Different Effects of Thymidine and 5-Fluorouracil 2'-Deoxyriboside on Biosynthetic Events in Cultured P 815Y Mast Cells

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1. Addition of 2mM-thymidine, although resulting in the cessation of cell division, allows the continuation of phospholipid and protein synthesis and results in an increase in mean cell volume for at least 15h. 2. 5-Fluorouracil 2'-deoxyriboside inhibits cell division but differs from thymidine by inhibiting the synthesis of phospholipid and protein in a more marked manner. 3. The relation between these results and the P 815Y cell cycle is discussed.

Thymidine at 2mM inhibits DNA synthesis (Morris & Fischer, 1963; Morris, Reichard & Fischer, 1963) and is used to obtain synchronous cultures of mammalian cells (Xeros, 1962; Puck, 1964). A much lower concentration of FUdR, $\dagger$ 1 μ M, inhibits DNA synthesis (Bosch, Harbers & Heidelberger, 1958; Paul & Hagiwara, 1962) and is used to obtain synchronous growth (Littlefield, 1966).

Previous work has shown that gross changes occur in the rates of synthesis of phospholipid, protein, RNA and DNA during the cell cycle of P 815Y cells (Bergeron, Warmesley & Pasternak, 1969). Also, the temporal sequence of the above events during the cell cycle is the same regardless of whether the method of synchrony is by means of 2mM-thymidine (Bergeron, Warmesley & Pasternak, 1970) or whether a purely physical method is used (Warmesley & Pasternak, 1970).

In this study, interrelationships between DNA synthesis and phospholipid and protein synthesis are analysed by the use of the DNA-synthesis inhibitors thymidine and FUdR.

EXPERIMENTAL

Chemicals. [*Me*- 14 C]Choline chloride (CFA 138; specific radioactivity 54 mCi/mmol) and L-[*G*- 3 H]proline (TRA 82; specific radioactivity 304 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Thymidine was obtained from British Drug Houses Ltd., Poole, Dorset, U.K. FUdR was a gift from Dr M. L. Lunt.

Cells. P 815Y neoplastic mast cells kindly given by Dr G. A. Fischer and Dr M. Fox were cultured as described by Pasternak & Bergeron (1970).

Incorporation of label. Incorporated radioactivity was determined by filtration, washing with cold 5% (w/v)

trichloroacetic acid and assaying the filters as described by Pasternak & Bergeron (1970).

Cell viability. The proportion of dead cells was determined by eosin staining (Eaton, Scala & Jewell, 1959; Watanabe & Okada, 1967). The cell suspension was mixed with 0.5 vol. of 1% eosin solution and the proportion of eosin-stained cells determined. At least 500 cells were counted for each determination.

Cell number and volume. Cells were removed and analysed for cell number by using a Coulter Counter model A as described by Pasternak & Bergeron (1970).

For measurement of cell volume, cells were removed from cultures and diluted 1:25 with phosphate-buffered saline, pH 7.4 (Krebs & Eggleston, 1940). The proportion of cells appearing at progressive threshold values on the Coulter Counter was determined as the distribution of cell sizes. The threshold value is proportional to volume and the mean cell volume was calculated as described by Bergeron (1969).

Synchronous growth. Cells were synchronized by the addition of 2mM-thymidine to an exponentially growing culture for 15h, which is the mean generation time. Cells were washed free of inhibitor and allowed to recover for approx. 7h, and thymidine was added for a further 15h. On removal of inhibitor, synchronous growth commences (Bergeron *et al.* 1969).

RESULTS

Effect of 2mM-thymidine. Incorporation of labelled choline and proline (Fig. 1) continues for at least 15h although cell growth is inhibited. The increased synthesis of phospholipid and protein is reflected in the increase in mean cell volume (Fig. 2), suggesting that the inhibition of DNA synthesis has no immediate effect on phospholipid and protein synthesis.

Effect of FUdR. The above results contrast with the cessation in incorporation of choline and proline, which takes effect 10h after the addition of FUdR (Fig. 3).

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$\dagger$ Abbreviation: FUdR, 5-fluorouracil 2'-deoxyriboside.

Fig. 1. Effect of thymidine on phospholipid and protein synthesis. (a) A cell suspension (50 ml; 2.3×10^5 cells/ml) was inoculated with $[^{14}\text{C}]$ choline ($0.5 \mu\text{mol}$; $10 \mu\text{Ci}$). Portions were removed and cell number and radioactivity incorporation into phospholipid determined as described by Pasternak & Bergeron (1970). The arrow marks the addition of inhibitor. $\blacktriangle$, Uptake of $[^{14}\text{C}]$ choline; $\bullet$, cell number. (b) A cell suspension (50 ml; 2×10^5 cells/ml) was inoculated with $[^3\text{H}]$ proline ($10 \mu\text{Ci}$; $304 \mu\text{Ci}/\mu\text{mol}$). Portions were removed and cell number and radioactivity determined as described for (a) and in the Experimental section. The arrow marks the addition of inhibitor. $\blacktriangle$, Uptake of $[^3\text{H}]$ proline; $\bullet$, cell number.

Fig. 2. Effect of thymidine on mean cell volumes. At the arrow, 2 mM-thymidine was added to a flask of exponentially growing cells. Portions were removed and analysed for cell volume as described in the Experimental section and by Bergeron (1969).

It is conceivable that the inhibition of DNA synthesis by FUDR may cause the cessation of phospholipid and protein synthesis by possible feedback mechanisms operating to maintain the values of

cellular protein and phospholipid within certain limits. This explanation has been offered to explain the inhibition of protein and RNA synthesis after treatment of cultured L cells with FUDR (Paul & Hagiwara, 1962).

Cell viability. The contrasting results of the two DNA-synthesis inhibitors prompted an analysis shown in Fig. 4 of the toxicity of thymidine and FUDR to P815Y cells.

It is noteworthy that the decrease in cell viability is not reflected by a decrease in cell number (Fig. 4) but is seen as a change in the mean cell volume (Fig. 5). The diminution of mean cell volume is observed as a loss of larger cells and their appearance as smaller units (Fig. 6), presumably as part of the degradative processes of the cell.

The destructive action of FUDR may be a consequence of the catabolism of the compound to 5-fluorouracil and incorporation into 'fraudulent' RNA. Evidence for this pathway has been presented (Harbers, Chaudhuri & Heidelberger, 1959) for Ehrlich ascites cells.

Thus 2 mM-thymidine is less toxic to P815Y cells than FUDR and this is the basis for the different

consequences of the two inhibitors on phospholipid and protein synthesis.

Removal of 2mm-thymidine. The analysis of cells after removal of a programmed thymidine block to obtain synchronous growth (Fig. 7) shows that cell size is more disperse than is so for normal cells (Fig. 8). Further analysis of Fig. 8 suggests that cells synchronized by thymidine remain abnormal for at least one generation time.

DISCUSSION

The continuation of synthesis of phospholipid and protein after inhibition of DNA synthesis and cell division by 2mm-thymidine suggests the lack of direct linking between these events. The results show that cells treated with an excess of thymidine are abnormal, and support the notion (Studzinski & Lambert, 1969) that synchronization studies involving chemical inhibitors should be avoided if other methods are available.

Fig. 3. Effect of FdR on phospholipid and protein synthesis. To 50 ml of a cell suspension was added [^3H]proline (10 μCi ; 304 $\mu\text{Ci}/\mu\text{mol}$) and [^{14}C]choline (5 μCi ; 0.5 μmol). Portions were removed and cell number and radioactivity determined as described in the Experimental section. The arrow marks the addition of inhibitor. ▲, Uptake of [^{14}C]choline; ■, uptake of [^3H]proline; ●, cell number.

Fig. 4. Effect of thymidine and FdR on cell viability. Identical flasks were inoculated with 2.5×10^5 cells/ml. At 20h (arrows) thymidine (2mm) was added to flask (a) and FdR (1 μM) to flask (b). To prevent exhaustion of nutrients or inhibitor, cells were resuspended into fresh medium and inhibitor at 30h and 75h. Cells were diluted to optimum cell densities for normal growth. Portions were removed and cell viability and cell number determined as described in the Experimental section. ○, % viable cells; ●, cell number.

Fig. 5. Inhibitors and the decrease in cell volume. Portions were removed from the flasks incubated as described in Fig. 4 and the mean cell volume was determined as described in the Experimental section. The arrow marks the addition of inhibitor. ○, Mean cell volume in thymidine-treated flasks; Δ, mean cell volume of cells incubated with FUdR.

Fig. 7. Synchronous growth after thymidine treatment. Cells were synchronized by the use of 2 mM-thymidine and cell numbers were counted as described in the Experimental section.

Fig. 6. Effect of thymidine and FUdR on the distribution of cell size. (a) Portions were removed from the flask incubated as described in Fig. 4(a) and the distribution of cells at various volumes was determined as described in the Experimental section and by Bergeron (1969). (b) Portions were removed from the flask incubated as described in Fig. 4(b) and the cell size distribution was determined as for (a).

Fig. 8. Distribution of cell size during synchronous growth. Portions of the culture described in Fig. 7 were removed and analysed for the distribution of cell volume as described in the Experimental section and by Bergeron (1969). (a) Synchronous growth; (b) exponential growth.

Nevertheless the changes in synthesis of phospholipid, protein, RNA and DNA during the cell cycle are similar regardless of whether the method of synchrony is by thymidine (Bergeron *et al.* 1969, 1970) or by a physical method (Warmesley & Pasternak, 1970). As thymidine-synchronized cells are larger and remain unbalanced during synchronous growth, whatever triggers the temporal sequence of events about the P 815Y cell cycle, it is not related to cell mass or volume.

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