Mitogenic potential inducible by He:Ne laser in human lymphocytes in vitro

Mohamed El Batanounya,*, Soheir Korraab, Osama Fekrya

aDepartment of Surgery, Faculty of Medicine and Department of Biological and Medical Laser Applications, National Institute for Laser Enhanced Sciences, Cairo University, Cairo, Egypt
bLaboratory of Environmental Mutagens, Department of Radiation Health, National Centre for Radiation Research and Technology, Atomic Authority of Egypt, Cairo, Egypt

Received 11 February 2002; received in revised form 6 March 2002; accepted 17 May 2002

Abstract

The objective of the study was to investigate the mitogenic and genotoxic effects of He:Ne laser irradiation (632.8 nm) on human peripheral lymphocytes in vitro. We used the cytokinesis-block micronucleus assay, which incorporates cytochalasin B to inhibit cytokinesis while karyokinesis proceeds normally leading to the appearance of proliferating lymphocytes as binucleated cells. Also micronuclei will appear in cases of genotoxicologically-affected cells. Buffy coat leukocytes were exposed to 10 mW He:Ne laser at energy densities of 1, 2, 3 and 5 J/cm². Cells were then cultured in media 199 without any supplementation for 24, 48, 72 and 96 h adding cytochalasin B 24 h before harvesting of cells. Our results showed that laser-induced lymphocytes proliferate throughout the four consecutive days post-laser irradiation. The difference in the frequency of micronuclei between pre- and post-laser irradiation indicates that a He:Ne laser at such energy densities 1, 2, 3 and 5 J/cm² does not induce micronucleus formation. These results shed some light on the mechanism encountered by lymphocytes in the process of He:Ne laser-induced biostimulation.

© 2002 Elsevier Science B.V. All rights reserved.

Keywords: Biostimulation; He:Ne laser; Lymphocytes; Micro-nucleus assay; Mitogenesis

1. Introduction

He:Ne laser irradiation at wavelength 632.8 nm is an interesting region of the electromagnetic spectrum with respect to its photobiological effects which renders it a beneficial clinical modality in enhancing the process of wound healing, pain relief, and inflammatory suppression in cases of rheumatic arthritis and Achilles tendinitis [1,2]. Athermic and non-destructive photobiological effects of He:Ne laser vary between biostimulation and bioinhibition of physiological, biochemical and proliferative phenomena in various cells, tissues, organs and organisms [3,4]. Most of the related current studies suggest that biostimulation is enhanced at energy densities between 10^3 and 10^4 J/m², while inhibition is exhibited at energy densities between 10^5 and 10^6 J/m² [5].

Research conducted to elucidate the vague mechanism encountered in the photobiostimulatory effects of He:Ne laser irradiation, used in vitro models of immunocompetent cells, particularly lymphocytes whose activation and proliferation are essential parts of the immune response. These models revealed that He:Ne laser irradiation of Hypaque-Ficoll separated lymphocytes induced cell proliferation [6] and cytokine production [7,8] only in the presence of chemical mitogens such as phytohaemagglutinin (PHA) and lipopolysaccharides and that in the absence of such chemical mitogens, lymphocytes do not proliferate in vitro [9]. Consequently, it was suggested that the therapeutic action of He:Ne laser irradiation in a wound healing process is to render lymphocytes sensitive to natural growth factors and antigens [8,9]. However, this in vitro model gives no consideration to the fact that activation of the immune functions of lymphocytes is to a certain extent dependent on their interactions with other immunocompetent cells such as macrophages and neutrophils. The latter participate in the immune response by producing large amounts of nitric oxide intermediates (NOI) [10], and reactive oxygen intermediates (ROI) in response to pro-inflammatory stimuli [11] and also in response to irradiation with a He:Ne laser [12–14]. Recent evidence
suggests that ROI and NOI in addition to being mediators of anti-microbial defences act also as important competence signals in T-lymphocytes inducing their activation and proliferation [15–17].

The aim of this present work was to investigate the mitogenic and genotoxic potential of He:Ne laser irradiation on lymphocytes in vitro when irradiated simultaneously with other leukocyte blood cells, in an attempt to reflect the in vivo situation as closely as possible.

The cytokinesis-block micronucleus assay is a well-established cytogenetic end point for identification of damage induced by physical and/or chemical agents on somatic chromosomes [18]. In such an experiment, mitogen-stimulated somatic cells (usually lymphocytes) are incubated with 4 μg/ml cytochalasin B (an extract from *Helminthosporium dematioideum*) that inhibits cytokinesis without interfering with karyokinesis [19]. Accordingly, proliferating lymphocytes, which have passed through mitosis form binucleated (BN) cells and hence become recognizable from cells that did not proliferate [20]. In cases where there is damage to chromosome material, micronuclei appear in the cellular cytoplasm of binucleated cells. Micronuclei are particles seen in cellular cytoplasm, which stain similarly to the main cell nucleus. They are formed in the cytoplasm when a whole chromosome oracentric fragments fail to be incorporated into the main nucleus during mitosis. They vary in size depending on the amount of chromosomal material that is included in their structure [21].

2. Material and methods

2.1. Preparation of blood samples

Heparinized blood was obtained at different times from six healthy, non-smoker volunteers who had no recent diagnostic or occupational exposure to ionising radiation, laser, or chemicals and had not had any experience of recent allergic responses or drug administration.

Buffy coats were separated and concentrated in plasma at a cell density of 2×10^7 cells/ml. Also whole blood from the same individuals was subjected to Histopaque 1077 (Sigma, St Louis, MO, USA) separation in order to separate lymphocytes according to standard methods. Lymphocytes were then washed with Hank’s balanced salt solution and suspended in its native plasma at a concentration of 2×10^7 cells/ml; 100-μl aliquots of cells were distributed in 96-well tissue culture plates (Nalge Nunc, New York, USA). To minimize cross-irradiation between cells, at least two empty wells separated each experimentally irradiated well. Plates were shaken immediately prior to irradiation to maintain homogeneity of cells in suspension. Every treatment of each of six individuals was investigated in duplicate.

2.2. Irradiation

Irradiation was carried out using the Levelaser M 300 (CSO-Med/Mogliano Vento, Italy). This apparatus is equipped with an automated computer, which after setting the required power density (J/cm²) and the required scanning area, the time (min) is calculated automatically and the laser beam falls vertically over the required area for the duration of the calculated time. Irradiation was carried out with a 10 mW He:Ne laser (wavelength 632.8 nm) at energy densities of 1, 2, 3 and 5 J/cm². This required 2-, 4-, 6- and 10-min irradiation for each well, respectively.

2.3. Cell culture

Immediately after laser irradiation, cells were transferred into 15-ml sterile plastic round-bottomed tubes containing only media 199 (Sigma, St Louis, MO, USA). Cells were incubated for 24, 48, 72 and 96 h, adding cytochalasin B 24 h before harvesting. In addition to the main experiment of laser-exposed cells, lymphocytes from each individual were exposed to the following irradiation and cell culture exposure conditions:

(a) Cells exposed to laser irradiation at the various energy densities without cytochalasin B treatment.
(b) Cells treated with the chemical mitogen phytohaemagglutinin (PHA) and not exposed to laser but incubated with and without cytochalasin B.
(c) Cells neither exposed to laser nor PHA but incubated with cytochalasin B.
(d) Histopaque separated lymphocytes exposed to laser without PHA and treated with cytochalasin B.

No foetal calf serum or antibiotics were added in order to eliminate the suspicion of growth factors or antigen stimulation of lymphocytes, instead media 199 was sterilized just before use with a 0.22-μm Millipore filter (Nalge Nunc, New York, USA).

2.4. Viability testing

Viability testing was carried out using the trypan blue dye exclusion assay. Interference with cell types other than lymphocytes was not suspected, based on the fact that monocytes and granulocytes adhere to tissue culture walls, while lymphocytes remain suspended in the nutrient media. Viability was estimated at 0, 24, 48, 72 and 96 h post irradiation.

2.5. Harvesting of cells

Twenty-four hours after the addition of cytochalasin B, cells were collected and treated with 0.8% sodium citrate
for 3–5 min and then fixed in 5:1 methanol/acetic acid. Fixed cells were dropped gently onto clean microscope slides, air-dried and stained with 4% Giemsa (Sigma, St Louis, MO, USA) using standard procedures.

2.6. Scoring under the microscope

Slides were scored at 100× magnification using a Leica Biomed microscope (Leica Lasertechnik, Heidelberg, Germany). Identification of cytokinesis blocked binucleated cells and the frequencies of micronuclei in such cells were estimated according to the criteria stated by Fennech [22]. Binucleated cells were selected on the basis of having a well-preserved cytoplasm with two distinct nuclei of approximately equal size, which may be attached by a fine nucleoplasmic bridge or alternatively be overlapped. The micronuclei scored were therefore located within the cytoplasm and were not refractile or linked to the main nuclei via a nucleoplasmic bridge. From each culture, the ratio of binucleated (BN) to mononucleated cells (MN) was determined by counting the number of BN per 2000 MN. Additionally 500 binucleated cells were scored for micronuclei.

3. Results

3.1. Mitogenesis

Table 1 displays the percentage of BN cells per 2000 MN cells at different times post He:Ne laser irradiation and under different conditions of cell culture. Lymphocytes that neither received laser irradiation nor PHA stimulation displayed no binucleated cells when treated with cytochalasin B.

The ratio of BN cells per 2000 MN cells (Fig. 1) reveals that PHA-stimulated cells displayed the highest percentage of proliferation at 72 h (mean=31.3±4.9%) post stimulation followed by cells irradiated with 1, 2 and 3 J/cm², respectively (mean=20.5±5.5, 19.8±4 and 18.9±2%, respectively). Irradiation with 5 J/cm² (mean=17.5±3%) displayed the lowest percentage. The ratio was significantly higher in PHA stimulated cells (P<0.001) when compared to laser-stimulated cells at all energy densities investigated using the Student’s t-test. Histopane separated lymphocytes without other blood cells exposed to laser at 1, 2, 3 and 5 J/cm² exhibited no BN cells when they were cultured in the absence of PHA and treated with cytochalasin B. Negligible amounts of BN cells were observed in cultures receiving no mitogen stimulation but cytochalasin B treatment in the range of 0–4 BN/2000 MN.

3.2. Percentage viability

Fig. 2 reveals the percentage viability of lymphocytes irradiated with a He:Ne laser. Mean percentage viability was 95±1.8, 90±3.5, 90±5.6, 90.5±5, 86.5±6, 88.5±5.2, and 47.5±8.9 for 1, 2, 3 and 5 J/cm², respectively, as well as for PHA without laser stimulation and for non-stimulated cells (all observed after 72 h in culture). There was

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Percentage of BN/MN after different culture times</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>Energy density</td>
<td></td>
</tr>
<tr>
<td>0 J/cm²</td>
<td>–ve</td>
</tr>
<tr>
<td>0 J/cm²</td>
<td>+ve</td>
</tr>
<tr>
<td>0 J/cm²</td>
<td>5±2.5</td>
</tr>
<tr>
<td>1, 2, 3 or 5 J/cm²</td>
<td>–ve</td>
</tr>
<tr>
<td>1 J/cm²</td>
<td>7.9±2</td>
</tr>
<tr>
<td>2 J/cm²</td>
<td>6.9±3.2</td>
</tr>
<tr>
<td>3 J/cm²</td>
<td>7.2±3.8</td>
</tr>
<tr>
<td>5 J/cm²</td>
<td>6.4±2.5</td>
</tr>
<tr>
<td>Histopane separated lymphocytes+1, 2, 3, or 5 J/cm²</td>
<td>0.04±0.05</td>
</tr>
</tbody>
</table>
no significant difference in percentage viability between lymphocytes irradiated with 1, 2, 3 or 5 J/cm², or cells stimulated with only PHA ($P>0.05$), using the Student’s t-test.

Cells irradiated with 5 J/cm² displayed the lowest percentage viability amongst the energy density stimulated cells. Lymphocytes, which did not receive any mitogen stimulation, had a significantly lower percentage viability when compared to cells which received either laser or PHA stimulation.

3.3. Micronucleus induction

Optimal levels of binucleated cells and micronuclei formation occur 72 h after starting the culture [22]. Mean frequency of micronuclei was $3.1\pm0.7$, $4.1\pm1.1$, $4.5\pm1.6$, $5.8\pm0.72$ and $5.8\pm1.8$ for 1, 2, 3, 5 J/cm² and PHA without laser stimulated cells, respectively (Fig. 3). Excluding cells irradiated with 5 J/cm², there was a significantly lower frequency of micronuclei formed in cells treated with different energy densities of laser when compared to cells stimulated to divide with PHA. Irradiation with 1 J/cm² displayed the lowest percentage of micronuclei.

4. Discussion

Human peripheral blood leukocytes offer an acceptable test system for studying cytogenetic, toxicological and immunological effects induced by exposure to physical and chemical external stimuli on human cells. Leukocytes are a heterogeneous mixture of monocytes, lymphocytes and polymorphonuclear granulocytes. Lymphocytes are subdivided into two main populations: (a) B-lymphocytes specified in humoral immunity and, (b) T-lymphocytes whose main functions are to induce, help, suppress and modulate immune functions. The main function of granulocytes is phagocytosis of tumour or parasite-bearing cells [23]. This is achieved by generating oxidant molecules such as nitric oxide intermediates (NOI) [24] and reactive oxygen intermediates (ROI) [11]. Oxidants if not physiologically controlled by endogenous antioxidant molecules and enzymes, can be a major source of damage to inflamed tissue and surrounding cells [25,26]. Chromosomal damage can be induced in mammalian cells due to exposure to substantial amounts of oxygen generated from stimulated phagocytes [27,28].

In multicellular organisms, general biological principles postulate that the organization of cells (particularly immune cells) depends on regulated cell surface interactions both with molecules on the surface of other cells and with immune mediators in the extracellular fluid. Such interactions have been shown recently to generate and/or require reactive free radicals or derived species to successfully transmit their signals to the nucleus [29]. An example is that neutrophils undergo massive prolonged generation of ROI at the command of macrophages and lymphocytes, which react with microbial products and antigens [30]. Lymphocytes have recently been shown to respond to ROI released by neutrophils [31], suggesting that ROI act as competence signals involved in the regulation of cell cycle entry and in the control of early gene expression in T-lymphocytes [16,17]. NOI have also been shown to activate human lymphocytes leading to an increased secretion of lymphokines and cytokines that enhance the inflammatory process [17]. Most recently, the He:Ne laser has been shown to induce the production of both ROI and NOI by human neutrophils in vitro [12] via a signal transduction-induced mechanism [32].

To investigate the mitogenic and genotoxic potentials induced in human lymphocytes when irradiated with He:Ne laser irradiation, it was necessary to construct an in
vitro model system that reflects the in vivo situation as closely as possible. Accordingly, in this present study lymphocytes were irradiated in the presence of their native neutrophils and plasma with an He:Ne laser in an attempt to approach the in vivo situation. Results indicated that the He:Ne laser (632.8 nm) at an output of 10 mW and energy densities of 1, 2, 3 and 5 J/cm² stimulated a percentage of lymphocytes to divide in vitro in the absence of any chemical mitogen, e.g. PHA. This phenomenon was observed from 24 h up to 96 h post laser irradiation and was maximal at 72 h but only when lymphocytes were irradiated in the presence of neutrophils. Histopaque separated lymphocytes did not exhibit this phenomenon. This result is not different from results obtained by Karu et al. [10], who showed that irradiating Hypaque-Ficoll separated lymphocytes with an He:Ne laser of power output 6 mW/m² and energy densities of 0.56 J/m² did not stimulate DNA synthesis in such cells measured in terms of radioactive thymidine uptake. Although there are differences in the magnitude of power output and energy densities used in this present study compared to their experiment, their experimental in vitro model system did not consider the role of other blood leucocytes as was done in the in vitro model of this study.

The results of this study also resemble those previously obtained by Stadler et al. [33], who showed that lymphocyte proliferation was significantly higher in samples irradiated in the presence of whole blood compared to lymphocytes irradiated after isolation from whole blood. They attributed the reason for this increase in cell proliferation to the presence of haemoglobin in their in vitro system, neglecting the presence of neutrophils and the stimulatory effect of He:Ne laser on such cells [32] in inducing the formation of oxygen free radicals [13]. Similarly, Yu et al. [34], showed that in vivo activation of lymphocytes by primed doses of microbial antigens followed by low energy density laser irradiation in vitro significantly enhanced the proliferation of lymphocytes in the presence of PHA compared to lymphocytes, which did not receive in vivo antigen stimulation [34]. In another study the differential blood count of in vivo laser-treated animals was characterized by significantly higher lymphocyte values and lower neutrophil values at 20 h among antigen boostered rabbits and 23 h in non-boostered rabbits post He:Ne irradiation [35]. The observed lymphocyte activation effect in both studies is most probably due to the in vivo presence of neutrophils. The latter upon antigen-induced stimulation produce substantial amounts of ROS and NOI [11,24]. Recent evidence suggests that ROI and NOI in addition to being mediators of anti-microbial defences act also as important competence signals in T-lymphocytes inducing their activation and proliferation [31].

The release of ROI- and NOI-induced immune mediators into the culture medium such as, cytokines [30] and/or arachidonic acid metabolites [32,36] in response to the stimulatory effects of laser irradiation on neutrophils [12] and consequently on lymphocytes seems to be the most probable reason for the observed proliferation of lymphocytes during the four consecutive days post laser irradiation. This was proved in this study by the fact that Histopaque separated lymphocytes in the absence of neutrophils did not respond to laser biostimulation. Concomitantly, the stimulation of only a small percentage of lymphocytes to divide in this present study provides the probability that there has been selective division of certain lymphocytes subsets. Yet this requires further investigation together with the identification of the immune mediators released in response to laser irradiation.

This present work, demonstrated the mitogenic potential of He:Ne laser irradiation on human lymphocytes in vitro. It was carried out using the cytokine-block micronucleus assay. It is clear from Table 1 that irradiated lymphocytes whose cultures were not treated with cytochalasin B developed no binucleated cells unlike the cells that were treated. This indicates that laser alone does not induce the formation of binucleated cells. The latter can be observed in cases of exposure to ionising radiation, anti-neoplastic drugs [37,38] and disease such as leukaemia [39]. Although, somatic cells containing two nuclei are present in tissues even at very low numbers compared to mononucleated cells, the presence of binucleated cells have been used by some authors as a criterion for cytological identification of tumour populations and in some cases these cells can be used as a marker of the malignant phenotype of tumour [40,41]. The presence and absence of binucleated cells in cultures treated with and without cytochalasin B, respectively indicate that binucleated cells were not originally present in the blood of these individuals but evolved as a consequence of mitosis of lymphocytes after being stimulated with different mitogens such as laser and PHA.

This present study also demonstrated that He:Ne laser irradiation under the conditions of the energy densities and power outputs used in this experiment is not genotoxic. Excluding cells irradiated with 5 J/cm², there was a significantly lower frequency of micronuclei formed in cells treated with different energy densities of laser when compared to cells stimulated to divide with PHA. There was a clear dose dependency that was present at the dose of 1 J/cm² and reached saturation at 5 J/cm² compared to PHA stimulated cells. This phenomenon could be due to the fact that the modulation effect of laser irradiation is a function of the dose applied. Stimulation of neutrophils has been shown to be effective within the dose range from 0.01 to 6.00 J/cm² [42]. At the lowest laser dose, oxidants are generated at lower concentrations, which increase gradually with increasing dose until a maximum is reached after which oxidant generation then decreases in a dose-dependent manner [12,43]. Thus at the highest dose (5 J/cm²) applied in this present study the oxidants generated from neutrophils are speculated to
cause an oxidative stress equal to that induced by PHA while stimulating lymphocytes to divide in vitro [44]. Meanwhile at 1 J/cm², the generation of oxidants was at a low level sufficient enough to enhance DNA repair [45]. The He:Ne laser has been shown to increase the induction of the frequency of sister chromatid exchanges in sheep peripheral blood mononuclear cells in a dose response-dependent pattern [46]. Sister chromatid exchanges are considered to be an indicator of DNA repair [47].

Lymphocytes are one of the most accessible of human cell types. They are highly sensitive to physical and chemical agents producing a cytogenetic response that is considered a biological dosimeter reflecting the effects of such agents [48]. Previous investigations demonstrated that argon laser (460–515 nm) irradiation at energy densities of 100 and 400 J/cm² induced chromosomal aberrations in Chinese Hamster cells [49]. This is inconsistent with the results of this present investigation, possibly due to the differences in wavelengths and to the high magnitude in energy density used in that experiment compared with this present study. Meanwhile, it has been shown that irradiating Friend erythro leukemia cells with a gallium aluminium arsenide laser (660 nm) at a power output of 12 mW and energy densities of 2–20 J/cm² induced no mutations [50]. This agrees with the results of this present study, although the laser and the in vitro systems are different in the two different studies.

In summary, we report here a phenomenon observed that irradiating human lymphocytes, in the presence of native neutrophils and plasma in vitro, by He:Ne laser irradiation at 1, 2, 3 and 5 J/cm² induced a certain percentage of lymphocyte proliferation as well as an increase in rate of survival in culture; suggesting that stimulation is essential for enhancement of cell survival and providing proof of the importance of red light for survival of organisms [51]. It can also be concluded that within the chosen experimental conditions of this present study, laser fluency of He:Ne irradiation ranging from 1 to 5 J/cm² induced minimal micronuclei formation indicating that this is not a genotoxic insult. Further investigations are required to identify the lymphocyte subsets stimulated to divide and the immune mediators liberated to enhance this cell proliferation.

References


