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BIOPHOTON RESEARH IN BLOOD REVEALS ITS HOLISTIC PROPERTIES

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Abstract

Monitoring of spontaneous and luminophore amplified photon emission (PE) from non-diluted human blood under resting conditions and artificially induced immune reaction revealed that blood is a continuous source of biophotons indicating that it persists in electronically excited state. This state is pumped through generation of electron excitation produced in reactive oxygen species (ROS) reactions. Excited state of blood and of neutrophil suspensions (primary sources of ROS in blood) is an oscillatory one suggesting of interaction between individual sources of electron excitation. Excited state of blood is extremely sensitive to the tiniest fluctuations of external photonic fields but resistant to temperature variations as reflected in hysteresis of PE in response to temperature variations. These data suggest that blood is a highly cooperative non-equilibrium and non-linear system, whose components unceasingly interact in time and space. At least in part this property is provided by the ability of blood to store energy of electron excitation that is produced in course of its own normal metabolism. From a practical point of view analysis of these qualities of blood may be a basement of new approach to diagnostic procedures.

Introduction

Ultra-weak photon emission (PE) in the optical spectral range from many cells is commonly thought to represent random imperfections accompanying normal physiological processes of oxygen consumption, and no biological function is usually ascribed to it. However evidence is accumulating that electron excited species are regular products of biochemical processes[1] and that energy of their relaxation may be used by living systems in different ways, such as provision of excitation energy for endergonic chemical reactions, photomodulation of enzyme activities, ets. [2]. Energy of electron excitation may be transmitted from the sites of its generation (donors) to the sites of its utilization (acceptors) both by radiation-less as well as by radiative mechanisms[3].

The major sources of electron excitation in living systems are the reactions with the participation of reactive oxygen species (ROS), in particular, the reactions of superoxide anion radical ($O_2^{\bullet -}$) recombination, yielding hydrogen peroxide (H_2O_2) and singlet (electronically excited) oxygen ($^*O_2$), reactions of H_2O_2 reduction to water and oxygen or of oxidation by it of chlorine ions, reactions of direct oxidation of carbonyl compounds with oxygen from which reaction products in a triplet excited state arise, etc. These reactions may go on non-enzymatically or be enzyme catalyzed and are under strict external regulation besides a probable ability to be self-regulatory[4]. A substantial portion of oxygen consumed by aerobic organisms is permanently used for generation ROS, thus, electronic excitation should also be permanently generated.

Among the mostly intensively studied biological sources of PE are stimulated neutrophils and other phagocyting cells. They react to multiple stimuli by a respiratory burst (RB) – strong intensification of ROS production followed with PE[5]. As intensity of this emission is very low, PE indicators, such as luminol or lucigenin are introduced into a cellular suspension to increase quantum yield[6]. Luminol and lucigenin are known to be indicators of different oxygenation activities. Lucigenin is regarded as a relatively selective probe for $O_2^{\bullet -}$, while luminol is less specific and reports of a variety of reactive oxygen species (H_2O_2, ClO^-, $OH^\bullet$, etc.) production[7]. Non-diluted blood is *a priori* considered to be completely non-transparent for visible light because of a very high hemoglobin` content, and it is practically never used for PE studies. However, here we show that significant photon emission can be registered from non-diluted human blood, both in a resting state in the presence of lucigenin and especially when agents inducing RB of neutrophils are added to it. Patterns of PE revealed due to continuous monitoring of it allowed to expose peculiar systemic properties of blood.

Materials and methods

Reagents

All reagents unless otherwise specified were obtained from Sigma Chemical Co., USA. Stock solution (10^{-1} M) of luminol was prepared in analytical grade dimethyl sulfoxide. It was diluted 50-fold in saline just before use and added to a blood sample to a final concentration of 10^{-4} M. Stock solution (10^{-2} M) of lucigenin was prepared in saline (0.9% sodium chloride solution). It was added to a blood sample to a final concentration of 10^{-4} M. Zymosan was opsonized with human blood serum by a routine procedure and was added to blood to a final concentration of 0,1 mg/ml.

Preparation and treatment of blood samples

Blood from healthy volunteers was obtained by venous puncture between 9 and 11 hours a.m. and was stabilized by heparin or sodium citrate. Blood was kept in 5 or 10 ml plastic disposable syringes without air bubbles at 20 ^oC or at 4 ^oC if it was stored for more than 6 hours. In these cases blood was kept at room temperature for 1 hour before the measurements. For experiments described in this paper blood of healthy donors (males, 20 - 51 years old) was used. Individual differences in PE kinetic curves progression, PE maximal intensity were noted, though they were reproducible in experiments with blood of each particular donor. General trends of PE from blood exemplified at figures presented in the paper were typical for blood of all the donors.

The ability of neutrophils to reduce nitro-blue tetrasolium (NBT), expressed as percent reducing neutrophils was evaluated by a common method [8]. In brief an aliquot of blood (50 mkl) was taken from a blood sample, mixed with 50 mkl of 0,1% NBT solution in 0,15 M Na-P buffer (pH 7,2), the mixture was incubated at 37 ^oC for 20 min and at 20 ^oC for 20 min. Blood was smeared on a slide, dried out, fixed with methanol for 10 min. Slides were dyed with methylene green. 100 neutrophils were counted at each slide. NBT test was considered positive when dark blue diformasan granules (the product of NBT reduction) occupied from 5 to 100% of neutrophils cytoplasm. All the cells were grouped in 6 ranks according to their activity: range 1 -- 0%, range 2 -- 5-7%, range 3-- up to 30%, range 4 -- 30-50%, range 5 -- 50-90%, range 6 -- 100% of cytoplasm is filled with reduced diformasan granules.

Detection of photon emission

PE from blood was registered either in a liquid scintillation counter Mark-II (Nuclear-Chicago, USA), equipped with photomultipliers EMI 9750QB/1 or on a single photon counter equipped with PMT type EMI 9558 QA, cooled to -20^0C +- 0,2 ^{0}C[9]. Mark II counter was used in the mode of single photon counting (out-of-coincidences mode) in a tritium window. The measurements were performed at room temperature (19-21 ^{o}C). PE was recorded as counts per 0.2 or 0.1 min. 1 ml Eppendorf polyethylene test tubes were used as blood containers. Test tubes were fixed in empty standard borosylicate glass vials for liquid scintillation counter in one and the same position. Vials and test tubes having short decay time of own luminescence after insertion into the counting chamber were selected. Dark counts with an empty test-tube in a counting chamber varied in the range of 40-50 counts/sec. All the operations were performed at dim ambient illumination. Sequence of addition of blood, luminol, lucigenin and zymosan are described in figure legends. Other experimental details are described in the section “Results". Single photon counter[7] was used in experiments where temperature dependence of PE from blood was measured. Blood was placed in standard disposable transparent plastic cuvettes for spectrophotometers, and a cuvette was fixed in a copper container with one transparent wall and equipped with the Peltier element for its heating and cooling. A thermistor coupled to the Peltier element was inserted to blood and using a special software temperature in blood could be changed and PE and blood temperature were simultaneously recorded.

Results

Photon emission from blood and its dependence on oxygen.

Addition of either lucigenin or luminol to blood as soon as 5 min after it had been taken out by a finger puncture or elbow vein drainage is followed with an increase of PE in the absence of RB stimulants (Figure 1). Fresh blood response to lucigenin was much more pronounced than that to luminol. During the first hours of blood storage after its withdrawal luminol-dependent PE (LM-PE) level was decreasing, but addition of luminol to 1 day old blood resulted in rapid and strong elevation of LM-PE (Fig 2a). Unlike complex pattern of LM-PE changes during blood storage, lucigenin-dependent PE (LC-PE) was not decreasing, but was rather gradually increasing during blood storage. Similar to LM-PE strong enhancement of LC-PE was observed in 1-day old blood (Fig 2b). Blood even in a resting state (without addition of inducers of RB) to which lucigenin was added continued to emit photons for many hours indicating that the process of ROS production and generation of electron excited species persistently proceeds in it.

The very fact that pronounced PE may be registered from non-diluted blood -- a highly opaque liquid due to very high concentration of hemoglobin -- indicates that hemoglobin packed in erythrocytes does not quench efficiently PE. However, if free hemoglobin is added to blood at a concentration of only 0,5% of already present in it, LC-PE practically disappears (Figure 2). Taking into account that concentration of hemoglobin in erythrocytes may reach as high value as 35-40% (hemoglobin can not reach such high concentration in a free solution) one may suggest that hemoglobin in erythrocytes is present in a liquid crystalline state. In such a form it may provide transfer of excitation energy over long distances without its dissipation, unlike hemoglobin in a solution that absorbs and dissipates energy of electron excitation.

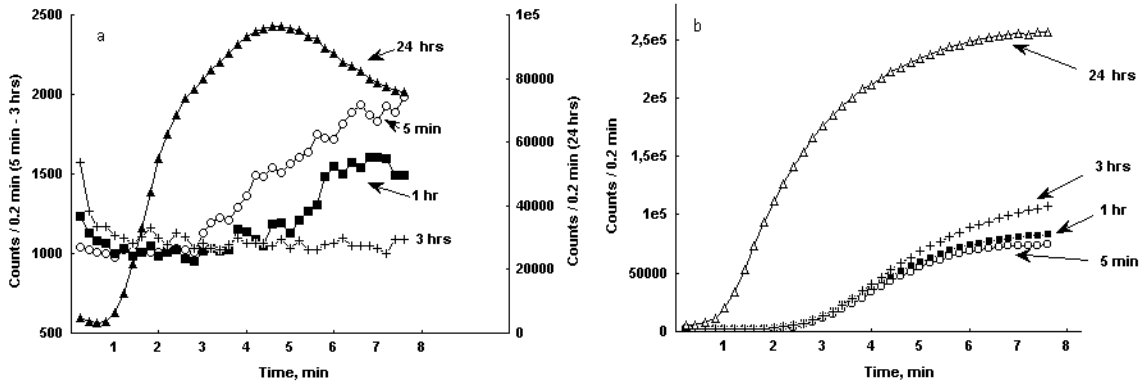

Figure 1. Photon emission changes in non-diluted blood (0.2 ml) supplemented with luminol (a) or lucigenin (b) in relation to time of blood storage. Aliquots (0.2 ml) for measurements were taken from this sample at time moments marked by inscriptions at each curve. Note, that the curves for 5 min., 1 hr, and 3 hours in (a) apply to the left ordinate and the curve for 24 hours -- to the right ordinate.

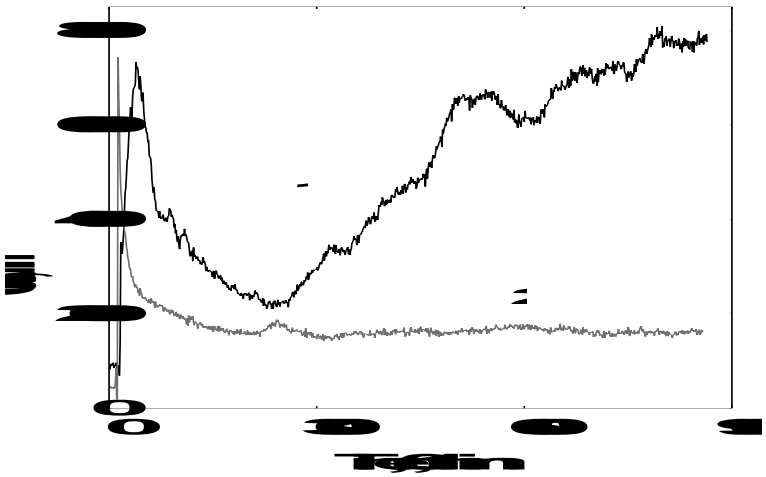

Figure 2. Lucigenin-dependent PE from blood diluted 1:1 with physiological saline (curve 1) or physiological saline to which human hemoglobin was added at a concentration of 2 mg/ml (curve 2).

High intensity of LC-PE in fresh blood indicated of a production of $O_2^{\bullet -}$ in it, and a weak response to luminol suggests that generation and/or accumulation of more reactive oxygen species does not occur. However, after zymosan addition to fresh blood strong LM-PE developed (Figure 3, curve 2). Figure 3 shows also that even in the absence of luminol PE response to induction of RB in the same blood is pronounced (curve 1). It should be noted that these two samples of blood were monitored simultaneously on two identical counters under same conditions differing only by the presence or absence of luminol in blood. Elevation of PE intensity in the absence of luminol indicates that blood contains own fluorophores that are able to transfer energy of electron excitation and that part of it is released as photon emission. Luminol as a very efficient fruorophore amplifies PE from blood nearly 100 x fold. However, it may be seen that besides amplification maximal intensity of PE in the presence of luminol is reached 30 min earlier than in blood without luminol

and that it decays faster. This may suggest that excessive PE from blood devastates its energy resources.

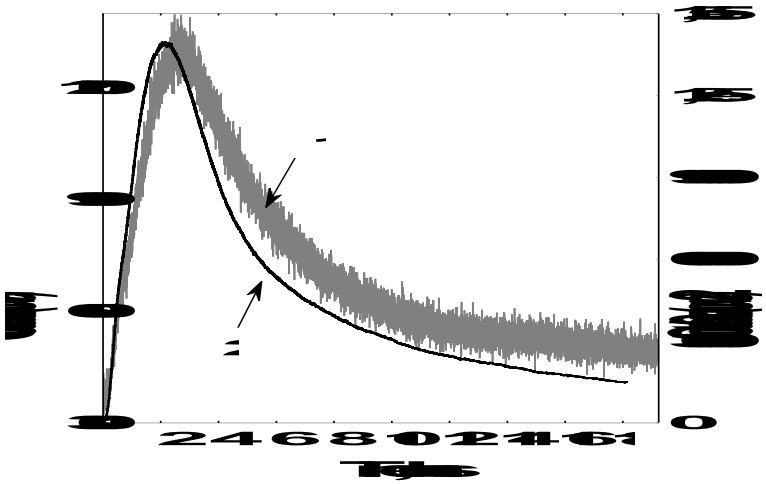

Figure 3. Photon emission from non-diluted blood (0.1 ml) without luminol (curve 1, left Y-scale) or with it (curve 2, right Y-scale) after induction of respiratory burst by addition of zymosan to blood (without zymosan and luminol PE from blood sample was 250-300 counts/6 sec).

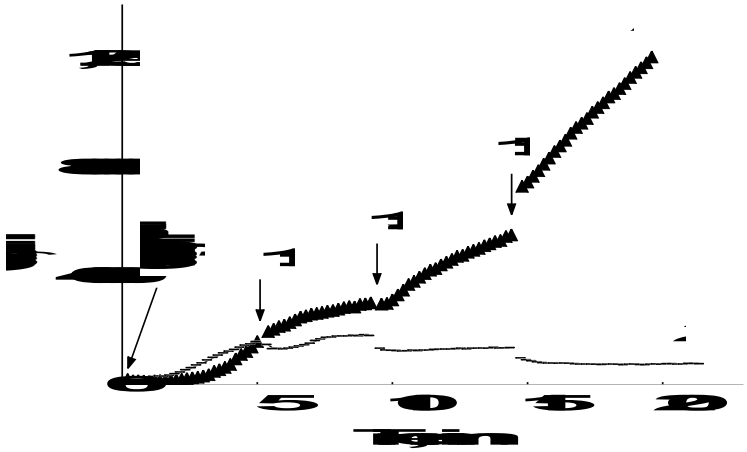

Figure 4. Effect of blood dilution with physiological saline upon photon emission from blood to which either lucigenin or luminol+zymosan were previously added. Initial blood volume – 0,1 ml. Dilution did not change cell contents in blood.

Comparison of PE from non-diluted blood and from suspensions of neutrophils isolated from the same blood and adjusted in cell concentration to that of concentration of neutrophils in whole blood revealed differences as well as similarities in PE patterns from these two experimental systems. We suggested that these differences could be at least in part be explained by different conditions of oxygen supply to neutrophils in blood and in neutrophil suspension. Concentration of dissolved oxygen due to its high affinity to hemoglobin is very low in blood plasma, and white blood cells, in particular neutrophils may get it for respiration only from erythrocytes. On the contrary, in neutrophil suspensions cells consume oxygen dissolved in cell medium. Thus it is

possible that high level of LC-PE in non-diluted blood may be related to interactions of neutrophils and erythrocytes in blood. In fact, when blood to which luminol+zymosan or only lucigenin had been added was diluted with saline (supplemented with either luminol or lucigenin) only LC-PE, but not LM-PE intensity was progressively declining with the increase of the degree of dilution (Figure 4). This result agrees with the suggestion that persistent LC-PE from blood is provided by a close contact of neutrophils and oxygen-donating erythrocytes in it.

Oscillatory behavior of PE from neutrophil suspensions and from blood.

Under certain conditions PE from suspensions of isolated neutrophils may gain oscillatory patterns with high amplitude of oscillations reaching up to 25% of the mean PE intensity. These conditions include lack of agitation of a suspension, an optimal buffered medium containing nutrients and access to air. As it can be seen in Figure 5 prominent oscillations under these conditions develop about 1 hour after RB initiation, and last for many hours. In the absence of air access oscillatory behavior can also be seen, however, PE fades much earlier and amplitudes of oscillations are smaller.

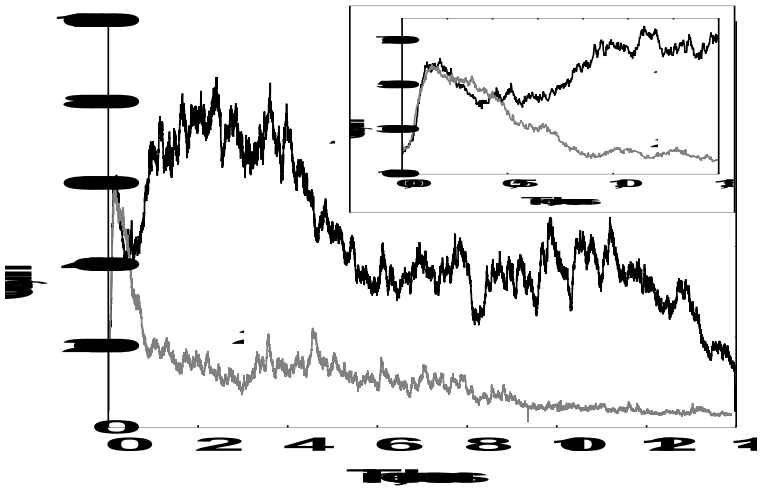

Figure 5. Sustained oscillations of LM-PE in neutrophil suspensions (20000 cells in 0.1 ml of M-PRM medium (ICN) after RB initiated with zymosan. Curve 1 – aerobic conditions, Curve 2 -- suspension is isolated fron air. Insert – the initial period of RB.

Oscillations of LC-PE can be also registered from non-diluted blood (figure 6). Significant oscillations could be seen at the shoulder of the major peak of PE, and then they appeared again at a new smaller peak long time later. It is interesting to note that some similarity may be seen between oscillatory behavior of neutrophils in suspensions and blood: in both cases there could be distinguished two macroscopic waves of PE, and the second wave was modulated by oscillations of rather large amplitude.

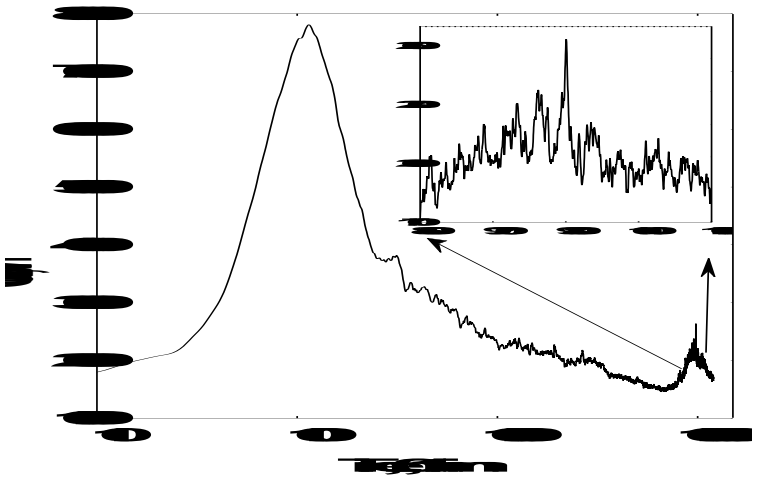

Figure 6. Oscillatory behavior of LC-PE from non-diluted blood (0,1 ml). Insert – oscillations at the second peak of PE arising about 16 hours after addition of lucigenin to blood and start of the experiment.

Thermal hysteresis of PE from blood

Dependence of LM-PE intensity during the development of zymosan-induced RB upon slow (period about 20 min) and regular temperature changes in the range of physiological temperatures is illustrated in Figure 6. During the lag-period preceding RB development and until PE reaches maximal intensity it practically does not depend upon temperature. It can be seen that at this stage PE elevates even when temperature declines that formally accounts for the "negative energy of activation" (Figure 7, A&B). Only after maximal intensity of PE is reached and starts to decline the major tendency of curves in Ahrrenius coordinates starts to approach a classical one (Fig. 7C), however, even at this stage a strong temperature hysteresis is still observed.

Violation of periodicity of temperature changes or its elevation to extreme values were followed with the paradoxical blood response (Figure 8). It can be see that blood reacts to changes in the gradient of temperature decrease (time points 1800, 3000 and 4200 sec) by elevation of PE intensity. On the other hand when temperature reaches 39,5 ^{o}C PE intensity drops abruptly, in spite of further temperature rise, as if blood is denaturized. However, this is not the case because as soon as temperature after reaching its maximum begins to decline PE from blood starts to elevate. Thus decrease of PE from blood is not caused by irreversible changes in it.

Effect of back reflected photons upon the development of RB in blood.

If PE from blood reflects functionally significant processes of electron excited states generation one can expect that irradiation of blood with low level intensity photon flux with the same spectral properties as emitted by it may modulate the character of the processes related to EES generation. In fact previously we[10], [11], and others[12] have demonstrated that two populations of neutrophils or blood being in an optical (but not chemical) contact with each other can influence intensity of oxidative processes in each other if in one of them RB is induced. It was interesting to see if back reflection of photons emitted by blood can influence the processes, that lead to photon

emission, in particular, because this experimental approach could demonstrate effect of ultra-weak irradiation of blood more convincingly than others.

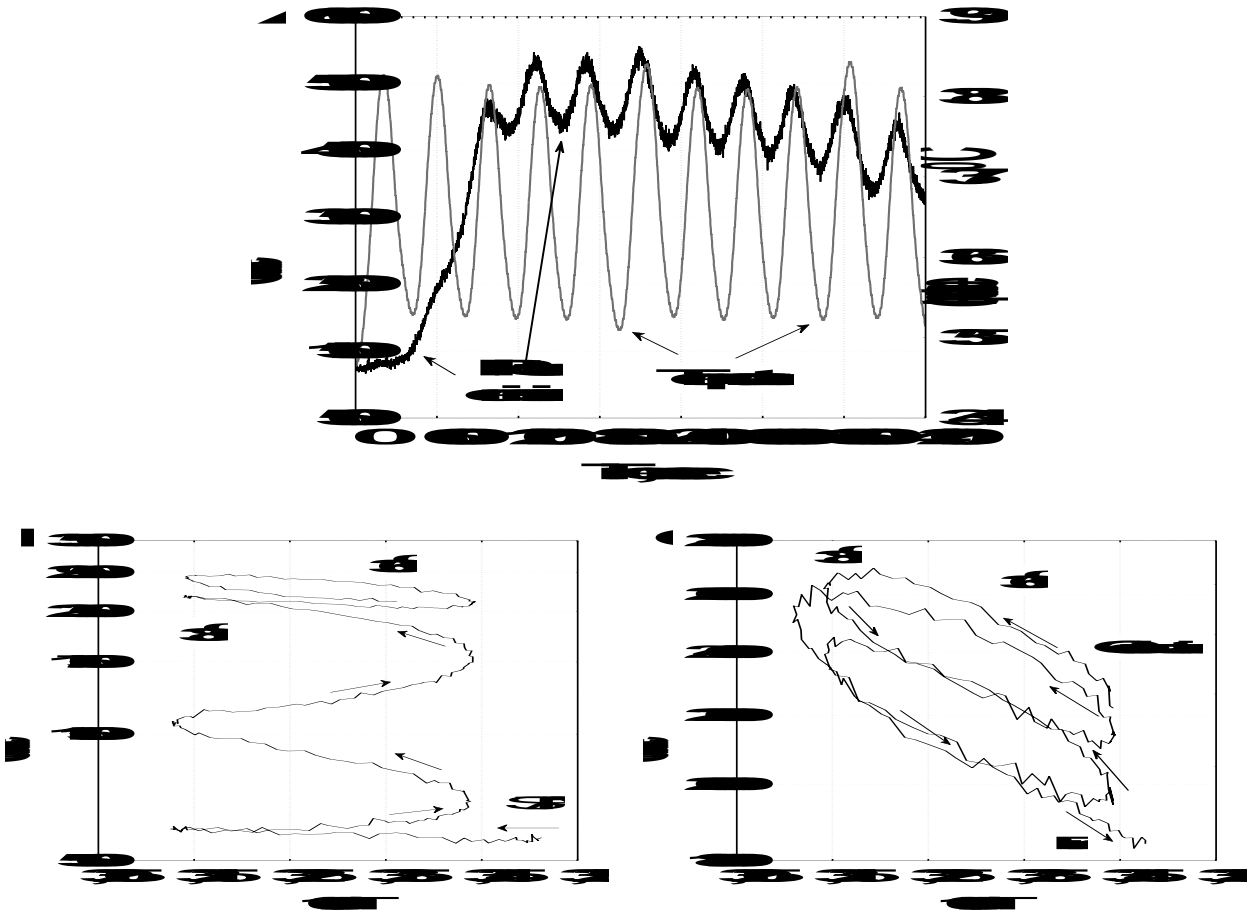

Figure 7. Dependence of LM-PE from blood in which RB is induced with zymosan upon temperature waves. (A) Original data. (B and C) Ahrrenius plots for the initial stage of RB (ca. up to 1700 sec) and later stage of RB, respectively.

To achieve high degree of reflection of photons emitted by blood back to it Eppendorf test tubes were wrapped in aluminum foil screens. As it is demonstrated in Figure 9 (A and B), kinetics of LM-PE development during RB induced by zymosan was different in test tubes with blood covered with aluminum foil and in control test tubes. The “sign” of the effect: enhancement or attenuation of PE intensity in experimental test tubes after the foil was moved away from the test tube depended upon the rate of PE development in blood. If PE was accelerating slowly (count rate in the control samples did not exceed 100 000 counts/0.2 min at the moment of foil removal) PE intensity in the initially screened sample just after a screen removal usually exceeded that of a control one 1.5-5 fold and continued to increase faster that in control samples (Fig. 9A). On the contrary, at high rates of PE development in control blood samples back reflection of photons either did not modify the process or even attenuated PE in wrapped samples in comparison to the control ones (Fig. 9B).

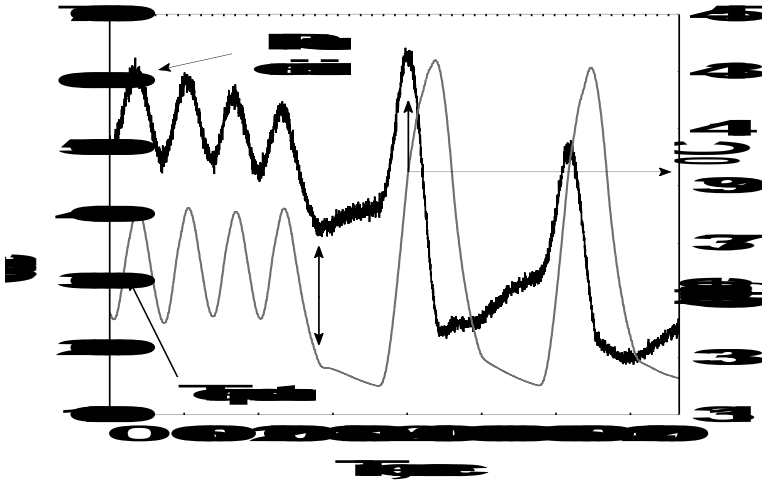

Figure 8. Effect of interruption of periodicity of temperature changes or its elevation to extreme values upon the patterns of LM-PE from blood in which RB was induced 2,5 hour before the time point 0 at this panel.

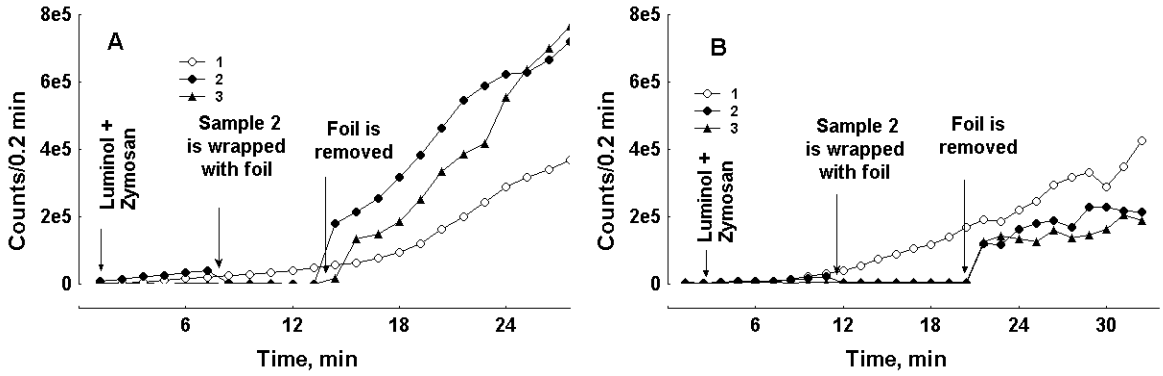

Figure 9. Effect of aluminum foil screen over the test tube with blood upon LM-PE development in zymosan treated preparations of blood with slow (A) and fast (B) PE development. Curve 1 – control test tube, curve 2 -- test tube was wrapped with foil at the moment indicated with the arrow, curve 3 -- test tube was wrapped with foil before luminol and zymosan addition. In each experiment a preparation of blood was distributed in 3 equal portions counted in the mode of rotation (in turns).

It can be seen from Figure 10 that effects of photons reflection from aluminum foil on LC-PE development are generally the same as those observed with LM-PE development after RB inducing in blood. In the sample with slow development of LC-PE screening accelerates photon emission (Fig. 10, A), and in a blood preparation with fast development of LC-PE its inhibition is observed (Fig. 10, B).

NBT test revealed the effect of foil screens upon activity of neutrophils in blood where RB was induced with zymosan and in which LC-PE was registered. In this experiment LC-PE was prominently higher after foil removal from the experimental sample than in the control sample (data not shown). It can be seen in the Figure 11 that after zymosan addition to blood the number of active neutrophils significantly increased in both samples. However, the total number of active cells in the sample screened with foil is larger than in the control sample (39% vs. 30%). It is notable that

the number of cells belonging to high ranks (more than 50% of cytoplasm is filled with diformasan granules) is twice as high in the former sample in comparison to the control one (compare the bars ##5 and 6 in 1c and 1f). Thus, the NBT test gives an independent confirmation that sample screening with aluminium foil at the initial stage of RB development enhances reactive oxygen forms generation by neutrophils in whole non-diluted blood.

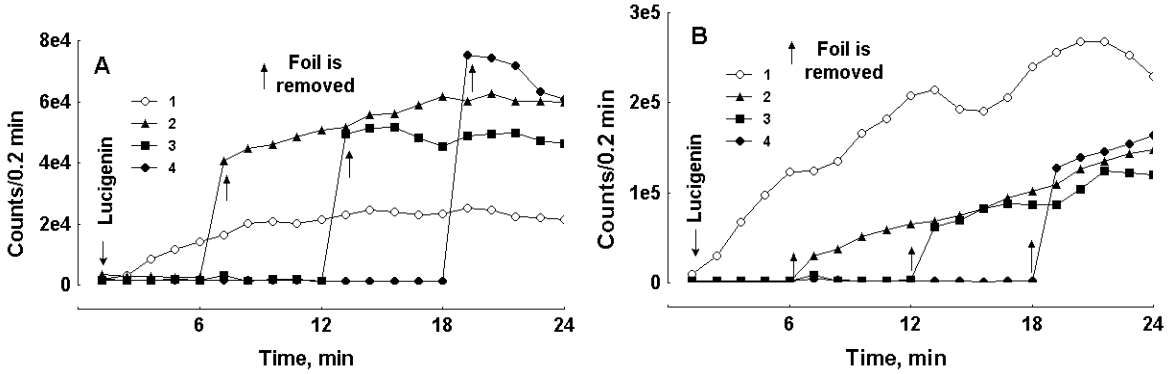

Figure 10. Effect of aluminum foil screen on LC-PE development in two preparations of blood with slow (A) and fast (B) PE development. Curve 1 – control test tube, curves 2-4 -- test tubes were wrapped with foil before lucigenin addition. Foil was removed from respective test tubes at moments indicated by arrows. In each experiment a preparation of blood was distributed in equal portions among 4 samples, which were counted in the mode of rotation. Note the difference in ordinate scale values in A and B.

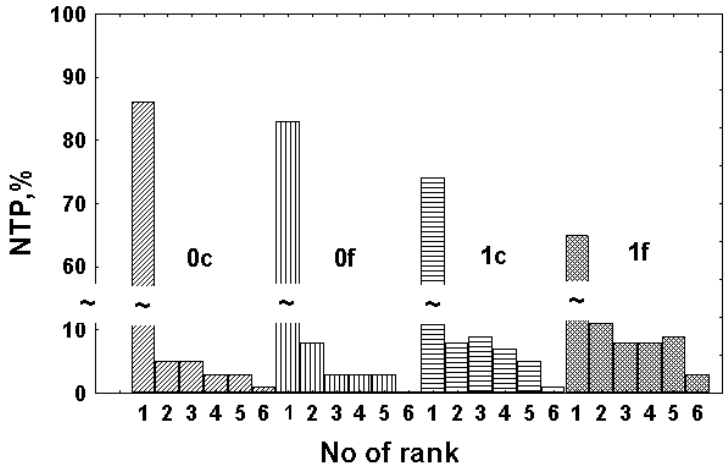

Figure 11. Effect of foil screening on neutrophil activity in whole blood evaluated by NBT test. 0c and 0f – distribution of neutrophils according to the ranges of their activity in a control and a foil screened samples just before the addition of zymosan and lucigenin to them.1c and 1f – same 6 minutes after addition of zymosan and lucigenin to the control and foil screened blood samples.

We used also another experimental set-up to reveal effects of back reflected photons upon RB patterns in blood. Eppendorf test tubes were fixed in glass vials as it is shown in the upper right corner of Figure 12. Part of light emitted from blood samples can be reflected from the glass wall of

a vial back to a sample. We compared the level of radiation registered by PMT from test tubes with blood inserted in empty vials and in vials filled with water. These measurements has shown that PE intensity levels measured from the same sample of blood were 16 ± 4% (mean ± s. d.) higher when a vial was filled with water than when a test tube was inserted in an empty (containing air) vial. Increased light emission from test tubes with blood positioned in water filled vials is due to lack of photon reflection from the inner wall of the vial back to its source due to the immersion effect of water (reflection coefficient of water and glass are similar unlike those for air and glass). In these experiments it was noted that even this small part of light that was reflected back and irradiated a test tube with blood had a measurable effect upon the kinetic parameters of PE response in blood during RB. As it can be seen in figure 12, when the sample was transferred to a water filled vial at the initial stage of PE development its intensity initially leaped by 16% due to the immersion effect and then its acceleration temporarily retarded (Fig. 12, A). Sharp leaps of PE intensity were observed when the sample was transferred from an empty vial to a water filled one and back at the quasi-stationary stage of LM-PE (Fig. 12, B). However, it can also be noted, that when after a few minutes of the sample stay in a water-filled vial it was returned back to a empty one the stationary level of LM-PE in it increased to a much higher level that could be expected if blood continued to stay in an empty vial. On the other hand, when sample transfers from an empty to a water-filled vial and back were made at the stage of PE decay (Fig. 12, C), PE intensity in water-filled vial was rapidly declining. After the sample was transferred back to an empty vial it appeared initially at a much lower value than previously, but began to rise up. Thus, the effect of irradiation of blood samples by back reflected photons may be opposite at different stages of RB which in turn differ in the absolute levels of PE and the direction of its change. Lack of self-irradiation depresses photon emission from blood at relatively low levels of PE (at the stage of PE development and even more prominently at the stage of its decay). On the other hand, at high levels of photon emission (stationary stage) back reflected photons seem to depress PE (Fig. 12, B).

Discussion

Blood provides vital functions for an organism as a whole. It carries gases, nutrients and evacuates by-products of metabolism, participates in humoral regulation of physiological functions of all other tissues and systems, supports water, salt and acid-base homeostasis, executes immunological defense, etc. A lot of knowledge about particular functions of blood components has been gained due to the relative ease of their isolation from whole blood. However, this approach does not allow to get information about interactions of blood components in whole blood, though their interactions are undoubtedly very significant for efficient blood functioning. In a frame of the dominating biochemical paradigm interactions of blood components are provided by chemical signaling through diffusion of bio-regulatory molecules such as cytokines and hormones. However, there may exist an alternative, or, to be more precise, a complementary route of regulation of blood functions – through a non-stationary biophotonic field, or, in other words, through a field of electronically excited states of molecular components of this tissue. It has been long ago stated that in complex and structured systems energy of electron excitation does not immediately dissipate into heat but rather may migrate along the common energy levels and perform chemical, mechanical and other forms of useful work[13, 14, 15, 16]. Such energy migration and storage may be provided by structural organization of a biological systems. However, most of these conclusions were made

basing on data obtained from experiments with external irradiation of a system under study and analysis of fluorescence or delayed photon emission data (e. g. [14-16]). It was only in studies of mitogenetic radiation by A.G. Gurwitsch and his school where internal oxidative metabolism was suggested to be the source of energy that pumps biophotonic fields of living organisms (see e. g. [17]).

It is shown here that addition of lucigenin – the probe for superoxide anion radical ($O_2^{\bullet -}$) to non-diluted blood is followed with PE of rather high intensity even in a resting state, indicating that ROS are permanently produced in blood (Fig. 1B). Due to high activity of superoxide dismutase in blood $O_2^{\bullet -}$ is rapidly converted into hydrogen peroxide, and the latter is immediately decomposed with catalase present in human blood. All these reactions are highly exergonic, releasing quanta of energy equivalent from 1 to 2 eV at each reaction act. Thus electron excited states are continuously generated in blood. When the immune reaction – respiratory burst of neutrophils – is induced in blood, intensity of PE dramatically increases (Fig. 3). It is known that under these conditions other highly reactive oxygen species appear, in particular, hypochlorite (ClO^-), the product of oxidation of Cl^- with hydrogen peroxide, in a reaction catalyzed with myeloperoxidase. Luminol amplifies PE under these conditions nearly 100-fold. However, PE elevation is registered even in the absence of luminol and sustains at high level for many hours. This again argues for the generation of electron excited state in native blood.

The very opportunity to register photon emission from such an opaque liquid is possible only because energy of electron excitation can migrate in blood at least partially without dissipation. We suggest that hemoglobin – the major candidate for dissipation of energy of electron excitation does no do it in intact blood because it is present in erythrocytes in a liquid crystalline state. In fact, hemoglobin dissolved in blood even in a very low concentration readily quenches photon emission (Fig. 2).

It has recently been shown that individual neutrophils produce ROS in a strictly oscillatory manner with a period of oscillations in the range of tens of seconds[18]. Spontaneous arousal of multi-period oscillatory regime including very low frequency oscillations of PE from neutrophil suspensions and whole blood indicates that production of ROS by individual cells manifested as generation of electronic excitation is to a certain degree correlated in systems containing tremendous quantities of these cells (Fig. 5&6). Such correlated behavior may be seen even in such a complex system as non-diluted blood in which only a tiny fraction of cells (granulocytes do not exceed together 0,1% of erythrocytes in quantity) is responsible for ROS generation. Such a correlated, cooperative behavior can be in principle provided by diffusible regulatory molecules that activate or inhibit granulocyte activity. However this explanation was ruled out in the experiments in which PE from blood was registered under the conditions of oscillatory temperature changes.

For the first time thermal hysteresis of PE from a living system (etiolated barley) was observed by Slawinsky and Popp[19]. Their data argued for the presence in living matter of a delocalized coherent electromagnetic field far away from thermal equilibrium. Here we confirmed and extended their observations. In our case PE from blood originated definitely due to a set of biochemical reactions induced in neutrophils and resulting in generation of electron excited species. Very prominent temperature hysteresis of PE especially at a stage of the development of RB suggests that either the kinetics of these biochemical reactions and/or the kinetics of the processes resulting in photon emission do not obey simple laws of chemical kinetics, in other words these processes are at least partially not determined by the rate of diffusion of reagents which should

strongly depend upon temperature. As soon as RB starts to fade, temperature dependence of PE more and more resembles of a "classical" one, though even many hours after RB was initiated pronounced temperature hysteresis is still observed . Of a special interest is the behavior of blood when the periodicity of temperature changes was violated or temperature was raised to extreme values. Paradoxical changes in PE -- elevation of its intensity when temperature continued to decline though at different rate than before, or abrupt drop in PE intensity when it continued to raise – indicate that blood has some peculiar mechanism sensing temporal variations of temperature. The nature of this mechanism is currently obscure.

Modulation of activity of neutrophils in whole blood by back reflected photons and changes of patterns of PE from it in response to this very low intensity irradiation (Fig. 10-12) also argues in favor of the functional role of the biophotonic field in this tissue. As it has been pointed out above, non-diluted blood is a system with a very high optical density and the ratio of neutrophils to erythrocytes in it is approximately 1:1000, so the question arises as how these very low intensity photon fluxes may have any measurable effect upon neutrophil activity it. It may be suggested that at least in part these effects may be related to specific ways by which neutrophils obtain oxygen for their respiration in blood. It was demonstrated here that LC-PE in blood is strongly dependent upon the interaction of neutrophils and erythrocytes, and that LC-PE depends on oxygen supplied by erythrocytes (Fig. 4). Taking into consideration that λ_{max} for luminol emission is 427 nm and for lucigenin emission it is 470 nm, and that several hemoglobin absorption maximums are not far from this spectral region, hemoglobin may be excited by back reflected photons. It is interesting to speculate that due to liquid crystalline state of hemoglobin in erythrocytes absorption of photons by few hemoglobin molecules may provide enough energy of excitation for dissociation of oxygen from multiple oxyhemoglobin molecules belonging to this crystal thus providing the mechanism for cascade amplification of a signal and additional substrate supply for generation of ROS by neutrophils and enhancement of PE intensity in blood.

However, these considerations cannot readily explain attenuation of PE intensity by back-reflected photons in blood preparations with initially high rate of PE intensity growth. Previously we observed that the high rate of LM-PE without artificial induction of RB in it is characteristic of blood of patients with cardiovascular diseases[20]. Besides it has been shown here that both LM-PE and LC-PE intensity is dramatically high after long storage of blood even of healthy donors'. After being stored in certainly unnatural conditions blood in a certain sense becomes "sick". It can be supposed, that in such blood preparations hemoglobin is already highly excited for the reasons discussed below, and that auxiliary irradiation of blood with back reflected photons does not provide an additional stimulus for oxygen release. On the other hand, additional irradiation of blood with weak photon fluxes originated from blood itself and reflected back to it, may play an organizing role so that less energy is lost from it, and the photon flux from such blood registered by a photomultiplier is lower after the reflective screen removal than the control sample.

The hypothesis that reactive oxygen species generation by white blood cells is enhanced due to an increase in oxygen availability, ensuing the enhancement of photon emission from "normal" blood, is supported by the results of our studies of the effect of carbon monoxide upon whole blood and isolated neutrophils[21], [22]. We found that while CO sharply intensified LC-PE in non-diluted blood; it had much weaker effect in saline-diluted blood, and inhibited LC-PE in a neutrophil suspension. CO is known to bind to heme 100-fold more tightly than O_2. In whole blood its major

target is deoxy-Hb. Sharp elevation of LC-PE after CO addition to blood is a strong indication of highly co-operative processes proceeding in blood. Presumably, high energy released when CO binds to heme of hemoglobin molecules packed in erythrocytes induces accelerated oxygen release from many HbO_2 molecules resulting in the similar effect upon PE from whole blood as that produced by blood self-irradiation. Inhibitory effect of CO upon LC-PE in neutrophil suspensions is most probably explained by its ability to bind to heme enzymes of neutrophils responsible for oxygen reduction[23]..

Thus, blood displays many features of an active physical medium, constituents of which are permanently present in an electronically excited state. Electronic excitation of blood constituents is provided by the reactions of ROS generation permanently proceeding in it and providing energy for pumping internal "biophotonic" field of blood. Efficient migration and storage of energy in blood is provided by its peculiar structure. It is reasonable to suggest that this energy field plays a very important role in efficient performance of all blood functions.

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