

# EFFECT OF THE RUBY LASER MICROBEAM ON MITOCHONDRIA OF KB CELLS SUPRAVITALLY STAINED BY PINACYANOL

YASUKAZU TANAKA

From the Institut de Pathologie Cellulaire, Hôpital de Bicêtre, Paris, France. The author's present address is the Veterans Administration Hospital, San Francisco, California 94121

## ABSTRACT

With pinacyanol as the supravital stain, a preferential effect on mitochondria of KB cells was achieved by the irradiation with the ruby laser beam. The observation confirmed the results of other workers using janus green B in the same experimental system. The preferential effect on mitochondria was noted in the area extending 8–10  $\mu$  beyond the nonpreferential damage of 4–5  $\mu$  in diameter. The opaque material associated with mitochondria possibly represented coagulated protein. The effect involved cristae mitochondriales without severe disarrangement of their structure. The opaque material could be interpreted as the result of direct interaction between mitochondria and the laser beam, even though the mitochondria were noted outside of the previously estimated focal spot size of about 3  $\mu$ . Within the thickness of 2–4  $\mu$  of monolayered cells, larger areas of damage can be accounted for by divergence of the beam which is focused by a microscope objective of very short focal length. A threshold of biologic effectiveness is probably also involved.

Selective mitochondrial damage in KB cells by a focused ruby laser beam has been demonstrated electron microscopically (1, 2). The phenomenon was only noted when cells were stained vitally by janus green B at low dye concentrations ranging from 1:80,000 to 1:1,000,000 (2). No similar result was obtained with other dyes such as cresyl blue, methylene blue, Nile blue sulfate, and toluidine blue (2), possibly because they had a less selective effect on mitochondria of living cells. For supravital staining of blood cells, pinacyanol has been recommended as a good choice for preferential mitochondrial staining in place of janus green B because of its lesser toxicity and stable staining effect (3, 4). The dye is relatively insoluble in water and stains the nucleus. The dark purple color of the organelles which was obtained by this dye is considered as a chromophore capable of absorbing red light. Thus the selective effect of a ruby laser

beam on mitochondria stained with this dye was expected. With the same laser equipment utilized in previous experiments by other authors (1, 2), mitochondria were damaged preferentially in KB cells stained with pinacyanol.

## MATERIALS AND METHODS

The material and methods were almost identical with those described in the previous publications from this Institute (1, 2), except for minor modifications in fixation techniques which were specified elsewhere (5). Briefly, KB cells were grown for 2 days in Leighton's tubes containing cover slips, previously marked with tiny circles of about 200  $\mu$  and coated with Formvar. The cells settled out on the cover slips were stained for 30 min at 37°C in saturated aqueous pinacyanol, diluted from 1:8 to 1:10 with culture medium. The cover slips were then inverted on microscope slides with a layer of fresh culture medium between the slide and the cover slip, and then they

were irradiated. A 100 $\times$  objective lens was used throughout the experiment. For electron microscopy, cells were fixed in precooled 0.5% glutaraldehyde in M/15 Sorenson's phosphate buffer, pH 7.3, for 5 min by running the fixative between the slide and cover slip. This process minimized the risk of losing the target cells before fixation. After brief fixation, the cover slips were detached from the microscope slides, placed in fresh glutaraldehyde fixative for an additional 10 min, and subsequently treated with 1% osmium tetroxide in the same buffer for 20–30 min. The embedding and sectioning procedures followed exactly the method recommended by the previous authors (1, 2).

The emitted wavelength of the laser was 6943 Å. The energy of the beam at the laser surface was 0.5 joule, and the pulse duration was approximately 500  $\mu$  sec. The energy imposed on the cells could be varied by means of a graded neutral density filter between the microscope and the laser head (2, 6, 7).

The maximum flux obtained without filter was used in all experiments with pinacyanol.

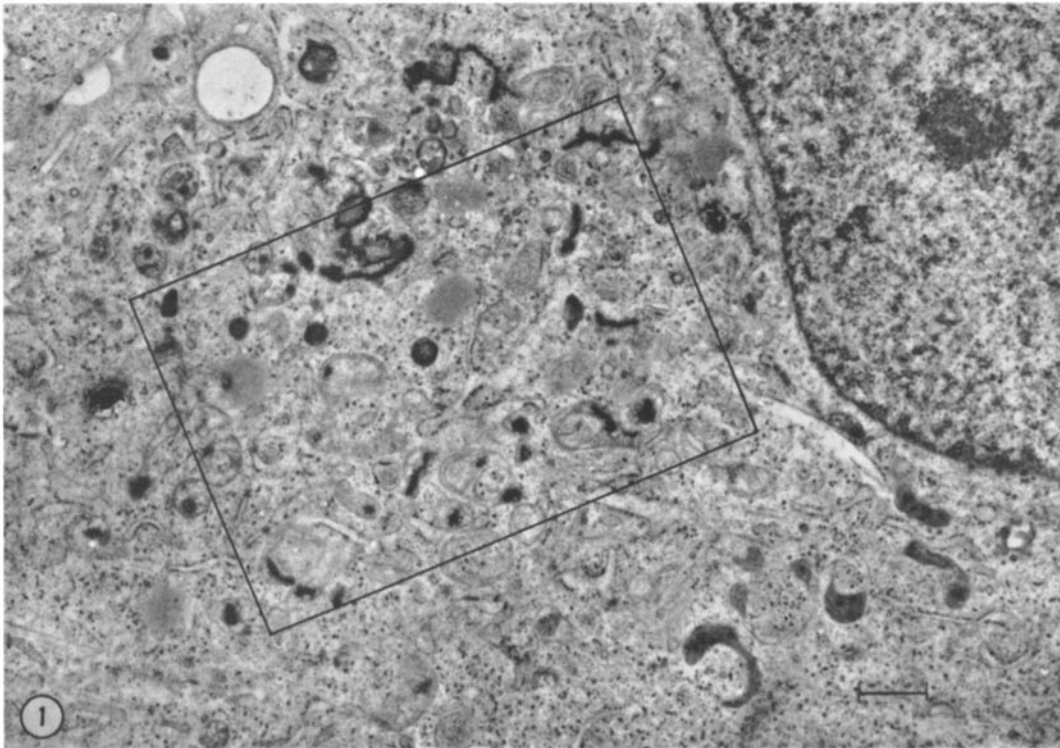
Cells stained with janus green B and Nile blue sulfate at dye concentrations from 1:100,000 to 1:200,000 were also subjected to irradiation and were examined with the electron microscope to compare the result with the results obtained with pinacyanol treatment. Nile blue sulfate was selected because it stained mitochondria more preferentially than did the other vital dyes except janus green B and pinacyanol. The blue color of this dye could also be a chromophore capable of absorbing the red light. The neutral density filter at the position between 5 and 3 (7) was used for irradiation of cells stained with janus green B and Nile blue sulfate.

Unstained cells with or without irradiation and stained cells without laser beam treatment were used as controls for all experiments.

## RESULTS

At a given dye concentration of pinacyanol, granular and filamentous structures of the cytoplasm, presumably mitochondria, were stained a dark purple color. The staining was fairly uniform within a single cell, but in cells of smaller colonies the mitochondria tended to stain more intensively. The nucleus was also stained slightly. In 15 trials, a preparation satisfactory for electron microscopic study was successful only once when two cells were targeted. Lack of success was primarily due to losing the targeted cells during the fixation and sectioning. Poor quality of either fixation or plastic blocks also contributed to failure. After irradiation the cells demonstrated no visible sign of degeneration under the phase-contrast microscope. They were fixed within 5 min after exposure. In

one cell the laser beam created a hole about 2  $\mu$  in diameter surrounded by a dark rim several micra in width. In another cell the laser beam failed to form a visible hole but left a dark oval spot several micra in its longer axis. Electron microscope examination of semiserial sections of the first cell revealed an irregularly shaped, opaque area about 4  $\mu$  in diameter which included a vacuole about 2  $\mu$  in diameter. In the second cell, a vacuole of about 1  $\mu$  was seen in the center of the opaque area of about 4  $\mu$ . These vacuoles were formed as the result of the interaction between the substance of the cell and the high energy beam. The perivacuolar rim consisted of moderately opaque material of floccular appearance and probably represented coagulated protein formed by the thermal effect at the site of maximum energy absorption. No structures were identified within this area except those composed of plasma membrane, such as multivesicular bodies. The area containing both vacuole and coagulated protein could be regarded as the zone of nonpreferential effect, and this area measured less than 5  $\mu$  in its maximum extension in both cells. Electron-opaque material of irregular shape was distributed in the cytoplasm at a distance which varied, in different sections, up to 10  $\mu$  from the outer border of the zone of nonpreferential effect in the first cell and up to 8  $\mu$  in the second cell. The opaque material in that area was associated exclusively with mitochondria. Examples are shown in Figs. 1 and 2. Images from semiserial sections from four and six electron-micrographs at two different levels from one cell and images from four micrographs from the other cell were traced on thin, transparent papers, and the overlapping images were studied. It became evident that the cytoplasmic alteration produced by the laser beam consisted of a central, nonpreferential zone and a concentric outer, preferential zone of damage. The maximum dimensions of the affected area for the two cells were roughly 17 and 23  $\mu$  in diameter, respectively, which was far greater than the estimated focal size of less than 3  $\mu$  (6). Though the previous authors concluded that the effect on mitochondria in the area beyond the focal size of the beam computed by them was produced by unknown mechanisms not related to the primary interaction between the tissue and the laser beam (2), the alteration could represent a direct interaction between the laser beam and the organelle for reasons which will be discussed in a later section.



Bars on each micrograph indicate  $1 \mu$ .

**FIGURE 1** A low-power view of a pinacyanol-stained cell with affected mitochondria. The micrograph represents a part of the cell near the edge of the nonpreferential zone of damage. The nucleus (at right) shows no degenerative signs. Note the presence of electron-opaque material at the middle of the micrograph. Comma-shaped structures of moderate opacity at the right lower corner are mitochondria with dye precipitation.  $\times 10,000$ .

The morphology of the mitochondria and of the opaque material described seemed to vary, depending on their distance from the center of irradiation. Structures noted near the center were difficult to identify, but the presence of characteristic double membrane around highly opaque material suggested that the changes were associated with mitochondria (Figs. 1, 2). At a few micra farther on from the zone of nonpreferential damage, electron-opaque material was identified definitely within mitochondria (Fig. 2). The opaque material did not occupy the entire organelle but localized at multiple sites within a single mitochondrion. Higher magnification revealed that the granular or filamentous structure involved cristae mitochondriales without any severe disarrangement of their structure (Fig. 3). Affected mitochondria showed no internal opacity

or dye deposition. Dye precipitates present in unaffected mitochondria could be readily distinguished from the opaque material in damaged mitochondria. All of the observations strongly suggested that there was a gradient of density distribution in the beam spot.

Similar observations were made on cells stained with janus green B at a dye concentration of 1:100,000, on two successive occasions out of nearly 20 trials. All other trials failed to give satisfactory results for reasons stated with respect to the previous experiments with pinacyanol. The morphology of such mitochondria was essentially similar as has been demonstrated by Storb et al. (see reference 2, particularly Fig. 12 *c*). Localization of electron-opaque material associated with mitochondria was generally more difficult to identify in cells stained with janus green B than

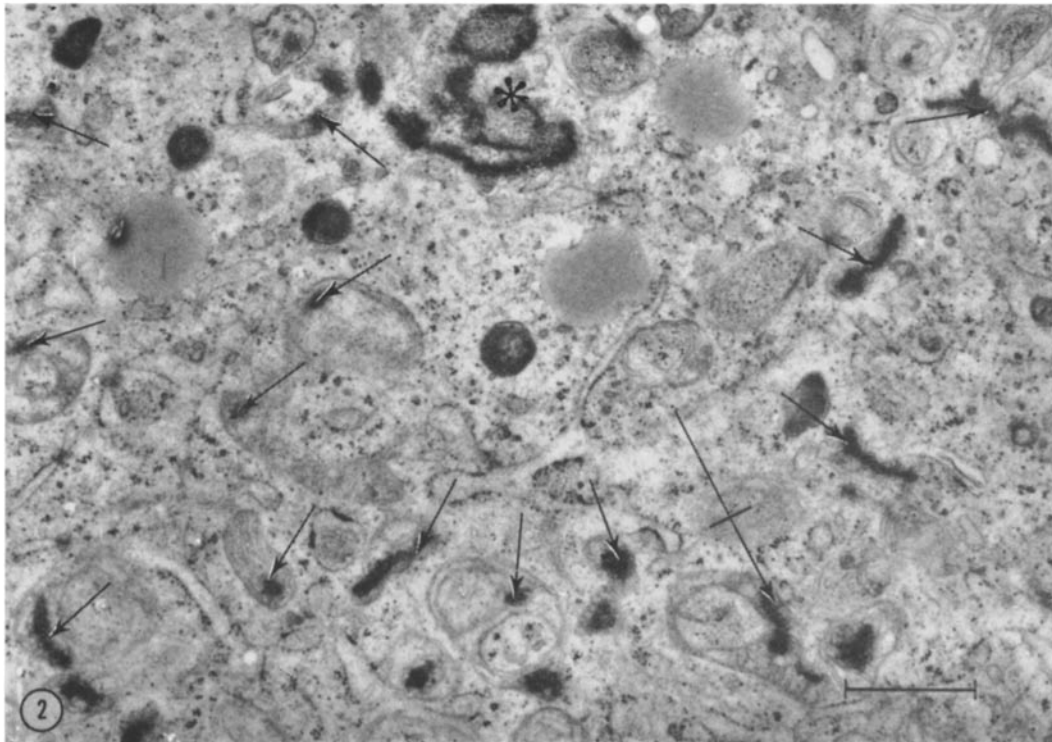


FIGURE 2 Details of the marked area in Fig. 1. The opaque material (arrows) is associated with mitochondria. The asterisk indicates the approximate position of the center of irradiation. The significant effect on mitochondria is noted near this area. The several round structures are cytoplasmic granules and can be seen in controls.  $\times 19,000$ .

in cells treated with pinacyanol. This difficulty could be due to shrinkage of mitochondria in cells treated with janus green B at the dye concentration employed (5). The secondary effect on mitochondria which was claimed by the previous authors (2) could not be confirmed in the two successful preparations we obtained. This is possibly accounted for by the failure of the previous authors to appreciate the dye precipitation in mitochondria (5) and the actual area of exposure which will be discussed in a later section. In two cells stained with janus green B, the preferential damage was present in a concentric ring  $2 \mu$  in width beyond the nonpreferential zone of about  $4 \mu$ . The modest size of the preferential zone derived from the use of the neutral density filter in this experiment.

The irradiation of cells stained with Nile blue sulfate did not produce any preferential effect on mitochondria in spite of the fact that a nonpreferential zone of similar size could be created by the

irradiation. No similar mitochondrial alteration was ever noted in any of the controls studied.

For an analysis of the actual effect of the laser beam on the cytoplasm outside of the area of focus, human red cells were chosen as the target, since they are known to be susceptible to the ruby laser beam. A small amount of fresh blood, enriched in its formed elements, was allowed to spread between a cover slip and a microscope slide so that the red cell formed a monolayer of suitable concentration to facilitate observation. Irradiation with a neutral density filter produced focal coagulation not exceeding a few micra in size in a single cell, and the target cell subsequently lysed and left a ghost with a dark spot, presumably coagulated hemoglobin. At maximum flux obtained without filter, the increased intensity could instantaneously destroy the whole target cell and even neighboring red cells at a distance of up to  $13\text{--}15 \mu$  from the axis of irradiation, i.e. in an area  $26\text{--}30 \mu$  in diameter. The phenomenon could

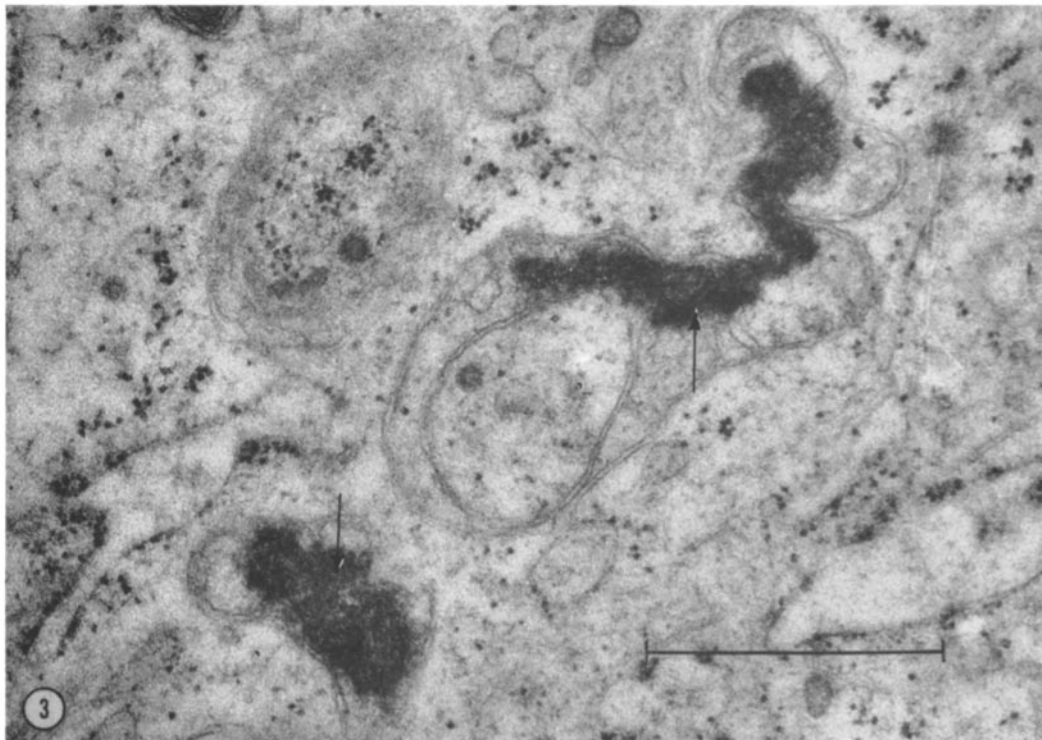


FIGURE 3 A high magnification of two mitochondria with opaque material (arrows) from the area indicated by a crossed arrow in Fig. 2, but from an adjacent section. Note the precise localization of opaque material in mitochondria, and note that the effect involves the cristae mitochondriales without any significant disarrangement in their structure.  $\times 44,000$ .

be reproduced repeatedly and had been considered as a secondary effect because it was assumed that no enlargement of the focus could result from merely increasing the intensity of the beam in the same optical equipment. However, when the beam was focused at a spot where neither red corpuscles nor any other formed elements were present in an area of at least  $3 \mu$  from the center of the irradiation, the same phenomenon could be observed, i.e. red cells up to  $13\text{--}15 \mu$  from the center were affected. During this experiment the pilot spot which indicated the axis of irradiation was carefully readjusted each time before irradiation. This experiment suggests that the effect on red cells outside of the presumed focus is primary rather than secondary to thermal conduction, since no cells capable of energy absorption were present in the target area. Furthermore, when white cells were embedded between red cells, they were usually undamaged. This result particularly favors the conclusion that the reaction between the

laser beam and red cells was direct rather than indirect because a conducted thermal effect would be expected to affect white cells as well as red cells. Thus, direct laser effects can be registered on biologic material in larger areas than the  $3 \mu$  diameter previously reported (6), particularly with strong fluxes.

#### DISCUSSION

This paper has presented data to indicate that pinacyanol sensitizes mitochondria to a ruby laser beam. The present observations confirm similar results which were obtained by others (1, 2) for janus green B in the same experimental system. The morphology of the laser effect on mitochondria appeared to be more clear-cut in material stained by pinacyanol than in that treated with janus green B.

The present results appear to differ from the previous results obtained with the identical laser equipment in that the area of direct effects is

larger. However, in a recent communication from Dr. Bessis' laboratory, M. Lutz (Personal communication) pointed out that calculations which gave a value of  $3 \mu$  for the focal spot size represented merely the lower limit of the spot under ideal conditions. When the plane of section is not coincident with the optical plane, there is an increase of approximately  $3.4 \mu$  in diameter of the spot size for each  $1 \mu$  deviation. This is of importance since the thickness of cells irradiated in the present experiment was  $2-4 \mu$ . Aberrations within the optical system, such as multiple reflections within the system and contamination at a lens surface, also act to increase the spot size. In addition, the value for divergence of the laser beam used in the computation was derived from experiments in which the area of a burned spot of a film emulsion was used to determine the size of the laser beam at 1 m from the laser. The burned area may, however, represent only the center of the actual spot for the following reasons.

M. Lutz (Personal communication) points out that a fall-off of energy from the center to the periphery must be expected because some of the rays will be emitted obliquely to the optical axis and hence will carry less energy due to the non-

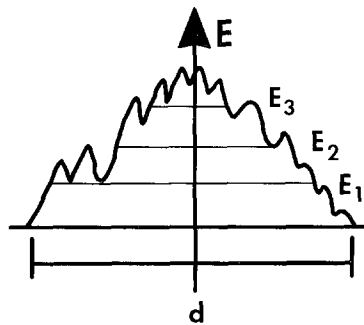


FIGURE 4  $d$ , diameter of spot size at maximum energy flux;  $E$ , energy distribution within beam at focal plane.  $E_1$ ,  $E_2$ ,  $E_3$  represent decreased spot size due to threshold effects or insertion of neutral filters.

spherical emission characteristics of the surface of the laser. Additionally, although not of a major concern here, the light emission is not uniform on the whole front face of the laser and consequently, from physical considerations alone, the energy distribution may be expected to be qualitative as shown in Figure 4. It follows that the size of the effective beam will be a function of the threshold of the detector for the effect to be measured. The higher the threshold (energy levels  $E_1$ ,  $E_2$ ,  $E_3$  in Fig. 4), the smaller will be the effective beam diameter. Because the threshold for burning of a film emulsion is likely to be higher than that for damage to a biologic structure, the larger area observed in the present experiments is not inconsistent with the diameter deduced from measurements on the film emulsion. By the same token, if neutral filters are used to decrease the intensity of the beam (e.g. to energy levels  $E_1$ ,  $E_2$ , or  $E_3$  in Fig. 4), the effective diameter of the beam will also be reduced. Conversely, the actual observation that the biologically effective spot size was reduced by insertion of a neutral density filter implies both non-uniform illumination of the type illustrated and a threshold for the biologic damage.

In view of the many factors enumerated which tend to increase the spot size and to which no specific values can be assigned without extensive further experimentation, no quantitative correlation between the calculable lower limits of the spot size and the observed biologic effect appears feasible at this time.

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