Ultraviolet Radiation Effects on Ehrlich Ascites Tumor Cells

Observations Using a Flying Spot Ultraviolet Microscope*

By JEROME J. FREED, Ph.D., JAMES L. ENGLE, B.S.E.E., GEORGE T. RUDKIN, Ph.D., and JACK SCHULTZ, Ph.D.

(From The Institute for Cancer Research, Philadelphia)

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ABSTRACT

A flying spot ultraviolet microscope, employing a fast scan and pulsed operation of the raster, has been used to induce radiation damage in ascites tumor slide cultures, and to study by time-lapse cinematography the progressive stages of cell damage. The cells observed came from a strain (EF1) of the Ehrlich ascites carcinoma. Irradiated cells were found to show a characteristic syndrome of damage, involving blebbing at the cell surface, while control cells in the adjacent areas of the preparation remained unchanged. The end of the blebbing period is marked by swelling of the cells, and the time taken for this phenomenon to occur was used as a measure of the severity of the damage. It was found that the time required for swelling is dependent on the size of the dose employed, as well as on the sensitivity of the cells. This latter sensitivity was found to decline as the physiological age of the tumor increased. If ultraviolet illumination below 255 mμ is excluded, no symptoms of damage occur, even when very large doses are used. These observations are discussed in relation to the nature of the system in the cell which is affected.

INTRODUCTION

The studies reported in this paper grew out of work designed to evaluate the usefulness, for observation of living cells, of a flying spot ultraviolet microscope constructed by the Philco Corporation. The flying spot microscope can serve both as a means of observing living cells and as a means of delivering a damaging dose of radiation, and then of observing the primary cytological manifestations of radiation damage. Such studies can contribute to an understanding of the processes of radiation damage, as well as to a definition of the conditions favorable for cytochemical studies of living cells in the ultraviolet.

While the desirability of using living cells in cytochemical studies with the ultraviolet microscope has long been recognized, the rapidity with which the ultraviolet has been found to damage the cells has made it necessary to use fixed material for all but the most rapid and limited studies (1, 4, 19). Thus, the distribution patterns and absorption curves for ultraviolet absorbing substances in cells found in the literature are with few exceptions derived from fixed preparations. Such studies have yielded much information on the nucleoprotein organization of the cell, and quantitative studies have been made of the amount of absorbing substances in cells, nuclei and chromosomes (2, 18). Nevertheless, it is known that the distribution of absorbing materials, and their apparent amounts as determined by absorption cytophotometry (3), are altered by the fixation process. Therefore, direct study of the sequence of events in living cells by time-lapse filming of ultraviolet micro-

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† Government and Industrial Division, the Philco Corporation, Philadelphia.

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scope images would have obvious advantages for understanding the physiological processes in which the ultraviolet absorbing structures participate.

The conventional ultraviolet microscope, using photographic emulsions, may be used to make as many as 12 photographs of a living cell exhibiting no signs of damage (4, 19), but it is found that the cell subsequently dies. If the study of cells over any considerable period of time is to be made possible, less radiation and hence more sensitive detectors must be employed. It has been estimated that devices using photo-emissive surfaces (17) may be made 30 to 100 times more sensitive than the best photographic emulsions available for work in the ultraviolet, and consequently a number of television techniques have been applied to the problems of ultraviolet microscopy.

Ultraviolet television microscopes are of two types. In the first type, the ultraviolet sensitive surface of a television image tube, e.g., the U.V. vidicon (5), is placed in the image plane of a conventional ultraviolet microscope, and the television signal applied to a picture tube, which may be observed or photographed. The more sensitive image orthicon tube has been applied to ultraviolet microscopy more recently, in a television version of the color-translation microscope (21). The second principle used, that of the flying spot microscope, was suggested by Roberts and Young (17), and applied to the ultraviolet by Montgomery, Bonner, and Roberts (14). In this microscope the object field is scanned by a special cathode ray tube used as the source, and the detector is a multiplier phototube (see below).

Using the flying spot ultraviolet microscope, Montgomery et al. have been able to make continuous observations on living cells for considerable periods of time (15). They have investigated the growth and division of cells in tissue cultures, and have studied the permissible levels of radiation for this work. Adequate picture quality may be obtained with intensities apparently consistent with normal behavior of the cells, including mitotic division (16).

The design of the flying spot microscope used in this work, while the same in principle, differs from that of Montgomery, Bonner, and Roberts, in certain respects, to be described below.

With this instrument, we have investigated the nature of the damage caused to ascites tumor cells by exposure to the ultraviolet radiation used in the instrument, and studied some of the factors influencing the subsequent development of the damage syndrome. Using deliberately damaging doses, we have prepared time-lapse films to record the occurrence of subsequent abnormalities, as observed with the flying spot ultraviolet microscope. The effects of the physiological age of the tumor, the spectral distribution of the radiation, and the size of the dose have been investigated. Mouse ascites cells served as a useful test system for study of cell behavior in the ultraviolet. These experiments will be discussed with reference to the significance of the syndrome observed, and in relation to the problems of ultraviolet microscopy of living cells.

Materials and Methods

Ascites Slide Cultures.—The cells used were a hypotetraploid subline (EF) derived from a clone of the Ehrlich ascites carcinoma. They were propagated by weekly serial transplantation of 0.2 ml. of ascitic fluid, containing approximately $14 \times 10^6$ cells, into female Swiss mice. After the 4th day of each transplant generation, a small volume of ascitic fluid was withdrawn for observation by peritoneal puncture with a small glass capillary, so that the same tumor could be sampled on successive days. The ascitic fluid was spread in a flat drop on a quartz coverslip which was placed with the fluid downward on the surface of a drop of paraffin oil on a quartz slide. Pieces of glass coverslip were used to support the coverslip bearing the cells, while the paraffin oil served to prevent evaporation. Such cultures, modified from the technic of Makino and Nakahara (12), are suitable for phase contrast or ultraviolet microscopy over a period of many hours. Mitotic divisions continue to occur, although there may be some delay in metaphase, as compared with the condition in vivo. For example, when a tumor was sampled 4 days after inoculation, an initial metaphase frequency of 0.9 per cent was noted. Counts made at intervals thereafter showed an increase in the metaphase frequency with a peak value of 3.2 per cent noted at 4.5 hours. Subsequently, the metaphase count declined, reaching zero at about 12 hours. The drop in frequency of metaphases is correlated with an increase in the count of anaphases and telophases; the divisions appear to proceed normally. The interphase cells are normal in appearance for 12 hours; after this time some dead cells begin to appear. Our time-lapse films were all completed in 9 hours or less.

The Flying Spot Microscope.—The principle of operation of a flying spot microscope is shown in Text-fig. 1. The light source is a scanner cathode ray tube of a special type producing a very small and intense spot of light (S) on the tube face. The spot (S) is completed in 9 hours or less.

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2 We are indebted to Mrs. M. L. Wilson for providing us with the tumor-bearing mice used in this work.
illuminates the microscope through a suitable filter (F); a mirror (M) is used to direct the light through the eyepiece (EP) and objective (OBJ). The microscope is thus used in the reverse of the usual direction, so that a strongly demagnified image of the spot at S is formed in the plane of the specimen (O). Deflection circuits are used to sweep the spot in a pattern of parallel lines, called a raster. The demagnified spot is thus made to trace out a small raster image in the specimen plane. As the image of the spot passes over the specimen, light losses occur according to its structure, and the photomultiplier (PM) tube placed below the specimen receives illumination varying in intensity with time. The resulting varying signal from the photomultiplier is amplified and applied to the grid of a television picture tube (monitor CRT), whose deflection circuits are synchronized to those of the scanner tube. The beam intensity of the picture tube is thus modulated in brightness to produce an enlarged image of the specimen (I). This image may be inspected visually, or recorded by means of a camera. In the microscopes under discussion, the spot S emits ultraviolet radiation, but very little visible light.

The instrument constructed by the Philco Corporation is comparable to that described by Montgomery et al. (14). The main differences derive from the use in the Philco instrument of the sweep rates standard for United States television broadcast equipment. As compared with the low sweep rate used by Montgomery et al., the high sweep rate used in the Philco system requires a brighter spot in order to override the greater thermal noise of the photomultiplier, and thus to provide a sufficiently high signal to noise ratio in the integrated television picture on the film. The intensity used is such as to damage cells rather quickly if continuous scanning is employed. To reduce damage, the Philco microscope is equipped with a pulsing system, so that the operation of the scanner tube may be confined to one or more integral frames per cycle, synchronized to the shutter of a 16 mm cine camera. The specimen need thus be exposed to ultraviolet only during the actual photographic exposure time.

With the present type of scanner tube, the grains of the phosphor appear superimposed upon the image of the specimen. They can be removed by a correcting circuit as follows: a second photomultiplier is placed to receive light from the scanner tube directly, and its signal is used to correct the picture signal for variations due to the granular structure of the phosphor. The effect is to prevent the phosphor structure from appearing to be in focus in the specimen plane; with objects of low contrast, the intelligibility of the image is much improved.

The earlier work with the flying spot ultraviolet microscope (14–16), used the N.A. 0.72 Grey reflecting objective, also employed for much of the work described here. In later experiments, the Grey reflecting objective, N.A. 1.00, was found to provide better resolution of the details within the cells.

For time-lapse photography, exposures on reversal film were made at a rate of 5 to 10 camera frames per minute, each camera frame being exposed to from 4 to 16 television frames. The brightness of the picture tube was adjusted by reference to an exposure meter, and a high level of video contrast was found to be desirable. Multiple exposure of each camera frame reduces the noise level in the picture, and it has been found advantageous to increase the number of television frames integrated in this way to the maximum consistent with the safety of the cells.

The reflecting optics were provided through the courtesy of Mr. A. Wexlin and Mr. G. Crebbin of the Bausch and Lomb Optical Company.
TEXT-FIG. 2. Spectral distribution of output of Philco L-2116 flying spot scanner tube. Solid curve was furnished by Philco Corporation. Dotted curve was calculated by applying to the solid curve the measured transmittance of the filter described in the text. Dashed curve was calculated by applying similarly curve 7-54 published by Corning Glass Company.

The dose rate for the ultraviolet radiation to which the cells are exposed is not easily calculated in absolute terms, but may be controlled from experiment to experiment, and measured in arbitrary units. The intensity employed is that obtained from a type L-2116 Philco experimental flying spot scanner tube, run with a beam current of 50 microamperes. Constancy of light output and stability of the transmission of the optics are controlled by calibration of the video channel with a standard light source. Under these conditions, the number of television frames used is a measure of the total dose delivered, through a given set of optical equipment.

Some control of the spectral distribution of the illumination is possible through the use of filters. Text-fig. 2 shows the unmodified output of the L-2116 scanner tube, with its peak at about 245 m. Corning filter 9863 (7-54) removes a considerable fraction of the short wavelength ultraviolet, and has been used for time-lapse photography. A filter consisting of 1 cm. of a solution of 0.6 gm. per liter of 2-methylthiophene in cyclohexane removes almost completely wavelengths shorter than 255 m. Unfortunately, this filter solution is not stable in the ultraviolet: after 30 minutes exposure to the scanner tube, the transmittance at 260 m. may drop as much as 50 per cent. With renewal at suitable intervals, it has been used to test for the effect of short wavelength ultraviolet on the cells.

When a filter is used, the intensity of radiation delivered to the cells may be made equal to that delivered without the filter, by increasing the beam current of the scanner tube until the signal in the calibrated video channel is raised to the standard operating level. This equalization is only an approximate one, due to the wavelength dependent response of the photomultiplier tube. However, the tube used has a sufficiently flat response so that the approximation is expected to be a reasonably good one.

Experimental Technic.—In experiments designed to study the effects of radiation on cells over considerable periods of time, it is important to be able to distinguish between effects due to radiation and those due to inadequacy of the culture conditions or other causes. To this end, we have evolved a technic called a split field experiment. The dose of radiation, the effect of which is to be investigated, is applied to a raster area in the layer of cells comprising the preparation. At the end of the dose, the preparation is moved one-half the width of the raster image. Subsequent time-lapse photography thus includes both irradiated cells, and...
adjacent normal controls. At the conclusion of the experiment, it is possible to move the preparation further and compare the cells exposed to the radiation used in pulsed time-lapse operation to those having had no previous irradiation.

When the dry objective is used, it is found that the control cells in such split field preparations remain indistinguishable from entirely unirradiated cells for long periods. The radiation damage appears only in those cells exposed wholly or in part to the initial dose. When the N.A. 1.00 objective is used, mild radiation effects have been seen to occur in control cells located near the edge of the initial raster image. This effect is possibly due to the high aperture of the objective, the large angle with which illumination strikes the coverslip, perhaps resulting in increased multiple reflection of radiation outside the raster image.

Irradiations of approximately one-half the area of a single cell were carried out by placing a small piece of electrical tape on the face of the scanner tube to act as a shield. After delivery of the dose, the cell was moved so that all of it could be seen, and the resulting damage recorded by time-lapse photography. In one such experiment, the cells were irradiated in the flying spot microscope and transferred to a phase contrast microscope for serial photomicrographic study.

**RESULTS**

The Radiation Damage Syndrome.—Thirty ultraviolet time-lapse films have been prepared to record the occurrence of radiation damage in ascites tumor cells exposed to the full spectral output of the scanner tube. That the abnormalities observed are radiation effects is established by the normal appearance of the control cells in the split field. Depending on the dose used, and on the sensitivity of the cells, the rate at which damage occurs may vary greatly, but the sequence of morphological changes occurs in a constant order. These morphological changes thus seem to constitute a definite syndrome, and may be described with reference to a typical experiment.

Undamaged ascites tumor cells appear in the flying spot microscope as smoothly outlined, highly absorbing bodies (Fig. 1). Since the cells are not flattened in the preparations used, the high absorption of the cytoplasm tends to obscure the position of the nucleus. When, after some hours in culture, some of the tumor cells flatten themselves against the coverslip, the nucleus appears and is seen to be relatively transparent except for the heavily absorbing chromatin blocks and the nucleolus.

After the delivery of a sufficiently high dose of radiation, or during the dose itself if it is sufficiently prolonged, the earliest evidence of radiation damage is seen in the form of blebbing (Figs. 2 to 5). This process consists of the repeated eruption and withdrawal of blunt protrusions of cytoplasm. These pseudopod-like protrusions may be extended for considerable distances, and appear to originate from all portions of the cell surface. The television image shows that the blebs contain ultraviolet absorbing material, which must be present in appreciable concentration if account is taken of their small diameter. In the phase contrast microscope, the blebs are seen to be hyaline in nature, lacking the granular elements found in the more central portions of the cell cytoplasm (Figs. 9 to 11).

The blebbing process is terminated by a swelling of the cell (see Figs. 6 and 7, cells in lower half of field). In time-lapse films, it appears that the blebs are no longer fully withdrawn but instead run together. In the swollen condition, the cells appear flatter, occupy a larger projected area, and are less strongly absorbing; the nucleus becomes prominently visible. The structure of the nucleus at this stage, as seen in the flying spot microscope, is almost spherical, with a clearly outlined absorbing margin, and contains prominent absorbing blocks, among which is the nucleolus.

Following the swelling process, the changes which occur consist of a contraction of the granular materials of the cytoplasm onto the nucleus, and a concomitant shrinkage of the nucleus itself (Fig. 8). At the same time, the cytoplasmic margin tends to become circular in outline, except for irregularities caused by contact with adjacent cells. Phase contrast examination at this time shows that the region between the contracted material at the center of the cell and the margin is almost structureless except for small particles in brownian motion.

These three phenomena constitute the radiation damage syndrome. Blebbing has been observed in at least one instance to be reversible: the cell returned to an apparently normal appearance after several hours of blebbing. Swelling, on the other hand, has never been observed to be reversed, and probably represents the “death” of the cell. The phenomena of nuclear contraction, etc., would then be considered to be postmortem changes. Swelling was found to be a convenient end point in studies designed to evaluate the effects of different doses, and of varying cell sensitivity. It occurs very suddenly, and is a striking change,
readily observed in the flying spot time-lapse films.

As a measure of the severity of the radiation damage, it has been found convenient to estimate the time elapsed from the start of the dose to the time when less than half of the cells still show blebs. The time ("time to swelling") is usually fairly uniform from cell to cell within the raster area, and as will be shown, it is correlated with the size of the dose, swelling occurring more rapidly with larger doses.

Effect of Varying Dosage.—The rapidity with which cells show damage is affected by the sensitivity of the cells themselves, by variation in radiation output of scanner tubes, as well as by the number of television frames used in the dose. A series of films made under comparable conditions, using cells from animals inoculated 7 to 9 days previously, is summarized in Table I. A single scanner tube, of constant output, was used in all of these experiments. Control cells in these split field experiments remained normal, that is, showed no differences from unirradiated cells. The time to swelling of the irradiated cells decreases as the time of the initial dose is lengthened.

In other experiments, using younger and therefore more sensitive cells (see below), 15-minute doses resulted in blebbing periods so brief that the swelling had occurred before the dose was completed.

Variation of the dose was also tested by changing the intensity. In a single experiment, using cells of 8 days' growth in which the intensity used for the initial dose was doubled by raising the scanner tube beam current to 150 microamperes, swelling of the cells occurred in 16 minutes. In a comparison experiment on the same preparation in which the standard intensity (50 microamperes beam current) was used for the same dose period, 58 minutes was required for swelling.

The swelling phenomenon thus requires time for its occurrence; the larger the amount of radiation used on the cell, the less time required.

Partial Irradiation of Cells.—Several experiments have been done to study the effect of masking approximately one-half the cell area during the delivery of the dose. Adjacent cells served to indicate the effect of irradiating whole cells at the same dose rate (Figs. 9 and 13). As may be seen from Table II, the protection of one-half of the cell increases the time taken for swelling to occur. Experiment 3, in which apparent recovery of the half-irradiated cell occurred, is illustrated in Figs. 9 to 12.

As a first step in analysis, the relation of the subsequent damage syndrome to the initial area irradiated has been examined. In the earliest time-lapse frames, made just after the end of the dose period, there is a distinct impression that blebs are emitted in greater numbers from the side of the cell that was irradiated. Selected frames from these experiments are shown in Figs. 13 and 14. After a few minutes, however, movement of the cell becomes pronounced, and the effect can no longer be localized, but blebs appear to be emitted in all directions. In the half-irradiated cell studied with phase contrast, a similar asymmetric effect may be observed (Figs. 10 and 11). This particular cell did not bleb very violently, and subsequently appeared to recover (Fig. 12).

Wavelength Dependence of Radiation Effect.—A series of split field experiments has been carried out to compare the effect of radiation including short wavelengths with that of radiation from which the wavelengths below 255 m\(\mu\) have been excluded. Adjacent raster areas were successively exposed to the same dose period with and without filtering, a dose being chosen of such size as to assure rapid

### TABLE I

<table>
<thead>
<tr>
<th>Date (min.)</th>
<th>No. of experiments</th>
<th>Swelling time</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>2</td>
<td>4 hrs.; 6 hrs.</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>11</td>
<td>58 min., average</td>
<td>44–104 min.</td>
</tr>
<tr>
<td>15</td>
<td>4</td>
<td>31 min., average</td>
<td>15–48 min.</td>
</tr>
</tbody>
</table>

Age of tumor, 7 to 9 days, constant scanner tube output. Cells blebbed continuously from end of radiation to swelling time noted.

### TABLE II

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Age of tumor</th>
<th>Swelling time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Half-irradiated cell</td>
<td>Fully irradiated cell</td>
</tr>
<tr>
<td>1</td>
<td>6 days</td>
<td>89 min.</td>
</tr>
<tr>
<td>2</td>
<td>6 days</td>
<td>97 min.</td>
</tr>
<tr>
<td>3*</td>
<td>7 days</td>
<td>†</td>
</tr>
</tbody>
</table>

* Examined under phase contrast subsequent to flying spot irradiation.
† Blebbing continued for 2 hours, apparent recovery after 6 hours.
damage by the unfiltered radiation. In two experiments, the intensity of the filtered radiation was made equal to that of the unfiltered radiation by raising the beam current. These experiments are summarized in Table III. It may be seen that even a 30-minute dose to the very sensitive cells used (4-day tumor), when filtered through 2-methylthiophene, caused no damage detectable in a film of 9 hours duration. This dose did, however, produce hemolysis of erythrocytes present in the irradiated area, those in the control area remaining unaffected. The protective effect of the filter is seen to exist when the tube brightness is increased so that approximately equal intensities are delivered with and without the filter. It is thus clear that the short wavelength ultraviolet is many times more effective in producing damage than the radiation in the region above 255 m\(\mu\).

**TABLE III**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Tumor age</th>
<th>Dose</th>
<th>Film duration</th>
<th>Swelling time*</th>
</tr>
</thead>
<tbody>
<tr>
<td>4/4</td>
<td>6 days</td>
<td>15 min.</td>
<td>286 min.</td>
<td>180 min.</td>
</tr>
<tr>
<td>5/1</td>
<td>8 days</td>
<td>30 min.</td>
<td>204 min.</td>
<td>30 min.</td>
</tr>
<tr>
<td>5/5</td>
<td>7 days</td>
<td>15 min.</td>
<td>58 min.</td>
<td>20 min.</td>
</tr>
<tr>
<td>5/6</td>
<td>7 days</td>
<td>10 min.</td>
<td>165 min.</td>
<td>20-160 min.</td>
</tr>
<tr>
<td>6/7</td>
<td>5 days</td>
<td>12 min.</td>
<td>85 min.</td>
<td>27 min.</td>
</tr>
<tr>
<td>6/6</td>
<td>5 days</td>
<td>12 min.</td>
<td>196 min.</td>
<td>30 min.</td>
</tr>
<tr>
<td>8/7</td>
<td>4 days</td>
<td>30 min.</td>
<td>9 hrs.</td>
<td></td>
</tr>
</tbody>
</table>

* Swelling time where damage syndrome occurred; ± indicates that only blebbing was observed; — indicates that no blebbing or other signs of damage was observed.

This experiment was carried out with a low output tube (CR 3300); the remaining experiments employed a tube having higher output (CR 3745).

Equal intensity delivered to filtered and unfiltered areas by increasing scanner tube beam current.

**Variation in Cell Sensitivity with Age of Tumor.—**

The speed with which the radiation syndrome developed after a given dose appeared in preliminary experiments to be related to the physiological age of the tumor. The age of the tumor is taken as the number of days after inoculation at which the tumor is sampled. As is well known (9), a number of biological properties of the cells are found to be dependent on the stage of growth of the tumor. A series of experiments was undertaken, using a single scanner tube of constant output, to determine the relationship between cell sensitivity and tumor age. Ten-minute doses of unfiltered radiation were delivered to preparations from tumors 4 to 13 days after inoculation, the same mouse being

![Text-Fig. 3. Sensitivity of tumor cells as a function of the number of days after inoculation at which the tumor sample was obtained. The time from beginning of irradiation to swelling of cells was determined from time-lapse films. Each point represents a single filmed experiment; 4 mice in all were used as donors of the cells; each mouse is represented by its special symbol.](image-url)
blebbing is terminated by the apparent death of receiving a lethal dose, and this instability or normally spread themselves out on a substrate in culture, show a marked instability of shape after ascites cells. The ascites cells, which do not contrasted with our own, on the normally rounded irradiated in the flattened condition may be con-
tact with the coverslip, and became relatively opaque. These observations on cells which are illuminated used, the cells rounded up losing in the "older" tumors did the swelling process occur during the actual time-lapse films.

Variations in sensitivity within a population of cells were also found to occur. In a number of time-

lapse films showing the occurrence of radiation damage, individual cells were observed which were either damaged much more rapidly than similarly irradiated neighboring cells, or were injured after doses which did not affect the bulk of the cells in the microscope field. In each of six cases which have been studied, it was found that these cells were larger than the average tumor cells. When the swelling process was completed, five of these were found to have been binucleate or trinucleate. In one case, the nucleus was of very large size, and may have been of a higher polyploid class.

DISCUSSION

The flying spot microscope serves as a particularly convenient means of delivering doses of radiation to very small areas and at the same time provides a means of observing the onset of very early stages of manifestation of damage. The damaging effects of ultraviolet light have been observed directly using the Kohler microscope (1, 8, 19), but the process of development of the radi-
tion damage syndrome has received little attention. Montgomery et al. have described the occurrence of ultraviolet radiation damage resulting from the flying spot illumination of tissue culture cells (15). They found that at low intensities pinocytosis is inhibited; stronger illumination causes retraction of pseudopodia; and, at the highest levels of illumination used, the cells rounded up losing contact with the coverslip, and became relatively opaque. These observations on cells which are irradiated in the flattened condition may be con-
trasted with our own, on the normally rounded ascites cells. The ascites cells, which do not normally spread themselves out on a substrate in culture, show a marked instability of shape after receiving a lethal dose, and this instability or blebbing is terminated by the apparent death of the cell. Thus both our observations and those of Montgomery et al. agree in that the earliest ob-
servable radiation effects involve the maintenance of the cell margin; damage leads to inability to maintain the normal cell form. It should be men-
tioned that preliminary experiments of our own,

used on successive days. Results of experiments with 4 mice are summarized in Text-fig. 3. It may be seen that after the 6th or 7th day much longer times were required for swelling to occur, indicating that the cells from the older tumor were less sensitive. Seven similar experiments using a 15-minute dose were also carried out; in experiments before the 7th day after inoculation, the cells usually swelled before the completion of the dose. Only in the "older" tumors did the swelling process occur during the actual time-lapse films.

Lettré (11) has observed similar instability of cell form in tissue culture cells, and has suggested a hypothesis to relate the maintenance of cell form to cell metabolism. It was found that tissue culture cells treated with mitochondrial poisons in interphase round up and exhibit blebbing similar to that normally occurring in anaphase and telophase in other materials. However, if cell division takes place in a medium containing added adenosineetriphosphate (ATP), it is found that the cells divide while remaining in the flattened condition characteristic of their intermitotic period. Accordingly, Lettré suggests that available high energy phos-
phate bonds are necessary for the maintenance of a stable cell form, and that during the division process the level of ATP drops due to requirements of the spindle mechanism. This results in the blebbing which occurs in a number of types of dividing cells.

The radiation damage syndrome observed in the ascites tumor cells is morphologically com-
parable to anaphase blebbing, but is of a more violent nature. Very similar blebs are described as an early response of the Ehrlich ascites tumor to the effects of ionizing radiation (20).

It is of interest that the time required for the death of the cell to occur is a function of the size of the dose. If a direct photolytic effect on the cell structure were involved, it might be supposed that the damage would occur as rapidly as the dose was delivered. The actual observations, on the other hand, suggest that damage is done to some system necessary for the survival of the cell, but either a product of the system takes time to be exhausted, or the system is not capable of being restored sufficiently rapidly to maintain the cell. Exploratory experiments designed to detect photoreactivation and to observe the effect of fractionated doses showed that recovery, if it occurs, does so at a very slow rate. The result is not surprising in view of the failure to detect photoreactivation of viability of Ehrlich ascites
tumor cells exposed to ultraviolet in vitro before inoculation into mice (13).

The system damaged in these experiments has not been identified, but some of its properties might be inferred from the experiments reported here. Partial shielding of the cell extends the time required for death of the cell, so that it is probable that the structures affected are distributed rather generally throughout the cell. However, the asymmetry of blebbing observed in these partially irradiated cells suggests that degree of damage is at least partially localized, as might be the case if, for example, the required substance were not freely diffusible. Alternatively, the localization of blebbing might indicate that direct damage to the cell surface is involved. More experiments are required to investigate the degree of localization of the sensitive structures.

The wavelength dependence of the radiation damage syndrome described here cannot be precisely described from our experiments, but the importance of short wavelength ultraviolet in producing the damage seems clear. Action spectra for ultraviolet effects on living systems are of two types (6). Spectra having a peak in the 260 m\(\mu\) region are found for systems in which mutation or inhibition of mitosis are measured (7); these are interpreted as being due to direct absorption by nucleoprotein. When direct lethal effect on the irradiated cells is measured, the action spectrum found has a peak in the 280 m\(\mu\) region, and is identified with absorption by proteins. However, such spectra, when studies are continued far enough into the short ultraviolet, show very high lethality in the region below 235 m\(\mu\), which is associated with end absorption of proteins. Thus, King and Roe (8), testing for damage to neutrophils under the Kohler microscope, found that 231 m\(\mu\) was 20 times more effective than either 257 or 275 m\(\mu\) radiation. Similarly, the killing of Paramecium is accomplished with high effectiveness at short wavelengths, according to a curve that may be interpreted as corresponding to absorption by unconjugated protein (6). It must also be remembered that the absorption of phospholipides is high at the very short wavelengths. The information available for the wavelength dependence of the effects we have observed can thus perhaps be summarized as suggesting the implication of materials other than nucleic acids as the direct absorbers of the damaging radiation. It is reasonable to look for the ultraviolet sensitivity in structures composed primarily of proteins, e.g., mitochondria, enzymes, cell membranes.

The physiological age of a tumor is apparently an important factor in determining the sensitivity of its cells to radiation. That there are important changes in the properties of the cells is known from the studies of Klein and Revesz (9), and others (10). As the tumor reaches its maximal growth, the rate of cell division decreases, and the ratio of RNA to DNA drops. At the same time (10), the total ultraviolet absorbancy of the cells at the nucleic acid absorption band decreases. What the relation is between possible changes in nucleoprotein (or protein) structures, and the nature of the decrease in sensitivity found in the cells from older tumors remains to be investigated.

The differential sensitivity of cells of large size may provide a hint as to the nature of the systems responsible for the development of the radiation syndrome. If, for example, the sensitive system were composed of separate particles not proportionately increased in number in binucleate cells, as compared with uninucleate ones, the larger cells would be more sensitive to a dose inactivating the same fraction of the total number of particles. A complicating factor to be resolved would be the decrease in self-shielding due to dilution of other components or of the absorbing particles themselves, in the larger cells. The same arguments could apply to the change in sensitivity with tumor age, if the average cell content of sensitive particles were decreased during rapid growth.

The preliminary dose-response data reported here are consistent with the hypothesis that the sensitive structures are particles. In the two sets of data in which dose was varied by changing intensity or time of irradiation, the magnitude of the effect did not increase linearly with increasing dose, but increased as at least the second power. This could be interpreted in one of two ways: either the inactivation curve of the sensitive particles is of the multi-hit type, and the time of death depends directly on the number of particles inactivated, or the inactivation is a single-hit process, and the time to death is a power function of the number of inactivated particles. The second possibility is rendered unlikely by the experiments in which the dose was halved by protecting half of the cell from the radiation. In that case, regardless of the shape of the inactivation curve, twice as many particles were irradiated in the unprotected cells as in the protected ones, and the
protected ones required twice the time to swell. The interpretation of the higher order relationship in the dose-response curve should therefore be considered in terms of a direct effect of the radiation on the particle. More precise determinations of the dose-response curves are obviously necessary.

Our information on the nature of the damage produced in ascites tumor cells by ultraviolet irradiation may be used to suggest a crude working hypothesis to serve as a guide to further experimentation. Damage to the cells is caused by absorption of ultraviolet radiation, probably not by nucleic acids or their derivatives. Doses of the appropriate size cause inactivation of a system required to maintain the normal shape of the cell, especially of the stability of the cell surface. Depending on how complete the inactivation is, the cell survives in a damaged condition for a longer or shorter time, but finally undergoes an irreversible change associated with the loss of motion, probably signifying cell death. The system damaged might, for example, be the mitochondria, thereby causing a loss of the ability to supply energy to the cell surface. Further experiments to examine the mitochondria of partially irradiated cells might thus be of interest.

From the point of view of the applicability of the flying spot microscope to the study of living cells in the ultraviolet, the experiments reported above indicate the possibilities of making rather detailed studies. With suitable filtering of the illumination, cells can be protected from visible abnormality following the administration of as many as $5 \times 10^4$ television frames delivered in the minimum time, 30 minutes. Therefore, if 15 TV frames are integrated on each camera frame, 3000 ultraviolet photomicrographs of useful quality could presumably be made without introduction of artifact. It should be emphasized that we are not in a position to make accurate comparisons between the ultraviolet flying spot microscope and other systems, e.g., the conventional ultraviolet microscope or the image orthicon, in terms of the damage to the cell resulting from the production of comparable photomicrographs. It will be necessary to make absolute measurements of the energies involved in producing comparable damage syndromes in the same type of cell, before an effective evaluation can be made.

Since the signal to noise ratio obtained in the flying spot microscope is to a considerable extent a function of the amount of energy striking the cell, the quality of the picture obtained, in each frame of a time-lapse film, is set by the allowable number of television frames. The pulsed time-lapse technic may thus be shown to allow films to be made, of quality approaching the limits set by the optics, for as long as the cells can be kept in good condition in explants of the type we have used. While damage to the genetic structures of the cells under study may occur, it is possible to avoid influencing the cytological appearance of the cells themselves. Since unirradiated cells within the preparation may subsequently be examined, adequate controls are available.

**BIBLIOGRAPHY**

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Explanation of Plates

PLATE 91

Figs. 1 to 8. Serial flying spot ultraviolet photomicrographs showing induction of radiation damage in ascites tumor cells. Tumor sampled 5 days after inoculation. Objective N.A. 1.00, Corning filter 9863, monitor photographed with a 20-second exposure. Magnification approximately 600. Position of typical blebs (bl) indicated by arrows in Figs. 2 to 4.

Fig. 1. Normal ascites tumor cells, about 10 minutes after removal from mouse.

Fig. 2. Raster area immediately after exposure to 5 minutes of unfiltered radiation.

Fig. 3. Seven minutes after start of dose. Preparation has been moved so that the original irradiated area now occupies the position below the white line.

Fig. 4. Eighteen minutes. Blebbing in irradiated cells.

Fig. 5. Twenty-six minutes. Blebbing continues.

Fig. 6. Forty-five minutes. Blebs are no longer withdrawn; swelling is in process, nuclear margins may be seen, as well as absorbing blocks in nuclei.

Fig. 7. Seventy-two minutes. Irradiated cells completely swollen. Control cells at top of picture are still normal; partially irradiated cell indicated by arrow just beginning to swell.

Fig. 8. Five hours. Nuclei of damaged cells have contracted, unirradiated cells show normal appearance.
(Freed et al.: Radiation damage in Ehrlich ascites tumor cells)
PLATE 92

Figs. 9 to 12. Serial phase contrast photomicrographs showing induction of radiation damage in ascites tumor cells irradiated in the flying spot microscope. Tumor sampled at 7 days, exposed to 15-minute dose. X 780.

Fig. 9. Twenty-one minutes after start of dose. Cell at left was shielded by tape on scanner tube face, placed to keep radiation from striking to left of the line indicated by arrows. Cell at right received full dose; note blebbing.

Fig. 10. Twenty-five minutes. Cell at left shows small blebs, which appear to occur to a greater extent on the irradiated side.

Fig. 11. Forty-nine minutes. Beginning of swelling of fully irradiated cell. Half-irradiated cell still shows blebbing, especially on irradiated side.

Fig. 12. Six hours. Half-irradiated cell resembles unirradiated cells in preparation, has strong halo. Apparent recovery.

Figs. 13 and 14. Selected frames from 16 mm. flying spot time-lapse films, showing radiation damage in half-irradiated cells. 0.72 N.A. objective, 2-methylthiophene filter, 8 television frames per camera frame, about X 1900. Tumor sampled at 6 days. The shielded area formed by the tape may be seen at the left of the figures; the position occupied by the cells during irradiation was indicated on the monitor tube with wax pencil.

Fig. 13. About 17 minutes after start of dose. Arrow indicates blebbing on irradiated half of cell. Lower cell in photograph was fully irradiated, shows violent blebbing.

Fig. 14. Another experiment, about 18 minutes after start of dose. Arrow indicates blebbing on irradiated half of cell. Asymmetry of blebbing is rather well shown.
(Freed et al.: Radiation damage in Ehrlich ascites tumor cells)