Simultaneous Evaluation of Chemiluminescence and Bioluminescence in a Phagocytic System

D. G. Deryabin and I. F. Karimov

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The spectra of luminol-dependent chemiluminescence of leukocytes, forming as a result of their oxygen-dependent bactericidal systems activation, and bacterial bioluminescence of *Escherichia coli* recombinant strain with cloned *lux* operon, used as the object of phagocytosis, are not identical. Mutual overlapping of these spectra reaches 87%, including overlapping of the photoemission maximums. However these spectra can be evaluated separatelely in the short-wave "arm" of the chemiluminescence spectrum (<420 nm) and the long-wave "arm" of the bioluminescence spectrum (>560). The kinetics of luminol-dependent chemiluminescence of phagocytes and of bacterial bioluminescence in their mixtures is characterized by mutually dependent phase-wise changes in the intensity of the analyzed parameters.

Key Words: *phagocytosis; chemiluminescence; bioluminescence; Escherichia coli*

Diagnostic and analytical methods, based on the use of the fluorescence phenomenon (fluorescence emerging during biochemical reactions *in vitro* and *in vivo*), are now widely used. It is largely explained by the fact that by the beginning of the 1980ies the technologies for detection of radiation in the optical range have reached the sensitivity when individual quanta can be counted. One more important advantage of the appropriate devices (luminometers) is their rapid work, sufficient for studies of the reaction kinetics in the real time mode.

The method for evaluation of luminol-dependent chemiluminescence (CL) of phagocytes [9,10] is one of well-known technologies based on fluorescent analysis of biological fluids and cell suspensions. It is based on the ability of luminophores (luminol, lucigenin, *etc.*) to be activated during oxidation by active oxygen forms, this activation resolving with emission of a light quantum. The intensity of the resultant fluorescence is directly proportional to the degree of activation of oxygen-dependent bactericidal systems of phagocytes.

One more well-known trend is based on the use of fluorescent sea microorganisms. The level of their fluorescence is in proportion with their metabolic activity or viability. Cloning of the respective *lux* operons in pathogenic or opportunistic microorganisms appreciably extended the sphere of bioluminescent analysis. Studies of the regularities of bacterial infection development [14] and of the status of humoral [7] and cellular immunity effectors, for example, the completeness of the phagocytic reaction [8], became possible.

This brings us to the development of an integral method for phagocytosis evaluation, combining the chemiluminescent and bioluminescent approaches and providing (on the base of fluorescence analysis) the data on the degree of activation of the phagocyte oxygen-dependent systems and the intensity of their bactericidal effect.

We studied the probability of separate valuation of CL and bioluminescence (BL) in the phagocytic system. Different chemical nature of the involved luminophores and the relevant probable

Orenburg State University, Russia. ***Address for correspondence:*** dgderyabin@yandex.ru. D. G. Deryabin

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differences in the fluorescence emission spectra [13] suggested this study.

MATERIALS AND METHODS

Recombinant *Escherichia coli* strain with *Photobacterium leiognathi* (a fluorescent sea bacterium) *luxCDABE* operon, cloned in *pUC18* vector, served the object of phagocytosis [2]. It was shown previously [4,7] that using this microorganism, it is possible to attain direct proportionality between bacterial target cell BL quenching and intensity of the developing bactericidal effect towards these bacteria. Before the study the bacteria were restored from lyophilized state by adding cold distilled water and 30-min exposure at 4°C for stabilization of fluorescence. Opsonization was carried out by mixing the bacterial biomass in equal volumes with commercial normal human immunoglobulin so that its final concentration was 6-10 mg/ml and the final concentration of bacteria 5×10^8/ml. The resultant mixture was exposed during 10 min at 37°C. Due to this opsonization procedure, early bactericidal effect, unrelated to phagocytosis, is ruled out. (This effect is possible if serum or plasma are used). In addition, opsonization provided a sufficient level of subsequent activation of phagocytes [3]. Microorganisms with heat-inactivated (10 min at 42°C) enzymatic system of fluorescence generation served as the object of phagocytosis in a special series of experiments for evaluation of luminol-dependent CL alone.

Human peripheral blood neutrophils were isolated by centrifugation in a double ficoll-verograffin density gradient with 1.077 and 1.112 g/ml densities, after which the cells were collected from the lower interphase, washed in cold saline, and resuspended in medium 199 to the concentration of 5×10^6/ml.

The phagocytic system was formed by mixing 1 volume of opsonized bacteria (5×10^8/ml) and 9 volumes of phagocytes (5×10^6/ml), so that the bacteria:phagocytes proportion was 10:1. Luminol (Sigma) was added into the system to the final concentration of 10^{-3} M and the mixture was incubated during 60 min at 37°C in a thermostat measuring cell of BLM8802M2C two-channel biochemiluminometer (Nauka Firm). The original design of this device (Fig. 1) allows simultaneous registration of fluorescence by two photoelectron multipliers [5], while the cuvette compartment has two independent outlets leading to them; photofilters can be placed between the sample and the recorders [6]. In a special series of experiments these studies were carried out without luminol for evaluating the kinetics of bacterial BL changes during phagocytosis of these bacteria.

Analysis of photoemission spectra, forming in bacterial BL or luminol-dependent CL, was carried out using a series of interference band photofilters (Photo-Optic Firm) with transmission maximums of 400, 420, 440, 460, 480, 500, 520, 540, and 560±5 nm. Summary fluorescence intensity of a sample was recorded in one channel and the photoemission spectrum portion, passing through a certain filter, was recorded in the other one. The final estimation of fluorescence intensity (I) was carried out using the formula $I=I_{sample}/T\times S$, where T is the individual characteristic of the photofilter, describing the degree of its transmission at appropriate wavelength, and S is the spectral sensitivity of the photoelectron multiplier at this wavelength.

The BL intensity (I_{BL}) in kinetic analysis of bacterial target cell BL during phagocytosis was calculated by the formula $I_{BL}=100\times(X_0-X_n)/X_0$, where X_0 is BL intensity at 0 sec and X_n is BL intensity at the n-th sec of the phagocytic system existence [8].

The results were statistically processed using Statistica 5.0 software.

RESULTS

Analysis of BL spectrum of *E. coli* strain with cloned *P. leiognathi luxCDABE* operon, carried out with a series of band interference photofilters, showed the characteristic distribution with the maximum at 480±5 nm, where its intensity was 15.41±0.75% of the summary photoemission (Fig. 2). Other characteristics of the spectrum were determined by the presence of a more steep short-wave (from 440 nm) and gently sloping long-wave "arm". Using the 560±5 nm interference filter, it was possible to register up to 1.31±0.40% of total fluorescence intensity. This indicated the presence of emission in a longer-wave (up to 600 nm) band. This spectrum is typical of the majority of natural and recombinant fluorescent microorganisms [15], utilizing 4a-hydroxyflavin as the photoemitter; its degradation is associated with release of excessive energy (blue-green light quantum) [12].

Co-incubation of fluorescent microorganisms and human peripheral blood neutrophils resulted in phase-wise changes in the summary fluorescence intensity (Fig. 3, *a*). The initial reaction consisted in short-term BL stimulation (by 131.18±11.75% of initial level), forming by sec 45-75 of the contact. This stimulation was presumably caused by accumulation of LPO products (forming as a result of interactions with active oxygen forms and serving as substrates for the bacterial luciferase) in the bacterial cell [1]. Further changes presented as progressive reduction of fluorescence intensity, reaching

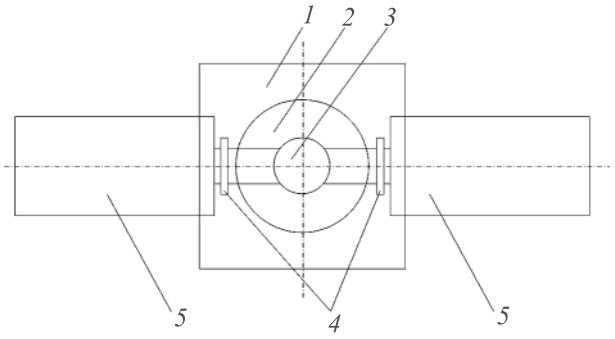

Fig. 1. Scheme of cuvette compartment of two-channel biochemiluminometer. *1*) all-metal body of cuvette compartment; *2*) cuvette holder; *3*) cuvette; *4*) slots for photofilters; *5*) photoelectron multiplier.

42.09±7.39% of initial level and correlating with the intensity of bactericidal effect forming under these conditions.

Study of luminol-dependent CL spectrum showed that it was not identical to that of BL with a shift towards the short waves. For example, the fluorescence maximum with a photofilter with a transmission band of 440±5 nm detected 22.09± 0.28% summary photoemission (Fig. 2). Chemiluminescence, in turn, was not detected in the spectrum of >560 nm, while due to use of the 400±5 nm photofilter it was possible to record up to 7.57± 0.42% summary photoemission, this suggesting the presence of fluorescence in the nearest ultraviolet spectrum as well. This distribution in general corresponds to the known characteristics of luminol-dependent CL mediated through the formation of oxidized stimulated intermediate product (3-aminophthalate), whose relaxation leads to emergence of fluorescence in the blue-violet spectrum.

During the formation of the phagocytic system the CL response of the peripheral blood neutrophilic leukocytes also demonstrated characteristic kinetics (Fig. 3, *a*), reflecting the intensity of active oxygen forms generation. Rapid increase in the fluorescence intensity was recorded during the first stage, with the maximum during min 5-6 of the phagocytic system existence, which was followed by stabilization of the signal.

The results indicate that the intensity of bacterial BL and luminol-dependent CL during phagocytic reaction undergoes characteristic phase-wise mutually dependent changes. The formation of CL maximum (indicator of activation of the leukocyte oxygen-dependent systems) is synchronous with the most pronounced fall of BL intensity (reflecting the development of bactericidal effect towards the target cells).

Due to different nature of photoemitters in BL (4a-hydroxyflavin) and CL (3-aminophthalate), the recorded spectra are not identical, though have a large (reaching 87%) overlapping zone. This rules out separate evaluation of BL and CL at their maximum values and creates such a possibility for measurements of the photoemission spectra not overlapping each other: short- and long-wave "arms". For example, the measurements are possible by using the band interference photofilters 420±5 nm for CL and 560±5 nm for BL evaluation. Simultaneous evaluation of fluorescence intensities in the same phagocytic system in two above spectral bands detected changes (Fig. 3, *b*), corresponding by their direction and time characteristics to the above-described kinetics of bacterial BL and luminol-dependent CL. However the fluorescence intensity in this case was by more than an order of magnitude lower, which fact necessitated the use of higher amplification coefficients with the relevant emergence of accessory "murmur".

Hence, the results indicate the principal possibility of separate evaluation of BL and CL in a phagocytic system. This task can be realized by

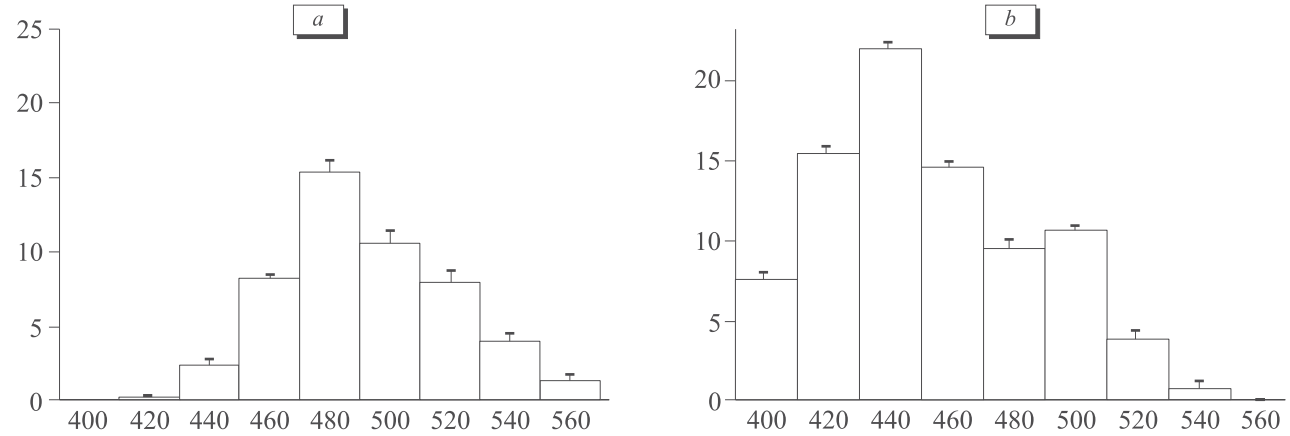

Fig. 2. Spectra of bacterial BL (*a*) and luminol-dependent CL (*b*). Abscissa: recorded fluorescence wavelength (nm); ordinate: fluorescence percentage of summary photoemission.

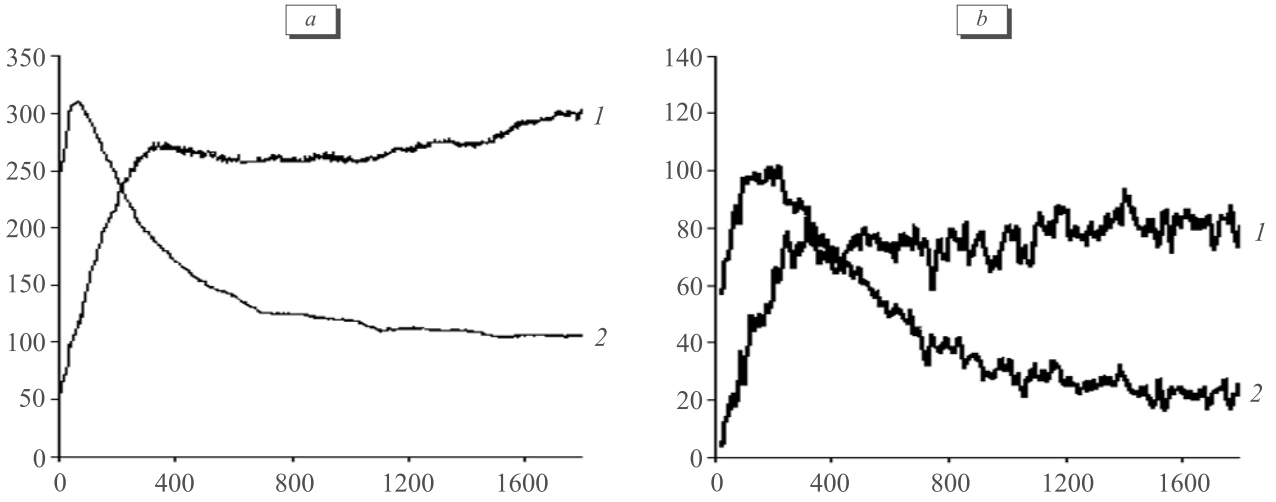

Fig. 3. Results of separate (*a*) and combined (*b*) measurements of CL (*1*) and BL (*2*) in the phagocytic system. Abscissa: duration of measurement (sec); ordinate: fluorescence intensity (arb. units).

using a special two-channel biochemiluminometer [5,6] with a set of photofilters. The fluorescence intensity can be evaluated in the real time mode in two spectral bands. Limitations because of low intensity of the signal can be eliminated by using longer periods of its averaging and use of limiting (but not band) photofilters, allowing registration of fluorescence in the <420 and >560 nm spectra. Complex solution of these tasks should form the base for creation of a new modern and technological method for evaluation of phagocytosis, one of the parameters of natural anti-infection resistance and immunological reactivity.

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