AN ELECTRON MICROSCOPE STUDY OF VITALLY STAINED SINGLE CELLS IRRADIATED WITH A RUBY LASER MICROBEAM

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ABSTRACT

An electron microscope study has been made of vitally stained single cells whose cytoplasm has been subjected to a localized ruby laser microbeam. Light and moderate laser absorption (the resultant of stain concentration and laser energy density) produced restricted selective damage of mitochondria in cells stained with Janus green B; heavy laser absorption resulted in mitochondrial damage, as well as in nonselective interaction with other cell structures. With four other basic vital stains, the polysomes, ergastoplasm, mitochondria and other organelles at the irradiated site were uniformly damaged. Unstained cells showed no morphological alterations. With light primary damage (that restricted to the irradiation site), no secondary effects of the incident radiation were observed. With moderate primary damage, however, secondary damage of the mitochondria in the unirradiated cell portions was produced, which was reversible within 4 hr after irradiation. Heavy primary lesions caused severe secondary alteration of all cell structures that was irreversible and cell death occurred within 2 hr. Surviving cells examined 24 hr after light and moderate irradiation could not be distinguished from unirradiated controls. The possible mechanisms involved in the production of laser-induced cellular alterations are discussed.

INTRODUCTION

Low-power ruby laser microbeam-irradiation of living, nonpigmented cells does not produce readily perceptible reactions; however, in naturally pigmented cells such as erythrocytes, certain infusoria and plant cells, or in vitally stained cells, a visible morphological effect is produced, presumably because of the absorption of laser energy by appropriate chromophores (3, 5, 6, 17, 28–30). It has been shown that extremely small, invisible amounts of dye are sufficient to produce this effect (3, 6). Bessis et al. (5) have postulated that a selective interaction of the laser beam with stained subcellular structures could be expected when adequately selective vital stains, proper stain concentrations, and laser intensities were chosen. Such a specific interaction with mitochondria in living tissue culture cells stained with Janus green B (JGB) has been demonstrated by electron microscopy (2).

The effect produced in a living cell by coherent, monochromatic light concentrated in a very small cytoplasmic area is of interest also, both with respect to primary effects produced at the irradiation site and with respect to secondary effects produced in nonirradiated parts of the cell. The latter phenomenon has been demon-

strated in other partial-cell irradiation experiments. Ulrich (33) showed that X-irradiation of the cytoplasm of *Drosophila* eggs can affect the cell nucleus. In chick fibroblasts subjected to localized alpha-particle irradiation, Clegg (8) observed spreading of the induced damage. Secondary effects of ruby laser irradiation in living cells have been studied with respect to cell survival or division rates (3, 6, 30), chromosomal abnormalities (29), the effect on RNA synthesis (35), and the activities of oxidative enzymes (32). In the investigations just cited, laser-induced damage has been studied with the light microscope; in the present work, such damage is investigated at the ultrastructural level. Single cells subjected to a localized ruby laser microbeam were studied with the electron microscope; the results are presented under the following headings: (1) the primary radiation effect in JGB-stained cells as a function of absorption (the resultant of stain concentration and laser energy density), the location and the extent of the primary damage being evaluated with the help of horizontal and vertical sections of irradiated cells; (2) the primary radiation effect in cells stained with other basic vital stains; (3) the secondary radiation effects evoked in unexposed portions of irradiated cells; (4) comparison of the morphology of treated cells at 2, 4, and 24 hr postirradiation.

MATERIALS AND METHODS

Laser Microscope

The apparatus employed has been described by Bessis et al. (5). The air-cooled Laser consisted of a ruby rod, 40 mm long and 3 mm in diameter, and an excitation xenon flash tube of silicon whose energy was supplied by a 160 μf. condenser charged to 2.6 kv. The total energy output of the laser was 0.5 joule with a pulse duration of approximately 500 μsec; the emitted wavelength was 6943 A. By the use of a 6-fold ocular and a 40-fold objective (numerical aperture, 0.65) one obtains a calculated spot diameter of approximately 5.8 μ (Dr. G. Nomarski, personal communication). The energy could be varied by means of a graded neutral density filter placed between the laser and the microscope. A beam of ordinary visible light, that traveled the same optical path as the laser beam, was used in selecting the target to be irradiated; alignment was made with the aid of a test object prior to the beginning of experimentation. All experiments were performed with a normal phase-contrast microscope; a long-focal length condenser was employed when Rose chambers were used. Observation of cells during the irradiation procedure was possible by means of integrally mounted television equipment. Photographs of each cell were taken before irradiation, with the spot of ordinary light marking the site to be irradiated, and after irradiation with a 16 mm film camera that was incorporated into the system (40 American Standard Association negative film). All experiments were done at room temperature.

Cells

KB epithelial cells (11) and BHK21-C13 fibroblasts (16) were used. The former were cultured in casein hydrolysate medium (14), and the latter in Eagle's medium (12). Both media contained 10% (20% for long term experiments) inactivated calf serum; phenol red was used in some of the experiments. The stock cultures were grown continuously by trypsinization every 10 days for the KB cells, every 8 days for the BHK21-C13 cells.

The cells used in the experiments were subjected to trypsin (0.25% trypsin of the Pasteur Institute, in buffered 0.9% NaCl, pH 7.2) for 30 min at 37°C. Two ml of a suspension with 50,000 cells/ml were put into individual Leighton tubes where they grew on slides whose preparation will be described below. The cells were kept at 37°C and used for the experiments 48 hr after preparation. Culture chambers (25, 26) filled with 2 ml of cell suspension (50,000 cells/ml) were used when cells were to be fixed for the electron microscope at 2, 4, or 24 hr after irradiation. The preparation of their cover slips will be described below.

A few experiments were performed with rabbit macrophages collected from the peritoneal cavity 12 hr after the injection of 200 ml of Subtosan (Rhône-Poulenc, Paris). They were resuspended in autologous plasma, then used directly.

Preparation of Slides and Coverslips (Fig. 1)

Three guide circles, each with a diameter of 3 to 4 mm, were made with a diamond marker on the lower surface of small glass slides (47 × 13 × 1 mm); the distance between them was such that only one circle could be covered by a single embedding capsule. A partial circle (Fig. 1 *a*), having a diameter of 200 to 400 μ, was then made with an eccentric diamond scribe on the slide's upper surface within the bounds of each guide circle. The slide was coated with Formvar (0.2% solution in dichloroethylene), then dried for at least 24 hr before use. The cover slips (Fig. 1 *b*) measured 32 × 11 × 0.1 mm. On both ends of their lower surface a thin layer of nail polish was applied to provide support.

The lower cover slip of each Rose chamber had four 200 to 400 μ circles cut on its inner surface; each of these, in turn, was marked with a guide circle drawn on its outer surface with a wax pencil. The

internal surface was covered with a Formvar sheet, fixed to the glass by an Epon ring, to prevent its removal by the culture fluid.

Vital Staining

Janus green B in a concentration of 8×10^{-4} to 4×10^{-6} was put directly into the Leighton tube or the culture chamber and the cells were stained for 30 min at 37°C. The concentrations for nile blue, toluidine blue, cresyl blue, and methylene blue were 6×10^{-4} to 5×10^{-5}.

photographs were taken at 1- to 4-hr intervals after irradiation (for details, see reference 3). Fixation was carried out at selected times as described below.

Preparation of the Cells for Electron Microscopy

Fixation, following a modification of the technique of Robbins and Gonatas (24), was carried out on the slide according to the following schedule: (*a*) 5 min, 1.25% glutaraldehyde in Tyrode's solution (pH 7.3); (*b*) 15 min, 1.0% OsO_4 in semi-isotonic Tyrode's

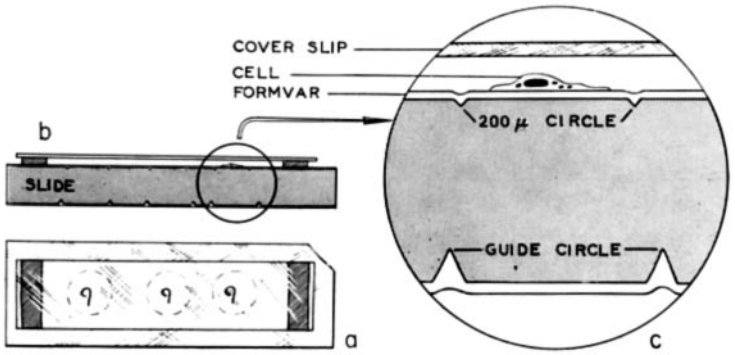


FIGURE 1 Diagrams of the slide–cover slip preparation. Fig. 1 *a*, as seen from above; Fig. 1 *b*, in lateral view; Fig. 1 *c*, enlarged portion of Fig. 1 *b*. The 3 to 4 mm guide circles (dashed line in Fig. 1 *a*) were cut in the lower surface of the slide with a diamond marking pencil. Within each, on the upper slide surface, a partial circle 200 to 400 μ in diameter was cut with an eccentric diamond scribe. Cells grow on the Formvar sheet covering the slide. The distance between slide and coverslip is less than 0.1 mm.

Irradiation Procedure

The slide containing the vitally stained cells was taken out of the Leighton tube and covered with a cover slip (Fig. 1). Under low power a group of 1 to 6 cells lying adjacent to one of the circles (Fig. 2) was chosen and a diagram showing its position with respect to the circle was made. Then, under higher magnification, the spot of ordinary light was focused on the part of the cell to be irradiated, the irradiation was carried out, and its effect, which could be seen immediately on the television screen, was recorded. As part of the routine observation procedure, each cell was photographed. On the average, the cells remained on the microscope stage 10 to 15 min. The cover slip, 30 to 60 sec after laser exposure, was floated off by dipping the preparation vertically into Tyrode's solution. Then the cells were fixed and processed for the electron microscope.

For the long term experiments the Rose chambers were inverted and put directly under the microscope. Thus, the cells to be treated were on the under surface of the cover slip which faced the objective. To facilitate later identification of the individual cells,

solution (pH 7.3); (*c*) 35 min, a mixture of 1.0% OsO_4, 2.0% C_2H_5OH, 1.0% Formalin in quarter-isotonic Tyrode's solution without Ca (pH 8.3; 4°C). After being washed in distilled water, the slides were placed in uranyl acetate for 1 hr, dehydrated with ethyl alcohols (30 min each, starting in 70%), then put through two changes (30 min each) of propylene oxide, and finally embedded in Epon (15). For final inclusion, the Epon-filled half of a gelatin capsule was inverted over the circle containing the irradiated cells. A second capsule on an adjacent circle served as a control. Polymerization was carried out for 16 hr at 37°C, 24 hr at 45°C, and 1 to 5 hr at 60°C. After the capsule had been removed from the slide, it was returned to 60°C for 48 hr. Dry ice was used in freeing the capsule from the slide after the excess Epon around the capsule's base had been removed. On the surface of the freed capsule, the impression of the circle and the irradiated cells can be seen in mirror image (Fig. 3). A truncated pyramid, 50 to 100 μ on an edge, with the irradiated cells on its surface, was cut from the capsule (Fig. 4). Unirradiated cells were removed during the trimming process. The surface of the pyramid was oriented so that it

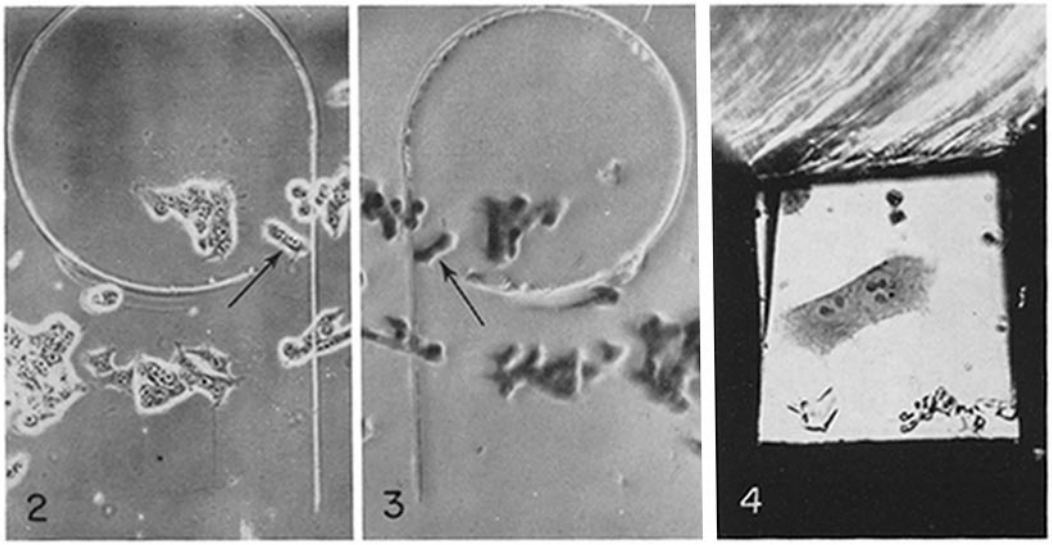


FIGURE 2 Low power view of living KB cells before irradiation adjacent to one of the circles (diameter 326 μ) shown in Fig. 1. (The circle is drawn with a tail to provide for more precise location of the treated cells.) Arrow: 2 cells to be irradiated. Phase-contrast, × 127.

FIGURE 3 The same cells as in Fig. 2 after irradiation, fixation, embedding in Epon, and polymerization, now shown in mirror image on the surface of the Epon capsule after the latter had been removed from the slide. The impression of the circle and the 2 irradiated cells (arrow) can be identified easily. Bright field, × 114.

FIGURE 4 The 2 irradiated cells on the surface of the cut pyramid before sectioning. At the upper left is a part of the circle impression. The pyramid surface is 71 μ square. Phase-contrast, × 456.

was parallel with the edge of the microtome knife, and horizontal sections were cut from the spread cells. Since the knife encounters the cell on its first pass, proper orientation of the capsule is of utmost importance. With an automatic feed it was possible to cut 30 to 60 sections (600 to 800 A thick) through each cell. The sections were poststained with lead citrate (22) and examined under the electron microscope. The exact area irradiated could be identified with the aid of the phase-contrast photograph and the diagram made earlier.

To study the depth and the size of the primary laser lesion, and to determine whether or not the cell membranes were affected, the above technique was modified slightly so that vertical sections of an irradiated cell could be made. After 24 hr of polymerization at 60°C, a prismlike block was cut from the Epon cylinder under the dissecting microscope. This was done in such a way that the irradiated cells were at the edge of the upper surface of the block (Fig. 5 *a*). The prism was reoriented and attached with Epon to the base of the cylinder (Fig. 5 *b*), then returned to 60°C for 24 hr. Next, a pyramid was cut as usual and the cells were sectioned under these conditions. The irradiated part of the cell was oriented towards the microtome knife.

RESULTS

Janus Green B

LIGHT PRIMARY DAMAGE[1] With low-level laser absorption we observed, under the phase-contrast microscope immediately after irradiation, one or two irregularly shaped black granules in the irradiated part of the cytoplasm; their greatest dimension was 2.8 μ. Fig. 6 shows an example of this type of damage; the granule covers 2.4 μ^2 which is approximately $\frac{1}{300}$ of the whole cell surface. (This fraction varied in other cells from $\frac{1}{300}$ to $\frac{1}{400}$.) There are no signs of secondary damage; the nucleus and other parts of the cell

[1] "Primary damage" is used to designate a morphological alteration produced at the irradiation site; "secondary damage" is defined as a change induced in parts of the cell that have not been subjected directly to the microbeam.

outside of the damaged region are obviously unchanged.

In this group, 4 cells were examined with the electron microscope. At the irradiation site one observes a striking alteration of the mitochondria. Their interiors were filled with electron-opaque material; cristae and inner and outer membranes were only partially preserved (Fig. 8 *a*, *b*, and *d*). In the section in Fig. 8 *b*, several mitochondria form a large black mass in which only remnants of double membranes are visible. A section taken from a region closer to the upper cell membrane shows only two altered mitochondria (Fig. 8 *d*), and the electron-opaque material is limited to them. The total number of mitochondria damaged by a single pulse is difficult to estimate. The other cell structures in the immediate vicinity of altered mitochondria or even between them, i.e. parts which have been exposed to the laser light, do not show striking morphological alterations. No secondary radiation damage can be detected; cell parts outside of the irradiated region are intact (Fig. 8 *c*).

MODERATE PRIMARY DAMAGE: In cells in which moderate laser absorption had occurred, phase-contrast examination showed that one or two black granules, up to 5.5 μ in their greatest dimension, were produced in the cytoplasm. In some cases the granules formed a mass with a light core that often contained a temporary gas bubble; in other instances the lesion was surrounded by a light halo. Light core or halo did not seem to be effects due to the optical characteristics of the phase-contrast since they retained their identity when changing the focal level of the microscope. This level of damage is illustrated in Fig. 7. The 2 granules, which measure 5.3 by 4.1 μ, cover an area that occupies approximately $\frac{1}{120}$ of the total cell surface. (This fraction in other cells varied from $\frac{1}{50}$ to $\frac{1}{120}$.) No signs of secondary damage are seen in other parts of the cell.

In this group, 32 cells were examined with the electron microscope. Figs. 9 and 10 are representative of findings in KB cells. In Fig. 9 *b* the primary damage is visible as a large electron-opaque mass on whose periphery remnants of outer and inner mitochondrial membranes are seen. Nearby,

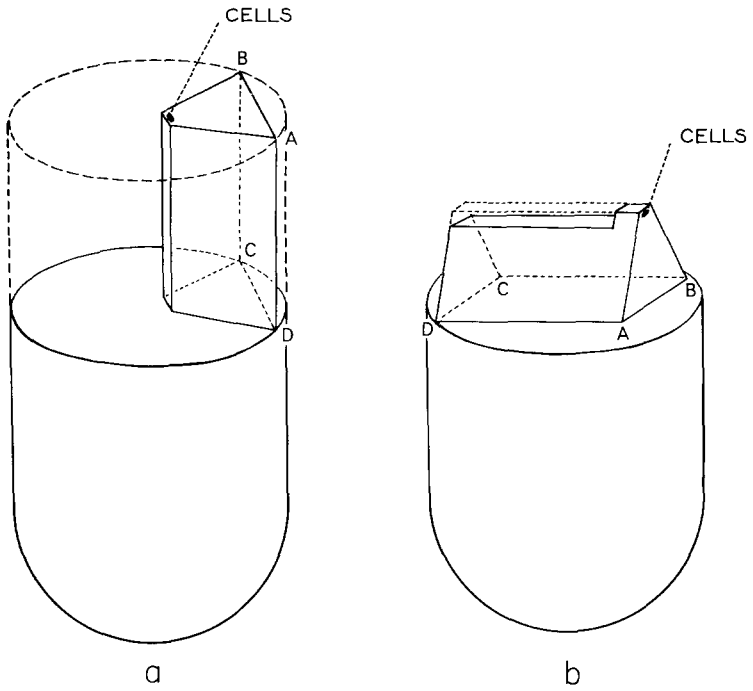


FIGURE 5 Diagrams showing how cells were reoriented for vertical sectioning. Fig. 5 *a* Epon cylinder out of which a prismlike block was cut. The irradiated cells can be seen on the upper surface of the block. Fig. 5 *b* The block is shown in its new position on the base of the cylinder with the pyramid cut on its upper right surface. Sections will be cut at right angles to the original upper surfaces of the cells.

three altered mitochondria, more or less filled with electron-opaque material, are recognizable. Ergastoplasmic lamellae and polysomes, which are between them and the central mass, are morphologically intact, as are parts of the nucleus lying in the irradiated zone. Primary damage, several sections closer to the upper cell membrane, is shown in Fig. 9 *c*. At least five mitochondria, whose contours are still discernible in the periphery of the electron-opaque material, are clumped together as a result of the irradiation; in one place cristae are evident. The restriction of the damage to the mitochondria is striking. In one place the nucleus, only 155 mμ from the lesion, is unaltered morphologically.

An analysis of all the sections through this particular cell showed that the primary damage was located some distance from the cell membrane. This was corroborated by a study of vertical sections through cells that exhibited similar reactions under the phase-contrast microscope. For example, in one cell 5.1 μ thick in the irradiated area, the damage began 1.1 μ below the upper cell membrane (the one closest to the objective during irradiation) and ended approximately 1.2 μ above the lower membrane. Both membranes were intact, as was the cytoplasm lying between them and the damaged organelles. In this case the damaged region constituted approximately $1/300$ of the volume of the whole cell.

With phase-contrast the primary damage appears larger than in a single, given electron microscope section. This is because the optical image of the damage is the sum of all ultramicroscopic planes. The extent of the damage in each plane is limited by the diameter of the impinging laser beam; within this limitation it depends on the number and position of the JGB-stained mitochondria present.

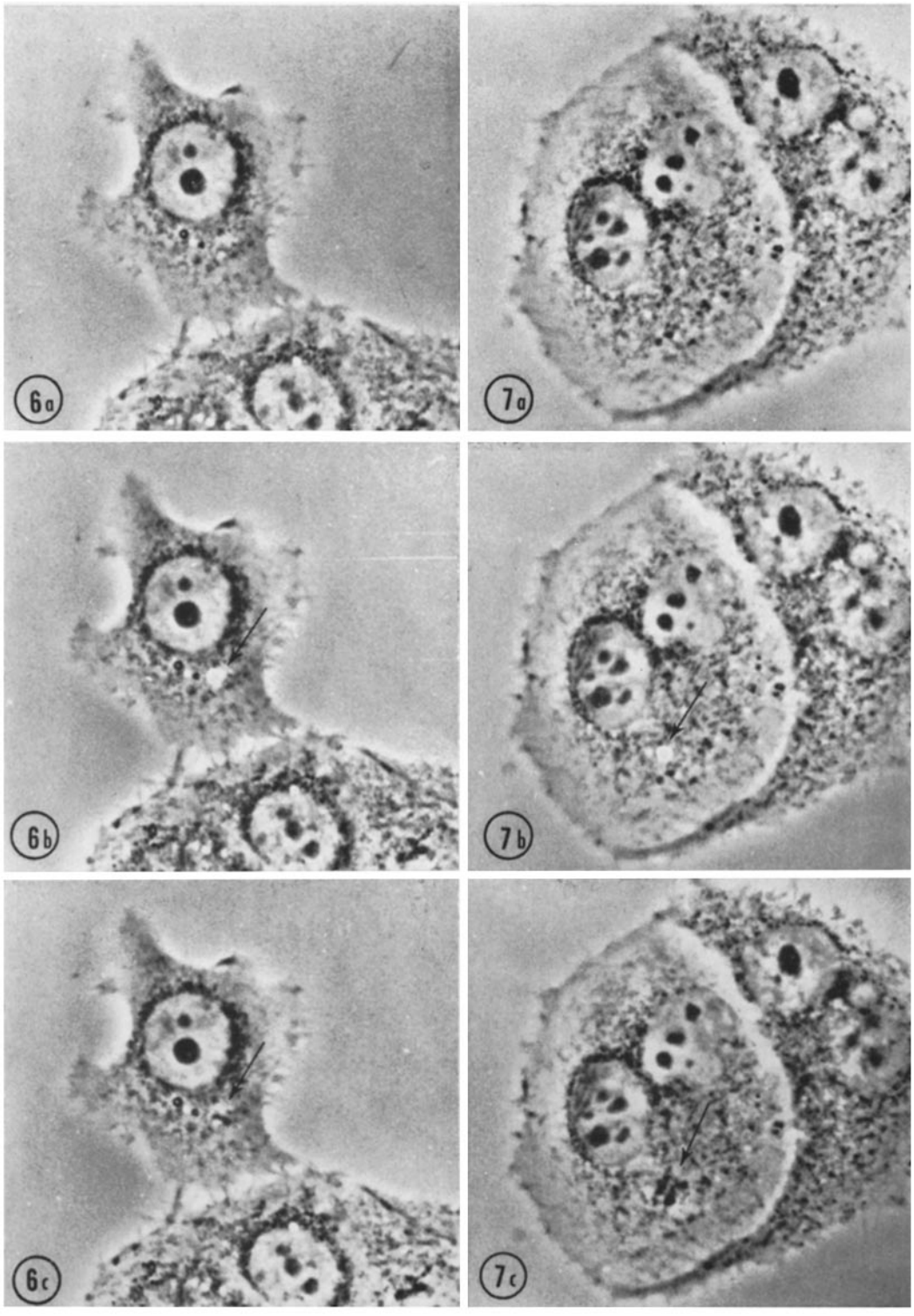
Most cell structures outside of the irradiated region (polysomes, ergastoplasm, Golgi apparatus, centriole, nucleus, nucleolus, and nuclear membranes) exhibited no signs of secondary radiation damage. By contrast, the mitochondria had undergone a striking change, being greatly swollen and having lost their cristae (Fig. 10). The matrix of each was light or had been transformed into granular, electron-opaque material condensed on the smooth inner membrane; the latter occasionally had fragments of cristae on its surface.

Results similar to those just described for KB cells were obtained also with laser-irradiated fibroblasts. Such a cell is shown in Figs. 12 and 13. The limitation of primary damage to the mitochrondria is clearly evident. In the nonirradiated region of the cell two different mitochondrial populations are seen. Most of the mitochondria show secondary damage similar to that already described for the KB cells (Fig. 12 *d*). In 3 cells, however, there were a few mitochondria possessing recognizable cristae as well as a great number of electron-opaque granules (Fig. 13).

MORPHOLOGY OF THE CELLS WITH MODERATE PRIMARY DAMAGE FIXED AT 2, 4, OR 24 HR POSTIRRADIATION: Long term studies with the phase-contrast microscope showed that cells with moderate damage survived the irradiation (3). To study primary and secondary mitochondrial damage as a function of survival times, we fixed cells for the electron microscope at different times after irradiation. Seven cells were examined 2 hr, and 7 cells 4 hr, after treatment. The results in both instances were similar. When compared with the results obtained directly after irradiation, two differences were evident.

FIGURE 6 Photomicrographs of vitally stained KB cells before irradiation Fig. 6 *a*, with spot of visible light (arrow) marking site to be irradiated Fig. 6 *b*, and immediately after exposure to the ruby laser microbeam (incident energy 0.026 joule) Fig. 6 *c*. The black mass (arrow in Fig. 6 *c*) characterized as light primary damage was produced at the irradiation site. JGB (1.6×10^{-5}) was applied 45 min prior to exposure. Phase-contrast, $\times$ 850.

FIGURE 7 Description as given in Fig. 6. Arrow in Fig. 7 b: Site to be irradiated. The two black granules (arrow in Fig. 7 *c*) which were produced by exposure to an incident laser energy of 0.062 joule are characterized as moderate primary damage. JGB (2×10^{-5}) was applied 50 min prior to irradiation. Phase-contrast, $\times$ 850.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7c

First, the electron-opaque material in the irradiated region was scattered over a large zone rather than occurring in clumps (Fig. 15 *b*). It was found partially in slightly vacuolized mitochondria with preserved cristae, partially in mitochondria without cristae but with preserved inner and outer membranes, and partially in loose contact with fragments of membranes. The second and more noteworthy difference was found in the mitochondria outside the irradiated region. The majority, even those in the immediate vicinity of the primary damage, were normal in structure with well preserved cristae (Fig. 15 *d*); a small number of the latter showed vacuolization. The prominent secondary mitochondrial damage visible directly after irradiation was missing. All other cell components showed typical structure.

In the 8 cells fixed 24 hr after irradiation, no alteration in the region of primary damage has been found. Such cells could not be distinguished from controls.

HEAVY PRIMARY DAMAGE: A cell that had just absorbed a large dose of laser radiation, when viewed with phase-contrast, exhibited what appeared to be a hole or a dark-rimmed cavity (about 10 μ in diameter) at the focal point of the beam. Other manifestations of damage often included the transient appearance of a gas bubble, the formation of blebs at the cell surface, the loss of contrast in both cytoplasm and nucleus, the arresting of intracellular movement and, in extreme cases, cell fragmentation. As might be expected, most cells died within 2 hr after being irradiated (3).

In this group of 10 cells examined with the electron microscope, there were prominent alterations in the irradiated region consisting of large electron-opaque masses with light, structure-poor centers. Remnants of double membranes were visible in such lesions. Other cell structures were often involved also, e.g., in four instances a small part of the nucleus and the nuclear membrane and, in one, the eccentrically located nucleolus were affected. In most cases the cell membrane was disrupted over the primary damage, and heavy secondary damage of all cell structures was observed. The cytoplasm had lost contrast and was partially empty, and the number of polysomes per surface unit appeared to be decreased compared with controls. Many mitochondria showed partial loss of cristae, as well as the alterations described above; often they were uniformly filled with dense material which obscured the cristae. The ergastoplasm was dilated and often disrupted; centrioles and fibers were preserved, while microtubules had disappeared. The nucleus had low contrast and its chromatin was condensed into small, scattered islands; its membrane was intact except where it had sustained direct damage. The nucleoli in 5 of the 10 cells were more compact than usual. In the one instance in which part of the nucleolus was involved in primary damage, its unirradiated portion showed essentially normal morphology.

Other Vital Stains

This group includes 12 cells vitally stained with nile blue, toluidine blue, cresyl blue, or methylene

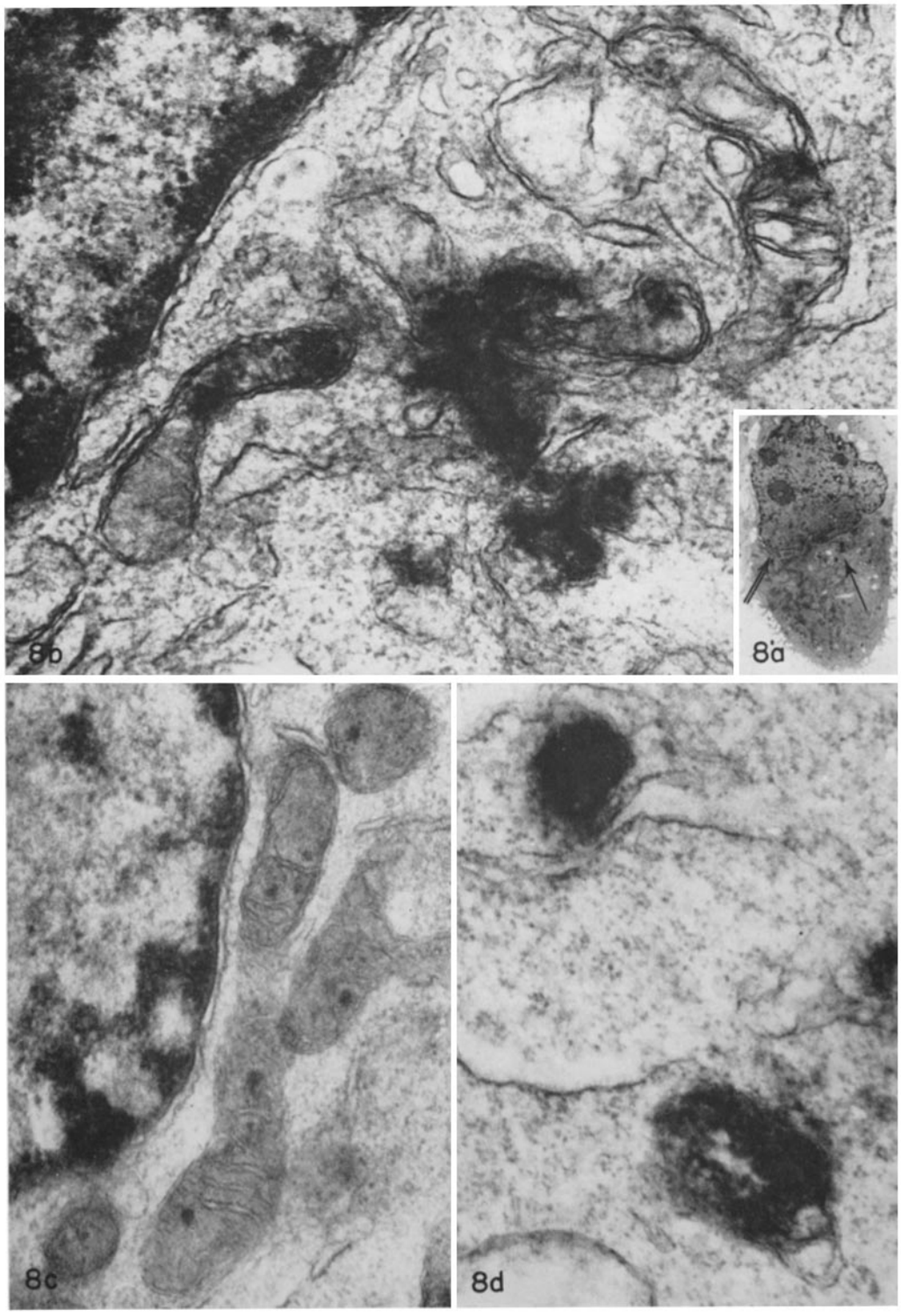
FIGURE 8 Electron micrographs of a vitally stained KB cell with light primary damage 2 min after laser irradiation (incident energy 0.01 joule). JGB (4×10^{-5}) 44 min prior to irradiation.

FIGURE 8 *a* A section of the whole cell in which the primary laser damage is visible as a black mass (arrow) in the cytoplasm. × 2670.

FIGURE 8 *b* An enlargement of the area marked by the arrow in Fig. 8 *a* showing electron-opaque material within the remnants of the mitochondria; portions of cristae are visible. The mitochondrion on the left is only partially altered. × 55,000.

FIGURE 8 *c* An enlarged portion of an unirradiated part (double-shafted arrow) of the whole cell shown in Fig. 8 *a*. Notice intact cell structures. × 50,000.

FIGURE 8 *d* The irradiated area in another section. The restriction of the primary damage to the mitochondria is clearly evident. × 58,500.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blue. In most cases no selective alterations of organelles were observed; the irradiated area was uniformly covered with loose electron-opaque material containing membrane fragments. Apparently all cell structures at the irradiated site were affected, including the polysomes. In one case the double lamellae of the Golgi apparatus that were situated in the periphery of the irradiated field were involved. In one fibroblast stained with cresyl blue, however, there seemed to be a selective conversion of the Golgi lamellae into electron-opaque material, whereas the centriole and other adjacent cell structures apparently were unaffected; all cell components in this particular case showed secondary damage. The secondary damage found in cells of this group was similar to that reported for JGB-sensitized cells.

Controls

Unirradiated cells, taken from the same slide as the irradiated ones that have been described, served as controls. Fig. 11 shows a portion of a typical KB control cell; Fig. 14, part of a typical fibroblast. All cells were morphologically intact, even in those cases where the irradiation of the neighbor cells was carried out very close to the control cell's membrane, i.e., the distance between the primary damage and the unirradiated cell was approximately five to ten times less than that to the distal pole of the irradiated cell.

Irradiation of Unstained Cells

In this group 4 cells, whose cytoplasm had been irradiated with maximum laser intensity (0.5 joule), were examined. All showed intact structures with no detectable signs of primary or secondary damage.

DISCUSSION

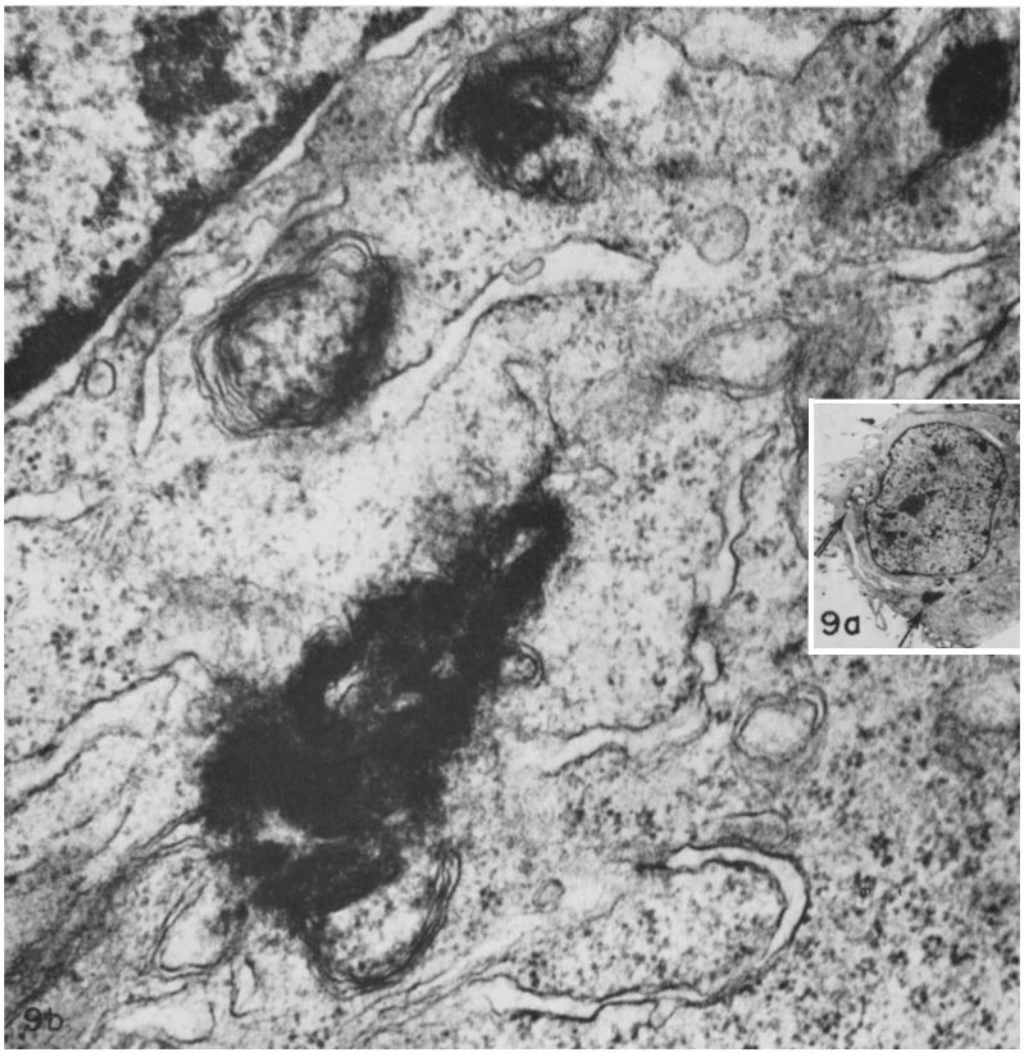
Different vital stains in nontoxic concentrations were used to mediate interactions between a localized ruby laser microbeam and organelles of single living cells. The selective conversion of mitochondria to electron-opaque masses in cells sensitized with JGB was due, almost certainly, to absorption of energy by the stained organelles, since irradiation of nonvitally stained cells with an even greater energy flux did not produce such alterations. JGB is reduced to its leuco form in a living cell by the nicotinamide-adenine dinucleotide flavoprotein system (9). Being a basic stain, it has an affinity for electronegative cell constituents, one of which is the mitochondrion. It is only in the mitochondrion, however, that leuco-JGB is reoxidized to the colored form by the cytochrome system. Under these conditions it becomes a chromophore capable of absorbing red light. If concentrations and low doses of laser energy are employed, their interaction is restricted to the mitochondria. In cases where only portions of mitochondria are damaged, presumably just the altered part of the organelle contained reoxidized dye. With strong dye concentrations and high laser flux, interactions with traces of unreduced stain in the hyaloplasm or in the nucleus of living leucocytes were observed (6). This same mechanism probably accounts for the involvement of the nucleus and other cell structures in the present study when heavy primary damage was produced in KB cells or fibroblasts.

FIGURE 9 Electron micrographs of a KB cell with moderate primary damage 1 min after laser irradiation (incident energy 0.092 joule). JGB (6×10^{-5}) 43 min prior to irradiation.

FIGURE 9 *a* The arrow points to a black mass in the cytoplasm produced as a result of the laser interaction. Part of the cell indicated by the double-shafted arrow is magnified in Fig. 10. × 3000.

FIGURE 9 *b* An enlargement of the primary damage shown in Fig. 9 *a*. Remnants of mitochondrial membranes are visible on the periphery of the central mass of electron-opaque material. Three other mitochondria containing smaller amounts of the electron-opaque substance are nearby. × 62,000.

FIGURE 9 *c* Another section through the irradiated site shows other (at least five) damaged mitochondria. × 52,000.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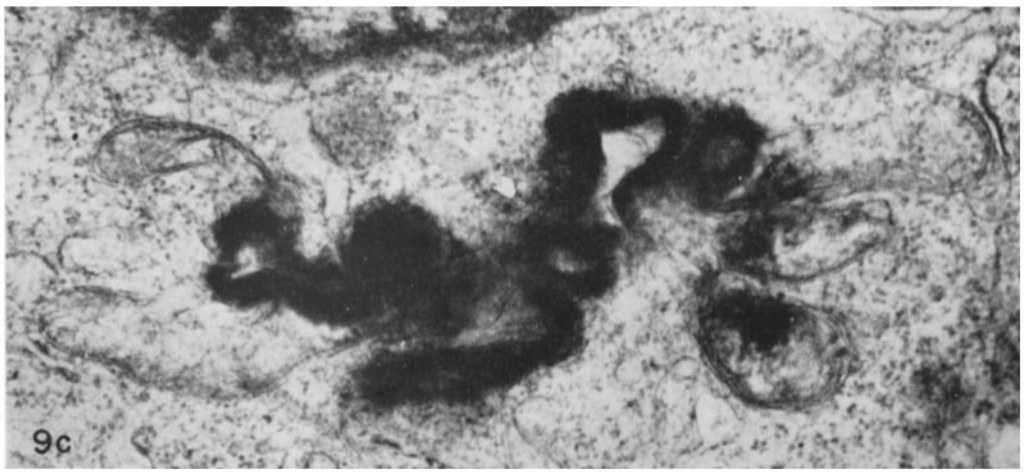
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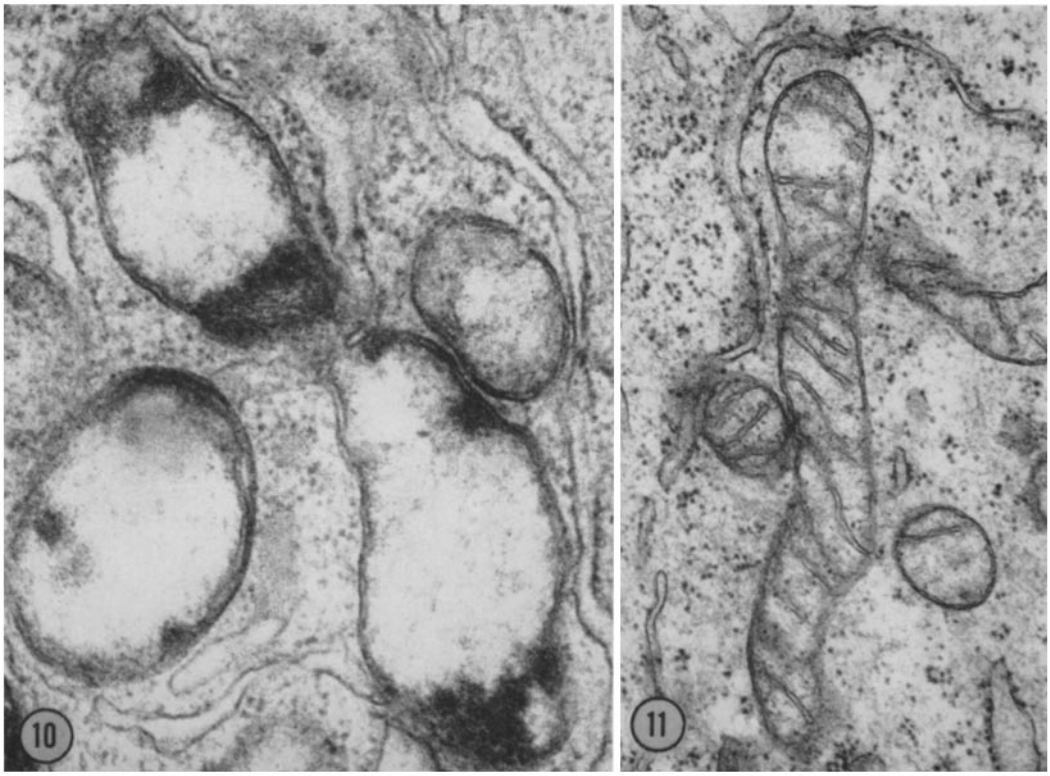


FIGURE 10 A portion of an unirradiated part of the cell illustrated in Fig. 9 *a* (double-shafted arrow) showing secondary mitochondrial damage. It is characterized by much swelling, loss of cristae, lightening of the matrix, and the occurrence of small electron-opaque masses on the inner membrane. × 45,500.

FIGURE 11 Typical mitochondria of an unirradiated control cell from the same slide as shown in Figs. 9 and 10. × 48,500.

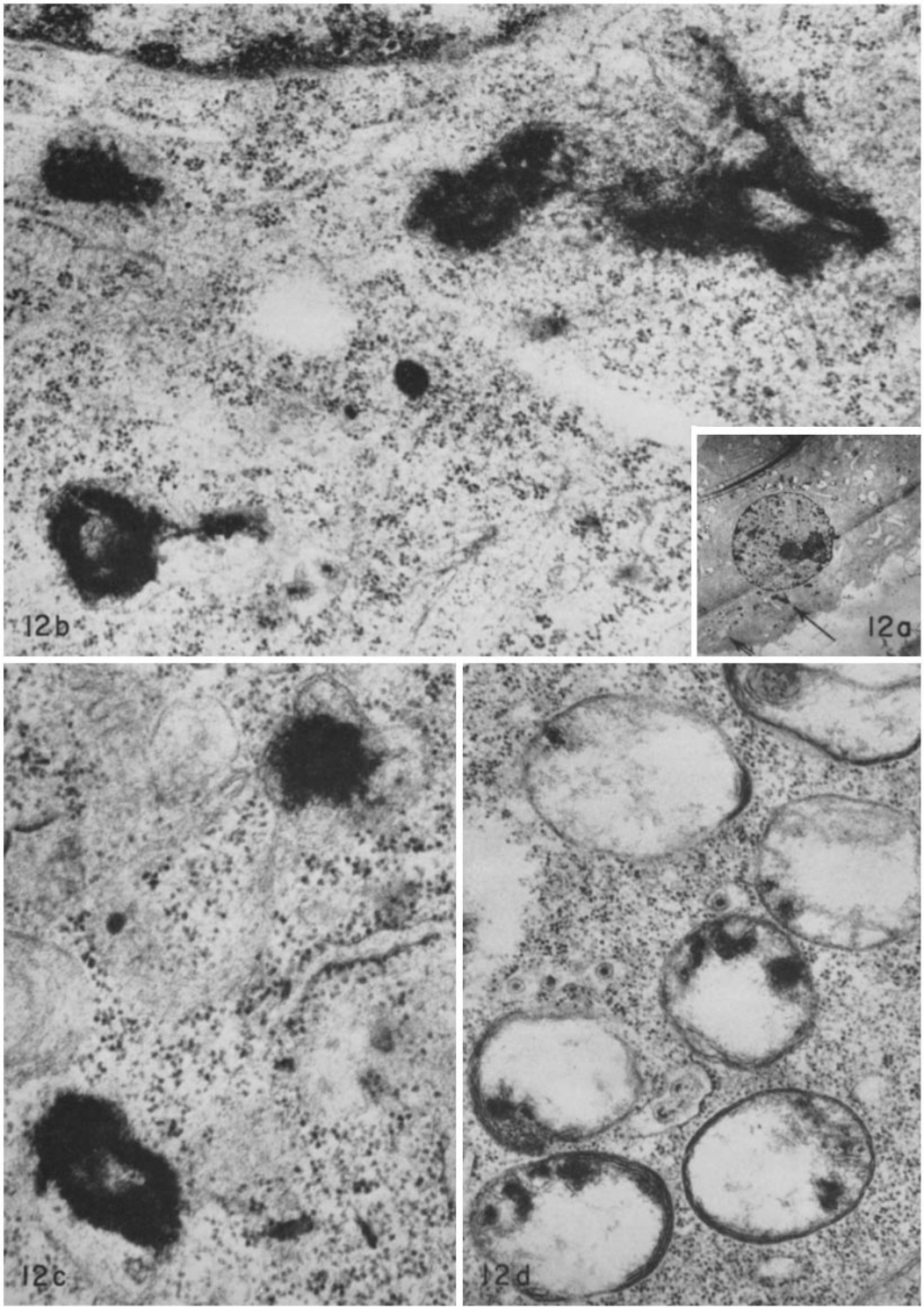
FIGURE 12 Electron micrographs of a BHK21-C13 fibroblast cell with moderate primary damage 1 min after laser irradiation (incident energy 0.020 joule). JGB (10^{-5}) 54 min prior to irradiation. (The viruslike structures in the ergastoplasm sacs are probably identical with the viruses described by Bernhard et al. (4).)

FIGURE 12 *a* In this section of the whole cell, the arrow points to black granules produced as a result of laser interaction. Part of the cell indicated by the double-shafted arrow is magnified in Fig. 13. × 2,175.

FIGURE 12 *b* An enlarged portion of the site of interaction showing several altered mitochondria; remnants of double membranes are recognizable. × 44,000.

FIGURE 12 *c* In another section only two mitochondria showing primary damage are visible. × 56,500.

FIGURE 12 *d* A nonirradiated portion of the cell shown in Fig. 12 *a*. Mitochondria exhibit secondary damage similar to that described for the KB cell in Fig. 10. Some fragments of cristae can be observed on the inner membrane. × 37,000.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12c
12d

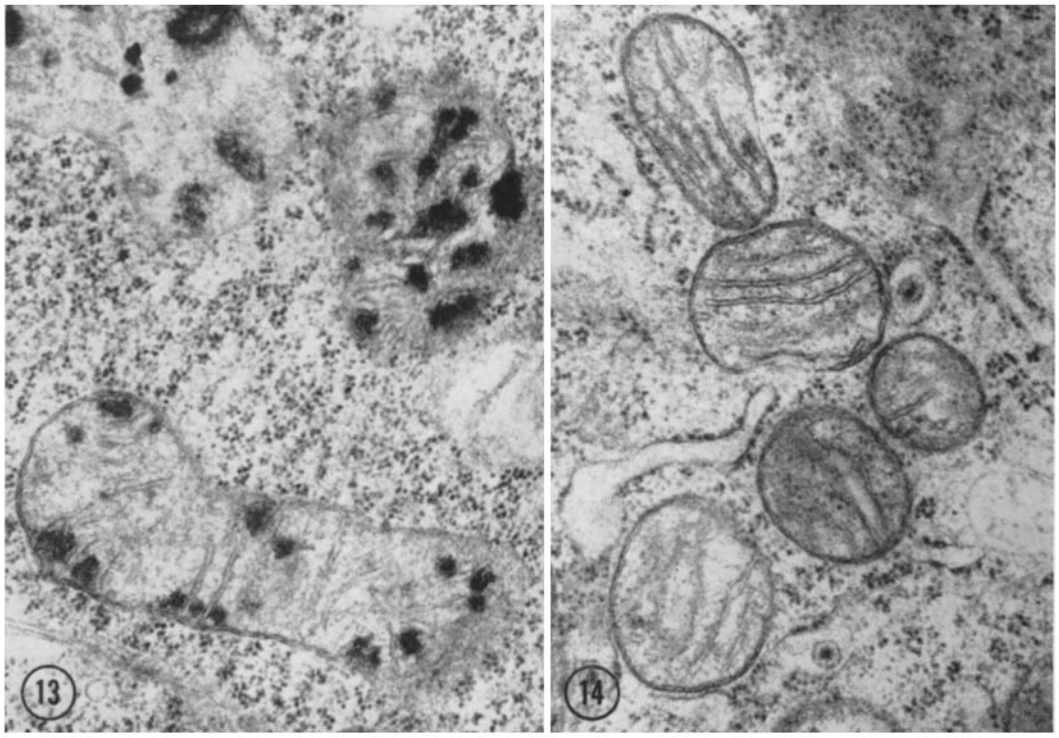


FIGURE 13 An enlarged portion of Fig. 12 *a* (double-shafted arrow) showing mitochondria with secondary damage having cristae but containing many electron-opaque granules. × 41,500.

FIGURE 14 Mitochondria of an unirradiated fibroblast from the same slide as shown in Figs. 12 and 13. × 47,500.

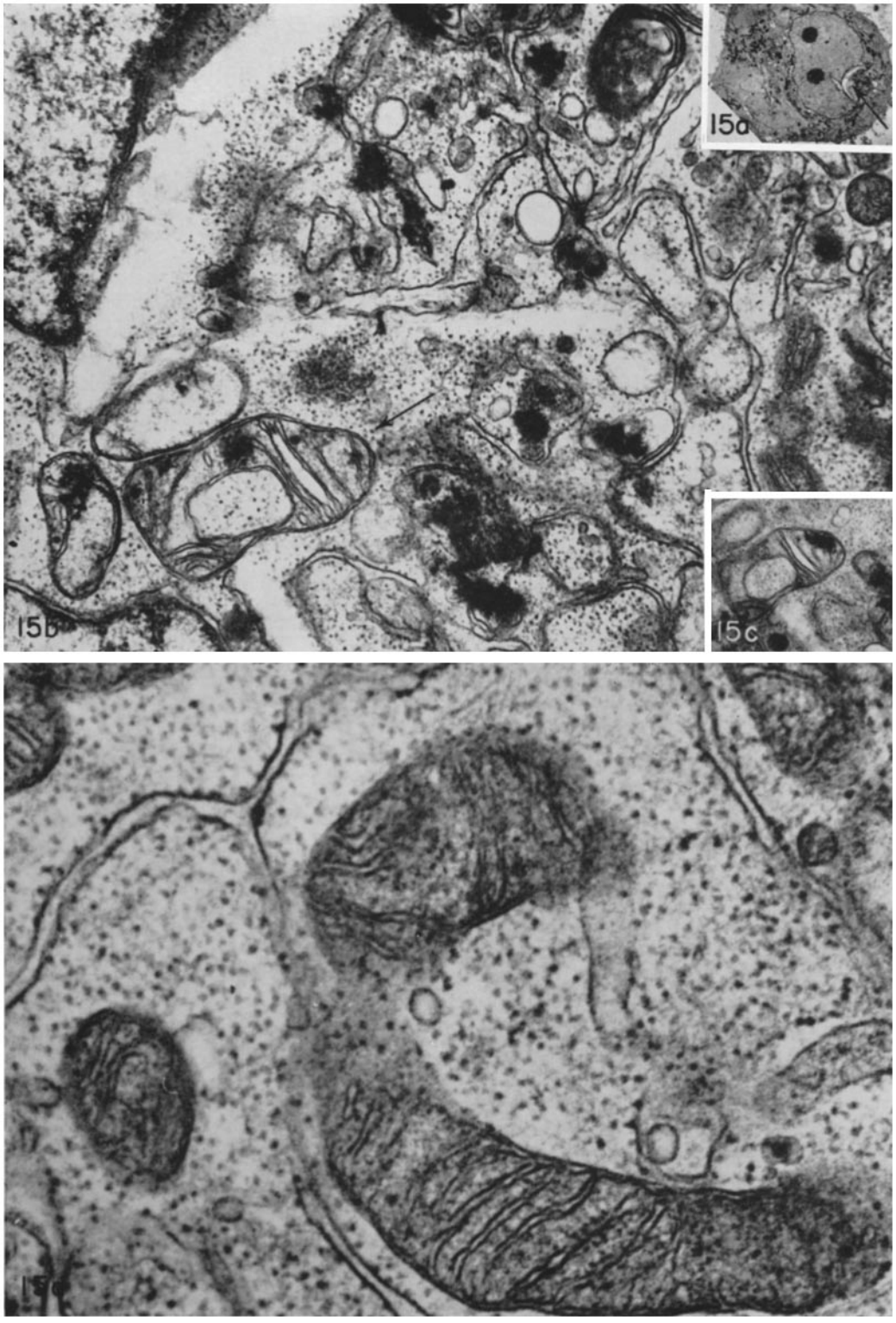
FIGURE 15 Electron micrographs of a KB cell with moderate primary damage 4 hr 22 min after irradiation (incident energy 0.091 joule). JGB (10^{-6}) applied 1 hr 5 min prior to irradiation.

FIGURE 15 *a* A section of the whole cell with the arrow pointing to the site of interaction. × 1300.

FIGURE 15 *b* An enlargement of the site of interaction several sections closer to the lower cell membrane than that shown in Fig. 15 *a*. The electron-opaque material is loosely scattered over the whole field; it is present in two mitochondria (lower left) with preserved cristae and in one mitochondrion (lower center) in which only inner and outer membranes are preserved. Other electron-opaque material is surrounded by simple membranes or is only loosely connected with membrane fragments. × 36,000.

FIGURE 15 *c* Another section of the mitochondrion in Fig. 15 *b* (arrow). In this plane, laser-stain interaction took place in another part of the organelle. × 18,500.

FIGURE 15 *d*. The mitochondria from an unirradiated part of the same cell have a typical appearance. Secondary damage observable immediately after irradiation is no longer visible. × 74,500.


15a
15b
15c

In most cases there was no selective interaction with given cell organelles when the other four basic vital stains were employed. All cell structures subjected directly to the beam seemed to absorb laser energy. This agrees with the concept that these dyes undergo an adsorptive binding with all electronegative cell constituents, e.g., nucleic acids, mucopolysaccharides, and phospholipids (for the literature see reference 31). Selective absorption by the Golgi lamellae was found in only one case, the organelle having apparently fixed greater amounts of dye than the adjacent cell structures.

The nature of the electron-opaque material induced by laser-stain interaction in the mitochondria is still unknown. Probably it consists of denatured lipids or proteins produced as a consequence of the development of intense heat caused by energy transfer in the area of laser absorption. The temperature and duration of the heat induced at the irradiation site in our system are not known. In melanoma tissue subjected to a high-power neodymium laser (337 joules) with a pulse duration of 500 μsec, temperatures from 85 to 395°C, enduring for 300 μsec, have been measured (19).

In cells with light primary damage, no secondary damage could be demonstrated. All cell structures other than the mitochondria at the site of interaction were morphologically intact. Most of these cells survived the irradiation but only 60% of them divided (3), indicating that there was a severe disturbance of cell metabolism which was not detectable morphologically. A slight regression of RNA synthesis could be shown (35), whereas several oxidative enzyme systems were intact (32).

In cells with moderate primary damage, secondary radiation damage, restricted to the mitochondria outside of the irradiated area, was observed. All such organelles, even those separated from the primary damage by the nucleus, had lost their conventional structure. In only 3 of 32 cells examined, a few mitochondria with cristae, but containing an abnormally large number of electron-opaque granules, were found. These mitochondria were similar to those that have been observed in the cells of the duodenal wall after increased absorption of sodium and potassium (34), in kidney tubule cells of mice after injection of albumin (23), and in pancreas cells of fasted guinea pigs (21). It seems that the secondary alteration is more than simple mitochondrial swelling. Rouiller (27) distinguishes between reversible swelling and more pronounced mitochondrial alterations which he thinks are irreversible. He divides the former type into two categories. In the first, the conventional structures are preserved and the organelles are easily recognized; the cristae are shorter than normal, and the matrix appears to be diluted. He regards water imbibition as the cause of this kind of swelling. In the second and less frequent type, the mitochondria are more dense due to the concentration of their substance. In Fig. 8 of Rouiller's article, cristae were rendered less visible by the increased density of the matrix. Normal and swollen mitochondria can be present in the same cell. The secondary damage in our irradiated cells seems more like that which Rouiller regards as irreversible. The latter mainly involves the cristae "which, through vesiculation, break up into granulations and disappear" and the matrix "which assumes a roughly granular, filamentous appearance or else is converted into osmiophilic masses." It is, therefore, surprising that the mitochondria in our irradiated cells apparently recover morphologically within 2 to 4 hr after irradiation.

The mechanism which evokes secondary mitochondrial damage within 30 sec after irradiation is difficult to explain. Mitochondria appear to be more sensitive to deleterious influences, which spread from a center of primary damage to nonirradiated regions, than other organelles. In a cell irradiated in a circumscribed area with a small dose of alpha particles from a focused microbeam, those mitochondria located in nonirradiated parts of the cell are the first cellular constituents to undergo alteration (8). The secondary mitochondrial damage in our system is apparently not linked to the use of the selective mitochondrial stain Janus green B, since it appears to be similar with all five vital stains used. This fact would exclude also the possibility that the observed secondary effects result from a "spreading" of the beam to an area greater than 6 μ. Further evidence for this contention is provided by the observation that, even if the primary damage is localized 4 to 6 μ from an unirradiated neighboring cell, this cell shows normal mitochondria; however, the mitochondria in the irradiated cell, often up to 50 μ from the site of the primary damage, show secondary damage.

The rupture of the cell membrane, observable in Clegg's cells (8), can be excluded as a contributing cause of the secondary damage seen in the present study. Vertical sections show that the

primary damage lies 1 to 2 μ from the upper and lower cell membranes, both of which are morphologically intact. Furthermore, no trypan blue penetration can be demonstrated (32).

An argument against the involvement of heat conduction in the production of secondary damage is the fact that the primary damage, i.e., the area to which short term (<500 μsec) heat development by energy transfer is restricted, is only $\frac{1}{200}$ to $\frac{1}{400}$ of the volume of the whole cell. Under these circumstances, conduction could result in a final average increase of the cell temperature, ranging from not more than 0.25° to 2.0°C and lasting a short period of time, if one uses the figures given by Minton et al. (19). Necessarily, the temperature must be higher in the immediate vicinity of the lesion; however, there is no morphological evidence for a heat gradient. Mitochondria in all cell parts show the same degree of secondary damage. Limitation of the heat to the site of interaction seems to be true even at the mitochondrial level. Mitochondria which had interacted only to a limited extent with the laser are not necessarily destroyed but can apparently recover. Several hours after irradiation they show normal morphology, aside from the small electron-opaque mass marking the site of primary damage. Further corroborative evidence comes from the work of Mendelson et al. (18), who made skin irradiations with a high-power ruby laser. They believe that the heat remains strictly limited to the site of interaction, in spite of its high intensity.

Pressure waves emanating from the irradiated area after exposure of living tissue to high-intensity ruby laser radiation have been observed (1, 18). Mendelson et al. (18) reported that these low-intensity waves are stopped rapidly by the surrounding tissue and thus produce no biologically important effect. In the cells used in the present study, one would expect that the adjacent portions of a control cell would be affected more severely by the eventual effects of the waves than the distal pole of the irradiated cell. This argument is valid only if one assumes that conduction through two cell membranes is as efficient as conduction through or around the nucleus which separates the distal pole from the primary damage site.

Shrinkage of the primary interacting mitochondria may produce mechanical stresses, but these, as well as other physical forces that might be involved, are difficult to evaluate.

Derr et al. (10), who irradiated the skin of black mice with a 100 joule ruby laser, found free radicals in the irradiated area. Since the probability of a free radical reacting with a cellular constituent is great, its range must be very limited. The latter was estimated to be 16 to 30 A in the case of irradiation with alpha particles (7, 8, 13). Secondary damage far from the site of laser interaction could hardly be attributed to this phenomenon.

Clegg (8), who irradiated chick fibroblasts with an alpha particle microbeam, discusses changes of pH, as a consequence of short term local heat development (to 100°C) at the site of irradiation, as possible causes of damage in distant cell structures. This may contribute to observed secondary damage in our cells. In addition, ammonia, CO, and CO_2, together with H_2O vapor, may be found in the temporary gas bubble often formed at the site of primary interaction. The rapid diffusion of these substances may have direct toxic effects on the organelles of the whole cell.

In spite of the extensive secondary mitochondrial damage observed in our irradiated cells, there is a normal histochemical activity of a series of oxidative enzymes in such cells (32). However, the mitochondrial alterations may be accompanied by a temporary uncoupling of phosphorylation and oxidation because of the close relationship between mitochondrial morphology and oxidative phosphorylation (for the literature see reference 20). This may be one explanation for the decrease of RNA synthesis in these cells (35). Although the mitochondria in cells with moderate primary damage have morphologically recovered from secondary radiation damage after 2 to 4 hr, there is strong evidence for irreversible cell damage at the molecular level. Four hours after moderate laser damage, only 35% of the cells returned to a normal rate of RNA synthesis. The rest showed either sharply decreased synthesis (10% of cells) or activity approaching zero (55% of cells) (35). Indeed, the majority of the cells die within the first 24 hr after irradiation (3); also, the cells that survived for 24 hr do not undergo division although their ultrastructure at 24 hr is virtually the same as that of unirradiated controls.

In cells with heavy primary damage, drastic secondary alteration of all cell structures except

centrioles and microfibrils was found. Such cells exhibit decrease or absence of oxidative enzyme activity (32), the incorporation of uridine-H^3 averages 27% of that of unirradiated controls (35); cell death in most cases occurs within 2 hr postirradiation (3).

The massive secondary damage of all cell structures in cells exhibiting heavy primary damage seems to be associated with the rupture of the cell membrane, which occurred in the majority of the cells, over the site of interaction. This is corroborated by trypan blue penetration studies (32). Whether or not the rupture is due to laser interaction with traces of unreduced JGB on the membranes is difficult to say. As a consequence of the membrane damage, water and ions penetrate the cell while cell substances (proteins) probably leave it. Such a phenomenon, also found in irradiated erythrocytes, parallels a loss of contrast seen in time lapse films. The resulting drastic change of the internal cell milieu probably causes the irreversible secondary damage of all cell structures.

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