0 mM IMPY should be able to effect this synchronization. However, when HeLa cells which had been cultured in the presence of 1·0 mM IMPY for 16 hr were resuspended in drug-free medium, they incorporated tritiated thymidine at essentially the same rate as cells in unsynchronized control cultures and did not show the sharp wave of DNA synthesis characteristic of a truly synchronous population. The results in Figs. 1 and 2 show that, although IMPY, at a concentration of 1 mM, arrested the growth of HeLa cell cultures for at least 16 hr, it had not completely blocked DNA synthesis throughout this period. This suggests that some cells might have resumed their progress through the S-phase and so diminished the degree of synchrony. It seemed possible therefore that a higher concentration of IMPY might more effectively inhibit DNA synthesis and perhaps thereby improve the synchrony.

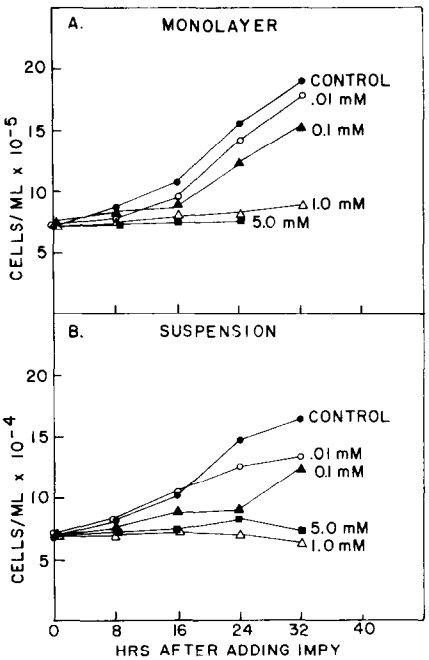

FIG. 1. Effect of IMPY concentration on growth of cultured HeLa cells. (A) Monolayer cultures: grown in 10 ml BEHM as described in Materials and Methods. At zero time, IMPY was added in 0·2 ml saline to give the final concentrations shown in A; 0·2 ml saline was added to the controls. At the indicated times the cells in duplicate cultures were trypsinized and counted in a Coulter counter. (B) Suspension cultures: grown in 60 ml of spinner medium (Materials and Methods). At zero time, IMPY was added in 1·2 ml saline to give the final concentrations shown in B; 1·2 ml saline was added to the control. At the indicated times, 1-ml portions were taken in duplicate for counting.

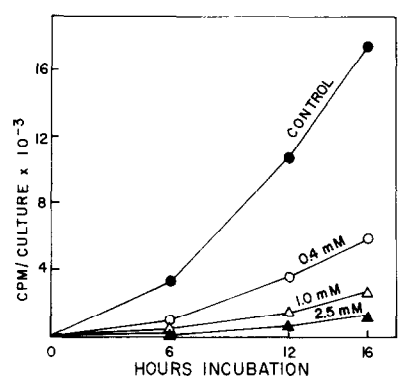

FIG. 2. Effect of IMPY concentration on incorporation of tritiated thymidine by HeLa cells. Cultures: monolayers grown in 10 ml BEHM as described in Materials and Methods. At zero time, IMPY was added in 0·2 ml saline to give the final concentrations indicated; 0·2 ml saline was added to the controls. All cultures received ^{3}H-thymidine (20 μg, 0·75 μCi), amethopterin (10^{-6}M) and adenosine (5×10^{-5}M). Amethopterin was added to block the endogenous synthesis of thymidylic nucleotides during the measurements of DNA synthesis. At the indicated times, the cells in duplicate cultures were washed with acid, dried and assayed for acid-insoluble radioactivity as described in Materials and Methods.

provided it did not at the same time lead to undesirable cytological or biochemical disturbances.

It was found (Fig. 2) that the tendency of cells to escape from the inhibitory effect of IMPY on DNA synthesis was substantially less at the high drug concentration of 2·5 mM. It also appears from the results in Fig. 3 that, at this drug

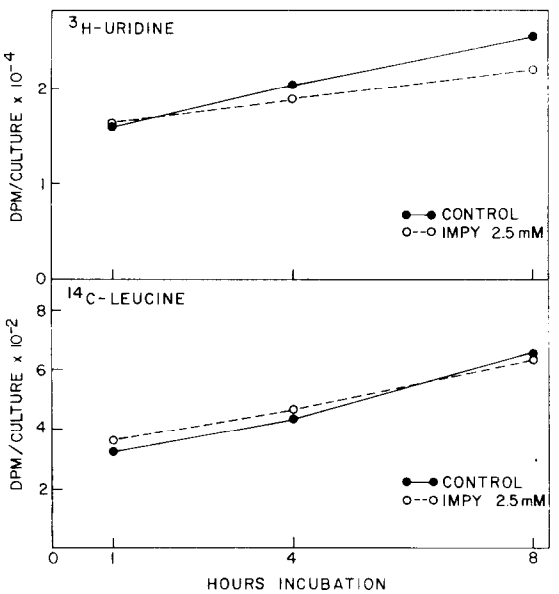

FIG. 3. Effect of 2·5 mM IMPY on incorporation of ^{3}H-uridine and ^{14}C-leucine into acid-insoluble material of HeLa cells. Cultures: monolayers grown in 10 ml BEHM (Materials and Methods); population at start of experiment, $1·75 \times 10^6$ cells/culture. At zero time, the cultures were divided into two groups; IMPY dissolved in 0·2 ml saline was added to one group to a final drug concentration of 2·5 mM; 0·2 ml saline was added to the other (control) group. At 0, 3 and 7 hr a mixture of ^{3}H-uridine and ^{14}C-leucine in 0·2 ml saline was added to representative duplicate cultures from each group (amounts per culture: ^{3}H-uridine, 6 μCi, 3 μg; ^{14}C-leucine, 0·25 μCi, 0·26 mg, including the leucine already in the medium). After incubation for 1 hr with the radioactive precursors, the cells were acid washed (Materials and Methods) and radioassayed in a liquid scintillation counter programmed to differentiate between tritium and carbon-14. Representative cultures were trypsinized and the number of cells was determined with a Coulter counter. Control cultures, ●—● ; IMPY-treated cultures, ○ ○. The data are plotted at the times corresponding to the end of the 1-hr pulses.

concentration, RNA and protein synthesis was still relatively unaffected for at least 8 hr. Beyond 8 hr, there was a gradual apparent restriction in the overall rate of RNA and protein synthesis in IMPY-treated cultures when compared to the rising level in the logarithmically growing control cultures. However, the control cells, in contrast to IMPY-treated cells, continued to undergo nuclear replication and cell division during this interval. Correcting the data to equivalent cell levels, it was found that cells treated with IMPY for 16 hr showed 92 and 123 per cent of the control rates for RNA and protein synthesis, as measured by the incorporation of ^{3}H-uridine and ^{14}C-leucine respectively. In accord with these findings, it was observed that cells which had been incubated with 2·5 mM IMPY for 16 hr were

somewhat larger than those in control cultures, but otherwise showed no obvious cytological changes. When restored to drug-free medium after this treatment, the cells immediately resumed DNA synthesis, incorporating tritiated thymidine at a rate which was, on a per cell basis, initially substantially greater than that of cells in control cultures (Fig. 4). The pattern of thymidine uptake, i.e. a sharp rise leveling off almost to a plateau after about 6 hr, is characteristic of cultures in which a high proportion of the cells first accumulate at or near the beginning of the S-phase then enter and pass more or less simultaneously through the S-phase.

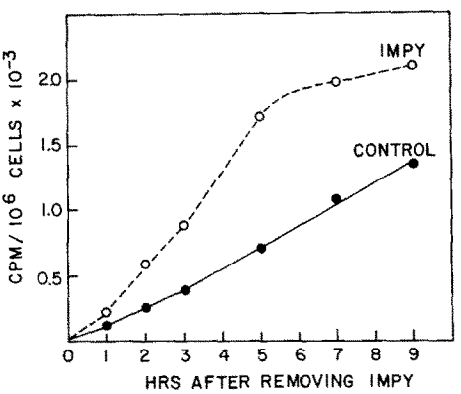

FIG. 4. Incorporation of ^{3}H-thymidine by HeLa cells after release from 16-hr of pretreatment with 2·5 mM IMPY. Monolayer cultures (starting population, 1·27 × 10^6 cells/culture) were incubated for 16 hr in either the presence or absence of 2·5 mM IMPY. At the end of this period, the medium in all cultures was replaced with 10 ml of fresh drug-free BEHM containing ^{3}H-thymidine (7 μCi, 12 μg), amethopterin (10^{-6}M) and adenosine (5 × 10^{-5}M). At the indicated times (above) after the medium change, the cells in duplicate cultures were acid washed and radioassayed as described in Materials and Methods. Control cultures (no IMPY treatment), ●——●; cultures pretreated with IMPY, ○——○. The data are expressed as cpm/10^6 cells based on the number of cells in the cultures at zero time.

The use of 2·5 mM IMPY for synchronizing monolayer and suspension cultures of HeLa cells is shown in Fig. 5. During the 16-hr period in which the cultures were blocked with the drug, there was very little increase in cell numbers. On replacing the IMPY medium with drug-free medium, the cells proceeded at a normal rate and as viable and highly synchronized populations through the S- and G_2-phases and mitosis into the next cycle. Thus DNA synthesis was essentially complete within 6–7 hr of releasing the cells from the block; there was no appreciable increase in cell numbers during the period of DNA synthesis, but shortly thereafter the cell populations rapidly increased to almost double the pre-replication values. The compound appears therefore to offer a useful alternative to other agents such as hydroxyurea,[6] amethopterin[3] or excess thymidine[7] for synchronizing mammalian cells in culture.

The results in Fig. 5 are consistent with a mechanism in which the drug permits the cells to reach the S-phase but then prevents their further progress through the cycle. In this regard, the drug appears to act on cells which are already staged for DNA synthesis. Thus studies with HeLa cell cultures which have been synchronized by blockade with amethopterin show that 2·5 mM IMPY immediately inhibits DNA

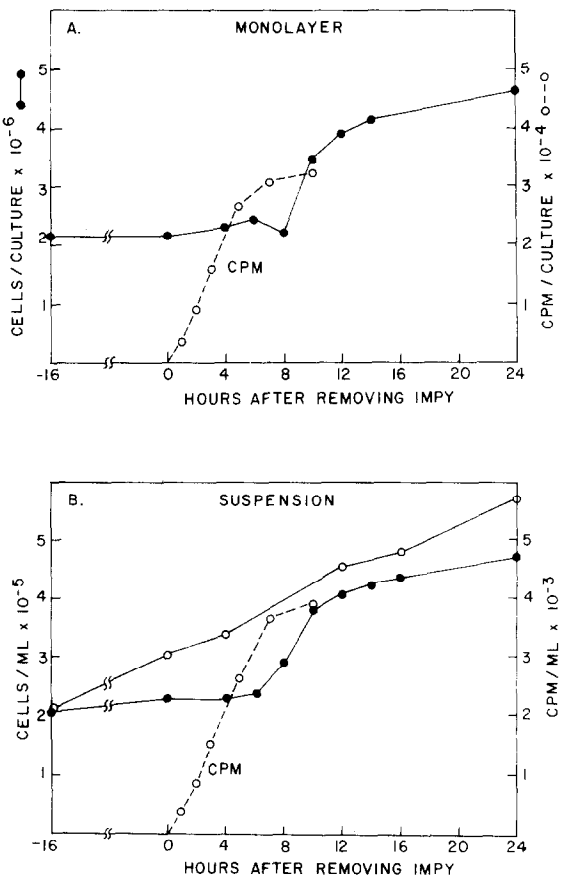

FIG. 5. Synchronization of monolayer and suspension cultures of HeLa cells with 2·5 mM IMPY. (A) Monolayer cultures: grown in 3-oz. pharmacy bottles in 10 ml BEHM (Materials and Methods) to a density of $2{\cdot}1 \times 10^6$ cells/culture. IMPY in 0·2 ml saline was then added to all cultures to give a final drug concentration of 2·5 mM. After incubation for 16 hr in the presence of the drug (−16 to 0 hr), the medium in all cultures was replaced with 10 ml fresh, drug-free BEHM; in addition, the medium in cultures to be used for monitoring DNA synthesis contained ^{3}H-thymidine (1 μCi, 20 μg/culture). At the times indicated, duplicate cultures were taken for the determination of cell numbers and the radioactivity incorporated into the acid-insoluble material of the cells. Cell numbers, ● ●; radioactivity, ○ ○. (B) Suspension cultures: A suspension culture of HeLa cells ($2{\cdot}02 \times 10^5$ cells/ml) in spinner medium (Materials and Methods) was divided into portions; 300 ml was incubated with 2·5 mM IMPY for 16 hr; 150 ml (drug-free) was used as a control culture. The culture containing IMPY was (after 16 hr) centrifuged and the cells were washed once with 5 ml medium and resuspended in 300 ml fresh, drug-free spinner medium. One-half of this suspension was used for monitoring changes in population on further incubation; duplicate 1-ml aliquots were counted at the times indicated. The other half of the culture was incubated in the presence of ^{3}H-thymidine (2 μg/ml, 0·1 μCi/ml); the accumulation of acid-insoluble radioactivity in the cells was determined on duplicate 4-ml aliquots taken at the times indicated (Materials and Methods). IMPY-treated culture: cell numbers, ● ●; radioactivity, ○ ○. Control culture (no drug treatment; no medium change at zero time): cell numbers, ○ ○.

synthesis irrespective of whether the cells are at the G_1 S boundary (i.e. at the time of release from the amethopterin blockade) or are well into the S-phase (Fig. 6).

Other experiments in this laboratory have shown that, in spite of its almost complete inhibition of DNA synthesis in intact cells, IMPY (2·5 mM) did not

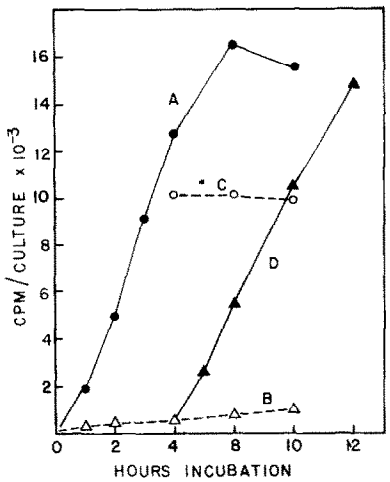

FIG. 6. Effect of 2·5 mM IMPY* on the incorporation of ^{3}H-thymidine by HeLa cell cultures pre-synchronized with amethopterin. Monolayer cultures were grown in 10 ml BEHM (3-oz. pharmacy bottles) to a density of $7·5 \times 10^5$ cells/culture. All cultures were synchronized by incubation for 16 hr in the presence of amethopterin (10^{-6}M) and adenosine (5×10^{-5}M).[2] At the end of this period, the cultures were divided into four groups (A–D) for further treatment and incubation as shown below. At the times indicated in this figure, the cells in duplicate cultures were acid washed and assayed for tritium (Materials and Methods).

Incubation time (hr)	Additions per culture*	Treatments			
		A	B	C	D
0	^{3}H-TdR (10 μg, 1 μCi)†	+	+	+	+
	IMPY (2·5 mM)	−	+	−	+
3	IMPY (2·5 mM)	−	−	+	−
4	Medium change (10 ml BEHM) + ^{3}H-TdR (10 μg, 1 μCi), amethopterin (10^{-6}M), adenosine (5×10^{-5}M)	−	−	−	+

* Additions were made in 0·2 ml saline.
† Thymidine addition (at zero time) releases all the cultures from amethopterin blockade.

inhibit the incorporation *in vitro* of deoxynucleoside triphosphates into DNA by nuclei isolated from cells in the S-phase. Similarly, the inhibition of DNA synthesis *in vivo* by IMPY was overcome immediately by isolation and incubation of the nuclei in the system *in vitro* described by Hershey *et al.*[8] It therefore seems most likely that, *in vivo*, the compound inhibits DNA synthesis by interfering with the supply of deoxyriboside triphosphates rather than directly with the process of DNA replication. IMPY might, for example, be an inhibitor of deoxynucleotide kinases or ribonucleotide reductase. With respect to the latter possibility, Ennis *et al.*[1] have reported that in L cells deoxyguanosine could, to a limited extent, reverse the inhibitory effect of IMPY on the labeling of DNA by radioactive thymidine. However, in other studies we have found that HeLa cells have a low capacity to

phosphorylate and utilize exogenous deoxyguanosine: this could explain the present experiments in which we were unable to reverse or prevent the inhibition by IMPY with deoxyguanosine and other deoxynucleosides. Since thymidine was similarly ineffective as an antagonist of IMPY, it also is unlikely that IMPY acts as an antifolic agent in the synthesis of thymidine nucleotides. It is apparent that the elucidation of the intracellular mechanism of IMPY action awaits an analysis of the deoxynucleotide levels in treated cells.

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