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COMPLEX SYSTEMS
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Effects of Exposure of Different Skin Areas to Low-power Laser Light

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Abstract—The effect of He–Ne laser light of extremely low power (632.8 nm, 0.2 mW/cm^2) on the immune status of mice bearing solid tumors was studied. The state of tumor-bearing animals was assessed taking into account the number of immunocompetent cells, concentration of cytokines (tumor necrosis factor and interleukin-2), production of nitric oxide, expression of heat shock proteins 70 and 90, and the activity of natural killer cells. The model of a solid tumor was formed by subcutaneous transplantation of Ehrlich ascite carcinoma cells to mice; the average lifespan of animals was approximately 55 days. Different areas of skin of tumor-bearing mice were irradiated with laser light either singly (1 min; dose, 0.012 J/cm^2) or repeatedly (1 min every 3 days over 30 days; total dose, 0.1 J/cm^2). It was established that long-term chronic exposure of mice bearing Ehrlich ascite carcinoma cells to low-power laser light in the thymus projection area and especially in the tumor projection area leads to a decrease in the natural antitumor potential, which is manifested in acceleration of tumor growth and a tendency to decrease in the lifespan of tumor-bearing mice. Conversely, stimulation of antitumor immunity was observed over several days after a single exposure to low-power laser radiation. The results suggest that it is expedient to continue studies of the immunomodulating effects of low-power laser light and demonstrate the necessity of monitoring the immune system in the course of laser therapy.

Key words: extremely-low-power laser radiation, He–Ne laser, tumor necrosis factor, heat shock protein 70, heat shock protein 90, interleukin 2, nitric oxide, natural killer cells, tumor-bearing mice, Ehrlich ascite carcinoma

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INTRODUCTION

Despite the broad spectrum of known anticancer drugs, the search for optimal methods of treating malignant diseases remains a relevant problem. A promising direction in anticancer therapy is immune therapy, which is aimed at activation of natural antitumor potential of an organism, because it is the immune status that determines the success of treatment. It is known that natural antitumor control at early stages of malignization is exercised by two groups of nonspecific immune effectors—cytotoxic macrophages or dendritic cells and natural killer cells (NKCs) [1, 2]. For this reason, the treatment of cancer, at least at the initial stages of carcinogenesis, should include the activation of the key components of antitumor resistance, stimulating the synthesis of cytotoxic agents, such as nitric oxide (NO), and anti-inflammatory cytokines, such as tumor necrosis factor (TNF) and interleukin 2 (IL-2), as well as increasing the activity of NKCs. Additionally, in the course of antitumor therapy, immunocompetent cells should be adequately protected from the "aggressive" influence of toxic agents produced by them and from anticancer drugs. Such protection is ensured by the unique family of proteins called heat shock proteins (HSPs) [3, 4].

Today, taking into account the immunomodulating effect of laser light, it has been suggested that low-power laser radiation (LPLR) be employed as the major or auxiliary instrument for treating malignant diseases. For example, the authors of [5, 6] demonstrated the ability of LPLR to stimulate the synthesis of anti-inflammatory cytokines and to activate NO synthase, which leads to an increased NO production by immune cells.

Today, there exists a large body of evidence corroborating successful employment of laser light in photodynamic therapy and adoptive immunotherapy of tumors. However, a substantiated method of selecting individual laser irradiation doses in laser therapy is still missing. It is believed to have been proven that thera-

Abbreviations: NKCs, natural killer cells; NO, nitric oxide; TNF, tumor necrosis factor; IL2, interleukin 2; LPLR, low-power laser radiation; LPS, lipopolysaccharide; FCS, fetal calf serum; HSP, heat shock protein.

peutic doses leading to activation of blood microcirculation do not exceed 10 J/cm^2. However, the biological effects of extremely low doses of laser light, which are tens and even hundreds of times lower than the recommended therapeutic doses, are insufficiently studied. In addition, there is still no comprehensive notion on the immune status of patients with malignant diseases who undergo long-term laser therapy.

Earlier, we discovered that the low-power red light of a He–Ne laser had a marked stimulatory effect on the production of TNF-α, IL-2, interferon γ, interleukin 6, and NO by isolated murine immunocompetent cells exposed to radiation [7]. The goal of this study was to investigate in vivo the effect of LPLR on mice bearing solid forms of Ehrlich ascite carcinoma, when different skin areas are irradiated. In the first case, the right hind limb at the tumor projection area was irradiated; in the second, the projection area of the thymus—one of the key organs of the immune system.

EXPERIMENTAL

Animals. The study was performed with mature male outbred NMRI mice weighing 20–25 g. This strain was a kind gift from Charles River Laboratory to the Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry (Pushchino Branch), Russian Academy of Sciences, and is kept at its nursery. During the entire exposure period, the animals were given food and water ad libitum. All measurements were performed individually for each mouse.

Tumor transplantation. The model of a solid tumor was formed by subcutaneously transplanting Ehrlich ascite carcinoma cells into the right hind limb of animals (2×10^5 cells in 0.2 ml saline per mouse).

Irradiation. An LGN-111 He–Ne laser operating at 632.8 nm was used as a source of LPLR. The incident radiation power was 0.2 mW/cm^2. Different skin areas—the tumor projection area and the thymus projection area—were irradiated through an optic fiber; prior to irradiation, these areas were depilated. During the irradiation session, animals were immobilized and shielded by white thick paper with a hole 1 cm in diameter over the area to be exposed. The animals housed under the same conditions and subjected to the same procedures, except for laser irradiation, served as a control. Irradiation sessions were performed in the morning at 20–22°C.

Immunological parameters were determined 12, 24, and 48 h after a single exposure to LPLR for 1 min (dose, 0.012 J/cm^2).

In the case of chronic irradiation, the animals were exposed to laser light for 1 min every 48 h for 30 days. Immunological parameters were determined on days 10, 19, and 31 from the beginning of the experiment (i.e., 48 h after the 3rd, 6th, and 9th exposure). The summary doses absorbed by irradiated animals during the time intervals specified were 0.036, 0.072, and 0.1 J/cm^2, respectively.

Isolation of immunocompetent cells. The animals were decapitated and all subsequent procedures were performed under sterile conditions in the cold. Macrophages were isolated from the peritoneal fluid as described in [8]. Peritoneal cells were pelleted, then washed three times in RPMI-1640 medium supplemented with 2 mM glutamine, gentamycin, and HEPES. The cells were counted after their staining with a mixture of 0.1% trypan blue and 0.1% eosin. The cells (1.5×10^6 cells/ml) were suspended in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), placed into 24-well plates (1 ml per well), and incubated in a 5% CO_2 atmosphere at 37°C for 1 h.

To isolate T-lymphocytes, the spleen was ground and the cells were suspended in DMEM medium (Sigma, United States) supplemented with 1 M HEPES (Serva, Germany) and 0.05% gentamycin. Washed cells were treated with a hemolytic solution containing 0.85% NH_4Cl and 0.01 M Tris–HCl (9 : 1) in 0.15 M NaCl (pH 7.2). Then, the cells were washed three times, placed in plastic Petri dishes, and cultured in a 5% CO_2 atmosphere at 37°C for 2 h to remove macrophages. Nonadhered cells were collected and counted in a Goryaev cell-counting chamber.

Determination of TNF. Macrophages or splenocytes (1.5×10^6 cells/ml) were resuspended in 1 ml of RPMI-1640 medium supplemented with 2 mM *L*-glutamine, gentamycin, HEPES, and 10% FCS. Then, macrophages were incubated in a 24-well plate in 5% CO_2 atmosphere at 37°C for 24 h. After incubation, the cells were lysed by freezing–thawing three times and stored at –20°C until determination of TNF concentration. The blood collected during decapitation was incubated at 4°C for several hours and centrifuged at a low speed (1000 rpm) for 20 min. The supernatant was transferred into glass tubes and stored for 1 day at 4°C. TNF concentration was measured using freshly prepared blood plasma. The concentration of TNF was determined by assessing the cytotoxic effect of samples (lysates of macrophages and splenocytes and blood plasma) on L929 target cells as described earlier [8]. The test for cytotoxicity was performed individually for each mouse in three independent experiments. For each sample, measurements were performed in nine to twelve replicates. In addition, the level of synthesis of TNF-α by murine macrophages was determined with the use of mouse TNF-α ELISA kits (OptEIA, Unites States).

Determination of NO concentration. Peritoneal macrophages were washed from the satellite cells with saline. The monolayer of macrophages that formed in each well of the plate was resuspended in 1 ml of DMEM medium supplemented with 1 mM sodium pyruvate, 2 mM *L*-glutamine, gentamycin, 15 mM HEPES, and 5% FCS, which contained no red indicator. To simulate macrophages, lipopolysaccharide (LPS

(Sigma, United States)) was added to the culture medium (0.5 μg per 10^6 cells). The activated macrophages were incubated in a 24-well plate in a 5% CO_2 atmosphere at 37°C for 19–21 h. The concentration of NO was determined by the concentration of nitrites, which are the final metabolic products of the short-lived compound NO [9, 10].

Determination of interleukin concentration. Splenocytes (1.5 × 10 cells/ml) were resuspended in RPMI-1640 medium supplemented with 2 mM *L*-glutamine, gentamycin, 25 mM HEPES, 5×10^{-5} M β-mercaptoethanol, and 10% FCS and placed in a 24-well plated for culturing. To stimulate lymphocytes, the culture medium was supplemented with 0.5 μg/ml phytohemagglutinin P (Difco Laboratories, United States), and the mixture was incubated in 5% CO_2 at 37°C for 72 h. The three-day-old culture of activated splenocytes was transferred from the plate to tubes and centrifuged at 3500 rpm for 10 min. Freshly collected supernatant was used to determine the concentration of interleukins (IL-2 and IL-3) by enzyme immunoassay using polyclonal rabbit antimouse antibodies to IL-2 and IL-3 (PeproTech, United States) as primary antibodies, antirabbit goat IgG conjugated with biotin (StessGen, United States) as secondary antibodies, and a complex containing biotin conjugated with horseradish peroxidase (IMTEK, Russia). Staining was performed using ABTS solution (Sigma, United States) in 0.05 M citrate buffer (pH 5.0) containing 0.01% H_2O_2. Optical density was measured at 405 nm with a spectrophotometer for plate reading.

Preparation of samples for electrophoresis. Lymphocytes (20×10^6 cells) were lysed by sonication with an ultrasound disintegrator on ice under constant stirring for 2 min. Then, cell membranes were pelleted by centrifuging them two times at 2000*g* for 5 min; smaller particles were pelleted by ultracentrifugation at 171000*g* for 40 min. Total protein concentration was determined according to Bradford [11] using a commercially available Bradford solution (Sigma, United States). Proteins were precipitated with acetone and mixed with a 2X solubilizing buffer containing 50 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate, 25% glycerol, 5% β-mercaptoethanol, and 0.1% bromphenol blue (ratio, 1 : 1). The samples were boiled for 5 min and stored at –20°C. Samples (final protein concentration, 1 mg/ml) were loaded into slots at 10 μl per slot. The commercially available HSP 72 (Sigma, United States) in solubilizing buffer was used as a marker and added in an amount of 0.5 μg.

Electrophoresis. The presence of heat shock proteins in samples was detected by PAGE in 10% polyacrylamide gel at a voltage of ~100 V in the concentrating gel and ~140 V in the separating gel. Electrophoresis was performed in a Tris–glycine buffer containing 25 mM Tris–HCl (pH 8.3), 0.1% sodium dodecyl sulfate, and 250 mM glutamine. Gels were stained with 0.25% Coomassie Brilliant Blue R-250 (Sigma, United States) in 5% acetic acid. The specificity of analysis was confirmed by immunoblotting.

Transfer of proteins and immunoblotting. The proteins were transferred from gel onto a nitrocellulose membrane using a transblot chamber filled with a transfer buffer containing 47.9 mM Tris–HCl (pH 7.4), 0.1% sodium dodecyl sulfate, 38.6 mM glutamine, and 20% methanol for 1 h at 350 mA. Then, the membrane with transferred proteins was incubated at 4°C overnight in a blocking buffer containing 10 mM phosphate buffer saline (pH 7.4), 2% dry milk, and 0.5% Tween-20. After blocking, monoclonal mouse antibodies to HSP 70 (HSP 72, clone SPA-812, StressGen Biotechnologies) (inducible form) and HSP 90α (HSP, clone SPA-828, StressGen Biotechnologies) were added to the membrane, which were diluted into the blocking buffer to a concentration of 0.01 mg/ml, and the membrane was incubated in the antibody-containing solution at 37°C for 2.5 h under constant stirring. Thereafter, the membrane was incubated for 1 h under the same conditions in a solution of antispecies antibodies IgG conjugated with biotin (goat polyclonal antirabbit antibodies conjugated with biotin, StressGen Biotechnologies; 1 : 500000 in phosphate buffer) and then with a complex containing streptavidin and horseradish peroxidase (Imtek, Russia; 1 : 300000 in 10 mM phosphate buffer saline). Determination was performed in an ECL system (Amersham, Sweden). At each stage, the membrane was washed repeatedly with phosphate buffer saline containing 0.05% Tween 20. The content of HSPs in cells was determined by comparing the staining intensities of the bands corresponding to the HSPs of interest and the bands corresponding to the purified HSPs, which were titered prior to electrophoresis. The proteins were quantified using Quapa software.

RESULTS

Characteristics of the malignant growth model. This study was performed with the mice bearing the solid form of Ehrlich ascite carcinoma. The average lifespan of animals in this case was approximately 55 days (Fig. 1, curve *1*). The results of assaying the activity of lymphoid cells in the organisms of tumor-bearing mice showed that the expression and direction of the immune responses to solid-tumor growth depend on the stage of disease. For example, the initial stage was characterized by a marked "mobilization" of immune responses, which manifested itself as a considerable increase in the TNF production by macrophages and splenocytes and an increase in the concentration of this cytokine in the blood serum (Fig. 2b). However, the concentration of IL-2 in the supernatants of T-lymphocytes and blood serum of tumor-bearing mice decreased relative to the control group (Fig. 2c). Additionally, splenic cells of tumor-bearing mice showed a high level of expression of HSP 72 and HSP 90 (Figs. 2f, 2g). Note that at the initial stage of neoplastic

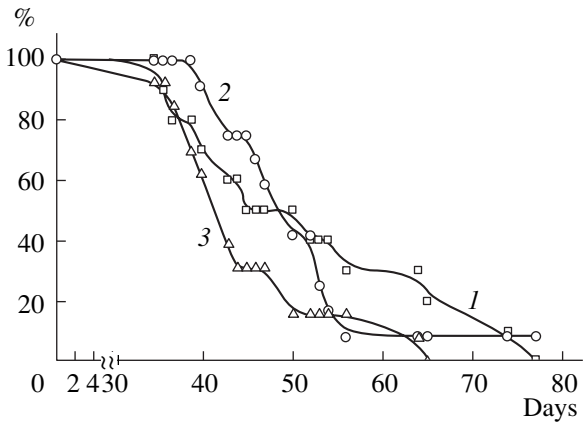

Fig. 1. Effect of LPLR on the time course of death and the average lifespan of tumor-bearing mice after irradiation of the thymus- or tumor projection areas over 30 days. Notations: (*1*) Nonirradiated tumor-bearing animals; (*2*) animals irradiated at the thymus projection area; (*3*) animals irradiated at the tumor projection area. The abscissa axis shows the time elapsed after the injection of tumor cells and beginning of irradiation treatment, days; the ordinate axis, the mice that survived, expressed as a percentage of the total number of animals in the group. The average lifespans (in days) were: (*1*) 54.9 ± 10; (*2*) 51.9 ± 12.4; (*3*) (45.5 ± 9.5)*.

*The differences from the control group (nonirradiated tumor-bearing mice) are statistically significant at $p < 0.06$.

growth, no profound change in the number of lymphoid cells was observed (Fig. 2a).

The next stage of neoplastic growth was characterized by a high TNF concentration in the blood serum (Fig. 2b) and an increased expression of heat shock proteins (Figs. 2f, 2g), as well as an increase in the number of peritoneal macrophages and splenic T-lymphocytes in tumor-bearing mice (Fig. 2a), elevated NKC activity (Fig. 2e), and increased TNF production by splenocytes (Fig. 2b). The level of IL-2 production by splenocytes, the concentration of this cytokine in the blood serum, and the production of TNF by macrophages did not differ from the control (Figs. 2b, 2c). Interestingly, the secretion of NO by macrophages of tumor-bearing mice on day 20 was significantly lower than in the control animals (Fig. 2d).

Finally, 30 days after tumor transplantation, the antitumor responses of mice, mediated by macrophages, diminished, as judged by a drastic drop in NO secretion, a decrease in the concentration peaks of TNF in the blood serum, and a decrease in the TNF-secreting ability of peritoneal macrophages of tumor-bearing mice compared to the initial stage of the disease. At this time, the main antitumor control was apparently exercised via the T-cell component of the immune system at the expense of activation of NKCs, and an increase in the total number of splenic lymphocytes and their ability to produce increased quantities of TNF. However, compared to the previous stages of tumor growth, the level of immune response was low, indicating, along with the decrease in the expression of HSPs by immune cells (Figs. 2f, 2g), a general weakening in the antitumor potential of the organism.

Effect of a single exposure to LPLR. Because the effects of laser light may manifest themselves several hours and even days after irradiation, in preliminary experiments we studied the effects of a single exposure of LPLR in the subsequent two days after irradiation of two different areas of mouse skin. Earlier, we discovered that it was the 1-min exposure to LPLR in vitro that produced the maximum stimulatory effect on cytokine production and NO secretion in immunocompetent cells [7]. According to these data, the activity of NKCs, NO secretion, TNF and IL-2 production, and expression of HSP 70 and HSP 90 in murine immunocompetent cells were evaluated 12, 24, and 48 h after a single irradiation with laser light for 1 min. A group of nonirradiated tumor-bearing mice served as the control. It was shown that irradiation of the thymus projection area caused nonmonotonic changes in NO production in macrophages of irradiated tumor-bearing mice. As seen in Fig. 3, the level of NO secretion increased 12 and 48 h after irradiation but decreased 24 h after irradiation. Interestingly, at the same time interval (i.e., 24 h after irradiation), the production of TNF in macrophages increased twice, whereas 12 and 48 h after exposure it did not differ from the control. The production of IL-2 and TNF in splenocytes of tumor-bearing mice irradiated at the thymus projection area did not differ from the control level. It can be seen that a single exposure of the thymus projection area caused a decrease in HSP 90 and HSP 70 production 12 and 24 h after irradiation; however, 48 h after irradiation, the level of expression of the stress proteins did not differ from the control (Figs. 4a, 4b, lanes *1–4*).

A single irradiation of the tumor projection area also resulted in nonmonotonic fluctuations in NO secretion and TNF production in macrophages, and expression of inducible HSPs in splenocytes. Note that NO secretion decreased 12 h after irradiation of animals and considerably increased 24 h and, especially, 48 h after exposure. The level of TNF production increased 12 h and 24 h after exposure but did not differ from the control 48 h after exposure. The changes in the production of stress proteins in splenocytes of animals irradiated at the tumor projection area were also nonmonotonic: the expression of HSP 70 and HSP 90 significantly decreased 12 and 48 h after irradiation but did not differ from the control 24 h after exposure. Interestingly, in contrast to the model with irradiation of the thymus projection area, stimulation of TNF and IL-2 production in murine splenocytes was observed 48 h after irradiation of the tumor projection area.

It should be emphasized that, against the background of such significant activation of the cytokine system caused by irradiation of both the thymus- and tumor projection area, a drastic suppression of the activity of NKCs was observed 48 h after irradiation,

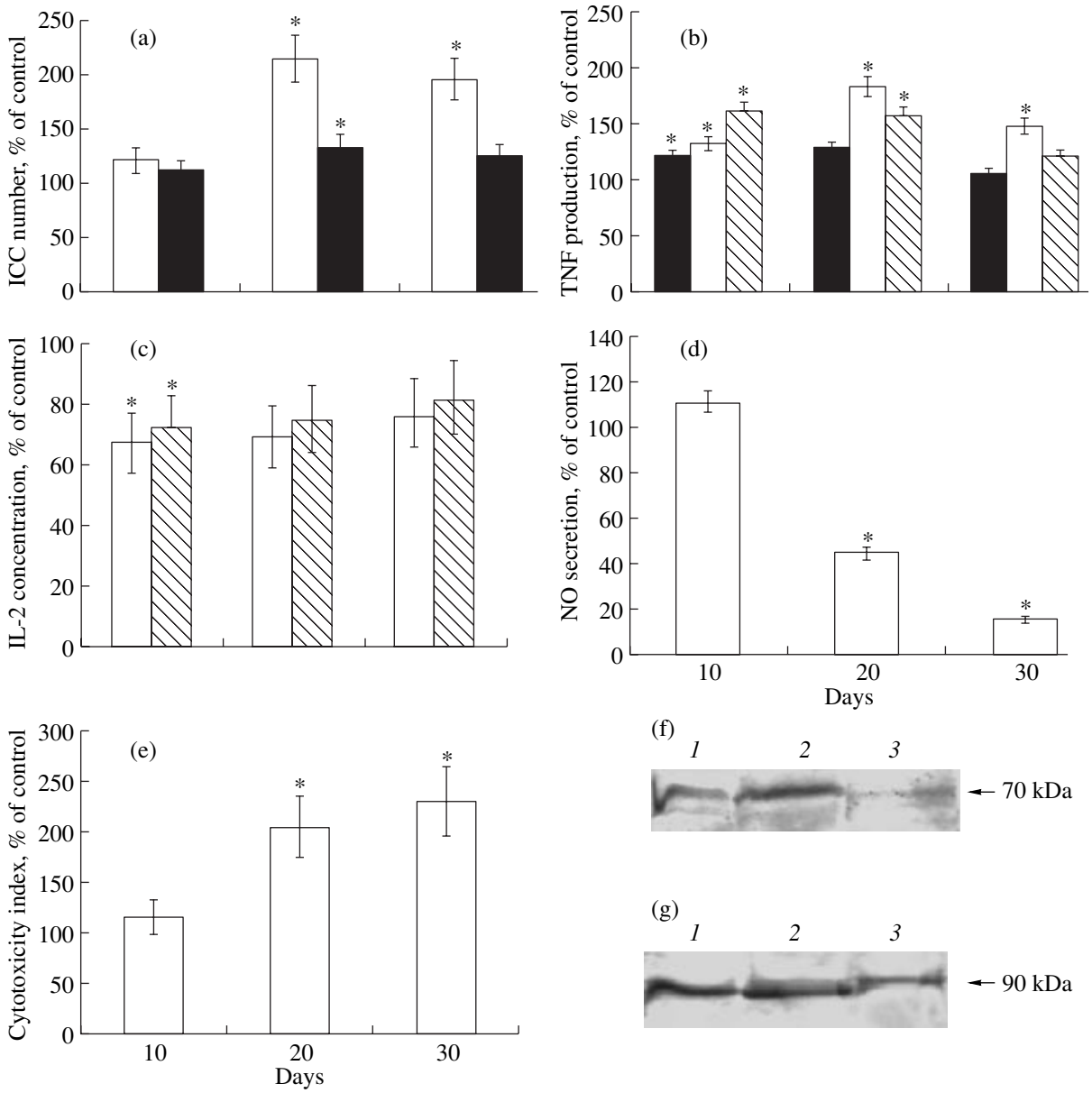

Fig. 2. Effect of transplantation of Ehrlich ascite carcinoma cells on changes in (a) the number of immunocompetent cells (ICC), production of (b) TNF and (c) IL-2, (d) NO secretion, (e) NKC activity, and expression of heat shock proteins (f) HSP 70 and (g) HSP 90 in mice. Notations: open columns, T-lymphocytes; filled columns, macrophages; crosshatched columns, bloods serum. In (f) and (g), numerals *1*, *2*, and *3* denote 10, 20, and 30 days after the injection of tumor cells, respectively. The abscissa axis shows the time elapsed after the injection of tumor cells, days.

* The differences from the control (intact animals) are statistically significant at $p < 0.05$.

indicating a decrease in antitumor defense (Figs. 3a, 3b, column 1).

Thus, it was shown that a single exposure to laser radiation of the thymus projection area or tumor projection area (the hind limb) causes nonmonotonic changes in NO secretion, TNF production, and expression of HSPs in immunocompetent cells of tumor-bearing mice 2 days after irradiation. The most pronounced effects produced by 1-min exposure were observed 48 h after irradiation. Note that the changes in cell activity produced by the irradiation of the tumor projection area had greater amplitude than the changes caused by the irradiation of the thymus projection area. In accordance with these results, in modeling long-term laser therapy, chronic irradiation of tumor-bearing mice was performed every 48 h.

Immunomodulating Effect of Chronic Irradiation

Effect of LPLR on the number of immunocompetent cells. In assessing the effect of a long-term exposure to LPLR on the number of immunocompetent cells in

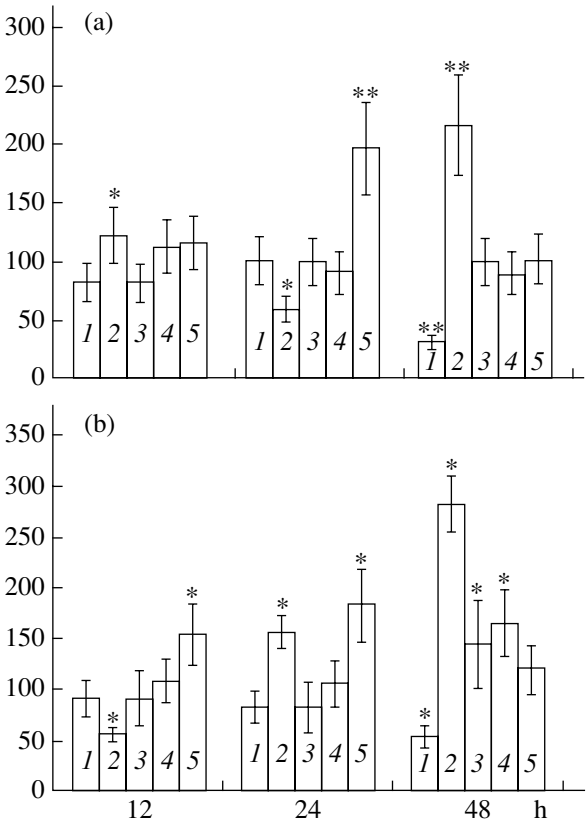

Fig. 3. Effects of a single exposure to LPLR on the immune status of tumor-bearing mice irradiated at (a) the thymus projection area or (b) the tumor projection area. Notations: *1*, NKC activity; *2*, NO secretion by peritoneal macrophages; *3*, IL-2 production by splenocytes; *4*, TNF production by splenocytes; *5*, TNF production by macrophages. The abscissa axis shows the time elapsed after the injection of tumor cells, h; the ordinate axis, % of the control group (nonirradiated tumor-bearing mice).

* The differences from the control are significant at $p < 0.05$.

** The differences from the control are significant at $p < 0.005$.

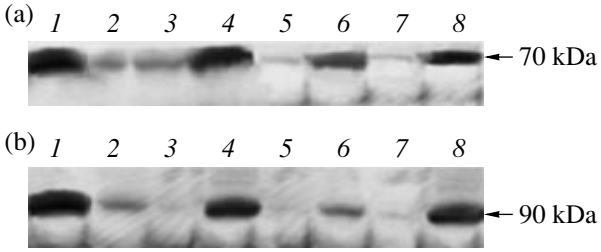

Fig. 4. Effects of a single exposure of LPLR on the production of heat shock proteins (a) HSP 70 and (b) HSP 90 in splenic cells of mice with transplanted Ehrlich ascite carcinoma. Notations: lane *1*, nonirradiated tumor-bearing mice; lane *2*, 12 h after a single irradiation of the tumor projection area; lane *3*, 24 h after a single irradiation of the tumor projection area; lane *4*, 48 h after a single irradiation of the tumor projection area; lane *5*, 12 h after a single irradiation of the thymus projection area; lane *6*, 24 h after a single irradiation of the thymus projection area; lane *7*, 48 h after a single irradiation of the thymus projection area; lanes *8* (a) and *8* (b), HSP 70 and HSP 90, respectively.

tumor-bearing mice, we found that irradiation with laser light primarily caused depopulation of lymphoid tissue in all groups studied (Fig. 5). Macrophages of tumor-bearing mice proved to be the most sensitive to long-term exposure to laser radiation (duration, 20–30 days; summary doses, 0.072 and 0.1 J/cm^2): their number significantly decreased as a result of irradiation of both the thymus- and tumor projection area. Exposure to laser light also caused a decrease in the number of splenic cells of tumor-bearing mice. Irradiation of the thymus projection area resulted in a decrease in the number of splenocytes after 20 days of exposure (summary dose, 0.072 J/cm^2); irradiation of the tumor projection area, after 10 days of exposure (summary dose, 0.036 J/cm^2). Other irradiation doses caused no changes in the number of splenocytes in mice bearing solid tumors.

Effect of LPLR on IL-2 production. Irradiation of tumor-bearing mice both at the thymus- and tumor projection area resulted in an increase in IL-2 concentration in blood serum of tumor-bearing mice; however, this effect was retained throughout the experiment only in the case of exposure of the tumor projection area (Fig. 6). The stimulatory effect of LPLR in the case of irradiation of the thymus projection area was observed only after 10-day exposure. Conversely, a 30-day course of irradiation significantly decreased the concentration of IL-2 in blood serum of irradiated tumor-bearing mice relative to the nonirradiated animals.

Interestingly, an increase in IL-2-producing activity of T-lymphocytes was observed only in the case of repeated irradiation of the tumor projection area. Conversely, repeated irradiation of the thymus projection area suppressed the secretion of this anti-inflammatory cytokine.

Effect of LPLR on TNF production. Data in Fig. 7 illustrate the effect of fractional exposure to laser light on TNF production in immunocompetent cells of tumor-bearing mice. It can be seen that irradiation of the thymus projection area suppressed TNF production by macrophages on day 10 and 20 of the experiment and did not cause any changes in the level of this cytokine synthesized by splenic T-lymphocytes of tumor-bearing mice compared to the group of nonirradiated animals with transplanted carcinoma (Fig. 7a). Laser irradiation of the tumor projection area induced opposite changes in the production of TNF by murine splenocytes and macrophages. An irradiation dose of 0.036 J/cm^2 (day 10 of the experiment) suppressed the TNF-producing activity of macrophages, whereas the level of production of this cytokine by splenocytes increased. Conversely, irradiation for 30 days (dose, 0.1 J/cm^2) suppressed the TNF-producing function of T-cells but had no effect on the concentration of this cytokine in macrophages (Fig. 7b). Irradiation for 10 days (dose, 0.036 J/cm^2) of both the thymus- and tumor pro-

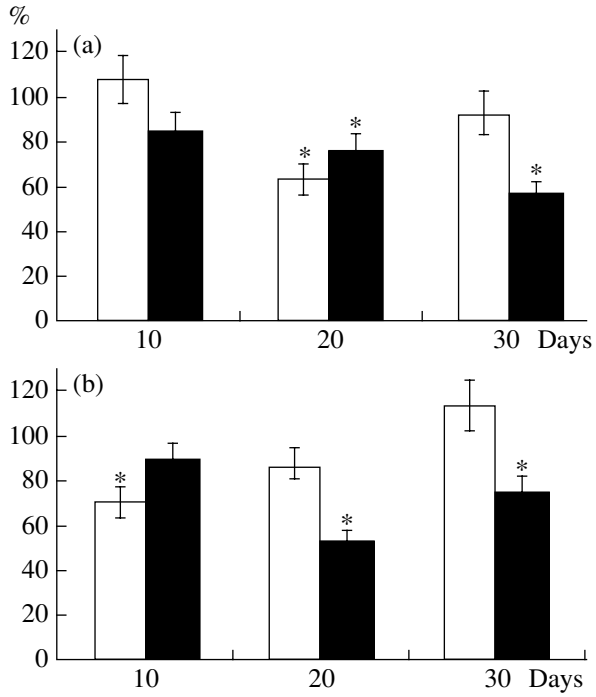

Fig. 5. Effect of LPLR on the number of immunocompetent cells in mice bearing transplanted Ehrlich ascite carcinoma, which were irradiated at (a) the thymus projection area or (b) the tumor projection area. For notations, see Fig. 2. The abscissa axis shows the time elapsed after the injection of tumor cells and beginning of irradiation treatment, days; the ordinate axis shows the number of immunocompetent cells, % of the group of tumor-bearing mice.

*The differences from the group of nonirradiated tumor-bearing mice are significant at $p < 0.05$.

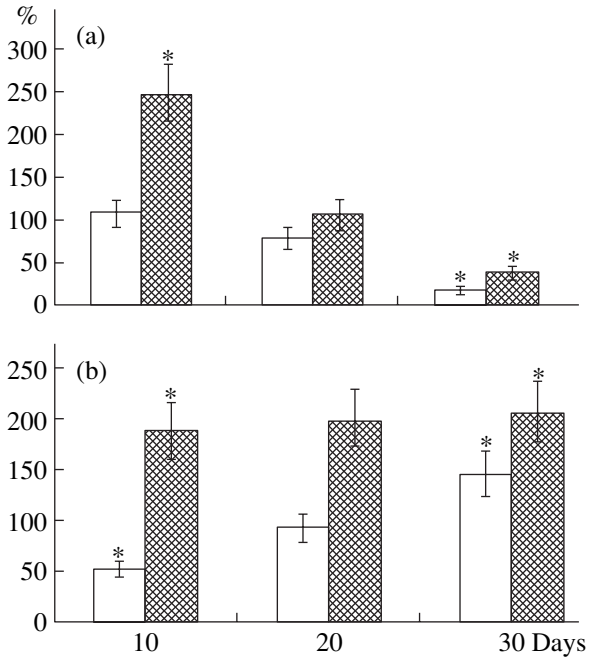

Fig. 6. Effect of LPLR on IL-2 secretion by splenic T-lymphocytes and the cytokine concentration in the blood serum of tumor-bearing mice irradiated at (a) the thymus projection area or (b) the tumor projection area. For notations, see Fig. 2. The abscissa axis shows the time elapsed after the injection of tumor cells and beginning of irradiation treatment, days; the ordinate axis shows IL-2 concentration, % of the group of tumor-bearing mice.

*The differences from the group of nonirradiated tumor-bearing mice are significant at $p < 0.05$.

jection area of tumor-bearing mice caused a significant increase in the concentration of TNF in blood serum (Figs. 7a, 7b, crosshatched columns). However, an increase in the irradiation dose to 0.1 J/cm^2 resulted in a twofold decrease in the amount of this cytokine in blood serum in both irradiation models. Interestingly, the employment of laser light resulted in complex correlation dependences between the total TNF content in blood of tumor-bearing mice and the ability of immune cells to produce this cytokine. The dynamics of changes in the concentration of TNF in blood directly correlated with the changes in the level of production of this cytokine by murine T-lymphocytes (correlation coefficient, 0.773) in the case of irradiation of the tumor projection area, whereas irradiation of the thymus projection area yielded an inverse correlation (correlation coefficient, –0.993) between the concentration of TNF in blood plasma and the production of this cytokine by macrophages of tumor-bearing mice.

Effect of LPLR on the concentration of nitric oxide. As shown in Fig. 8, irradiation of the thymus projection area of tumor-bearing mice with LPLR for 10 days suppressed the NO-producing activity of macrophages. However, on days 20 and 30 of the experiment, opposite effects were observed. For example, at later stages of the experiment, stimulation of nitric oxide production by the peritoneal macrophages of tumor-bearing mice irradiated both at the thymus- and tumor projection area was observed. Interestingly, when the tumor projection area was irradiated, the expression of the stimulatory effect was directly proportional to the absorbed dose (Fig. 8, open columns).

Effect of LPLR on NKC activity. As seen from Fig. 9, irradiation of the thymus projection area of tumor-bearing mice on day 10 of the experiment resulted in a nearly complete suppression of the cytotoxic activity of NKCs; the latter partly recovered on day 20 and did not differ from the control on day 30. A similar pattern was observed under exposure to LPLR of the hind limb of animals with experimental tumors, except for the fact that the decrease in the activity of NKCs on day 10 of laser therapy was not as significant, and on day 30 the cytotoxicity index remained significantly decreased (Fig. 4). At later stages of tumor growth, the activity of NKCs in irradiated animals nearly returned to the level characteristic of nonirradiated tumor-bearing mice.

Effect of LPLR on production of HSPs in immunocompetent cells. Figure 10 shows the results of a study how the irradiation of two different skin areas of tumor-

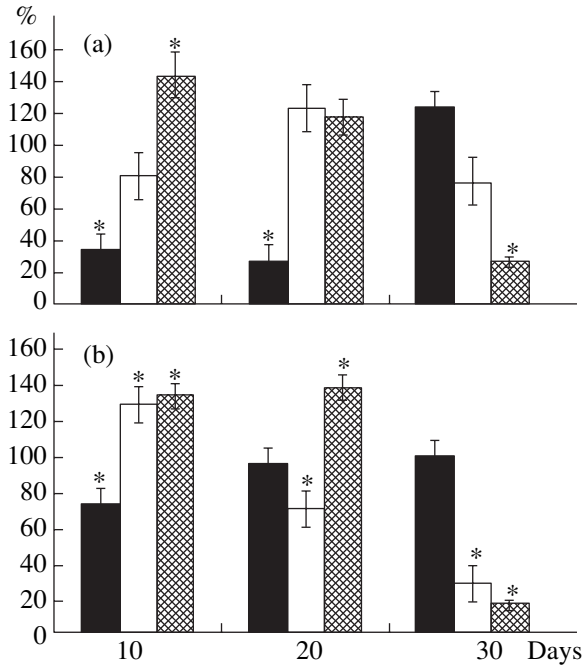

Fig. 7. Effect of LPLR on TNF secretion by immune cells and the cytokine concentration in the blood serum of tumor-bearing mice irradiated at (a) the thymus projection area or (b) the tumor projection area. For notations, see Fig. 2. The abscissa axis shows the time elapsed after the injection of tumor cells and beginning of irradiation treatment, days; the ordinate axis shows TNF concentration, % of the group of tumor-bearing mice.

*The differences from the group of nonirradiated tumor-bearing mice are significant at $p < 0.05$.

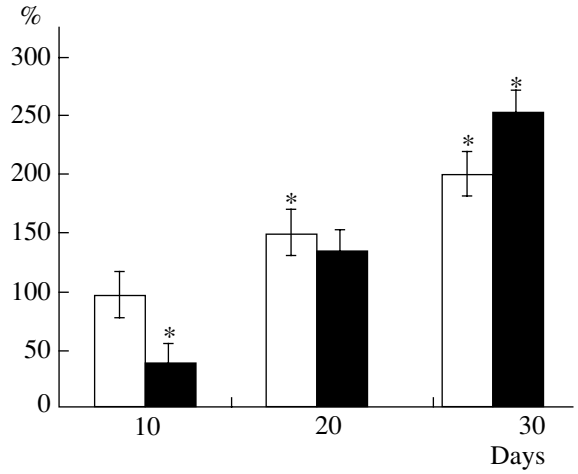

Fig. 8. Effect of LPLR on NO secretion by macrophages of mice bearing Ehrlich ascite carcinoma, which were irradiated at the thymus- or tumor projection area. Notations: open columns, irradiation of the thymus projection area; filled columns, irradiation of the tumor projection area. The abscissa axis shows the time elapsed after the injection of tumor cells and beginning of irradiation treatment, days; the ordinate axis shows NO concentration, % of the group of tumor-bearing mice.

*The differences from the group of nonirradiated tumor-bearing mice are significant at $p < 0.05$.

bearing mice affected the expression of HSP 70 and HSP 90 in splenic lymphocytes. It appeared that splenic cells of tumor-bearing mice irradiated both at the thymus- and tumor projection area on day 10 of the experiment expressed a somewhat lower amount of HSP 70 and HSP 90 compared to the nonirradiated tumor-bearing mice (compare Fig. 10 and Figs. 2f, 2g). However, a more long-term repeated irradiation of the thymus projection area caused no changes in the expression of HSP 70 and HSP 90 in splenic cells of the irradiated tumor-bearing mice compared to the nonirradiated ones.

Effect of LPLR on tumor size and average lifespan of mice bearing Ehrlich ascite carcinoma. Figure 11 shows the changes in the tumor size in mice bearing Ehrlich ascite carcinoma induced by exposure to LPLR of the thymus- or tumor projection area. It can be seen that chronic irradiation of the tumor projection area accelerated tumor growth. Furthermore, the average lifespan of animals irradiated at the tumor projection area tended to decrease compared to the nonirradiated tumor-bearing mice (Fig. 1). Irradiation of the thymus projection area caused no significant changes in the tumor growth rate and average live span of mice with experimental tumors (Figs. 1 and 11, curves *2*).

DISCUSSION

The effect of extremely-low-power laser light on the immune status of mice bearing solid forms of Ehrlich ascite carcinoma was studied. The general state of the animal's organism was assessed taking into account the concentration of cytokines in blood serum. In this study, a solid-tumor model was used, when the average lifespan of tumor-bearing animals was 55 days, with the maximum lifespan reaching 77 days, as opposed to the rapid process in the case of the ascites model, when animals die within 10 to 12 days. The model of tumor growth used was characterized by pronounced natural antitumor responses of the animal on day 20 of neoplasm development, which were expressed as a stimulation of TNF production by splenocytes, an increase in the concentration of this cytokine in blood serum, and an increase in NKC activity, against the background of the general suppressive effect of the tumor on the organism. For example, neoplastic growth considerably suppressed the secretion of TNF and NO by macrophages and secretion of IL-2 by T-lymphocytes and stopped the increase in the number of macrophages observed at the initial stage of disease. In addition, at the initial stage of tumor development, increased HSP 70 and HSP 90 production was observed at the initial stage of tumor development in the cells of nonneoplastic tissue, which then decreased at later stages of neoplastic growth.

The results of our work demonstrated a significant modulating effect of LPLR on the functioning of the

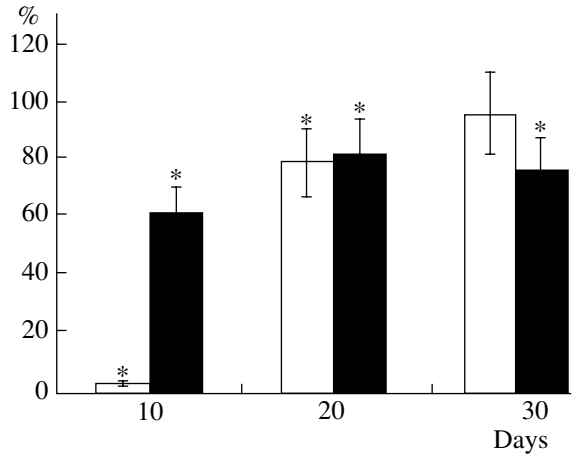

Fig. 9. Effect of LPLR on the activity of NKCs in mice bearing Ehrlich ascite carcinoma, which were irradiated at the thymus- or tumor projection area. For notations, see Fig. 8. The abscissa axis shows the time elapsed after the injection of tumor cells and beginning of irradiation treatment, days; the ordinate axis shows NKC activity, % of the group of tumor-bearing mice.

*The differences from the group of nonirradiated tumor-bearing mice are significant at $p < 0.05$.

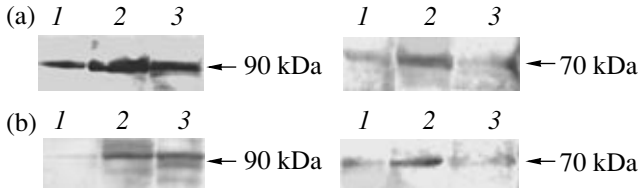

Fig. 10. Effect of LPLR on production of HSP 70 and HSP 90 by splenic cells of mice bearing Ehrlich ascite carcinoma, which were irradiated at (a) the thymus projection area or (b) the tumor projection area. Numerals *1*, *2*, and *3* denote 10, 20, and 30 days after the injection of tumor cells, respectively.

immune system of irradiated mice bearing Ehrlich ascite carcinoma, with the dependence of effects on the absorbed dose having a complex pattern. However, despite a slight activation of the immune system of tumor-bearing mice after a single exposure, repeated irradiation with LPLR, irrespective of the location of the irradiated area, did not increase the antitumor potential of the organism.

Although it was reported earlier that low-power laser irradiation can have a stimulatory effect on the thymus and lymph nodes [12], in our experiments irradiation of the thymus projection area in tumor-bearing mice caused a decrease in TNF production and NKC activity on days 10 and 20 and a decrease in TNF concentration in blood serum and suppression of IL-2 secretion on day 30. Despite a slight stimulation of TNF production and NKC activity at later stages of carcinogenesis, the organisms of tumor-bearing mice were so weakened by this time that these changes had no marked effect on the effectiveness of antitumor defense. Furthermore, irradiation of the tumor projection area resulted in a profound suppression of nearly all components of anticancer defense at the initial stages of carcinogenesis, when an adequate antitumor response is crucial for protecting the organism from the pathogenic action of malignant growth. For example, on day 10 of the experiment, irradiation of the tumor at the site of its location significantly decreased the secretion of NO and TNF by macrophages, suppressed IL-2 production and expression of HSP 70 and HSP 90 in splenocytes, decreased NKC activity, and reduced the total number of splenic cells. After a 30-day course of irradiation with LPLR, the state of antitumor defense in the organism was suppressed as well. By this time, TNF production in splenocytes and the total concentration of this cytokine in blood serum significantly decreased, the activity of NKCs remained low, and the level of NO secretion by macrophages sharply increased, which is known to facilitate tumor progression at the late stages of disease [13, 14]. Such a dramatic inhibition of antitumor resistance under chronic exposure of the tumor projection area to LPLR accelerated tumor growth and decreased the lifespan of tumor-bearing mice.

Thus, the results of this study do not provide grounds for optimistic prognosis of employment of LPLR as an antitumor tool. Nevertheless, combination of laser light with other methods yields good results. For example, LPLR is used for treating various oncological diseases as a tool of photodynamic therapy [15–18], adop-

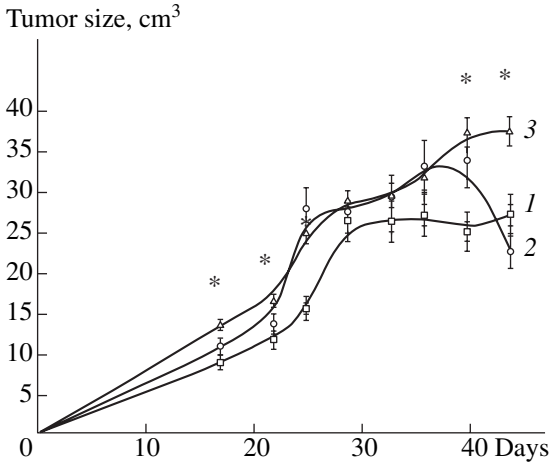

Fig. 11. Effect of LPLR on the changes in the tumor size in tumor-bearing mice irradiated at the thymus- or tumor projection area. Notations: (*1*), Nonirradiated tumor-bearing animals; (*2*) animals irradiated at the thymus projection area; (*3*) animals irradiated at the tumor projection area. The abscissa axis shows the time elapsed after the injection of tumor cells and beginning of irradiation treatment, days; the ordinate axis, shows the tumor size, cm^3.

*The differences from the group of nonirradiated tumor-bearing mice are significant at $p < 0.05$.

tive laser immunotherapy [15, 19], or extracorporal laser irradiation of a patient's blood.

Photodynamic therapy of tumors is based on the action of laser light on the tumor provided that specific photosensitizers are injected immediately into the tumor tissue. This method makes it possible to increase the antitumor immunity of the organism, primarily at the expense of an increase in tumor immunogenicity. Adoptive photoimmune therapy is based on extracorporal irradiation of packed white cells isolated from patient's blood and its subsequent reinfusion into the bloodstream. Another approach to treating oncological diseases with the use of lasers is endovascular laser irradiation of blood [20–23]. Unfortunately, all proposed methods are too complex and may lead to complications; in addition, long-term and uncontrolled irradiation with LPLR, as was noted long ago, may lead to suppression of immunity and development of immunodepressive conditions [19, 24, 25].

Thus, our results showed that long-term chronic irradiation of the skin of mice bearing solid tumors with extremely-low-intensity light of a He–Ne laser at the thymus projection area and especially in the tumor projection area decreases the natural antitumor potential, which enables tumor progression and tends to cause a decrease in the average lifespan of tumor-bearing mice. Nevertheless, a short-term single exposure to LPLR results in the stimulation of antitumor immunity for several days after irradiation. This indicates that further studies in this field are promising and should include both investigation of the primary mechanisms of interaction of laser light with a biological system and optimization of the conditions and parameters of laser therapy. In general, the results of this study, together with the published data, show the expedience of further study of the immunomodulating effects of low-power laser light and the necessity of monitoring the immune system in using any type of laser therapy.

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