The effects of low-level laser light irradiation are debatable and the mechanisms of its action are still unclear. This study was conducted to test the effects of low-level laser irradiation on human blood cells: erythrocytes, granulocytes, and lymphocytes. Whole blood obtained by phlebotomy was irradiated at 632.8 nm by using energy fluences 0.6 J/cm². An analysis of blood gases revealed an increase in $PO_2$ and $SaO_2$ ($P<0.001$) in irradiated blood. No shifts in $PCO_2$ and pH were recorded. Spontaneous synthesis of DNA in T and B blood lymphocytes decreased significantly after laser irradiation ($P<0.02$ and $P<0.04$, respectively). Phytohemagglutinin (PHA)-induced proliferation of T cells and SAC proliferation of B cells, expressed as a stimulation index, were statistically higher in the samples of irradiated than in non-irradiated blood ($P<0.01$). Chemiluminescence of fMLP-stimulated granulocytes from irradiated blood increased in comparison with non-irradiated samples ($P<0.001$). No changes of spontaneous and stimulated chemiluminescence kinetics in irradiated samples were observed. These results reveal the influence of photodynamic reactions on the ability of blood to transport oxygen and on immunomodulatory effects on leukocytes.

Key words: chemiluminescence, erythrocytes, granulocytes, laser irradiation, lymphocytes

INTRODUCTION

A wide use of lasers in clinical (wound healing, tissue repair, and vascular restenosis) and experimental medicine requires detailed information on the
mechanisms of their biological effects (1, 2). The emitted laser light is polarized and coherent and may be absorbed by different tissues (3). Tissue biostimulation is only possible if irradiated cells possess molecular photoacceptors that absorb the light and enter into state of excitation triggering intracellular cascade of signals leading to a measurable biological effect (3, 4).

It is generally accepted that the mechanism of laser biostimulation is based on the absorption of monochromatic light by components of the cellular respiratory chain (3, 4). Mitochondrial NADH-dehydrogenase at the beginning of the respiratory chain and cytochrome c oxidase - terminal respiratory-chain oxidase are among of primary cellular photoacceptors. It has also been documented that irradiation of HeLa cells increased DNA synthesis and this effect is regulated by ATP and redox-sensitive transcription factors NF-κB (3, 4). NADPH-oxidase is responsible for non-mitochondrial respiratory burst of phagocytic cells. This enzyme constitutes a redox chain that generates reactive oxygen species in response to activation and can react to laser irradiation (3, 4).

Several animal models have shown that low-power laser irradiation modulates immune cell functions, such as cell-mediated hypersensitivity reactions and inflammatory process (3) and induces non-mitochondrial respiratory burst measured by chemiluminescence (4). Laser light also influence human blood cells. Irradiation of red blood cell lysate with a neonium laser (337 nm) induces oxidation of hemoproteins (5). This process shows a strong dependence on the concentration of red blood cell lysate and the dose of radiation. Experiments with deoxygenated red blood cell lysate rule out the involvement of reactive oxygen species and suggest that the process is not a photodynamic reaction (5, 6).

The aim of the present study was to assess the influence of light emitted by a low energy helium-neon (He-Ne) laser on oxidative metabolism of human blood erythrocytes, granulocytes, and on T and B lymphocyte function. Blood granulocytes are the cells of the first line of defense against bacterial infections, and it has been documented that their dysfunction may both predispose to and result from infections (7). T and B lymphocytes are responsible for a specific line of defense against viruses and foreign tissue antigens. As activities of these cells are best expressed in optimal environments that are created by a stable pH and proper O_{2} saturation we also decided to verify the influence of laser irradiation on acid-base and oxygen balance (8).

MATERIAL AND METHODS

Heparinized venous blood (5 ml) was taken by phlebotomy from 15 healthy volunteers. All subjects gave informed consent for participation in the study and the study protocol was approved by a local Ethics Committee. Every sample was divided into two equal parts: laser irradiated and control sample.
Laser irradiation

A 632.8 nm (red light) laser beam from a He-Ne laser (Amber LBK; Poland) was used. The power of a laser on the samples was of 10 mW. During the experiments, the laser beam was delivered to the tubes of blood samples with an irradiation spot of a 5 mm diameter. The fluency rate was 1.5 mW/mm². The 1 minute irradiation applied energy of 0.6 J/cm². Blood samples were irradiated at room temperature (23 ±2°C).

Proliferation of lymphocytes

Mononuclear cells (MNC) from 15 blood samples were isolated by centrifugation on Hypaque 1077 (Sigma Aldrich, Poznan, Poland). After washing in 0.9% NaCl, MNC were suspended in culture medium containing RPMI 1640 with bicarbonate (2g/l) supplemented with 10% FCS, Hepes buffer (10mM), L-glutamine (2mM), 2-mecaptoethanol 5x10⁻⁵M and gentamycin (25µg/ml); all reagents were purchased from Sigma Aldrich (Poznan, Poland). MNC cultures were established in triplicate with 1x10⁵ cells/well in flat bottomed microtiter plates using T cell (phytohemagglutinin (PHA), 10µg/ml) or B cell (S. aureus, Cowan, SAC Pansorbin, Calbiochem; at a final concentration of 1x10⁻⁴) mitogens. After 48-h incubation at 37°C in air supplemented with 5% CO₂ and 95% humidity , ³H-thimidine was added (1µCi/well) and incubation was continued for another 16 h. Radioactivity of incorporated H³-thimidine was counted in a liquid scintillation counter Wallac1409 (Wallac, LKB, Finland). The results were expressed as a mean number of counts per minute (cpm) in control cultures of MNC and in cultures of MNC stimulated with PHA or SAC, before and after laser irradiation. For both groups of results a stimulation index (SI) was calculated, according the formula: SI = (cpm after PHA or SAC stimulation – cpm in control culture)/cpm in control culture.

Chemiluminescence of granulocytes

Chemiluminescence of granulocytes was studied in full blood according to the method described earlier (1). Granulocytes concentration was adjusted to 1000/µl using Hanks medium (Sigma Aldrich, Poznan, Poland). Luminol – dependent chemiluminescence was assessed before and after stimulation with fMLP (10⁻³M). Light emission was measured in scintillation counter (Wallac 1409, LKB, Finland) with an option for chemiluminescence. Results were shown as a number of impulses per minute (imp/min) (Fig. 1).

Blood gases analysis

Blood gases analysis was performed in two heparinized capillaries. One capillary was treated as a control, another was irradiated. Each pair of capillaries was analyzed at the same time. Measurements of baseline partial pressure of carbon dioxide (PCO₂), partial pressure of oxygen (PO₂), bicarbonate concentration HCO₃⁻, bases excess, and oxygen saturation (SaO₂) were performed with a GemPremier 3000 (Instrumentation Lab, USA) which was calibrated daily. All measurements were performed by trained technicians from the central hospital laboratory.

Statistics analysis

A paired t-test and Mann-Whitney non-parametric test were used to evaluate the difference between the irradiated and non-irradiated samples. All statistical analyses were performed with a Statistica package.
RESULTS

At each time point studied, spontaneous chemiluminescence of neutrophils after laser irradiation was significantly higher compared with a control (P<0.01). Addition of fMLP peptide to irradiated blood caused an appreciable increase of the light emitted by granulocytes compared with non-irradiated samples (P<0.001). Fig. 1 illustrates these changes in spontaneous and fMLP-induced chemiluminescence under the influence of laser irradiation.

Fig. 1. The influence of laser irradiation on spontaneous and fMLP-stimulated chemiluminescence of granulocytes. Grey squares - spontaneous chemiluminescence; grey circles - chemiluminescence of cells after laser irradiation; open squares - chemiluminescence stimulated by fMLP; open circles - chemiluminescence stimulated by fMLP after laser irradiation. Black arrows - statistical difference at P<0.001.

Spontaneous synthesis of DNA in T and B blood lymphocytes decreased significantly after laser irradiation (P<0.02 and P<0.04; Fig. 2A and Fig. 3A, respectively). PHA-induced proliferation of T cells, expressed as SI, was statistically higher in the irradiated samples when compared with non-irradiated blood (P<0.01) (Fig. 2B). However, the absolute values (expressed in cpm) of [3H]thymidine uptake in PHA-stimulated lymphocytes showed only an upward tendency in the laser-irradiated samples compared with those in the non-irradiated controls (26881 ±14122 vs. 28161 ±10380 (P<0.06, Mann-Whitney test)).

Fig. 2. The influence of laser irradiation on: A - spontaneous DNA synthesis in T lymphocytes, and B - on PHA-induced T cell proliferation assessed as the stimulation index (SI, see text for explanation).
Spontaneous synthesis of DNA in T and B blood lymphocytes decreased significantly after laser irradiation (P<0.02 and P<0.04; Fig. 2A and Fig. 3A, respectively). PHA-induced proliferation of T cells, expressed as SI, was statistically higher in the irradiated samples when compared with non-irradiated blood (P<0.01) (Fig. 2B). However, the absolute values (expressed in cpm) of [3H]thymidine uptake in PHA-stimulated lymphocytes showed only an upward tendency in the laser-irradiated samples compared with those in the non-irradiated controls (26881 ±14122 vs. 28161 ±10380 (P<0.06, Mann-Whitney test)).

As it is shown in Fig. 3B, [3H]thymidine uptake of B-lymphocytes from the laser-irradiated blood significantly increased compared with that in control blood (P<0.05). In this experiment, the absolute values of [3H]thymidine uptake in SAC-stimulated B lymphocytes were significantly higher after laser irradiation in comparison with the non-irradiated samples (5523 ±6410 vs. 7198 ±9099 P<0.04; Mann-Whitney test).

An analysis of blood gases revealed an increase in the PO2 (31.4 ± 3.7 mmHg in irradiated samples vs. 25.3 ± 3.3 mmHg in control samples; P<0.001) and in the SaO2 (54.6 ± 7.3% in irradiated samples vs. 42.4 ±7.7% in control samples; P<0.001). No appreciable shifts in PCO2 (51.0 ±14.1 mmHg vs. 49.6 ±9.1 mmHg) or in pH (7.34 ± 0.05 vs. 7.35 ±0.03) in laser-irradiated vs. non-irradiated blood samples were recorded. These results are presented in Fig. 4.

**DISCUSSION**

Exposure to laser light is widely applied in therapy. A commonly used source of light is a He-Ne laser with radiation at 632.8 nm (red light). The main reason for using the lasers radiating in the red and near infrared spectral regions is the fact that hemoglobin does not absorb light in this region and thus light can penetrate deeply into living tissue (3). Low-energy laser is capable of producing so low energy density that all biologic effects are the result of a direct irradiation and not of thermal events (3, 4). In this system, temperature elevation in the irradiated tissue is limited to less than 0.1–0.5°C (3, 4).

In the present study we examined the effects of low-energy He-Ne laser irradiation on three types of primary target blood cells: erythrocytes, granulocytes and lymphocytes. Erythrocytes demonstrated a rapid increase of oxygen saturation of hemoglobin and an increase of oxygen tension in blood. Both PaCO2 and pH showed no significant changes after irradiation. These results reveal the influence of photodynamic reactions on the ability of erythrocytes to transport oxygen. Similar observations were made by Brutinell et al (9). The purpose of their report was to assess changes in arterial blood gases in patients with airway malignancy treated with Nd-YAG laser (9). These results are not fully comparable with ours as the authors used a different type of laser. Nd-YAG laser effects on cells are not only limited to biostimulation, but also include thermal effects on tissues. Moreover, those observations were made in vivo. Many other authors have stated that laser light has a powerful influence on red blood cells. According to Glassberg et al (10) erythrocytes demonstrate rapid, dose-dependent lysis, as determined by release of free

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**Fig. 3.** The influence of laser irradiation on: A - spontaneous DNA synthesis in B lymphocytes, and B - on SAC-induced B cell proliferation assessed as stimulation index (SI, see text for explanation).

**Fig. 4.** Blood gases analysis before and after laser irradiation.
An analysis of blood gases revealed an increase in the PO$_2$ (31.4 ±3.7 mmHg in irradiated samples vs. 25.3 ±3.3 mmHg in control samples; P<0.001) and in the SaO$_2$ (54.6 ±7.3% in irradiated samples vs. 42.4 ±7.7% in control samples; P<0.001). No appreciable shifts in PCO$_2$ (51.0 ±14.1 mmHg vs. 49.6 ±9.1 mmHg) or in pH (7.34 ±0.05 vs. 7.35 ±0.03) in laser-irradiated vs. non-irradiated blood samples were recorded. These results are presented in Fig. 4.

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In the present study, laser irradiation of granulocytes led to increases of both spontaneous and fMLP-stimulated chemiluminescence reactions. Our results are
not in accord with those of Fuyimaki et al (14) who examined the effects of low-
level laser therapy on production of reactive oxygen species by human neutrophils
(14). After irradiation, production of reactive oxygen species by neutrophils was
measured using luminol-dependent chemiluminescence. The CL response of
neutrophils was reduced by laser irradiation at 60 min prior to the stimulation with
opsonized zymosan and calcium ionophore (14). Those differences may result
from different models used. We estimated an immediate reaction to laser light in
response to fMLP, which is a selective granulocyte stimulant. Fumiyaki et al (14)
have measured chemiluminescence 60 min after the stimulation with opsonised
zymosan and in the presence of calcium ionophore. According to many data, the
influence of laser irradiation on oxidative stress metabolites of cells is quite
complicated. It is generally explained by three reactions: (i) photodynamic action
on membranes accompanied by intracellular calcium increase and cell stimulation;
(ii) photoreactivation of superoxide dismutase (SOD) protecting tissues from
damage, and (iv) photolysis of the metal complexes of NO with its release. It has
been postulated that these three effects are responsible for indirect bactericidal,
regenerative, and vasodilatory action of laser radiation (3, 4, 15). All these
effects may be related to intracellular calcium concentration changes. An increase
of intracellular calcium is observed under irradiation of lymphocytes and
granulocytes with He-Ne laser light (3), which results, in turn, in cell activation
and proliferation. Biological consequences of increased Ca\(^{2+}\) concentration in
the cytoplasm will differ depending on the type of cell exposed to laser radiation. It is
generally accepted that laser radiation acts on mitochondria or even whole cells (3,
17, 18). The results of the some studies suggest that more reactive oxygen species
are generated in the electron transport system of the cell. Mitochondria are the
major source of intracellular free radicals (3, 17-19).

In the present study, we showed a stimulatory effect of laser light on B
lymphocyte proliferation and a slight effect on T lymphocytes. This action of
laser irradiation is the result of initiation of primary, free radical reactions
inducing activation of cells. Other authors have also observed stimulatory effects
of laser irradiation on mitogen-induced proliferation of B and T lymphocytes (3,
20, 21). Our results are in agreement with the observation of Karu (4) that
radiation with a He-Ne laser does not lead to the blast transformation of isolated
lymphocytes but enhances the blast transformation-induced phytohemagglutinin
(PHA), and also leads to an increase of the mitotic index on the 3\(^{rd}\) and 4\(^{th}\) day
after radiation with low doses. He-Ne irradiation acts through an increased
content of intracellular calcium in lymphocytes (3). Also, other authors indicate
that cellular proliferation could be triggered by the action of the He-Ne laser (20,
21). Hu et al (22) suggest that proliferation effect is due to the mitochondrial
photoacceptor-cytochrome c oxidase. Using the melanoma A2058 cell as a model
for cell proliferation, the photobiologic effects induced by the He-Ne laser were
studied (22). He-Ne irradiation immediately induced an increase in mitochondrial
membrane potential, ATP, and cAMP via enhanced cytochrome c oxidase activity.
It ultimately leads to A2058 cell proliferation (22). Under the action of light, singlet oxygen is released, which, in turn, can stimulate such processes as the synthesis of RNA and DNA (20-23). All these observations suggest the improvement of respiratory chain functioning. An increased level of ATP in cells also was observed after irradiation of human blood lymphocytes with the light of an infrared (diode) laser at radiation wavelength of 820 nm. These changes in mitochondria indicate the preparation of the cell for division (3).

An immediate biological consequence of prestimulation of leukocytes (granulocytes and lymphocytes) by laser irradiation of blood is activation of phagocytosis and proliferation leading to the destruction of pathogens by these cells (3). As already stated, a positive action of laser radiation is observed when using it for treatment of persistent wounds (1, 2). It can be supposed that the sequence of events under laser irradiation in this case is the following: (i) absorption of a photon by an endogenous photosensitizer followed by lipid peroxidation; (ii) entering of calcium ions into cells; and (iii) activation of intracellular processes (3). Laser light acts on cellular immunity. Laser therapy produces an immunomodulating action on T-lymphocytes and an immunostimulating one on B-lymphocytes, potentiating phagocytic ability of neutrophils (22). A beneficial action of laser irradiation is the result of the initiation of primary, free radical reactions whose induction is expressed in increased bactericidal activity, production of proteins and cytokines, and cell proliferation. All these events are the basis for the therapeutic action of laser therapy (3).

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