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REPORT NO. 3

THE EFFECT OF LASER ENERGY ON CELLS IN TISSUE CULTURE

(Annual Report)

Donald E. Rounds, Ph. D
Department of Laser Biology
Pasadena Foundation for Medical Research
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ABSTRACT

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OBJECTIVE

To evaluate the response of cells in tissue culture and biochemical systems following treatment with laser power at 2650 A; and to describe the type of injury produced by an argon microbeam laser focused on various cellular organelles.

METHODS

Multiple flashes from a frequency-quadrupled neodymium laser was directed at mammalian cell suspensions or monolayer cultures, as well as LDH and DNA solutions. Changes in morphology and rates of growth of cells, frequency and types of chromosomal aberrations, and rates of enzyme and DNA template activities were measured. The microbeam laser was used to injure individual nucleoli, mitochondria, and chromosomal regions in living cells. Their structural and functional changes were described.

RESULTS AND CONCLUSIONS

The ultraviolet laser (UVL) produced different degrees of effects on cells of different types. Hamster (DON-C) cell populations were reduced in number following treatment, but recovered numerically, morphologically, and in rates of ^{3}H-TdR uptake. Human malignant (CMP) cells did not show recovery. Human lung (WI-38) cells and DON-C cells showed loss of nucleolar and ribosomal components, while mitochondria were the most sensitive structure in CMP cells. Multiple exposures caused a reduction in DNA template activity during RNA synthesis, and it inhibited rates of LDH catalysis. The UVL produced all known types of chromosomal aberrations in DON-C cells, but human lymphocytes were primarily killed, but showed no aberrations in survivors.

A concentration of 0.1 μg/ml of quinacrine was not toxic, but could photosensitize nucleoli of CMP cells to the blue wavelengths from the argon laser. Mitochondria of myocardial cells contained natural chromophores, the cytochromes. Acridine orange-stained newt lung chromosomes showed both an 0.5μ diameter paling and a loss of function following argon microbeam laser treatment. Recent studies showed a relationship between the nucleolar organizer and the mitotic process.

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I. BIOLOGICAL EFFECTS OF A FAR ULTRAVIOLET LASER BEAM

A. Introduction

Our experience with lasers operating in the visible region of the electromagnetic spectrum has indicated that: (a) energy must be absorbed in accordance with Beer's law before a biological effect can be produced, and (b) high power densities can break molecular bonds, while high energy systems primarily produce thermal effects. The ionizing radiation literature has described a large number of photo-chemical and biological effects from ultraviolet radiation, using a wavelength of 2536 Å from germicidal lamps. The established absorption characteristics of purine, pyrimidines, and amino acids permits the prediction that laser energy at 2650 Å would produce effects in these classes of chemicals whether they are isolated or incorporated into nucleic acids or proteins. The unresolved question, prior to this study, was whether the high power densities available from a Q-switched laser operating at 2650 Å could produce effects which were different from those described from high UV energies available from conventional sources. Moreno (Exp. Cell Res. 65: 129-139, 1971) reported that a UV laser microbeam produced lesions in the nucleoplasm of interphase nuclei which were repaired in situ, as demonstrated by a localized incorporation of tritiated thymidine. In contrast, a similar treatment with conventional UV energy showed no such repair process. Therefore, a study was conducted to survey the effect of UV laser energy on the morphology, biochemistry, and cytogenetics of mammalian cells in tissue culture.

B. Materials and Methods

The ultraviolet wavelength of 2650 A was generated by a Q-switched neodymium laser with a rated output of 125 Mw at 1.06μ. About 10 percent of the power at this frequency was doubled to 5300 Å by transmission through a potassium dihydrogen phosphate crystal, which was carefully oriented in the path of the beam to index match the velocities of the primary and second-harmonic frequencies. A second doubling crystal converted about 1.5 percent of the green wavelength to a far ultraviolet wavelength of 2650 Å. After removal of the infrared and green frequencies with appropriate filters, the UV powers ranged from 150-300 kw, while the energy densities in the unfocused beam ranged from 2.3 - 4.6 j/cm^2. The pulse width was 15 nsec. The UV laser energy was measured, using malachite green leucocyanide as an actinometer.

The cells used in this study included both a hamster cell line and three types of human cells. The hamster cells were derived from a clone of an established line of Chinese hamster fibroblasts (DON-C), having a modal value of 23 chromosomes. The human cells were: (1) primary cultures of human buffy coat cells; (2) a strain of human embryonic lung fibroblasts called WI38, and (3) and established line of human adenocarcinoma cells, designated CMP. The latter cell type had a modal chromosome number of 69, while the other two had the normal diploid number of 46.

The cells were generally exposed to an unfocused beam of the UVL in a quartz well having a capacity of 0.2 ml and a radius of 12 mm. The UVL energy was delivered in one or more pulses at a rate of 1 pulse per minute. Prior to each pulse, the cells were mixed to ensure a uniform

exposure. In the case of the electronmicroscopic investigations, the cells were seeded in colonies, ranging from 8-12 mm in diameter in 50 mm plastic Petric dishes which were relatively transparent at 2650 Å. The cells were exposed to single pulses of UV laser power of variable power densities. In this series, the power output of each pulse at 5300 Å was monitored with a fast pulse detector used in combination with a Tektronix oscilloscope. The application of the conversion efficiency, as a correction factor, allowed the calculation of the power at 2650 Å.

C. Morphological Effects

Control DON-C hamster cells in vitro showed a fibroblastoid morphology with relatively smooth cytoplasmic margins. The cells tended to grow in colonies. The average mitotic index was about 3.0-3.5% in the 2- to 3-day cultures. Treated cells showed different degrees of damage, depending upon the imposed number of UV laser flashes. Although there appeared to be a dose-dependent suppression of cell growth on the second post-treatment day, the cell populations showed a recovery in the growth rate of cells surviving each of the treatment schedules (Fig. 1a), as measured with the Coulter cell counter. The parallel form of the 2- and 4-day survivor curves (Fig. 1b) is consistent with the concept that cells which survived were capable of increasing at the control rate of growth. The morphology of these survivors indicated that they had sustained a sublethal injury, however. The uniform, mononuclear morphology of control cells was often replaced with bizarre pleomorphic cells. The types of morphological change associated with UV laser treatment included nuclear abnormalities, cytoplasmic vacuolization, giant cell formation, zeiotic blebbing and cell death.

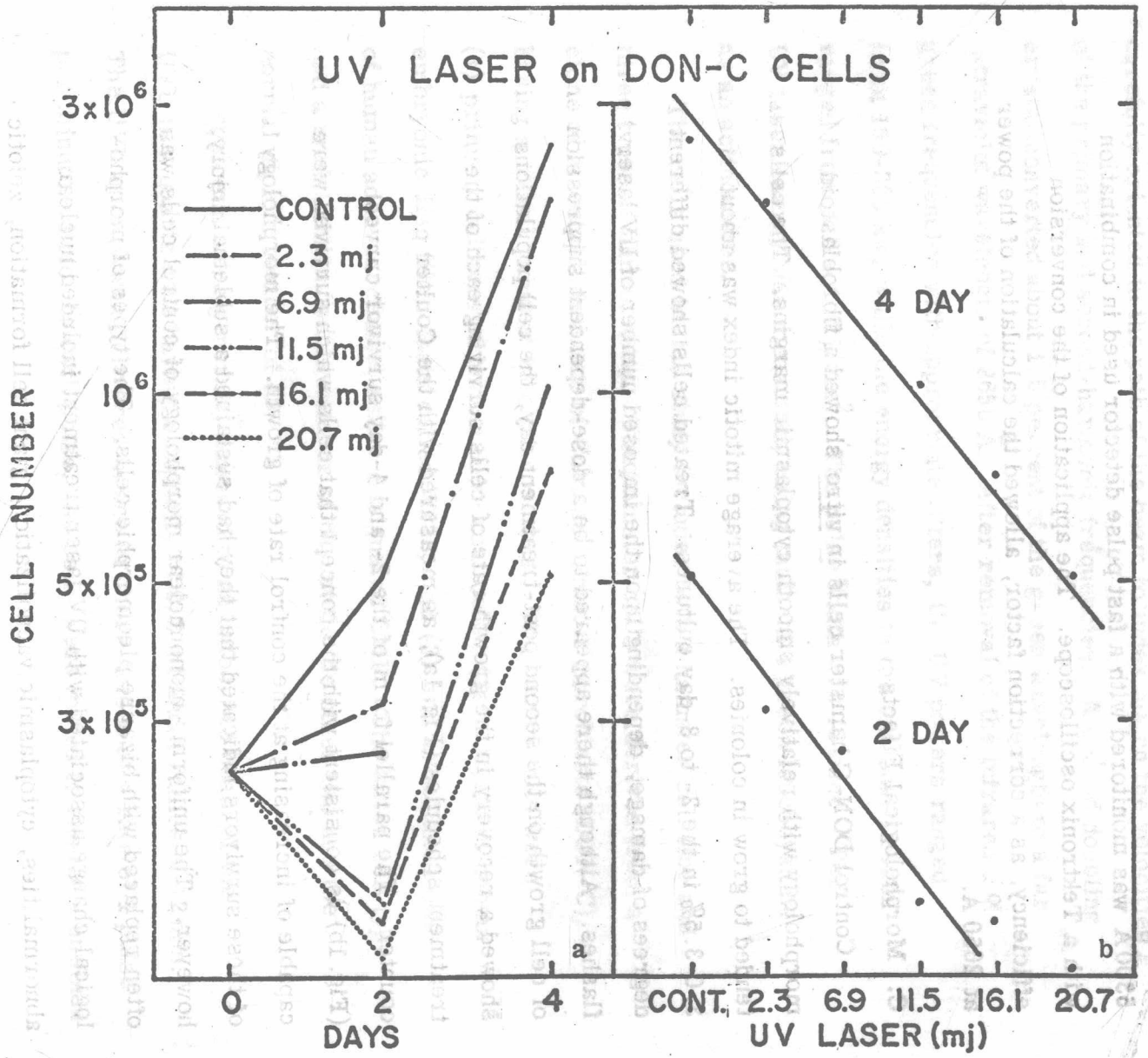
UV LASER on DON-C CELLS
CELL NUMBER
3x10^6
10^6
5x10^5
3x10^5
CONTROL
2.3 mj
6.9 mj
11.5 mj
16.1 mj
20.7 mj
a
0
2
4
DAYS
4 DAY
2 DAY
b
CONT.
2.3
6.9
11.5
16.1
20.7
UV LASER (mj)

Figure 1

The data in Figure 2a indicate that an accumulated energy of 6.9 mj/cm^2 reduced the frequency of mitotic figures at 2- and 4-days post-treatment, as compared with the mitotic indices of control populations. At 6 days, the UV laser-treated populations showed a mitotic index which was nearly equivalent to that of the controls, and complete recovery was observed by the 8th post-treatment day. A reciprocal pattern was noted, in the same cell populations, when the frequency of nuclear abnormalities were scored (Fig. 2b). The greatest frequency of aberrant cells (more than 6% of the cell population) was observed 2 days after exposure to 6.9 mj/cm^2 of UV laser energy density. This percentage diminished to 3.6% of the treated population by the 8th post-treatment day, although it was still significantly higher than the average of 2.3% abnormal cells recorded for the control populations throughout the 4-8 day period following subculture.

Ultrastructural changes were also studied in the DON-C, WI-38, and CMP cell types following UVL treatment. The cells were maintained under standard culture conditions for 2-3 days prior to laser irradiations. The DON-C cells were exposed to multiple (1-9) pulses of the laser beam in suspension in 0.2 ml volumes in a quartz well. Laser irradiations with CMP and WI-38 cells were done in situ. Cells were grown in special plastic Petri dishes (Permanox, about 50% transparent at 265 nm) and exposed to one pulse of the UVL beam. Following exposure to UVL, the cells were washed twice in balanced salt solution (BSS) and maintained on normal growth medium. The cells were incubated for a further period of 4 days and observed under

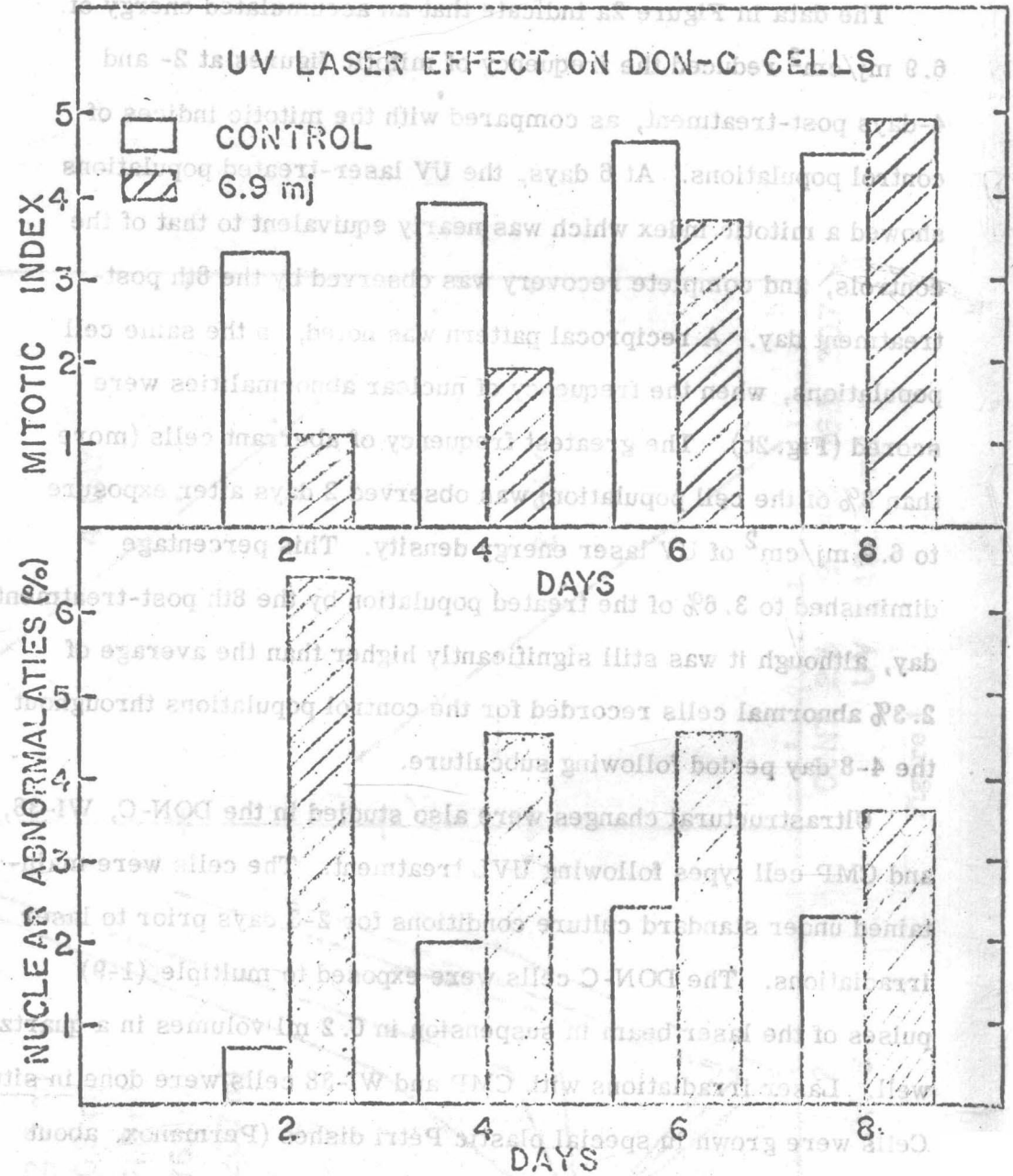
UV LASER EFFECT ON DON-C CELLS
CONTROL
6.9 mj
MITOTIC INDEX
DAYS
NUCLEAR ABNORMALITIES (%)
DAYS

Figure 2

the phase microscope periodically. Cells for electronmicroscopic observations were fixed in situ 20 min., 6 hrs., 18 hrs., 24 hrs., and 96 hrs. following UVL irradiation. Fixation, processing, and embedment of the cells were done according to established procedures in electronmicroscopy.

At an output power of the UVL in the range of 200-280 kw, DON-C and WI-38 cells showed a better survival rate as compared to the CMP adenocarcinoma cells. Very few of the CMP cells (less than 25%) survived for 4 days following exposure to the UVL beam in these experiments. In an effort to determine the critical lethal dose (power output) of the UVL beam, the CMP cells were irradiated in another experiment with one pulse of the UVL at 8 different power output settings in the range of 65-250 kw. The results of this study showed the CMP cells generally survived exposures of the UVL at power outputs of less than 100 kw for up to a period of 72 hours. Approximately 40-50% of the cells appeared to be attached to the Petri dish at 72 hours following UVL treatment at a power output of 165 and 175 kw. In general, ultrastructural changes in DON-C cells attributable to the UV laser irradiation were evident in a majority of cells fixed 24 hours and 48 hours after irradiation. In contrast, very few cells fixed 20-30 minutes after irradiation showed detectable alterations in structure.

Among the most striking alterations noticed in cells fixed soon after irradiation were zeiotic cells showing pycnotic nuclei (margination of chromatin, loss of nucleolar components), vacuolated cytoplasm with

loss of many of the cytoplasmic structures. A less striking but also consistent effect was "paling" of the perinuclear cytoplasm that contained, for the most part, fine, closely-packed fibers.

A greater percentage of the irradiated DON-C cells showed drastic alterations in or loss of the cellular components when fixed after growing for 24 hours or 48 hours after laser irradiation. The cytoplasm appeared more drastically altered in comparison to the nucleus. Cytoplasmic damage varied from highly vacuolated cytoplasm with many cristae-less mitochondria, smooth vesicles, and a few ribosomes,to absence of polyribosomes and a few vacuoles. Nucleoli often showed segregation and/or loss of its ribonucleoprotein components. Occasionally, the nucleolar structure was devoid of all components except for a ring of nucleolus-associated chromatin.

DON cell populations fixed and studied after 5 days in culture, following laser irradiation, showed cells apparently in the process of recovery. Such cells showed a large number of polysomes in the cytoplasm. Nucleoli were still loosely structured, but had all the components found in normal cells.

WI-38 cells showed obvious damage ascribable to the UV laser in the protein synthetic machinery (endoplasmic reticulum, nucleoli) of the cell. There was only an occasional cell drastically damaged by the laser beam and obviously dying or dead. This was the case in the population of cells examined either immediately (one-half hour following) or a few hours after the UV laser irradiation. The more frequently observed changes were the dilation and fragmentation of the endoplasmic reticulum, as well as loss of ER ribosomes. Many

areas of the cytoplasm contained, to a large extent, many small vesicles and smooth membrane or fibrillar components. Mitochondria did not show detectable damage and appeared to be of normal morphology. Cells with abnormal nucleolar ultrastructure (suggesting inhibition of ribosomal RNA synthesis and/or processing and transport) were particularly evident 18-25 hours following irradiation. Cells with fragmented or segregated nucleolar components were visible even 72 hours following UV laser irradiation, but none were detected in cell populations fixed immediately following laser irradiation. Many apparently healthy cells with normal morphology and rather large nucleoli were seen at 24-72 hours following UV laser treatment, which suggested either an overabundance of cells that escaped UVL treatment, or a significant recovery of the laser damaged cells. The methodology used in these experiments did not allow us to clearly differentiate between these two alternatives.

CMP cells fixed one-half hour and 6 hours following UVL treatment showed striking alterations in cytoplasmic structures, particularly the mitochondria. Some cells showed extensive blebbing or cytoplasmic components. Other cells showed obvious damage in the cytoplasm by the presence of non-membrane bound clear areas (except for a few scattered fibrils) and damaged mitochondria (swelling, breakage, and loss of cristae, loosening and separation of the outer membrane from the inner membrane). At 6 hours and 24 hours, areas of cytoplasm in many cells consisted of osmiophilic dense bodies of numerous small, smooth vesicles. At 48 hours such cells were also

seen, but many dead cells or cell debris were present. There were a few cells that showed some loss of nucleolar components, but none were drastically affected. Observations on CMP cells fixed 74 hours and later were not conclusive because of the presence of extensive cell debris or too few cells to permit embedment and examination in the electronmicroscope.

In general, ultrastructural studies on the three types of cells indicated the damage to the CMP cells to be primarily in the energy-regenerating system (mitochondria). The damage incurred by the WI-38 and DON-C cells appeared to be primarily in the protein synthetic machinery (nucleoli, ribosomes) of the cells.

D. Reproductive and Synthetic Responses

The UV laser was tested for its effect on the colony forming or reproductive capacity of cells, and on their rates of nucleic acid synthetic activity.

Colony-forming ability was measured by clonal growth of cells after UVL irradiation. Cells in log phase were trypsinized, centrifuged, and resuspended twice in colorless balanced salt solution (C. B. S. S.) before making a cell count with a Coulter counter. An aliquot of cells was suspended in C. B. S. S. so that between 3. 5 and 4. 5 x 10^4 cells were contained in about 0. 2 ml for irradiation. Following irradiation, the cells were dispersed by aspiration and resuspended in a synthetic nutrient containing fetal calf serum. Appropriate dilutions for plating cells into Petri dishes (60 mm) were made after a cell count. The plating efficiencies of the cell lines were known before so that the cell concentration on the plastic dish was

adjusted to yield approximately the same number of colonies (100-300) at all dose levels. Four replicate dishes were plated for each dose level of which 3 were scored. The Petri dishes with single cells were incubated at 37°C under 2% CO_2 and air for a period of 10-14 days. After this time, the colonies were fixed with acetic acid and methanol (1:3), and stained with Giemsa. A cell was considered to have retained proliferative capacity if it gave rise to 50 or more cells in the colony. The total number of colonies on each Petri dish were counted three times with a micro-colony counter, and a statistical evaluation was made within and between each dose group.

The survival curves, as measured by colony formation following several UVL exposures, for the DON-CII and CMP cell lines, both show an exponential response resembling that of a single-hit phenomenon with an extrapolation number very similar to one. The mean lethal dose or the percentage necessary to reduce survival by 37% (LD_{37}) was 8.3 mj/cm^2 for the DON-CII line and 3.9 mj/cm^2 for the CMP line.

Colony size of the CMP cell line 14 days after UVL irradiation was found to be inversely proportional to dose. Although the average number of cells per colony decreased with dose, only a small increment of nuclear volume change was noted, unlike that reported for UV microbeam and X irradiation.

The rate of DNA and RNA synthesis was determined by the labeling index of cells after pulse and continuous labeling with tritiated thymidine (^{3}H-TdR, 0.36 Ci/mM) and tritiated uridine (^{3}H-UdR, 1.5 Ci/mM). Cells were plated into Petri dishes following irradiation, and 1 μCi/ml of ^{3}H-Tdr or ^{3}H-UdR was added to the nutrient at various intervals thereafter.

Cells were fixed one-half or one hour after the addition of the isotope. Liquid emulsion was added as a thin layer over the cells to form autoradiographs. The cells were stained after development with May-Grunwald-Giemsa, and air-dried. The number of labeled cells and the grains per cell were counted. The rate and duration of DNA and RNA synthesis was determined from these counts.

The number of DON-CII cells in DNA synthesis were recorded after pulse labeling with ^{3}H-TdR following 6.9 mj/cm^2 and 11.5 mj/cm^2. A decrease in the uptake of ^{3}H-TdR was observed following both doses over a 6-hour period, and the effect was greatest with the higher dose. The maximum effect was seen 4.5 hours after exposure and was 82% of the control following 6.9 mj/cm^2, and 70% after 11.5 mj/cm^2. The effect diminished with time and returned to control values 24 hours after 6.9 mj/cm^2; however, it remained depressed by 24 hours (84% of controls) after 11.5 mj/cm^2. The average number of grains per cell for the same doses were enumerated, and it was found that there was a significant decrease in the irradiated cells up through 3.5 hours after exposure compared to controls; however, there was a return to control values by 6.5 hours. The maximum decrease was seen 2.5 hours after exposure for both doses. There was very little difference between the numbers of the grains per cell in comparing the two doses.

The rate of RNA synthesis in DON-CII cells was determined by the incorporation of ^{3}H-UdR (pulse labeling) following 11.5 mj/cm^2. There was about a 50% reduction in the number of cells utilizing ^{3}H-UdR 3 and 4 hours after irradiation compared to the controls.

However, by the 5th hour a 25% increase was observed, and a return to control values was reached by 7 hours. Grains per cell decreased to between 50% and 60% of the controls 4 and 5 hours after irradiation, followed by an increase thereafter, reaching a plateau (74%) by 7 hours and remaining at this level (75%) 24 hours after exposure. These data have been published in Volume 19, pp. 157-165 of the International Journal of Radiation Biology, 1971.

E. Biochemical Changes

Studies on the effects of the UV laser on biochemical systems fell into 3 categories: (1) DNA template activity; (2) enzyme inhibition, and (3) photoproducts of purines and pyrimidines.

Several investigators have described the effect of ultraviolet energy on calf thymus DNA, used as a primer for nucleic acid synthesis in the presence of either DNA polymerase or RNA polymerase. For example, it has been reported that (a) relatively low doses of UV caused a loss of DNA primer activity; (b) moderate UV doses denatured the DNA molecule; and (c) intense UV energies produced a decrease in the optical density of DNA at 2650 Å. A preliminary survey of the response of DNA to the quadrupled frequency of a Q-switched neodymium laser was made to determine if any unique effects could be detected.

Calf thymus DNA was dissolved in 0.01 M Tris-HCl (pH 8.0) at a concentration of 1 mg/ml. The DNA solution was extruded from the siliconized tip of a micropipette in 0.015 ml drops which were irradiated while clinging to the pipette tip through surface-tension forces. Following multiple exposures of the drops to the focused UV laser beam at room