ARGON LASER MICRO-IRRADIATION OF NUCLEOLI

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Laser micro-irradiation of cell inclusions has become a useful method in cell biology (1). A pulsed ruby laser microbeam system has been successfully used to study morphological and functional relationships within mitochondria of tissue culture cells vitally stained with Janus green B (2–6). Recently, an argon ion gas laser microbeam has been used to place lesions on mitotic chromosomes of salamander lung cells in tissue culture; these cells were photosensitized by short pretreatment (5 min) incubation with a dilute (2×10^{-6}% w/v) solution of acridine orange (7, 8). The same argon system is also being used to selectively place lesions in the large sarcosomes (mitochondria) of beating rat myocardial cells in tissue culture without prior pretreatment with a photosensitizing agent (unpublished data). Generally, the rapid pulse repetition rate and/or continuous wave capabilities, multiple or single wavelength capacity, wide power and energy range, wavelengths in the biologically active region of the spectrum, and the existence

of several nontoxic, specific organelle-binding blue-green absorbing vital dyes give the argon laser decided versatility over other laser systems.

It is the purpose of this paper to present data on the potential usefulness of the argon microbeam system for selective morphological and functional alteration of nucleoli of tissue culture cells. The two initial goals of this preliminary research were amply met: (*a*) that nucleoli could be altered structurally and functionally with the laser microbeam; (*b*) a reliable technique for recovery and assay could be developed.

MATERIALS AND METHODS

The laser microbeam system was the same one used in earlier studies (7, 8). An argon gas laser with principal wavelengths of 5145 and 4880 A was focused by the optics of a phase-contrast microscope to an effective spot diameter of 0.6–0.7 μ. Peak power output of the laser was 1 w, the pulse duration was 50 μsec, and the pulse repetition rate was 60 pulses/sec. Total irradiation time of an individual nucleolus was 250–300 μsec. Irradiation was accomplished by locating the desired cells in the microscope field, with the nucleolus to be irradiated directly under a cross hair located in the optical system. Prior alignment established that the laser beam was focused at the point indicated by the crosshair. Though peak power was used for all irradiation, it was not possible to measure the power or energy density at the focused spot.

In order to sensitize the nucleoi to the laser wavelengths, the cells were treated with the amino acridine, quinacrine hydrochloride (Gamaleja et al., unpublished data) dissolved in Gey's balanced salt solution (1×10^{-5} M). Immediately before placing the culture chamber on the microscope stage, the normal incubating medium was removed aseptically with a syringe, and the quinacrine solution was added. Preliminary experiments indicated that exposure to quinacrine for more than 30 min could affect the cells permanently, but cells could completely recover from exposures of less than 20 min. All irradiation on the nucleoli was performed while the cells were in the quinacrine solution. Irradiation was done during the period of not sooner than 2 min nor later than 20 min following quinacrine addition. Following irradiation, the cells were washed twice with balanced salt solution, photographed, and either discarded or used in radioautography experiments. In the latter procedure, the location of the irradiated cells was precisely indicated by placing several marks directly over the cells on the chamber cover glass, by means of a "Sanford Sharpie" permanent waterproof marking pen. This marking system plus the photographs permitted precise relocation and matching of the irradiated cells following the radioautography procedures. The cells were continuously incubated by a constant air-curtain maintained at 37°C.

Radioautography was conducted with the labeled compound, uridine-^{3}H, at a concentration of 5 μc/ml of Eagle's medium. The most frequently used incubation time was 30 min in medium containing the labeled uridine. However, prior to incubation in this medium, the cells were incubated for 30 min in normal, unlabeled Eagle's medium. This step was necessary because preliminary experiments (unpublished data) indicated that the quinacrine temporarily blocked incorporation of uridine. After incubation in the labeled medium, the culture chamber was disassembled and the cover slip containing the cells was subjected to standard stripping film radioautography.

The cell types used in all experiments were prepared by standard tissue culture procedures. Individual myocardial and endothelial cells harvested from trypsinized ventricles of newborn rat hearts were established in Rose multipurpose chambers, according to the method of Mark and Strasser (9). The medium was prepared aseptically and the pH was adjusted to 7.4, but no phenol red was used as an indicator. Primary diploid cultures of human embryonic skin were obtained from Microbiological Associates, Inc., Bethesda, Md. (Catalog No. 72–176). Cells were trypsinized from T-60 flasks, suspended in Eagle's medium with 10% fetal calf serum, and injected into Rose chambers. After 3–4 days of incubation at 37°C, the cells were growing as a monolayer. Similar procedures were followed in establishing monolayer cultures of the CMP cancer cell line. This latter cell type was established at the Pasadena Foundation for Medical Research and is routinely maintained and subcultured in our laboratory.

RESULTS

Lesions could be placed easily on nucleoli of all four cell types. Fig. 1 *a* illustrates the types of lesions obtained when human CMP cells in 1×10^{-5} M quinacrine hydrochloride were irradiated with the laser microbeam. It should be noted that both the irradiated nucleoli and the subsequent lesions are morphologically different. The phase-dark, more dense nucleolus (Fig. 1 *a*) had a halolike (light center with dark periphery) lesion (Fig. 1 *b*). The lighter, more granular nucleolus (Fig. 1 *c*) had a dark, localized type of lesion (Fig. 1 *d*). Similar morphological results were observed with the other cell types.

The ability to alter the morphology of nucleoli selectively raised the obvious question of functional change. In Fig. 2, phase-contrast pictures

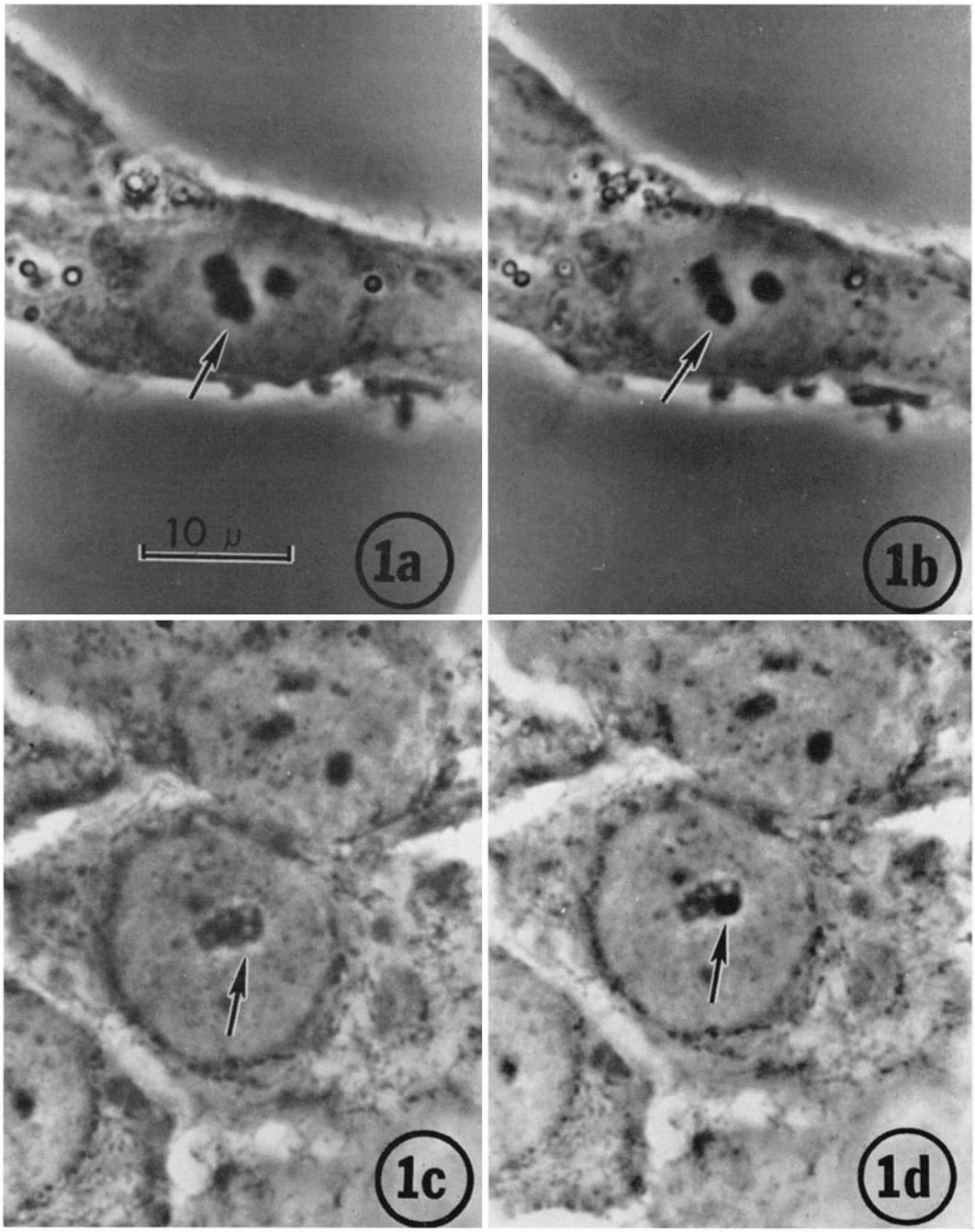

FIGURE 1 Phase-contrast photomicrographs of living CMP cells in 1×10^{-5} M quinacrine hydrochloride: (*a*) phase-dark dense nucleolus prior to irradiation (arrow); (*b*) halolike lesion following irradiation (arrow); (*c*) phase-light, granular nucleolus (arrow); (*d*) dark, localized lesion following irradiation (arrow). × 3000.

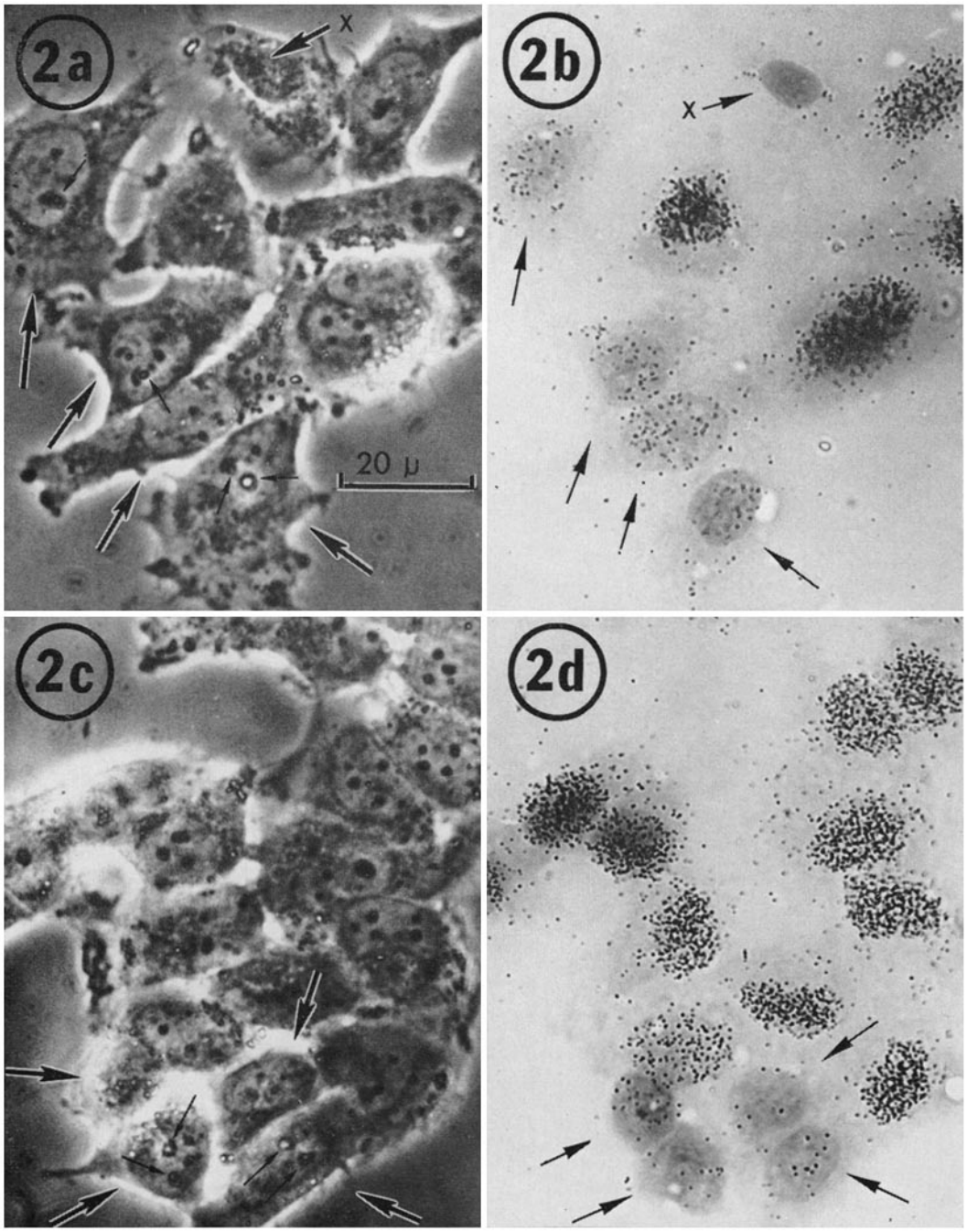

FIGURE 2 Phase-contrast photomicrographs of living CMP cells following laser irradiation, and radioautographs of same cells: (*a*) living CMP cells following laser irradiation of each nucleolus in the four cells indicated (large arrows): small arrows indicate some of the more visible lesions; (*b*) radioautograph of same groups of cells. Note the dead cell, *x*; (*c*) CMP cells following irradiation; (*d*) radioautograph of same cells. × 1500.

both of the living cells immediately following laser irradiation and of the same cells following uridine-^{3}H labeling and processing are presented. In each group of cells, four cells had all their nucleoli irradiated with the laser microbeam. In many of the living cells the lesions were clearly visible and halolike. Because of the low magnification of the photographs, lesions were not visible on some of the small nucleoli. The corresponding radioautographs clearly demonstrate that incorporation of the uridine label was greatly reduced in the irradiated cells. Despite the irradiation, however, all the cells incorporated some of the label. In addition, a nonirradiated pycnotic dead cell (*x*, Fig. 2 *b*) is clearly visible. Note the dark-stained nucleus and the total lack of label over it. The laser-irradiated cells do not show the characteristic staining or the lack of label demonstrated by the dead cell. In addition, the heavy label over the control cells indicated that the concentration and duration of exposure of quinacrine did not seriously affect the cells.

DISCUSSION

Two types of lesions have been described when a quinacrine concentration of 1×10^{-5} M was used: a halolike lesion and a small, dark, localized lesion. An attempt was made to correlate the type of lesion with the appearance of the nucleolus; i.e., the halolike lesion with the phase-dark, dense nucleolus and the small, dark lesion with the less dense, phase-light nucleolus.

It is interesting to compare our results with similar observations made by Amy et al. (3) on Janus green B–stained mitochondria. By varying the concentration of Janus green, six different categories of lesions were obtained, ranging from a small, darkened type of lesion obtained with the lowest dye concentration to a fragmentation of the cell obtained with the highest dye concentration. With moderate dye concentrations, a darkened lesion with a small light zone in the center was obtained. This type of lesion strongly resembles the halolike lesion we obtained. However, it is difficult to make meaningful comparisons until experiments are conducted in which both dye concentration and laser power are varied.

The labeling experiments with uridine-^{3}H demonstrate that the laser microbeam can be used to alter selectively the function of a single nucleolus. Perry et al. (10) were able to do this by using an ultraviolet microbeam system. Their results strongly supported the hypothesis that the major portion of cytoplasmic RNA is synthesized in the nucleolus. The laser microbeam system could offer an important extension to the technique of nucleolar micro-irradiation devised by Perry et al. (10). Not only can entire nucleoli be "turned off" functionally, but, with the capacity for creating morphologically visible lesions as small as 0.5 μ, individual parts of a single nucleolus can be altered and viewed at the light microscope level. Subsequent labeling experiments should be most useful in further elucidating nucleolar function. In addition, the high specificity of the laser beam for only those structures that are selectively photosensitized eliminates many of the secondary effects caused by absorption by other cell components. However, before the technique becomes more fully applied, more comprehensive studies on the effects of the photosensitizing agents must be carried out, radioautography experiments must be conducted following laser irradiation of nonnucleolar portions of the cell, and electron microscope studies must be performed in order to define the full extent of morphological alteration caused by the laser microbeam.

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