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LOW-LEVEL CHEMILUMINESCENT ANALYSIS OF NONDILUTED HUMAN BLOOD REVEALS ITS DYNAMIC SYSTEM PROPERTIES

Vladimir L. Voeikov, Cyrill N. Novikov, and Natalia D. Vilenskaya
Lomonosov Moscow State University, Department of Bioorganic Chemistry, Faculty of Biology, Moscow 119899, Russia

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ABSTRACT

Lucigenin- and luminol-dependent chemiluminescence [(LC-CL) and (LM-CL)] in nondiluted human blood was studied. LM-CL was low in fresh blood and disappeared after its storage for 3 h, though the respiratory burst (RB) stimulation in blood was followed by high intensity and long-lasting LM-CL. LC-CL was high in fresh blood and was steadily increasing with blood storage. Blood dilution with saline resulted in LC-CL attenuation and LM-CL elevation. LC-CL did not depend on air supply to blood, while LM-CL elevation during RB needed constant blood aeration. The results suggest that besides a well-known mechanism of reactive oxygen species production by neutrophils during RB, another process of electron excited state generation reflected by LC-CL operates in blood. It needs blood integrity for its manifestation and uses oxygen supplied by erythrocytes. Dynamic system properties of blood were revealed also in experiments with blood transfer from one sample to another in the course of RB. Highly nonlinear changes of CL intensity both in a ''donor'' and in a ''recipient'' sample resulted in strong differences in CL levels in two samples, one of which was prepared by blood subtraction, and another by blood addition. We suggest that CL data from measurements on nondiluted blood may be informative of integrative properties of blood tissue in addition to its being a measure of some sort of oxidative metabolism in it. [S1083-3668(99)00601-2]

Keywords whole blood; chemiluminescence; luminol; lucigenin; respiratory burst.

1 INTRODUCTION

Neutrophils are among the most intensively studied biological sources of ultraweak and low-level chemiluminescence (CL). They react to multiple stimuli by strong enhancement of oxygen consumption [respiratory burst (RB)]. Oxygen is reduced by NADPH oxidase to superoxide anion radical ($O_2^{\bullet -}$),[1] which initiates a series of reactions in the course of which other reactive oxygen species (ROS) appear.[2] Production of ROS during RB correlates with CL in preparations of neutrophils.[3] CL is supposed to result from the immediate relaxation of the electron excited states that emerge in course of the free radical oxidative reactions. In particular it is explained by singlet oxygen and its dimers (eximers) fluorescence[4,5] and by phosphorescence of excited carbonyls—products of dioxethanes and tetroxides decomposition.[6] To increase the quantum yield of CL luminol (5-amino-2,3-dihydro-1,4-phtalazinedione) or lucigenin (10.10′-dimethyl-9,9′-biacridinium dinitrate) are usually used.[7] Lucigenin is a relatively selective probe for $O_2^{\bullet -}$, while luminol is less specific and reports of a variety of ROS (H_2O_2, $O_2^{\bullet -}$, $OH^{\bullet}$, $OCl^{\bullet -}$, $NO^{\bullet}$) production.[2]

The intensity and the kinetics of CL correlate with the activity of neutrophils of individuals undergoing stress and disease.[8,9] However, the diagnostic value of the neutrophil CL measurements is detracted by possible artifacts: in the course of purification procedures the neutrophils may be primed, and some already activated cells may be lost due to clumping and adhesion. Besides, a lot of factors present in whole blood important for neutrophil proper functioning are lost.[10] Therefore, a lot of studies of CL are performed on the so-called ''whole blood'' preparations, that represent blood diluted to a different extent in order to avoid photon absorption by hemoglobin and other blood constituents.[11,12] It is implicitly supposed that blood dilution should not interfere with the neutrophil functions, though the disturbance of neutrophil interactions with other blood constituents may not be indifferent for their biological activities as well as for activities of other blood constituents.

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Address all correspondence to Vladimir L. Voeikov. Tel: 007(095)939 1268; Fax: 007(095)939 2788; E-mail: vvl@ecol.msu.ru

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We speculated that if CL arising due to the processes of constitutional and stimulated oxidative reactions in blood might be registered in nondiluted blood, it could reflect some properties of blood in its native state. The present study investigates CL from nondiluted human blood in the absence and presence of luminol and lucigenin. It is demonstrated here that although nondiluted blood is a highly opaque substance it may be a source of relatively strong photon emission. The character of nondiluted blood CL demonstrates some features that are not easily interpreted in the frame of the general model explaining photon emission from biological liquids as an immediate result of the excited state relaxation. Rather the chemiluminescent properties of nondiluted blood reflect its behavior as of a nonlinear dynamic cooperative system.

2 MATERIALS AND METHODS

2.1 REAGENTS

All reagents unless otherwise specified were obtained from Sigma Chemical Co., USA. The stock solution (10^{-1} M) of luminol was prepared in analytical grade dimethyl sulfoxide. It was diluted 50-fold in saline (0.9% sodium chloride solution) just before use and added to a blood sample to a final concentration of 10^{-4} M. The stock solution (10^{-2} M) of lucigenin was prepared in saline. It was added to a blood sample to a final concentration of 10^{-4} M. RB in blood was stimulated by zymosan that was opsonized with human blood serum by a routine procedure and was added to blood to a final concentration of 0.1 mg/ml.

2.2 PREPARATION AND TREATMENT OF BLOOD SAMPLES

Blood from healthy volunteers (males, 20–51 years old) was obtained by venous puncture or by a finger puncture between 9 and 11 a.m. and was stabilized by heparin. If venous blood was stored before use for more than 6 h it was kept in 5 or 10 ml plastic disposable syringes without air bubbles at 4 °C. In these cases blood was equilibrated at room temperature for 1 h before measurements. Capillary blood from a finger was taken into heparinized polypropylene Eppendorf test tubes and kept there until use. The individual differences in CL kinetic curves progression, CL maximal intensities were noted, though they were reproducible in experiments with blood of each particular donor. The general trends of CL from blood exemplified at figures presented below were typical for blood of all the donors.

2.3 DETECTION OF CHEMILUMINESCENCE

CL from blood was registered in a liquid scintillation counter Mark-II (Nuclear-Chicago, USA), equipped with photomultipliers EMI 9750QB/1. It 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 °C). CL was generally recorded in counts per 0.2 min. All the operations were performed at dim ambient red light illumination. The sequences of addition of blood, luminol, lucigenin, and zymosan are described in figure legends. In most experiments the Eppendorf polypropylene or polyethylene test tubes were used as blood containers. The test tubes were fixed in empty standard borosylicate glass vials for the liquid scintillation. The vials and the 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 500–700 counts/0.2 min.

The measurements were made in two different modes. In one experimental setup a sample was inserted into the counting chamber and sequential readings of the count rate were made. This setup will be referred to as a ''continuous mode of counting.'' In another type of experiments two or more samples were continuously counted in rotation. Each sample was mildly agitated manually after the count reading for 0.2 min and extraction from a counting chamber while another one was counted. Such a mode of measurement allowed to compare CL from two or more samples practically simultaneously. This setup will be referred to as a ''consecutive mode of counting.'' In the experiments in which the effects of air supply to blood upon CL were studied 1 or 2 ml disposable plastic syringes were used as blood containers. Neither the time course of CL development in blood nor its intensity depended in a significant degree upon the material of blood containers (glass, polyethylene, polypropylene, acryl). Other experimental details are described in Sec. 3.

3 RESULTS

3.1 PHOTON EMISSION FROM NONDILUTED BLOOD IN THE ABSENCE AND IN THE PRESENCE OF LUMINOL OR LUCIGENIN

No significant increase of the photon-count-rate over background was registered without luminol or lucigenin from healthy donors' blood both in the absence and in the presence of RB stimulants within several hours after it had been taken. However, after overnight blood storage at 4 °C and warming it to room temperature (20 °C) a weak photon emission from the blood sample exceeding background level could sometimes be registered (Figure 1). Zymosan addition to blood resulted in a steady elevation of CL intensity indicating that neutrophils did not lose their activity in 1 day old blood. However, zymosan-stimulated CL in 1 day old blood usually did not exceed of 3–8 dark count levels in the absence of luminol or lucigenin.

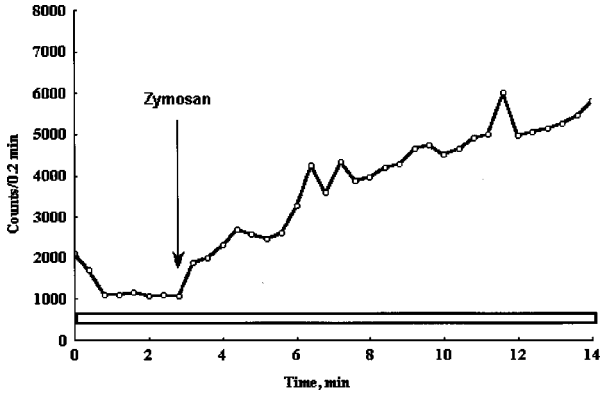


Fig. 1 Photon emission from nondiluted blood (1 ml) 24 h after it was taken by venous puncture before and after addition of zymosan (arrow). Shadowed rectangle—level of a count rate with an empty test tube in the measuring chamber.

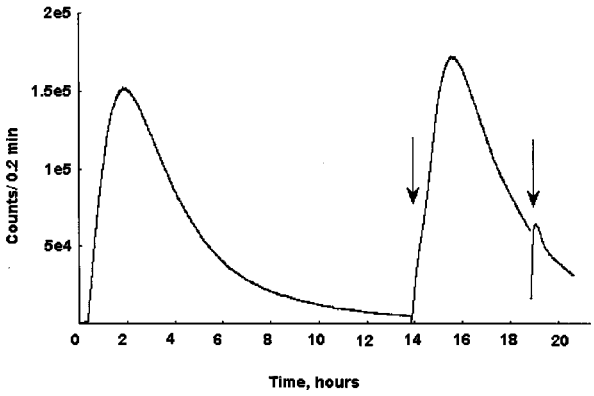
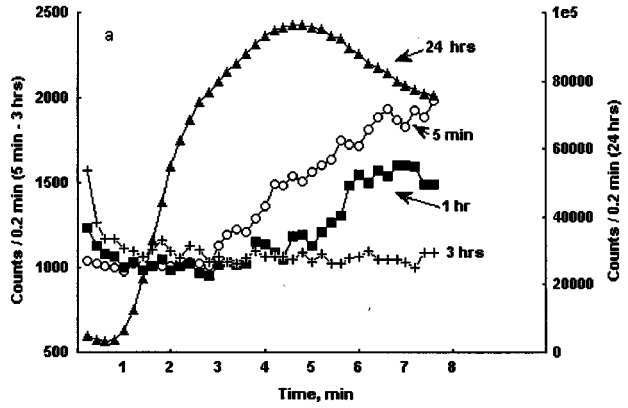


Fig. 3 LM-CL following RB induced by zymosan in nondiluted healthy donor's blood (0.2 ml) 1 h after it has been taken. At time points indicated by arrows several air bubbles were blown through the sample.

Figure 2 shows that addition of either lucigenin or luminol to blood 5 min after it had been taken is followed with an increase of CL in the absence of RB stimulants. Fresh blood response to lucigenin was much more pronounced than to luminol. During the first hours of blood storage LM-CL level was decreasing, but 24 h later its response to luminol appeared again. Addition of luminol to 1 day old blood resulted in rapid and strong elevation of LM-CL [Figure 2(a)]. This result agrees with data presented in Figure 1, where the slight photon emission from 1 day old blood can be seen. Unlike the complex pattern of LM-CL changes during blood storage, LC-CL was not decreasing, but rather slightly increasing during the first hours of blood storage. Similar to LM-CL strong enhancement of LC-CL was observed in 1 day old blood [Figure 2(b)].

The high intensity of LC-CL in fresh blood indicated the production of $O_2^{\bullet -}$ in it, but the weak response to luminol suggests that no accumulation of other reactive oxygen species occurs in fresh preparations of blood. However, after zymosan addition to fresh blood strong LM-CL developed in it (Figure 3). Both the duration of LM-CL growth to maximal values and the duration of its fading away were much more sustained than those observed in purified neutrophil populations.[13,14] Also, unlike RB in an isolated neutrophil population, which cannot be reinitiated after its termination, a new ''wave'' of LM-CL of nearly the same amplitude and duration as the first one could be provoked in blood by just passing several bubbles of air through it. As a test tube with blood was open and blood had free access to air, this effect was surprising. Besides, reinitiating LM-CL elevation by passing fresh air through blood could be gained only after a deep CL decline. When air was bubbled through blood on the descending slope of the CL ''wave,'' only a slight and transitory increase of CL intensity was observed followed by the relaxation of CL along the previous trajectory.



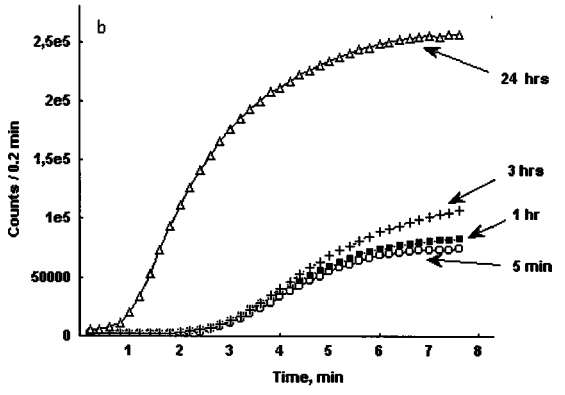


Fig. 2 Chemiluminescence changes in nondiluted blood (0.2 ml) supplemented with luminol (a) or lucigenin (b) in relation to time of blood storage. Aliquots (0.2 ml) for measurements were taken from this sample at time moments marked by inscriptions at each curve. Note, that the curves for 5 min, 1 h, and 3 h in (a) apply to the left ordinate and the curve for 24 h—to the right ordinate.

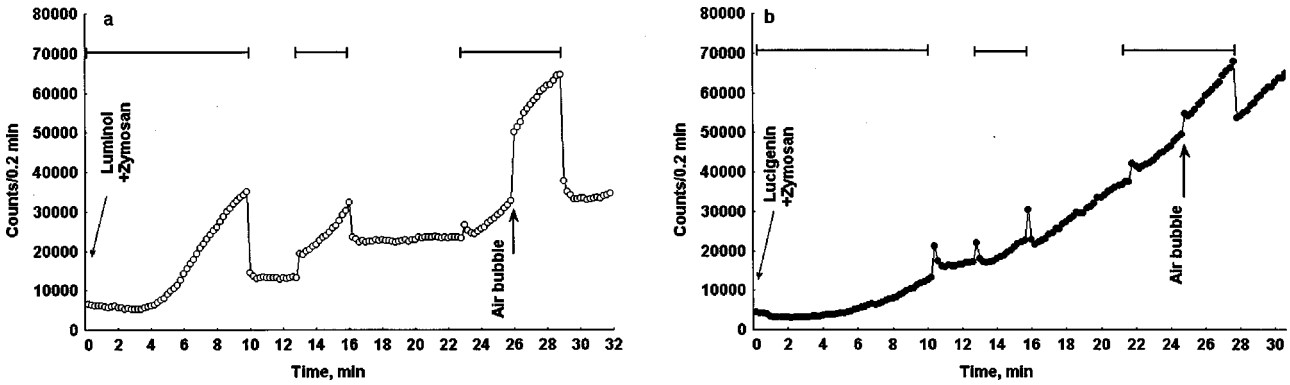


Fig. 4 Dependence of LM-CL (a) and LC-CL (b) during RB induced by zymosan in fresh nondiluted blood (1 ml) on air access to the blood sample. 2 ml syringe with 1 ml of blood supplemented with zymosan and either luminol or lucigenin and 1 ml of air was fixed in the counting chamber of the photon counter. Blood was aerated at periods marked by the straight lines above the curves; gaps between the lines correspond to the periods when air was evacuated.

3.2 OXYGEN SOURCES FOR LM-CL AND LC-CL IN NONDILUTED BLOOD

The peculiar dependence of LM-CL upon air supply may be considered in relation to the fact that only a minor part of oxygen in whole blood is dissolved in plasma. Oxygen in blood is mostly bound to hemoglobin and it is not easily accessible for activated neutrophils. This contrasts with the situation in isolated neutrophil suspensions or diluted ''whole blood'' preparations, where oxygen dissolved in a cell suspension medium is drawn for the RB. It is possible, that during RB development in nondiluted blood complex and dynamic interactions between the oxygen consuming cells and the oxygen sources take place. To reveal these interactions we studied the effect of blood segregation from ambient air upon CL progression curves in the course of zymosan stimulated RB. For these experiments 1 ml of blood was put into a 2 ml plastic syringe and placed into a measuring chamber of the photon counter. Air could be completely evacuated from the syringe or sucked into it by moving the syringe piston. As it can be seen in Figure 4(a), if air was evacuated from the syringe shortly after the onset of RB, the counts rate immediately dropped down and did not increase until the blood sample got access to air. When air was evacuated from the syringe for the second time, LM-CL level dropped down not so deeply and again began to increase when blood got access to air. When an air bubble (50 μl) was introduced into blood at this stage, CL level jumped up, and continued to grow. Air evacuation at this stage again suppressed CL intensity, though its level stayed relatively high and had a tendency for elevation. Under the same conditions LC-CL responded to air evacuation in a different manner. Figure 4(b) demonstrates that neither the counts rate level, nor the slope of the kinetic curve are affected by air evacuation from the syringe up to 30 min after lucigenin and zymosan addition to blood. Though air evacuation at a later stage was followed with a drop in LC-CL intensity, the slope of the kinetic curve was not changed. Thus, LC-CL was significantly less dependent upon ambient oxygen availability than LM-CL.

Relative independence of LC-CL on ambient air supply to blood allowed us to speculate that $O_2^{\bullet -}$ production in nondiluted blood indicated by LC-CL is provided by close interaction of ROS producing cells and erythrocytes. If this is the case, then blood dilution should result in LC-CL attenuation. The results of a representative experiment with blood dilution with saline are demonstrated in Figure 5. In order to compare the effects of blood dilution upon LM-CL and LC-CL the blood samples after 24 h storage were used [Figure 5(a)], because, as is shown in Figure 2, LM-CL by this time is significant without stimulation of RB. Blood was diluted with saline supplemented with chemiluminescence indicators to maintain their constant concentration. Dichotomy between LC-CL and LM-CL became evident already after the first dilution, and with further dilution LM-CL continued to increase, while LC-CL was decreasing. The effects of dilution on LM-CL and LC-CL during RB induced with zymosan in a fresh blood preparation [Figure 5(b)] were generally similar to those observed in aged blood, though in blood, where RB was developing LC-CL dropped not to such a low level than in blood without zymosan addition. Strong attenuation of LC-CL with blood dilution supports our suggestion that close interaction between blood components, in particular between neutrophils and erythrocytes, provided in nondiluted blood, is needed for the development of LC-CL. It also turns out that LM-CL depends upon blood integrity to a much lower degree than LC-CL.

3.3 DEPENDENCE OF CL PARAMETERS DURING RB IN NONDILUTED BLOOD ON SAMPLE VOLUME

To study the dependence of CL intensity during RB on blood volume the following experimental setup

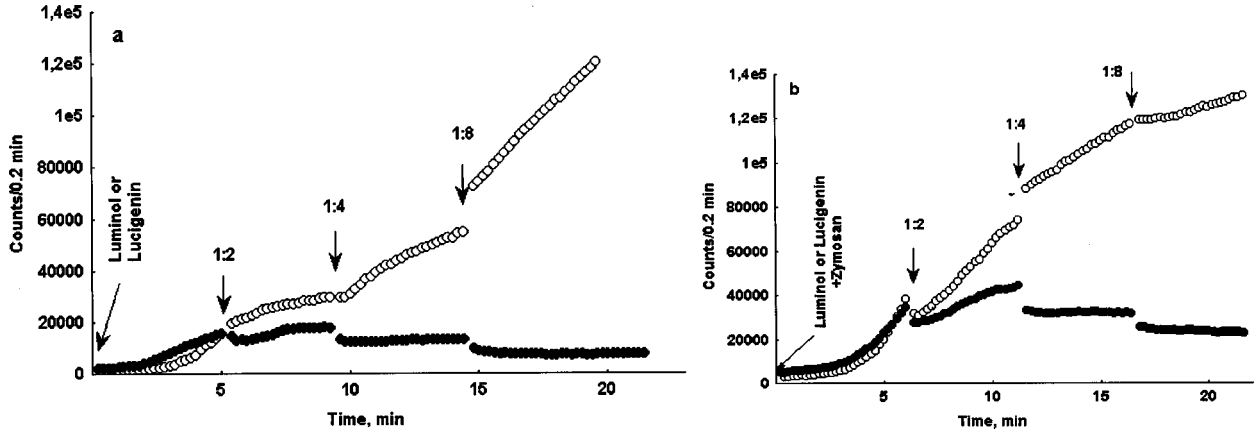


Fig. 5 Effect of blood dilution upon CL kinetics in the presence of luminol (open circles) or lucigenin (closed circles). Arrows mark saline addition to blood (initial volume 0.1 ml) to provide the indicated dilution factors. (a) Blood after 24 h storage. Saline is supplemented with either luminol or lucigenin to maintain initial concentration of indicators (0.1 mM). (b) Blood after 1 h storage to which zymosan was added.

was used. Blood was introduced into a test tube and luminol plus zymosan were added to it. This sample (a ''donor'' sample) was counted in a consecutive mode of counting with the second test tube (a ''recipient'' sample), which was initially empty. After CL intensity began to increase aliquots of blood from a donor sample were sequentially transferred to a recipient sample. Figure 6(a) demonstrates the results of an experiment with 3 ml of blood, and Figure 6(b) shows LM-CL progression curves in samples containing initially 1 ml of blood. Unexpectedly, when after several aliquots transfer the volumes of blood in both samples became equal (Sec. D), CL intensity in donor samples was severalfold higher than CL intensity in recipient samples. As illustrated in Figure 6(a) this difference remained after further blood transfer: CL intensity in the donor sample exceeded that in the recipient sample even when blood volume in the latter was five times as high as that in the former (Sec. F). Back transfer of blood from the recipient sample to the donor one resulted in acceleration of CL intensity growth in the former and in retardation of its growth in the latter. Although when the volumes of blood in two samples became equal again, the difference in photon count rate still persisted (Sec. H). Figure 6(b) compares two modes of blood transfer from the donor to the recipient sample. In one case (curve 1) blood was taken out of the donor sample in 0.1 ml portions (Secs. B–F). In the other case (curve 3) 0.1 ml portions of blood were extracted from the donor sample and returned back (Secs. B–E) and only at 22 min time point 0.5 ml of blood was transferred to the recipient sample. Comparison of curves 1 and 3 shows, that CL intensity was growing faster in the sample from which small portions of blood were sequentially taken away, than in the sample, in which the imitations of blood

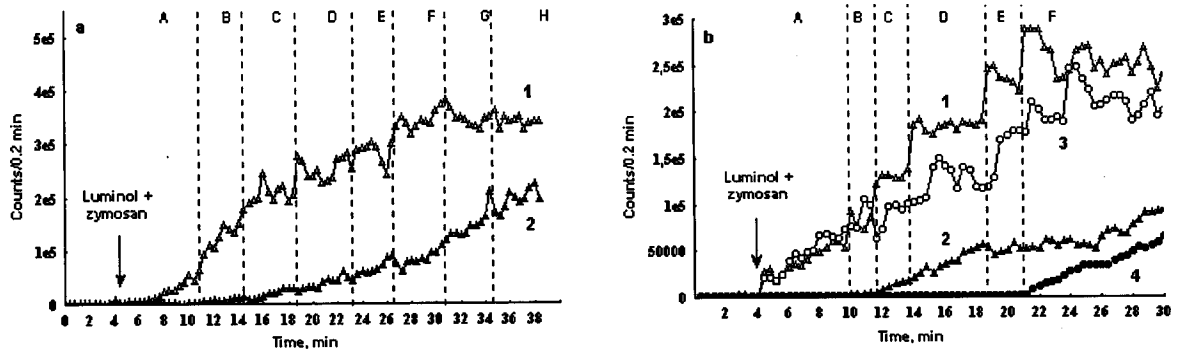


Fig. 6 Progress curves of LM-CL in blood in the presence of zymosan in samples from which blood aliquots were sequentially taken (odd numbered curves) and to which these aliquots were sequentially added (even numbered curves). (a) Luminol and zymosan were added to 3 ml of 24 h old blood (curve 1, open triangles) and after initial CL elevation (Sec. A) 0.5 ml aliquot was transferred to the initially empty test tube (curve 2, closed triangles). Volume ratios (ml/ml) for samples 1 and 2 were (A) 3.0/0, (B) 2.5/0.5, (C) 2.0/1.0, (D) 1.5/1.5, (E) 1.0/2.0, (F) 0.5/2.5; (G) 1.0/2.0 (back transfer of blood was done), (H) 1.5/1.5. (b) Luminol and zymosan were added to 1 ml samples of 12 h old blood (curves 1 and 3, open triangles and circles). After initial CL elevation (Sec. A) 0.1 ml aliquots were transferred from sample 1 to the empty test tube (curve 2, closed triangles). Volume ratios for samples 1 and 2 (ml/ml) were (A) 1.0/0, (B) 0.9/0.1, (C) 0.8/0.2, (D) 0.7/0.3, (E) 0.6/0.4, (F) 0.5/0.5. In the sample marked by curve 3 (open circles) 0.1 ml aliquots were extracted and immediately returned back in Secs. B–E; in (F) a 0.5 ml aliquot was taken and transferred into an empty test tube (curve 4).

withdrawal were done. Lack of smoothness of CL progression curves in Figure 6 is due to intermittent agitation of samples with blood counted in the consecutive mode of counting.

Paradoxical difference in CL properties in a donor and a recipient blood samples was observed only if nondiluted blood preparations were used in this kind of experiments. When the same experimental protocol was applied to saline diluted blood (1:100), aliquot extraction from the first sample resulted in a drop of CL intensity in it, and addition of this aliquot to a recipient sample was followed with an increase of CL intensity in this sample (data not shown).

4 DISCUSSION

Spontaneous as well as luminol- and lucigenin-dependent chemiluminescence in nondiluted human blood was studied here systematically for the first time. The results demonstrate that CL-indicators dependent photon emission from blood might be confidently registered. The spectral distribution of photon emission was not studied here, but as the chemiluminescence detector used by us is most sensitive in the blue region of the spectrum and its photoelectric efficiency drops drastically at $\lambda > 600$ nm it is most probable that a significant part of photon emission is in the blue-green region. This should be expected for both LC-CL and LM-CL (λ_{max} for luminol CL is 427 nm and for lucigenin CL is 470 or 510 nm in case of its fluorescence due to energy transfer).[5] It is probable also that at least a part of weak photon emission that may be registered from 1 day old blood upon addition of zymosan to it (cf. Figure 1) is also in the blue region of the spectrum though this question needs a more careful examination.

Our results indicate that the parameters of CL in nondiluted blood depend upon its physiological rather than on its physical properties as of a highly opaque body. For example, changes in the characteristics of LM-CL and LC-CL in blood during its storage are unlikely to be explained by changes of light transmission properties of blood. Evidently, they depend upon some nonlinear evolution of the state of this complex living tissue. LM-CL was decreasing during the first hours of blood storage though it readily responded to RB stimulation with zymosan. On the contrary, LC-CL in all the cases strongly exceeded LM-CL in fresh blood and its intensity monotonously increased during blood storage. Exceptionally high levels of LC-CL and LM-CL were observed in blood 24 h after it has been taken. Enhancement of CL after zymosan addition to blood argues that neutrophils do not lose their viability after its storage for at least 24 h. Blood response to passing several air bubbles through it by a new intensive, though slow ''burst'' of CL activity 14 h after initiation of the first RB in it is also indicative of high viability of ROS-producing cells in nondiluted blood. These and several other features of oxidative metabolism of ROS-producing cells in native blood are in contrast to those observed in purified neutrophil suspensions and even in diluted whole blood.

The high levels of LC-CL that were comparable to or even exceeding the levels of LM-CL both in nonstimulated blood and after RB stimulation in it, contrasts with the data obtained in the experiments with purified neutrophil suspensions. It is well known that unless neutrophils are deficient of myeloperoxidase activity LM-CL accompanying RB in neutrophil preparations develops more rapidly and comes to higher levels, than LC-CL.[2,9,15] On the other hand, we demonstrated that blood dilution results in strong attenuation of LC-CL without significant effect upon LM-CL. Thus, after simple operation like blood dilution some features characteristic for the unperturbed tissue are lost.

The discrepancy between the high levels of LC-CL and the lack of LM-CL in fresh nonstimulated healthy donors' blood allowed us to suggest that LC-CL reports of some process of ROS production that differs from ROS production by stimulated neutrophils.[1] Significant generation of LC-CL due to intracellular production of $O_2^{\bullet -}$ was reported for hepatocytes, whose cytochrome P450 system was induced by barbiturates.[16] Recently it was demonstrated that topical application of lucigenin onto the rat brain *in vivo*, or loading of lucigenin in a neuronal cell culture *in vitro* allows to register CL burst following the brain or the neuronal cell culture reperfusion. LC-CL correlated with intensification of intracellular $O_2^{\bullet -}$ production without superoxide release from cells.[17] How can lucigenin report of radical reactions that occur in another compartment? There are indications that lucigenin belongs to the class of substances capable of nonradiative energy transfer of a Forster type.[16,18] In some model systems lucigenin may accept energy of the product of its oxidation, excited N-methyl acridone, and radiate it in the region characteristic of its fluorescence. It is also known that the acceptors of a nonradiative energy transfer need not necessarily be in the same phase as the donors.[19] If lucigenin is accepting energy from the appropriate excited donors appearing due to intracellular radical reactions, then LC-CL may indicate a constant generation of electron excited states in blood. Such process is different from the electron excited states generation during RB, and it occurs most effectively in blood under the conditions when the interactions between all its components are least perturbed.

As it is illustrated in Figure 4, the maintenance of blood ''organization'' allows it to preserve for some time its oxidative metabolism reflected by LC-CL even without ambient air supply. On the other hand, cessation of air supply to blood drastically reduces LM-CL, which predominantly reflects the generation of ''late'' reactive oxygen species. It is

interesting to speculate that neutrophils in peripheral capillary or venous blood continuously generate intracellular $O_2^{\bullet -}$ and/or other ROS in the course of their oxidative metabolism, and oxygen for its generation is supported by erythrocytes. However, the physiological role of erythrocyte-dependent ROS production by nucleated blood cells giving rise to electron excitation, that can be revealed due to presumably nonradiative energy transfer to lucigenin, is currently unknown.

Experiments with blood transfer from one sample to another during the development of RB in blood revealed another peculiar property of this complex system. The extraction of blood from the sample is followed by an acceleration of CL intensity growth rather than by a reduction of its level. On the other hand, the sequential addition of the new portions of blood to the recipient sample retarded the elevation of CL in it. As a result when the donor and the recipient samples contained equal volumes of one and the same blood CL intensity in the former significantly exceeded that in the latter. The same trends as illustrated in Figure 6 were consistently reproduced with different nondiluted blood preparations. We noted also that the difference between CL progression curves in the donor and recipient samples was especially pronounced in cases when healthy donors' blood after 12–24 h of storage was used. In such preparations LM-CL without addition of RB stimulants was already noticeable. The only difference between the two samples containing equal volumes of blood after its transfer is that in the first one it was reached by blood extraction and in another—by its summation. Thus, it may be proposed that the strong difference in CL levels in two samples reflects blood reaction as an integral dynamic system upon its dissecting.

It seems premature to suggest at this stage of investigation any specific mechanisms of blood integration that are reflected in its chemiluminescent properties. Nevertheless CL data from the measurements on nondiluted blood may be informative of the integrative properties of blood tissue in addition to its being a measure of some sort of oxidative metabolism in blood.

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