DEREPRESSION OF THE GENOME AFTER IRRADIATION OF HUMAN LYMPHOCYTES WITH HE–NE LASER

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Mitogenic Activation of Lymphocytes

Small lymphocytes can circulate in the peripheral blood of mammals for months or years without replicating their DNA or undergoing mitosis. During this time the cells remain small, with a condensed nucleus and scanty cytoplasm (e.g. see Figure 7A). However, when confronted with an immunological stimulus, the lymphocyte shifts from this resting (G_0) state to one of rapid enlargement, culminating in DNA synthesis and mitosis. This process is called blast-transformation, and the agents responsible for blast-transformation are called mitogens.

The first-discovered and most widely used mitogen is PHA, a specific mitogen for T-lymphocytes (70–80% of all population).[1,2] Characteristics of biochemical and morphological reactions in lymphocytes under the action of PHA are well-studied (see e.g. references 1–4). The addition of PHA to resting lymphocytes produces an increased level of activity in many cellular reactions. Some of these reactions are presented in Figure 1.

Cellular responses to a mitogen can be divided into short-term responses without *de novo* synthesis occurring during the first seconds, minutes and hours after the addition of the mitogen; and long-term ones connected with the synthesis of protein and occurring from hours to days after the beginning of stimulation (Figure 1).

We provided parallel experiments with PHA and irradiation and compared the results for these two experimental groups with each other and with the intact control.

As the short-term responses, Ca^{2+} influx, RNA synthesis and accessibility of chromatin to acridine orange were studied. DNA synthesis after 72 h was measured as a long-term response to the treatment.

Short-term Responses of Lymphocytes to PHA and Irradiation with He–Ne Laser

Changes in the Cellular Membrane

A rapid increase in intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) in first minutes after PHA treatment is one of the most important early mitogenic signals in lymphocytes.[5,6] We measured the $[Ca^{2+}]$; 2,3 and 6 min after irradiation.[7,8]

The lymphocytes were separated from uncooled blood of healthy male human donors in a Ficoll-vecografin density gradient as described in the papers of Fedoseyeva *et al.*[9,10] The cells obtained were resuspended in 199 medium in a concentration of 1×10^6 cells/ml. The cells were incubated for 1 h with quin 2/AM (Calbiochem-Boehring, San Diego, U.S.A.) at a final concentration of 10 μM (stock 20 mM in DMSO) at 37°C in a saline solution (1.45 mM NaCl, 5 mM KCl, 1 mM $NaHPO_4$, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 5 mM glucose, 10 mM HEPES, pH = 7.6). After 1 h, the cells were washed twice with saline medium warmed to 37°C. The pellets were resuspended in warm saline medium plus 10 mM EGTA at 7×10^6 cells/ml. Samples (2 ml) were transferred into quartz cuvettes and PHA at a concentration of 2 μg/ml was added, and the experimental group of samples were irradiated with the He–Ne laser at a dose of 56 J/m^2. The fluorescence was recorded with a spectrofluorometer 'Spectrofluo JY3D' (Jobin Yvon, France) 2, 3 and 6 min after the irradiation or PHA treatment. Excitation was set at 339 nm and emission at 492 nm. The intracytoplasmic concentration was counted as described in the paper of Watman *et al.*[6]

The baseline concentration of Ca^{2+} in our freshly prepared lymphocytes was 122–137 nM (Table 1). Addition of PHA induced a rise in $[Ca^{2+}]_i$ from the baseline to 254–297 nM after 2 min, and to 173–208 nM after 3 min. In time point 6 min the value of $[Ca^{2+}]_i$ was very close to the value of $[Ca^{2+}]_i$ in intact cells (Table 1). These results are in agreement with the data in the literature.[5,6,11]

The kinetics in the changes of $[Ca^{2+}]_i$ after the irradiation of cells with red light at $\lambda = 632.8$ nm at a dose of 56 J/m^2 is rather similar to that after PHA treatment of the lymphocytes. There was 2–3-fold increase in $[Ca^{2+}]_i$ at 2 min after the irradiation, but no statistically significant difference at time points 3 and 6 min (Table 1).

The experiments were performed in triplicate with blood of three donors.

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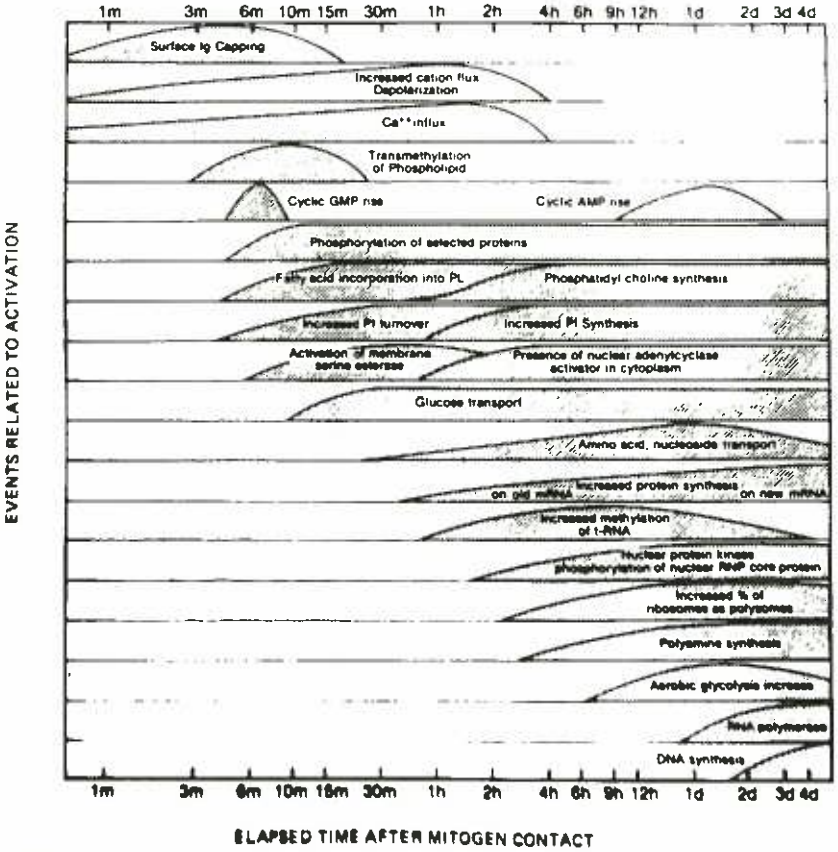


Figure 1. Kinetic relationships of lymphocyte activation events. Shaded lozenges indicate the approximate time course of the mitogen-induced activation events. Some activation events are evident for a brief period and others persist throughout the S-phase. There is considerable asynchrony of activation events within cell populations, and no guarantee that the order of events depicted here holds for all subpopulations or species. Time is on a log scale. Adapted from the paper of Ashman[2]

Table 1. Concentration of cytosolic calcium ($[Ca^{2+}]_i$) in nM in lymphocytes from blood of three different donors after irradiation of the lymphocytes with He–Ne laser (56 J/m^2) or treatment with PHA (2 μg/ml)

Time	2 min			3 min			6 min		
Donor	Intact control	PHA	He–Ne	Intact control	PHA	He–Ne	Intact control	PHA	He–Ne
I	124 ± 6	254 ± 3	422 ± 8	125 ± 3	195 ± 6	82 ± 2	122 ± 9	150 ± 1	92 ± 8
II	135 ± 8	297 ± 4	319 ± 6	137 ± 2	208 ± 8	121 ± 4	131 ± 3	133 ± 7	129 ± 4
III	129 ± 3	263 ± 8	270 ± 4	123 ± 6	173 ± 3	115 ± 9	122 ± 5	132 ± 3	119 ± 6

Early Nuclear Changes

A number of nuclear alterations have been reported which follow rapidly upon the addition of PHA to lymphocytes, including increased acridine orange binding[12] and RNA synthesis.[13,14] Increased acridine orange (AO) binding would reflect the greater accessibility of the activated chromatin.[12,15] As a rule, the enhancement of the binding of AO reflects the changes in the structure of the chromatin correlating with its template activity.[2–4] This increase, about 50–70% above control values is characteristic of practically all kinds of G_0 cells stimulated by different ways.[4]

In our experiments we measured the AO binding to DNA, RNA synthesis rate during the first hours, and performed a series of electron-microscopic studies of nucleoli of cells irradiated with the He–Ne laser or treated with PHA human lymphocytes.

Binding of Acridine Orange to DNA. Lymphocytes were separated from uncooled blood in the Ficoll-verografin density gradient (ζ = 1.077 g/cm^3). Blood from 30 healthy male human donors was used.[9,10]

The cells thus obtained were washed twice and then resuspended in 199 medium at a concentration of 7.5 × 10^5 cells/ml. The microscopic analysis of

the suspension showed that it contained mainly lymphocytes. Vital cells determined by the trypan blue exclusion test amounted to no less than 95%. The suspension was applied to cover glasses for irradiation. Cover glasses were put into flasks with a bottom diameter of 30 mm. Nutrient medium covering the glasses was ~ 1 mm deep.

The source of light was a He–Ne laser (Model LG-52) 6 mW in output power. The source beam was defocused with a short focal length positive lens to the diameter of the sample. Irradiation was effected in a dim scattered light at room temperature. Irradiation times were from 5 to 15 s. In the experiments aimed at determining the properties of chromatin, the cells after irradiation were incubated at 37°C for various periods of time in the range of 45–360 min and then fixed with a 1:1 mixture of alcohol and acetone and stained with acridine orange by the Rigler method[16] without acetylation, as reported in paper of Zelenin and Kushtch.[17] The accessibility of DNA to acridine orange (AO) was determined by measuring the fluorescence of the AO–DNA complex (λ = 530 nm) formed in protein-free sections of DNA. At the same time experiments were conducted under the same conditions with the use of the mitogen PHA (Bacto phytohemagglutinin P, Difco Laboratories, Detroit, Michigan, U.S.A.) at a concentration of 2 μg/ml and also control experiments without the use of any stimulant.

Measuring of the chromatin template activity is based on determination by fluorescence (Figure 2) of amount of acridine orange intercalated in chromatin. The number of chromatin primary-binding sites for dye molecules is proportional to chromatin template activity and characterizes the regions which are possible sites of transcription of the chromatin.[18]

In the first series of experiments, we compared chromatin binding of AO in intact lymphocytes (not exposed to any treatment), and lymphocytes which 45 min before the analyses were irradiated with the He–Ne laser or treated with PHA. The choice of this incubation time for the cells after exposure to the action of the laser light or PHA was governed by the fact that, as demonstrated earlier in paper of Zelenin and Kushtch,[17] it was exactly after this period that substantial changes occurred in the chromatin of lymphocytes treated with PHA.

Figure 3 shows the accessibility of the lymphocyte chromatin DNA to AO as a function of the light dose. It can be seen that as the dose is increased from 28 to 56 J/m^2, the degree of binding of the dye to the chromatin increases. A further increase of the light dose does not produce any further increase in the degree of the dye binding to the DNA, but conversely, some reduction in the degree of binding occurs. Such a characteristic of the dose curve points to the existence of some specific action of the He–Ne laser light on the lymphocytes within a relatively narrow dose interval.

The results of these experiments point to the fact that 45 min after irradiating the lymphocytes with the He–Ne laser the lymphocyte chromatin became more accessible to the low-molecular ligand AO,

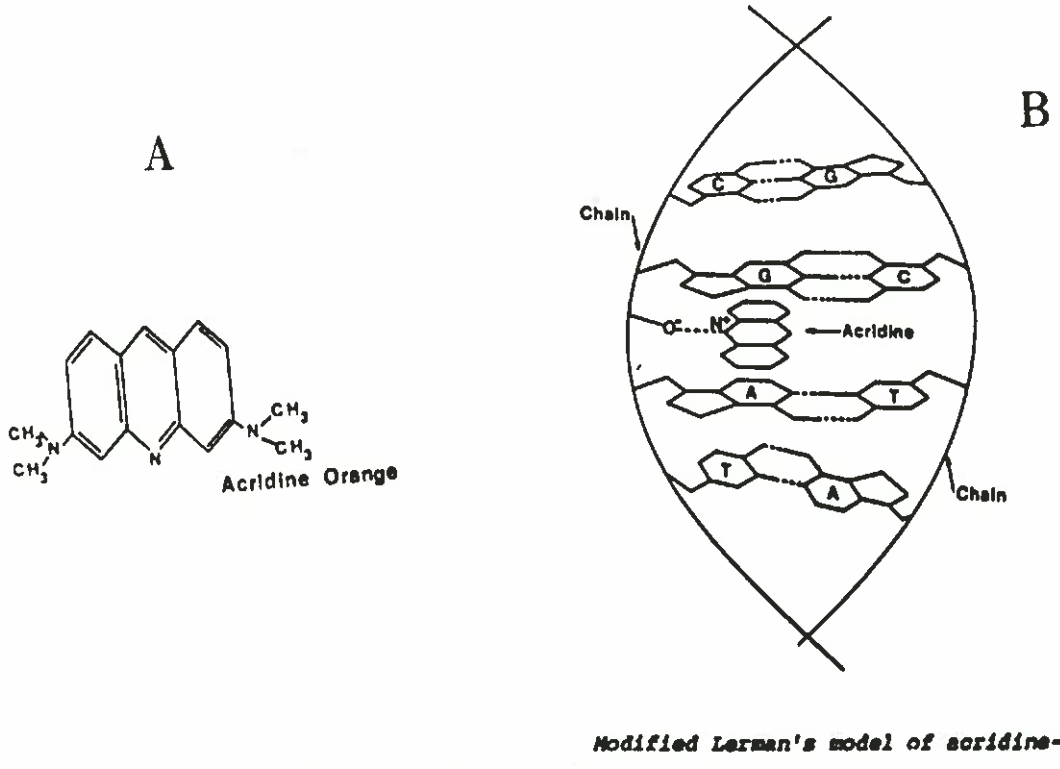


Figure 2. A, structure of acridine orange (AO); B, binding of AO to DNA

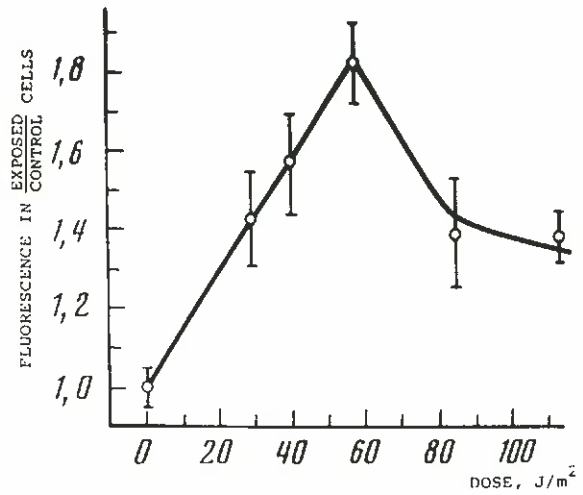


Figure 3. Fluorescence intensity of the chromatin–AO complex 45 min after the irradiation as a function of He–Ne laser light dose

the increase in the degree of binding as compared with the control lymphocytes being close to that obtained for the cells treated with PHA. This suggests that by this time the irradiated cells had undergone some changes in their chromatin structure similar to those caused by PHA.

In the second series of experiments, we studied changes of the chromatin structure as a function of the period of time elapsed after irradiation. Similar experiments were run simultaneously with the lymphocytes treated with PHA. The change of the chromatin structure in response to irradiation with the He–Ne laser or treatment with PHA turned out to be dependent on the time elapsed after the moment the cells were exposed to the action of these agents (Figure 4). Both curves are similar in shape: the binding of AO to DNA is observed to increase during 45–90 min after exposure, then the accessibility of DNA to the dye decreases reaching

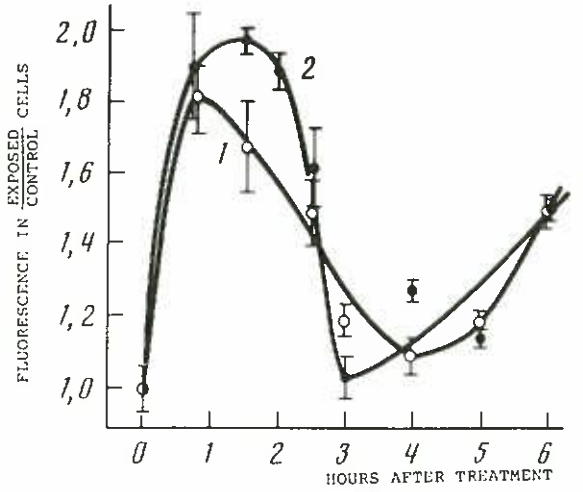


Figure 4. Fluorescence intensity curves of the chromatin–AO complex. 1, Irradiation with He–Ne laser (D=56 J/m²), 2, treatment with PHA (2 μg/ml)

the control level in 3–4 h after exposure, and then the binding of the dye to DNA increases once more. Such a three-stage character of the chromatin structure changes in the resting cell induced to proliferate by a variety of stimuli differing radically in nature is apparently universal.[19] All increases in chromatin template activity always occur in the first 4 h after the application of the stimulus to proliferate, i.e. many hours before the onset of DNA synthesis. While at the stage of the increase in chromatin template activity, the cells can return to the state of rest. And only after some critical point in this period has been passed, the G_1 and S periods become irreversible. In the case of PHA-stimulated lymphocytes the interval between the application of the stimulus and the onset of DNA synthesis is ~ 48 h.[19]

Thus, the results of the above experiments show that the irradiation of lymphocytes with He–Ne laser brings about alterations in the chromatin structure similar to those caused by PHA.

In our study, the maximum change in the lymphocyte chromatin structure 45 min after the irradiation was attained at a comparatively low light dose of 56 J/m². The fact that increasing the dose to over 56 J/m² decreased the chromatin structure change following irradiation is direct evidence that the enhancement of the binding of AO to the chromatin DNA of irradiated lymphocytes is not due to some light damage to the cells. Otherwise, as the light dose is increased, more and more cells would be damaged and the average per-cell degree of the dye binding would grow proportionately. A direct indication that the He–Ne laser light caused no damage to the lymphocytes was obtained when studying the morphology of the irradiated cells. In our experiments, the lymphocytes had rounded or slightly bean-shaped nuclei with a delicate fluorescent dye-round chromatin structure and surrounded by a thin rim of weakly luminous cytoplasm. The viability of the irradiated lymphocytes was also borne out by the fact that after 4 days' incubation the amount of dead cells in the control and irradiated samples was approximately the same and did not in any case exceed 20%.

It is interesting to compare the optimal light dose in our experiments causing the lymphocyte chromatin structure to change following irradiation, with data of the other authors,[20–23] who have studied the action of He–Ne laser light on lymphocytes. In those works the incorporation of [³H]-thymidine in DNA was measured. They obtained indications that red light at a dose of 2.6×10^4 J/m² had an immunosuppressing effect,[21] and described changes occurring in the immunological characteristics of healthy and oncological patients following their irradiation with the He–Ne laser at a dose of 2.7×10^4 J/m².[23] It was concluded that irradiation with He–Ne laser at a dose of 7.5 J/m² [20] or 10^7–10^8 J/m² [22] did not in itself cause blast-transformation, but

substantially enhanced it when the cells after the irradiation were treated with PHA.[20] It should be noted that in these works a single light dose was used, no attempt being made to study the dose dependence of the effects observed. It is quite possible that these doses were not optimal. Comparison between these doses and the dose curve obtained in our work (Figure 3) shows that they either exceeded the optimum dose interval by several orders of magnitude[21–23] or failed to reach it.[20] The literature available to us contained no data about the changes of chromatin template activity after the irradiation with He–Ne laser.

We studied the possibility of modifying the effect of enhancement of the AO-chromatin binding by means of the reducing agent cysteine. If cysteine is added in various concentrations to lymphocytes after their irradiation with the He–Ne laser or treatment with PHA, the binding of AO to chromatin decreases. The further increases of the cysteine concentration causes the binding effect to vanish altogether. Cysteine in a concentration of 5 mM even inhibits the binding of AO to chromatin as compared with the unexposed control lymphocytes (Figure 5).

The property of cysteine to cancel blast-transformation caused by $NaIO_4$ was described in the literature.[24] In this case, the effect is believed to be due to the reduction of receptors for mitogens on the plasma membrane. To establish what mechanism is responsible for the disappearance of the effect in the case of irradiation with the He–Ne laser, requires further investigation. At present, we can only draw attention to the fact that whatever the agent the lymphocytes are exposed to, such as PHA (Figure 5) or $NaIO_4$[24] or He–Ne laser radiation (Figure 5), the effect of cysteine remains in principle the same — to retard the stimulatory action of these factors.

RNA Synthesis. At selected times (from 30 min to 12 h) following irradiation or after adding the PHA, [^{14}C]-uridine (3 μCi/ml; 54 mCi/mM, Isotop, Leningrad, USSR) was added to the cell suspension and the solution was incubated for 40 min.[8,25,26] After killing the cells with ice-cold 7% trichloracetic acid, $HClO_4$, precipitated materials were collected on filters (0.2–0.4 μm, Sinpor, Czechoslovakia), fixed in 100° ethanol, dried in air, and the radioactivity was counted in a 'Intertechnique SL-4' (France) liquid scintillation counter.

Figure 6 shows the incorporation of [^{14}C]-uridine as a function of the time elapsed after the irradiation or beginning of the treatment with PHA. It can be seen that in both cases the incorporation of [^{14}C]-uridine increases within the first 1.5–2 h, then decreases and reaches the control level 3–4 h after the irradiation or treatment with PHA. After 5 h the [^{14}C]-uridine incorporation reaches a new peak and then decreases.

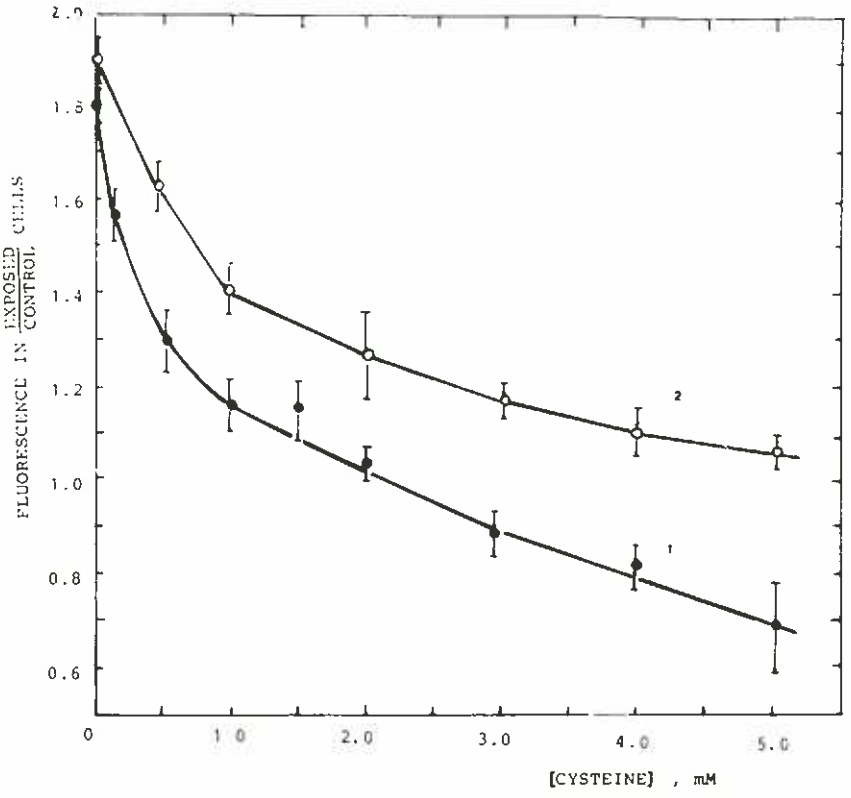


Figure 5. Fluorescence intensity of the chromatin–AO complex of lymphocytes treated with cysteine. 1, Irradiation with He–Ne laser (D=56 J/m^2, incubation time after irradiation, 60 min); 2, treatment with PHA (4 mg/ml, incubation time after treatment, 60 min)

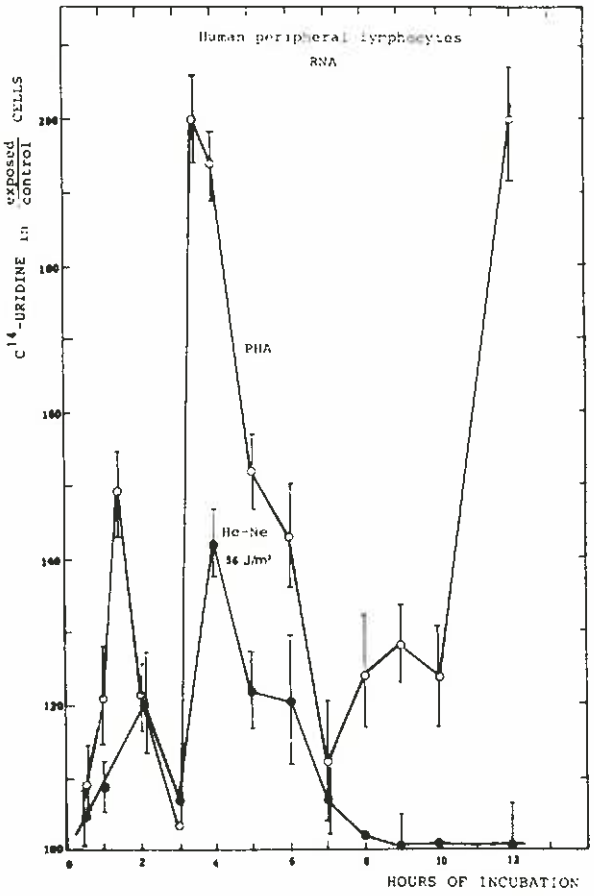


Figure 6. [^{14}C]-uridine incorporation into RNA of human peripheral lymphocytes after adding PHA (2 μg/ml) or after irradiation of lymphocytes with He–Ne laser at a dose of 56 J/m²

It should be noted that both PHA treatment and laser irradiation produce qualitative similar changes in rate of RNA synthesis. However, the maximum values for the PHA-treated cells are more significant (152 and 190%, respectively) than those for the irradiated cells (142 and 123%, respectively).

There is a difference in the shapes of two curves at 7 h after irradiation or adding PHA. In PHA-treated lymphocytes a fresh dramatic increase of [^{14}C]-uridine incorporation occurs but in irradiated cells, RNA synthesis drops on the control level without following changes. These results are summarized from experiments with blood from 15 donors.

Ultrastructural Changes in Nucleoli. The data of the dose-dependent decondensation of total nuclear chromatin, and an increase in RNA synthesis rate speaks about the activation of the transcription function of chromatin and possible derepression of the genome. The task of our electron-microscopic studies[27,28] was an investigation into the changes of nucleoli which perform synthesis of ribosomal RNA (rRNA), and this way to confirm possible activation of the transcription of r-genes as a result of He–Ne laser irradiation. Circulating lymphocytes are convenient objects for this investigation because the changes in their nucleoli after PHA-activation have been studied by electron microscopy.[29–31]

Lymphocytes were separated from uncooled blood of male donors in a Ficoll-Paque (Pharmacia, Sweden) gradient and irradiated with a He–Ne laser. The power density at the sample was 5.6 W/m^2, the dose used was 56 J/m^2, and an irradiation time of 10 s. These parameters were found earlier to be optimal to cause changes in the structure of lymphocyte chromatin (Figure 3).

The electron-microscopic morphometry was performed 1 h after the irradiation or adding PHA.

Cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2–7.4) for 1 h at 4°C, washed with the same buffer and postfixed with 1% solution of OsO_4 in 0.1 M phosphate buffer (pH 7.2–7.4) for 1 h at 4°C.

The cells were dehydrated in a series of ethanol solutions with increasing concentration, rinsed in propylene oxide and embedded in Epon. Ultrathin sections (~ 80 nm) were cut with an LKB ultratome. Ultrathin sections were contrast-stained with uranyl acetate and lead citrate, and then studied with a JEM100 CX electron microscope.

The profiles of the lymphocytes on negatives were projected at a final magnification of 308000 X onto paper and the contours of the cells, their nuclei and mitochondria were outlined using a pen. These images were processed by a Hewlett Packard computer (model 9830A) and digitizer (model 986A). Morphometric analysis included measuring the following parameters on a cell section: cell area, cytoplasm area, the total and individual areas of mitochondria, number of mitochondria, distance between two nearest mitochondria in the zone of their accumulation, and number of mitochondria contacts relative to the total number of mitochondria.

There were 50 random cell sections investigated for each of the three groups, (control, irradiated with He–Ne laser, and treated with PHA cells). Each group consisting of 50 cell sections was collected from five experiments. Repeated cutting and photographing of the same cell at various depths was excluded.

The final data was processed by a computer for the statistical analysis. The results were compared using Student's *t*-test.

Lymphocytes are easily distinguished from other white blood cells present in the suspension. The lymphocytes are characterized by the presence of azurophylic granules and single cytoplasmic ribosomes as well as by the localization of mitochondria, Golgi complex and centrioles in the broad part of the cytoplasm (Figure 7A). There are few polysome complexes which is evidence of a low level of protein synthesis in these resting (G_o) cells. One of the characteristic features of lymphocytes is the presence of large chromatin masses situated in the periphery of the nucleus. Intact lymphocytes in

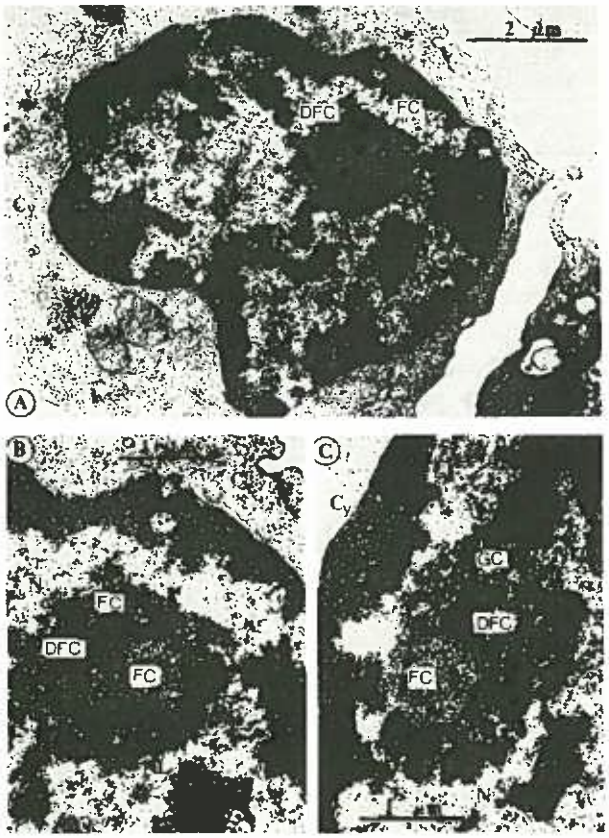


Figure 7. Ultrastructure of intact human circulating lymphocytes: A, general view; B, ring-shaped nucleolus with one round FC; C, ring-shaped nucleolus with a developed granular component. Notations: Cy—cytoplasm; M—mitochondria; N—nucleus; Nl—nucleolus; GC—granular component; DFC—dense fibrillar component; FC—fibrillar component

our experiments have been found similar to those described in literature (see e.g. reference 32).

Intact Lymphocytes. Electron microphotographs of intact lymphocytes are presented in Figure 7 (A, B, C). A lymphocyte contains a ring-shaped nucleolus with a single round or oval fibrillar centre (FC) characterized by moderate density and fibrillar texture. The FC is surrounded by a dense fibrillar component (DFC) which consists of electron opaque RNP-fibrils approximately 4 nm in diameter. The peripheral zone of the nucleolus is formed by a granular component (GC) situated in close proximity to the DFC. The GC is represented by RNP-granules approximately 15 nm in diameter resembling cytoplasmic ribosomes.[33-35] Ring-shaped nucleoli of intact lymphocytes do not contain vacuoles (interstices) or intranucleolar chromatin blocks which have been found in nucleoli of some other cells.[33,35] Figure 8A presents a scheme of the ring-shaped nucleolus of an intact lymphocyte and Figure 8B shows the general location of nucleic acids in this nucleolus.

According to current concepts of the structure and functions of nucleoli,[33,36] ribosomal genes (r-genes) are localized in the FC on the boundary with DFC and the transcription is believed to occur in the periphery of FC. The products of rDNA transcription are RNP-fibrils which are precursors of the RNP-granules (GC). Biochemical investigations demonstrated that DFC contains pre-rRNA (45S RNA), and GC contains processing intermediates and 28S ribosomal RNA.[34] Nucleolar RNP-granules are considered to be preribosomal particles. The ring-shaped nucleolus of the resting lymphocyte is considered to be a nucleolus with a low level of rRNA synthesis.[37,38]

A scheme of production of rRNA in animal cells is shown in Figure 9. A large precursor (45S) is transcribed from DNA (rDNA). During transcription the molecule is methylated at various points. Cleavage into 32S and 18S segments occurs. Further cleavage of the 32S to 28S RNA follows. The 28S and 18S rRNA's are transported to cytoplasm, in combination with a large number of proteins, to

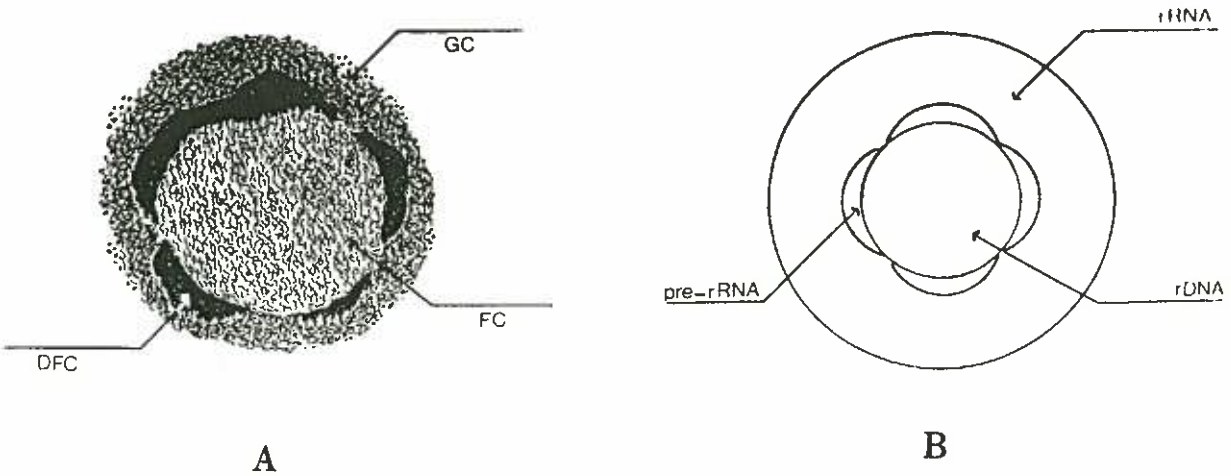


Figure 8. Schemes of (A) structural organization of a ring-shaped nucleolus, and (B) localization of nucleic acids in nucleolar components. Notations are the same as in Figure 7

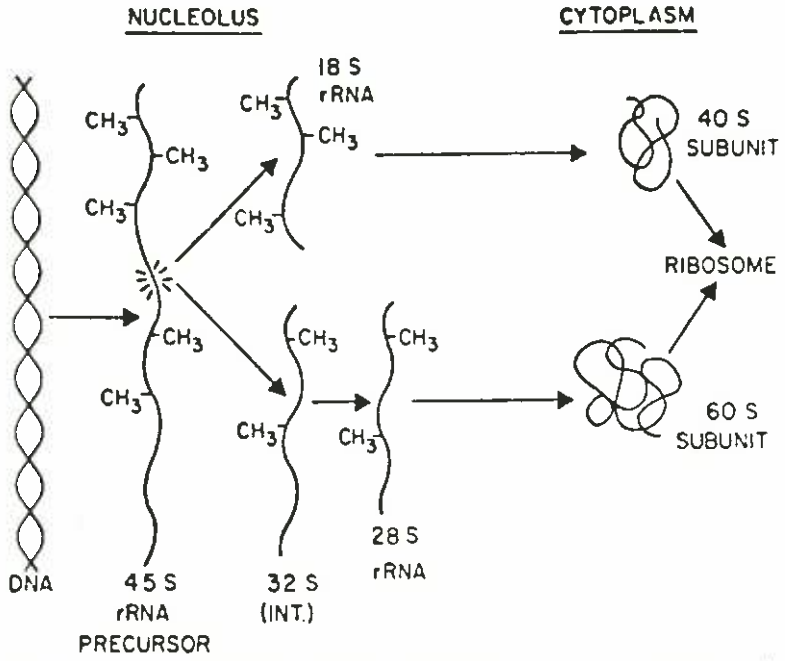


Figure 9. A scheme indicating synthesis, processing and transport of ribosomal RNA in animal cells (adapted from paper of Cooper[1])

form the ribosomal subunits which are active in protein synthesis.[1]

PHA-Treated Lymphocytes. As a rule, the structural changes in the nucleoli of human peripheral blood lymphocytes (circulating lymphocytes) under PHA action have been studied in late periods after the beginning of PHA stimulation.[29,30,32,39] Nucleolar changes 1 h after the introduction of this agent have been described for rat circulating lymphocytes.[31]

In the present experiments, modifications of the nucleolar organization of human circulating lymphocytes were observed soon after the beginning of PHA-treatment (Figure 10A, B). As a rule, the area of the nucleoli of stimulated cells increased a little. FC became irregular in shape (Figure 10A). In some nucleoli, several discrete FCs (two or three), appeared. The size of such individual centres was decreased as compared with a single FC. Such changes result in an increase of the total outer surface of several FCs. Simultaneously, the number of DFC zones and their total area increased insignificantly.

Sometimes several small distinct vacuoles appeared in nucleoli (Figure 10B). As a result, in some PHA-stimulated lymphocytes, strand-like nucleoli caused by an increase in the number of FCs as well by an appearance of the vacuoles may be revealed.

Irradiated Lymphocytes. We found that ~ 70% of the irradiated lymphocytes responded to irradiation, like those shown in Figures 10C, 11A, B, C. One h after irradiation of human lymphocytes *in vitro*, the nucleoli were characterized by the appearance of a FC of an irregular shape (Figure 11A). In some cases there were from two to four discrete FCs, each of them being smaller than a single FC (Figure 11C). Similar changes, *viz.* a fragmentation and reduction in the dimensions of an individual FC, were observed many times in different experimental models as rRNA synthesis was activated.[29,40] The formation of several FCs reduced in their dimensions in some cells as compared to a large single FC in a ring-shaped nucleolus, is considered to be related to the increased content of transcriptionally active rDNA localized at the periphery of the FC.[33,34,36] Moreover, the nucleoli of irradiated lymphocytes were characterized by an increase in the quantity of the DFC zones, and their area enhanced (Figure 11A, B). Since the DFC contains newly synthesized pre-rRNA, a similar modification is usually considered to be the result of the intensification of pre-rRNA synthesis.[33,36]

Another important feature of the nucleoli of irradiated lymphocytes was an increased number of RNP-granules uniformly distributed over the whole body of the nucleolus (Figures 11C), and not over the nucleolar periphery as it was observed in intact lymphocytes of Figure 7B, C). The increase in the GC area is usually explained by an activation of pre-rRNA processing, however the decreased transport of RNP-granules from the nucleolus is also not excluded.[33]

The nucleoli of some irradiated lymphocytes were characterized by the appearance of vacuoles (Figure 11B, C). These structures were attributed to a special vacuolar component of the nucleolus which

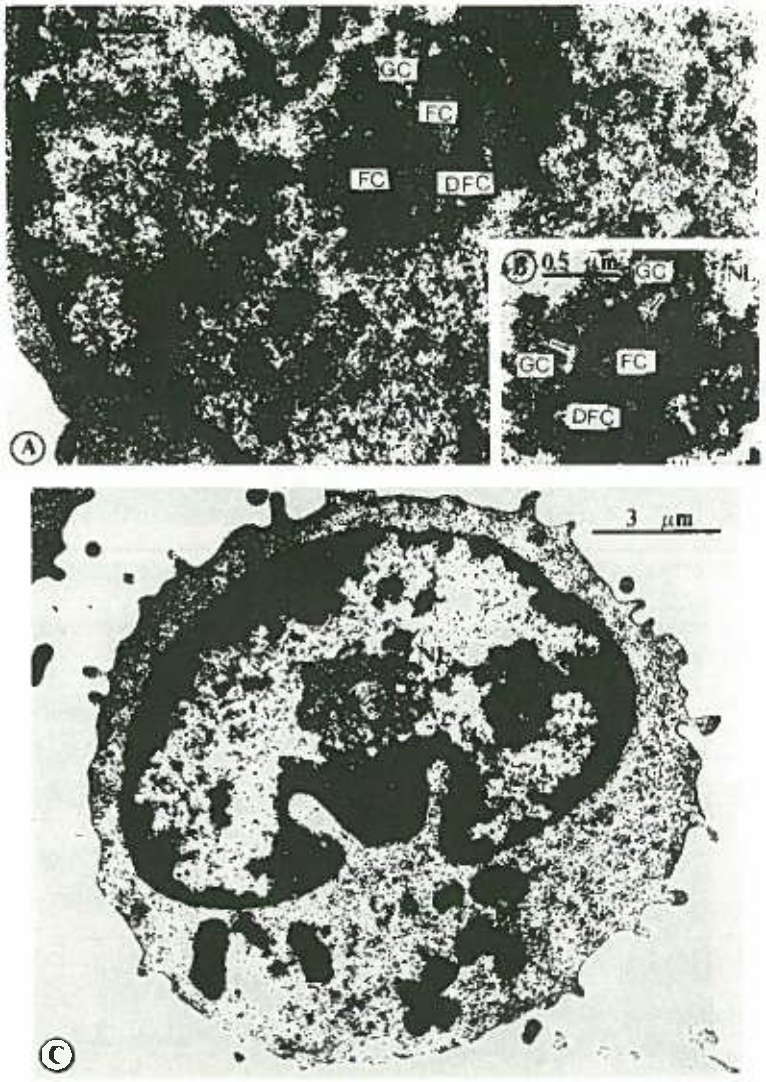


Figure 10. Ultrastructure of irradiated lymphocytes or lymphocytes treated with PHA: A and B, ring-shaped nucleoli (a developed vacuolar system is denoted by arrows in B; C, general view of an irradiated lymphocyte. Notations are the same as in Figure 7

take part in the transport of preribosomes.[33,34,41]

A well-developed vacuolar component has been described for cells with a high level of RNA and protein syntheses, for example hepatocytes.[41] The appearance of distinct vacuoles in our experiments points to the intense transport of the preribosomes actively formed in the nucleoli of irradiated lymphocytes.

Note that in some irradiated lymphocytes reticular nucleoli appeared (Figure 11C). These nucleoli consisted of strands of DFC and GC, several FCs and vacuoles. Such reticular (or nucleolonema-like) nucleoli were larger as compared to ring-shaped nucleoli.

As a whole, changes in the nucleolus 1 h after the irradiation of the lymphocytes *in vitro* have been found to be similar to the changes revealed 1 h after the beginning of PHA-treatment. This point will be analysed in the next section.

Similarity in Activation of Transcription of r-Genes under PHA Action and He-Ne Laser Irradiation. The lymphocyte nucleolus contains r-genes synthesizing pre-rRNA. Pre-rRNA is synthesized here and subsequently processed first into intermediated molecules and then into their ribosomal subunits.[36] It is also known that the morphology of the nucleolus reflects its functional activity.[33,40]

The ring-shaped nucleoli of circulating human lymphocytes investigated in our experiments are characterized by low activity of r-genes. They turned out to be good subjects for revealing the effects of He-Ne laser irradiation. One h after the irradiation of human circulating lymphocytes with He-Ne laser (56 J/m^2, t = 10 s) or adding PHA (2 μg/ml) to the cell suspension, significant changes in the nucleoli of the treated lymphocytes were found as compared with the intact cells. The changes were considered to be similar for both experimental groups: appear-

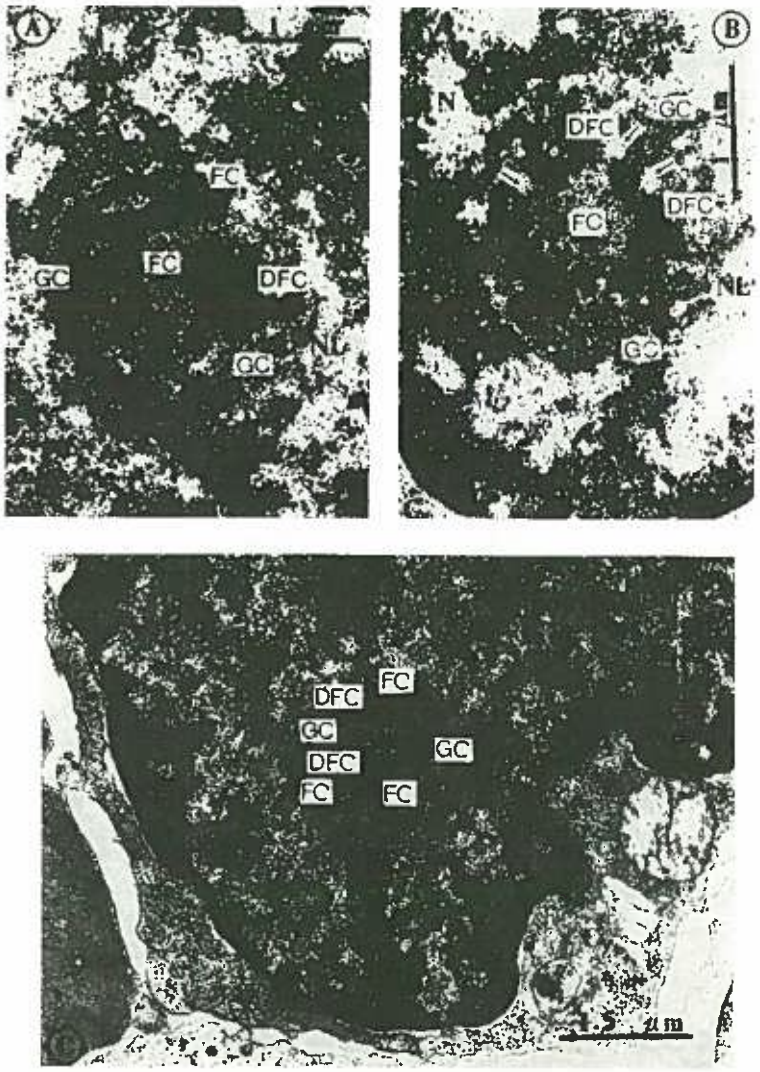


Figure 11. A, B, and C, ultrastructure of the ring-shaped nucleoli of irradiated lymphocytes. Widened vacuoli are marked with arrows. Notations are the same as in Figure 7

ance of a FC of irregular form; fragmentation of single FC to two or three smaller FCs; the increase in the total area of DFC; increase in the number of RNP-granules; and appearance of small vacuoles which are absent in the nucleoli of the intact lymphocytes.

Figure 12 presents general rearrangements of a lymphocyte nucleolus 1 h after the beginning of PHA-treatment, as it was proposed by Manteifel and Chelidze.[31] In the present experiments we found that the irradiation caused similar changes in the nucleoli (previous section). In this schematic number 1 presents an intact ring-shaped nucleolus characterized by low activity of the r-genes. Both irradiation and PHA-treatment caused the appearance of two main modifications in the nucleolus (numbers 2 and 3). Based on the current knowledge of the activational rearrangement of the nucleoli of G_0 cells, one can propose the transformation from the typical ring-shaped nucleolus (1) to a ring-shaped nucleolus with an irregular fibrillar centre (2), followed by the transformation to a strand-like nucleolus with several FCs and enlarged DFC and GC (3). Direct transformation from 1⟶3 is also possible.

One should emphasize that similar changes in nucleoli had been observed earlier in PHA-treated circulating lymphocytes in the rat.[31]

A possible functional interpretation of the changes observed in the nucleoli of irradiated cells is as follows (for better understanding see also Figure 9).

First, the lengthening of the outer FC contour (an irregular enlargement of its shape and an increase in the number of FCs) can be interpreted as an expression of new r-genes because actively transcribed rDNA is situated (as it was described above) in the periphery of FCs.[35] There also exists a correlation between the number of FCs and the level of transcription of r-genes.[40]

Second, the increase in the total area of the DFC in the irradiated cells might reflect an enhancement of pre-rRNA production. It is known that DFC

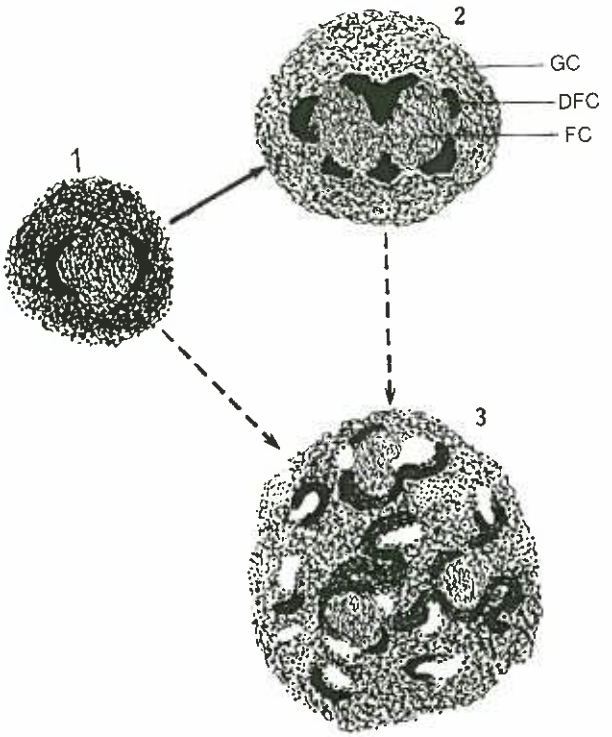


Figure 12. A schematic of main modifications to a nucleolus activated by He–Ne laser radiation or PHA-treatment (adapted from paper of Manteifel and Chelidze[31]: 1, ring-shaped nucleolus of the intact lymphocyte; 2, changed nucleolus with a complex FC; 3, strand-like nucleolus with several FC. Notations are the same as in Figure 7

consists of newly synthesized pre-rRNA molecules.[33,37]

Third, the increase in the number of RNP-granules observed in our experiments probably can be related to the enhancement of the pre-RNA processing and the preribosome production.

Finally, the appearance of vacuoles can be related to the activation of transport of the r-RNP synthesized by the transformed nucleolus as it was suggested earlier for PHA-activated rat lymphocytes.[31]

The results of our experiments demonstrate the similarity in the nucleolar modifications of human lymphocyte in nucleoli under the action of two different factors — PHA and laser radiation. On the whole these changes can be interpreted as an activation of rRNA metabolism including its synthesis, processing and transport. These results are in agreement with the increase in the total RNA synthesis rate in the human lymphocytes during the first hours after both types of treatment (Figure 6). The results of the present experiment show that the intensification of rRNA synthesis contributes to the increase of total RNA synthesis described in a previous section.

Ultrastructural Changes in Mitochondria

In case of cell cultures (both human and animal origin), as well as microorganisms, primary photoacceptors are believed to be components of the respiratory chain.[42–46] The redox mechanism for signal transduction from mitochondria via the cellular membrane and cytoplasm is also proposed in these papers. The question concerning primary lymphocyte photoacceptors has not been investigated. As chromatin and other components of the cell nucleus do not have chromophores with preferential absorption at a wavelength of 632.8 nm, and He–Ne laser irradiation caused reactions in the lymphocyte nucleus one should look for possible photoacceptors in other parts of the lymphocyte. For this reason it would be interesting to study the morphological changes in lymphocyte mitochondria. This was the purpose of our experiments.[28,47]

Lymphocytes were separated, irradiated and studied electron-microscopically as described previously.

The mitochondria of lymphocytes resemble the mitochondria of other eukaryotic cells.[48] In ultrathin sections, the profiles of mitochondria are rounded or elongated (Figure 7A). A majority of mitochondria profiles are concentrated in the widened part of the cytoplasm. The mitochondria are surrounded by an external membrane situated near the internal one, the latter forming cristae inside the mitochondria. There are from two to 16 mitochondrial profiles in the widened part of the cytoplasm, some of them in close proximity to each other. Some other mitochondrial profiles (from 1 to 6) are spaced apart from the main mitochondrial accumulation in the narrow part of the cytoplasm.

According to visual electron microscopy, the distribution of the mitochondria as well as their shape in PHA-treated lymphocytes was found to be similar to those in intact cells (Figure 7A).

Table 2 presents quantitative characteristics of the mitochondrial profiles. As seen in Table 2, the total area of the profiles of the PHA-treated mitochondrion increased as compared with the respective values for mitochondria of intact control cells. These changes were accompanied by a slight decrease in the number of mitochondrial profiles and by an increase in the area of individual profiles.

Table 3 presents data characterizing the distribution of mitochondria in cell profiles. The mean distance between the nearest mitochondria in treated cells was found to increase slightly. At the same time, the number of mitochondria in physical contact decreased. These results show that there was only a tendency of deaggregation of mitochondrial profiles in lymphocytes 1 h after the beginning of PHA-treatment.

Visual electron microscopy did not reveal any changes in mitochondria of irradiated lymphocytes relating to any damage such as lysis of membranes, swelling of the matrix and so on. The shape of the mitochondria in irradiated cells was found to be similar to those in the intact cells. The distribution of mitochondria was very similar

Table 2. Quantitative characteristics ($M \pm$ SEM) of mitochondrial profiles in a cell section

Characteristics Experiment	Number of profiles per cell section	Total area of profiles per cell section (μm^2)	Average area of a profile (μm^2)	Mean diameter (maximal:minimal)
Control	5.66 ± 0.37	0.643 ± 0.065	0.134 ± 0.006	1.857 ± 0.061
PHA	6.61 ± 0.54	0.867 ± 0.085	0.142 ± 0.013	1.827 ± 0.059
	$p < 0.9$	$p > 0.95$	$p < 0.4$	$p < 0.3$
			$p' < 0.8$	$p' < 0.6$
He–Ne laser	6.81 ± 0.44	0.739 ± 0.078	0.123 ± 0.005	1.891 ± 0.068
	$p = 0.95$	$p < 0.7$	$p < 0.9$	$p < 0.4$

p = Significance of differences from control samples.
p' = Significance of differences from irradiated samples.

Table 3. Distribution of mitochondrial profiles on cell sections

Characteristics Experiment	Distance between nearest profiles (μm)	Relative number of contacting profiles (%)
Control	2.56 ± 0.29	42.2 ± 5.7
PHA	3.34 ± 0.57	33.1 ± 5.5
	$p < 0.8$	$p < 0.8$
	$p'' > 0.99$	
He–Ne laser	2.93 ± 0.29	36.7 ± 5.3
	$p < 0.7$	$p < 0.6$

p = Significance of differences from control samples according to t-test of Student.
p'' = Significance of differences from control samples according to Kolmogoroff–Smirnoff test.

to the respective distribution in the intact cells (Figure 7A).

Quantitative data is presented in Tables 2 and 3. The irradiation of lymphocytes with He–Ne laser resulted in a statistically significant increase in the number of mitochondrial profiles per cell section (Table 2), accompanied by a decrease in area of the individual mitochondrial profiles. The total area occupied by all mitochondrial profiles in a cell section remained practically the same. The main diameter (relation of maximal to minimal diameter) is the same as is the control group (Table 2).

As seen in Table 3, the relative number of two contacting neighbouring mitochondrial profiles was decreased. This decrease was less expressed in irradiated cells compared with PHA-treated cells, although the changes were statistically insignificant for both experimental groups.

The results of our experiments demonstrated that 1 h after the irradiation of the lymphocytes or after the beginning of their stimulation with PHA, changes in the structure of the mitochondria as compared to the mitochondria of the intact cells were revealed (Tables 2,3). These changes were not similar in both experimental groups. In the irradiated cells, the significant increase in the number of mitochondrial profiles was not accompanied by an increase in the total area of the mitochondrial profiles whereas in the case of PHA stimulation the number of mitochondrial profiles increased in parallel with the considerable increase of their total area (Table 2).

In electron-microscopic morphometry of different cellular structures performed at random (not chosen by the experimenter) ultrathin sections allowes us to expand the two-dimensional analysis into three-dimensional ones.[49] For example, according to this method, a 35% increase in the average total area of the mitochondrial profiles on a cell section observed in PHA-treated cells (Table 2) was equal to a 56% increase in the volume of these organelles in whole cells. In other words, PHA stimulation leads to the essential increase in the mitochondrial mass 1 h after the beginning of the treatment. On the contrary, in irradiated cells the increase in the mitochondrial mass was insignificant.

The $G_o \longrightarrow S$ transformation of the lymphocytes induced by the mitogen PHA probably includes proliferation of mitochondria similar to other G_o-cells stimulated to proliferation.[50] In so far as irradiation with the He–Ne laser does not induce the $G_o \longrightarrow S$ transformation of human lymphocytes (see section on DNA synthesis), the difference in the early changes of mitochondrial mass after the PHA-treatment and He–Ne laser irradiation can be connected with the specificity of the mode of action of these two factors as well as with their different ability in mitogenic activation.

An early increase in the number of mitochondrial profiles on ultrathin sections was observed during the activation of macrophages by phagocytosis,[51] in hepatocytes during restoration after starvation,[52] in the hepatocellular carcinoma.[53] In some cases there was also an increase in mitochondrial mass. A possible explanation of this data was given by Rohr and Riede.[52] The increase in the number of mitochondrial profiles was proposed to be connected with 'incomplete proliferation' of mitochondria as compared to the 'completed proliferation' when the increase in the number of profiles was accompanied

by an increase in the mitochondrial mass. However, the term 'incomplete proliferation' was neither clear nor completely defined from either the functional or the morphological point of view.

How can one explain an increase in the number of mitochondrial profiles per cellular section without changes in total mitochondrial area, as demonstrated in the irradiated cells (Table 2)? It has been found by three-dimensional reconstruction of the small lymphocyte from mouse lymph nodes that there are one to four little oval and two to three giant branched mitochondria present in this cell.[54] Taking into account these data, it is possible to suggest that numerous mitochondrial profiles on a ultrathin section of the circulating lymphocyte in our experiments do not correspond to the real number of mitochondria in the cell since one section across a cell can cut several times across one branched (and possibly twisted) mitochondrion. Thus, it may be assumed that the increase in the number of mitochondrial profiles per cellular section in the case of irradiated lymphocytes may be connected with more branched and extended mitochondria. This possibility is confirmed by some decrease in average area of a mitochondrial profile (Table 2). Clearly other experimental approaches are needed to explain this phenomenon.

It has been demonstrated that the enhancement of cellular metabolism is accompanied by an increase in the number of mitochondrial profiles per cell section.[51–53] From a functional point of view these changes have been interpreted as an activation of the mitochondrial function.

The assumption about possible functional activation of mitochondria in irradiated lymphocytes is supported by two experimental findings. First, it has been found that the isolated hepatocyte mitochondria irradiated by He–Ne laser have been characterized by an enhanced ATP level.[55] This metabolic shift has been accompanied by ultrastructural transformation from the orthodox to a condensed conformation revealed by electron microscopy.[56,57] It should be kept in mind that resting lymphocytes differ drastically from hepatocytes. Moreover, the irradiation of mitochondria *in vitro*, as it was performed in the work of Passarella *et al.*[55,56] differs from the irradiation of mitochondria *in situ* as in our case.

The second experimental finding follows. The irradiation of human circulating lymphocytes with a diode laser emitting at $\lambda = 820$ nm increased the ATP level measured immediately after irradiation.[58] One should note a difference in the wavelength used in our work and in the work of Herbert *et al.*[58] (632.8 and 820 nm, respectively). One should emphasize that light with $\lambda = 820$ nm can also be absorbed by porphyrin components of the respiratory chain.[44–46]

Our suggestion concerning metabolic activation of mitochondria in lymphocytes as a result of He–Ne laser irradiation is supported by some increase of the distances between nearest mitochondrial profiles and a decrease in the number of profiles in contact. In other words, the irradiation resulted in a deaggregation of mitochondrial profiles. The deaggregation of mitochondria in other cells was earlier connected with the possibility of enhanced respiration.[59]

Based on the data described here we conclude that the increase in the number of mitochondrial profiles with a simultaneous increase in mitochondrial mass observed 1 h after the beginning of PHA-stimulation of the lymphocytes is probably connected with mitochondrial proliferation which is necessary for transition of G_0-cells to the active cell cycle. At the same time, the increase in the number of mitochondrial profiles without changes in their mass in irradiated cells is not to be associated with the division of these organelles but related to their functional activation taking place during the interphase.

Long-Term Responses to Irradiation

DNA Synthesis

To find out whether the passage of the early stages preparatory to leaving G_0-phase of the cell cycle (changes in chromatin structure, changes in RNA synthesis rate and in $[Ca^{2+}]_i$ are followed by reaching the S-phase, we measured the intensity of the incorporation of [^{14}C]-thymidine in DNA of the lymphocytes after their exposure to the He–Ne laser or PHA.[7,8,26]

The separated lymphocytes were washed twice with phosphate buffered saline (120 mM NaCl, 14 mM Na_2HPO_4, 2.64 mM KH_2PO_4, pH = 7.2–7.4), and then resuspended in 199 medium which contained 5% homolytic serum of blood group IV (inactivated for 40 min at 56°C), and 75 units/ml of both streptomycin and penicillin. After resuspension and before the experiments the cell suspension was incubated for 1.5 h at 37°C (5% CO_2).

To estimate the DNA synthesis rate, the irradiated (56 J/m^2 as described above), PHA-treated (2 μg/ml) or control samples were incubated at 37°C (5% CO_2) for various periods seen in Figure 13. [^{14}C]-thymidine (1 μCi/ml, 56.8 mCi/mM, 'Isotop', Leningrad, USSR) was added 24 h before the end of the incubation. After the incubation, the cells were put in an ice bath and treated as described above for the measurement of RNA synthesis. The viability of PHA-treated and irradiated cells was practically the same during all period of incubation. The number of dead cells did not exceed 20% even after 8 days of incubation.

As can be seen in Figure 13, a noticeable increase in the incorporation of [^{14}C]-thymidine occurs 2–3 days after treating the cells with PHA. This result

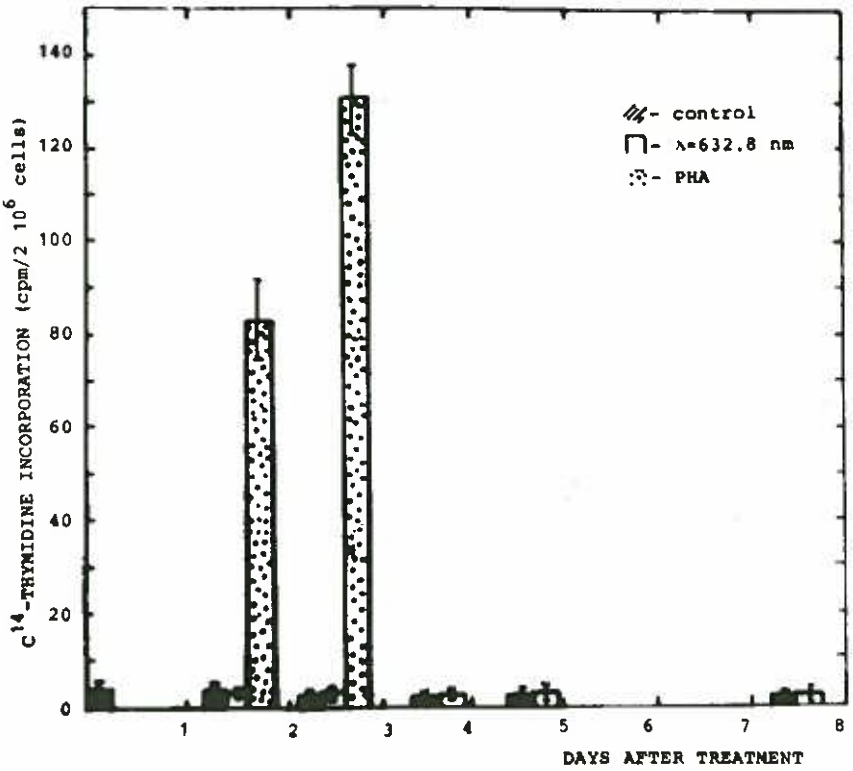


Figure 13. [^{14}C]-thymidine incorporation into DNA of lymphocytes after irradiation the cells with He–Ne laser (D=56 J/m^2) or treatment with PHA (2 μg/ml)

is in accordance with the literature data.[2–4] In the irradiated cells, no differences compared to the controls can be observed even 7–8 days after the exposure (Figure 13).

In order to check whether a repeated irradiation can have an effect, we irradiated the cells once more 4 h after the first exposure (4 h being that time within which the AO-chromatin binding was observed to be at its minimum (Figure 4). In some of the experiments, the cells were irradiated three times (4 h and 6 h after the first exposure, Figure 14). As seen in Table 4, no increase in the incorporation of [^{14}C]-thymidine after 72 h was observed in these experiments either.

Blood from 20 donors (measurements in triplicate) was used in this series of experiments.

Evidently there is no increase in DNA synthesis level after the irradiation of the lymphocytes with He–Ne laser (Figure 13, 14; Table 4). As a next step in our studies we tried to get an answer to the question: if the irradiation could increase the level of DNA synthesis when PHA is added after the irradiation. The cells were irradiated at a dose of 56 J/m^2, and after the irradiation, PHA in concentrations of 0.5, 1, 2 or 4 μg/ml was added. DNA synthesis rate was measured after 72 h.[26]

Experiments were performed in triplicate using lymphocytes from the blood of six different donors. Results are shown in Table 5 as indices of incorporation of [^{14}C]-thymidine (index of incorporation =

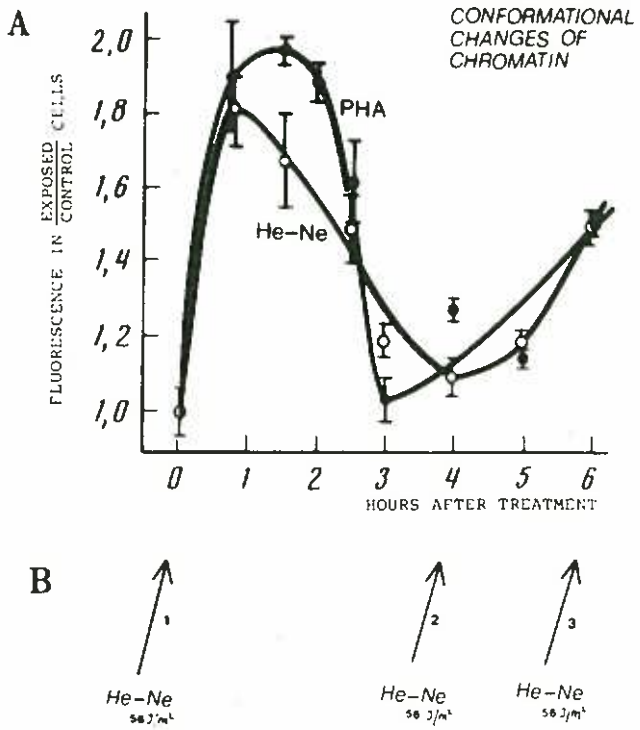


Figure 14. A, conformational changes of chromatin (intensity of fluorescence of acridine orange–DNA complex) after treatment with PHA (4 μg/ml) or irradiation with He–Ne laser (56 J/m^2); B, a schematic indicating the time points when the lymphocytes were irradiated with He–Ne laser in the experiment where DNA synthesis rate was measured: one time (arrow 1, results are shown in Figure 13), two times (arrows 1 and 2, results in Table 4, and three times (arrows 1, 2 and 3, results in Table 4)

Table 4. Incorporation of [^{14}C]-thymidine in DNA (cpm per 2 × 10^6 cells) after double and triple irradiation with He–Ne laser (D = 56 J/m^2) of human peripheral lymphocytes

Experiment	Control	Double irradiation (4 h interval between treatments)	Triple irradiation (4 h and 6 h after first treatment)
1	837	610	642
2	570	730	520
3	487	948	650
4	633	540	938
	721 ± 130	707 ± 198	688 ± 200

cpm of treated sample/cpm of intact control). The effect of irradiation is indicated as percentage efficacy (rate of thymidine incorporation index in the case of joint action of irradiation and PHA and in the case of PHA alone). It means that in all cases when the percentage efficacy is higher than 100, the irradiation elevates the DNA synthesis level as compared with action of PHA alone.

As seen in Table 5, the inducibility of DNA synthesis by PHA varies from donor to donor, i.e. it is not similar for all subjects. PHA is the specific mitogen for T-lymphocytes[2] and the level of induced proliferation depends on the rate of T-helpers and T-suppressors.[60] In work of Korneeva *et al.*,[60] it was shown that the decrease of the number of T-helpers and the increase of the number of T-suppressors causes the lowering of PHA-induced DNA synthesis level. Further experiments are needed to explain if this could be the case in our experiments also.

As a rule, the irradiation increased the effect as compared with the action of PHA alone (Table 5). Maximal effect of irradiation was found for PHA concentrations 1.0 and 2.0 μg/ml. At very low (0.5 μg/ml) as well as at higher (4.0 μg/ml) PHA concentrations the effect of irradiation was less pronounced.

The irradiation elevated the DNA synthesis level as compared with the action of PHA alone (Table 5). For example, in cases when PHA concentration was suboptimal (1 μg/ml), the effect was especially noticeable, exceeding the effect of PHA alone in the optimal concentrations (2 μg/ml). The mechanism of this boosting effect of irradiation is not clear. First, one can explain this with the fact that part of metabolic processes connected with activation occurs under the action of light as well. In this way, the effective dose of PHA per cell decreases and more cells can be activated by PHA. Second, in so far as He–Ne laser radiation induces ATP extrasynthesis,[55] this energy can be successfully used for increasing some ATP-dependent reactions in the early stages of signal transduction (e.g. patching/capping of components expressed by PHA receptors).

Combined Action of Interleukin-2 and Irradiation

Quiescent (G_0) lymphocytes isolated from peripheral blood constitute approximately 70–80% of the T-lymphocytes present.[4] Upon stimulation with T-cell specific mitogenic lectin PHA, those cells enter the G_1-phase of the cell cycle where they produce T-lymphocyte growth factor interleukin-2 (IL-2) and express its receptors (IL-2R).[2] Individual T-cell growth is regulated both by IL-2 and by the presence on T-cells of IL-2R which are induced by PHA and IL-2 itself.[2,6]

Our previous experiments clearly showed that irradiation with He–Ne laser induced some early changes in lymphocytes similar to those caused by PHA (activation of transcription; Ca^{2+} influx). At the same time, the irradiation itself did not cause the proliferation of lymphocytes (see section on DNA synthesis), but elevated the proliferation level induced by PHA (Table 5).

Table 5. Indices of [^{14}C]-thymidine incorporation*: Irradiation of lymphocytes with He–Ne laser (56 J/m^2) and treatment with PHA in various concentrations

	0.5 μg/ml PHA			1.0 μg/ml PHA			2.0 μg/ml PHA			4.0 μg/ml PHA		
Donor	PHA	He–Ne+ PHA	%†	PHA	He–Ne+ PHA	%	PHA	He–Ne+ PHA	%	PHA	He–Ne+ PHA	%
I	40.8±3.4	48.4±3.5	118.6	85.4±11.0	129.4±3.9	151.5	102.4±6.7	143.5±28.0	140	—	—	—
II	—	—	—	82.9±4.5	142.2±7.8	171.6	84.0±3.0	84.9±8.2	101.1	—	—	—
III	—	—	—	59.1±8.5	60.2±11.7	101.9	88.5±5.4	120 ±5.1	136.5	—	—	—
IV	—	—	—	22.8±3.0	39.8±4.6	174.0	47.5±7.8	73.6±11.3	154.9	—	—	—
V	—	—	—	—	—	—	100.5±12.3	123.5±7.0	122.9	85.6±6.6	87.6±7.3	102.3
VI	—	—	—	—	—	—	118.1±15.5	121.0±5.3	102.4	85.4±7.9	111.9±7.6	130.7

*Index of [^{14}C]-thymidine incorporation = $CPM_{experiment}$: $CPM_{intact\ control}$.
†% of efficacy of irradiation = $Index_{He-Ne+PHA}$:$Index_{PHA}$.

If PHA is removed from the cellular suspension within 6–7 h of its addition, the activated T-lymphocytes return to G_0-phase of the cell cycle. However it is sufficient to incubate the lymphocytes with PHA for less than 1 h and then force them to proliferate by adding IL-2.[6,61,62] The reason for such a result is the expression of IL-2R during the short contact with PHA. To explain if the IL-2R are expressed by irradiation, we performed a series of experiments, adding IL-2 to irradiated cells.[7,8]

The experiments were performed with blood from two donors. There were 10 experimental groups and the results in Table 6 are given as $M \pm m$ from three samples. IL-2 was added to intact cells, to PHA-activated (for 45 min) cells or to cells irradiated with selected doses of He–Ne laser.

Recombinant human IL-2 (Biogen, Riga, Latvia) with a concentration of 150 units/ml was used. The lymphocytes under investigation were (a) intact; (b) treated with IL-2; (c) stimulated by PHA (2 μg/ml); (d) stimulated by PHA (2 μg/ml) for 45 min, then washed with medium 199; (e) stimulated by PHA (2 μg/ml) for 45 min with subsequent washing with 199 medium and addition of IL-2; (f) irradiated with He–Ne laser (doses from 7 to 745 J/m^2) and IL-2 added after the irradiation; (g) irradiated with doses from 7 to 745 J/m^2. The incorporation of [^{14}C]-thymidine into DNA was measured after 72 h.

As seen in Table 6, the treatment of intact cells with IL-2 caused practically no effect (the increase of DNA synthesis index to 6.4 or 9.9 is considered not to be connected with proliferation.[3] Indeed, in intact cells the IL-2R are not expressed.[6] The level of DNA synthesis was high in PHA-treated cells even in this case when the PHA was removed 45 min after the beginning of the incubation. At the same time, 45-min incubation with PHA without IL-2 had practically no effect, the index of DNA synthesis being 9.4 (Table 6). These results show that it is sufficient to incubate cells for 45 min with PHA as events occur in the lymphocytes which render them susceptible to the action of IL-2 (i.e. the expression of IL-2R).

The response of irradiated lymphocytes to IL-2 was found to be comparable in all cases to the response of the control cells to IL-2 (Table 4). This result was confirmed using various concentrations of IL-2 from 100 to 700 U/ml (data not shown).

The results of these experiments allow us to conclude that the irradiation of lymphocytes with He–Ne laser does not increase the expression of IL-2R.

Laser Irradiation as a Ligand-Independent Type of Treatment

Specific binding between a surface receptor and a ligand (e.g. lectins like PHA or Concanavalin A) is required for lymphocyte activation and replication.[3] Interactions between lymphocyte surface receptors and various ligands initiate a signal which is transduced from the cellular membrane to the nucleus and subsequently induces proliferation.[2–4] Despite different chemical structures of the activating ligand, the signal transduction is considered to be more or less similar in all cases including a complicated cascade of changes in parameters of cellular homeostatis in seconds and minutes (e.g. K^+ and Ca^{2+} influx and Na^+ efflux, changes in cell membrane electrical potential), hours (e.g. RNA and protein synthesis) as well as days (DNA synthesis) after the beginning of stimulation (see Figure 1). This type of activation is a ligand-dependent treatment. The activation of lymphocytes by laser radiation is a ligand-independent treatment. According to the laws of photobiology, in this case as a first step the light should be absorbed by some molecules—photoacceptors. Effects following irradiation with low incident doses of laser energy are considered to be photobiological phenomena: from the absorption of light in mitochondria, and only after that has occurred, the signal is transduced to the cellular membrane and then to the nucleus.[44–46] It is not clear if that is also the case in lymphocytes. An action spectrum reflecting the absorption spectrum of the primary photoacceptor(s), is absent. On the other hand, the changes in structure of chromatin, which does not have absorption bands at 632.8 nm, after irradiation clearly show the existence of a signal transduction chain from some photoacceptor to the nucleus. The

Table 6. DNA synthesis level* 72 h after irradiation of lymphocytes with He–Ne laser in various doses or treatment with PHA (2 μg/ml) in presence of interleukin-2 (150 U/ml)

	Control		PHA (2 μg/ml)			Irradiated in various doses				
Donor	Intact	Intact + IL-2	72 h	45 min	45 min + IL-2	56 J/m^2	56 J/m^2 + IL-2	7 J/m^2 + IL-2	112 J/m^2 + IL-2	754 J/m^2 + IL-2
I	1.0±0.2	9.9±1.2	168±11.1	9.4±0.8	85.1±10.1	1.0±0.1	10.2±0.8	5.0±1.2	8.4±1.3	4.8±0.9
II	1.0±0.1	6.4±0.4	205.9±8.3	—	105.9±3.1	1.0±0.1	7.3±0.6	—	—	—

*As index $CPM_{experiment}:CPM_{intact\ control}$.

question, if these photoacceptors are located in the mitochondria, needs further explanation. Electron-microscopical investigations show that there are changes in mitochondria in short time periods after He–Ne laser irradiation.

Our experiments have shown that the chromatin of human lymphocytes irradiated with red light at λ = 632.8 nm at first minutes and then hours after the irradiation was subjected to changes similar to those observed following mitogenic stimulation with PHA.

Firstly, there is the similarity in conformational changes of chromatin after both types of treatment during the first 6 h (Figure 15). Such a three-stage character in the changes of the chromatin structure in resting cells induced to proliferate by a variety of stimuli differing in nature is apparently universal,[4,63] and is believed to reflect the changes in chromatin template activity.[2,4] The changes in chromatin template activity should be accomplished by consequent changes in the RNA synthesis rate as in the case for PHA.[3,62] In the case of irradiated cells, the changes in the RNA synthesis rate during the first 7 h after irradiation are quantitatively similar to the respective changes in PHA-treated cells (Figure 15).

Second, the rapid increase in the concentration of free calcium in the cytosol both after adding

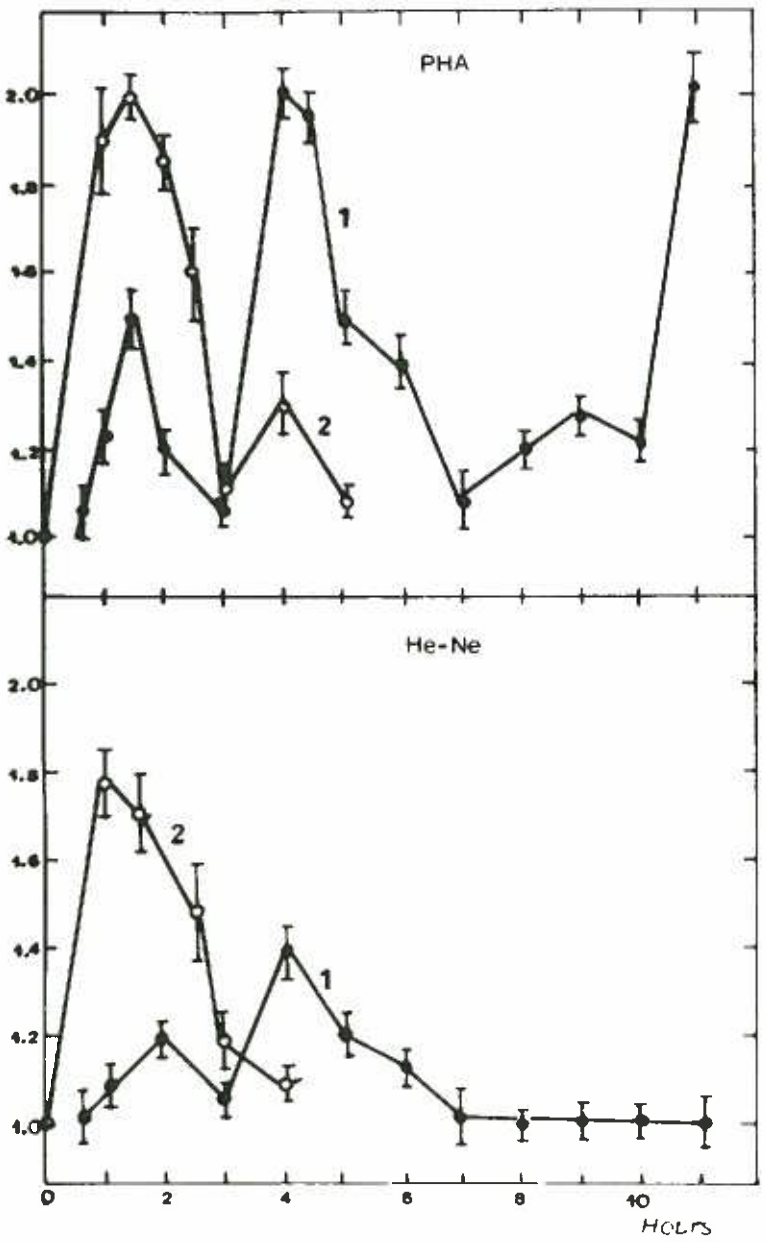


Figure 15. Comparison of the kinetics of two activation events stimulated by PHA (4 μg/ml) or He–Ne laser radiation (56 J/m²) in human lymphocytes. 1, Isotope uptake; 2, AO binding by the cells are presented as the ratio of the values obtained with stimulated and control cells

PHA to, or following laser irradiation of the cells, (Table 1) confirms the possibility of light acting like a proliferative stimulus. In the case of PHA treatment such an increase of $[Ca^{2+}]_i$ is considered to be an obligatory event in T-lymphocyte activation. It activates a protein kinase which takes part in RNA synthesis.[5,6]

The similarity in early responses of the lymphocytes to the action of two principally different agents, PHA and He–Ne laser radiation, is of considerable interest for the reason that PHA acts on cells during 1 h, because it is constantly present in the cell suspension, whereas the laser irradiation action lasts only for the first 10 s.

A ligand-independent method of lymphocyte activation is described in the paper of Bourguignon *et al.*[64] High-voltage pulsed galvanic (electrical) stimulation of mouse splenic T-lymphocytes increased the Ca^{2+} influx, and induced the expression of IL-2R during the 5 min of activation (IL-2R expression reached maximum levels 20 min after the stimulus). The capping of IL-2R and the synthesis of DNA were also induced. Whenever IL-2R capping was blocked, there was always a reduction in DNA synthesis. There was no S-phase reached when IL-2 was not added but, contrary to our experiments, IL-2R were expressed.[64] The authors conclude that the exposure and subsequent patching/capping (redistribution of receptors into small clusters and pig aggregates) of at least one hormone receptor is required for the activation of T-lymphocytes. This is one difference when comparing the lymphocyte responses to electrical and laser stimulation, and this difference could explain the absence of DNA synthesis in our case (Figure 13).

The mechanisms by which ligand-dependent agents elicit their cellular effects include the rapid transcriptional activation of a set of genes. Our electron-microscopic studies showed evidence of expression of rRNA genes 1 h after both ligand-dependent (PHA) and ligand-independent (laser) treatment.

The rapid increase in the RNA synthesis rate after the beginning of PHA treatment is connected with the activation of certain genes encoding not only r-genes but also growth factors and their receptors, as well as with activation of some protooncogenes. A marked increase within 10 min in the levels of mRNA's for c-fos and c-myc, within 6 h for IL-2R and IL-2 mRNA's, within 14 h for c-myb, p53, N-ras and TFR mRNA's has been detected.[62,65]

It is not excluded that there is an activation of genes like c-fos (e.g. elevation in c-fos and β-actin RNA's is detectable within minutes after stimulation, peaks at 10–20 min and returns to prestimulated level within 2h), and c-myc (c-myc RNA does not peak until 1–2 h after stimulation and decays over a longer period).[65] A cohort of genes that behave like c-fos are required for transition from G_o-phase to G_1-phase but they are not essential for passage through the rest of the cell cycle.[66] The gene c-fos is also extremely sensitive to cellular conditions and can be transcriptionally activated by a wide variety of agents, including stress.

Our data do not allow the exclusion of the possibility that irradiation with monochromatic (red) light transcriptionally activates some of the so-called early response genes, including protooncogenes like c-myc and c-fos.

It is important to note that despite the similarities in early cellular responses to both kinds of treatment (increase in intracellular Ca^{2+} concentration, total RNA synthesis and template activity of DNA) the irradiation did not result in blast-transformation of the lymphocytes. This difference was explained by the absence of interleukin-2 receptor expression in irradiated T-lymphocytes. Therefore, the reaction of the expression of r-genes can be considered as an activation of genes within the G_o-phase of the cellular cycle, whereas similar reactions in PHA-treated cells connected with the $G_o \longrightarrow S$ transition.

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