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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. 2. Using the method of speckle-microscopy with high spatial resolution

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***Abstract*. The method of speckle microscopy was adapted to estimate the reactogenicity of the prototypes of vaccine preparations against extremely dangerous infections. The theory is proposed to describe the mechanism of formation of the output signal from the super-high spatial resolution speckle microscope. The experimental studies show that bacterial suspensions, irradiated in different regimes of inactivation, do not exert negative influence on the blood microcirculations in laboratory animals.**

Keywords: *speckle-microscopy, blood microcirculation, monitoring, brucellosis, tularaemia.*

1. Introduction

Essential changes in the blood motion may arise under the use of different prophylactic preparations. The study of such changes, occurring, e.g., in the mesentery vessels of Guinea pigs, can be used as a base for the screening of these preparations or for studying their reactogenicity.

At present the diffraction of focused beams is used in diagnostics of the state of microvessels and the blood motion in them. Laser Doppler microscope was first applied to measure the blood flow in a separate vessel of the eye retina in early seventies of the past centenary [1, 2]. In Refs [3, 4] the statistical properties of biospeckles, formed due to the light scattering by a single blood vessel, are considered [5, 6]. Papers [7, 8] are devoted to the study of the statistical characteristics of biospeckles, arising due to the diffraction of sharply focused laser beams. The process of formation of speckle-modulated biospeckles and their statistical characteristics are considered in paper [9].

It is worth noting, that the diffraction of focused coherent beams is often used in the measuring systems (speckle-microscopes [10], Doppler microscopes [2], retinal blood flow meters [1], etc.). The study of microvessels by means of lasers requires precise focusing of the beam to provide the required spatial resolution of the optical system. In this case the speckles are formed from a small number of scatterers. The number of papers, specially devoted to the study of such speckle fields, is rather small. At the same time, many phenomena, accompanying the formation of such speckles, require a more detailed analysis. The development of classical methods of speckle microscopy and the analysis of the output characteristics of a speckle-microscope are presented in [10 – 12]. The results, presented in these papers, are obtained using the assumption that the statistics of the speckle fields is Gaussian. However, the studies of microvessels, whose size is smaller than that of a red blood cell, require focusing of the laser beam into a spot, whose diameter should be comparable with the wavelength of light. Such tight focusing may be achieved using a $90^{\times}$ microscope with the numerical aperture 1.25.

In the present work the speckle-microscope was modified to achieve the ultimate spatial resolution and the theory was proposed to describe the mechanism of the output signal formation in the speckle-microscope with super-high spatial resolution.

2. Speckle-microscopy with high spatial resolution

It is obvious that the scattering of a focused laser beam by a moving phase screen causes time-dependent phase modulation of the diffracted beam intensity. This results in the appearance of temporary fluctuations of the scattered wave intensity in the far-field region of diffraction. Within the approximations of the scalar diffraction theory the equation for the amplitude fluctuations of the dynamical speckle field in the far-field region for the normal incidence of a focused Gaussian beam onto a moving phase screen can be written in the form [13]:

$$U_{\rm s}\left(\frac{vt}{\lambda}\right) = \text{const}\int_{-\infty}^{\infty} U_0\left(\frac{vt}{\lambda}-\alpha\right)G(\alpha)\mathrm{d}\alpha, \qquad (1)$$

where

$$G(\alpha) = \exp\left(-\frac{2\pi \mathrm{i} X_0}{Z_0}\alpha\right)\exp\left(-\frac{\alpha^2}{W_0^2}\right); \qquad (2)$$

t is the time; v is the velocity of the blood flow, simulated by the moving phase screen; X_0 is the coordinate of the point

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of observation of the field fluctuations, normalised to the laser radiation wavelength λ; Z_0 is the distance between the scattering plane and the plane of observation; W_0 is the beam-waist radius; $U_0(vt/\lambda)$ is the field amplitude in the scattering plane under the illumination of the screen with a plane wave. All quantities entering Eqns (1) and (2) are dimensionless. The parameters having the dimension of length are scaled to λ, and the field amplitudes in the equations are scaled to the field amplitude in the waist centre of the focused Gaussian beam.

Obviously, when the light is scattered by the vessels whose diameter is of the order of the red blood cell size, a specular component is present that dominates over the scattered field. It may be easily shown (see the relevant derivation in [13]) that the expression for the correlation function of the fluctuation component of the field intensity can be written in the form:

$$\Psi_I\left(\frac{vt}{\lambda}\right) \propto \exp\left[-\frac{(vt/\lambda)^2}{2W_0^2 + L_c^2}\right], \quad (3)$$

where L_c is the correlation length of the spatial irregularities of the screen, simulating the blood flow in a microvessel.

It is worth noting that, strictly speaking, relation (3) is valid only for Gaussian statistics of the speckle field, which is not the case for a small number of scatterers. Direct numerical simulation and checking the hypothesis about the form of the distribution show that the intensity fluctuation statistics for the scattered field in the far-field region (the speckle forming in the speckle-microscope with high spatial resolution) obeys the Nakagami-*m* distribution [14]. However, the validity of Eqn (3) is fully confirmed by the computer simulations even in those cases, when the simplifying assumptions, used to derive it, become inapplicable.

It is important to emphasise, that the correlation time of the intensity fluctuations depends not only on the velocity of the scattering object, but also on the beam-waist radius and the correlation length of the spatial inhomogeneities of the scattering screen.

The principle of operation of the speckle-microscope with high spatial resolution is the following. A radiation beam from a 633-nm, 1-mW He – Ne laser is tightly focused with a 90$^{\times}$ microscope objective into a small-radius spot (of the order of the radiation wavelength) on the microvessel of interest. The blood flows in the microvessels cause intensity modulation of the tightly focused beam in the waist plane. This gives rise to the formation of dynamical speckles in the far-field diffraction region. To vary the illumination intensity we used the rotary NDC-100C-4M attenuator (Mounted Continuously Variable na Filter Thorlab, USA). The biospeckles dynamics was recorded using the silicon PDA 10A photodetector (Thorlab, USA) and a preamplifier. The detector area was 0.78 mm^2, the frequency band was 150 MHz, and the noise characteristic was 5.5×10^{-11} W Hz$^{-1/2}$. The amplified signal was applied to the linear input of the two-channel A-to-D card (NI USB-5133, 8 bit, band 50 MHz, National Instruments, USA), connected to the computer via a USB-port.

Besides the laser, the speckle-microscope comprises the source of white light and the optical imaging system, including the Biolam microscope (LOMO, Russia) and the monochrome digital WinCamD CMOS-camera (DataRay, USA) with the resolution 1024×1024 pixels. The camera was connected to the computer via another USB-port and allowed visual observation of the blood flows in capillaries and determination of the vessel contour position in real time.

The spectral functions of intensity fluctuations in biospeckles typically have a complicated form and may have one or several local maxima. Their appearance may be caused by the pulsing character of the blood flow in microvessels or by the contraction of the vascular walls. A measure of the velocity of blood microcirculation in an isolated vessel is provided by the width of the spectrum of the intensity fluctuations of the dynamic speckles, arising under the scattering of a Gaussian beam, focused onto the microvessel under study. An indirect characteristic of the blood flow velocity is the mean square deviation σ of the output signal of the speckle-microscope, namely, the greater the σ, the higher the blood microcirculation velocity.

3. Estimating the reactogenicity of bacterial suspensions after their irradiation in different regimes of photoinactivation.

3.1 Harmlessness estimation

The harmlessness of the cultures of vaccinal strains of tularaemia and brucellosis, both non-irradiated and irradiated with the low-coherence light ($\lambda_0 = 650$ nm, $\Delta\lambda = 10$ nm, $P = 0.2$ mW), was tested by administering them to a group of Guinea pigs. For this purpose we placed the suspensions of two-day-old bacterial cultures into the plate wells of the setup (250 μL into each well) and irradiated the cells during six hours for the tularaemia vaccine and three hours for the brucellosis one.

The degree of harmlessness of *B. abortus* 19BA and *F. tularensis* 15 before and after the irradiation was determined in Guinea pigs according to Refs [15, 16]. For this purpose, after a subcutaneous administration of *B. abortus* 19 BA in the doze of 2×10^9 microbe cells (m.c.) mL^{-1} and *F. tularensis* 15 in the doze of 5×10^9 m.c. mL^{-1} the animals were observed during 15 and 30 days, respectively.

It was noticed in the experiment that the Guinea pigs easily sustain the subcutaneous administration of high dozes of non-irradiated and irradiated cultures of *F. tularensis* 15 and *B. abortus* 19 BA. We observed neither diseases nor death of the probe animals, nor any reduction of their activity.

3.2 Studying the blood microcirculation changes in laboratory animals under the administration of photoinactivated bacterial suspensions on a tissue level

As mentioned above, the spectral functions of the intensity fluctuations of biospeckles are usually complicated and may have one or several local maxima.

A characteristic view of the output signal from a speckle-microscope is presented in Fig. 1a. The spectrum of the intensity fluctuations (corresponding to the output signal, shown in Fig. 1a) is presented in Fig. 1b.

The speckle-microscope was used to estimate the blood microcirculation abnormalities in a single vessel, caused by the action of photoinactivated cells of vaccinal strains. The bacterial suspensions of laser-processed vaccinal strains of tularaemia and brucellosis were administered to the mesentery of Guinea pigs using the application method. The suspension concentration was 10^9 m.c. mL^{-1}. The observa-

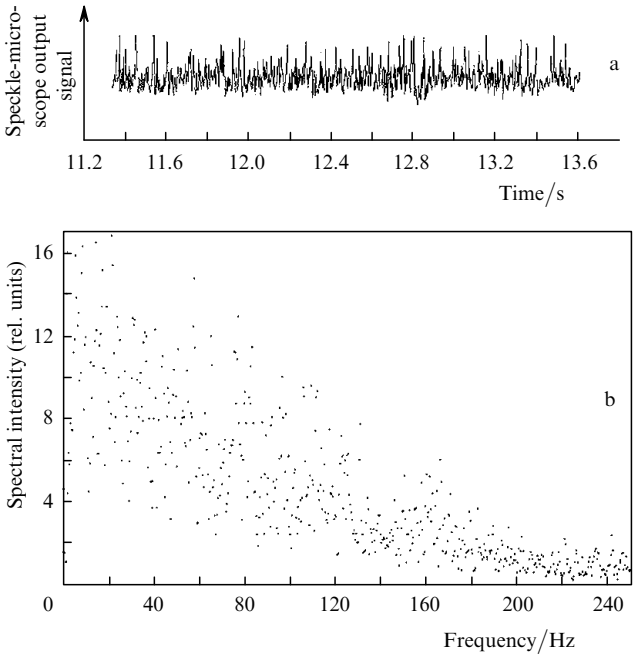

Figure 1. The results of the study of blood microvessel in the initial state: a typical output signal of the speckle-microscope with the duration 2 s (from the 11th to the 13th second of observation) (a) and the spectrum of the output signal of the speckle-microscope (b).

tion was performed during 40 min after the administration of preparations.

As the experimental studies have shown, the application of laser-processed suspension of cells of vaccinal strain of tularaemia caused almost immediate changes in the blood flow, which became essentially slower, up to complete stopping. The vessels came to the pre-stasis state. This effect was observed under the action of vaccinal strains, irradiated in different photoinactivation regimes and at different concentrations of the photosensitiser. If in the normal state the spectral width of the output signal, corresponding to the vessel of interest was 160 Hz, then after the application of the preparation the spectral width immediately decreased to 10 Hz. This means the reduction of the blood flow by 16 times. The blood flow dynamics (temporal variations of velocity) is presented in Fig. 2a. A similar behaviour is demonstrated by the mean-square deviation σ of the speckle-microscope output signal (Fig. 2b). One can see from Fig. 2b that the blood flow is practically stopped (this usually occurs at the 10th second after the use of preparations). Then after some time ($\sim$ 5 s), the blood flow resumes but with irregular behaviour (Fig. 2c). After about 5 minutes the blood flow becomes regular again (Fig. 2d), being still slower in comparison with the initial state. The blood flow velocity restores its initial value 10 minutes after the preparation use.

The experiments carried out allow us to conclude that the vaccinal strain of the tularaemia causative agent conserves its reactogenicity for the blood vessel tissue after the photoinactivation. However, the resulting blood flow changes fall within the permissible variation limits, since the microcirculation irregularities, caused by the action of the preparation, are reversible and the blood flow completely restores after 10 minutes.

As shown by the experimental studies, the application of photoinactivated brucellosis cells to the mesentery of

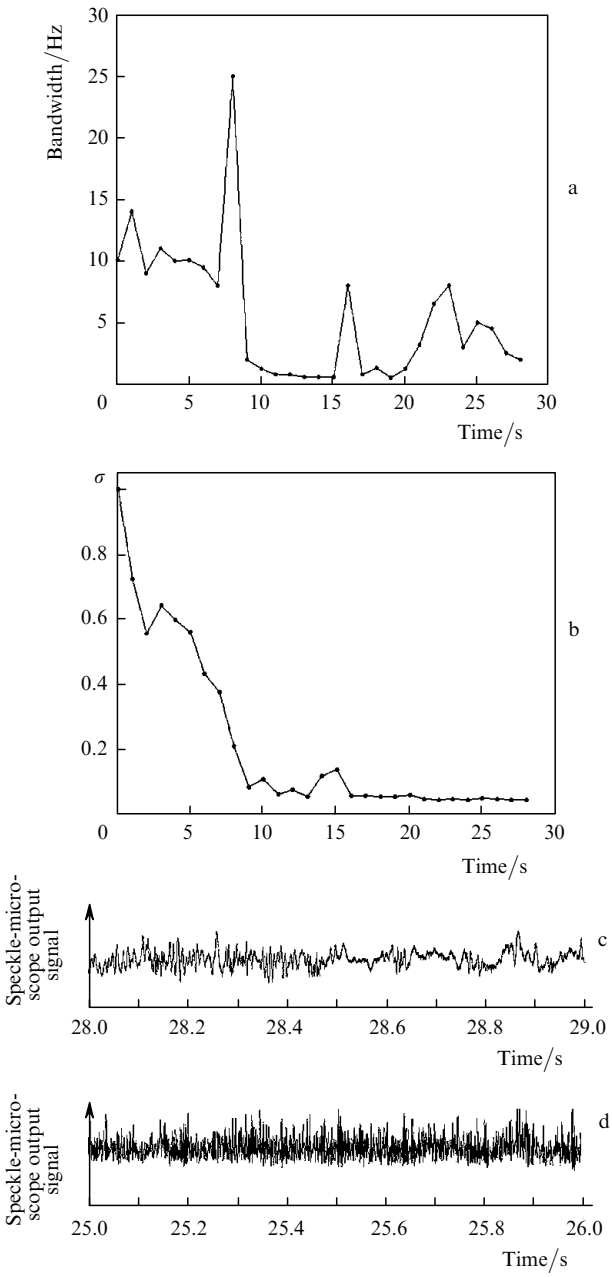

Figure 2. Blood flow dynamics after the application of photoinactivated *F. tularensis* 15 cells to the Guinea pig mesentery: time dependence of spectral band width of the speckle-microscope output signal (a) and mean square deviation σ of the speckle-microscope output signal, normalised to the mean-square deviation of the signal, recorded immediately after the use of the preparation (b); the speckle-microscope output signal, demonstrating the irregular character of the blood flow from the 28th to the 29th second after the application of the preparation (c), and the speckle-microscope output signal with the duration 1 s, recorded 5 min after the use of the preparation (the regular blood flow restored) (d).

Guinea pigs not only caused no decrease in the blood flow velocity (as with tularaemia bacteria), but, quite the contrary, increased it significantly. For example, if the spectral width of the speckle-microscope output signal before the action of the preparation was 9 Hz (Fig. 3a), then after the application of photoinactivated brucellosis bacterial cells this width increased up to 45 Hz (Figs 3b, c). This is an evidence of a quintuple increase in the blood flow velocity due to significant reduction of the vascular lumen.

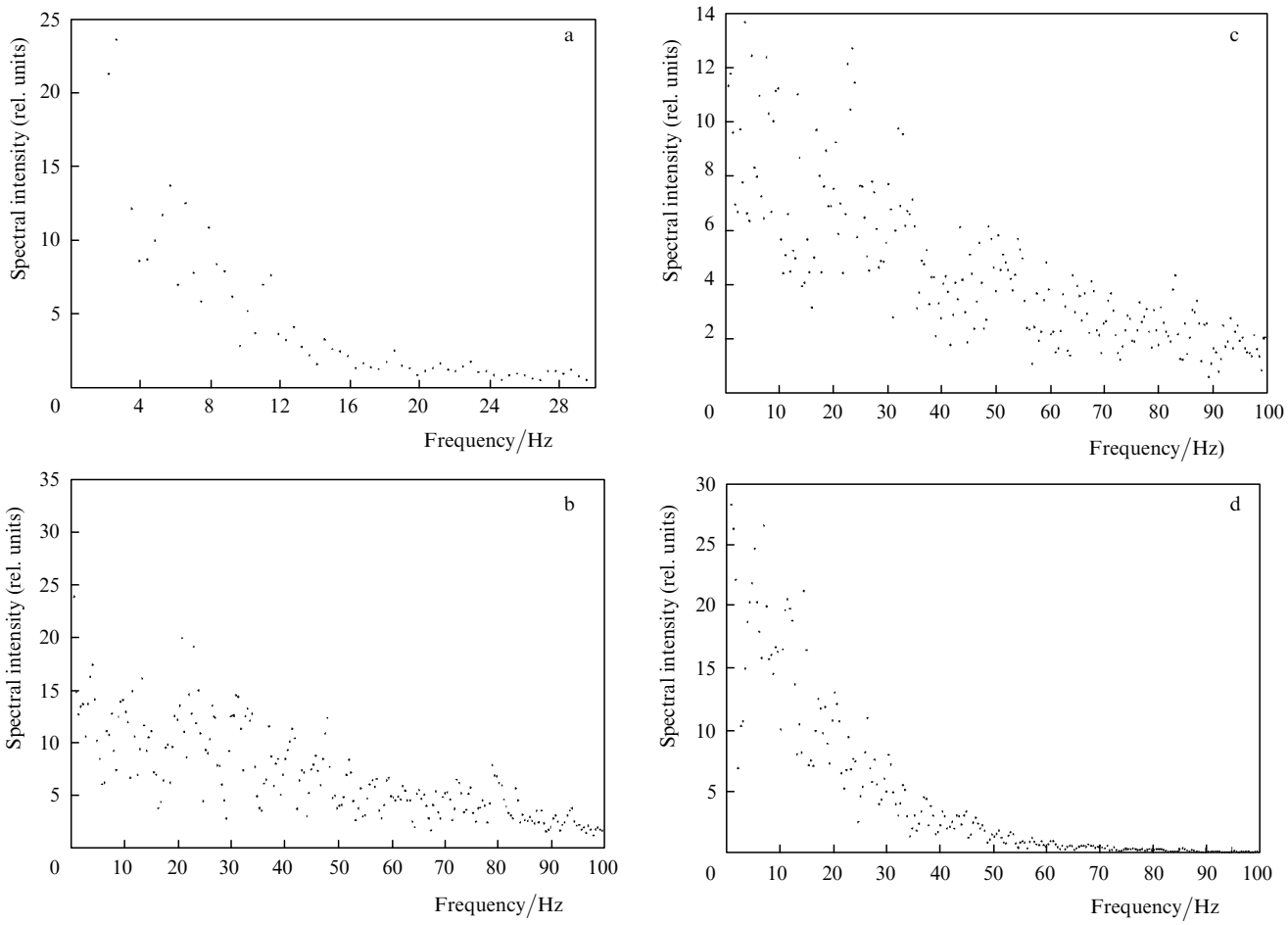

Figure 3. Spectra of the speckle-microscope output signal, recorded before the application of the photoinactivated cells of *B. abortus* 19 BA to the mesentery of a Guinea pig (a) and after 1 (b), 2 (c) and 3 min (d) after the application of the preparation. The width of the spectrum is 9 (a), 45 (b), 40 (c), and 20 Hz (d).

This is the reactogenic action of the preparation on the vascular tissue. With time the spectral width and, correspondingly, the blood flow velocity gradually decrease (Fig. 3d). The blood flow characteristics become completely normal during 3–7 min after the preparation application.

4. Conclusions

The studies performed show that the speckle-microscopy is much more sensitive and informative than the laser speckle contrast analysis (LASCA) in application to the problems of estimating the reactogenicity of prophylactic preparations.

Using the speckle-microscopy technique we estimated the effect of photoinactivated suspension of tularaemia vaccinal strain bacteria on the blood microcirculation in a single vessel of the mesentery. It is shown that the use of the preparation causes deceleration of the blood flow. The present experiments allow us to conclude that after two hours of photoinactivation the vaccinal strain of the tularaemia causative agent remains reactogenic for the vascular tissue. However, the abnormalities of microcirculation, caused by the preparation, are reversible.

Application of inactivated cells of the vaccinal brucellosis strain to the mesentery of a Guinea pig caused significant increase in the blood flow velocity (in many cases by 5 times). This is due to a significant reduction of the vascular lumen (just this is the reactogenic action of the preparation). With time the spectral width and, correspondingly, the blood flow velocity gradually decrease and completely restore to the normal values during 5–7 min after the application of the photoinactivated bacterial suspension.

Using the method of speckle-microscopy we managed to determine not only the duration of the reactogenic action of the photoinactivated vaccines on the vascular tissue, but also its character. The authors' opinion is that, obviously, the tularaemia and brucellosis vaccines subjected to photoinactivation excite the nerve endings of different subdivisions of the nervous system. The tularaemia vaccine affects the parasympathetic nervous system, causing the dilatation of the mesentery vessels. The brucellosis vaccine excites the nerve endings of the sympathetic subdivision of the nervous system, which is manifested in the pronounced constriction of the mesentery vessels.

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