The Effect of X-Radiation on the Yoshida Ascites Tumor

I. Inhibition of Mitosis by a Single Irradiation*

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The ascites tumors are particularly well suited to radiobiological research. Since the cells float and grow freely in an ambient medium, there is no stroma to be damaged and thus complicate the interpretation of radiation effects on the tumor cells. If radiation is carried out at an early stage of tumor growth, there is such a small volume of ascitic fluid that adequate diffusion of oxygen and nutrients to the cells may be assumed. Furthermore, it is possible to remove small aliquots of fluid containing tumor cells repeatedly from the same animals without apparent harm to host or tumor. Thus, the same tumor may be followed throughout its life span in the same host, both before and after radiation.

Yoshida ascites tumor cells in their free state resemble monoblasts. The solid tumor has the histological appearance of reticulum-cell sarcoma. Although the origin of the tumor is uncertain, it is probably derived from the reticuloendothelial system (31, 32).

This paper will describe the first discernible effect of x-radiation on the Yoshida tumor, the inhibition of mitosis. An attempt will be made to explain this phenomenon and to determine which cells of the irradiated population have been affected.

MATERIALS AND METHODS

We used male rats of the Harvard strain (a Wistar derivative) which weighed about 100 gm. at time of inoculation. Intraperitoneal inoculation with 15 million tumor cells, diluted in 0.5 cc. of cold normal saline, produced ascites in 90 per cent of mice. The ascites contained tumor cells which weighed about 100 gm. at time of inoculation. Infection of the irradiated population have been affected.

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The errors in counting are analyzed in Table 1. It should be noted that the coefficient of variation due to random sampling would be 8.8 per cent for a count of 140 mitoses in 4000 cells. Since only one of the morning counts was less than 4000 cells, the 9.0 per cent coefficient of variation found indicates a remarkable constancy of mitotic indices in different animals at this time of day.

The phases of mitosis are defined as follows: Prophase begins with the first discernible separation and thickening of chromosomes and continues as long as the nuclear membrane is visible. Metaphase begins with the disappearance of the nuclear membrane and continues until separation of the chromatids at the equatorial plate can be seen. Anaphase covers the period from end of metaphase until pinching off of the cytoplasm begins. Telophase continues from this point until the chromosomes enter the cytoplasm of the daughter cells.
daughter cells separate and the chromosomes reform into nuclei. Many pairs of cells could be found without cytoplasmic connection but with identical nuclei having the characteristic appearance of late telophase. These were considered to be newly separated daughter cells and classified as a single telophase.

RESULTS

Preradiation mitotic rates.—The average of the preradiation mitotic indices, counted 3 days after inoculation of the tumor, is given in Table 2. There is a definite diurnal variation. This is an obvious source of error in calculating percentage of a preradiation mitotic index, but no attempt has been made to adjust for this, since the variation is relatively small in comparison with the major changes produced by x-rays. Table 3 shows that at 3, 4, and 5 days after inoculation of the tumor (0, 1, and 2 days after radiation) the mitotic indices in control tumors were constant at the same times of day.

Host survival.—Mean survival time of the inoculated rats was 7 days after radiation. Survival time was the same in radiated animals, nonradiated controls, and animals inoculated at the same time but subjected to no further experimental procedures.

Postradiation mitotic rates.—The effect of x-radiation on mitotic rate fell into two stages: (a) rapid disappearance of mitotic figures, followed by a period of complete mitotic inactivity; (b) prolonged mitotic recovery, at the end of which the preradiation mitotic index was equaled or exceeded.

Stage 1 (mitotic disappearance): Chart 1 shows the rapid disappearance of mitoses following 425 r total-body radiation. The straight line drawn through the points was derived by the least squares method. When extrapolated, it intersects the 100 per cent line at 3 minutes and the 0 per cent line at 54 minutes after the midpoint of radiation.

The relative proportions of the four phases to one another during the rapid decrease in mitotic index are shown in Chart 2. It can be seen that prophase figures decreased first, as would be expected if no new mitoses were appearing (28). As long as cells in prophase progressed into metaphase, the latter remained constant in number; but, between 20 and 30 minutes after radiation, metaphase figures began to disappear as they went into anaphase without further replacement by cells from prophase. Eventually the same occurred with each phase, leaving only a few visible mitoses at the end of 1 hour, predominantly in telophase.

This stepwise decline reflects not only the disappearance of mitotic activity in the tumor, but also the orderly fashion in which it occurs. Each cell in mitosis at the moment of radiation seems to complete the process normally.2 Those few cells in

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TABLE 1

COEFFICIENTS OF VARIATION IN DETERMINING A SINGLE MITOTIC INDEX IN THE YOSHIDA ASCITIS TUMOR

<table>
<thead>
<tr>
<th>Method</th>
<th>Coefficient of Variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) In counting (based on 6 counts of 2000 tumor cells each from the same smear)</td>
<td>±8.9 per cent</td>
</tr>
<tr>
<td>b) In the same tumor over a brief time span (based on single counts of 2000 tumor cells from each of ten samples taken from two rats over a 2-hour period)</td>
<td>±10.2 per cent</td>
</tr>
<tr>
<td>c) In different tumors (based on single counts of 2000-4000 tumor cells from 34 different animals at 8 times of day but same interval, 8 days, after inoculation of tumor)</td>
<td>7:00-8:00 A.M. ± 9.0 per cent</td>
</tr>
<tr>
<td></td>
<td>2:30-3:30 P.M. ± 15.0 per cent</td>
</tr>
</tbody>
</table>

TABLE 2

AVERAGE PRERADIATION MITOTIC INDICES, 3 DAYS AFTER TUMOR INOCULATION, SHOWING DIURNAL VARIATION

<table>
<thead>
<tr>
<th>Time of day</th>
<th>No. animals</th>
<th>Mean mitotic index</th>
<th>Standard error of the mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>7:00-8:00 A.M.</td>
<td>11</td>
<td>0.0857</td>
<td>0.0010</td>
</tr>
<tr>
<td>2:30-3:30 P.M.</td>
<td>23</td>
<td>0.0820</td>
<td>0.0010</td>
</tr>
</tbody>
</table>

(This difference is probably significant: P = 0.02.)

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TABLE 3

MITOTIC RATE IN CONTROL TUMORS AND IN TUMORS IRRADIATED 3 DAYS AFTER INOCULATION

<table>
<thead>
<tr>
<th>Hours after radiation (MEAN)</th>
<th>No. ANIMALS</th>
<th>MEAN MITOTIC RATE*</th>
<th>STANDARD ERROR OF THE MEAN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:7</td>
<td>8</td>
<td>97.2</td>
<td>2.4</td>
</tr>
<tr>
<td>25</td>
<td>12</td>
<td>100.2</td>
<td>6.4</td>
</tr>
<tr>
<td>49</td>
<td>8</td>
<td>102.3</td>
<td>5.9</td>
</tr>
<tr>
<td>74</td>
<td>5</td>
<td>88.6</td>
<td>7.9</td>
</tr>
<tr>
<td>98</td>
<td>4</td>
<td>74.7</td>
<td>15.6</td>
</tr>
<tr>
<td>102</td>
<td>2</td>
<td>34.7</td>
<td></td>
</tr>
<tr>
<td>Radiated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.7</td>
<td>10</td>
<td>1.8</td>
<td>0.4</td>
</tr>
<tr>
<td>5.7</td>
<td>7</td>
<td>12.5</td>
<td>1.4</td>
</tr>
<tr>
<td>10.3</td>
<td>6</td>
<td>51.5</td>
<td>4.7</td>
</tr>
<tr>
<td>16.0</td>
<td>7</td>
<td>86.6</td>
<td>7.0</td>
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<tr>
<td>22.0</td>
<td>14</td>
<td>110.5</td>
<td>5.3</td>
</tr>
<tr>
<td>28.0</td>
<td>9</td>
<td>104.1</td>
<td>1.8</td>
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<tr>
<td>38.0</td>
<td>4</td>
<td>112.8</td>
<td>3.3</td>
</tr>
<tr>
<td>41.0</td>
<td>5</td>
<td>91.6</td>
<td>2.7</td>
</tr>
<tr>
<td>46.0</td>
<td>9</td>
<td>95.6</td>
<td>4.1</td>
</tr>
<tr>
<td>64.0</td>
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<td>8.4</td>
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<tr>
<td>75.5</td>
<td>5</td>
<td>93.1</td>
<td>5.6</td>
</tr>
<tr>
<td>96.0</td>
<td>7</td>
<td>75.8</td>
<td>8.6</td>
</tr>
<tr>
<td>120.0</td>
<td>4</td>
<td>40.3</td>
<td>7.9</td>
</tr>
</tbody>
</table>

* Per cent of preradiation mitotic indices.
Cancer Research

telophase still present just before mitosis disappeared probably represent cells which were in early prophase when irradiation occurred. The time elapsed between irradiation and final disappearance must, therefore, approximate the time required for the cell to divide, the mitotic time (MT) (17, 30). Examination of the extrapolated line in Chart 1 shows that the average duration of mitosis in the Yoshida tumor is 51 minutes. Stage 2 (mitotic recovery): Approximately 4 hours after radiation, mitosis reappeared in the tumor and increased gradually. This is illustrated in Chart 3, in which the slope of recovery is derived by the least squares method from all points between 2 and 24 hours after radiation. It will be seen that the preradiation mitotic rate was not reached until 20.3 hours after radiation and that by 24 hours the mitotic rate in some tumors had continued to increase beyond the preradiation level.

The individual points in Chart 3 have been pooled in Chart 4. Mitotic rate throughout the next four days is also shown (Table 3). Although

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Chart 1.—Disappearance of mitosis in 1st hour after radiation. Each point represents count from a single animal. A total of six radiated and three control animals were used in three separate experiments. Broken line has been extrapolated to 100 per cent and 0 per cent of preradiation mitotic index. Interval between intercepts equals mitotic time. (See text.)

Chart 2.—Changing proportions of the phases of mitosis to one another during the period of mitotic disappearance. Data from the same animals as used in Chart 1; 1721 mitotic figures counted in 98,000 tumor cells.

Chart 3.—Mitotic recovery in the first 24 hours after radiation. Each point represents count from a single animal. A total of fourteen radiated and twelve control animals were used in four separate experiments. Broken line is same as shown in Chart 1. Cross-hatched area is equal to the proportion of the total cell population which succeeded in dividing during a period in which an untreated tumor population would be expected to have doubled. (See text.)

Chart 4.—Mitotic rate in radiated and control tumors from time of radiation until 5 days later. Solid line is same as shown in Chart 3. Points taken from data in Table 3, showing standard errors of the means. In some, these are too small to be drawn.

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Sato et al. (26) timed the duration of mitosis in the Yoshida tumor in vivo at longer than 47 minutes and less than 1 hour.
the mitotic rate in the radiated tumors between 22 and 32 hours after treatment appeared to be higher than that in the controls and at 46 hours appeared to be slightly lower, the differences are not statistically significant. From then on until the deaths of the hosts there was no difference between mitotic activity in the radiated and control animals.

**Population analysis.**—It must be emphasized that the cells represented in Charts 1 and 2 were the only cells in mitosis which were exposed to ionizing radiation. The mitotic index shows that they constituted less than 4 per cent of the total cell population at the time of radiation. At the end of 1 hour they had divided, producing the first daughter cells of the irradiated generation. Ninety-six per cent of the cell population was irradiated in the resting phase. It is with these cells that we are primarily concerned.

Hoffman (10) showed that, if one knows the mitotic time (MT) and the mitotic index (MI), it is possible to calculate the duration of the intermitotic time, or interphase (IP). In an exponentially increasing tissue, such as this rapidly growing tumor, the natural logarithm of 2 is introduced:

$$IP = \frac{-\ln 2 \times MT}{MI}$$

When the known MT (51 min.) and the diurnal extremes of MI (0.0357–0.0320) are introduced in the above equation, IP is found to vary between 16.4 and 18.4 hours. It is assumed that this averages out over the day to around 17\(\frac{1}{2}\) hours.

This means that, even if the daughters of those cells radiated while dividing developed normally, they would not have passed through their interphase and be ready to divide again until at least 17\(\frac{1}{2}\) hours after radiation. Furthermore, the daughters of those few cells which entered mitosis early in the recovery phase (beginning 4 hours after radiation) would not divide into grand-daughter cells until at least 4 plus 17\(\frac{1}{2}\), or 21\(\frac{1}{2}\), hours after radiation. Thus, during at least the first 21\(\frac{1}{2}\) hours of the experimental period the mitotic figures found in the tumor must be in that 96 per cent of cells from the preradiation generation which were radiated in the resting stage.

The life span of the undamaged Yoshida tumor cell would equal IP plus MT, or approximately 18\(\frac{1}{2}\) hours. This means that in the undamaged tumor, where mitotic activity is continuous around the clock in spite of a small diurnal variation, 100 per cent of the viable cells would divide within an 18\(\frac{1}{2}\)-hour period (the generation time).\(^4\)

In view of the marked mitotic inhibition which follows radiation, it would be impossible for 100 per cent of the cell population to divide in the first 18\(\frac{1}{2}\) hours after radiation. Assuming that radiation has not changed MT, the segment of the cell population which has succeeded in dividing within 18\(\frac{1}{2}\) hours after radiation is equal to the area under the recovery curve within that span of time (cross-hatched area, Chart 3). This is about 96 per cent. If radiation has prolonged MT (which is uncertain in other tissues \([9b, 27]\) and not yet known in this tumor) the percentage of the population which divided within 18\(\frac{1}{2}\) hours would be even less.

The mitotic hyperactivity which follows inhibition is thought to be owing to the simultaneous division of many of those cells which had previously failed to divide on a normal schedule (7, 16). It is quite possible that those cells which completed division within 18\(\frac{1}{2}\) hours of radiation were also delayed; i.e., they might have been ready to divide just at the moment of treatment. However, Carlson (5) has observed in grasshopper neuroblasts that some newly formed cells, the progeny of mitoses completed shortly before radiation, would "catch up" with those cells which had been almost ready to divide at the time of radiation and would enter mitosis ahead of the more mature cells. They presumably divided at the end of an interphase of normal duration while the older cells were still recovering from radiation damage. Possibly some of the 96 per cent of cells which divided in the normal generation time of the tumor fall into this category. One can definitely state, nonetheless, that 94 per cent of the population, the area above the curve of recovery, was prevented from entering mitosis on schedule.

**DISCUSSION**

Progressive inhibition of mitosis, followed by recovery, was described by Strangeways and Oakley in 1923 (28). Since that time, the response of mitosis to ionizing radiation in a variety of plant and animal tissues has been studied, both in vivo and in vitro (1, 2, 3, 6, 7, 10–18, 20, 23, 25, 30). Most authors have agreed with Strangeways and Oakley’s theory that the fall in mitotic rate was owing to arrest of those cells which were on the verge of mitosis at the moment of radiation, while those cells already in mitosis go on to completion.

\(^4\)Hoffman et al. (11) have analysed the relationship between the duration of interphase and the generation time of a tissue. The greater the difference in the time it takes individual cells in the tissue to go through interphase, the wider the variation in the generation time. Since at least a 2-hour variation in IP is known in the Yoshida tumor, the given figure of 18\(\frac{1}{2}\) hours must be viewed in this light and considered an approximation rather than an absolute figure.
of division. The data summarized in Chart 2 also confirm this theory.

When working with fixed preparations, it is difficult to remember that mitosis is a dynamic process which sweeps through the entire population of cells very much as the crest of a wave moves across a body of water. Thus, cessation of mitotic activity for any length of time will mean that a large proportion of the population has failed to enter mitosis, although in each sample the effect seems limited to the small proportion of cells which would be dividing at that moment. It is probably for this reason that the attention of many investigators has been concentrated on those cells which were actually in mitosis or were about to divide at the moment of radiation. Particular emphasis has been placed on the sensitivity of the preprophase to mitotic inhibition (4, 16, 19), and, although it has never been said that mitosis is not inhibited in other stages of cell development, the tendency has been to ignore the cells which were not actually dividing or about to divide.

On the other hand, Lea (21) subjected the experimental results of other workers to mathematical analysis and arrived at this conclusion: "It is not sufficient to ask which stage of a cell's life cycle is most sensitive; it is necessary to specify the effect which is being considered. If the effect is delayed division, then obviously the effect of radiation can only be discovered when the cell reaches the stage at which it is due to divide. It is probably safe to say that a given dose of radiation produces the same physical and chemical changes in a cell due to divide an hour hence as in one due to divide 5 hours hence, yet, on account of the times available for recovery being different, the one cell finds itself unable to commence division at the proper time while the other cell enters mitosis entirely according to programme. Thus it is quite true to say that if one wishes to demonstrate a visible effect of radiation upon a cell one is most likely to be successful if one chooses to irradiate it shortly before it is due to enter mitosis, but it is hardly correct to say that a cell has a period of special sensitivity shortly before it is due to divide."

Friedenwald and Sigelman (6) studied the effect of x-radiation on mitosis in the rat corneal epithelium and concluded that all cells in the radiated population were affected, except at very low dosages. Friedman et al. (7) have also emphasized the effect of x-ray and radiomimetic agents on a large part of the cell population.

The application of autoradiography to radiation biology has made it possible for the first time to detect dynamic changes during the interphase (14, 29). The work of Howard and Pelc (15, 24) on Vicia faba suggests that there may be cells at a certain stage in the interphase upon which radiation will have a particularly marked inhibitory effect when the time comes for them to divide. Furthermore, there may be another stage during which DNA synthesis is most easily disrupted. These two manifestations of radiation effect do not seem related.

These authors themselves have emphasized that the detection of P³² in a cell nucleus still has not been proved to be directly associated with the actual process of nucleoprotein synthesis and chromosome reduplication. In a discussion of Howard's most recent paper on this subject (13), G. B. Brown said that there is evidence that tagged purines might go in and out of metabolically active nucleoprotein molecules without having anything to do with the synthesis of the latter. Hence, one cannot yet accept without qualification the existence of discrete synthetic stages during the interphase which are postulated on the basis of P³² and adenine-C¹⁴ autoradiographs.

The experimental evidence we have presented neither confirms nor disproves the existence of an interphase stage during which mitosis is more easily interfered with. It is our purpose to emphasize that only a moderate dose of x-radiation is sufficient to postpone mitosis in at least two-thirds of the cells in the total population. This figure is derived from the area over the mitotic recovery curve. Since Friedenwald and Sigelman (6) showed a direct relationship between dose and duration of mitotic inhibition, one would expect that with larger doses mitosis would be delayed in nearly 100 per cent of the cell population. Thus, for all practical purposes, one might consider this sensitivity to be characteristic of most cells in the tumor, with the exception of those actually in mitosis. Although variation in sensitivity of cells to mitotic inhibition might well exist, it is probably of significance only at very low dosages.

That mitosis can be inhibited in an entire cell population is not surprising, since Loveless and Revel (22) have shown that there is a wide variety of physical and chemical agents which will inhibit mitosis for a short time. For example, after immersion of a root tip in ethyl alcohol there is a transient mitotic inhibition. They reach the conclusion that it is relatively easy to upset so complex and delicate a cellular process as mitosis. In the case of most of these agents, however, if the cell is not killed outright, recovery of normal function is complete.

Although it is possible that mitotic inhibition is
due to the same chemical or physical reaction that is lethal to the cell, it is equally possible that entirely different modes of action are involved (19). Indeed it has been shown (9a) that the actual killing of cells could not be correlated with the degree or duration of mitotic inhibition. Furthermore, the rapid return of mitotic activity suggests that the effect of mitotic inhibition is transient and, insofar as further growth is concerned, rather slight. We are continuing our studies in an attempt to determine whether mitotic inhibition is a biologically important effect of radiation or whether it is merely a nonspecific reaction such as can be produced by a variety of noxious chemical or physical agents.

SUMMARY

1. The average duration of mitosis in the Yoshida ascites tumor is 51 minutes. The duration of the interval between is approximately 17 hours. Thus, during the first 5 days of growth, when mitotic rate is high, the cell population should double every 18 hours.

2. Total-body x-radiation with 425 r will produce complete inhibition of mitosis in 1 hour which is sustained for another 3 hours.

3. There is no inhibition of those cells which are already dividing when irradiation occurs. These go on to completion of mitosis. It is emphasized that they constitute less than 4% of the total cell population. The important effect of radiation or whether it is ionizing raditions.

4. Of greater importance is the effect of radiation on the 96 per cent of cells treated while in the resting stage. Mitosis is inhibited in at least 64 per cent of the entire cell population. The important point is not that some interphase cells might be more liable to mitotic inhibition than others, but that with moderate dosages most of the cells radiated while in interphase have mitosis delayed.

5. There is slow recovery from mitotic inhibition. The preradiation mitotic rate is regained about 20 hours after radiation.

6. The biological significance of mitotic inhibition on tissue growth still remains to be clarified.

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