Chemiluminescence as a Method for Detection and Study of Free Radicals in Biological Systems

Yu. A. Vladimirov, E. V. Proskurnina*, and D. Yu. Izmailov*

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Supplement 2, pp. 13-20, April, 2007
Original article submitted February 18, 2006

Chemiluminescence observed during LPO or reactions of nitric oxide and oxygen radicals and was named "ultraweak luminescence". In the presence of chemiluminescence activators (luminol, lucigenin, rhodamine G, or coumarine C-525) the appearance of radicals is associated with intensive fluorescence; the registration of this fluorescence is widely used in biomedical and clinical studies.

Key Words: *chemiluminescence; radicals; kinetics; disease; antioxidants*

Russian scientist Alexandre Gurvich was presumably the first who noted that free radical reactions are responsible for fluorescence of biological objects; in his experiments this fluorescence manifested in UV spectrum and was called mitogenetic [1]. From chemical viewpoint radicals are very active particles and their reactions are associated with release of energy, often sufficient for the formation of products in electron-excited state. In this case chemical reaction is accompanied by fluorescence, or chemiluminescence (CL). Fluorescence in biological systems can be observed directly during reactions of radicals; in this case it is called "natural CL"; however, CL is often observed only in the presence of certain fluorescent substances, called activators [19,20]. Activators, in turn, can be divided into chemical (participating in reactions with free radicals) and physical (not participating in chemical reactions, but amplifying the quantum yield of radiation at the expense of electron excitation energy transfer from the excited product molecule to the activator molecule) [8,27]. Development of sensitive measuring equipment (chemiluminometers) and application of CL activators led to wide use of the CL method in biomedical studies in all cases when the level of free-radical processes or antioxidants (AO) in human and animal cells, tissues, and organs was to be evaluated [8,27].

Ample data on the use of the CL method for studies of free radical reactions and evaluation of their intensities in humans and animals cannot be reviewed in a single paper. We shall offer only examples with emphasis on the studies carried out at our laboratory. The papers in which the reader will find additional information are offered in references.

Equipment

The intensity of CL of living cells and tissues without activators is extremely low, and hence, in order to detect natural CL of these objects, photoelectron amplifiers (PEA) cooled with liquid nitrogen for reducing dark current were previously used; the fluorescence was called "ultraweak" [5]. Modern PEA are sufficiently sensitive and their dark current is low even at ambient temperature, due to which the fluorescence of cells (for example, phagocytes) or tissue fragments can be measured without PEA cooling. The chemiluminometer amplifier, adapted for measuring the CL kinetics, amplifies and filters the pulses transmitted from the photocathode under the effect of falling photons (Fig. 1). Importantly that a thermostat is attached to the device, as the CL

Institute of Physicochemical Medicine, Federal Agency for Health Care and Social Development, Moscow; *Faculty of Fundamental Medicine, M. V. Lomonosov Moscow State University

0007-4888/07/1443-0390

thermal coefficient is usually very high, and therefore the fluorescence at ambient temperature can be many times lower than at body temperature; in addition, slight fluctuations of temperature lead to significant changes in the level of fluorescence. If the initial stages of rapid reactions are to be measured, rapid introduction of reagents during the experiment should be provided. Unfortunately, chemiluminometers often do not meet these requirements.

Processing of the results of measurements is no less important, particularly when dynamic information on the time course of CL is needed. For example, potent PowerGraph software (http://www.powergraph.ru) is attached to the Lum-5773 device (Interoptika), due to which the CL curves can be recorded in different modes, the noise can be leveled, derivative functions can be obtained, maximum and minimum values can be found, and hundreds of other useful operations with experimental records are possible.

Fig. 1. SmartLum chemiluminometer. *1*) body with elements of electronics and feeding and a thermostat for cuvette; *2*) removable block with mixer; *3*) mixer electric motor; *4*) cuvette with the mixer inside; *5*) PEU. Elements *2-5* are inside the body or removable block; *6*) catheter for introduction of reagents.

Natural CL ("ultraweak luminescence")

Luminescence accompanying chain oxidation of lipids. Our knowledge of the mechanism of chain oxidation of lipids in biological membranes and plasma lipoproteins are largely based on measurements of the CL kinetics. From the classical chemistry viewpoint, LPO reaction consists in inculcation of an oxygen molecule into polyunsaturated fatty acid molecule with the formation of hydroperoxide at the level of α-carbon atom (for double bond):

$$LH+O_2 \rightarrow LOOH.$$

Actually the reaction is realized according to a well-known scheme as a chain process starting and continuing with participation of radicals [3]; the main oxidation chain represents multiple repetitions of two alternating reactions:

$$L_n + O_2 \rightarrow L_nOO^{\bullet}.$$

The chains branch in the presence of Fe^{2+} as a result of degradation of hydroperoxide (chain process product):

$$LOOH+Fe^{2+} \rightarrow Fe^{3+}+OH^{-}+LO^{\bullet}$$
$$LO^{\bullet}+LH \rightarrow LOH+L^{\bullet} \rightarrow \text{new oxidation chain.}$$

Combination of these reactions with the reactions of chain initiation and breaks, which have several pathways, provides an intricate system of interrelated processes, the main participants of which are radicals. But radicals are inaccessible for common chemical analysis because of their extreme activity and therefore instability, and hence, it is impossible to isolate or analyze them by routine methods. But it is the $LOO^{\bullet}$ radical reaction that is associated with CL, and hence, the CL intensity (I_{CL}), level of radicals ($LOO^{\bullet}$), and hence, LPO rate (w) are in unambiguous direct relationship:

$$I_{CL}=Aw^2=B[LOO^{\bullet}]^2,$$

where A and B are proportionality coefficients.

This allows monitoring of LPO reaction rate by measuring the CL kinetics in a nonstationary mode, for example, after addition of Fe^{2+} salts to suspensions of phospholipids, mitochondria, lipoproteins, cells, *etc.* [3,24]. Study of CL kinetics, O_2 consumption, accumulation of LPO products, and Fe^{2+} oxidation to Fe^{3+} not only confirmed the scheme of branched chain lipid oxidation reactions, but also helped experimentally determine the effective rate constants for the three basic reactions: chains continuation, branching, and break [18,24]. When we know these constants and the initial concentrations of the main participants in the process: oxidized substrate (polyunsaturated fatty acids), O_2, and Fe^{2+} ions, we can calculate (from differential equations) the kinetics of not only these substance concentrations, but also of the reaction products (hydroperoxides) and intermediates, *e.g.* free radicals. Today this procedure is essentially simplified due to development of Kinetic Analyzer software [10,11].

The complex curve of Fe^{2+}-induced CL of mitochondrial or phospholipid vesicle (liposome) sus-

pension undergoes characteristic changes under the effect of AO: the "slow flash" of CL is inhibited, the degree of this inhibition directly depends on the concentration of added AO and its activity [3,21]. The simplest indicator of AO activity is delay of attaining slow flash maximum after addition of Fe^{2+} salts to the lipid-containing sample. More informative data can be obtained only by combined use of measurements of the CL kinetic curves after addition of Fe^{2+} and mathematical simulation (Fig. 2). Due to this procedure it is possible to more precisely evaluate the constants of AO reaction with radicals maintaining the oxidation chain and evaluate the fate of the AO radicals [10]. To make the short story long, let us note that evaluation of CL kinetics in lipid systems is more informative after CL activation by C-525 coumarine: in this case side effects in the reaction do not interfere with the data interpretation, except the dioxide radical disproportioning reaction. Measurements of the kinetics help to understand the mechanism of AO effect. For example, the AO effect of ascorbic acid in a system containing Fe^{2+} salts is due to recycling (reduction) of Fe^{2+} from Fe^{3+}, when Fe^{2+} (but not ascorbic acid proper) captures free radicals in the system [10]. AO effect of carnosine is due to its capacity to form complexes with Fe^{2+} ions, this preventing their branching of oxidation chain, but not due to its function as a radical trap [14].

Fluorescence in reactions of active oxygen and nitrogen forms. Very weak fluorescence during the formation of active O_2 forms, such as H_2O_2, superoxide radicals, and hydroxyl is presumably caused by the formation of singlet and triplet oxygen complexes (eximers) [1]. The fluorescence is more intense during reaction of peroxynitrite with proteins, but the nature of this fluorescence is little studied. No practical application of these weak fluorescences is known [20].

A flash of fluorescence is observed in exposure of protein-containing systems to sufficiently high concentrations of H_2O_2; it is presumably caused by the formation of O_2 eximers and organic radicals in the system [27].

Natural CL as a marker of oxidative stress. The term "oxidative stress" was introduced by H. Ziss in 1991 and was included in the Pubmed dictionary in 1995. Oxidative stress is a shift of the prooxidant-AO balance in favor of AO. Oxidative stress manifests by accumulation of damaged DNA bases and products of protein oxidation and LPO, as well as by reduction of AO level and increased sensitivity of membrane lipids and lipoproteins to prooxidant effects (including Fe^{2+} ions or H_2O_2), associated with reduced AO level.

Measurements of natural CL of the plasma, serum, or erythrocyte suspension after addition of Fe^{2+} salts or H_2O_2 are widely used in clinical laboratory analysis [27]. CL responses to Fe^{2+} increase 2-3-fold in inflammatory diseases, due to which, for example, phlegmonous forms of appendicitis can be differentiated from catarrhal forms. The am-

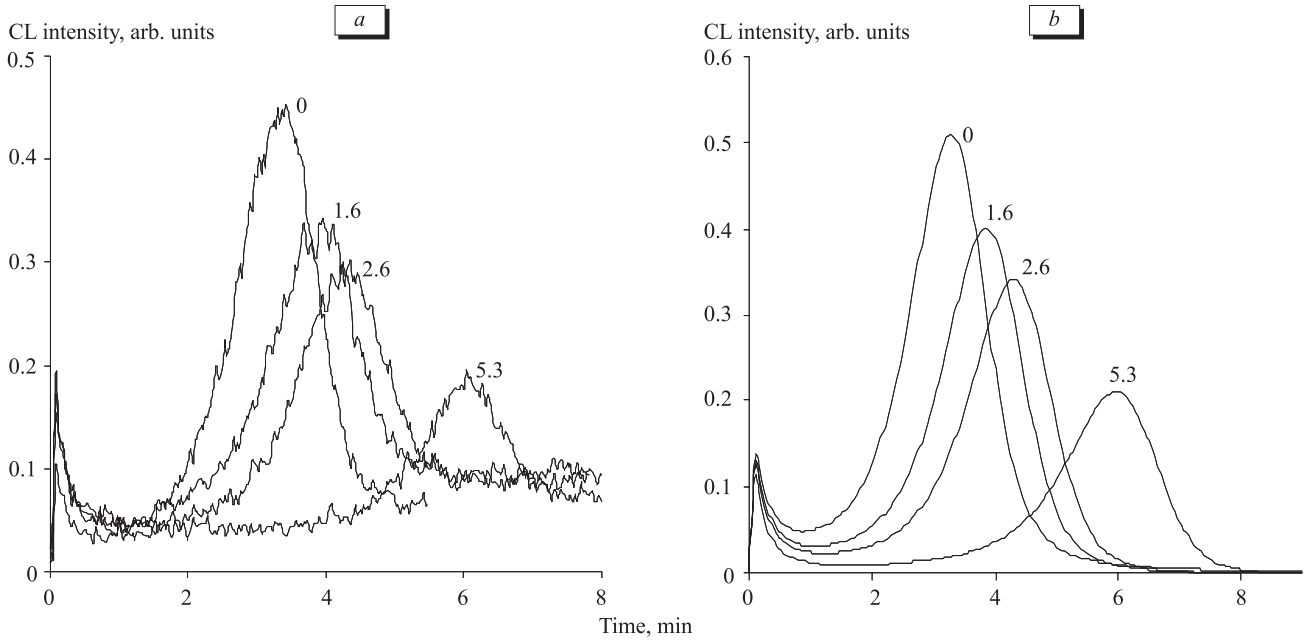

Fig. 2. Kinetics of CL in phospholipid liposome suspension at different initial concentrations of β-carotene (according to [10]). *a*) experimental data; C-525 coumarine was used as physical activator of CL; *b*) mathematical simulation. Figures at the curves show β-carotene concentrations (μM).

plitude of CL sharply increases in pancreatitis. A significant decrease in the amplitude below the normal in deep hypoxia serves as an alarming prognostic sign [27]. An appreciable intensification of Fe^{2+}-induced CL of the plasma and lipoproteins isolated from it is observed in hypercholesterolemia: it is caused primarily by increased level of LPO substrate (LDL) and increase in LDL oxidation [27]. Accumulation of oxidized LDL is an atherosclerosis risk factor [17].

One more method for evaluation of oxidative stress and treatment efficiency, in addition to evaluation of Fe^{2+}-induced CL, is measurement of H_2O_2-induced fluorescence of the plasma, erythrocyte suspension, and wound exudation [27]. Unfortunately, the nature of the reactions during these processes remains little studied, and hence, this method cannot yet be recommended for practice.

Addition of Fe^{2+} to the urine leads to CL flash, presumably caused by the appearance of tryptophan oxidation products in the urine, which can serve as an indicator of oxidative stress. The flash is reduced in renal failure and rheumatoid arthritis and increased in appendicitis [27].

Activated CL

Chemical activators, luminol-dependent CL. Luminol is the best known CL detector or a chemiluminogen probe [14]; it induces intensive fluorescence in the system with generation of active oxygen forms or on-going LPO. The mechanism of luminol reactions leading to the formation of exitated molecule of the product is intricate and includes luminol oxidation as the first step, followed by reduction of oxidized product by superoxide radical. Oxidation can be realized either by hydroxyl radical or by any other strong oxidant, for example, hypochlorite. Great number of possible oxidation reactions for luminol creates certain difficulties in interpretation of results of luminol-dependent CL in complex systems, to which living cells, no doubt, belong.

Luminol can be also oxidized in peroxidase-catalyzed reactions, presumably as a result of interaction between luminol molecule (LumH) with compound I ($^{\bullet}Px(Fe4^{+})$-OH):

$$Px(Fe^{3+})+H_2O_2 \rightarrow {}^{\bullet}Px(Fe^{4+})\text{-}OH+ H_2O_2$$
$$^{\bullet}Px(Fe^{4+})\text{-}OH+LumH \rightarrow Px(Fe^{3+})+Lum(Ox).$$

Reduction of oxidized luminol (by superoxide or hydrogen peroxide) leads to the formation of electron-excited product (P*) and emission of a photon:

$$Lum(Ox)+{}^{\bullet}O^{-} \rightarrow ...P^{*} \rightarrow P+photon.$$

We used luminol-dependent CL for studies of peroxidase activity of cytochrome *c* associated with cardiolipin [7]. The kinetics of CL (initial elevation of fluorescence and its subsequent drop) confirms

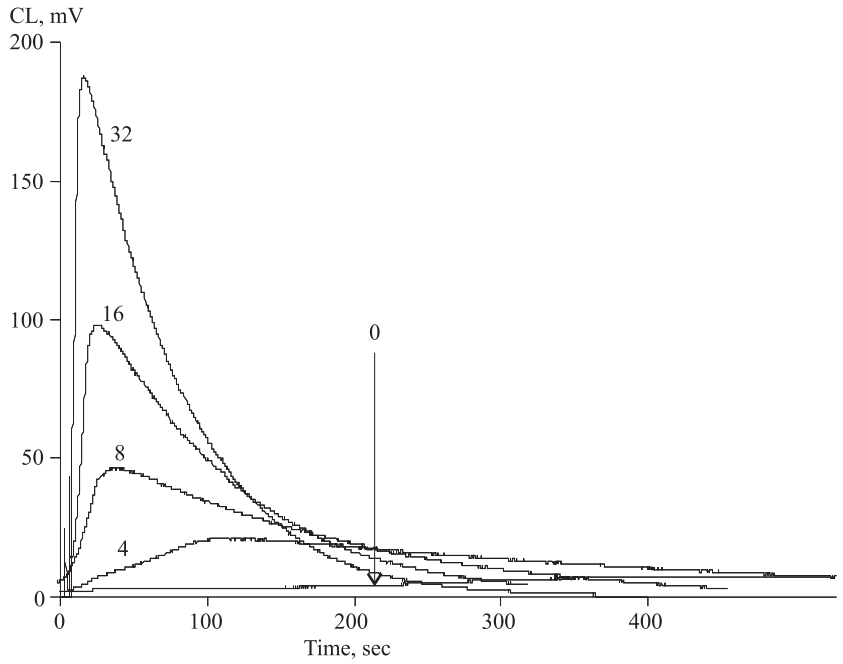

Fig. 3. Effect of tetraoleoyl cardiolipin on peroxidase activity of cytochrome *c* (according to [7]). Figures at the curves: cardiolipin/protein molar ratio. Peroxidase activity was evaluated by the intensity of luminol-dependent CL. Two phases of the curve (elevation and drop) correspond to two luminol reactions.

the two-staged pattern of the reaction (Fig. 3). Cardiolipin specifically potentiated the initially low peroxidase activity of cytochrome *c* many times stronger than free fatty acids or anionic detergents. Activation of the cytochrome *c* peroxidase activity is now regarded as the initial stage of apoptosis [7].

Radicals of organic compounds, and not only hydroxyl radical, can trigger the cascade of luminol oxidation reactions associated with CL. For example, this phenomenon underlies the method for measurements of AO in a studied system. It includes measurement of CL kinetics in solutions to which a source of radicals, for example 2,2*-azobis(2-amidinopropane, ABAP) is added. Thermal (spontaneous) degradation of ABAP leads to the formation of radicals oxidizing luminol and development of CL reaching the constant level. Antioxidants, such as ascorbic acid or α-tocopherol, effectively compete for radicals, and hence, CL in the system is suppressed until complete oxidation. The duration of this delay in the development of CL (latent period) is proportional to AO content in the cuvette. This method is widely used for evaluating the content of AO in foodstuffs, vine, tea, *etc.* In our laboratory this method is used for the analysis of AO content in patients' sera.

Lucigenin-dependent CL. Lucigenin (bis-*N*-methylacridinium) is widely known in practical biochemistry as a specific CL probe for a superoxide radical. The mechanism of reactions associated with CL includes the following stages:

1) single-electron reduction of lucigenin (a bivalent cation):

$Luc^{2+}+e^{-}\rightarrow Luc^{+}$ (lucigenin cation-radical);

2) cation-radical reaction with superoxide:

$^{\bullet}Luc^{+}+{}^{\bullet}OO^{-}\rightarrow Luc{=}O$ (lucigenin dioxetane);

3) dioxetane degradation with the formation of excited product molecule, N-methylacridone, and photone emission:

$$Luc{=}O\rightarrow ...MA^{*}+photon.$$

Measurements of CL can be used for evaluation of superoxide radicals in biological systems. Two circumstances should be taken into consideration in this case. First, the first-stage reaction can be realized by different reducing agents with different efficiency. The superoxide proper also can realize it:

$$^{\bullet}OO^{-}+Luc^{2+}\rightarrow {}^{\bullet}Luc^{+}+O_2.$$

However, the rate of this reaction is low, and it is highly possible that lucigenin cation-radical in biological systems forms due to functioning of electron carriers. Therefore, the fluorescence intensity, determined by not only superoxide content, but also the content of lucigenin cation-radical, presumably depends on the work of these transmitters.

The second, even more important circumstance, is that both, lucigenin and the product of its single-electron reduction, carry positive charges and are therefore accumulated inside cell structures with a negative electric potential. It is surprising, that this fact is neglected by scientists investigating the formation of superoxide in the mitochondria and whole cells by lucigenin-dependent CL. But according to Nernst's equation, 60-mV difference of potentials at the membrane determines 10-fold difference in cation-radical concentrations and 100-fold difference in lucigenin concentrations. The membrane potential of energized mitochondria is about 180 mV, while the cell potential adds 60-70 mV more. All this means that lucigenin concentration in mitochondria can be 100,000 times higher than its concentration in the cell and 1,000,000 times higher than its concentration in the extracellular environment. Hence, lucigenin-dependent CL of cells and tissues is virtually completely determined by the content of superoxide in the mitochondrial matrix. Changes in the work of the mitochondrial respiratory chain lead to dramatic changes in the lucigenin fluorescence in suspensions of mitochondria and living cells not because of changed rate of the superoxide production, but because of minor modulations of the mitochondrial membrane potential. For example, reduction of O_2 concentration sharply reduces the lucigenin-dependent CL of animal tissue sections.

Activated fluorescence in chain reactions of lipid oxidation. Activators of CL in LPO have been described long ago [1], but their efficiency was poor and, importantly, these were not physical activators, but chemiluminogenic samples, such as luminol [27]. The first very effective activator was rhodamine G [23], which was then used for studies of Fe^{2+}-induced plasma CL (Table 1). The most potent activators are europium complex with chlortetracycline and coumarine C-525 dye [19,26]. Coumarine C-525 in nanomolar concentration led to more than 1000 times increase of CL flash after addition of Fe^{2+} to the suspension of phospholipid liposomes, the kinetics of CL remaining unchanged, similarly as the volume of LPO products forming in the system. This means that C-525 acts as a physical activator. Without activator the fluorescence in the system, in which chain oxidation of lipids is in progress, is realized as a result of formation of excited ketone molecules:

$$LOO^{\bullet}+LOO^{\bullet}\rightarrow LOH+O_2+L=O^*.$$

Quantum yield of ketone emission is very low (of the 10^{-4} order of magnitude), which determines low intensity of CL in general. The presence of physical activator A leads to electron excitation energy transfer:

$$L=O^*+A\rightarrow L=O+A^*,$$

as well as subsequent emission of the photon with high quantum yield. The intensity of CL stimulated by C-525 approaches its theoretical threshold, which is attained after 100% energy transfer and quantum yield of the activator fluorescence, equal to 1.

Unfortunately, coumarine-activated CL is not widely used in studies on biological membranes, though fluorescence of coumarine-stained cultured cells with activated LPO was detected [22]. The use of C-525 as a CL activator was effective in studies of LPO in liposome suspension and AO effect on this process [10,11].

Cell CL

Natural CL of blood leukocytes, observed in stimulated cells, was discovered in 1972 [15]. Registration of natural leukocyte CL was little used in practice because of its low intensity, but after some time the same authors suggested luminol for intensification of the same fluorescence, that is, as a "chemiluminogenic probe" [15]. The intensity of blood leukocyte CL response in the presence of luminol is several thousand times higher than without it [25]. Unfortunately, the relationship between luminol-dependent CL of stimulated blood leukocytes and tissue macrophages, on the one hand, and the content and ratio of several products released by stimulated cells (superoxide radical, hydroxyl radical H_2O_2, and hypochlorite), on the other, is intricate and depends on intracellular or extracellular location of reduced pyridine nucleotide oxidases [8].

We cannot discuss all applications of cellular CL in a short review. Applications of cellular CL, described in publications before 1989, are discussed previously [8].

We showed that luminol-dependent CL of blood leukocytes, stimulated by opsonized zymosan, is sharply increased in rats with isoprenaline cardiomyopathy and after myocardial ischemia [12]. Chemiluminescence increased 2-3-fold in coronary patients (Table 2), 10-fold and more in myocardial infarction [13]. Cell activity in these cases increased as a result of increase in the number of receptors on the surface of polymorphonuclear leukocytes. Active oxygen forms, released by stimulated leukocytes, induced LPO in plasma lipoproteins; on the other hand, oxidized lipoproteins induced prestimulation (priming) of phagocytic cells [25]. Later it was shown that water-soluble LPO products also caused priming [16]. These studies formed the basic for the development of two trends of research. The first of them is application of cellular CL for evaluating the level of inflammatory processes. The highest CL response of cells to a stimulus (latex particles) is characteristic of patients with a pronounced inflammatory process (acute pneumonia, myocardial infarction, *etc.*). By contrast, chronic hypoxia leads to suppression of leukocyte activity below the normal. Similar data were obtained in studies of whole blood: barium sulfate (adherent cell precipitator/activator [9]) was added to diluted blood; a sharp increase of luminol-dependent CL was observed in patients with more pronounced inflammatory process [27]. The usefulness of the CL method directly for disease diagnosis is limited by several applications; for example, myocardial infarction can be detected in acute cardialgia in cases when the ECG changes are poorly pro-

TABLE 1. Rhodamine G-Activated Fe^{2+}-Induced CL of Sera from Cardiovascular Patients (according to [27]; $M\pm m$)

Group of patients	*n*	CL amplitude, 10^6 quanta×sec^{-1}×4π
Healthy volunteers	10	6.2±1.6
Type II hyperlipoproteinemia	25	26.0±1.5
Coronary disease or atherosclerosis of lower limb arteries	35	12.7±0.5*+

Note. $p<0.05$ compared to *healthy volunteers, +type II hyperlipoproteinemia.

TABLE 2. Amplitude of CL Response of Isolated Blood Polymorphonuclear Leukocytes in Patients (according to [25]; $M\pm m$)

Group of patients	*n*	CL amplitude, arb. units
Healthy volunteers	13	2.3±0.5
Acute pneumonia	22	7.1±1.0*
Acute myocardial infarction	18	6.0±0.8*
Lung cancer	11	6.7±0.8*
Mastopathy	17	5.6±0.7*
Achlorohydria	6	4.9±0.7*
Chronic coronary disease	29	4.0±0.7*
Atherosclerotic cardiosclerosis	16	3.4±0.6*
Asthma	5	1.3±0.4*
Chronic pneumonia	13	0.9±0.5*

Note. *$p<0.05$ compared to healthy volunteers.

nounced; however the method is obviously useful for evaluating changes in patient's status during disease development and treatment.

The second trend of research with the use of cellular CL is study of the mechanism of the biostimulatory effect of low-energy laser. It was found that low-dose exposure to He-Ne laser caused leukocyte priming, while higher doses led to reduction of phagocyte activity [2,4,6]. These and many other data obtained using luminol-dependent CL confirmed the hypothesis on the photodynamic mechanism of laser action, according to which the process starts from LPO photoinduced by endogenous hematoporphyrin, leading to Ca^{2+} entry into cell and activation of metabolic processes.

The study was supported by the Russian Foundation for Basic Research (grants Nos. 05-04-49765-a, 06-04-08278-ofi, 06-04-48451-a, and 06-04-81052-Bel_a).

REFERENCES

1. Yu. A. Vladimirov, *Ultraweak Luminescence in Biochemical Reactions* [in Russian], Moscow (1966).
2. Yu. A. Vladimirov, *Efferent Medicine* [in Russian], Moscow (1994), pp. 51-66.
3. Yu. A. Vladimirov and A. I. Archakov, *Lipid Peroxidation in Biomembranes* [in Russian], Moscow (1972).
4. Yu. A. Vladimirov, G. I. Klebanov, G. G. Borisenko, and A. N. Osipov, *Biofizika*, **49**, 339-350 (2004).
5. Yu. A. Vladimirov and F. F. Litvin, *Ibid.*, **4**, No. 5, 601-605 (1959).
6. Yu. A. Vladimirov, A. N. Osipov, and G. I. Klebanov, *Biokhimiya*, **69**, 103-113 (2004).
7. Yu. A. Vladimirov, E. V. Proskurnina, D. Yu. Izmailov, *et al.*, *Biofizika*, **71**, 1215-1224 (2006).
8. Yu. A. Vladimirov and M. P. Sherstnev, *Chemiluminescence in Animal Cells* [in Russian], Moscow (1989).
9. Yu. A. Vladimirov, M. P. Sherstnev, and A. P. Piryazev, *Biofizika*, **34**, 1051-1054 (1989).
10. D. Yu. Izmailov and Yu. A. Vladimirov, *Biol. Membran.*, **20**, 349-358 (2003).
11. D. Yu. Izmailov and Yu. A. Vladimirov, *Ibid.*, **19**, 507-515 (2002).
12. G. I. Klebanov, M. V. Kreinina, V. M. Pozin, *et al.*, *Byull. Eksp. Biol. Med.*, **106**, No. 9, 297-299 (1988).
13. I. M. Korochkin, G. I. Klebanov, I. I. Chukayeva, *et al.*, *Ter. Arkh.*, **56**, No. 8, 29-31 (1984).
14. Kh. I. Li, Yu. A. Vladimirov, and A. I. Deyev, *Biofizika*, **35**, 82-85 (1990).
15. R. C. Allen, *Methods Enzymol.*, **133**, 449-493 (1986).
16. L. V. Koval'chuk, G. I. Klebanov, S. R. Ribarov, *et al.*, *Biomed. Sci.*, **2**, No. 3, 221-231 (1991).
17. O. M. Panasenko, T. V. Vol'nova, O. A. Azizova, and Yu. A. Vladimirov, *Free Radic. Biol. Med.*, **10**, No. 2, 137-148 (1991).
18. Yu. A. Vladimirov, *Free Radicals, Aging, and Degenerative Diseases*, New York (1986), pp. 141-195.
19. Yu. A. Vladimirov, *Free Radicals in the Environment, Medicine, and Toxicology*, London (1994), 345-373.
20. Yu. A. Vladimirov, *Free Radicals. A Practical Approach*, Oxford, New York, Tokyo (1996), pp. 65-82.
21. Yu. A. Vladimirov, *ISNA*, New York (1996), pp. 125-241.
22. Yu. A. Vladimirov, A. Arroyo, J. M. Taylor, *et al.*, *Arch. Biochem. Biophys.*, **384**, No. 1, 154-162 (2000).
23. Yu. A. Vladimirov, T. B. Atanayev, and M. P. Sherstnev, *Free Radic. Biol. Med.*, **12**, No. 1, 43-51 (1992).
24. Yu. A. Vladimirov, V. I. Olenev, T. B. Suslova, and Z. P. Cheremisina, *Adv. Lipid Res.*, **17**, 173-249 (1980).
25. Yu. A. Vladimirov, S. R. Ribarov, P. G. Bochev, *et al.*, *Gen. Physiol. Biophys.*, **9**, No. 1, 45-54 (1990).
26. Yu. A. Vladimirov, V. S. Sharov, E. S. Driomina, *et al.*, *Free Radic. Biol. Med.*, **18**, No. 4, 739-745 (1995).
27. Yu. A. Vladimirov and M. P. Sherstnev, *Soviet Medical Reviews. Sect. B. Physicochemical Aspects of Medicine*, London (1991), Vol. 2, Pt. 5, pp. 1-43.