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Estimation of reactogenicity of preparations produced on the basis of photoinactivated live vaccines against brucellosis and tularaemia on the organismic level. 1. Using the LASCA method

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***Abstract*. A new method of photoinactivation of bacteria aimed at producing prototypes of vaccine preparations against extremely dangerous infections is described. The reactogenicity of the new prophylactic preparations was studied using the laser speckle contrast analysis (LASCA). The performed experimental studies show that bacterial suspensions, irradiated using different regimes of photoinactivation, do not cause detrimental effect on the blood microcirculation in laboratory animals.**

Keywords: *speckles, contrast, LASCA, blood microcirculation, monitoring, brucellosis, tularaemia.*

1. Introduction

At present vaccination is performed using live, killed, chemical, and recombinant vaccines. Unfortunately, all of them are not free of drawbacks. Recombinant vaccines include the new class of vaccines, produced using different methods of genetic engineering. They are derived using recombinant proteins, lipopolysaccharides, fragments of proteins or lipopolysaccharides of pathogenic microbes, synthesised in the cells of laboratory strains of bacteria, viruses, yeasts. Many recombinant vaccines cause the immune response, insufficient to induce a long-term specific immunity. Probably, the cause is that such preparations contain a monomeric form of the protein with linear epitopes instead of conformational ones, which are immunogenic. Living vaccines are derived from divergent or attenuated strains of microorganisms, possessing a weakened virulence for a human, but containing a full set of antigens. Administration of a living vaccine causes reproduction and generalisation of microorganisms stimulating a pronounced immune response in the human organism. Vaccinal process differs from the inflectional one by the benign course. However, the usage of living vaccines keeps the probability of their reversion into a virulent form, which may cause a disease in the vaccinated person. Inactivated vaccines are enough stable and safe, since they are unable to cause reversion. As a rule, these vaccines are less immunogenic and, therefore, require the administration of multiple doses (booster immunisations), which often causes a definite level of their reactogenicity.

Practically, as a rule, the greater the efficiency, the more frequent the complications of the vaccination. Therefore, improving the methods of producing vaccines, safe both for the whole organism and, particularly, for its immune system, is an urgent problem at present.

The goal of the present study is to investigate the possibility in principle to create a new class of vaccine preparations based on the photoinactivated bacteria against such extremely dangerous infections as tularaemia and brucellosis. Preliminary analysis of the protective properties of the prototypes of prophylactic preparations is presented in Ref. [1]. In particular, it is shown that the photoinactivated cells of the bacteria *Francisella tularensis* 15 NIIEG and *Brucella abortus* 19 BA conserve the antigen structure under some irradiation regimes in the presence of a photosensitiser in small concentrations. The present paper is devoted to the study of reactogenicity of these preparations using coherent optical methods.

2. Materials and methods

In the present work we used the vaccinal strains of Gram-negative bacteria *B. abortus* 19 BA and *F. tularensis* 15 (SIU Microgen, Omsk, Russia). The cells of *F. tularensis* 15 were cultivated at the temperature $T = 37\,^{\circ}\mathrm{C}$ during 48 hours on the FT agar substrate with pH 7.0 (FSIS SRCAMB, Obolensk, Moscow region). The cells of *B. abortus* 19 BA were kept in a thermostat at the same temperature during 5 days on the erythritol agar substrate with pH 7.2 ('Nutrient media' Scientific-Production Association, Makhachkala, Russia).

The bacterial suspension was prepared in the saline using the two-day-old agar culture. The suspension to which an exogenous photodynamic dye (methylene blue) was added was subjected to irradiation by 650-nm light-emitting diodes. The treatment of bacteria was carried out using the setup for the inactivation of microorganisms [2]. The

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setup was built on the standard immune-enzyme assay plate with removable strips. Each well of the plate was equipped with an individual light-emitting diode ($\lambda = 650$ nm). The photosensitiser concentration was varied from 0.0005 % to 0.05 %, the irradiation power was from 0.2 to 1.4 mW. The time of exposure for the bacterial suspension was varied within the wide limits from 2 to 360 minutes. After irradiation the suspension was again seeded onto the dense nutrient substrate and cultivated at $T = 37\,^{\circ}\mathrm{C}$ during 14 days.

In the course of the experiment 0.1 mL of methylene blue was introduced into each of 96 wells of the plate. Then 0.1 mL of the suspension, containing 10^9 microbe cells (m.c.) in 1 mL of the *F. tularensis* 15 or *B. abortus* 19 BA culture was added into each well. The plate was put onto the setup for photoinactivation closed with a shutter to prevent the contamination by the air microflora. The viability of the culture was estimated by the number of grown colonies.

Three control experiments were performed parallel to the main experiment. In the first one the control of the number of microbe cells was implemented. 0.1 mL of the mixture from the test tube, containing 10^3 m.c. mL^{-1} of *F. tularensis* 15 or *B. abortus* 19 BA, was seeded on a dense nutrient medium and cultivated in a thermostat at 37 °C. Then the number of newly arisen colonies was counted. In the second experiment the influence of methylene blue on the number of microbial cells was estimated. In this case irradiation was excluded. As already mentioned, 0.1 mL of methylene blue was placed into each well of the plate. Then 0.1 mL of the *F. tularensis* 15 or *B. abortus* 19 BA suspension was added into each well from the test tube, containing 10^9 m.c. mL^{-1}. After the exposure, corresponding to the conditions of the main experiment, the culture was seeded onto the dense nutrient medium and cultivated in a thermostat at $T = 37\,^{\circ}\mathrm{C}$. In the third experiment the culture irradiation was controlled. The influence of the photosensitiser was eliminated, and only the action of the irradiation on the bacteria was taken into account. Instead of the photosensitiser, 0.1 mL of saline and then 0.1 mL of bacterial suspension (10^9 m.c. mL^{-1}) was placed into each well of the plate, so that the total volume (0.2 mL) of the irradiated mixture was unchanged. The duration and the power of irradiation corresponded to the main experiment. The preparations with the concentration 10^9 m.c. mL^{-1} were injected intramuscularly to the laboratory animals in the volume of 0.5 mL. Previously Nembutal was administered for anaesthesia following the standard scheme. The animals were observed during an hour after the administration of preparations.

The reactogenicity of bacterial suspensions in Guinea pigs was determined via the variation in cerebral blood flow after the intramuscular injection of 0.5 mL of the suspension, containing 10^9 m.c. mL^{-1} of irradiated or intact cultures of the vaccinal strains of tularaemia or brucellosis, respectively. To study the changes in the cerebral blood flow, we used a relatively new method, namely, the method of laser speckle contrast analysis (LASCA) [3–13]. A detailed review of the studies in this field is presented in paper [14].

The LASCA method allows visualisation of brain microvessels *in vivo*, avoiding the craniotomy of laboratory animals. A detailed description of the setup used in the experiments is given in [15].

There exist two modifications of LASCA. The first one is based on the analysis of a single realisation of statistical speckles [3]. In this case the whole realisation of the speckle field is divided into small regions, as a rule, 5×5 or 7×7 pixels each. For each of the selected regions the local value of the statistical speckle contrast is calculated, and then the image is constructed. In the present work the second modification of LASCA was exploited, based on the processing of dynamical speckles [16]. A detailed comparison of the two speckle processing approaches is presented in [5].

The principle of speckle-field processing in the dynamical LASCA method is the following. At each point of the dynamical speckle pattern the contrast of dynamical speckles is calculated using the formula

$$V = \frac{\sqrt{\langle (I - \langle I \rangle)^2 \rangle}}{\langle I \rangle} = \frac{\sigma_I}{\langle I \rangle}, \tag{1}$$

where I is the instantaneous intensity of the dynamical biospeckles; σ_I is the standard deviation of the intensity fluctuations; the angular brackets mean time averaging. Obviously, the lower the contrast, the higher the local velocity of the blood flow.

LASCA is typically used to study brain microcirculation in white rats. It is important to emphasise that the study of brain blood flow in Guinea pigs is a much more difficult problem. The thickness of the head bone tissues in Guinea pigs is much greater, than in white rats, which significantly worsens the quality of registered images. Usually the rat's head is fixed with respect to the setup by means of rigid fastening. However, the use of this technique for the study of blood microcirculation in Guinea pigs leads to negative results. The thicker bone tissue of the Guinea pig's head suffers greater deformation under its fixation in the setup. This causes additional speckle dynamics and, consequently, lower signal-to-noise ratio. Moreover, Guinea pigs are essentially larger than white rats. A greater depth of breathing in Guinea pigs may cause significant displacements of the animal's head in the course of measurements.

To improve the sensitivity of the method, the algorithm for processing the dynamical images was essentially modified [17]. At the first stage of the processing the two-dimensional function of cross-correlation between each two subsequently registered images of the speckle field was calculated. The position of the maximum of this function allows high-precision determination of the relative displacement of the images. At the next stage the correction of the speckle-field image displacements, caused by breathing of the laboratory animal, was performed. Then the time-dependent contrast of the dynamical speckles was calculated at each point of the registered two-dimensional images, followed by the construction of its two-dimensional distribution. For reconstructing the image of the microvessels to be visualised, the averaging over 300 frames of the dynamical speckle field, registered by means of the monochrome Phoenix 1280 USB DC CMOS camera (MuTech, USA), was carried out. The field of view was 6×6 mm, the optical system magnification was 1.6 and the spatial resolution was 5.8 μm. In the course of correcting the position of individual frames in the regions, where the motion of red blood cells is observed, the speckle contrast will be also reduced. Obviously, in this case the two-dimensional distribution of speckle contrast completely

reproduces the view of the capillary network. According to estimates, this improved technique allows visualisation of brain microvessels up to the size of ~ 15 μm.

In the experiment for monitoring the changes, occurring in the cerebral blood flow, we calculated the correlation coefficient of the visualised brain microvessel images, obtained before and after the administration of preparations, obtained on the base of photoinactivated bacterial suspensions. Since in the experiment the correlation coefficient was close to 1, besides the statistical processing, an additional visual comparison of the analysed images was carried out.

The studies were performed in the Research Bacteriologic Laboratory of the Chernyshevsky National Research Saratov State University, Saratov, Russia.

3. Monitoring the blood microcirculation changes in laboratory animals under the administration of bacterial suspensions, irradiated in different photoinactivation regimes

Figures 1 and 2 show the time-averaged speckle-field images, the distributions of mean-square deviation of temporary fluctuations of the intensity and the contrast of the speckles before and after the administration of the irradiated suspension of cells of vaccinal strain of tularaemia, respectively. The spatial distribution of the speckle contrast in the image plane reflects the spatial localisation of deeply localised brain vessels. The images of averaged speckles and the spatial distribution of the mean-square deviation of the temporary intensity fluctuations of the biospeckles are less informative and mainly reflect not the localisation of microvessels in the capillary network of the brain, but the surface irregularity of the head bone tissue of the animal under the study (Fig. 2).

Comparing Figs 1 and 2 one can notice that the field of view is shifted. This effect is due to the displacement of the animal in the process of breathing. As already mentioned above, it is very difficult to eliminate this effect in the experiment.

The visual comparison of the images shows that the topology of the capillary network is practically the same before and after the administration of the suspension of photoinactivated cells *F. tularensis* 15. No changes in the number of microvessels with intense blood flow were observed. This means that the reactogenic effect of the studied preparation on the Guinea pig organism is small.

Similar results were obtained in the study of the reactogenic effect, caused by the suspension of photoinactivated cells of the vaccine strain of brucellosis.

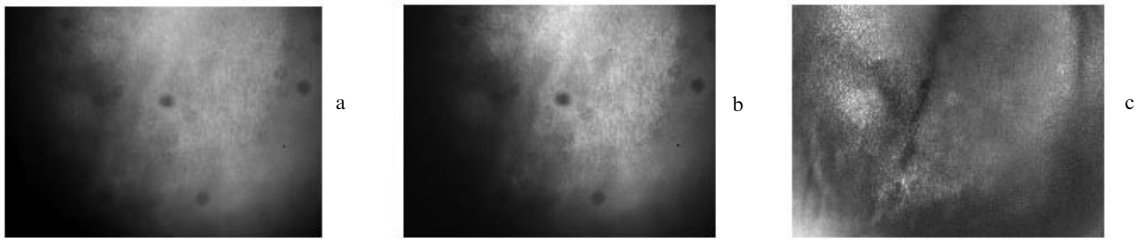

Figure 1. Visualisation of the Guinea pig brain vessels before administration of photoinactivated bacterial cells: time-averaged dynamical speckles (a) and spatial distributions of the mean-square deviation of temporary intensity fluctuations of the biospeckles (b) and the contract of dynamical speckles (c) in the image plane.

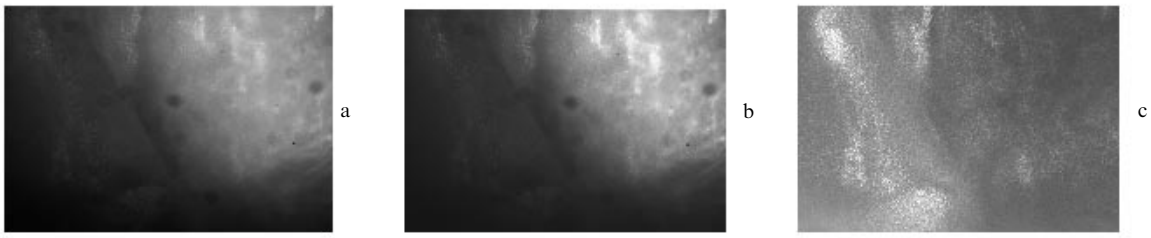

Figure 2. Visualisation of the Guinea pig brain vessels after administration of photoinactivated *F. tularensis* 15 cells: time-averaged dynamical speckles (a) and spatial distributions of the mean-square deviation of temporary intensity fluctuations of the biospeckles (b) and the contract of dynamical speckles (c) in the image plane.

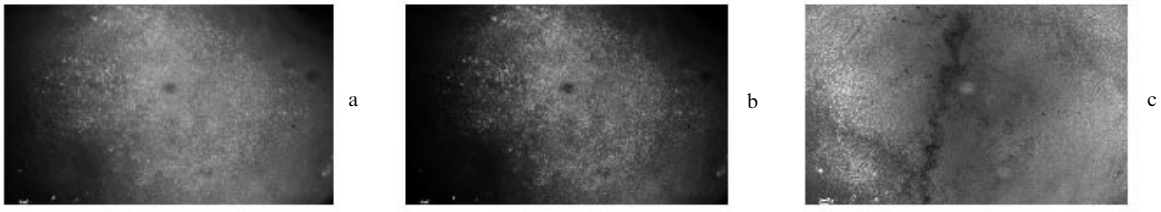

Figure 3. Visualisation of the Guinea pig brain vessels after administration of photoinactivated *B. abortus* 19 BA cells: time-averaged dynamical speckles (a) and spatial distributions of the mean-square deviation of temporary intensity fluctuations of the biospeckles (b) and the contract of dynamical speckles (c) in the image plane.

Figure 3 presents the images, corresponding to Fig. 1 and registered after the administration of the reparation, obtained on the basis of *B. abortus* 19 BA. No visible changes of the blood vessel network topology were found.

It was demonstrated in the experiment that Guinea pigs easily sustain the subcutaneous administration of non-irradiated and irradiated cultures of *F. tularensis* 15 and *B. abortus* 19 BA in high concentrations. No disease of laboratory animals or lowering of their activity was observed. Therefore, we can conclude that photoinactivation by low-coherence light does not increase the reactogenicity of vaccinal strains.

4. Conclusions

Monitoring of blood flow changes in the brain microvessels in Guinea pigs has shown that the topology of the capillary network is virtually identical before and after administration of the suspension of photoinactivated cells of vaccine strains of tularaemia and brucellosis. No change in the number of microvessels with an intense blood flow has been observed. This means that the reactogenic effect of the studied preparations on the blood vessels is small. As a result of the performed study we have found found that the intramuscular administration of the experimentally obtained bacterial suspensions does not exert long-term negative influence on Guinea pigs.

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