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CELL BIOPHYSICS

Effects of Weak Laser Radiation (632.8 nm) on Isolated Mouse Immune Cells

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Abstract—The dose dependence of in vitro effects of low-intensity radiation of a He–Ne laser (632.8 nm, 0.2 mW/cm^2) on the functional activity of peritoneal macrophages and lymphocytes of mouse spleen was studied. The exposure of isolated cells was varied from 5 to 180 s. If the exposure did not exceed 60 s, stimulation of secretory activity was observed: increased production of interleukin 2, interferon γ, and interleukin 6 in lymphocytes; increased production of tumor necrosis factor α, nitric oxide, and interleukin 6 in macrophages; and enhanced activity of natural killer cells. A longer exposure (up to 180 s) either had no effect on the synthesis of certain cytokines (interleukin 2 in lymphocytes and interleukin 6 in macrophages) or inhibited it, which was expressed in decreased production of interleukin 6 and interferon γ in lymphocytes and nitric oxide in macrophages, as well as in suppression of the activity of natural killer cells. Conversely, the production of interleukin 3 decreased after a short-term exposure but increased after 180-s irradiation. The high sensitivity of cells to extremely weak laser light also manifested itself as a considerable increase in expression of the inducible heat shock protein 70; this effect was observed at all doses studied, including the 5-s exposure. In contrast, expression of the heat shock protein 90 slightly decreased after irradiation of cells with laser light.

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Key words: extremely weak laser radiation, He–Ne laser, tumor necrosis factor α, heat shock protein 70, heat shock protein 90, interleukin 2, interleukin 3, interleukin 6, interferon γ, nitric oxide, natural killer cells

INTRODUCTION

It is known that the priority in developing laser technology and studying the biological effects of laser radiation belongs to Russian scientists [1–6]. In the years that passed after the creation of laser, the primary mechanisms of interaction of laser light with living matter have been studied. The greatest contribution to solving this problem was made by the representatives of the school of thought of Yu.V. Vladimirov [7, 8].

A large number of studies of the biological effects of laser radiation have been performed with the use of He–Ne lasers with a wavelength of 632.8 nm; in these studies, endogenous porphyrins and flavonoids were regarded as the primary red-light chromophores [2, 9]. The authors of some papers provided convincing evidence for the immunostimulatory activity of weak laser irradiation [10, 11]. These properties of laser light determined, to a certain extent, the development of laser therapy, which received wide acceptance in various areas of clinical practice [12], including photodynamic therapy for malignant neoplasms [13]. However, although laser therapy is widely used today, there is yet no generally accepted

Abbreviations: TNF, tumor necrosis factor; IFN, interferon, HSP, heat shock protein; NK, natural killer.

concept regarding the primary mechanisms of interaction of laser light with biological objects. In addition, there are problems with establishing a safety level of laser radiation power used in clinical practice. There is no doubt that high doses of laser radiation, used to produce heat effects, cause immunosuppression. For example, it was shown that high-power laser radiation at a dose of 37.8 J/cm^2 drastically suppressed the activity of immunocompetent cells exposed in vivo, which was expressed in diminished production of tumor necrosis factor (TNF), interferon γ (IFN-γ), and interleukin 2 (IL-2) [14]. Today it is commonly believed that laser radiation doses used for therapeutic purposes should not exceed 10 J/cm^2 [2, 7]. However, there are grounds to assume that, in some cases, the recommended therapeutic doses may also cause detrimental side effects associated with the high sensitivity of immune cells to external effects. For example, we have shown recently that fractionated irradiation of limited skin areas of mice with a He–Ne laser (total dose, 0.1 J/cm^2) considerably suppressed the activity of natural killer (NK) cells and the antitumor immunity of animals with experimental tumors [15].

The main goal of this study was to investigate the dose–effect dependences with respect to production of some cytokines (IL-2, IL-3, IL-6, IFN-γ, and TNF-α) and nitric oxide, the activity of NK cells, and expression of heat shock proteins (HSP 70 and HSP 90) in cells exposed in vitro to extremely weak laser radiation (0.2 mW/cm^2). In our opinion, the demonstration of the sensitivity of immunocompetent cells to laser radiation at doses that are several orders of magnitude lower than the recommended therapeutic doses will help attract greater attention to the problem of safety of laser therapy.

EXPERIMENTAL

Experiments were performed with mature male outbred NMRI mice weighing 20–25 g.

An LGN-111 He–Ne laser operating at 632.8 nm was used as a source of weak laser radiation. The incident radiation power was 0.2 mW/cm^2. Cells were irradiated for 5 to 180 s in plastic culture flasks (in the case of spleen lymphocytes) or in 24-well plastic plates (in the case of macrophages) obtained from Corning Costar (United States). Cell suspensions ($1.5 \cdot 10^6$ cells per ml RPMI 1640 medium) were irradiated through an optic fiber. Nonirradiated cells served as a control.

Isolation of lymphocytes. Mice were sacrificed by cervical dislocation. All further procedures were performed under sterile conditions. The spleen was homogenized in a glass homogenizer, and the homogenate was centrifuged at 1500 rpm for 5 min. After selective hemolysis of erythrocytes in an isotonic solution of ammonium chloride, lymphocytes were washed three times with DMEM.

Isolation of macrophages. Macrophages were isolated from mouse peritoneal exudate. Peritoneal cells were pelleted and washed three times in DMEM medium. Then, cells ($1.5 \cdot 10^6$ cells/ml) were suspended in RPMI-1640 supplemented with gentamycin, HEPES, and 10% fetal calf serum, placed in 24-well plates (1 ml per well), and allowed to incubate at 37°C for 2 h in a 5% CO_2 atmosphere. After incubation, the supernatant was carefully discarded, the adhered cells were washed with RPMI-1640, and the monolayer of macrophages was allowed to incubate in 1 ml of the medium at 37°C for 24 h in a 5% CO_2 atmosphere. Then, the supernatants or cells lysates obtained by three freezing–thawing cycles were assayed.

Determination of production of TNF-α, interleukins, and IFN-γ. Lymphocytes were resuspended in RPMI-1640 supplemented with 1% *L*-glutamine, HEPES, 0.5% gentamycin, $5 \cdot 10^{-5}$ M β-mercaptoethanol (Sigma, United States), and 10% fetal calf serum. Then, lymphocytes ($1.5 \cdot 10^6$ cells/ml) were incubated at 37°C for 72 h in 24-well plates in a 5% CO_2 atmosphere. After incubation, the samples were centrifuged, and the supernatants were stored at –20°C.

The concentration of cytokines (IL-2, IL-3, IL-6, and IFN-γ) in the supernatants of lymphocytes or in the lysates of macrophages that were incubated for 24 h (TNF-α, IL-6) was determined using enzyme immunoassay. For this purpose, tested samples were dispensed in a 96-well plate for enzyme immunoassay (100 μl per well) and allowed to incubate at 4°C overnight. Then, a blocking buffer containing PBS (pH 7.4) supplemented with 0.05% Tween 20 and 5% dry milk was added to the plate (200 μl per well) and incubated at 37°C for 60°C. After blocking, primary antibodies (100 μl, 0.5 mg/ml) were added to wells, and the plate was incubated at 37°C for 2.5 h. For this purpose, we used a series of polyclonal rabbit

antimouse antibodies (specifically, antibodies to TNF-α, IL-2, IL-3, IL-6, and IFN-γ). All antibodies and cytokines were from PeproTech (United States). Then, secondary antibodies (antirabbit goat IgG conjugated with biotin; StressGen, Canada) were added (100 μl per well), and the plate was incubated at 37°C for 1 h, whereupon a solution containing horseradish peroxidase-conjugated biotin (IMTEK, Russia) was added. After the incubation with the secondary antibodies, the green substrate solution ABTS (Sigma, United States) in 0.05 M citrate buffer (pH 5.0) containing 0.01% H_2O_2 was added (100 μl per well), and the mixture was allowed to react at room temperature. Staining developed within 10 to 40 min, whereupon the reaction was stopped by the addition of 1.5 mM NaN_3 dissolved in 50 mM citrate buffer (pH 4.0). Optical density was measured at 405 nm in a plate reading spectrophotometer. Each stage of enzyme immunoassay was accompanied by washing the wells with PBS containing 0.05% Tween 20.

Determination of NO concentration. Macrophages ($1 \cdot 10^6$ cells) were resuspended in 1 ml of DMEM without Phenol Red bur supplemented with 1 mM sodium pyruvate, 25 mM HEPES, 2 mM *L*-glutamine, and 3% fetal calf serum, and the mixture was incubated in the presence of lipopolysaccharide (5 μg/ml) at 37°C for 20 h in a 24-well plate in a humid atmosphere of 5% CO_2. The production of NO was determined by the concentration of nitrites–-the final products of its metabolism. The amount of nitrites in supernatants was determined using the Griess reagent containing a mixture of 0.1% *N*-naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid and 1% sulfanilamide in 2.5% phosphoric acid in equal volumes. Samples of supernatants obtained from macrophages cultured for 20 h were placed in a 96-well plate (100 μl per well), whereupon freshly prepared Griess reagent was added (100 μl per well), and the plates were incubated at room temperature for 10 min. Optical density was measured at 546 nm in an MCC/340 Titertek Multiscan plate-reading spectrophotometer (Flow Laboratories, Finland). The calibration curve was obtained using the standard $NaNO_2$ solutions.

Determination of heat shock proteins by SDS-PAGE and immunoblotting. To prepare samples, lymphocytes ($20 \cdot 10^6$ cells), which were incubated for 8 h after treatment, were lysed using an ultrasonic disintegrator; this procedure was performed for 2 min on ice under constant stirring. Cell membranes were pelleted by centrifugation at 2000 *g* for 5 min two times; smaller particles were pelleted by ultracentrifugation at 17 000 *g* for 40 min. The total protein concentration was determined by the method of Bradford using the commercially available Bradford solution (Sigma, United States). Protein was precipitated with acetone and mixed with an equal volume of 2× solubilizing buffer containing 50 mM Tris–HCl, 2% SDS, 25% glycerol, 5% β-mercaptoethanol, and 0.1% Bromphenol Blue (pH 6.8). The samples were boiled for 5 min and stored at –20°C. The final concentration of protein in the samples was 1 mg/ml; each slot contained 10 ml of samples. The commercially available proteins HSP 72 and HSP 90 (0.5 μg; StressGen) in the solubilizing buffer were used as markers. The presence of heat shock proteins in samples was detected by PAGE in 10% polyacrylamide gel at a voltage of ~60 V in the concentrating gel and ~100 V in the separating gel. Electrophoresis was performed in a Tris–glycine buffer (25 mM Tris-HCl, 0.1% SDS, and 250 mM glycine, pH 8.3). The specificity of analysis was verified by 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, 0.1% SDS, 38.6 mM glycine, and 20% methanol (pH 7.4). Then, the membrane with transferred proteins was incubated at 4°C overnight in a blocking buffer containing 10 mM sodium phosphate buffer (pH 7.4), 5% dry milk, and 0.05% Tween 20. After blocking, monoclonal mouse antibodies to one of the heat shock proteins–-either HSP 70 (HSP 72, the inducible form; clone SPA-812, StressGen) or HSP 90 (HSP 90α, clone SPA-828, StressGen)–-were added. The membrane was incubated in the antibody-containing solution at 37°C for 1 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; titer 1:300 000 in phosphate buffer) and then with a complex containing streptavidin and horseradish peroxidase (Imtek, Russia; 1:100 000 in 10 mM sodium phosphate buffer). Determination was performed in an ECL system (Amersham, Sweden). At each stage, the membrane was washed repeatedly with a sodium phosphate buffer containing 0.05% Tween 20. The content of HSPs in cells of samples was assessed by comparing the staining intensities of the bands corresponding to

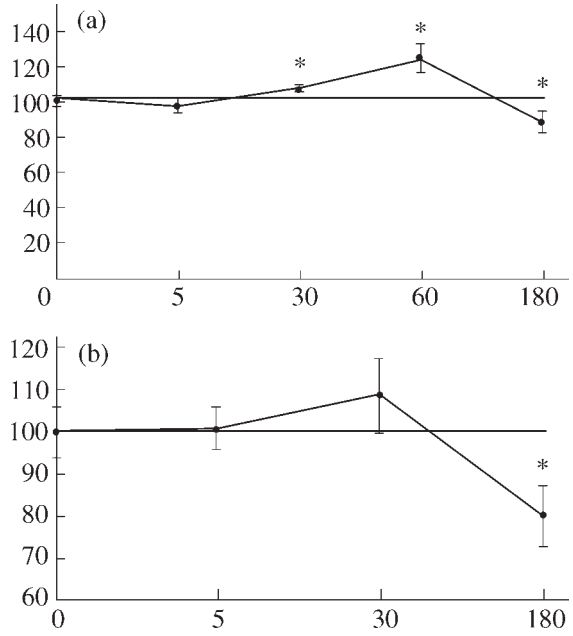

Fig. 1. Effect of weak laser irradiation in vitro on (a) the production of NO by macrophages and (b) the activity of natural killer cells. Each value is the mean of three or four independent experiments; all experiments were performed in 12 replicates. The abscissa axis shows the duration of exposure (s); the ordinate axis shows the percentage of the control (nonirradiated cells). Asterisks mark the values significantly different from the control at $p < 0.05$.

the protein markers and the bands corresponding to the purified HSPs, which were titrated prior to electrophoresis. The proteins were quantitated using the Qapa software.

Determination of NK activity. The activity of NK cells from mouse spleen was determined using [^{3}H]uridine-labeled K562 cells as described earlier [16].

Statistical analysis was performed using the Student's *t*-test.

RESULTS

This study was performed with isolated mouse cells (peritoneal macrophages and spleen lymphocytes), which were irradiated for 5–180 s. The effects of laser light were tested at four doses of incident light: 10^{-3}, $6{\cdot}10^{-3}$, $1.2{\cdot}10^{-2}$, and $3.6{\cdot}10^{-2}$ J/cm^2, which were received by the animals during exposure for 5, 30, 60, and 180 s, respectively.

Determination of NO production by peritoneal exudate macrophages showed that irradiation of isolated cells for 30 and 60 s induced a small yet significant increase in the NO production. Conversely, the NO production in irradiated macrophages was significantly lower than in the control cells when the duration of exposure increased to 180 s (Fig. 1a). As can be seen, a similar dependence of the effect on the irradiation dose was observed when measuring the activity of NK cells . Despite the fact that the suppression of NO production and NK activity as a result of irradiation of cells (macrophages and spleen lymphocytes, respectively) was not very strong, the tendency was obvious–-cell activity was stimulated by smaller doses and suppressed by higher doses of laser irradiation.

The dose dependence of changes in the production of IFN-γ in irradiated macrophages demonstrates this tendency more clearly (Fig. 2b). Indeed, marked stimulation of the production of this cytokine was detected after irradiation with laser light for 5 and 30 s, whereas higher irradiation doses (exposure for 60 or 180 s) significantly suppressed the IFN-γ secretion by macrophages. More profound changes (by a factor of 1.5–2.5) were observed when measuring TNF-α production in exposed macrophages (Fig. 2b). It can be seen that irradiation with laser light activated TNF-α production at all four doses used; however, the degree of activation was much greater with 30-s and 60-s than with 180-s exposure. Importantly, the production of TNF-α and IFN-γ was markedly activated already after 5-s exposure (dose, 10^{-3} J/cm^2), which is indicative of very high sensitivity of the cells to extremely low doses of laser radiation.

Figure 3 shows the changes in the production of IL-6, which was measured in two populations of cells—peritoneal macrophages and spleen lymphocytes. The results of measurements showed that the production of IL-6 in macrophages was stimulated immediately after 5-s exposure, whereas higher doses of laser radiation were not stimulatory (Fig. 3a). Under the same conditions, the production of IL-6 in spleen lymphocytes decreased nearly by half after 60-s exposure, whereas the decrease in the production of this cytokine after 180 s of irradiation was uncertain (Fig. 3b).

The results of changes in the dose dependences for the production of IL-2 showed that the synthesis of this cytokine almost did not change under exposure to laser light; only slight activation of IL-2 secretion was observed after irradiation of lymphocytes for 60 s

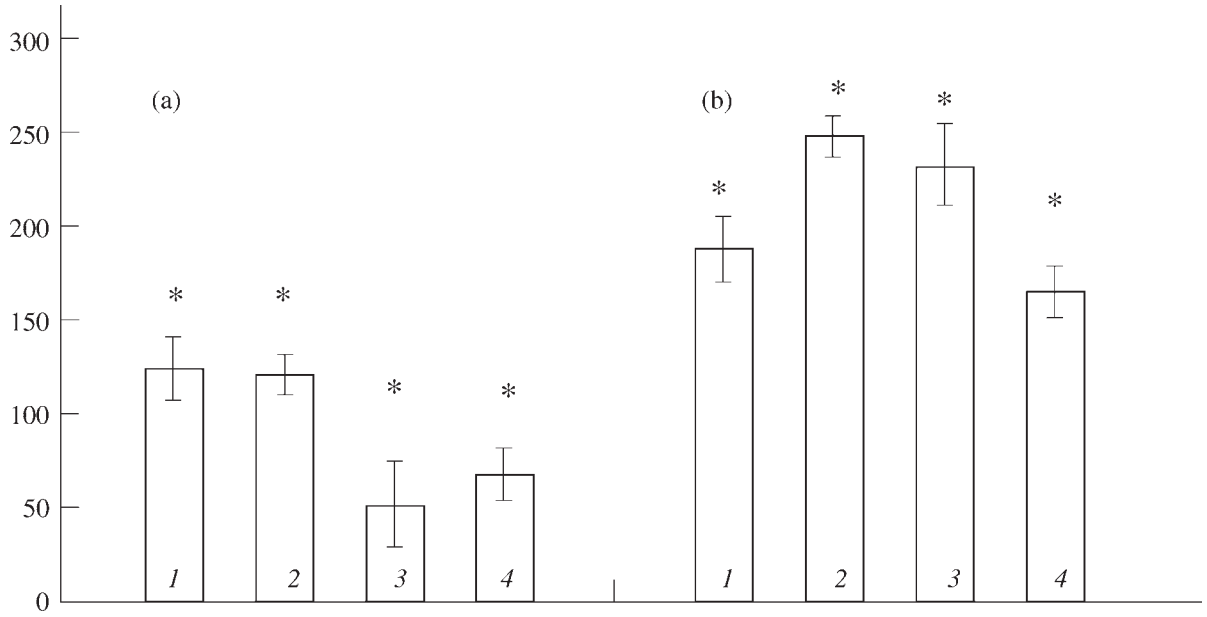

Fig. 2. Effect of weak laser irradiation in vitro on the production of (a) IFN-γ and (b) TNF-α by isolated mouse macrophages. Numerals *1*, *2*, *3*, and *4* designate exposure of cells for 5, 30, 60 and 180 s, respectively. Each value is the mean of three or four independent experiments; all experiments were performed in 12 replicates. the ordinate axis shows the percentage of the control (nonirradiated cells). Asterisks mark the values significantly different from the control at $p < 0.05$.

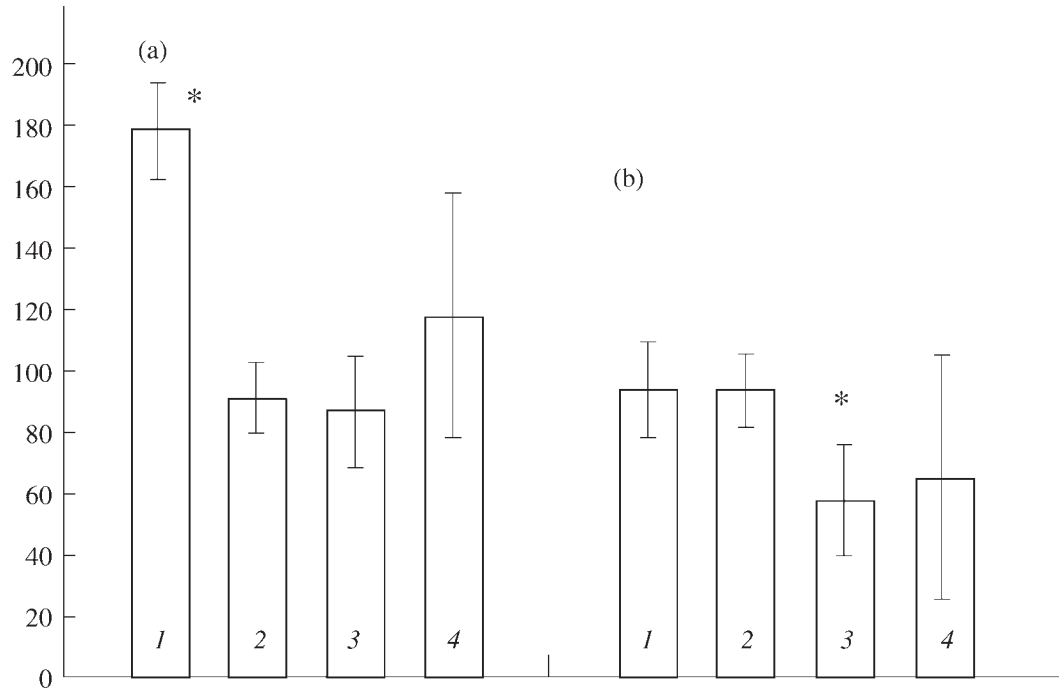

Fig. 3. Effect of weak laser irradiation in vitro on the production of IL-6 by isolated mouse (a) macrophages and (b) lymphocytes. For designations, see Fig. 2.

(Fig. 4a). Interestingly, the pattern of changes in the IL-3 production differed from that for other cytokines. It can be seen that irradiation of cells with lower doses (5- and 30-s exposure) led to a significant (in the case of 30-s exposure) suppression of IL-3 production, whereas a higher irradiation dose (180-s exposure) stimulated the production of this cytokine by a factor of 1.5 (Fig. 4b).

It should be emphasized that the dose dependences of the effect of red laser light in vitro were

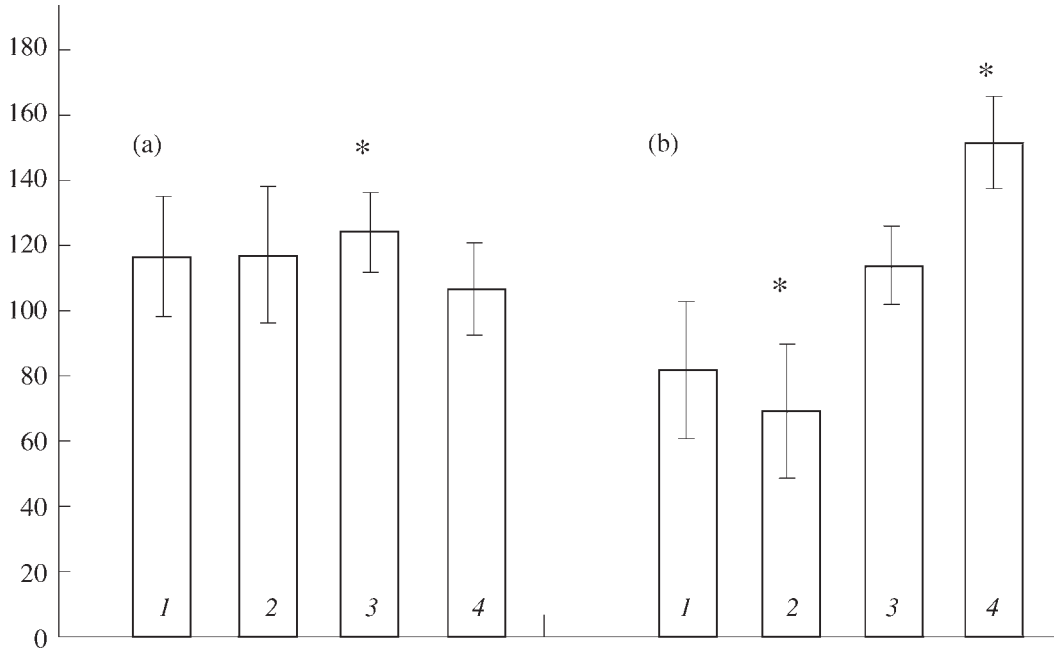

Fig. 4. Effect of weak laser irradiation in vitro on the production of (a) IL-2 and (b) IL-3 by isolated mouse macrophages. For designations, see Fig. 2.

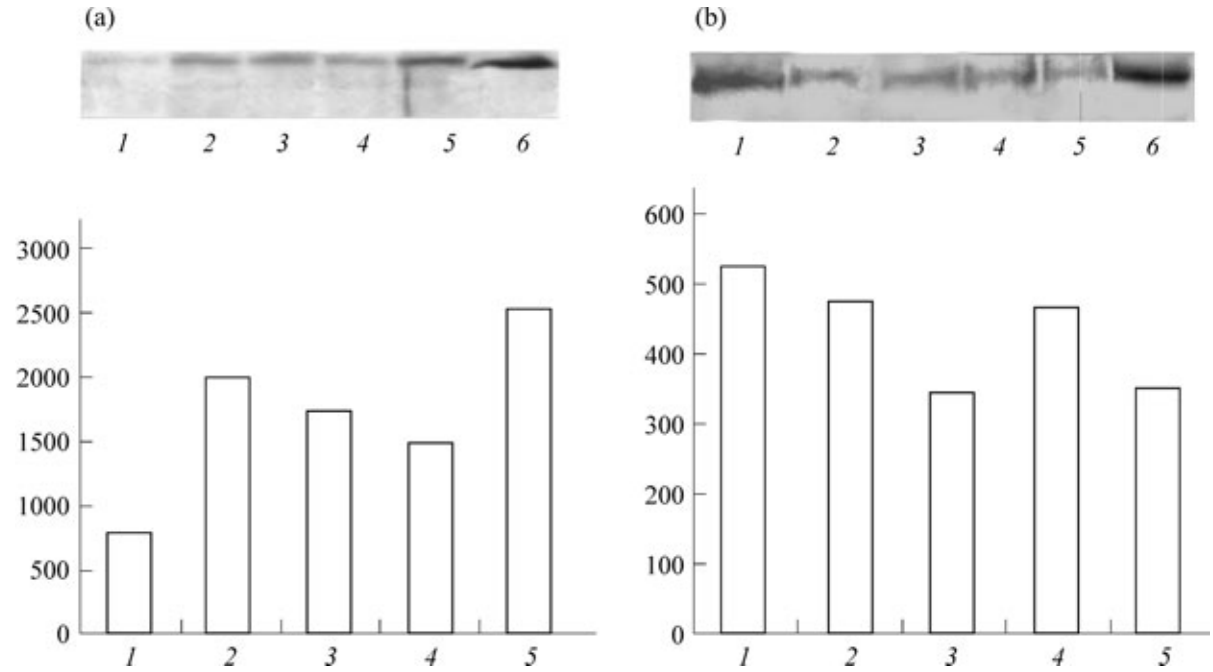

Fig. 5. Effect of weak laser irradiation in vitro on the expression of heat shock proteins (a) HSP 70 and (b) HSP 90 in isolated mouse lymphocytes. Notations: lane *1*, control; lanes *2*, *3*, *4*, and *5*, exposure of cells for 5, 30, 60 and 180 s, respectively; lane *6*, recombinant proteins (a) HSP 70 and (b) HSP 90. The results of quantification of (a) HSP 70 and (b) HSP 90 in samples, obtained using the Qapa software and expressed in arbitrary units, are shown below.

determined in this study in the range of extremely low doses, which could not appreciably increase the temperature in the culture medium. Despite this fact, expression of the heat shock protein HSP 70 in the irradiated lymphocytes was detected (Fig. 5a). It can be seen that the inducible protein HSP 70 was expressed after irradiation at all the four doses used; however, according to the results of measurement of

protein concentration, the highest level of HSP 70 expression was observed when the cells were exposed to laser light for 180 s.

A study of expression of HSP 90, a heat shock protein belonging to another family, did not reveal any marked effects of laser light (Fig. 5b). Furthermore, the results of estimation of the protein content showed that the HSP 90 expression in the exposed cells tended to decrease.

DISCUSSION

In this work, the effect of weak laser radiation was studied in vitro, when isolated cells were irradiated in an aqueous medium. These conditions, in contrast to the effects at the organismal level, when inevitable integrative processes between different structures within the organism take place, made it possible to estimate directly the sensitivity of immune cells to laser light. Taking into account the discrepancy in data regarding the immunomodulating effect of laser light, we determined the dose–effect dependence within quite a broad range of doses of incident light (three dozen times). In addition, the incident beam power was constant, and the irradiation dose was changed by varying the duration of exposure. In this study, the absorbed dose was not assessed; however, with regard for the weak power of monochromatic incident light, we assumed that the absorbed dose was determined by the incident light power.

Without touching on the primary acceptors of He–Ne laser radiation, we assessed the effect of weak laser light on the functional activity of cells irradiated in culture by their secretory and cytotoxic activity. The novelty of this study consisted in the fact that the average radiation power (0.2 mW/cm^2) was several orders of magnitude lower than the values used in the majority of works devoted to applied aspects of laser therapy and investigation of the immunomodulating effect of weak laser light with the use of biological models (in our study, the doses of incident light ranged from 10^{-3} to $3.6\cdot10^{-2}$ J/cm^2).

The results of this study showed that laser radiation at extremely low doses causes significant changes in the production of cytokines, NO, and HSP 70 in the exposed cells. Additionally, under these conditions, the activity of NK cells—a small population of cells exhibiting high cytotoxicity with respect to foreign cells and viruses—also changed significantly. With a small exception, the pattern of dose–effect dependences was similar: the production of cytokines and NO and the NK activity of cells in exposed macrophages increased when the dose of laser light was quite small (10^{-3} or $6\cdot10^{-3}$ J/cm^2, 5- and 30-s exposure, respectively). As the irradiation dose increased, the effect was inverted: the NK activity and the production of NO and some cytokines (IFN-γ and IL-6) was suppressed when the cells were irradiated at doses $12\cdot10^{-3}$ and/or $36\cdot10^{-3}$ J/cm^2 (60- and 180-s exposure, respectively). However, the production of TNF-α was stimulated at all irradiation doses used.

Note that Klebanov et al. [17] demonstrated the stimulatory effects of laser irradiation in vitro on the production of cytokines and NO in human blood cells and in rat macrophages. In the dose range from 0.1 to 0.6 J/cm^2 and at the same wavelength that we used in this study, they also observed nonmonotonic dose–effect dependences. However, the doses they used [17] were two orders of magnitude higher than in our study. This similarity of dose effects of weak laser irradiation in two different dose ranges on cytokine production is suggestive of a polymodal pattern of dose–effect dependences under these conditions. The general principles of occurrence of such unique dose–effect relationships under exposure to extremely weak physical and chemical factors have been formulated by E.B. Burlakova et al. [18].

Returning to the results obtained in this study, we can state that the demonstrated stimulation of the key components of cell immunity under exposure to extremely weak doses of laser light and the appearance of the opposite effect (suppression) with an increase in irradiation dose reflect the mechanism of occurrence and subsequent weakening (in the case of long-term exposure) of an adaptive response of lymphoid cells. This pattern makes it possible to conclude that cells exhibit a stress response to extremely weak laser radiation. This was the first study to demonstrate that the weak laser radiation induces the expression of the inducible heat shock protein HSP 70, the main marker of cell stress, which is a direct experimental corroboration of the above conclusion. It is known that the inducible forms of heat shock proteins, which are produced under the influence of many damaging factors, such as high temperature, ionizing and nonionizing radiation, neurotoxins, and heavy metals, are the main intracellular factor of defense against these

impacts. It has been shown recently that HSP 70 is expressed under exposure to high-power laser radiation that causes heat effects [19].

The mechanism of the protective effect of heat shock proteins is based on their chaperone activity, manifested in their ability to protect functional proteins from degradation and polypeptide adhesion. Unlike other chaperons, HSP 70 and other proteins of the HSP family are expressed under stress and perform motor functions in relocating improperly folded proteins, enable folding of proteins, transfer proteins between intracellular components, and facilitate the formation of protein multimers—i.e., are involved in the processes aimed at repairing the protein component of the cell. In addition, it has been established in recent years that heat shock proteins play a pivotal role in the induction of immune response of the organism to viral, microbial, and tumor antigens [20, 21].

This study made it possible to discover the previously unknown fact of involvement of HSP 70 in the cell response to irradiation with weak laser light, which causes no heat effects. It is of interest that in this work we did not detect any marked changes in the expression of another inducible heat shock protein, HSP 90, which is classified into another family. As shown earlier, HSP 90 principally differs from other molecular chaperones in that it exhibits a high affinity for only certain substrates. For example, the majority of substrates of HSP 90 are proteins of the signal transduction system (e.g., receptors of steroid hormones and signal cascade kinases [22]). The fact that the production of HSP 90 is not increased as a result of irradiation indicates that signal proteins are not actively involved in the cell response to extremely weak laser radiation.

To conclude, it can be noted that evidence for a high sensitivity of immune cells to extremely weak laser light has been obtained in this study and that the response of cells to such treatment can be regarded as a stress response. Changes in the production of cytokines and NO and in NK activity depending on the irradiation dose are described by largely similar dose–effect curves. The stress-type response of cells implies that, in the case of prolonged irradiation, the adaptive nature of their response to laser light, consisting in the stimulation of cell activity, may change its sign for the opposite and lead to suppression of their functional activity. This fact points out the necessity of a thorough parallel monitoring of the activity of the main regulatory systems of the organism, including the immune status, in the course of laser therapy. Additionally, further studies on refining the safety standards in using therapeutic doses of laser radiation are required.

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