LIGHT AND ELECTRON MICROSCOPY OF LASER MICROIRRADIATED CHROMOSOMES

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INTRODUCTION

In earlier laser microbeam studies (Berns and Floyd, 1971; Berns et al., 1970; Berns and Salet, 1972), it was suggested that in salamander cells it was possible to alter selectively either DNA or histone protein in an irradiated chromosome region of less than a micron. This was possible by varying the energy of the laser in combination with the application (or absence) of the vital dye acridine orange (which binds selectively to DNA).

Though much was learned with this approach, questions dealing with secondary damage to adjacent organelles and cell regions could not be

resolved. In addition, it could not be ascertained clearly that the small chromosome lesion (0.25–0.5 μm in diameter) was indeed limited to the "paled" spot resulting upon irradiation and observed with the phase microscope. A detailed ultrastructural analysis would resolve these questions.

If, as suggested by the earlier cytochemical studies, laser microirradiation was able to alter selectively either DNA or protein, this selectivity might be reflected in two classes of ultrastructural damage. Demonstration of this would further strengthen the conclusions based upon light microscopy.

This study presents an analysis by both light and electron microscopy of the laser lesions. In addition, the chromosome damage produced with a new tunable organic dye laser microbeam will be compared with the damage produced with the argon laser microbeam.

MATERIALS AND METHODS

In these studies, cells of the rat kangaroo line (PTK_2) were used. The cells remain flat during the entire process of mitosis, thus permitting selective microirradiation of any chromosome. The large acrocentric chromosome no. 1 generally was selected for irradiation. Stock cultures of cells were grown in T30 flasks in modified Eagle's medium as described in earlier publications (Berns et al., 1972). Two days before irradiation, cells were trypsinized from the flasks, resuspended in culture medium, and injected into Rose multipurpose culture chambers. The coverglass plates of the Rose chambers were coated with 0.1% silicone. This was necessary to facilitate separation of the cells from the glass after flat embedding for electron microscopy.

Within 30 s after irradiation, appropriate fixative was injected directly into the Rose chamber. The cytochemical method for staining DNA was a standardized Feulgen procedure (Deitch, 1966), and that for staining basic protein (histone) was the alkaline fast green procedure (Alfert and Geschwind, 1953). Monolayer cultures were fixed *in situ* after irradiation and fixed and embedded according to the method of Brinkley et al. (1967).

Epon disks obtained by the procedure described above were examined using a × 16 phase objective lens. The irradiated cell was scored with a diamond marker, cut from the Epon disk with a cork borer, and cemented on an Epon blank. Serial sections in the silver range were cut on an LKB Ultratome III, collected on copper slotted grids coated with formvar, and examined in a Siemens Elmiskop 1A microscope operated at 80 kV. Serial sections through the lesion area of four cells at each energy level were examined.

Microirradiation was performed with an argon laser (488 and 514 nm) or an organic dye laser (440–460 nm). Both microbeam systems have been described in detail elsewhere (Berns, 1971; Berns, 1972). Energy density in the focused spot varied from 50 μJ to 1,000 $\mu J/\mu m^2$ for the argon laser. Direct measurements were not made on the dye laser, but a comparative sensitivity irradiation of red blood cells permitted an approximate calibration of the dye laser to the argon laser. By placing various calibrated neutral density filters in front of the laser beam, it was possible to attenuate the energy so that "phase-paling" lesions could be categorized as "severe" or "threshold." Threshold and severe lesions were produced on chromosomes in cells that had not been treated with acridine orange and in cells that had been treated with acridine orange (0.1 μg/ml for 5 min). Irradiation of acridine orange-treated cells was performed after fresh culture medium had been placed in the chamber. Identical acridine orange and nonacridine orange experiments were conducted with the dye laser microbeam and argon laser microbeam. Cytochemical study was performed on the argon laser cells and electron microscopy on both the dye laser cells and the argon laser cells.

RESULTS

The cytochemical and ultrastructural data are summarized in Table I. With respect to cytochemistry, the severe, high-energy, nonacridine orange lesion showed negative Feulgen and negative alkaline fast green staining. The threshold, moderate-energy (500 $\mu J/\mu m^2$), nonacridine orange lesion showed positive Feulgen and negative fast green staining.

The acridine orange-treated cells exhibited very different staining properties than the nonacridine cells. With moderate laser energy (300 $\mu J/\mu m^2$), the irradiated region exhibited a severe lesion which stained negative for DNA (Feulgen procedure) and positive for histone (alkaline fast green procedure). With low energy levels (50 $\mu J/\mu m^2$), a threshold lesion was obtained and the same staining properties were observed (negative for DNA and positive for histone).

The following ultrastructural observations were made. Under severe laser energy conditions, the lesion area in the nonacridine orange-treated cells appeared as a discrete area containing numerous electron-dense aggregates 0.08–0.19 μm in diameter and a prominent central mass perhaps formed by a fusion of the smaller peripheral masses (Fig. 1). The threshold lesions were similar in morphology, but lacked the central mass (Fig. 2). In addition to the aggregates, the lesion area at both energy levels contained less dense material perhaps representing remaining chromatin. Chromatin im-

TABLE I

Summary of Cytochemical and Ultrastructural Data

	Pretreatment acridine orange 0.1 μg/ml 5 min	Wavelength	Energy	Light microscope morphology	Electron microscope morphology	Alkaline fast green	Feulgen reacton
		nm	*μJ/μm²*				
Argon Laser	–	488 514	1,000	Large pale region	Central electron dense mass + peripheral aggregates	–	–
	–	488 514	500	Small pale region	Electron dense aggregates 0.08–0.19 μm	–	+
	+	488 514	500	Large pale region	Numerous electron dense aggregates 0.05–0.15 μm	+	–
	+	488 514	50	Small pale region	Numerous electron dense aggregates 0.05–0.15 μm	+	–
Dye Laser	–	460	not available	Large pale region	Central electron dense mass + peripheral aggregates	not available	not available
	–	460	not available	Small pale region	Electron dense aggregates 0.08–0.19 μm	not available	not available
	+	460	not available	Large pale region	Numerous electron dense aggregates 0.05–0.15 μm	not available	not available
	+	460	not available	Small pale region	Numerous electron dense aggregates 0.05–0.15 μm	not available	not available

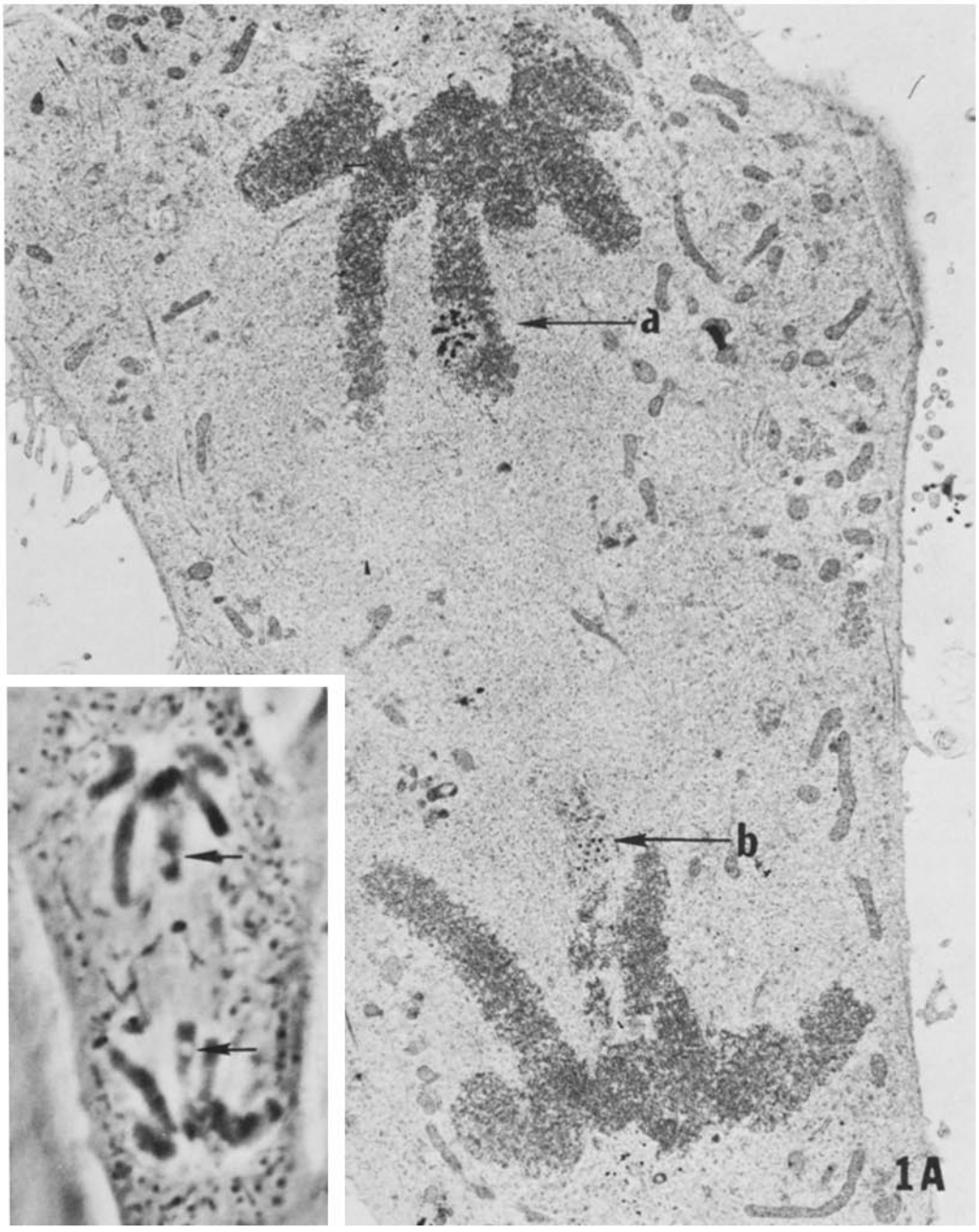
mediately adjacent to the lesion area displayed normal morphology.

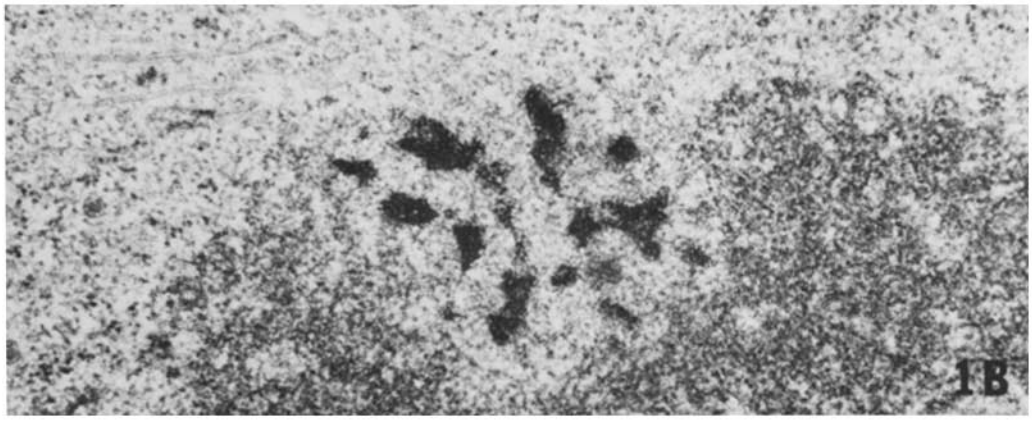
The lesion area of the acridine orange-treated cells was characterized by a diffuse array of electron-dense material that appeared smaller (0.05–0.15 μm) and more spherical in nature than in the nonacridine orange cells (Fig. 3). The lesion ultrastructure was identical for both the severe and threshold lesions. However, the severe lesions appeared more extensive.

When the organic dye laser was used to irradiate the chromosomes, the lesion appeared, by light microscopy, to be identical to the lesions produced with the argon laser. Similarly, in their ultrastructure the lesions produced with both types of lasers were identical: discrete area of numerous electron-dense aggregates without acridine orange sensitization, and smaller more spherical electron-dense masses with acridine orange.

In all the classes of lesions, the damaged chromosome region when examined by electron microscopy, was perfectly correlated with the light microscope phase-paling spots (see insets of Figs. 1–3). There did not appear to be any extension of lesion material along the chromosome. In addition, the cytoplasm adjacent, above, and below the damaged chromosome region appeared normal. Undamaged microtubules were often observed

FIGURE 1 Argon laser—1,000 $\mu J/\mu m^2$ energy, no acridine orange. (A) Low power micrograph illustrating two sites of chromosome damage. (*a*) Central portion of lesion area characterized by a large electron-dense aggregate. (*b*) Peripheral portion of second lesion characterized by numerous electron-dense aggregates 0.08–0.19 μm in diameter. (Inset: phase micrograph. Lesions appear as paled regions within the chromosome.) × 6,000. (B) High magnification of lesion area. × 40,000.


a
b
1A


1B

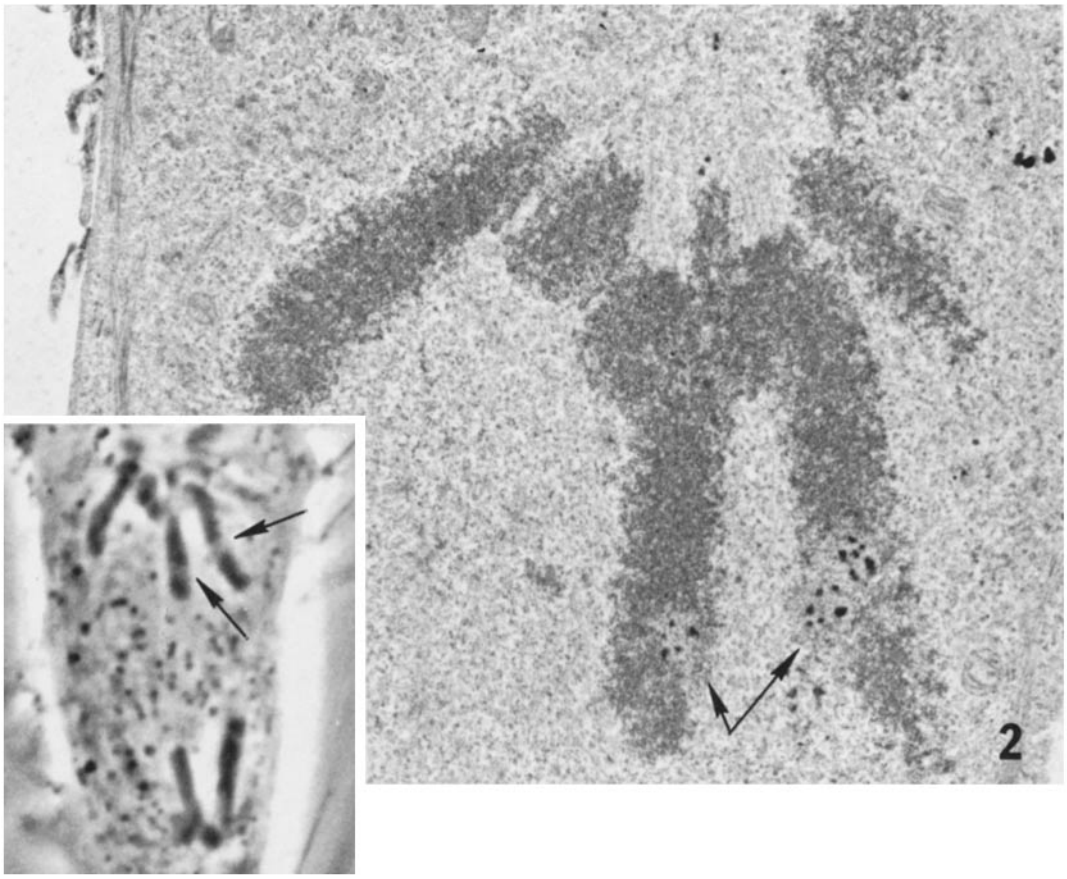


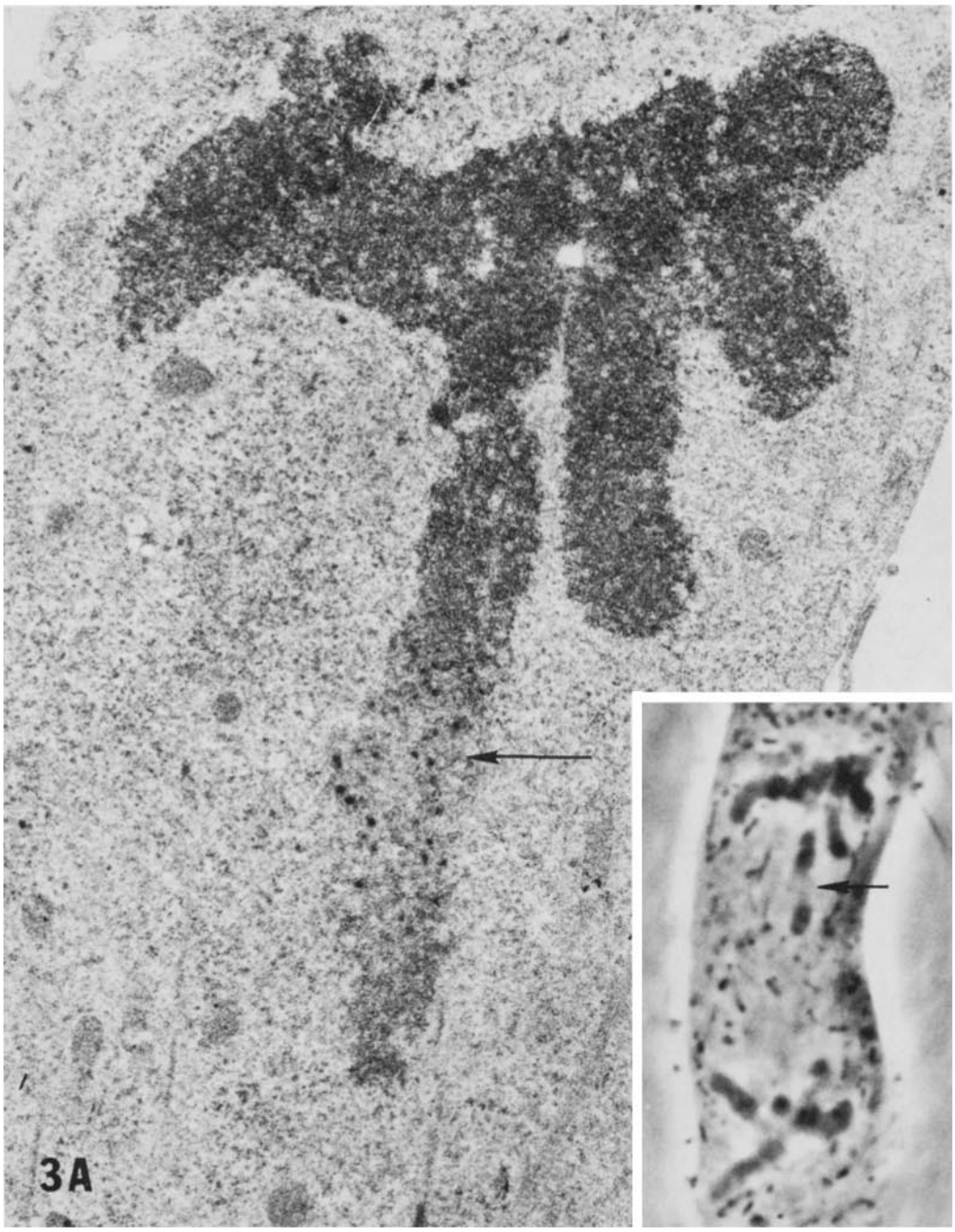
FIGURE 2 Argon laser—300 $\mu J/\mu m^2$ energy, no acridine orange. Low power micrograph illustrating sites of chromosome damage. No prominent central mass is present at this energy level. However, smaller aggregates are still present (0.08–0.19 μm). (Inset: phase micrograph. Lesion appears as paled region within the chromosomes.) × 11,000.

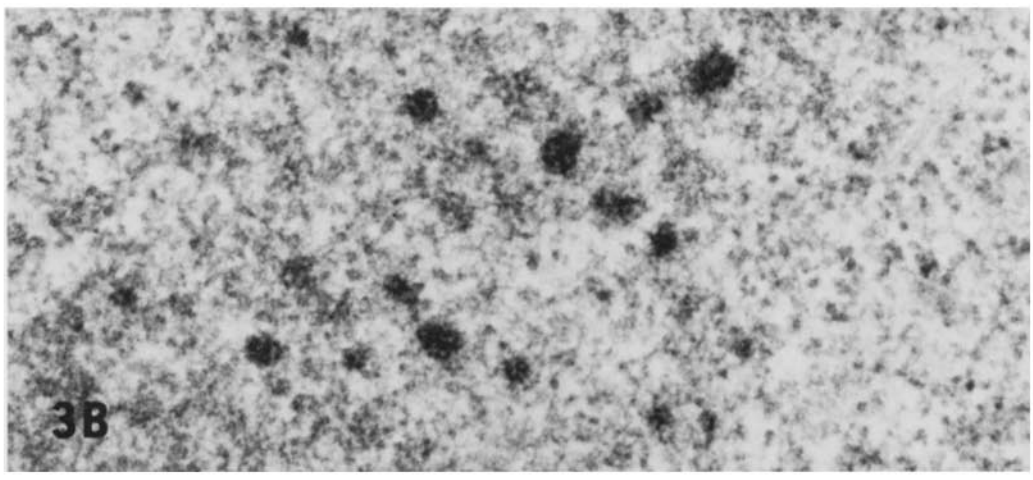
passing very close to or through the lesion site. Normal-appearing mitochondria were frequently detected near the irradiated chromosome.

DISCUSSION

The cytochemical data (negative fast green staining of the nonacridine orange severe and threshold lesions) are consistent with those of earlier studies (Berns and Floyd, 1971) which suggested that laser microirradiation without presensitization with a vital dye (acridine orange) resulted in damage to chromosomal basic protein. Similarly, the positive fast green staining of irradiated chromosomes pretreated with acridine orange demonstrated that, under those conditions, the basic protein component in the lesion area was not affected. The Feulgen staining experiments demonstrated that DNA could be affected under the high-energy, nonacridine orange conditions and under all of the acridine orange irradiation conditions.

FIGURE 3 Argon laser—500 $\mu J/\mu m^2$ energy, acridine orange. (A) Low power micrograph; site of chromosome damage appears as a diffuse mass of electron-dense aggregates 0.05–0.15 μm in diameter. (Inset: phase micrograph. Lesions appear as paled regions within the chromosomes.) × 13,000. (B) High magnification of lesion area. × 60,000.


3A


3B

Ultrastructural analysis of all the lesion types revealed two distinct classes of damage. The larger interconnected aggregates of electron-dense material were consistently found in all the lesions produced under the nonacridine orange conditions. This type of lesion, therefore, could be correlated with the negative fast green-staining reaction. The smaller electron-dense spherical bodies were found in the lesions produced under the acridine orange conditions. These lesions consistently stained negative for DNA and positive for histone.

The one class of lesions that stained negative for DNA but also exhibited the large aggregate type of lesion in the electron microscope was the high-energy, nonacridine orange type. This result was not unexpected when one considers that the irradiation energy was very high, 1,000 $\mu J/\mu m^2$. The cytochemical data indicated that both DNA and protein were affected under these irradiation conditions. The large aggregates of electron-dense material observed with the electron microscope could easily have obscured or contained the smaller electron-dense spherical bodies.

Lesions with exactly the same ultrastructure were produced with the organic dye laser. These results indicated that either the dye laser or the argon laser could be used for selective chromosomal damage. The ability to obtain any wavelength throughout the visible spectrum with the dye laser makes this instrument far more versatile. However, it must be pointed out that the dye laser was far more inconsistent in energy output. One must certainly bear this fact in mind, because it has been demonstrated that the type of lesion produced in the nonacridine orange experiments (in terms of the cytochemical staining) does depend upon the amount of laser energy in the focused spot.

It is tempting to conclude that the large aggregates of electron-dense material represent the damaged histone component of the chromosome, and the smaller electron-dense spherical bodies the damaged DNA component of the chromosome. These conclusions would be consistent with the cytochemical and ultrastructural data. However, final conclusions of this nature must await additional ultrastructural cytochemical studies.

The ultrastructural studies do demonstrate that the lesion area on the chromosome and within the cell is confined to a very limited area corresponding precisely with the phase microscope lesion and the size of the focused spot. This fact is reflected in the high survival rate of irradiated cells and the success we have had with cloning of single irradiated cells (Basehoar and Berns, 1973; Berns, 1974).

SUMMARY

Small chromosome lesions, 0.25–0.5 μm in diameter, were produced with argon laser and dye laser microbeams and analyzed by cytochemical staining with the light microscope and ultrastructurally with the electron microscope. When acridine orange was employed as a photosensitizing agent (prelaser), all lesions stained negative by the DNA-Feulgen procedure. Only the higher energy irradiation resulted in a lesion that stained negative for basic protein using the alkaline fast green procedure. Ultrastructurally, the acridine orange laser lesions all appeared similar; they consisted of small, spherical aggregates of electron-dense material (0.05–0.15 μm in diameter). Irradiation of chromosomes in cells that had not been treated with acridine orange resulted in lesions that stained negative for both DNA and histone when high energies were used and negative for histone but positive for DNA when low energy was employed. The ultrastructural changes under both energy conditions were identical; they consisted of dark, interconnected aggregates, 0.08–0.19 μm in diameter. There was no apparent secondary damage to nonirradiated chromosomal or cytoplasmic regions.

We express our gratitude to Ms. Rita Nep and Marti Witter for their assistance in preparation of tissue culture materials.

This research has been supported by National Science Foundation grant GB 36646, National Institutes of Health grant HL 15740-01, and a grant from the University of California Cancer Research Coordinating Committee.

Received for publication 18 December 1973, and in revised form 5 March 1974.

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