On-Line Determination of Serum Bactericidal Activity Using Recombinant Luminescent Bacteria

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 142, No. 8, pp. 200-204, August, 2006
Original article submitted November 17, 2004

Intensity of luminescence quenching in recombinant strains of *Escherichia coli* with cloned lux-operones by human blood serum is directly proportional to the degree of bactericidal effect assessed by nephelometric and bacteriological methods. This correlation was most characteristic of *E. coli* with luminescence genes from *Photobacterium leiognathi*, which substantiates its use in the development of the kinetic bioluminescent method to determine of serum bactericidal activity. The possibility of using this method for evaluation of activity of classic and alternative pathways of compliment activation was demonstrated by using zymosan or EGTA-Mg^{2+}-treated sera and C1-C5-deficient sera.

Key Words: *bacterial luminescence; Escherichia coli; blood serum bactericidal activity*

Construction of recombinant strains of *E. coli* carrying lux-operons of natural luminescent (LS) bacteria [4,6] extended the application area of LS-bioanalysis to the assay of biological fluids from humans and animals [2], where *Escherichia coli* is a persistent symbiont. In this way, LS-bioanalytical methods were developed to determine blood serum bactericidal activity (BSBA), which is routinely tested with *E. coli* strains [5]. A system for the LS-analysis of BSBA based on recombinant strain *E. coli* with cloned luciferase genes of *Pyrophorus plagiophthalamus* insect was described [9]. However, the molecular features of the structure of *Pyrophorus plagiophthalamus* insect LS-system result in a peculiar bell-shaped curve of luminescence intensity during the action of blood serum (BS), which is explained by facilitation of substrate diffusion to luciferase after perforation of the plasma membrane with serum factors. Application of the substrate at late terms of the contact [10] restored proportionality between biological LS and bactericidal potency, but made impossible kinetic measurements of BSBA and on-line analysis of this parameter.

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It seems promising to study the possibility to using other recombinant strains of *E. coli* with cloned lux-operons of marine and soil LS-bacteria, whose reaction to BS was previously investigated by us [1].

Our aim was to compare the intensity of BS-induced quenching of bioLS produced by various *E. coli* recombinant strains with the degree bactericidal effect of the serum in order to develop a kinetic LS-biological assay to test BSBA.

MATERIALS AND METHODS

The study used recombinant strains of *E. coli* "Ekolyum-5" and "Ekolyum-8" (M. V. Lomonosov Moscow State University) containing plasmids constructed on the basis of pUC18 vector. Ekolyum-5 strain carried LS-genes of marine bacterium *Photobacterium leiognathi,* while Ekolyum-8 strain had lux-operon from soil microbe *Photorhabdus luminescens* ZM1 [4].

The pool of BS from 20 healthy donors was used as the source of bactericidal agents. Special experiments were performed with the same BS pool incubated at 56°C for 30 min for complete inactivation of the complement system. The classical

0007-4888/06/1422-0234

pathway of complement activation in BS pool was inactivated by application of 10 mM EGTA with 2.5 mM $MgCl_2$ at 37°C for 60 min [7]. The alternative pathway was inactivated with zymosan (5 mg/ml) applied at the same temperature and exposure time; zymosan particles were then removed by 5-min centrifugation at 13,600 rpm [8]. In some experiments commercial samples of BS deficient by one of C1-C5 complement components were used (Institute of Hematology and Blood Transfusion, Kirov). Recombinant LS-strains of *E. coli* (10^9 CFU/ml, 0.5 ml) were mixed with equal volumes of 10-100% normal BS (preliminary dissolved with 0.85% NaCl) and BC deficient by various complement components. NaCl saline (0.85%) served as the control. The intensity of bacterial LS was measured in kinetic mode using a BLM-8820M bioluminometer (SCTB Nauka) coupled with a pen-recorder. During measurements, the strain was placed in a thermostat at 37°C for 30 min. LS was presented as percent of control, background LS was subtracted.

In parallel, BSBA was tested by nephelometric or bacteriological methods using the corresponding recombinant strains of *E. coli* as the target objects.

The data were processed routinely.

RESULTS

E. coli strain Ekolyum-5 with LS-genes from *Photobacterium leiognathi* first reacted to addition of BS by small (112.1±4.6% baseline level) and short-term (5-8 min) luminescence. Then LS of this strain was characterized by progressing dose-dependent quenching of luminescence proportional to BS concentration, which attained a plateau 25-30 min after the contact (Fig. 1, *a*). Parallel nephelometric (Fig. 1, *b*) and bacteriological testing of BS pool using *E. coli* strain Ekolyum-5 as a test object showed that bactericidal activity of BS determined by these methods strongly correlated (r=0.984, p<0.01). Moreover, they were characterized by high and significant correlation with the degree of luminescence quenching. For example, in paired experiments, where luminescence quenching was compared with nephelometric or bacteriological testing of BSBA, the corresponding values were r=0.859 (p<0.05) and r=0.815 (p<0.05).

E. coli strain Ekolyum-8 with cloned lux-operon from *Photorhabdus luminescens* ZM1 demonstrated a pronounced stimulation of luminescence (140.1±7.7% baseline level). The time needed for attaining maximum luminescence increased in proportion to the decrease in BS concentration (Fig. 1, *c*). Further development of LS-reaction was characterized by rapid drop of luminescence to >50% baseline level. In this period, luminescence was proportional to BS concentration. Hence, parallel nephelometric (Fig. 1, *d*) and bacteriological testing of BS pool using *E. coli* strain Ekolyum-8 as the test object could not demonstrate parallelism between changes in BS-induced luminescence of this strain and BSBA values. This was seen from pronounced decrease of correlation coefficients for both bacteriological (r=0.066, p>0.05) and nephelometric (r=0.343, p>0.05) methods.

Analysis of optical density of the bacterial cultures affected by BS, which is the basis of nephelometric method of BSBA determination, provided an explanation of peculiarities of LS-response of *E. coli* Ekolyum-8 strain. Initial stages of the contact of this test strain with BS were characterized by a pronounced and long-term increase in optical density, probably related to antibody-induced agglomeration of the bacteria. Parallel pronounced increase in the concentration of bacteria in some microvolumes of the probe and the corresponding potentiation of "quorum sensing" effect [3] can underlie the initial stimulation of biological LS. This was also observed during the action of BS on *E. coli* strain Ekolyum-5, where this phenomenon was short-term and far less pronounced. A specific peculiarity of Ekolyum-8 strain was greater stability to the bactericidal factors in BS (Fig. 1, *b*, *d*), which could contribute to the kinetics of biological LS.

Thus, the peculiarities of BS-induced luminescence of the recombinant strains are greatly determined by the nature of cloned LS-system, while the parameters of this reaction reflect individual features of the response of the natural donor strain [1]. Among serum-sensitive recombinant strain of *E. coli* only variants demonstrating proportionality between the decrease in spontaneous LS and the percent of cells subjected to bactericidal action under the same conditions can be used as the test-object for LS-based probing of BSBA. These requirements are met by recombinant strains of *E. coli* with cloned lux-operons from marine luminescent bacterium *Photobacterium leiognathi* (*E. coli* strain Ekolyum-5).

Taking into consideration the leading role of serum complement system in bactericidal potency of a particular biological fluid, further studies tested the possibility of using *E. coli* recombinant strain Ekolyum-5 to test BS with special disturbances in this complement system. In addition to the initial pool of normal BS, we used the same pool, where the complement system proteins were completely inactivated by heating. The variants of BS pool with selective inactivation of classical [7]

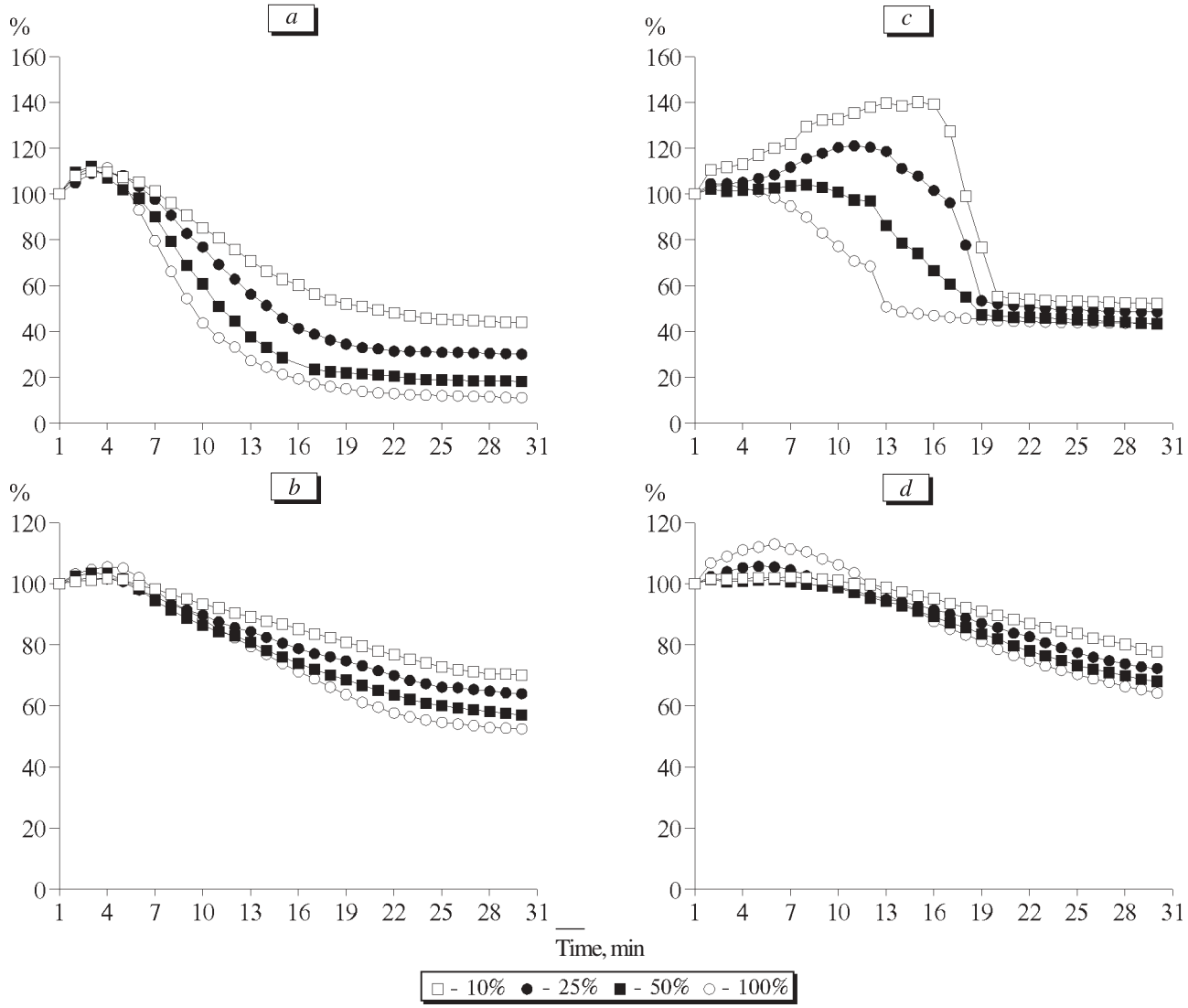

Fig. 1. Kinetics of biological LS (*a*, *c*) and optical density (*b*, *d*) of the recombinant strains *E. coli* strain Ekolyum-5 (*a*, *b*) and Ekolyum-8 (*c*, *d*) under the action of BS in various concentrations.

or alternative [8] pathways of complement activation were also used.

The application of normal BS pool to Ekolyum strains resulted in progressive decrease of LS accompanied by proportional drop in optical density. By contrast, the use of thermally inactivated BS demonstrated less pronounced quenching of luminescence (less than 50% effect observed with normal BS, Fig. 2, *a*), which was accompanied by minimal changes of nephelometric parameters. The results of testing BS pool with inactivated classical or alternative complement-activation processes attested to intermediate character of their quenching effects, which did not attain those of normal BS, although surpassed quenching characteristic of the thermally inactivated BS. When zymosan-treated BS was used, the quenching was less pronounced compared to BS treated with EGTA-Mg^{2+}, which confirmed greater importance of the alternative pathway of complement activation for the development of the bactericidal effect in respect to the used bacteria. It should be noted that in many regions of the kinetic curves, the sum of the effects of EGTA-Mg^{2+}- and zymosan-treated BS approximated 100% effect of normal BS pool. In other words, this combined treatment adequately assessed total activity of the alternative and classical pathways of complement activation.

Under the action of C1-, C2- and C4-defficient BS (these elements are involved in the initial reactions of classical complement activation pathway), the kinetics of LS was similar to the initial relatively long-term increase of LS followed by a drastic drop on minutes 21-26 of the contact (Fig. 2, *b*). Probably, this peculiarity of biological LS resulted from complement activation developed via a parallel alternative pathway after the formation of

TABLE 1. Parameters of BSBA Assays Based on Recombinant LS-Bacteria

Method	M. Virta *et al.*, 1997 [9]	M. Virta *et al.*, 1998 [10]	Original method
Recombinant bacterium	*E. coli*	*E. coli*	*E. coli*
Genetic source of LS-system	Insect *Pyrophorus plagiophthalamus*	Insect *Pyrophorus plagiophthalamus*	Marine bacterium *Photobacterium leiognathi*
Application of LS-reaction substrate	+ (before BS application)	+ (after BS application)	—
Determination of kinetic parameters	+	—	+
Direction of BS-induced LS changes	Increase followed by a decrease	Decrease	Decrease
Proportionality of the effect to BSBA determined by routine methods	± (partial: time of maximum effect is inversely proportional to BS concentration)	+	+

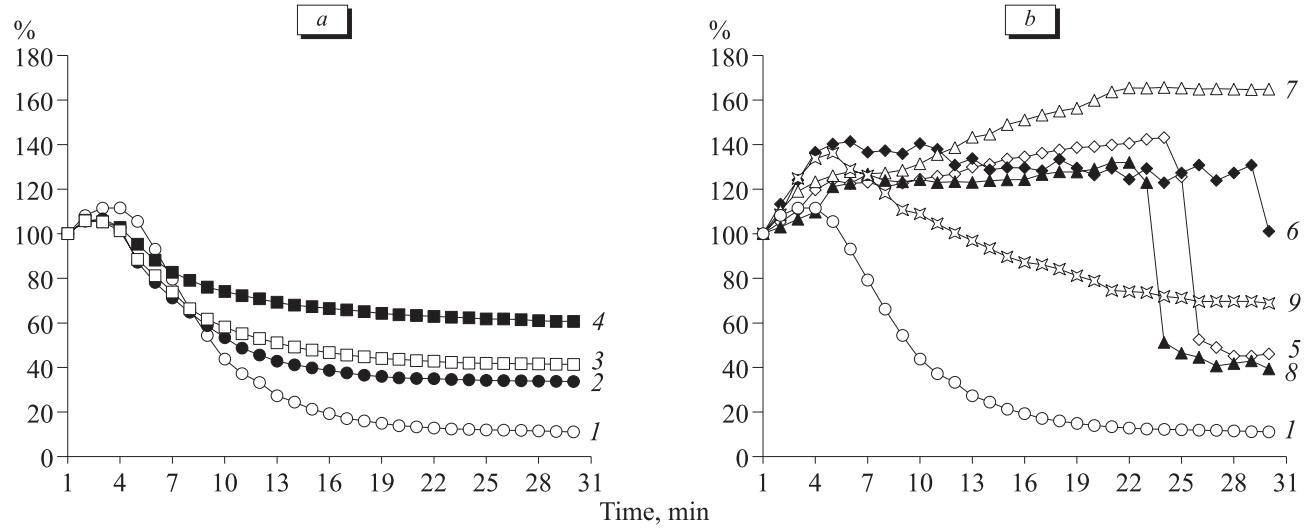

Fig. 2. Kinetics of bioLS of the recombinant strains *E. coli* strain Ekolyum-5 (*a*, *b*) under the action of BS with inactivated complement system. *1*) 100% BS; *2*) EGTA-Mg^{+2}-treated BS; *3*) zymosan-treated BS; *4*) thermally inactivated BS (56°C); *5*) C1-deficient BS; *6*) C2-deficient BS; *7*) C3-deficient BS; *8*) C4-deficient BS; *9*) C5-deficient BS.

membrane-attacking C6-C9 complex damaging the target cells. The use of BS deficient by C3 complement component located at the intersection of classical and alternative activation pathways completely prevented the development of bactericidal effect and stimulated LS of the test bacteria to 165.7±5.5% relatively to the initial level. The deficiency of C5-component of the complement system was characterized by the least depression of bactericidal potency.

Thus, recombinant *E. coli* strain Ekolyum-5 with cloned lux-operon of *Photobacterium leiognathi* is the key element for the development of a novel method of kinetic determination of BSBA by quenching biological LS with a possibility of automatization and parallel testing of a large number of the specimens. In comparison with available similar methods (Table 1), the important advantage of the present approach is direct proportionality between the intensity of luminescence quenching and bactericidal effect, which makes it possible to carry out on-line data procession. In all studies of normal and complement-deficient BS, the kinetics of LS-response was more contrast and pronounced in comparison with that observed in the parallel BSBA tests carried by the nephelometric method. Therefore, in contrast to routine methods of BSBA testing, the LS-based probing not only quantitatively describes bactericidal activity of blood serum, but provides more detailed information on the peculiarities of the development of the bactericidal effect.

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