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Effect of visible light on some cellular and immune parameters

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Summary The biological effect of visible light of low energy density was investigated in this study. The effects of diffuse (DL) and linearly polarized (LPL) light were compared on models *in vitro* and *in vivo*.

Experiments *in vitro* were performed on human lymphocytes to study their blast-transformation and rosette-formation abilities. Both DL and LPL increased the number of blast-transformed cells even in a lymphocyte culture without PHA, and reduced rosette-formation of T lymphocytes. LPL had a more pronounced effect.

In vivo exposure to DL and LPL of the spleens of tumour-bearing mice caused the appearance of factor(s) in their serum, inhibiting the incorporation *in vitro* of [^{3}H]-thymidine into the tumour cells obtained from non-exposed animals. In the other series of experiments serum samples were taken from tumorous animals after the exposure of their spleens to LPL. Following the daily administration of these sera to another group of non-exposed tumorous mice a decreasing tendency of the mitotic kinetics of ascites tumour was observed.

The application of visible (preferably linearly polarized) light for the stimulation of human immune competent cells, and clinical trials with extracorporeal irradiation of blood for the promotion of natural defences of an immune-repressed organism are suggested.

Key words: [^{3}H]-thymidine, ascites tumour, blast transformation, human lymphocytes, mitotic index, rosette formation, serum treatment, spleen irradiation, visible light.

Introduction

In the early 1980s it was suggested hypothetically that the changes in the cell membrane induced by laser light in the visible spectrum played an important role in the promotion of wound healing.[1] This biostimulating effect of laser is attributed to its polarized character. Similar biostimulation has also been achieved with polarized light. For instance, the promoted wound healing induced by polarized light has been explained by the increased amount of immune proteins in the exudate of wounds.[2] Favourable biological effects of visible light have been widely experienced in surgery, gynaecology, rheumatology and dermatology.[3-5]

During the past decade, data on the biological effects of visible light observed on cellular level have been gathered.[6-12] Changes on cell surfaces were also observed: the treatment of cultured human fibroblasts with laser, diffuse or polarized light caused the accumulation, to a varying extent, of negative charges.[13,14]

Some results, however, are in disagreement, describing opposite effects. Several authors found that visible light had an inhibitory influence on cell growth[7,12] and human lymphocyte stimulation.[9] In contrast, others revealed biostimulation phenomena induced by visible light of low intensity in the proliferation of HeLa cells,[8] Chinese hamster cells[6] or acceleration of the growth of Ehrlich tumour.[11] In different tumour cells the increase of colony formation capability *in vitro* was also found.[15] These facts motivated the continuation of studies on the response to light exposure of cells, including immune competent ones.

The aim of our current experiments was to answer the question, whether the exposure to light of immune competent cells leads to changes in their functional condition and, consequently, to the stimulation of the immune system, manifesting itself in the increased defence capacity of an organism. Biostimulation by this physical factor would be of particular practical value in cases of repressed immune system (malignant processes, radiotherapy and so on). Similar attempts giving promising results with laser[2,10] as well as UV irradiation[16-19] have been reported.

Thus, in one series of our experiments the effects of diffuse light (DL) and linearly polarized light (LPL) of 4 J/cm^2 energy density on the functional condition of human lymphocytes were studied.

The other series involved experiments *in vitro* and *in vivo* on tumour-bearing mice, the spleens of which were exposed to DL and LPL. The proliferation parameters of ascites tumour were determined on another group of mice after the administration of serum taken from the previous group.

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Materials and methods

Separation of human lymphocytes

The functional condition of non-exposed (control) and light-exposed human lymphocytes was studied *in vitro*. The effect of light on the blast-transformation ability of cells was examined according to morphological criteria. The changes on the surface of T lymphocytes were detected by a rosette-formation test.

Heparinized blood was collected from healthy donors and patients with lung small-cell carcinoma. Lymphocytes were separated by centrifuging the blood samples on Ficoll-Uromiro gradient of 1.077 g/mL density. The gradient was prepared by mixing Ficoll 400 (Pharmacia, Uppsala, Sweden) and Uromiro 60% (Bracco, Milan, Italy) to have the stock solution of 1.120 g/mL density. Briefly, 33.7 g Ficoll 400 was dissolved in 250 mL warm (50-60°C) distilled water, then 88.3 mL Uromiro 60% was added, and the final volume of 340 mL was prepared by adding distilled water.

For the experiments the stock gradient was diluted to 1.077 g/mL density. The lymphocytes were separated under isosmotic conditions and pH 7.2-7.3. After three washings the cells were resuspended in Parker medium 199 (National Institute of Hygiene, Budapest, Hungary) and used in blast-transformation and rosette-formation tests.

Light treatment of human lymphocytes

The cells were treated by DL and LPL using a halogen light source designed for test purposes (Bild-system AB, Malmo, Sweden) emitting in a broad spectrum ranging from 400 to 800 nm. LPL was produced by the use of a polar filter (Type HN 38 according to E. Kasemann; the dichroic polarizer was produced in Sweden). The loss of energy density across the filter was compensated through electronic control, therefore the energy density of both DL and LPL generated was equal. Cell suspensions of 1 mL volume (1 x 10^7 cells/mL) were exposed in Petri dishes of 3.5 cm diameter, with a 6.5 cm distance between the polar filter and the bottom of the dishes.[14] Thus the total surface of the Petri dish was exposed to the beam of polarized or diffuse light. The height of nutrient solution was 2.5 mm. During irradiation the covers were removed. The duration of exposure was 7 min. The power was measured for the bottom of the Petri dishes by a power meter (Quantronix Model 502, Smithtown, NY, USA), and the values measured were divided by the surface of the exposed area to obtain the power density. Energy density was calculated taking into consideration the duration of exposure. Under such conditions it was equal to 4 J/cm^2.[20]

Determining the blast-transformation ability of human lymphocytes

In the blast-transformation experiments lymphocytes of three healthy individuals were examined. The donors were young males (21-23 years old). The lymphocyte suspensions exposed to DL and LPL and the unexposed ones were incubated at 37°C, 5% CO_2 in 5 mL Eagle MEM medium completed with 10% autologous serum, 2 mol/L L-glutamine, 100 IU/mL penicillin and 100μg/mL streptomycin. One part of the cell suspensions was stimulated with 5 μg/mL PHA (Difco, Detroit, MI, USA), the other part was cultured without PHA. Blast-transformation of lymphocytes was evaluated according to morphological criteria by examination in a light microscope. For each case determinations were done with three parallels, and 500 cells were examined for each time-point. The corresponding results were pooled and statistically analysed (Table 3).

Rosette-formation test on human T lymphocytes

In the rosette-formation test, T lymphocytes of four healthy individuals and four patients with lung tumours were studied. The healthy donors and oncological patients were males of middle age between 47 and 65 years.

The ability of human T lymphocytes to form rosettes with sheep erythrocytes was tested using a method already described.[21] For the purposes of our tests the rosette was a lymphocyte binding at least three RBC. All measurements were performed with three to four parallels, for each variant and each time-point 500 cells were counted and the proportion of rosette-forming cells was determined. The corresponding results were pooled and statistically analysed (Table 4).

In vitro *testing of the effect of sera on [^{3}H]-TdR incorporation*

Adult DBA_2 male mice (LATI, Godollo, Hungary) of 22-25 g weight were divided in six groups of 10-12 animals each (Table 1). The mice in groups II-V were operated to reach the spleen. Serum samples were collected from groups I-V and pooled on the 1st, 3rd and 7th days after the operation. It means that three to four animals were killed at each of these times, and their sera were pooled. Ascites tumour cells were taken from animals of group VI. [^{3}H]-TdR (Amersham, Buckinghamshire, UK) was incorporated *in vitro* into these cells in the presence of the corresponding serum samples.

Five day old P388 ascites tumour cells were used in concentration of 0.5 x 10^6 cells/mL Parker medium 199. The final serum dilution was 1 : 10. The labelling with DNA precursor (1 *μCilmL*, at 37°C, 5% CO_2) lasted 6 h. After washing out the unbound [^{3}H]-TdR, the level of radioactivity incorporated in the tumour cells was measured in a liquid scintillation spectrometer. The results were expressed as percentage of the values for group V, mean values ± s.d. Group V served as a control in which animals were operated only.

Table 1 Murine groups in the [^{3}H]-TdR incorporation tests

Group no.	Treatment				Comment
	Tumour*	Operation**	LPL***	DL***	
I	+				Serum donor
II	+	+			Serum donor
III	+	+	+		Serum donor
IV	+	+		+	Serum donor
V		+			Serum donor
VI	+				Tumour cell donor

*Intraperitoneal inoculation with P388 murine ascites tumour (4 x 10^6 living cells in 0.5 mL physiological saline).

**Operation to reach the spleen was performed under narcosis on the day after inoculation with tumour.

***Treatment of the spleen with DL and LPL of 4 J/cm^2 energy density.

In vivo *testing of the effect of sera on the mitotic kinetics*

The serum samples collected on the 7th day after operation from 10 mice in each of groups I and II were pooled and kept at - 20°C before use (Table 2). This time was chosen on the basis of our earlier experiments *in vitro* showing the highest indices of [^{3}H]-TdR incorporation inhibition in the presence of the 7th day sera (Fig. 1). P388 ascites tumour cells (4 x 10^6 cells/mL) were preincubated at 37 °C, 5% CO_2 with sera of groups I and II (final dilution 1 : 10), then inoculated intraperitoneally in 0.5 mL volume to mice of groups IVA and IVB, respectively. On the following 5 days 0.1 mL of the corresponding pooled serum was given to groups IVA and IVB. Group IVC received only tumour cells from group III, without any serum treatment. The tumour-bearing and serum-treated mice were killed on the 1st, 3rd and 7th days. Thus, three to four animals were involved in one sub-group for each treatment and time-point. The smears of ascites tumours were prepared, fixed and stained according to May-Grünwald-Giemsa. By visual examination in a light microscope of 1500 tumour cells, the cells in mitosis (prophase, metaphase, anaphase and telophase) were counted, and the mean values ± s.d. of mitotic indices were determined and expressed in percentages.

Since the efficacy of LPL in relation to DL was shown to be significantly higher,[14] in our experiments on tumorous mice *in vivo* only LPL was used for treatment.

Results

Blast-transformation test of human lymphocytes

Table 3 summarizes the results of morphological evaluation of cultured human lymphocytes exposed to DL and LPL. Our findings show that in the presence of PHA there was some difference between the blast-transformation ability of lymphocytes in the control and LPL-exposed cultures as detected for the 8- and 24-h points. The intensifying effect of LPL (0.35) and DL (0.23) was observed after 24 h for samples cultured without PHA compared to the corresponding control sample (0.15). The effect persisted after 48 h while the control values remained the same.

Rosette-formation test of human T lymphocytes

The ability of human T lymphocytes to form rosettes with sheep erythrocytes was studied after the exposure of cells to DL and LPL. The cells originated from healthy persons and patients suffering from lung carcinoma (Table 4). It was found that both kinds of visible light decreased the number of rosette-forming lymphocytes. The proportions for LPL and DL were 0.51 and 0.55, respectively, while in the control this value was 0.72. A similar tendency was found for oncological patients: the proportions of rosette-forming cells for LPL and DL were 0.52 and 0.60, respectively, compared to the control value of 0.68.

Table 2 Murine groups in the mitotic kinetics tests

	Treatment			Comment
Group no.	Tumour	Operation	LPL	
I		+		Serum donor A
II	+	+	+	Serum donor B
III	+			Tumour cell donor
IVA	+			Recipient A
IVB	+			Recipient B
IVC	+			Recipient C*

*Recipient C received only tumour cells from group III, without any serum treatment.

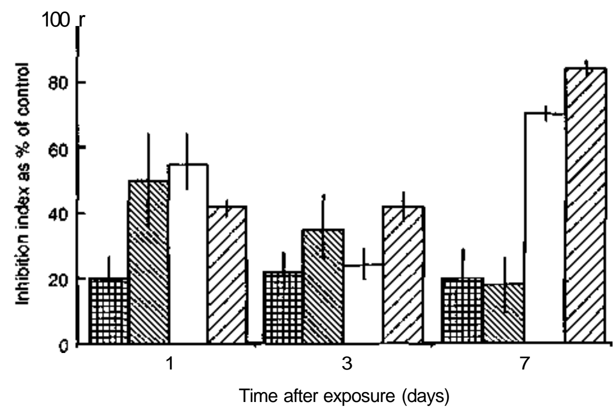

Figure 1 Effect of sera collected on days 1, 3 and 7 after treatment of various groups of mice upon the incorporation *in vitro* of [^{3}H]-thymidine into the tumour cells of untreated animals. The inhibitory effect of serum samples is expressed as percentage of the control. Mean values ± s.d. (▦) P388; (▧) P388 + operation; (□) P388 + operation + LPL; (▨) P388 + operation + DL.

Incorporation of[^{3}H]-TdR into the tumour cells

Serum samples were collected on the 1st, 3rd and 7th days after treatment with light (Table 1). We investigated the effect of sera on the incorporation *in vitro* of [^{3}H]-TdR into the tumour cells of untreated mice (Fig. 1). Our results demonstrate that sera taken from group I caused an inhibitory effect of 20-25% during the whole period of observation. The 1st day serum samples taken from the other three groups inhibited the incorporation of DNA precursor in a larger degree: inhibition indices varied from 42 to 52%. Practically, there was no significant difference between the effects of the 3rd day sera of all groups: the average values measured were between 27 and 40%. As for the 7th day serum samples, the inhibitory effect of sera taken from groups I and II was similar; that is, the indices measured were approximately 20%. However, remarkable inhibition was detected in groups III and IV (70-80%). The effect of DL seemed more pronounced.

Mitotic kinetics

Serum samples were collected on the 7th day after operation from group I and II (Table 2). P388 tumour cells taken from groups III were preincubated with these serum samples for 30 min at 37°C, 5% CO_2. The preincubated cells were inoculated intraperitoneally in groups IVA and IVB. In the following 5 days groups IVA and IVB received

Table 3 Blast-transformation of human lymphocytes exposed to light

Duration of culturing (h)	Treatment	Proportion (p) of blast-transformed cells in the presence of PHA	Calculated |z| statistic	*P* value of α for s.d. (*) between the respective proportions	Proportion (p) of blast-transformed cells[9] in the absence of PHA	Calculated |z| statistic	P value of α for s.d. (*) between the respective proportions
	None (control)	0.17			0.13		
8	LPL	0.20	2.12	*P= 0.042	0.20		*
	DL	0.19	1.43	P = 0.144	0.15	1.58	P=0.115
	None (control)	0.27			0.15		
24	LPL	0.34	4.17	*P=0.0001	0.35		
	DL	0.27			0.23		
	None (control)	0.47			0.15		
48	LPL	0.46			0.38		*
	DL	0.44	1.67	P=0.099	0.35		*

Data of three separate experiments containing observations of 500 cells each were pooled. The suspected differences between means of the control and exposed populations were tested for significance by the normally distributed z statistic, i.e. by the quotient of $\hat{P}_{control} - \hat{P}_{exposed}$ and the s.e. of the difference of binomial proportions. Statistical decision was made at the $\alpha = 0.005$ level.

Table 4 Rosette-formation test of human T lymphocytes

Treatment	Proportion of rosette-forming cells		Calculated |z| statistic	P value of α for s.d. (*) between the respective proportions
	Healthy persons	Patients with lung tumour	Patients with lung tumour	Patients with lung tumour
None (control)	0.72	0.68		
LPL	0.51	0.52		
DL	0.55	0.60	5.27	P<0.0001

Data of four separate experiments containing observations of 500 cells each were pooled. For the conditions and criteria of statistical analysis, see footnote to Table 3.

0.1 mL of the corresponding serum every day. Group IVC received no serum. The mitotic indices of growing tumours were determined on smears prepared on the 1st, 3rd and 7th days (Fig. 2). On the 1st day the values measured for groups IVA and IVC were equal (about 21%). On the 3rd and 7th days the mitotic indices decreased gradually to 14 and 11%, respectively. The decrease of the mitotic indices in group IVB relative to groups IVA and IVC was observed throughout the entire period of observation, that is, 17, 11 and 8.5% on the 1st, 3rd and 7th days, respectively.

Discussion

The favourable effect of visible light on wound healing was already published.[3,22] In our current experiments the energy density of light used was 4 J/cm^2, because in current clinical practice the promotion of the healing process of burns, ulcera cruris and other chronical epithelial injuries is experienced at this value.

Our results demonstrate that cells participating in the development of immune response could also be affected by visible light when exposed *in vitro*. Thus, the blast-transformation ability of human lymphocytes increased by the evidence of morphological studies of cultures incubated without PHA. Inhibition of lymphocyte inducibility by mitogens is also described in the literature.[9] The disagreement between the experimental results is possibly attributable to the age of cultures at the time of examination. The results also reflect that exposure to LPL produced a more pronounced stimulative effect than DL.

The response of lymphocytes to LPL and DL can also be measured by rosette-formation with sheep erythrocytes. We suggest that the reason for this phenomenon is the light-induced accumulation of negative charges on the surface of lymphocytes[14] that inhibits the binding of negatively charged red blood cells.

The spleen plays an important role in the immune system. Exposure to light of the spleens of mice *in vivo* caused the release of mediator(s) into the serum that inhibited the incorporation of [^{3}H]-TdR *in vitro* into mouse ascites tumour cells taken from unexposed animals. Preliminary examinations have excluded TNF-α as a possible inhibiting factor.

The next series of experiments revealed that the average value of the mitotic index decreased in tumours

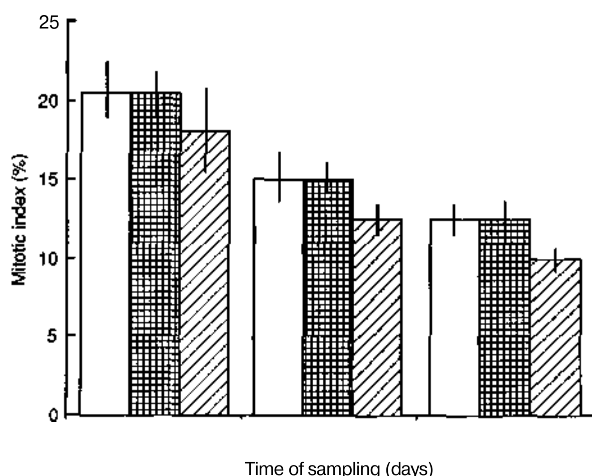

Figure 2 Impact of sera collected from mice whose spleens were exposed to LPL on the mitotic kinetics of tumour cells in untreated mice. (□) P388 (group IVC); (▦) control (group IVA); (▨) LPL (group IVB). Sera were collected on the 7th day after the operation (see Table 2: serum donor B, recipient B). The proportion of dividing cell forms was determined on days 1, 3, 7 and compared with the mitotic indices measured after the administration of control sera (see Table 2: serum donor A, recipient A), and with those without serum treatment (see Table 2: recipient C). Mean values for three mice ± s.d.

treated with LPL serum relative to unexposed serum and untreated tumours.

Other authors described the release of substances from human lymphocytes upon UV irradiation with 254 nm wavelength.[16] For the study of possible mechanisms of the stimulative effect, leucocyte-enriched plasma irradiated by UV light was added to the human bone marrow cells. The authors observed an increase in the colony-forming activity,[18] and stimulation of the phagocytic activity of monocytes and granulocytes in the whole volume of mixed blood composed of a sample irradiated with UV light and the untreated blood of the same donor.[17]

In vitro UV irradiation of blood followed by re-infusion for the stimulation of regenerative processes in various pathological cases is already known in clinical practice.[19,23-25] Together with the favourable experiences in therapy, the UV radiation-induced suppression of natural killer cell activity has also been described.[26] On the basis of experiments *in vivo* with UV, visible and infrared radiation, other authors suggest that mediators should play a crucial role in signal transduction from the skin to the peripheral blood cells.[27] The most likely candidates for connecting links are IFN-γ, granulocyte-monocyte-CSF and TNF. After HeNe laser irradiation of mononuclear cell cultures of human peripheral blood transient increase of various cytokines could also be measured *in vitro*.[28]

In our experiments we have shown that the effect of LPL on some parameters investigated is more pronounced than that of DL. On the basis of findings published earlier and our own data we suggest that visible light provokes the release of some biological mediators (cytokines) from the immune competent cells and in this way stimulates the natural resistance of an organism. Similar to UV radiation and without the negative effect of suppressed natural killer cell activity, the application of visible light, preferably LPL, for the extracorporeal exposure of human blood is suggested.

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