

## YEARLY REVIEW

### EFFECTS OF VISIBLE RADIATION ON CULTURED CELLS

#### *Introduction*

The developing therapeutic role of (laser) phototherapy in treating patients with various skin diseases (Parrish and Deutsch, 1984; Parrish, 1989) has aroused interest in the effects of visible light on cultured cells. The need to examine the action of different visible radiation wavelengths upon cellular cultures stemmed from the knowledge that disorders which respond to laser phototherapy, such as indolent wounds and trophic ulcers (see reviews of Mester, 1981; Gamaleya, 1977; Karu, 1989a) may be associated with increased proliferation of the cells surrounding the injuries (Karu, 1987).

The body of information about the action of visible-light wavelengths on cultured mammalian (both human and non-human) cells is rather scanty compared with the data available on the action of UV radiation. Some reasons for such ignorance were indicated by Smith (1980). In his opinion, there are some common misconceptions about visible light. First, visible light is natural and therefore safe, and second, visible light is not as photochemically active as UV radiation.

Without any doubt, the determination of the biological action of light in the visible region will be useful not only from the standpoint of the photo-medicine, but also from that of understanding and predicting the overall biological action of such a polychromatic light source as the sun.

The studies reviewed in the present paper pursue three aims. First, most papers are devoted to investigations into the mechanism of low-power laser therapy (sometimes called laser biostimulation). Second, since the phototherapy can be employed for postoperative treatment of patients with tumors, the problem of interest is whether light treatment can stimulate the proliferation of residual tumor cells, if present. In these studies, red (632.8 nm), far red (694.3 nm) and near IR (890-904 nm) wavelengths have been used.

The coverage of this review is not restricted to publications which appeared only recently for the reason that the subject has not been covered in the Yearly Reviews.

#### *Historical Perspective*

Because this topic has not been reviewed previously, it seems appropriate to begin with an expla-

nation of why the laser light action on cellular cultures should be considered from a photobiological point of view.

The therapeutic action of red light was well known in the last century, and much experimental work was done in the first third of this century (see reviews of Karu, 1987, 1989a). Red light was used in medicine even in ancient times, and curing with red light was among the methods used by Finsen, the father of contemporary phototherapy (Finsen, 1899, 1988). It is very likely that by the time the first lasers made their appearance, this old knowledge was forgotten. The He-Ne laser was the first commercially available source of coherent red light. It is no wonder that the stimulating effects of light, red light in particular, were rediscovered when coherent light sources were used. The observed effects were attributed to the high coherence of He-Ne and ruby laser radiation. Actually, there are no physical grounds for such a conclusion. The specific characteristics of laser light, such as coherence and polarization, are unimportant in the case of biological applications of low-intensity light. For example, the specific effects of light coherence in condensed media at 300 K are possible at intensities of  $s = 2 \times 10^{15} \text{ W/m}^2$  (Karu, 1987). The typical laser light intensities used in clinical practice and in experiments with cellular cultures range from 1 to  $10^2 \text{ W/m}^2$ . This means that under normal physiological conditions, the lasers are used as monochromatic light sources, whose radiation obeys the same laws of physics and chemistry as the light of the same wavelengths from conventional sources. Of course, the laser as a light source offers many benefits as a handy tool for laboratory and clinic (mono-chromaticity, high intensities, possibility of transport by means of fibers).

#### *Action on Proliferative Activity*

This topic is a controversial one: in experiments (mostly performed with exponentially growing populations), the radiation from visible light sources, lasers included, has been found to stimulate, be indifferent, or even inhibit the proliferation of cellular cultures. One should emphasize that there is as yet no universally accepted method for studying the stimulation of proliferative activity by light. The Puck's method for evaluating the surviv-

ing fraction of cells is good when studying the damaging effect of irradiation. The investigation of growth curves by counting daily the number of cells has some serious limitations, e.g. the increased attachment of irradiated cells. Some authors irradiate a monolayer of cellular culture, and some, a cellular suspension. So, the final results of the experiments may be different. They may also vary because of the differences in the handling of cells (e.g. in the dark or light before the irradiation), or owing to different laboratory techniques (e.g. the nutrient media chosen), not to mention that a very wide spectrum of normal and malignant cells is used. For this reason, it is very difficult, if not impossible, to compare the experimental results obtained by different authors.

The main parameter used here to systematize the data will be the wavelength of the light source (going from shorter to longer wavelengths). If one and the same cellular culture was used by different authors, we will try to give their data side by side.

In the *violet-blue spectral region*, the lethal action of wavelengths up to 434 nm was studied by Tyrrell *et al.* (1984) using a human lymphoblastoid cell-line. The action spectrum was found to be the same as the previously determined spectra for both prokaryotes and eukaryotes. The mutagenic and lethal action of the broad-band blue light emitted by different types of fluorescent light sources was investigated by Burki and Lam (1978), Yostes *et al.* (1977), Wang and Nixon (1978).

A stimulatory influence on the proliferation and the extension of the lifespan of diploid fibroblasts derived from adult skin (HU-274), minced whole fetus (HU-278), and fetal lung (WI-38) was established after irradiating the cells with a broad-band  $\lambda > 500$  nm one to three times weekly. The experimental conditions included a relatively high inoculum size and low population doubling level. The fluences were  $< 10^4$  J/m<sup>2</sup>. Irradiation with higher fluences ( $10^5$ - $10^6$  J/m<sup>2</sup>) appeared to be cytotoxic (Parshad and Sanford, 1977 a,b). An increased life span, but no enhanced proliferation rate in human embryonic lung fibroblasts, was established when the cells were irradiated with cool fluorescent light for 2 h daily over a period of 150 days: irradiated cells went through 70 divisions, while nonirradiated cells went through 53 divisions. The cells exposed constantly to cool fluorescent light died within 2-3 days (Litwin, 1972).

The changes in the mitotic index and the number of chromosome aberrations of human embryonic fibroblasts were established 5 h after irradiating the cells with continuous wave (CW) He-Cd ( $\lambda = 441.6$  nm) and He-Ne ( $\lambda = 632.8$  nm) lasers or pulsed ruby ( $\lambda = 694.3$  nm or  $347.15$  nm,  $\tau_{\text{pulse}} = 3 \times 10^{-8}$  s) and dye lasers ( $\lambda = 741$  nm,  $\tau_{\text{pulse}} = 3 \times 10^{-8}$  s) (Stepanov *et al.*, 1977). The authors studied the fluence dependences of changes in the mitotic index and the number of chromosome aberrations. The changes (increase or decrease) of these

parameters after irradiation were found to be fluence- and wavelength-dependent.

Marchesini *et al.* (1989) attempted to evaluate whether the colony forming ability of the human cancer cells HT29, MCF7, M14 and JR1 *in vitro* could be modified by argon laser radiation ( $\lambda = 488$  nm, fluences from  $4.2 \times 10^3$  to  $1.5 \times 10^5$  J/m<sup>2</sup> and intensities between 35 and 500 W/m<sup>2</sup>). The analysis of 35 experiments showed that in 4 of them there was an increase ( $P < 0.05$ ) and in 2, a decrease ( $P < 0.05$ ) in the number of colonies. Nevertheless, the trend of most of the data was towards an increase in colony formation after irradiation. Wilcoxon's matched-pair signed-ranks test indicated that an increase ( $P < 0.05$ ) occurred in the cell culture growth.

*Green light* was used only by two groups of authors. Irradiation of Chinese hamster fibroblasts in suspension with the 2nd harmonic of a Nd<sup>+</sup>YAG laser ( $\lambda = 532$  nm,  $1 \times 10^3$ - $9 \times 10^3$  J/m<sup>2</sup>,  $\tau_{\text{pulse}} = 1 \times 10^{-8}$  s) increased the colony forming ability of the cells to 130% at an optimal fluence of  $2 \times 10^3$  J/m<sup>2</sup> (Abdvakhitova *et al.*, 1982). Klein and Edsall (1967) irradiated a HeLa cell suspension grown in a mono-layer for 7 days before the irradiation. Radiation was supplied from incandescent and fluorescent lamps with filters. Growth stimulation (116%) was found to occur when green light around 564-579 nm ( $0.5$  W/m<sup>2</sup>) was used. Increasing the light intensity was found to inhibit the growth by 45% at the intensity of 3 W/m<sup>2</sup>. Irradiation and other experimental conditions are not described exactly in this paper. The same authors (Klein and Edsall, 1967) also cultivated HeLa cells for a week with alternating periods of irradiation and, darkness within a repeating 1-h cycle. The suppression of growth caused by green light was not autorepairable by a period within the limits of the 1-h alternation, nor by even extended periods of darkness following a short period of irradiation.

Most of the data in the *red part of visible light spectrum* are related to the use of CW He-Ne ( $\lambda = 632.8$  nm) or pulsed ( $\tau_{\text{pulse}} = 3 \times 10^{-8}$  s) ruby ( $\lambda = 694.3$  nm) lasers.

Irradiation of two types of human fibroblasts with a He-Ne laser (15 min, intensity 1 W/m<sup>2</sup>) significantly increased the growth rate of populations in exponential phase, as well as enhanced the attachment of cells to the substrate (Boulton and Marshall, 1986). It is interesting to emphasize that the attachment stimulation was monitored only for embryonic foreskin fibroblasts which in control experiments exhibited a considerably lower plating efficiency than adult skin fibroblasts.

He-Ne laser ( $\lambda = 632.8$  nm) irradiation ( $0.5$  W/m<sup>2</sup>, 15 s) increased the mitotic index of L cells on the 3rd and 4th days after irradiation (Gamaleyeva *et al.*, 1983). After the choriocallantoic cultures of the avian retinal epithelium were irradiated once with a He-Ne laser ( $5 \times 10^4$  J/m<sup>2</sup>, 83 W/m<sup>2</sup>, 10 min), a slight but significant increase

in the mitotic index was established (Yew *et al.*, 1982).

Belkin and Schwartz (1989) measured the mitotic indices of corneal epithelium cells. The debrided rabbit epithelium was irradiated with a 5 mW He-Ne laser ( $\lambda = 632.8$  nm) using an 0.8-12-mm beam expander giving an irradiance of  $4.42 \times 10^{-4}$  W/m<sup>2</sup>. The left eye was exposed to 3 min daily, sequentially for 3 days, while the right eye received sham radiation at a similar fluence in a similar exposure pattern. This treatment resulted in a left/right eye mitotic index ratio of 1.73. A longer daily irradiation reduced the mitotic index ratio, and daily 30-min irradiation actually reduced it below unity, i.e. the epithelium was damaged. It was also reported that irradiation with a He-Ne laser reduced the number of lens epithelium cells in prophase 30 h after the irradiation (Poon and Yew, 1980).

A bone marrow cell suspension from the C57 BL/10 mice irradiated with a He-Ne laser at fluences from  $6 \times 10^2$  to  $1.52 \times 10^5$  J/m<sup>2</sup> formed a higher number of microscopic spleen colonies than the intact bone marrow after transplantation into isogenic recipients. The stimulation of the colony forming capacity of the bone marrow persisted in the suspension for as long as 90 min after the irradiation. The fluence giving the maximal positive effect (approx. 170%) was  $5.08 \times 10^4$  J/m<sup>2</sup> at a time interval of 15 min between irradiation and transplantation (Vacek *et al.*, 1982).

He-Ne laser ( $\lambda = 632.8$  nm) irradiation at an intensity of  $4 \times 10^2$  W/m<sup>2</sup> (up to the fluence of  $5.8 \times 10^6$  J/m<sup>2</sup>) induced ultrastructural changes but no significant cytotoxic effects in rat kidney epithelial cells grown in a monolayer. In irradiated cells the rate of PHLthymidine incorporation into DNA was decreased 6-9 h after irradiation (Schneede *et al.*, 1988). Fluence-response studies showed that the repeated He-Ne laser irradiation ( $4 \times 10^2$  W/m<sup>2</sup>) once a day in the fluence range between  $1.19 \times 10^5$  and  $1.41 \times 10^6$  J/m<sup>2</sup> significantly inhibited the growth of the culture, while daily irradiation with a fluence of  $4.7 \times 10^4$  J/m<sup>2</sup> had no effect. The microscopic examination of nuclear spreads revealed an increased number of cells in mitosis after a single irradiation of a confluent culture with a fluence of  $1.42 \times 10^6$  J/m<sup>2</sup> (Gross and Jelkmann, 1990). Measurements of the temperature in the irradiated culture excluded thermal effects of irradiation.

The [<sup>3</sup>H]thymidine uptake increased in cultures of chick retinal cells after a He-Ne laser ( $\lambda = 632.8$  nm) irradiation with a fluence of  $5 \times 10^4$  J/m<sup>2</sup> and intensity of  $83$  W/m<sup>2</sup> (Tsang *et al.*, 1986).

Human embryonic fibroblasts obtained from healthy pregnancy interruption were exposed once, or repeatedly at 24-h intervals, to the He-Ne laser radiation at a fluence of  $1 \times 10^4$  J/m<sup>2</sup> ( $2.1 \times 10^3$  W/m<sup>2</sup>) (Kubasova *et al.*, 1984). A single irradiation with a fluence of  $1 \times 10^4$  J/m<sup>2</sup> was found to cause neither functional nor micromorphological alterations of the cell surface. This fluence, however,

applied four times at 24-h interval, changed the [<sup>3</sup>H]glucosamine uptake, and the [<sup>3</sup>H]concanavalin A and cationized ferritin binding. At the same time, the scanning and electron microscopy of laser-irradiated cells did not reveal any micromorphological or ultrastructural alterations. The authors conclude that the plasmatic membrane is involved in the laser stimulated processes in fibroblasts.

Cultured human fibroblasts of 3rd to 4th and 13th to 14th passages were irradiated daily with a 0.9-mW He-Ne laser ( $24.7$  W/m<sup>2</sup> for 60 s) over a period of 5 days. Neither stimulative nor inhibitive effect was found (Hallman *et al.*, 1988).

Investigations have proved that the He-Ne laser irradiation of 25-mW output had a direct influence on human fibroblasts of 32nd passage (Bosatra *et al.*, 1984). The synthesis of fibrillar material was stimulated, but collagenogenesis was not influenced. On the contrary, Lam *et al.* (1986) obtained a substantial (maximum 36-fold) collagen synthesis increase without any enhancement of the cell reproduction rate by irradiating human skin fibroblasts (initiated from normal skin obtained from surgical specimens) with He-Ne ( $\lambda = 632.8$  nm) or GaAs ( $\lambda = 904$  nm) lasers at fluences from  $5.5 \times 10^2$  to  $1.6 \times 10^4$  J/m<sup>2</sup> or from  $1.9 \times 10^{-3}$  to  $5.8 \times 10^{-2}$  J/m<sup>2</sup>, respectively. In these experiments, cells were irradiated with a single daily exposure during a period of up to 4 days and the temperature of the cell cultures during laser treatment (1-30 min) was monitored by a microprobe connected to a telether-mometer. No changes in the temperature of the cell cultures were noted at any of the energy fluences tested. It is interesting to mention that a particularly dramatic enhancement of the collagen production was obtained with He-Ne laser radiation using fibroblast lines which initially showed a very low level of collagen production. On the other hand, fibroblast lines which have already committed a large fraction of their total protein synthesis capacity to procollagen production failed to respond to stimulation by the He-Ne laser. The enhancement of collagen production was dependent on the treatment schedule: maximal stimulation was achieved after multiple exposures, while a single treatment utilizing the same fluence did not cause any enhancement. In the case of the GaAs laser, a 2-3 fold stimulation was achieved with fluences considerably lower than those required for stimulation with the He-Ne laser. What is interesting, is that a response to the GaAs laser was noted also in a fibroblast line which was initially a high-producer of collagen and which did not respond to the He-Ne laser. The differences in the biological effects of the He-Ne and GaAs lasers probably reflect differences in their physical parameters, mostly in wavelength. Also, the He-Ne laser used in this study was a CW device, while the GaAs laser was a pulsed laser. Control experiments demonstrated that the He-Ne laser at the same fluence which caused a marked stimulation of the collagen synthesis, had

no effect on the cell proliferation measured as [ $^3\text{H}$ ]thymidine incorporation into DNA. On the other hand, GaAs laser radiation inhibited DNA replication. Later, the same group of authors (Saperia *et al.*, 1986) found that irradiation with a He-Ne laser enhanced the procollagen gene expression: the elevated type I and type III procollagen mRNA levels were demonstrated. The increased collagen synthesis was also demonstrated after ruby laser ( $\lambda = 694.3$  nm) irradiation (Mester and Jaszagy-Nagy, 1973).

In many experiments, various malignant cells have been used. By investigating the viability and clonogenicity of HeLa (carcinoma of cervix) cells, the plateau phase cultures were irradiated with a He-Ne laser ( $\lambda = 632.8$  nm) and replated at various intervals after the irradiation (Kam *et al.*, 1984c, 1987). The growth stimulation (increase of the number of cells in exponential phase of growth) was observed to last 6-7 days when the interval between the irradiation ( $100 \text{ J/m}^2, 10 \text{ W/m}^2$ ) and plating was 30 min or more (Karu *et al.*, 1984c). The number of clonogenic cells as well as clone size distribution was also studied (Karu *et al.*, 1987). The part of clonogenic cells in non-irradiated culture was  $45.2 \pm 0.4\%$  and this number increased after the irradiation, being  $50.4 \pm 5$ ,  $58.3 \pm 0.4$  and  $54.5 \pm 0.9\%$  by irradiation fluences of  $10, 10^2, 10^3 \text{ J/m}^2$ , respectively. This increase of the plating efficiency was supposed to be connected with increased attachment of cells caused by irradiation. The histograms of the frequency distribution of the clone sizes indicated that the per cent of abortive clones (diam.  $< 0.25$  mm) practically did not change after the irradiation as compared with the nonexposed control. The per cent of small clones ( $0.25 < d < 0.35$  mm) decreased, and the number of middle ( $0.35 < d < 0.5$  mm) and big clones ( $d > 0.5$  mm) increased in a fluence-dependent manner. These data show that the stimulating effect of the He-Ne laser irradiation is most noticeable on the proliferative activity of the slowly-growing subpopulations (the slowly dividing cells give small and middle clones). These authors noticed that after the irradiation, the clone-size distribution became more homogenous. For example, when irradiating with the fluence of  $100 \text{ J/m}^2$ , percent of small, middle and big clones was almost equal (near 30% each). In the control experiment, the distribution was 42:36:11% (Karu *et al.*, 1987).

Irradiation with a He-Ne laser ( $\lambda = 632.8$  nm) of HT 29 cells (carcinoma of colon) at fluences from 1 to  $10^3 \text{ J/m}^2$  ( $0.1$  and  $1 \text{ W/m}^2$ ) did not induce any marked effects on the reproduction rate of the cells. DNA synthesis rate was found to be slightly greater than that of the control cells when the cells were irradiated at a fluence of  $10 \text{ J/m}^2$  with an intensity of  $1 \text{ W/m}^2$  (Fava *et al.*, 1986). Swanson *et al.* (1989) failed to alter the proliferation of HT1080 (fibrosarcoma) and HT29 cells by a He-Ne laser radiation in the fluences from  $5 \times 10^2$  to  $1.6 \times 10^4$

$\text{J/m}^2$ . In their experiments, the uptake of [ $^3\text{H}$ ]thymidine from 1 to 16 h after the irradiation was used as the parameter to evaluate the rate of the proliferation. One must emphasize that these two groups of authors (Fava *et al.*, 1986; Swanson *et al.*, 1989) used the same culture (HT1080) and the same laser (He-Ne), but the experimental conditions including the fluences and the intensities of the light as well as the incubation periods were different. It is quite possible that different results were due to variations in the culture handling as well as in the irradiation methods.

No changes in the survival of mouse lymphoma cells after the irradiation with 5- and 10-mW He-Ne lasers were noticed (Kovacs *et al.*, 1982).

The irradiation with a He-Ne laser ( $50 \text{ W/m}^2, 30$  or  $60$  min), increased the number of dividing and DNA-synthesizing cells in the Ehrlich ascites tumor. The cells were irradiated *in vitro* and then injected in mouse (Kozlov and Moskalik, 1984).

The effect of a ruby laser ( $\lambda = 694.3$  nm) radiation on the growth of the Ehrlich ascites cells was also investigated (Mester *et al.*, 1971). The growth rate of the tumor, the mitotic index of the tumor cells, and the survival of the test animals were compared to corresponding parameters of the control group (injected with tumor cells from the same donor but not treated with the laser radiation). In six series of experiments, the body weight values of the animals injected with irradiated cells surpassed those of animals in the control group by 10-16%, and the number of tumor cells was 19-30% higher in the experimental group than in the control group. The differences proved to be statistically significant in three series of experiments.

The stimulation of the proliferation of murine fibroblasts, melanoma cells and skin tissue culture was reported after the irradiation of these cultures with low fluences ( $< 100 \text{ J/m}^2$ ) of ruby laser ( $\lambda = 694.3$  nm) radiation (Carney *et al.*, 1967; Hardy *et al.*, 1967; Jamieson *et al.*, 1969; Rounds *et al.*, 1965). Jamieson *et al.* (1969) found an increase in the division of melanoma cells and murine fibroblasts by irradiating the cells with a ruby laser at the fluences of  $(4-40) \times 10^4 \text{ J/m}^2$ . Jamieson *et al.* (1969) performed experiments with lightly pigmented B16 melanoma cells and heavily pigmented melanoma cells. After 1 pulse of the ruby laser radiation ( $0.6 \text{ J/pulse}$ ,  $\tau_{\text{pulse}} = 6 \times 10^{-6} \text{ s}$ ), the mouse melanoma cells showed a significant increase in the growth rate over the controls during the period from 24 to 92 h. After the fluence was increased, the effect was lost and the growth rate was found to be on control level. At a high radiation fluence (20 pulses,  $0.6 \text{ J/pulse}$ ), the proliferation rate of the culture was below the control level. These results showed, first, that next generations of irradiated cells were affected, and second, the existence of a fluence-dependence of the radiation effect.

Human epidermal cells, retinal epithelium cells from young rabbits and mice, and human melano-

cytes were irradiated with a pulsed ruby laser at  $\lambda = 694.3$  nm (Rounds *et al.*, 1965). The authors found that a low fluence ( $15 \text{ J/m}^2$ ) produced cytoplasmic retractions and pyknosis of the nuclei of melanin-containing cells. Higher fluence values ( $1 \times 10^3 \text{ J/m}^2$ ) produced immediate death of cells. Nonpigmented cells derived from human skin, mouse lung, hamster melanoma or albino rabbit retinal epithelium showed no morphological changes following exposure to fluences up to  $1 \times 10^7 \text{ J/m}^2$ . Rabbit endothelial cells showed no significant difference in mitotic indices when exposed to fluence of  $2.8 \times 10^5 \text{ J/m}^2$  as compared with nonirradiated cell populations. A high degree of chromosomal clumping by irradiation with a fluence of  $2.3 \times 10^5 \text{ J/m}^2$  was observed. In these experiments, the effects of radiation were clearly melanin-dependent and more pronounced in heavily pigmented cells.

In experiments of Hardy *et al.* (1967), mouse fibroblasts L929 were irradiated with a ruby laser ( $\lambda = 694.3$  nm) at fluence of  $1 \times 10^5 \text{ J/m}^2$ , transferred to a fresh medium, and the number of cells was counted daily. Nonirradiated control cells underwent the lag-phase of growth for one day and then the phase of exponential growth for 2 days. The irradiated cultures remained in lag-phase for 2 days but then underwent the exponential growth at a markedly higher rate than the control ones, yielding 5 times more cells on the fourth day of growth as compared to the control. These results show that radiation prolongs the lag-phase and stimulates the mitotic activity in following exponential phase of growth.

Far red light, near 750 nm in wavelength, was used by Gordon *et al.* (1971) and Chen and Gordon (1972). The interest of the authors in this particular wavelength was connected with studies on the biological role of the phytochrome. One should emphasize that there is still no evidence that phytochrome is present in mammalian cells. Exposure of a monolayer of the pig kidney cells to a far red light near 750 nm was found to inhibit the incorporation of pHlthymidine into nuclei of these cells (Chen and Gordon, 1972), and to increase the incidence of the chromatoid aberrations and the number of aberrant cells (Gordon *et al.*, 1971) with a similar dose-response maximum ( $5 \times 10^2 \text{ J/m}^2$ ). Lowering the temperature of the cells during the light exposure increased the yield of aberrations (Gordon *et al.*, 1971). When the cells were exposed to far red light ( $\lambda = 750$  nm) at various times after subculture, they showed peaks in the aberration yield when the intervals were about 16 and 22 h. These maxima corresponded to the early S- and G<sub>2</sub>-stages of the cell cycle.

In connection with the growing use of near IR diode lasers (typical wavelengths from 890 to 904 nm) in phototherapy instead of the He-Ne laser, first studies into the action of these lasers on cellular cultures have appeared. Some experiments of the

group of Dr. Uitto were mentioned earlier in connection with the synthesis of collagen (Lam *et al.*, 1986).

The irradiation of keratinocytes with a pulsed diode laser ( $\lambda = 904$  nm, fluence from  $2.5 \times 10^3$  to  $4 \times 10^4 \text{ J/m}^2$ , pulse duration 200 ns, repetition rate 1205 or 3125 Hz) stimulated the proliferation of both subconfluent and confluent cultures (Steinlechner and Dyson, 1990). Variation in light pulsing frequency was found to be insignificant when the energy density was maintained constant. It is important to emphasize that a greater response was obtained in keratinocytes whose growth rate has been reduced before irradiation. The delay in onset of proliferation normally observed in keratinocytes obtained from confluent cultures was reduced by laser radiation.

The exposure of chondrocytes to 1, 30, or 60 J of Nd:YAG laser radiation ( $\lambda = 1066$  nm, 10 Hz) in the presence of saline resulted in a significant stimulation of the DNA synthesis at periods of 72 and 144 h after the irradiation (Herman and Khosia, 1988).

#### Action Spectrum

An action spectrum of the lethal effect of visible light exists only for blue-violet region up to 434 nm (Tyrrell *et al.*, 1984).

For the activation of proliferation, the action spectrum exists for both exponentially growing and plateau-phase HeLa cells in whole visible region (Karu *et al.*, 1982, 1984 a,b; Karu, 1989a). In these studies, the rate of DNA and RNA synthesis was taken as a criterion of proliferative activity. As a first step, the dependences on the fluence and intensity for various visible light wavelengths were studied. In the red and far red regions, the optimal fluence was equal to  $100 \text{ J/m}^2$ , and it was relatively insensitive to the wavelength. In the blue-ultraviolet region, the fluence causing maximal effect was lower by about an order of magnitude (e.g. for  $\lambda = 404$  nm,  $10 \text{ J/m}^2$ ) (Karu *et al.*, 1984a). In other words, achieving the maximum effect in the violet-blue region required approximately an order of magnitude lower fluence than that required to achieve the same effect with red or far red light. All fluence-effect dependences had a bell-shaped form with a threshold, maximum and a phase of decline.

The influence of the light intensity on the DNA synthesis stimulation effect in HeLa cells was also studied (Karu *et al.*, 1984a). In experiments with CW light at  $\lambda = 633$  nm, the radiation fluence was achieved by varying the intensity of light and the duration of the irradiation. The fluence was constant and amounted to  $100 \text{ J/m}^2$ . The stimulation of DNA synthesis rate was found to be sensitive to the irradiation time and to the intensity of light. The maximum stimulation effect was observed when the light intensity was from 8 to  $10 \text{ W/m}^2$  and the irradiation time 10-12 s. It decreased rapidly on increase in the irradiation time or on reduction in

the light intensity. The threshold of effect in our experimental conditions was  $7 \text{ W/m}^2$ .

The stimulation of the DNA synthesis in the exponentially growing HeLa cells was detected in wavelength ranges of 32(M50 nm, 600-720 nm, 750 and 840 nm with maxima near 400, 630,680 and 760 nm (Karu *et al.*, 1984a). In near IR region, there was a structure in a form of a peak near 820 nm. The action spectrum of stimulation of RNA synthesis was very close to the corresponding action spectrum of stimulation of DNA synthesis. The maxima in this action spectrum corresponded to the wavelengths of 400, 615, 680, 780, and 820 nm. In both action spectra, there were no statistically significant changes from control level in spectral range from 450 to 580 nm.

The action spectra for stimulation of DNA and RNA synthesis in plateau-phase HeLa cells were measured in the range of wavelengths from 580 to 860 nm (Karu *et al.*, 1984b). The action spectra obtained were found to be similar to respective spectra for proliferating HeLa cells described above.

An action spectrum for the stimulation of the mitotic activity of L cells after the irradiation in range from 400 to 700 nm was published by Gamaleya *et al.* (1983). The maxima were found near 415, 550 and 633 nm.

#### *Which Point in the Cell Cycle is Affected?*

The results of experiments in which the exponentially growing HeLa cells were pulse-labeled with [ $^3\text{H}$ ]thymidine at various times after the irradiation with a He-Ne laser in fluence of  $100 \text{ J/m}^2$  indicated that the number of DNA-synthesizing cells increased during the first 3-4 h after the irradiation and then diminished to control level. On the basis of this curve it is possible to suppose that the increased number of S-phase cells originated from a part of the G<sub>1</sub>-phase population ready for passing to S-phase. The stimulative effect of the irradiation on the progression of G<sub>1</sub>-phase cells into S-phase was confirmed in autoradiographic experiments with continuous labeling (Karu *et al.*, 1987).

To answer the question, does the irradiation influence the rate of the DNA synthesis in S-phase cells, the number of grains were counted on the labeled nuclei. The average grain count on the nuclei of exponentially growing cells increased after the irradiation, being above control level from 3 to 6 h after the irradiation. The distribution of grain number per individual nuclei indicated that the number of cells with higher grain counts increased following irradiation (Karu *et al.*, 1987).

On the basis of these data it was possible to conclude that the enhancement of DNA synthesis rate established in experiments of Karu *et al.* (1982, 1984a, 1989a) was due to the intensification of DNA synthesis in S-phase cells as well as due to enhanced transition G<sub>1</sub> → S of a part of the population. Different kinetics of both processes after the irradiation

made it possible to suggest that in the first hours after the irradiation the increased incorporation of [ $^3\text{H}$ ]thymidine is caused mainly by increased number of S-phase cells, and in longer terms after the irradiation (e.g. 6 h in our experiments) by enhanced DNA synthesis in S-phase cells.

Percent of the cells in mitosis (M) did not change during first hours after the irradiation. The only significant difference from control level was noticed 6 h after the irradiation (Karu *et al.*, 1987).

Irradiation of rat kidney epithelial cells in S-phase with inhibiting the proliferation fluences of a He-Ne laser temporarily arrested the cells in mitosis (Schneede *et al.*, 1988). In addition, electron microscopy of the irradiated cultures revealed a relatively high number of the cells in mitosis.

Irradiation of pig kidney cells with far-red light at 750 nm caused maximal yield of chromosome aberration when the synchronized population was irradiated in early S- and G<sub>2</sub>-stages of the cellular cycle (Gordon *et al.*, 1971).

Irradiation with a He-Ne laser ( $50 \text{ W/m}^2$ , 30 or 60 min) of Ehrlich ascites cells shortened the mitotic cycle of cells in all its phases and increased the growing fraction of the population. An increase in number of pathological mitoses was observed (Moskalik and Kozlov, 1984).

#### *Dichromatic Irradiation*

The stimulative effect of red light ( $633 \pm 7 \text{ nm}$ ) on the DNA synthesis rate was shown vanishing when a monolayer of HeLa cells was irradiated simultaneously with red light and light of broad bandwidth from the blue-yellow region (Karu *et al.*, 1984a).

To explain whether the disappearance of the stimulative effect was associated with the spectral range of the wide-band light or whether it was due to the specificity of the wavelengths involved, the experiments with dichromatic irradiation were performed. Since there were several maxima in the action spectra of stimulation of proliferation (Karu *et al.*, 1984a,b; Kara, 1989a) in the experiments of dichromatic irradiation, those wavelengths which were found to give maximal response were used (Karu *et al.*, 1985).

HeLa cells in the form of a monolayer were simultaneously irradiated with a red light ( $\lambda = 632.8 \text{ nm}$ ) and a variable-wavelength monochromatic light ( $\lambda_{\text{add}}$ ) (Karu *et al.*, 1985). The fluence of red light was always kept fixed at  $100 \text{ J/m}^2$ . The fluence for the variable-wavelength radiation was taken at  $100 \text{ J/m}^2$  in the range from 600 to 800 nm, and 10 or  $25 \text{ J/m}^2$  in the range from 400 to 570 nm. DNA and RNA synthesis rates were measured 1.5 h after the irradiation. In the red and far red regions of the new action spectra ( $\lambda_{\text{add}} = 600\text{-}820 \text{ nm}$ ), the maximum at 620 nm remained the same as in case of monochromatic irradiation whereas that at 760 nm disappeared. With the irradiation dose for the vari-

able-wavelength light  $\lambda_{\text{add}}$  set at  $10 \text{ J/m}^2$ , the maximum in the blue region of the spectrum, which was observed at 400 nm, now occurred at 450 nm, i.e. it shifted into a region of longer wavelengths. With the irradiation dose for  $\lambda_{\text{add}}$  increased to  $25 \text{ J/m}^2$ , the maximum in the blue region at  $\lambda = 400 \text{ nm}$  vanished and a new maximum appeared in the green region ( $\lambda = 550\text{-}570 \text{ nm}$ ). Both DNA and RNA synthesis rates were inhibited in the green region when the irradiation dose for  $\lambda_{\text{add}}$  was set at  $10 \text{ J/m}^2$ .

When the cells were subjected to consecutive dichromatic irradiation, the sequence  $\lambda_{\text{add}} + 633 \text{ nm}$  with a 60 min interval between the two irradiation events, the effects described above vanished and the respective action spectra became similar to those obtained earlier for the wavelengths equal to  $\lambda_{\text{add}}$ . -It means that only the first irradiation event proved to be effective, and by the onset of the second irradiation event with  $\lambda = 633 \text{ nm}$ , the system had changed into another state from which no new changes were possible.

The consecutive dichromatic irradiation with wavelengths of 760 and 633 nm (fluences of  $100 \text{ J/m}^2$ ), varying the time interval between the irradiation events over a broad range (from 1 to 2 h), confirmed the importance of the time interval between two irradiation events. With short (1-10 s) time intervals, the rate of DNA synthesis did not deviate from the control level. As the interval grew longer, the rate of DNA synthesis changed, the sense of the effect depending on the sequence of dichromatic irradiation wavelengths. The irradiation first with far red light ( $\lambda = 760 \text{ nm}$ ) and then with red light ( $\lambda = 633 \text{ nm}$ ) stimulated the DNA synthesis, whereas that in the reverse order (633 + 760 nm) inhibited it. These effects reached their maxima when the time interval between the successive irradiation events was from 1 to 3 min and became progressively less pronounced with further increase in the interval. When the cells were subjected to consecutive irradiation with light of the same wavelength (two times with  $\lambda = 633 \text{ nm}$  or two times with  $\lambda = 760 \text{ nm}$ ), the time interval between the irradiation events amounting to a few seconds, the net effects were the same as for the one-fold irradiation effects for both DNA and RNA synthesis rate.

The irradiation of the cells with red (633 nm) and blue (404 nm) light with different time intervals (from 1 s to 2 h) between the irradiation events and in different wavelength sequences had also different effects on the DNA and RNA synthesis rates. Irradiation in the sequence of 633 + 404 nm had either no effect at all (in the case of DNA) or a considerably weaker effect as compared with that in the sequence of 404 nm + 633 nm. Irradiation in this latter sequence (404 + 633 nm) was observed to stimulate the synthesis of both DNA and RNA, irrespective of the time interval between the irradiation events.

These data agree with the data of the consecutive dichromatic irradiation of the culture of human embryonic fibroblasts (Bogush *et al.*, 1982). The authors noticed no changes in the mitotic activity of the cells in the case of irradiation in the sequence of 633 + 441 nm, whereas in the case of the irradiation in the sequence of 441 + 633 nm, the mitotic index was observed to increase by 40-60% within a 5 min interval between the two irradiation events. The nonadditive stimulation was also noticed in cases in which one of the two irradiation components had an inhibitive effect when used individually (Bogush *et al.*, 1982).

Klein and Edsall (1967) established the red-light photoreactivation of induced by the green light suppression of HeLa cells.

Combined dichromatic irradiation of human embryonic fibroblasts with  $\lambda = 347.15 \text{ nm}$  and with other wavelengths (441.6, 694.3, or 741 nm) in both sequences increased nonadditively the number of chromosome aberrations as compared with monochromatic irradiation (Stepanov *et al.*, 1977). The maximal increase (10 times above control level) was found when wavelengths 347.15 and 693.3 nm were used in sequence of 694.3 + 347.15 nm. In contrast, when one of the components of dichromatic irradiation was red light of  $\lambda = 632.8 \text{ nm}$  (other components: 347.15, 694.3, or 741 nm), the mutagenic effect was reduced to the control level, or even more, in some cases a weak protective effect of red light was established.

#### Concluding Remarks

As discussed in previous parts of this paper, the radiation from visible light sources has been found to stimulate, be indifferent, or inhibit the proliferation of cellular cultures. How to explain this controversy? And second, does the light treatment stimulate the proliferation of tumor cells?

#### Controversy stimulation—inhibition

Systematic studies with cells of varying complexity levels (prokaryotic and primitive eukaryotic cells, see reviews of Karu, 1987, 1988, 1989a,b, as well as complex eukaryotic cells like cellular cultures, see this review and references therein) clearly indicate that such parameters of light as wavelength (monochromaticity within absorption bandwidth of molecules), fluence, and intensity play the most important roles in both stimulation and inhibition of cellular metabolism. For stimulative effects there exists a rather narrow and well-defined range of parameters of light. The inhibitive fluence and intensity ranges are not so exactly determined and are much broader. For example, increasing the stimulative fluence by two or three orders of magnitude inhibits the cellular metabolism, or in some cases (irradiation with blue light) can even have a lethal effect. This conclusion is drawn from

examples given in the "Action of proliferative activity" section above.

It was proposed that the primary photoacceptors for both stimulative and inhibitive effects are the same, components of the respiratory chain (see reviews of Karu, 1988, 1989a,b). The fact that irradiation of cells with visible light of the same wavelength and absorption of this light by the same molecules has a positive effect (enhancement of proliferation) and a negative one (damage to intra-cellular systems and even death of cells) was explained by different reactions prevailing in cases of lower and higher fluences due to different quantum yields of the respective reactions involved after electronic excitation of photoacceptor molecules. At lower fluences and intensities the redox reactions predominate (the result being the redox control of cellular metabolism), and at higher fluences and intensities the products of photosensitizing reactions cause damage (and lethality) of cells (Karu, 1988, 1989a). For achieving the stimulative effects, a functioning electron transport system is required. In case of inhibitive and lethal effects, the electron transport is inhibited or even disrupted. A possible scheme (signal transduction and its amplification in a cell) explaining the stimulation of proliferation was also proposed (Karu, 1988, 1989b). This scheme is based on the consideration that monochromatic visible light is one of the nonspecific environmental factors influencing the proliferation *via* alterations in cellular homeostasis. This scheme contains the usual and known in cellular biology pathways which cells use when their proliferative activity is influenced by external factors. Specificity of light action is featured by absorption of quanta by components of respiratory chain.

#### *Controversy stimulation—inference*

As seen from this review (see "Action on proliferative activity" above), in some experiments the action of visible light radiation was found to be indifferent, i.e. there was neither stimulative nor inhibitive effects on proliferation. On the other hand, in some experiments reviewed above, one can see that the effect of radiation was more pronounced in the cells in which metabolic activity (e.g. rate of proliferation, rate of collagen synthesis) was not high. Analysis of these data gives us the possibility to suppose that the magnitude of stimulation effects depends on the physiological state of cells at the moment of irradiation (Karu, 1987). It means that there is little possibility, if any, to increase the proliferation of fast growing cellular cultures in the cases when the proliferation occurs at a more or less maximal rate in these particular experimental conditions. This problem is discussed by Karu (1987, 1989a). Tiphlova and Karu (1989) made an attempt to find some quantitative laws of this phenomenon for a simpler model, *Escherichia coli*. They showed, first, the existence of a maximal specific rate of the

exponential growth, and second, the dependence of the magnitude of stimulated by irradiation specific rate of exponential growth on growth rate of this culture before irradiation. One should emphasize that reports of experiments designed with an aim to understand this problem are still absent, and the key question seems to be: which are the affected (e.g. retarded) metabolic reactions in cells making them sensitive to irradiation.

#### *Normal cells-malignant cells*

Experimental data gathered so far show no differences between normal and malignant cells in sensitivity to visible light wavelengths. The proliferation of both normal and tumor cells can be stimulated depending on light parameters and proliferation rate at the moment of irradiation. This circumstance should be taken into consideration in the clinical practice of (laser) photomedicine.

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