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BIOCHEMICAL STUDIES OF THE DIVISION CYCLE OF MAMMALIAN CELLS: EVIDENCE FOR THE PREMITOTIC PERIOD

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Abstract—In cultures of a murine mast cell tumor DNA synthesis was blocked by the addition of amethopterin, hypoxanthine, and glycine; administration of thymidine 6 hr later resulted in a partial synchronization of cell reproduction. In these partially synchronized cultures, mitotic indices and DNA synthesis, as expressed by the rate of incorporation of labeled thymidine, were determined at regular intervals. The results indicate that there is a distinct time interval (premitotic period) of approximately 1 hr between completion of DNA synthesis and onset of mitosis. This finding is in good agreement with earlier reports based on microphotometric or radioautographic methods.

PRESENT knowledge of the time relationship between the formation of deoxyribonucleic acid and mitosis is based on experiments using microphotometric or radioautographic techniques. With the aid of these methods it has been shown that DNA synthesis takes place during interphase within a distinct period of time, and that before and after mitosis there are two periods of the division cycle (termed G_2 and G_1 respectively) during which synthesis of DNA is not observed[1–3] (see Fig. 1). Both microphotometry and radioautography are, unfortunately, not very specific with regard to the chemical identification with DNA of the stained or labeled material. This

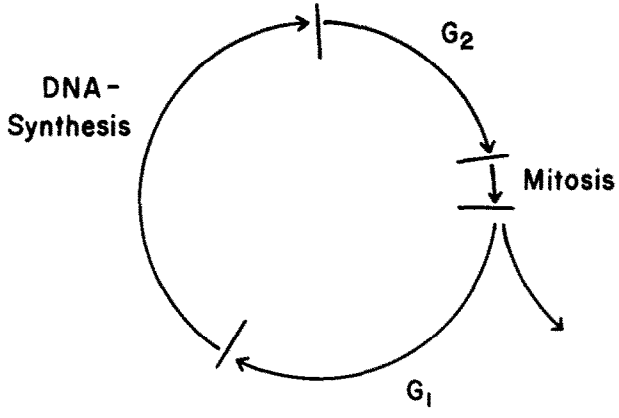

FIG. 1. The division cycle of mammalian cells.

objection can hardly invalidate the conclusions concerning the postmitotic G_1-period. On the other hand, radioautographic evidence for the premitotic G_2-period is based on the assumption that the label observed after fixing the cells may be ascribed exclusively to DNA. It has been reported, however, that a considerable proportion of the labeled material within the cell is represented by precursors of DNA of relatively low molecular weight;[4] similar arguments also may be raised against microphotometric data. In experiments with bone marrow cells *in vitro*, which are characterized by a rather long generation time, histochemical techniques were used to remove the label present in non-DNA cell components.[1] On the other hand, in similar studies on rapidly multiplying cells in culture, proof of the identity of the label with DNA was not obtained. It seemed desirable, therefore, to provide further evidence for the premitotic period in mammalian cell cultures on the basis of biochemical rather than radioautographic techniques.

A possibility for biochemical studies of the premitotic period is based on the effects of inhibitors of the biosynthesis of the thymine component of DNA, such as folic acid antagonists or 5-fluorouracil deoxyribonucleoside (FUDR). In mammalian cell cultures the inhibition of cell reproduction by the folic acid antagonist, amethopterin, is prevented by the addition of glycine, a purine, and thymidine to the culture medium.[5] Similarly, in the presence of amethopterin, the P-815-X_2 cells used in our studies exhibit a requirement for glycine, a purine (e.g. hypoxanthine), and thymidine. The addition of amethopterin in combination with glycine and a purine results, therefore, in a specific thymine deficiency and an interruption of the synthesis of DNA; similarly, FUDR acts as a specific inhibitor of the biosynthesis of thymidine 5'-phosphate, and the toxicity of the analogue in mammalian cell cultures is prevented by thymidine.[6] If thymidine is added to the cultures several hours later than the inhibitors, a partial synchronization of cell reproduction is observed.[7–10] After the addition of the inhibitor to cultures of neoplastic mast cells, the mitotic index (percentage of cells in mitosis) of the cell population does not change for approximately 1 hr and then falls to very low values. After thymidine has been added, the mitotic index, with the same lag of 1 hr, begins to rise rapidly and, after 3 to 5 hr, reaches a maximum that exceeds the values observed under steady-state conditions[8] (Fig. 2). From the time parameters of this synchronization the duration of the premitotic period and of the period of DNA synthesis may be determined. The same objection that was mentioned for the radioautographic and microphotometric data, however, is applicable to these synchronization experiments: the possibility that thymidine may be accumulated in the form of precursors of relatively low molecular weight, prior to DNA synthesis, cannot be excluded on the basis of mitotic indices alone. On the other hand, studies of the time course of DNA synthesis in such partially synchronized cultures should provide conclusive evidence as to the existence of the premitotic period. In the present communication, experiments are described in which synchronization of reproductive activity in cell cultures was combined with measurements of the rate of incorporation of thymidine-^{3}H into DNA.

MATERIALS AND METHODS

Cultures *in vitro* of a transplantable murine mast cell tumor (cell line P-815-X_2) were used. This cell line was derived from the original P-815 tumor[11] by a selection and two consecutive cloning procedures.[12, 13] The culture techniques and the medium

have been previously described.[12] Additional components of the medium, per 100 ml of medium, were: L-asparagine (10 μmoles), L-serine (10 μmoles), and Tris buffer (3 ml of 2% Tris in a 0·15 N solution of hydrochloric acid); furthermore, citrovorum factor was replaced by folic acid (10 mg/l), and the horse serum used was dialyzed prior to its addition to the medium. In this medium, generation times (time required for doubling the cell population) of 10 to 18 hr were observed. Cell reproduction in the cultures was determined by hemocytometer counts. In order to measure mitotic indices, the cells were fixed by mixing an aliquot of the suspension culture with an equal volume of ethanol:acetic acid:water (5:2:3, vol/vol); subsequently, the cells were centrifuged and resuspended in a small volume of 0·025% crystal violet in 1% acetic acid; in this suspension the percentage of cells in metaphase and early anaphase (subsequently termed "mitotic index") was determined by hemocytometer counts. The duration of prophase was calculated from the prophase index, observed in asynchronous cultures and found to be much shorter (less than 5 min) than the duration of metaphase-anaphase, which usually ranged from 10 to 12 min.

Partial synchronization of the cultures was obtained as follows: approximately 25×10^6 cells were suspended in 200 ml of medium and incubated at 37°; after 18 hr, 10 ml of a solution containing amethopterin (0·002 mM), hypoxanthine (0·6 mM), and glycine (2 mM) were added to the culture in order to block the synthesis of DNA. After 6 hr, 10 ml of a thymidine solution (0·6 mM, not labeled) were added, and thereafter the mitotic index was measured every 30 min. In order to determine the rate of synthesis of DNA, every 30 min an aliquot of 10 ml (containing approximately $2·5 \times 10^6$ cells) was withdrawn from the culture and incubated for half an hour with 5 μc of thymidine-^{3}H. Since the specific activity of the labeled thymidine used was high (1·9 curies/mmole, obtained from Schwarz Bio-Research, Inc.), the original concentration of thymidine (0·03 mM) in the culture medium was not changed appreciably by the addition of the tracer amounts of labeled compound; immediately after incubation with thymidine-^{3}H the cells were centrifuged and washed repeatedly with cold non-radioactive medium. Subsequently, 2 ml of a cold 5% solution of trichloroacetic acid was added, and the cellular material was fractionated according to the procedure of Schneider;[14] this permitted the separation of thymine derivatives of low molecular weight (acid-soluble fraction) from the products of nucleic acid hydrolysis. The radioactivity of the fractions was measured in a windowless flow counter; before pipetting the samples onto planchettes, the TCA was extracted from the solutions with ether. In order to determine the degree of self-absorption, small amounts of a standard solution of thymidine-^{3}H of high specific activity were added to aliquots of the various fractions prior to drying on the planchettes. Because of the minute amounts of cellular material, counting efficiencies of approximately 20%, as compared with those obtained with "carrier-free" thymidine-^{3}H, were obtained. The results given in Fig. 2 are expressed in terms of percentage of the total radioactivity added to each 10-ml aliquot of the culture; they are from a typical experiment that is representative of several others.

RESULTS

As in our earlier observations,[8] the mitotic activity of the cultures was very low 6 hr after DNA synthesis had been inhibited by the addition of amethopterin. After the addition of thymidine to the inhibited cultures, the mitotic index, as seen in Fig. 2,

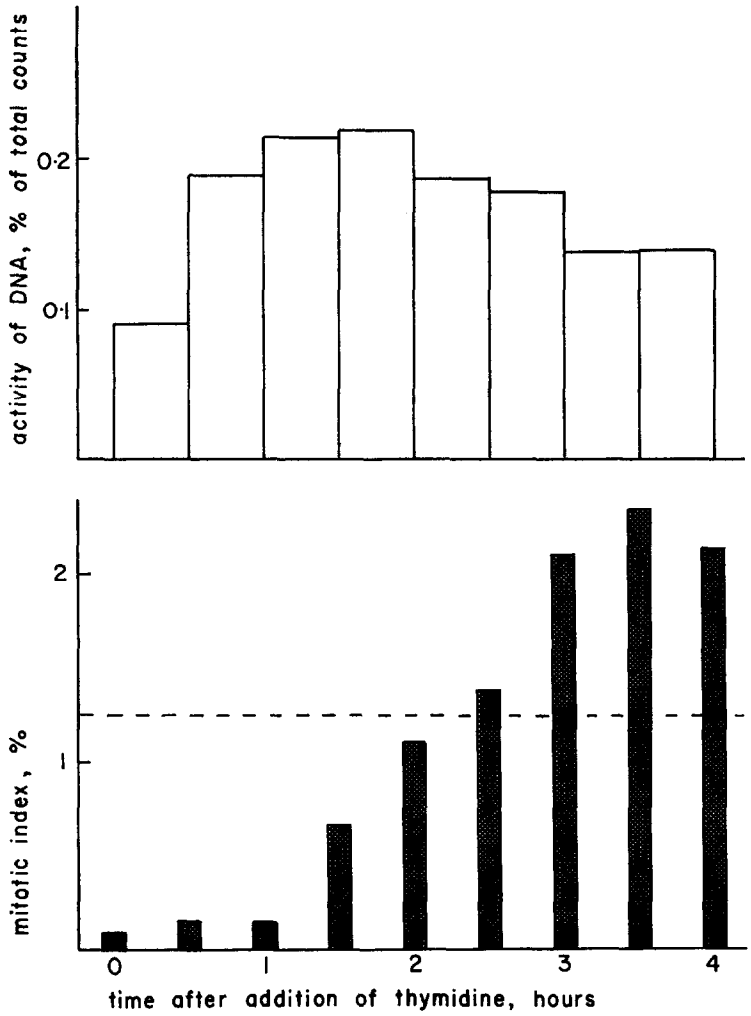

FIG. 2. Time course of mitotic activity and of DNA synthesis in a partially synchronized cell culture. Dotted line: mitotic index before addition of amethopterin.

remained at this low level for 1 hr and then began to rise in a more or less linear fashion during a period of approximately 2 hr. Cell multiplication in the partially synchronized cultures, as determined by cell counts, was resumed after the addition of thymidine at an over-all rate approximately equal to that observed prior to the inhibition by amethopterin (increase in cell number by factors of 2·0 to 3·3 during the 18 hr after the addition of thymidine). Similarly, in control cultures to which amethopterin, hypoxanthine, glycine, and thymidine, at the concentration indicated, were added simultaneously, no inhibition of cell reproduction was observed. In contrast to the mitotic activity, a lag in the incorporation of labeled thymidine into DNA was not observed. As early as 30 min after the addition of (unlabeled) thymidine, the incorporation rate (thymidine-^{3}H incorporated during 30 min) reached

a plateau, which was maintained for about 2 hr; after this time a gradual decline of the incorporation rate was observed, and this may be interpreted as reflecting the partial synchronization of the culture. In an analysis of some samples that were incubated with thymidine-^{3}H for 60 instead of 30 min, the radioactivity found in DNA was approximately equal to the added activities of the two corresponding samples incubated with the labeled precursor during 30 min each. This finding excluded the possibility that the radioactive isotope taken up by the cells might have inhibited further DNA synthesis.

DISCUSSION

The results indicate that the synthesis of DNA was resumed very rapidly when thymidine was added to cultures in which the biosynthesis of thymidine 5′-phosphate had been blocked by amethopterin. On the other hand, mitotic activity appeared in the cultures after a lag period of only 1 hr. Our interpretation of these findings is as follows: the interruption of DNA synthesis by amethopterin causes an accumulation of cells at the end of the G_1-period; as a result, very few cells eventually remain in premitosis and mitosis. Those cells which were in the period of DNA synthesis at the time the inhibitor was added remain in this period with only part of their DNA synthesized. The synchrony of such a culture, therefore, is only partial, because of the distribution of the cell population over the period of DNA synthesis and the end of the G_1-period, but almost complete with respect to the absence of cells in the G_2-period and in mitosis; as soon as thymidine is added to the inhibited culture, however, the synthesis of DNA is resumed without appreciable lag. Those cells which have almost completed DNA synthesis when the inhibitor is added rapidly build the rest of their DNA complement and then enter the G_2-period; after the time required to go through the G_2-period, these cells are the first to enter mitosis. This is reflected in the observed rise of the mitotic index after a lag of 1 hr. Since such a lag in the synthesis of DNA is not observed, it follows that the lag found for the mitotic activity represents the G_2-period.

The lower rate of DNA synthesis during the first 30 min after the addition of thymidine merits further comment. Since the inactive and the labeled thymidine were added simultaneously at the beginning of this first incorporation period, no preformed intracellular thymine derivatives were present that could have diluted the label after its uptake by the cells; in consequence, therefore, a higher rather than a lower incorporation, as compared with the following 30-min periods, should be expected. It appears that thymidine has to trigger some other process required for the synthesis of DNA before this synthesis is carried out. Other experiments in which the DNA content of HeLa cells was determined under similar conditions have led to the same conclusions.[9]

The demonstration of a distinct time interval between DNA synthesis and mitosis raises the question as to the biochemical events taking place during this premitotic period. These processes are of especial interest because they may be intimately related to the mechanisms that are responsible for triggering the onset of mitosis.

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