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DNA Synthesis and Incorporation of P^{32} in Irradiated Ehrlich Ascites Cells.* (22861)

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The experiments to be reported are part of a series of studies on the disturbances of desoxyribose nucleic acid (DNA) metabolism after irradiation. The interpretation of biochemical changes observed in irradiated mammalian tissues is complicated by the fact that they are generally composed of mixed cell populations(1). To circumvent this difficulty, many investigators have recently used ascites tumors since they can be obtained as almost pure cell suspensions(2,3,4).

It has been suggested(5,6,7) that while incorporation of precursors into DNA normally occurs only at the time of DNA synthesis, incorporation after irradiation might be due to turnover of existing DNA not accompanied by net synthesis. This seemed an important point which warranted further investigation.

Materials and methods. Ehrlich ascites tumor used was kindly donated by Dr. E. N. Sassenrath and had been carried in C_3H mice for several years. For each experiment 20 to 30 C_3H mice were inoculated intraperitoneally with ascitic fluid containing approximately 2 x 10^7 cells. All measurements were carried out between the fourth and sixth day after transplantation. Irradiated and control groups were always run simultaneously, and a minimum of 5 but frequently 10 mice were used for each point. The total body irradiation (800 r or 1400 r) was carried out as previously described(1). Cell number per mouse for the growth curves was calculated from the total volume of ascitic fluid and cell concentration in the ascitic fluid. To determine the volume of ascitic fluid 2 μc of radioiodinated (I^{131}) serum albumin (RISA, Abbott Laboratories) were injected intraperitoneally after the mouse had been sacrificed. After a 3 minute mixing period, ascitic fluid was withdrawn and counts in a 20 μl aliquot were compared to the injected dose. Cell concentration was determined by counting duplicate dilutions in a hemocytometer. DNA content and total nucleic acid content were determined in duplicate aliquots of ascitic fluid from each mouse. After the cells were centrifuged, their nucleic acids were extracted by the hot perchloric acid method of Ogur and Rosen(8). Total nucleic acid content of the extract was determined by ultraviolet absorption and the DNA content by Ceriotti's indole method(9). For calculation of mean nucleic acid content per cell the concentration of tumor cells was determined by "large cell counts"(10) in a hemocytometer. Since the proportion of non-tumor cells was consistently low (around 5% as determined by enumeration of small cells in the hemocytometer or differential counts on stained smears), no correction was made for their presence. Mean cell volume was calculated from the cell concentration and the "hematocrit" as measured in micro-hematocrit capillary tubes. Separate groups of mice were used for the incorporation studies. They were injected intraperitoneally with 20 μc $Na_2HP^{32}O_4$ at varying times after irradiation together with unirradiated controls and sacrificed 2 hours after injection. Ascitic fluid from 2 mice was pooled, and the cells sepa-

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TABLE I. Representative Values* from Experiments on Ehrlich Ascites Tumor 4 Days after Transplant.

DNA/cell, pg	Total nucleic acid/cell, pg	Cell vol, μ^3	No. of mice used	No. of determinations
24.9 ± .6	60.7 ± 1.2	2242 ± 61	5	10

DNA specific activity†	DNA specific activity as % inorganic phosphate	DNA specific activity as % acid soluble organic phosphate	No. of mice used	No. of determinations
.272 ± .012	3.54 ± .24	6.05 ± .57	10	5

* Mean values ± stand. error of mean.
† Specific activity expressed as counts/min./mg phosphorus divided by c.p.m. inj./g mouse. Two-hr P^{32} incorporation period.

rated from supernatant fluid. The cells were homogenized in 10% trichloroacetic acid (TCA) for extraction of acid soluble phosphorus compounds. The inorganic phosphate was precipitated from the TCA extract by the method of Le Page(11) and the remaining fraction was designated as the organic acid soluble fraction. DNA was isolated from the original TCA precipitate by a previously published method(12), and specific activities were determined. DNA content of individual nuclei was measured microspectrophotometrically on Feulgen stained smears by a previously described technic(13). Sixty cells were measured in both irradiated and control groups.

Results. Table I lists representative control values for mean DNA content and total nucleic acid content per cell and mean cell volume. Cell volume and total nucleic acid content per cell agree well with values reported by Klein and Forssberg(2), but the DNA content is considerably higher. Both the biochemical determinations and the Feulgen data (Fig. 3) yield a mean DNA content approximately 70% above the theoretical tetraploid DNA value. It is not known whether this high value is due to a large number of cells which are in the process of DNA synthesis or whether it represents a high proportion of hypertetraploid cells in our tumor line.

Table I also lists the DNA specific activity after a 2-hour P^{32} incorporation period, expressed in absolute units and as percent of the specific activity of intracellular inorganic phosphate and acid soluble organic phosphate.

In a few experiments DNA specific activity was determined 2, 4, and 6 hours after administration of P^{32}. When rate of formation of DNA was calculated using the specific activity of acid soluble organic phosphate as the precursor specific activity, the results were somewhat variable but averaged 1.7% per hour.

In most experiments 800 r total body irradiation was administered on the fourth day after transplanting when the cell number per mouse was approximately 3 x 10^8. During the 2 days following irradiation the cell number did not change measurably, while it increased in controls at the rate of 1.5% per

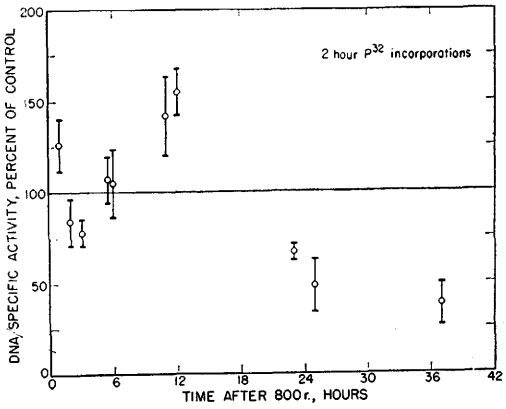

FIG. 1. 2-hr incorporation of P^{32} into DNA at varying times after radiation.*

* Values are mean specific activities as % of control. Vertical lines represent stand. errors of the ratios.

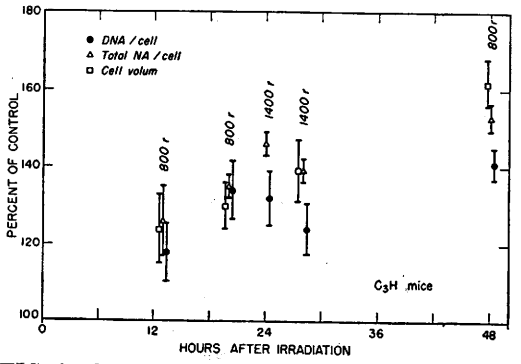

FIG. 2. Mean nucleic acid contents/cell* at varying times after radiation.

* All values expressed as % of controls. Vertical lines represent stand. errors of the ratios.

hour. At 4 days the control mitotic index was 1.2 ± 0.2%. After irradiation no mitotic figures were seen for the 2-day experimental period.

Fig. 1 represents DNA specific activities measured when P^{32} was injected at varying times after 800 r and the mice sacrificed 2 hours later. Each point is the mean of 5 determinations (10 mice) expressed as percentage of the control value measured simultaneously. As is evident from the standard error of the ratio there was considerable variability. However, the data indicate that the P^{32} incorporation into DNA was essentially normal for the first few hours after irradiation and above normal at 12 hours. Between 23 and 36 hours after irradiation the DNA specific activity was depressed to about half the control value. No significant alteration in the specific activity of cellular inorganic phosphate or organic acid soluble phosphate was observed throughout this time.

If the normal rate of incorporation of P^{32} into DNA after irradiation represented synthesis of DNA, there should be an increase in the total amount of DNA in the tumor. However, since there was no cell division and no increase in cell number during this time, the amount of DNA per cell should increase. Fig. 2 presents results of biochemical determinations of the mean DNA content per cell at several time intervals after 800 r. It also includes the mean total nucleic acid content per cell and mean cell volume. All values are expressed as percentage of controls measured simultaneously. A clear increase in all 3 quantities was observed after irradiation. The increase in mean cell volume and mean nucleic acid content per cell agree with the observations of Klein and Forssberg(2). Since the pronounced increase in the mean amount of DNA per cell did not agree with their findings (when they used 1200 r), 2 further experiments were performed with 1400 r which are included in Fig. 2.

The rate of increase in mean DNA content per cell during the first day post irradiation was 1.5% per hour. This is in agreement with the rate of increase in cell number in the unirradiated tumor during this time interval. It also compares well with the estimated rate of DNA formation of 1.7% from incorporation data in unirradiated cells. These data support the conclusion that incorporation of P^{32} into DNA of irradiated Ehrlich ascites cells is due to continuation of DNA synthesis during the first day post irradiation.

During the second day after irradiation the incorporation of P^{32} was markedly depressed (Fig. 1), and there was little further increase in mean DNA content per cell. To determine whether, during this time interval, a discrepancy existed between incorporation data and net synthesis of DNA, many more experiments would have to be carried out. In particular it would have to be shown that not even a small percentage of cells underwent mitosis or died.

Fig. 3 is a histogram of the distribution of individual DNA values (in arbitrary Feulgen units) in nuclei of control and irradiated ascites cells. It demonstrates a definite increase, after irradiation, in the proportion of cells with a high DNA content, confirming the biochemical findings. Whereas in the control tumor 32% of the cells had a DNA content above 11 (arbitrary units), 60% of the irradiated cells were above this value. It would be of interest to know whether the cells which still had the lower DNA content would have synthesized more DNA if given sufficient time, or if they represented cells damaged by irradiation or by the normal course of the tumor's development(14).

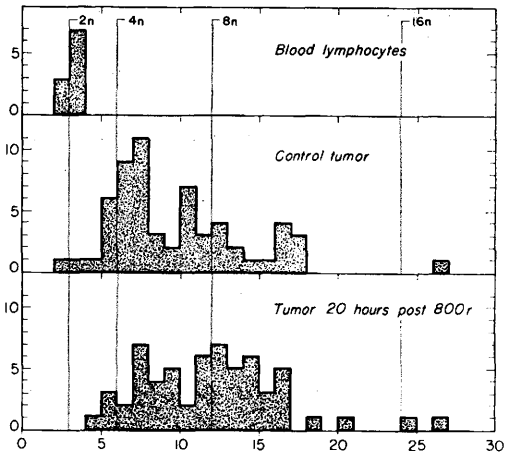

FIG. 3. Distribution of individual DNA values* in Ehrlich ascites cells.

* Abscissa: DNA content in arbitrary Feulgen units. Ordinate: No. of cells with given DNA content. Using 2n (diploid) value for DNA content of blood lymphocytes as reference point, other n values calculated in arbitrary Feulgen units.

The present experiment confirms once more the fact that mitosis may be inhibited after irradiation without simultaneous inhibition of DNA synthesis. The results would be consistent with the idea that although the cells are unable to go through mitosis, they continue to synthesize DNA until the majority of cells have reached the premitotic DNA content at which time the rate of DNA synthesis decreases. The Feulgen data, however, serve to emphasize the complexity of the cell population and the need for caution in interpretation of changes in mean values measured biochemically. The recent results of Harrington and Lavik(15) further suggest that there may be indirect factors involved.

Summary. Experiments were carried out 4 to 6 days after inoculation of approximately 2 x 10^7 Ehrlich ascites tumor cells in mice. When the 2-hour incorporation of P^{32} into DNA was measured at various times after irradiation (800 r total body x-irradiation), no significant depression in DNA specific activity was observed until 1 day. Measurements of mean cell volume, mean DNA content per cell, and mean total nucleic acid content per cell at 13 and 20 hours revealed that all 3 quantities increased at approximately the same rate, closely matching the growth rate of the unirradiated tumor and rate of DNA formation estimated from the incorporation data. The increased DNA content per nucleus after radiation was confirmed by Feulgen microspectrophotometry. At 48 hours after irradiation cell volume and total nucleic acid per cell had risen even higher while DNA per cell showed little further increase. The data suggest that the irradiated cells continue to synthesize DNA until they reach the premitotic DNA content (octoploid in Ehrlich cells) and are arrested there because they are unable to go through mitosis.

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