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*INHIBITION OF DNA SYNTHESIS IN EHRLICH ASCITES CELLS BY ACTINOMYCIN D, II. THE PRESYNTHETIC BLOCK IN THE CELL CYCLE**

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The interval between midpoint of mitosis in the parent cell and midpoint of the subsequent mitosis in the daughter cell is called the cell cycle[1] and is schematically represented in Figure 1. After completion of mitosis, the cell enters a presynthetic phase, the G_1 phase. It then synthesizes DNA (S phase), and when DNA synthesis ceases, it goes through a premitotic phase G_2 which is followed by a new mitosis, M.

In a previous paper,[2] we have shown that small doses of actinomycin D (0.016 μg/gm body weight) produce two distinct effects in Ehrlich ascites cells growing in the peritoneal cavity of mice: (1) a prompt 50 per cent inhibition of RNA synthesis; and (2) a delayed 50 per cent inhibition of DNA synthesis. The present communication shows that the delayed inhibition of DNA synthesis is due to a presynthetic block in the G_1 phase of the cell cycle and that the RNA mostly affected by these small doses of actinomycin D is ribosomal RNA. The over-all results then indicate that there is an actinomycin D-sensitive step in the G_1 phase of the Ehrlich ascites cells and that inhibition of this step prevents the entrance of tumor cells into the S phase of the cell cycle.

Methods and Materials.—These have been described in detail in the previous paper.[2] All experiments were performed on 5-month-old Strong A female mice on the 6th day after an intraperitoneal injection of Ehrlich ascites cells.

Autoradiography: Smears of Ehrlich ascites cells were fixed in methanol and autoradiographs

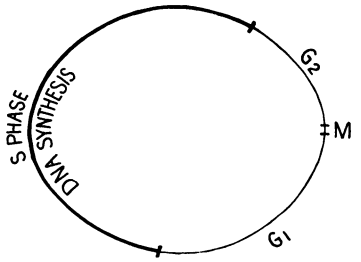

FIG. 1.—The cell cycle (see explanation in text).

were prepared by the dip-coating method of Joftes and Warren[3] using Eastman Kodak NTB emulsion. The percentage of labeled cells and the mean grain count per labeled cell were determined as previously described.[4]

Sucrose gradient analysis of RNA: For these studies, nuclei, ribosomes, and 105,000 × *g* supernatants were prepared according to the method of Hecht *et al.*[5] Packed tumor cells were homogenized in distilled water containing 1% bentonite, and concentrated solutions were added to the homogenate to give a final concentration of 0.25 *M* sucrose, 0.005 *M* $MgCl_2$, 0.025 *M* KCl, and 0.05 *M* Tris buffer, pH 7.6. The homogenate was centrifuged for 10 min at 600 × *g* and the pellet constituted the nuclear fraction. The supernatant was centrifuged for 2 hr at 105,000 × *g*, the sediment constituted the ribosomal fraction, and the supernatant the 105,000 × *g* fraction. RNA was extracted from each of these fractions according to the method of Hiatt.[6] Nuclei were resuspended and homogenized in 3 vol of a solution containing 0.03 *M* Tris buffer, pH 7.6, 0.25 *M* sucrose, 0.003 *M* $CaCl_2$, 10 μg/ml of sodium dextran sulfate, and 1% bentonite. Sodium dodecyl sulfate was added to the homogenate to a concentration of 0.4%, and after addition of an equal volume of 90% aqueous phenol containing 0.1% 8-hydroxyquinoline, the mixture was stirred mechanically at 4°C for 30 min. Following centrifugation, the aqueous phase was re-extracted with $^1/_2$ vol of phenol, and RNA was precipitated with sodium chloride and ethanol added to final concentrations of 0.1 *M* and 67%, respectively. The precipitate, redissolved in 0.01 *M* Tris, pH 7.4, containing 0.001 *M* $MgCl_2$, was treated with pancreatic DNase, 5 μg/ml, and the RNA purified as described by Hiatt.[6] Linear sucrose gradients, 5–20% in Tris, pH 7.4, 5 × 10^{-3} *M* containing 10^{-4} *M* Mg acetate, were prepared in 5-cc cellulose tubes by the method of Britten and Roberts,[7] and about 5 OD units of purified RNA were layered on top of the tube. The tubes were centrifuged at 39,000 rpm for 4 hr in the SW 39 rotor of the Spinco model L centrifuge. About 30 fractions were collected from the punctured bottom of the tube, and the OD and radioactivity of each fraction were determined as described by Starr and Fefferman.[8]

Materials: Actinomycin D (a gift of Merck, Sharp and Dohme) was dissolved in saline. The following radioisotopes were used: thymidine-methyl-H^3, 6.7 c/mM; cytidine H^3, 3.68 c/mM; uridine-H^3, 3.73 c/mM; orotic-6-C^{14} acid hydrate, 5 mc/mM; orotic-5-H^3 acid, 255 mc/mM (all from New England Nuclear Corp.); and uridine-2-C^{14} (Schwarz BioResearch), 125 μc/mg.

All injections were given intraperitoneally. RNase-free DNase was purchased from Worthington. All other chemicals were of reagent grade.

Results.—Groups of tumor-bearing mice received, every 2 hr, intraperitoneal injections of actinomycin D, 0.5 μg per injection (0.016 μg/gm body weight).[2] At intervals from 30 to 750 min after the first injection of actinomycin D, groups of mice were killed and 0.5-cc aliquots of Ehrlich ascites tumor were incubated for 30 min at 37°C either with 1 μc of H^3-thymidine or 2 μc of H^3-cytidine.[2] At the end of the incubation period, smears of tumor cells were made for autoradiography.

In Figure 2 the line with open triangles indicates that the mean grain count per labeled cell, as determined by autoradiography, is essentially the same in control and actinomycin-treated animals. The solid line with open circles, however, shows that the percentage of cells labeled by a 30-min exposure to H^3-thymidine begins to decrease at 270 min after the first injection of actinomycin D and reaches a minimum (48% of control value) at 510 min. This curve parallels the curve obtained by determining the uptake of H^3-thymidine into DNA and given in the previous paper.[2] These results, i.e., decreased incorporation of H^3-thymidine into DNA and decreased percentage of labeled cells, together with an unchanged thymidylic acid pool and an increase in thymidine kinase activity, as described in the previous paper,[2] support the conclusion that actinomycin D, after a lag period of

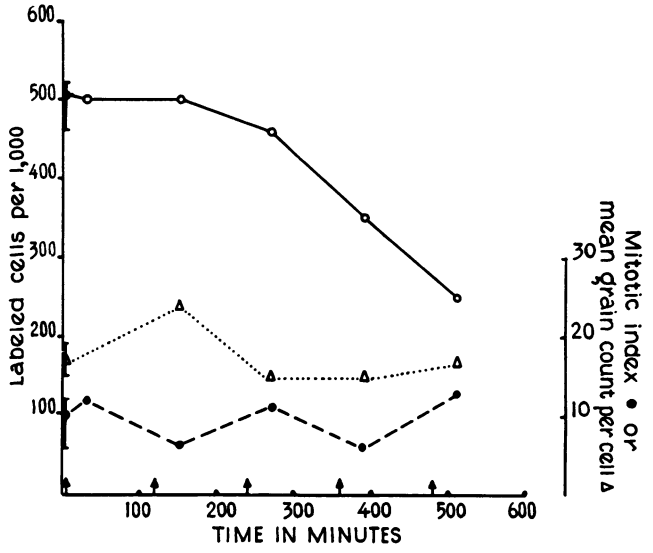

FIG. 2.—Effect of actinomycin D on the flow of Ehrlich ascites cells along the cell cycle. On the abscissa is time in minutes. O——O, Percentage of cells labeled by a pulse-exposure to H^3-thymidine; Δ....Δ, mean grain count per labeled cell; ●----●, mitotic index. The arrows indicate time of actinomycin D injections.

about 3 hr, inhibits DNA synthesis in Ehrlich ascites tumor by decreasing the number of cells synthesizing DNA. The rate of DNA synthesis in cells still capable of synthesizing DNA is apparently unaffected by actinomycin D, as indicated by the fact that the mean grain count per labeled cell is the same as in control animals.

Effect of actinomycin D on the various phases of the cell cycle: In theory, a decrease in the percentage of cells labeled by a brief exposure to H^3-thymidine can be produced by a metabolic block in any phase of the cell cycle represented in Figure 1. For instance, a mitotic block, in which the cells are arrested in metaphase, can eventually lead to a decrease in the number of cells synthesizing DNA by simply interrupting the flow of cells from mitosis to G_1 and therefore to S. To distinguish between the various possibilities, the following determinations were carried out: (*a*) mitotic index; (*b*) percentage of labeled mitoses at various intervals after pulse labeling with H^3-thymidine; and (*c*) percentage of tumor cells labeled by two pulse-exposures to H^3-thymidine. It should be noted at this point that the doubling time of Ehrlich ascites tumor growing in the peritoneal cavity of Strong A female mice is 36 hr.[4] On the 6th day of growth, 50–55 per cent of the tumor cells are labeled by a single injection of tritiated thymidine, and all cells can be labeled by repeated injections of tritiated thymidine properly spaced over a period of 20 hr. The G_2 period is 5.5 hr, and mitosis, calculated from the mitotic index, lasts 0.5 hr. These data allow us to estimate T_c, the length of the cell cycle, as 36 hr; T_s, the length of S phase, 19 hr; and T_{G_1} 11 hr. These estimates take into account the corrections for exponential growth, as given by Johnson[9] and by Puck and Steffen.[10]

(a) *Mitotic index:* Figure 2 (dashed line with closed circles) shows that the mitotic index (mitoses per 1,000 cells) of Ehrlich tumor cells does not change appreciably after actinomycin D.

(b) *Percentage of labeled mitoses:* Ehrlich ascites tumor cells were labeled by a

TABLE 1

PERCENTAGE OF LABELED MITOSES IN EHRLICH ASCITES CELLS 390 MIN AFTER A SINGLE INJECTION OF H^3-THYMIDINE*

Interval between H^3-Tdr and act. D	Per cent of labeled mitoses mean (range)
No act. D	61 (50–80)
30 Min	71 (50–94)
30, 150, 270, and 390 Min	72 (58–84)

* 20 μc of H^3-Tdr per mouse. Actinomycin D, 0.016 μg/gm body weight. See text for details.

single intraperitoneal injection of H^3-thymidine. Some mice were then injected with actinomycin D (with the schedule and dose given in Table 1), and others were kept as controls. The animals were killed 390 min after H^3-thymidine, and the percentage of labeled mitoses was determined by autoradiography. The results (Table 1) show no appreciable differences in the percentage of labeled mitoses between controls and actinomycin-treated animals, indicating that actinomycin D in these amounts does not block the flow of cells through the G_2 period. Together with the mitotic index, these findings indicate that the flow of cells through G_2 and mitosis is not affected by actinomycin D at the dose level used in the present experiment.

(c) *Percentage of tumor cells labeled by two pulse-exposures to H^3-thymidine:* The experiments described under (*a*) and (*b*) and the fact that the number of cells in DNA synthesis does not decrease immediately after actinomycin D suggest that the metabolic block may take place in the G_1 period. However, these findings can also be explained in the following manner: if actinomycin D inhibits the synthesis of a metabolite that is necessary to the continuation of DNA synthesis (S phase), and if the need for this metabolite becomes apparent only after 4 hr, then cells in the late part of the S phase will not be able to synthesize DNA, while G_1 cells may continue to flow at a regular rate into S. The result will be a decrease in the number of cells in DNA synthesis, undistinguishable from a G_1 block. The following experiment was used to decide between the two alternatives. Groups of animals were injected with H^3-thymidine, and after 30 min the number of labeled cells was determined by autoradiography on a sample of ascites tumor aspirated with syringe and needle from the peritoneal cavity. The animals were then divided into groups: one group received actinomycin D, and the other group served as control. Eight hours after the first injection of H^3-Tdr, all mice were reinjected with H^3-Tdr, killed after 30 min, and autoradiographs were prepared as above. The results are shown in Table 2. In the interval between the two injections of H^3-thymidine, cells in S phase have gone into G_2, while G_1 cells

TABLE 2

EFFECT OF ACTINOMYCIN D ON THE PERCENTAGE OF EHRLICH ASCITES CELLS LABELED BY TWO PULSE-EXPOSURES TO TRITIATED THYMIDINE*

	Labeled Cells per 1,000	
	After one injection	After two injections
Controls	550 (520–580)	708 (685–720)
Act. D	545 (490–600)	588 (505–650)

* Tumor-bearing mice were injected with 10 μc of H^3-Tdr, and an aliquot of tumor was aspirated from the peritoneal cavity after 30 min. After another 30 min, half of the animals were injected with actinomycin D (0.016 μg/gm body weight); injections were repeated every 2 hr for a total of four injections. One hour after the last injection of act. D, all mice were reinjected with 10 μc of H^3-Tdr, and killed after another 30 min. Smears for autoradiography were prepared from each mouse after the first injection of radioisotope and again after killing.

have entered S. The number of cells labeled by two injections of H^3-thymidine is equal to the number of cells labeled by the first injection plus the number of cells that have entered the S phase in the interval between the two injections. With an interval between the two injections of 8 hr, on the basis of the cell cycle times given above and using the equation Appendix (4) of Puck and Steffen,[10] one can calculate that the percentage of labeled cells in control mice should increase from 550 per 1,000 after the first injection to 760 per 1,000 after the second injection. If actinomycin D inhibits cells in the late phase of S, the percentage of labeled cells would be approximately equal to the controls, since cells in late S have been labeled by the first injection and the flow of cells from G_1 to S is not affected. If the block were in G_1, the number of labeled cells would increase only slightly, from 550/1,000 to 590/1,000. The results in Table 2 indicate that the percentage of labeled cells in actinomycin-treated animals increases only slightly from 545/1,000 after the first injection to 588/1,000 after the second injection. The increase in the control animals is less than the prediction would call for, but it can be explained on the basis of a slight degree of synchrony of the Ehrlich cells (unpublished data). The results, therefore, indicate that actinomycin D blocks the cells in the G_1 period.

Nature of RNA whose synthesis is inhibited by actinomycin D: In this experiment, tumor-bearing mice treated with actinomycin D were given, 30 min after actinomycin, an injection of 100 μc of uridine-H^3, while untreated mice were given 25 μc of uridine-2-C^{14}. Actinomycin injections were repeated in the former group every 2 hr, and all mice were killed 4 hr after the radioisotope. Treated and untreated tumors were homogenized together, and the RNA was extracted by the phenol method from three subcellular fractions: nuclei, microsomes, and 105,000 $\times$ g supernatant. The sucrose gradients in Figure 3, therefore, are a combination of treated and untreated tumors. The OD profiles belong to both, the H^3 radioactivity is from the actinomycin-treated RNA, and the C^{14} radioactivity from the control RNA. The results (Fig. 3*a*, *b*, *c*) indicate that the small amounts of actinomycin D used in this investigation inhibit the synthesis of ribosomal RNA in both the nuclear and microsomal fractions. Similar results were obtained using orotic acid as the RNA precursor, but only the sucrose gradient of ribosomal RNA is shown in Figure 3*d*.

Discussion.—The results indicate that there is a step in the G_1 phase of Ehrlich ascites tumor cells that is sensitive to very small amounts of actinomycin D (0.016 μg/gm body weight). Inhibition of this step causes an arrest of cells in the G_1 period, while the DNA synthesis phase, the G_2 period, and mitosis are not directly affected. Eventually, the G_1 block prevents cells from entering the S phase and thus causes a decrease in the number of cells in DNA synthesis. This G_1 block, on the basis of cell cycle times and Figure 2, can be located at a point about 2 hr before the initiation of DNA synthesis. Since actinomycin D is considered a specific inhibitor of DNA-dependent RNA synthesis,[11] one can speculate that the G_1 step whose inhibition prevents the entrance of cells into the S phase can be identified with the synthesis of some kind of RNA. If this is correct, the results of sucrose gradient analysis indicate that the RNA most inhibited by these small amounts of actinomycin D is ribosomal RNA. A similar situation has been described by Lieberman and his associates[12, 13] in rabbit kidney cells cultured directly from the animal and in regenerating rat liver. In primary explants from rabbit kidney cor-

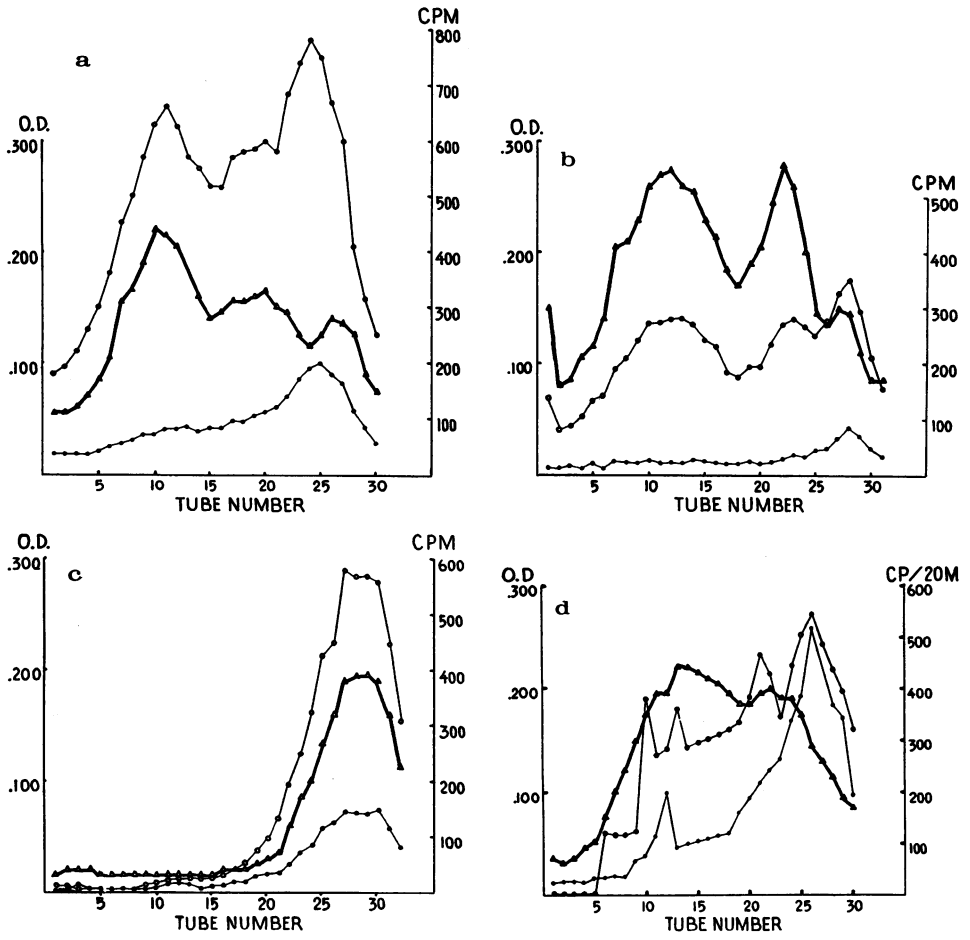

FIG. 3.—Sucrose gradient analysis of RNA phenol-extracted from Ehrlich ascites cells (see text for explanation). Δ, Optical density; ○, C^{14} radioactivity; ●, H^3 radioactivity. (*a*) Nuclear RNA labeled with uridine; (*b*) ribosomal RNA labeled with uridine; (*c*) sRNA labeled with uridine; (*d*) ribosomal RNA labeled with orotic acid.

tex, cells underwent a lag period of about 32 hr before DNA synthesis began. RNA synthesis during this lag period could be divided into three stages: a first stage (from 0 to 12 hr) characterized by an increase in the formation of stable ribosomal RNA, a second stage (from 12 to 20 hr) in which there was a sharp increase of the turnover rate of nuclear RNA, and a third stage (from 20 to 32 hr) in which RNA synthesis leveled off at its new, elevated rate. The second stage could be blocked by low doses of actinomycin D, and inhibition of this second stage prevented the cultured cells from entering DNA synthesis at 32 hr. Substantially the same sequence of events was described in the regenerating rat liver by Lieberman and his associates.[14]

The inhibition of ribosomal RNA synthesis in these experiments is in agreement with the findings of Perry *et al.*,[15] of Samarina,[16] and of Harel *et al.*[17] that small doses of actinomycin D selectively inhibit ribosomal RNA synthesis. These findings are also in agreement with those of Tsukada and Lieberman,[18] who found an increase of RNA polymerase activity in liver nucleoli after partial hepatectomy. Nucleoli

are considered the site of ribosomal RNA synthesis.[15, 19, 20] It is difficult to visualize at the present moment why ribosomal RNA should be necessary to the initiation of DNA synthesis. However, Levine *et al.*[21] reported an almost complete disappearance of polyribosomes in tissue cultures of human fibroblasts in which cell division and DNA synthesis were markedly depressed, and Colobert and Louisot[22] found that the peaks of ribosomal RNA were absent from sucrose gradient analyses of KB cells in the stationary phase and reappeared when cell division was resumed.

The results leave little doubt that low doses of actinomycin D block Ehrlich cells in the G_1 phase, while the other phases of the cell cycle are not affected. However, larger doses of actinomycin D can produce a metabolic block in the G_2 stage, at least in tissue cultures.[23]

The present communication confirms in tumor cells the presence of an actinomycin D-sensitive step, previously shown in tissue cultures and in regenerating liver,[12–14, 24] which precedes DNA synthesis. In addition it gives the first demonstration that this step is temporally located in the G_1 phase of the cell cycle.

Summary.—Mice bearing intraperitoneal Ehrlich ascites tumors were injected every 2 hr with actinomycin D (0.016 μg/gm of body weight). A 50 per cent inhibition of RNA synthesis was evident within 30 min after the first injection of actinomycin D, whereas DNA synthesis was not affected for 270 min and reached a maximum inhibition only after 510 min. Autoradiographic studies indicated that actinomycin D, at this low level, inhibited a step in the G_1 phase of the cell cycle, preventing the initiation, but not affecting the continuation, of DNA biosynthesis. The activity of thymidine kinase and DNA polymerase was not affected under these conditions. Sucrose gradient analysis indicated that actinomycin D had inhibited the synthesis of ribosomal RNA. The results can be interpreted as indicating that in the G_1 phase of the Ehrlich ascites cells is an actinomycin-sensitive step whose inhibition prevents the entrance of cells into the DNA synthesis phase.

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THE REGULATION OF NUMBERS OF PRIMITIVE HEMOPOIETIC CELLS

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It is generally accepted that blood cells are derived from a species of primitive undifferentiated cell in the bone marrow which are usually designated as stem cells. Morphological characterization of stem cells is impossible on other than arbitrary, authoritarian grounds, since the stem cell can be identified morphologically only by the absence of any feature characteristic of one of the many cell lines giving rise to mature blood cells. So long as such a negative definition must be employed, any primitive cell tentatively identified as a stem cell may already be a differentiated cell insofar as yet unknown genetic and biochemical processes of cell differentiation have already occurred, although differentiation has not yet reached the point where morphological characteristics can be observed. Investigations of stem cells dependent on morphological features therefore appear destined to be limited and suspect.

The properties, characteristics, and biological features of stem cells are, however, too important to await advances in histological and histochemical techniques, and in recent years, functional characteristics of stem cells have come under investigation. These studies have all been consequences of the monumental experiments of Jacobson *et al.*[1] in which animals receiving otherwise lethal doses of ionizing irradiation survived, provided that the spleen was shielded from injury. It is likely that the mechanism underlying this survival is the recolonization of the system by primitive cells carried in the bloodstream from the protected spleen.[2, 3] A host of radiobiological investigations and a vast literature, culminating in the classic demonstration of spleen cloning, have been direct outgrowths of these experiments. McCulloch and Till[4] first demonstrated that stem cells, or colony-forming units, may be assayed by the number of discrete clones demonstrable in the spleens of lethally irradiated mice ten days after irradiation. Over a wide range, the number of clones is proportional to the number of normal bone marrow cells injected after irradiation.

Another method of investigating primitive marrow cells has been developed in our laboratory.[5] Since morphological evidences of erythropoiesis disappear in the transfusion-induced plethoric mouse, and are manifest again after a single injection of erythropoietin, it was concluded that erythropoietin acts upon stem cells, inducing