A STUDY OF NUCLEIC ACID SYNTHESIS IN ASCITES TUMOR CELLS BY TWO-EMULSION AUTORADIOGRAPHY

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INTRODUCTION

Dividing cells go through a mitotic cycle that can be divided into four phases (Lajtha, 1957): (1) a postmitotic gap or rest period, called G_1, which varies from one type of cell to another and may last from a few hours to several months; (2) a period of more uniform duration, during which new deoxyribonucleic acid (DNA) is synthesized, called the S phase; (3) a postsynthetic gap or rest period, called G_2, lasting only a few hours; and (4) a new mitosis, probably lasting from 20 to 40 minutes (Schultze and Oehlert, 1960).

In a given cell population, the number of cells in DNA synthesis at any given time and the median duration of the S phase can be established by autoradiography with tritium-labeled thymidine (Hughes *et al.*, 1958; Quastler and Sherman, 1959). Other metabolic activities in the course of the mitotic cycle, such as synthesis of ribonucleic acid (RNA) or proteins, can be timed in bacteria (Burns, 1959), protozoa, and tissue cultures, which can be made to divide synchronously (Mazia and Prescott, 1954; Rueckert and Mueller, 1960). Synchronous division of mammalian cells *in vivo* has not been obtained yet; but the timing of various metabolic processes during the course of the mitotic cycle can be studied by an autoradiographic technique which allows the simultaneous observation of two distinct metabolic processes in the same cell (Baserga, 1961). Briefly, the technique consists in injecting an animal with two different radioactive precursors, labeled respectively with H^3 and C^{14}. Samples of tissue are then autoradiographed with two layers of sensitized emulsion separated by a thin layer of inert coating. The emulsion layer closer to the specimen will register beta particles both from H^3 and from C^{14} but the second layer, about 5 micra away from the labeled locus, will register only the more energetic C^{14} particles. The present communication reports our studies on the timing of RNA synthesis during the mitotic cycle of the Ehrlich ascites tumor cell.

MATERIALS AND METHODS

Strong A male mice, 6 months old and weighing from 30 to 35 gm, were used. The Ehrlich ascites tumor (EAT) was a hypotetraploid subline which

has been propagated in this laboratory for over five years by weekly intraperitoneal injections to healthy carriers.

The tritiated thymidine and the uridine-2-C^{14} used (both from New England Nuclear Co., Boston) had specific activities, respectively, of 5.21 c/mmole and 0.65 mc/mmole. All injections were given intraperitoneally. The total number of tumor cells was determined by the method of Klein and Revesz (1953); for autoradiographic studies, smears of ascitic fluid were fixed in methanol and autoradiographed by a two-emulsion technique (Baserga, 1961) which is a modification of the technique described by Krause and Plaut (1960). The slides were dipped in NTB emulsion (Eastman Kodak) and exposed for 20 days. After photographic processing, the smears were stained with Mayer's hematoxylin and eosin and then coated with a 1:2 dilution in alcohol-ether of Tissue Embedding Solution A-4700 (Randolph Products Co., Carlstadt, New Jersey). The slides were then dipped again in NTB emulsion, exposed for another 20 days, and finally developed and fixed. The thickness of the first emulsion and of the celloidin coating is not critical, because more than 90 per cent of the beta particles from tritium are arrested by a 1 μ layer of sensitized emulsion. Micrometric measurements have consistently shown that the silver grains in the lower emulsion are separated from the silver grains of the upper emulsion by an interval of 5 to 6 micra.

The percentage of labeled cells was determined on 3000 tumor cells, and the mean grain count per labeled cell on 200 cells in interphase. Only grains directly above the cells were counted. In animals receiving the same treatment, these values are usually within narrow ranges. The percentage of labeled cells, in animals of the same group, varies by not more than 15 per cent on either side of the mean of the group, and the mean grain count, in similar conditions, by not more than 20 per cent. In some experiments, the RNA of the ascitic cells was removed by extraction with 10 per cent perchloric acid for 5 hours at room temperature (Feinendegen *et al.*, 1960), or by digestion with RNase (Swift, 1955).

RESULTS

Specificity of the Label

When smears of EAT cells from mice injected with H^3-thymidine were treated with perchloric acid, the intensity of the label was unaffected. When smears of EAT cells from mice sacrificed 1 hour after injection of uridine-2-C^{14} were extracted with perchloric acid or RNase, about 90 per cent of the label was removed, *i.e.*, the mean grain count dropped from about 30 to 3 grains per labeled cell. This was still above background, which was less than 1 grain per cell. These findings are in agreement with the radiochemical determinations of Feinendegen *et al.* (1961), who found that cells in tissue culture incorporated 20 times more uridine into RNA than into DNA. It was therefore decided to consider a cell RNA-labeled when the grain count was at least 25 per cent of the mean grain count. These corrections have been taken into account in the following paragraphs.

RNA Synthesis during the Mitotic Cycle

Five mice, given an intraperitoneal injection of 2.4×10^6 EAT cells, received on the 5th day of tumor growth a single injection of 30 μc of H^3-thymidine (DNA precursor), followed, after 2 minutes, by an injection of 50 μc of uridine-2-C^{14} (RNA precursor). The animals were sacrificed after 1 hour, the total number of tumor cells was determined, and two-emulsion autoradiographs were made of the ascitic cells. The tumor cells were divided into four groups (Fig. 1): (1) cells with grains limited to the lower emulsion layer; (2) cells with about the same number of grains in both layers; (3) cells with a much higher number of grains in the lower than in the upper emulsion; and (4) unlabeled cells. Cells in groups 1 and 3 were designated as DNA-labeled cells, and those in groups 2 and 3 as RNA-labeled cells.

In this experiment, the number of DNA-labeled cells varied between 52 and 55 per cent. All cells in interphase were RNA-labeled; cells in early prophase were also RNA-labeled, but cells from late prophase to telophase were unlabeled. The EAT cells have a mitotic cycle of about 20 hours, of which 13 hours are spent in DNA synthesis, 6 hours in G_2 and early prophase, and about 1 hour in mitosis (Hornsey and Howard, 1956, confirmed by our own unpublished data). Because EAT cells lack a G_1 period, it is possible to study the rate of RNA synthesis in the G_2 period as compared with the S phase. For this purpose, grain counts were made, in the second emulsion layer registering only the beta particles from uridine-2-C^{14}, of EAT cells divided into two groups: (1) cells labeled with both DNA and RNA precursors, and therefore in the S phase of the mitotic cycle; and (2) cells labeled with RNA precursor only, and therefore in the G_2 plus early prophase stage of the mitotic cycle. The mean grain count per labeled cell was the same in both groups, indicat-

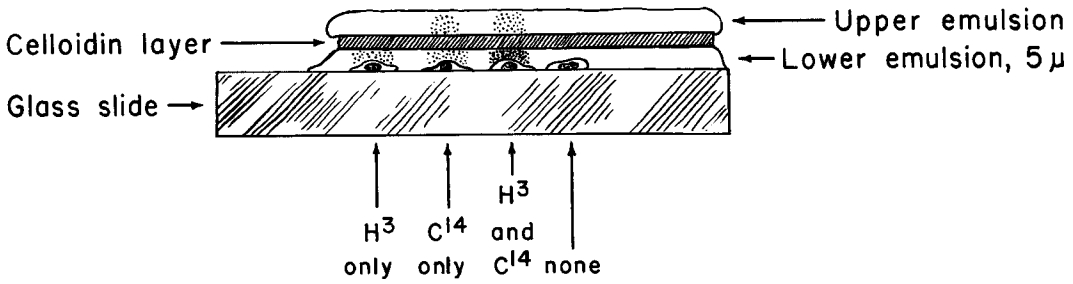

FIGURE 1

Diagram of two-emulsion autoradiography. Four cells are drawn schematically above the glass slide, each cell representing one of the four possible combinations of isotope uptake. The intensity of the label is given by the stippling around and above each cell. Grain counts on each cell are obtained by focusing the microscope alternately on the upper and lower emulsions.

ing that, in EAT cells growing in the peritoneal cavity of the mouse, RNA synthesis proceeds at the same rate from completion of mitosis to early prophase.

RNA Synthesis at Different Stages of Tumor Growth

It is known that the EAT, injected into the peritoneal cavity of mice, grows exponentially for about a week or until the tumor cells in the peritoneum number about 600 × 10^6, then grows slowly until it reaches a practical standstill when the tumor cells number about 2 × 10^9 per mouse (Klein and Revesz, 1953; Patt and Blackford, 1954). In order to study DNA and RNA synthesis in these various phases of tumor growth, 15 mice were injected with 2.1 × 10^6 EAT cells intraperitoneally. They were then randomized into five groups that were sacrificed at various intervals after tumor inoculation, as shown in Table I. One hour prior to sacrifice, the mice received 30 μc of tritiated thymidine and 50 μc of uridine-2-C^{14}, 2 minutes apart. The number of tumor cells per mouse was determined, and two-emulsion autoradiographs were made of smears. The results are shown in Table I. It can be seen that, whereas DNA synthesis, and therefore cell division, decreases progressively, RNA synthesis remains practically constant for the duration of the experiment. Grain counts on RNA-labeled cells on the 13th day showed that there was no difference in the rate of RNA synthesis between cells that were only RNA-labeled and those that were also DNA-labeled.

TABLE I

Uptake of H^3-Thymidine and Uridine-2-C^{14} by Ehrlich Ascites Tumor Cells at Various Intervals after Inoculation into the Peritoneal Cavity of Mice

		DNA-labeled cells		RNA-labeled cells	
Days after inoculation	No. of tumor cells per mouse × 10^6	Per cent labeled	Mean grain count per labeled cell	Per cent labeled	Mean grain count per labeled cell
4	73.5	54	56	98	22
5	159.0	54	61	98	17
7	618.0	47	40	97	18
10	1,315.0	33	19	98	19
13	1,758.0	19	10	97	23

DISCUSSION

Two-emulsion autoradiography allows the simultaneous investigation, in the same cell, of two distinct metabolic processes. In the present study, the number of cells synthesizing RNA and the rate of RNA synthesis in the various phases of the mitotic cycle were studied in Ehrlich ascites tumor cells growing asynchronously in the peritoneal cavity of mice.

The results indicate that, in EAT cells growing exponentially, RNA is synthesized continuously and at the same rate for the entire length of the mitotic cycle, with the exception of the brief period extending from late prophase to telophase. However, as the EAT cells lack a postmitotic rest period (G_1), the present results simply indicate

that RNA synthesis continues at the same rate from the beginning of DNA synthesis to early prophase.

The results of our studies on different phases of tumor growth show that, whereas DNA synthesis decreases rapidly after the cessation of the exponential phase of tumor growth, RNA synthesis continues at the same rate for the duration of the experiment. It has often been assumed that the declining rate of tumor growth is due to a general stopping of the metabolic processes of the cells, caused by a lack of nutrients providing energy and building blocks for metabolic and synthetic activities (Klein and Revesz, 1953; Patt and Straube, 1956). The present results are at variance with such a hypothesis, as it would be difficult to conceive that tumor cells may be lacking nutrients and energy for DNA synthesis but not for RNA synthesis, when the two processes have very much the same energy requirements (Kornberg, 1960; Ochoa *et al.*, 1961). As noted above, on the 13th day of tumor growth, when DNA synthesis is considerably reduced, the rate of RNA synthesis, as judged by grain counts, is similar in the few cells that are also synthesizing DNA and in those that are not. This further confirms that it is not a general lack of nutrients that determines the decrease in DNA synthesis. In this respect, EAT cells on the 13th day of tumor growth behave like bacteria and tissue culture cells growing in a thymidine-free medium (Barner and Cohen, 1956; Burns, 1959; Rueckert and Mueller, 1960): in thymidine-deficient cultures of bacterial or mammalian cells, DNA synthesis stops while other synthetic activities, including RNA synthesis, continue at an unchanged rate. Similarly, when EAT cells are irradiated *in vivo*, DNA synthesis decreases although RNA and protein synthesis are unaffected (Klein and Forssberg, 1954).

SUMMARY

Ehrlich ascites tumor cells from mice injected simultaneously with H^3-thymidine and uridine-2-C^{14} were autoradiographed by a two-emulsion technique that allows one to distinguish cells labeled with H^3 only from those labeled with C^{14} only or with both H^3 and C^{14}. With the use of the above-mentioned radioactive compounds, it was possible to distinguish cells synthesizing DNA only from those synthesizing only RNA or both RNA and DNA. It was found that in tumor cells, in the exponential phase of growth, the uptake of RNA precursor (uridine-2-C^{14}) proceeds at the same rate for the entire length of the mitotic cycle, with the exception of a brief period from late prophase to completion of telophase. At the cessation of the exponential phase of tumor growth, DNA synthesis, as revealed by the uptake of H^3-thymidine, decreases rapidly, but RNA synthesis continues at the same rate at least until the 13th day after an inoculum of 2 million tumor cells.

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