Absence of DNA Synthesis in One-Half of a Population of Mammalian Tumor Cells Inhibited in Culture by 5-Iodo-2′-Deoxyuridine

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SUMMARY

After a single doubling in culture with 5-iodo-2′-deoxyuridine (2×10^{-4} M), about one-half of a population of murine mast tumor cells (P815Y) fails to carry out DNA synthesis, as evidenced by the lack of grain formation in radioautographs of the cells after incubation in medium with ^{3}H-thymidine. These findings support the conclusion that only about one-half of the hybrid DNA of such a culture can replicate.

In the presence of high concentrations of 5-iodo-2′-deoxyuridine (IUdR) or 5-bromo-2′-deoxyuridine (BUdR), cultured mammalian cells undergo only one doubling of their DNA and of their cell number (1, 2). Although DNA does not increase in amount, we reported recently that DNA synthesis continues in cultures of murine mast tumor cells (P815Y) inhibited by IUdR in such manner that only a single doubling of cell number occurs (1). It was concluded that only about one-half of the hybrid DNA of these cultures can replicate.

The present study was designed further to elucidate these findings by means of radioautography by determining whether all IUdR-treated cells synthesize DNA at about one-half of the normal rate or whether the synthesis of DNA is carried out at a normal rate by only one-half of the cell population.

The culture medium, cell line (murine neoplastic mast cells, P815Y) and general techniques have been described previously (3). Cultures (87,500 cells/ml) in logarithmic growth were either treated or not treated with IUdR (2×10^{-4} M), and the increase in cell number was determined at intervals by means of the Coulter cell counter. In the drug-treated cultures, the cell number doubled exactly once and stopped increasing. Immediately afterward, the cells were sedimented by centrifugation, freed of medium, and quickly resuspended at their original cell concentration in fresh warm medium containing ^{3}H-thymidine (^{3}H-TdR) (Schwarz, 100 μC/μmole, 10^{-5} M final medium concentration). After the cells were incubated with the ^{3}H-TdR for the various times indicated in Table 1, they were sedimented again, resuspended, and washed twice with 0.9% saline solution to remove residual noncellular radioactivity. They were resuspended in a solution of sodium chloride, 0.18%, for 10 minutes to facilitate uncoiling of the chromosomes, fixed in 95% ethyl alcohol:glacial acetic acid (3:1) for 15 min, and then washed twice in the fixative to remove intracellular acid-soluble radioactivity. After the final washing, the cells were sedimented and resuspended in about 0.25–0.5 ml of fixing solution, and about five drops of this mixture were placed on a glass slide and dried in air. The slides were dipped in Kodak NTB Nuclear Track Emulsion and exposed in the dark for periods varying from 3 to 6 days before development. The cells were stained with Wright and Giemsa stains.

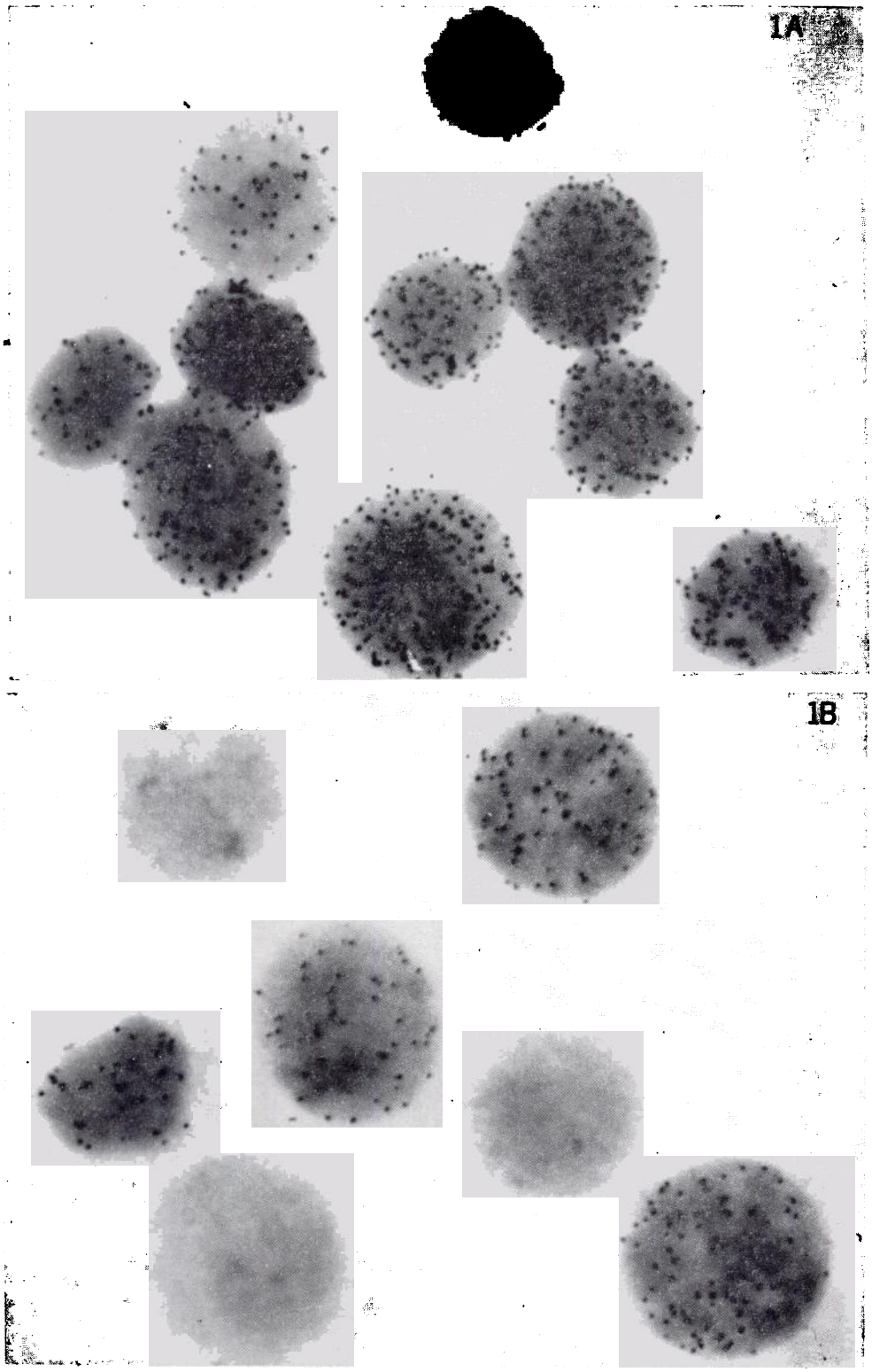

FIG. 1. *Radioautographs of control (1A) and IUdR-treated (1B) cells incubated with ^{3}H-TdR for 13.5 hr after exposure to IUdR*

For the determination of cloning efficiency the cells were harvested by centrifugation at the intervals indicated in Table 2 and prepared for cloning by the method of Schindler (4).

After one division in culture, only about one-half of the IUdR-treated cells given ^{3}H-TdR subsequently formed grains in the photographic emulsion of the radioautographs (Fig. 1B and Table 1). In three different experiments the percentage of drug-treated cells that incorporated the ^{3}H-TdR ranged from about 38% to about 62%, while in the cells untreated with IUdR the values ranged from about 96% to about 99% (Table 1). The number of drug-treated cells that were labeled was neither increased nor altered appreciably by varying the time of incubation in the presence of ^{3}H-thymidine from 5.5 hr to 13.5 hr (Experiment 1, Table 1). The radioactive thymidine was not extensively metabolized by the cells; after 13.5 hr, 77% of the supernatant radioactivity of the medium could be recovered as ^{3}H-TdR by ascending paper chromatography in ethyl acetate : water : formic acid 60 : 30 : 10, a system that separates thymidine, thymine, and dihydrothymine (5).

The amount of DNA synthesized by the IUdR-treated cells that became labeled in the presence of ^{3}H-TdR was essentially the same as that synthesized by the untreated cells. This was established by a comparison of the distribution curves derived from the grain counts of the individual IUdR-treated and control cells. It is seen from Fig. 2 that the treated and control cell cultures both gave essentially the same pattern of distribution of grains per cell.

TABLE 1

Radioautographic labeling with ^{3}H-thymidine of P815Y cells inhibited by IUdR

The cells were placed in fresh medium with ^{3}H-TdR (1×10^{-5} M; 100 μC/μmole) after a single division in medium with 2×10^{-4} M IUdR.

Experiment number	Time of exposure to ^{3}H-TdR (hr)	Number of ^{3}H-TdR-labeled cells[a]	
		IUdR-treated (%)	Control (%)
1	5.5	46.0 (1.0)[c]	96.8 (2.1)[c]
	7.5	NC[b]	95.7 (1.4)
	9.5	50.2 (0.8)	96.0 (1.3)
	10.5	37.9 (1.0)	98.3 (1.6)
	11.5	NC[b]	97.2 (1.5)
	13.5	42.2 (0.8)	98.7 (2.3)
1-TC	12.5	59.2 (1.9)	96.8 (2.1)
3-TC	12.0	61.7 (2.4)	95.2 (0.7)

[a] At least 1000 cells were counted for each time point.

[b] Not counted: the number of cells per slide was too low for counting.

[c] The numbers within parentheses are the percentages of the labeled cells that formed mitoses.

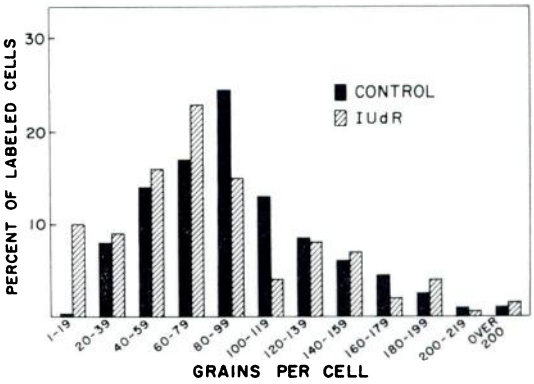

FIG. 2. *Distribution curves derived from the grain counts of individual IUdR-treated and control cells after incubation with ^{3}H-TdR for 13.5 hr*

The mitotic activity of the IUdR-treated cells that labeled with ^{3}H-TdR was somewhat less than that of the control cells (Table 1). No mitoses were observed in the IUdR-treated cells that did not label with ^{3}H-TdR. From this it appears that cells that failed to synthesize DNA subsequently failed to undergo mitosis.

These findings support the conclusion of an earlier report (1), namely, that only about one-half of the gross hybrid DNA of the IUdR-inhibited cell culture can replicate. The new findings demonstrate that the synthesis of DNA is indeed carried out by only one-half of the cells, and that in these cells this occurs at an essentially normal rate. Two schemes possibly could account for this. First, that each cell cultured with IUdR forms two IUdR-labeled cells, only one of which can double its DNA. The replication

scheme[1] of the DNA of such cells is indicated in sequence 1.

$$\boxed{\text{THY-THY}} \xrightarrow{\text{IUdR}} \begin{matrix}\boxed{\text{THY-IU}} \\ \boxed{\text{IU-THY}}\end{matrix} \xrightarrow{\text{THY*}} \begin{matrix}\boxed{\text{THY-THY*} + \text{THY*-IU}} \\ \boxed{\text{IU-THY}}\end{matrix} \tag{1}$$

Second, that about one-half of the cells treated with IUdR form IUdR-labeled cells, both of which can double their DNA. And that the other one-half of the cells form IUdR-labeled daughter cells, neither of which can double its DNA. This scheme is indicated in sequence 2.

$$\begin{matrix}\boxed{\text{THY-THY}} \xrightarrow{\text{IUdR}} \begin{matrix}\boxed{\text{THY-IU}} \\ \boxed{\text{IU-THY}}\end{matrix} \xrightarrow{\text{THY*}} \begin{matrix}\boxed{\text{THY-THY*} + \text{THY*-IU}} \\ \boxed{\text{IU-THY*} + \text{THY*-THY}}\end{matrix} \\ \boxed{\text{THY-THY}} \xrightarrow{\text{IUdR}} \begin{matrix}\boxed{\text{THY-IU}} \\ \boxed{\text{IU-THY}}\end{matrix} \xrightarrow{\text{THY*}} \begin{matrix}\boxed{\text{THY-IU}} \\ \boxed{\text{IU-THY}}\end{matrix}\end{matrix} \tag{2}$$

The net result in either case is the production of the following types of DNA: IU-THY, IU-THY*, and THY-THY*. The proposed schemes correspond well with our previously reported data (1) and account for our finding that the ^{3}H-TdR appears in both the IU-THY DNA and the THY-THY DNA bands in a cesium chloride density equilibrium gradient of the DNA prepared from IUdR-treated cells and that the specific activity of the IU-THY DNA is always about one-half of that of the THY-THY DNA.

The data, however, do not permit any definite conclusions regarding which of the two above schemes may actually represent the pattern of DNA replication in the cell population. It would seem that scheme 1 would be the most likely choice, since scheme 2 would imply that the cell population is divided into two dissimilar halves for no apparent reason. Scheme one also lends itself to the interpretation of Kubitschek on the mechanism of DNA replication, namely, that one strand of the DNA double helix is a master strand controlling the replication of both new strands of DNA, whereas the other strand controls the replication of the messenger RNA (6). If it is assumed that the replication of a specific messenger RNA is required to carry out a second round of DNA replication, and if this RNA is assumed to be transcribed from only one of the two strands, then the strand with THY might yield a true copy of the messenger RNA and the other with IU might not. In other words, DNA synthesis might be blocked in those cells having IUdR substituted for thymidine in the "messenger" DNA strand, but might not be blocked necessarily in those cells substituted in the "master" strand. It should be emphasized, however, that the effect of IUdR substitution on the replication of the genome would be expected to be more complex in the mammalian cell than in bacteria because of the higher ploidy (diploid versus haploid) of the mammalian cell compared with the bacterial cell, the increased number of chromosomes (40 versus 1) and the attendant possibilities for random segregation of the IUdR-substituted chromosomes of the mammalian cell. A simple extrapolation from Kubitschek's conclusions on the mechanism of DNA synthesis in the microbial cell to that of the mammalian cell must be viewed therefore with great caution.

From reports in the literature it is known that only a small fraction of cells survive one division in the presence of BUdR (7). In our experiments the survival of the cells was drastically reduced by exposure to

[1] The DNA of a cell before replication with IUdR is designated THY-THY and after replication with IUdR as THY-IU or IU-THY to designate the lineage of the different DNA strands. Cell lineage is indicated by enclosure of the DNA within boxes. The designation THY-THY or IU-THY is not meant to designate thymine- or iodouracil-thymine base-pairing, but is used to distinguish a thymine-containing strand from an iodouracil-containing strand. A DNA strand replicated with ^{3}H-TdR is designated as THY*.

TABLE 2

Time-dependent decrease in cloning efficiency of P815Y cells after growth with IUdR

The cells were transferred to fresh medium after growth in the presence of 2×10^{-4} M IUdR for the period indicated.

Time of exposure to IUdR (hr)	Treatment of culture	Number of cells per tube	Average number of clones per tube[a]	Cloning efficiency (%)	Normalized cloning efficiency[b] (%)
5	Control	72	49.6	70	100
	IUdR	1800	114.3	6.3	9.0
	IUdR	900	40.8	4.5	6.4
9	Control	72	39.7	55	100
	IUdR	7200	40.3	0.6	1.0
	IUdR	3600	28.0	0.8	1.4
13	Control	72	45.0	63	100
	IUdR	36,000	70.5	0.19	0.3
	IUdR	18,000	23.1	0.12	0.2

[a] Averages from 4 to 6 tubes.

[b] Normalized to a control value of 100%.

IUdR (Table 2). Growth with the drug for only the first 5 hr of the 13-hr doubling period was sufficient to reduce the number of survivors to between 6% and 9% of the population; exposure for the first 9 hr reduced the yield to 1.0%–1.4%; exposure for the full 13-hr period reduced it to 0.2%–0.3%. Presumably, the surviving cells are derived from those cells that can synthesize DNA. In the case of the nonsurviving cells that synthesize DNA, i.e., about 50% of the population, it is clear that DNA production per se does not alone ensure their survival.

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