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USE OF BIOLOGICS

Effect of Antigens Obtained from the Recombinant Strain *Bacillus anthracis* 55ΔTPA-1Spo⁻ on Organs and Tissues of Immunized Animals

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Abstract—The recombinant *B. anthracis* strain 55ΔTPA-1Spo⁻ was used for the development and trial of a method for the simultaneous production of immunogenic anthrax antigens, a protective antigen, and the EA1 protein of the S layer, which are components of a prototype anthrax vaccine. The proposed method includes inoculum preparation, cultivation in liquid medium without antibiotics, sterilizing filtration, concentration, diafiltration, and chromatographic purification on various carriers. This method provides for a high yield of both target products. The purified antigens (alone or in combination with each other) were shown to have no toxic effect on organs and tissues of vaccinated laboratory animals in amounts that were several times higher than immunizing doses. The minor changes revealed by histological examination reflect the adaptation-compensatory reactions of the macroorganism and tend to normalize. The response of immune-competent organs corresponded to moderate development of immunogenesis. The addition of EA1 protein to the recombinant protective antigen resulted in an increase in the expression of the genes determining TLR of innate immunity. Immunization of laboratory animals with the combined preparation caused more pronounced immunobiological alterations in lymphoid organs than the use of only the protective antigen.

Keywords: Bacillus anthracis, anthrax, anthrax vaccines, recombinant protective antigen

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INTRODUCTION

Bacillus anthracis is a gram-positive sporogenous microorganism that causes anthrax, a highly dangerous zooanthroponous infectious disease. Until the middle of the last century, the mass mortality of farm animals from this infection and the outbreak of disease among humans were a serious problem for veterinary and public health. In the second half of the 20th century, the situation tended to improve due to the spread of preventive veterinary and sanitary activities, as well as antiepidemic measures. However, even with the maintenance of a relatively safe situation, anthrax outbreaks occur continuously in endemic areas [1]. Humans are usually infected after contact with diseased animals or infected food and raw materials of animal origin. Spores of the pathogen can persist for a long time and accumulate in the environment. The data from monitoring of the epidemic situation in Russia indicate a steady expansion of the problem anthrax areas; their number is now about 35000 [2].

Expansion of the nosoareal of a pathogenic microorganism can occur due to climatic changes. Thus, an anthrax outbreak emerged due to a large epizootic among deer in the Yamal-Nenets Autonomous Area in 2016. The situation was provoked by an extremely hot summer, which contributed to the thawing and unearthing of the forgotten cattle burial ground [3]. Elimination of the consequences required a significant investment of resources. Thus, 946 people participated in preventive activities and over 13000 people were immunized against anthrax [4]. The risk of the infection transfer from the territory of neighbor countries, the activation of soil foci in areas of natural disasters, and the use of the microorganism for terroristic purposes are not excluded [5].

A key role in the prevention of anthrax is played by the vaccination of the prescribed contingent. The licensed, successfully used, live vaccine based on the attenuated strain *B. anthracis* STI-1 invaluably con-

Abbreviations: CL, culture liquid; PCR, polymerase chain reaction; rPA, recombinant protective antigen; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; TLRs, placebo-like (toll-like) receptors.

tributed to the improvement of the epidemiological situation [6]. Nevertheless, global practice has shown that the development of safer and more effective vaccines for the specific anthrax prevention is an urgent task. The research in this field has been carried out for several decades. The greatest success has been achieved in the development of chemical immune preventive measures. In 2016, three experimental vaccines based on the recombinant protective antigen (rPA) were at various stages of clinical trials in the United States. According to modern views, rPA is a basic component of the developed anthrax vaccine; immunogens and substances activating the structures of innate immunity can be added to it [7].

The main criteria for selecting candidates for vaccine development are high immunogenicity (the ability to induce a pronounced immune response in the vaccinated organism after a single or double immunization) and low reactogenicity (lack of damage to organs and tissues of the macroorganism). A prototype of the recombinant anthrax vaccine containing rPA as the main immunogenic component and EA1 protein of the S layer as an additional component was obtained at the Microbe Russian Antiplaque Scientific Research Institute.

The goal of the research was to develop and test a common technological scheme for the production of rPA and EA1 by the recombinant producer strain *B. anthracis* 55ΔTPA-1Spo$^-$ and to study the effect of the obtained antigens on the immunological reactivity of the macroorganism, organs, and tissues of immunized laboratory animals.

EXPERIMENTAL

Strains

The recombinant asporogenous strain *B. anthracis* 55ΔTPA-1Spo$^-$, which produces rPA (KM97, RF patent no. 2321629), was used. The phenotypic characteristics and plasmid composition of the strain were the following: Cap$^-$(pXO2$^-$), Tox$^-$(pXO1$^-$), and Km^r(pUB110PA-1). The strain was provided by the State Collection of Pathogenic Bacteria (Microbe Russian Antiplaque Scientific Research Institute).

Laboratory Animals

The experiments were carried out with BALB/c mice (20 ± 2 g) and guinea pigs (300 ± 20 g) from the nursery of the Microbe Russian Antiplaque Scientific Research Institute. The biomodels were kept on a standard diet with sufficient amounts of water in accordance with the requirements for the humane housing and use of animals in experimental studies.

Nutrient Media

Hottinger's agar and broth (Microbe Russian Antiplaque Scientific Research Institute) were used. Kanamycin (25 μg/mL) (Sigma, United States) and tryptone (20 mg/mL) (Roeper, Germany) were added when required.

Cultivation of the Asporogenous Recombinant Strain

The strain *B. anthracis* 55ΔTPA-1Spo$^-$ was plated from ampoules onto dishes with Hottinger's agar (pH 7.2–7.4) containing selective antibiotic (kanamycin) at a concentration of 25 μg/mL and incubated at 37°C for 18 h.

One microbial loop of an 18-h agar culture of the producer strain was added to the Hottinger's agar slant tubes containing kanamycin (25 μg/mL) and grown at 37°C for 8 h to obtain the bacterial culture of the first passage. The biomass was further washed off with 1 mL of the cooled solution of 0.85% sodium chloride (pH 7.2) added to each tube.

The obtained microbial suspension of *B. anthracis* 55ΔTPA-1Spo$^-$ strain was transferred to the Hottinger's agar slant tubes containing kanamycin (25 μg/mL) and incubated again at 37°C for 18 h in order to obtain bacterial culture of the second passage. The microbial mass was then washed off the agar surface via the addition of 3 mL of cooled 0.85% sodium chloride solution (pH 7.2) to each vial. The cell suspension collected after both passages was transferred to the same sterile vial and diluted with a 0.85% solution of sodium chloride (pH 7.2) to an approximate concentration of 50×10^9 cells/mL. The producer culture was subsequently used as inoculum for the fermenter in the amount of 10 mL of cell suspension per 1 L of medium.

Cultivation of the *B. anthracis* 55ΔTPA-1Spo$^-$ strain was carried out in large volumes in an experimental fermenter with a working volume of 14 L and automatic maintenance of the ambient parameters. Prior to cultivation, the standard procedures for preparation of the bioreactor, filters, and purification system were carried out. The nutrient medium (Hottinger's broth, 5 L) was introduced with a pump at a vacuum depth of 40–53 kPa. Sterilization was carried out at 121°C under a pressure of 110 kPa for 30 min. After sterilization and the setting of a temperature of 37°C in the bioreactor, the tryptone sterile solution was added to the nutrient medium according to the aseptic rules to a final concentration of at least 20 mg/mL. While the culture was grown at 37°C, the broth in the reactor was continuously aerated via stirring with a mechanical stirrer at a rate of 150 rpm. The amount of supplied air was 0.5–0.6 L/min per 1 L of the nutrient medium. Stirring and air supply were stopped after 16 h, and 2 mM EDTA (Sigma, United States) per 1 L of the medium was added, thereby ending the culture growth.

Cell Biomass Separation and Sterilization of the Culture Filtrate

Small volumes of CL (up to 1 L) were sterilized with a 500-mL Nalgene filtration device (United States) with a 0.2-μm filtration membrane and separated into the fraction of the culture filtrate containing rPA and the cell mass fraction containing EA1 (supernatant obtained from biomass). Separation of the cell biomass and simultaneous sterilization of the obtained culture filtrate were carried out with a device with a 0.2-μm Vivaflow 200 microfiltration membrane (Sartorius, Germany) when larger volumes of the culture of the producer strain were used. For this purpose, CL was fed to the container of the filtration unit after cultivation ceased, and the concentration (filtration) process was carried out at an operating pressure of 250 kPa until the volume of the processed product containing the cell mass decreased by at least ten times. The separated sterile culture filtrate containing PA and the cell biomass concentrate containing EA1 were subjected further to processing after the specific sterility control.

Isolation and Chromatographic Purification of the Protective Antigen

Sterile culture filtrate was diluted to a ratio of 1 : 1 (vol/vol) with buffer containing the following: 50 mM sodium chloride solution (Neva-Reaktiv, Russia), 25 mM diethanolamine (Sigma), and 2 mM EDTA; pH 8.9. The solution was then concentrated approximately 20 times by filtration (approximate operating pressure of 250 kPa) with a Vivaflow 200 (Sartorius) unit with a nominal 30 kDa molecular weight elimination. Diafiltration was performed with the same unit with a tenfold volume of the aforementioned buffer, which was added to the container with the concentrated product.

Subsequent purification was carried out with a BioLogic Duo Flow™ chromatographic system (BioRad, United States). A chromatographic column (90 × 90 mm) was filled with 100 mL of Macro Prep 50Q resin (BioRad), degassed, and washed with an equal volume of a buffer containing 1 M sodium chloride solution. The column was then equilibrated by passing ten sequential volumes of the buffer (pH 8.9) containing 25 mM diethanolamine, 50 mM sodium chloride, and 2 mM EDTA and one volume of the same buffer (pH 8.9) supplemented with 30 mM potassium chloride solution at a flow rate of 10 mL/min. The concentrated culture filtrate after dialysis (the volume for the resin binding) was applied onto a column with Macro Prep 50Q. The proteins that did not bind to the carrier were collected. The resin was then washed with diafiltration buffer containing 30 mM potassium chloride in a volume equal to the volume of the carrier. Both factions were combined.

The obtained preparation was concentrated ten times as described above. Diafiltration was carried out successively with a tenfold volume of buffer (pH 8.9) containing 50 mM sodium chloride, 25 mM diethanolamine, 2 M EDTA, and a tenfold volume of buffer (pH 10.0) containing 145 mM ammonium acetate and 2 mM EDTA. The preparation was then placed in small containers and stored at –70°C.

At the next step, a chromatographic column (25 × 800 mm) was filled with a Sephacryl-HR300 carrier (BioRad, United States) for gel filtration and equilibrated with three volumes of the buffer solution (pH 8.0) containing the following: 0.1 M Tris, 1 mM EDTA, and 50 mM sodium chloride. The system was programmed for a flow rate of 2 mL/min. The preparation was thawed and applied to the column (the whole sample at one time) in a volume not exceeding 3% of the gel volume. The protein concentration in the initial preparation did not exceed 2.5 mg/mL. The protein yield was controlled spectrophotometrically at a wavelength of 280 nm. Samples containing PA were collected and combined after 10–15 chromatographic cycles.

At the final step, rPA was concentrated (by approximately ten times) using a Vivaflow 200 ultrafiltration membrane with a nominal 30 kDa molecular mass elimination or Vivaspin-20 (Sartorius) concentrators with the same pore diameter.

The degree of PA purification was determined according to the results of electrophoresis under denaturing conditions in a 10% polyacrylamide gel. The protein concentration was measured by optical density on a spectrophotometer at a wavelength of 280 nm. The purified and lyophilized rPA was stored at –70 and 0–8°C, respectively.

Isolation and Purification of the EA1 protein of the S layer

The cell mass collected by centrifugation was washed from the remaining medium with a 0.85% solution of sodium chloride in order to isolate EA1. The biomass (500 mL) was mixed with 50 mL of the extracting buffer, which contained the following: 5 mM Tris hydrochloride, 1% SDS, and 5 mM 2-mercaptoethanol. The mixture was then heated on a water bath at 70°C for 30 min, cooled, and centrifuged at 7000 *g* (4°C, 30 min).

The obtained supernatant was sterilized on the filtration unit with a 0.2-μm Vivaflow 200 microfiltration membrane under an operating pressure of approximately 250 kPa. Control of the specific sterility was performed by plating of the supernatant onto a solid medium or inoculation into the liquid medium (0.1 mL per a dish with Hottinger's agar or per a tube with Hottinger's broth) and subsequent incubation at 37°C.

At the next step, the cell extract was subjected to diafiltration with ten volumes of the buffer (pH 8.0) containing 0.1 M Tris hydrochloride and 2 mM EDTA using a Vivaflow 200 unit with a nominal 30 kDa

molecular mass elimination. The intermediate product containing EA1 was stored at –70°C.

EA1 protein was purified with a BioLogic Duo Flow™ chromatographic system. A chromatography column (26 × 200 mm) was filled with preliminarily decanted hydroxyapatite (Sigma, USA) and equilibrated with ten free volumes of the buffer (5 mM K_2HPO_4/KH_2PO_4, pH 6.8) at a flow rate of 10 mL/min until a stable baseline was registered. The intermediate product was thawed and applied to a column. The protein was then eluted by the passage of three free volumes of a 0–1 M linear potassium phosphate gradient (K_2HPO_4/KH_2PO_4) through the column. The protein yield was spectrophotometrically controlled (at a wavelength of 280 nm). The fractions containing EA1 were collected and combined after 10–15 purification cycles.

The second step of purification was carried out on the same carrier by a similar procedure. The purified EA1 protein was diafiltered with ten volumes of bidistilled water at 4°C with a Vivaflow 200 (30 kDa) unit. The EA1-protein preparation was concentrated when required), placed in small containers, and stored at –70°C. The lyophilized preparations were stored at 8°C.

Protein Electrophoresis

Electrophoretic separation of proteins was performed in a 10% polyacrylamide gel with SDS. Each sample (20 μL) was mixed with a buffer(pH 6.8) containing the following: 0.125 M Tris hydrochloride, 4% SDS, 20% glycerol, 2% 2-mercaptoethanol, and 0.03 mM bromophenol blue. The ratio of the sample and buffer was 1 : 1. The mixture was subsequently heated at 100°C for 90 s and introduced into polyacrylamide gel wells (15 μL per well). Electrophoresis was performed in a vertical chamber (Amersham, USA) in the buffer (pH 8.3) containing 25 mM Tris, 192 mM glycine, and 3.5 mM SDS. The gel was stained in solution containing 0.3 mM Coomassie brilliant blue, 40% ethanol (Bryntsalov A-Ferein), and 7% glacial acetic acid (Panreac, Spain) for 1–2 h. The gel was then placed into a solution containing 5% ethanol and 7% glacial acetic acid and incubated with continuous shaking until a visible result was obtained.

Determination of the Concentration of the Protein Preparations

The protein concentration in the preparations was determined spectrophotometrically [8].

Immunoelectron Microscopy

The labeling of antibodies with colloidal gold was performed according to the procedure described earlier [9]. Preparations were prepared for immunoelectron microscopy according to the procedure described by J. Farchaus et al. [10] with our modifications [9]. The preparations were analyzed with a Hitachi HU-12A electron microscope (Japan).

Immunization of Laboratory Animals

Immunization of the laboratory animals was performed subcutaneously in the zone of the inner surface of the thigh once or twice with a two-week interval. A single dose was injected to guinea pigs and line mice in a volume of 0.5 and 0.2 mL, respectively. A sterile 0.9% solution of sodium chloride (0.2 mL for mice or 0.5 mL for guinea pigs) was injected to the animals of the control groups.

Isolation of Lymphocytes of the Thymus and Spleen

Line mice were divided into groups consisting of six animals each immunized with tested preparations. The biomodels of the control group remained intact. The animals were sacrificed 4 h after immunization by cervical dislocation in accordance with the Directive no. 2010/63/EC "On the Protection of Animals Used for Scientific Purposes" of the European Parliament and the Council of the European Union dated September 22, 2010. Lymphocytes were isolated by conventional methods [11].

Isolation of RNA from Lymphocytes of the Thymus and Spleen, Reverse Transcription, and PCR

Lymphocytes from the spleen and thymus, stored in Hanks solution at a temperature of –70°C, were thawed to room temperature. RNA from the cells was isolated with a RIBO-sorb reagent kit (InterLabServis, Russia) according to the manufacturer's recommendations. The reverse transcription was carried out with a Reverta kit (InterLabServis) according to the manufacturer's instructions. The cDNA preparation was stored for no more than 12 months at a temperature no higher than –70°C. PCR was carried out with primers to the fragments of the genes encoding β-actin (positive control) and TLR of types 2, 4, and 6. The PCR primers used in the study had the following sequences [12]:

β-actin-F 5'-TGGAATCCTGTGGCATCCATGAAAC-3'

β-actin-R 5'-TAAAACGCAGCTCAGTAACAGTCCG-3'

TLR2-F 5'-CAGCTTAAAGGGCGGGTCAG-3'

TLR2-R 5'-TGGAGACGCCAGCTCTGGCTCA-3'

TLR4-F 5'-AGTGGGTCAAGGAACAGAAGCA-3'

TLR4-R 5'-CTTTACCAGCTCATTTCTCACC-3'

TLR6-F 5'-AGTGCTGCCAAGTTCCGACA-3'

TLR6-R 5'-AGCAAACACCGAGTATAGCG-3'

Oligonucleotide primers were synthesized in the Microbe Russian Antiplaque Scientific Research

Institute on a DNA ASM-800 automatic synthesizer (Biosset, Russia). Amplification of the gene fragments was carried out with a Tertsik programmable thermocycler (DNK-tekhnologiya, Russia) according to the following profile: 1 cycle, 95°C, 5 min; 30 cycles, 95°C, 1 min; 1 cycle, 60°C, 1 min; 1 cycle, 72°C, 1 min; and the final cycle, 5 min, 72°C. A horizontal chamber (BioRad) was used for electrophoretic analysis of the PCR products in a 2% agarose gel.

Determination of the Proliferative Activity of Immunocompetent Cells by Flow Cytometry

Lymphocytes of the thymus and spleen of laboratory animals fixed in ethanol were collected by centrifugation at 250–300 g for 10 min. Potassium phosphate buffer (pH 7.3 ± 0.1) containing 50 μg/mL propidium iodide (Thermo Fisher Scientific, United States), 100 μg/mL RNase (Fermentas, United States), and 0.1% Triton X-100 (Sigma), was added to the obtained precipitate. After 15–20-min of exposure, samples of the mixture were analyzed on a Dako Cytomation Cyan ADP flow cytometer (Dako Cytomation, Denmark). The results were analyzed according to the protocol of the manufacturer of equipment and reagents.

Apoptosis was assessed by the accumulation of hypodiploid cells (<2C DNA per cell) in a peak located to the left of the peak corresponding to diploid cells. The activation of immunocompetent cells was determined by their number at the proliferative stage, which included the $S + G_2 + M$ stages of the cell cycle (>2C DNA per cell). The ratio of the number of cells at the stage of apoptosis and proliferation was also determined. This index value below 1 indicated a lack of the damage to the studied preparations on immunocompetent cells.

Samples of whole defibrinated human blood (healthy donor, 3 mL) with added anthrax antigens (rPA or EA1) (10 μg) in sterile physiological saline were used as a positive control during the determination of the cytotoxicity of antigenic preparations in vitro. The tubes with negative control contained physiological solution instead of antigens. Incubation was carried out for 1 day at 37°C. Cytofluorimetric control was performed as described above.

After incubation of the antigenic preparations with defibrinated blood, leukocytes were isolated and stained with a solution containing mithramycin and ethidium bromide (Sigma).

Morphological and Histological Studies

Immunized guinea pigs were sacrificed with chloroform within the defined time intervals (three individuals at a time) in order to determine the toxicity by morphological indicators. After autopsy, an examination was carried out, and a macrometric study of the mass and size of the animal organs was conducted.

Tissues of the liver, heart, lungs, kidneys, adrenal glands, skin, thymus, spleen, and local and distant lymph nodes of animals were selected for histological examination. The histological material was fixed in a 10% aqueous solution of neutral formalin. Further treatment was carried out according to the standard method [13]; the prepared semithin sections were stained with hematoxylin and eosin (Merck, USA). The material was examined under an Olympus CX31 microscope at a magnification of 40–200 times. The morphometric characteristics were assessed with the density-morphometric program of the MEKOS-TS (version 2.1.0.0) software package.

The average number of glomeruli was determined in the field of view of the section of kidneys. The number of stellate reticuloendotheliocytes (Kupffer cells) was determined in the field of vision of the liver section. The lymphatic follicles in lymphoid organs were enumerated.

The number of renal corpuscles (glomeruli) in the altered functional state (plethora of the capillary network, wrinkled glomeruli, edema of the vascular loops) was presented as the percentage of the glomeruli without visible changes.

The activity of lymphoid organs was characterized by a semiquantitative method (in points from 0 to 3). The lack of activity was assigned 0 points; poorly expressed activity was 1 point; moderately pronounced activity was 2 points; and significantly pronounced activity was 3 points. The test for follicles of lymph nodes (B zones) was assessed for 3 points if these zones had so-called light centers rich in blast forms of cells at the stage of mitosis and in macrophages containing fragments of nuclei. The average level of follicle activity (2 points) was indicated by an increase in the number of epithelioid cells in the light centers and a clear peripheral rim composed of small lymphocytes around the centers. In follicles with weak activity (1 point), the light centers contained few cells (characterized by "empty zones") and were surrounded by a wide mantle zone of small lymphocytes. Follicles without light centers were considered inactive (0 points).

The activity of medullary cords of B zones and T zones (paracortical zones) was considered to be zero activity (0 points) if no cellular hyperplasia was observed. The prevalence of blast forms indicated 3 points of activity; mixed composition (blasts and nonactivated lymphocytes or mature plasma cells) indicated 2 points of activity; and the prevalence of nonactivated cells with a small number of blast elements was estimated as 1 point of activity.

A similar approach was used to assess the activity of T and B zones in the spleen, where an increase in hyperplastic activity may be accompanied by an increase in the size of these structures, as in lymph nodes.

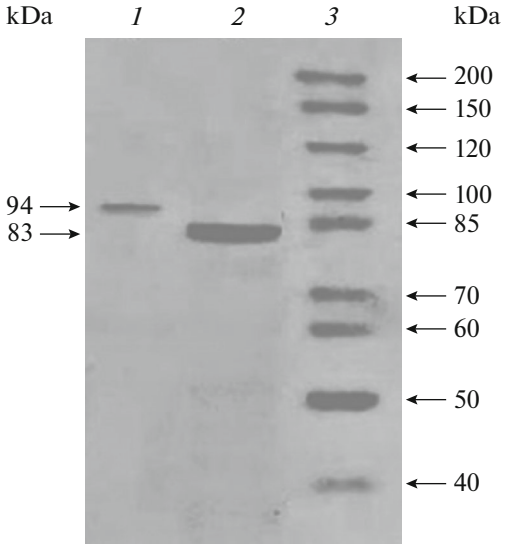

Fig. 1. Electrophoresis of purified preparations of EA1 (lane *1*) and rPA (lane *2*) isolated from *B. anthracis* 55ΔTPA-1Spo$^-$ strain; lane *3*, molecular mass markers (PageRuler^{TM} Unstained Protein Ladder, Thermo Scientific).

Statistical Methods

Statistical computing of the experimental data was carried out by standard methods: calculation of the arithmetic mean of absolute and relative values (M), the mean error of the arithmetic mean (m), and the confidence coefficient of the difference in means (t).

RESULTS AND DISCUSSION

Isolation and Purification of rPA and EA1 from the Asporogenous Recombinant Producer Strain

The *Bacillus anthracis* 55ΔTPA-1Spo$^-$ strain, which does not form spores and does not synthesize the pathogenicity factors of the anthrax microbe (edema and lethal factors, capsule), was stored in a lyophilized state at the State Collection of Pathogenic Bacteria. This strain was inoculated from ampoules and grown during two successive passages on media with kanamycin to obtain a sufficient biomass amount. Subsequent cultivation was carried out without the addition of selective antibiotic, which is important, since the target products are promising for use in human vaccinations. Cultivation of the producer was carried out for 16 h at a temperature of 37°C in the Hottinger's broth containing tryptone as an additional ingredient. These conditions were selected by us earlier for the efficient production of rPA and for the reduction of the risk of degradation of protein molecules under the action of the proteolytic enzymes of the strain [14].

At the next stage, separation of the culture filtrate and the cell mass occurred during sterilizing filtration. The sterile culture filtrate was used to isolate rPA, and EA1 protein was isolated from the concentrated biomass. A purified preparation of rPA was obtained by successive steps of concentration, diafiltration, and two-step chromatography on an ion exchange carrier and a carrier for gel filtration. The isolation of EA1 protein from the supernatant obtained after centrifugation of the disrupted cell mass and EA1 purification were carried out by successive stages of diafiltration and a two-step ion exchange chromatography with hydroxyapatite as a carrier.

The electrophoretic mobility of the protein obtained from the culture filtrate corresponded to the molecular weight of the PA of the anthrax microbe (83 kDa) (Fig. 1). The degree of protein purification was at least 85%; its concentration in the purified concentrated preparation was 10 mg/mL, and the yield was 128 mg of PA per 1 L of CL of the recombinant producer strain.

The electrophoretic mobility of the protein extracted from the supernatant of the disrupted cell mass corresponded to the molecular weight of the EA1 protein of the anthrax microbe (94 kDa) (Fig. 1); the degree of purification was 95%. The protein concentration in the purified preparation was 3.4 mg/mL, and the yield was 50 mg of the EA1 protein per 1 L of CL of the producer. In the immunoblot reaction with specific antibodies to PA and EA1, the formation of a detectable immune complex was observed at the level of electrophoretic mobility of proteins. The immunoreactivity of the purified proteins was also confirmed by the results of enzyme immunoassay with antibodies to PA and EA1 (data not shown).

We have previously isolated [9] EA1 protein from the plasmid-free derivative of the vaccine strain *B. anthracis* STIΔT. There is strong evidence that it belonged to the structures of the S layer of the causative agent of anthrax. Amino acid analysis demonstrated the ratio of polar and nonpolar amino acids typical for bacterial S layers (66 and 34%, respectively) and equal proportions (20 and 20.8%, respectively) of acidic and basic amino acids, as well as a lack of methionine and cysteine, which is also common for S-layer proteins of the anthrax microbe. The study of the ultrastructure revealed a paracrystalline composition of EA1 protein with a symmetrical pore arrangement [9].

To confirm the aforementioned characteristics of the EA1 protein obtained in the present work from the recombinant *B. anthracis* 55ΔTPA-1Spo$^-$ producer strain, immunoelectron microscopy with immunoglobulins labeled with colloidal gold and specific to EA1 protein was performed. Cells of the strain *B. anthracis* SterneΔT after interaction with these immunoglobulins were completely stained with a label impermeable for electrons; at the same time, the internal structures became indistinguishable (Fig. 2). Individual granules were visible only in the rarefied areas, mainly along the edge and in the intercellular space,

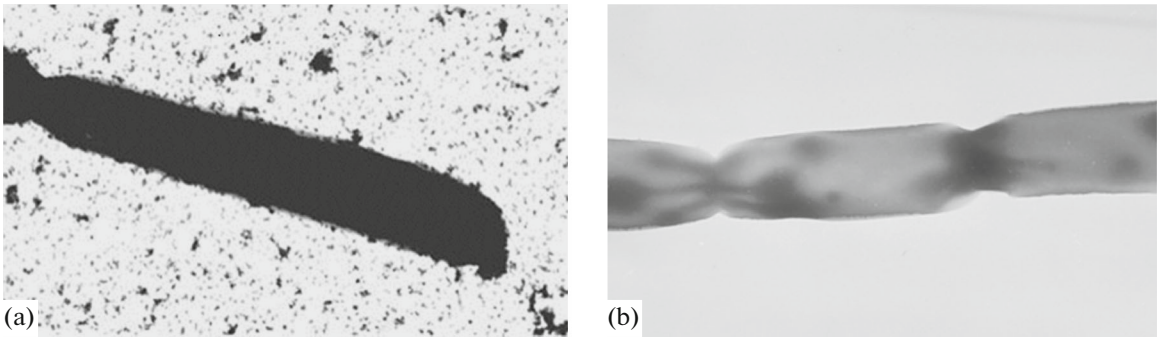

Fig. 2. Immunoelectron microscopy of cells of the *B. anthracis* SterneΔT strain with labeled antibodies for EA1 protein (isolated from *B. anthracis* 55ΔTPA-1Spo$^-$ strain) (a) and the same cells without treatment with the aforementioned antibodies (b). Magnification ×20000.

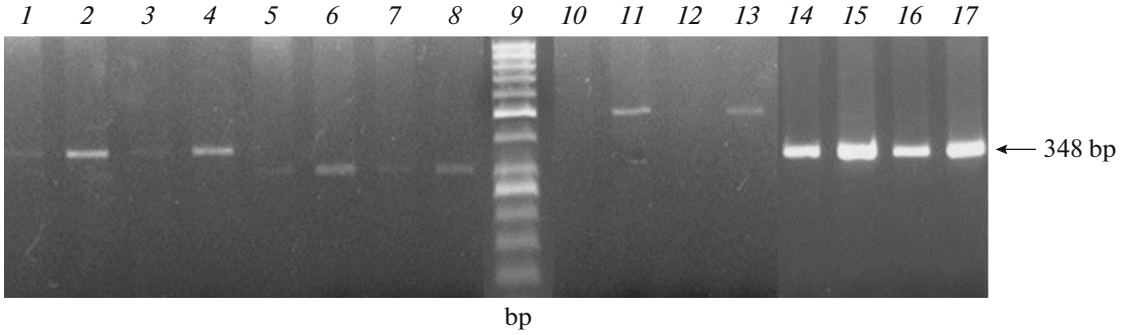

Fig. 3. PCR analysis of the effect of the obtained antigens on expression of the genes encoding TLRs of innate immunity in thymus and spleen cells of mice immunized with rPA (lanes *1*, *3*, *5*, *7*, *10*, *12*, *14*, and *16*) or rPA + EA1 (lanes *2*, *4*, *6*, *8*, *11*, *13*, *15*, and *17*). The results of PCR with primers to the fragments of the genes encoding TLRs of the type 2 (lanes *1–4*, amplified fragment of 380 bp); TLRs of the type 4 (lanes *5–8*, 311 bp); TLRs of the type 6 (lanes *10–13*, 520 bp); and β-actin (lanes *14–17*, 348 bp, positive control). Molecular mass markers (lane *9*, O' GeneRuler^{TM} 100 bp DNA Ladder Plus ready-to-use).

and only on the conglomerates of another protein of the S layer (Sap), which is produced by the anthrax strain in the medium and possesses a high degree of homology to EA1.

Thus, a common technological scheme for obtaining rPA and EA1 from the recombinant *B. anthracis* 55ΔTPA-1Spo$^-$ producer, which does not form spores or synthesize the pathogenicity factors of the anthrax microbe, has been successfully tested. The use of the method provides for a high yield and quality of both target antigens.

Effect of Antigens Obtained from the Recombinant Strain on the Immunological Reactivity of the Macroorganism

The defense of a macroorganism against pathogens is mediated by the coordinated action of two components of the immune system: innate and acquired. Stimulation of the structures of the innate immunity initiates a cascade of reactions, which trigger the development of the adaptive immunity. Thus, the expression of the genes encoding toll-like receptors (TLRs) was studied in immunocompetent cells of experimental animals immunized with anthrax antigens.

BALB/c mice were immunized once with rPA (10 μg) or with rPA and EA1 (10 μg + 5 μg). Lymphocytes were isolated from the thymus and spleen of the biomodels 4 h after immunization to determine the ability of preparations in order to interact with the structures of innate immunity. The material obtained after reverse transcription was used in PCR with primers to the TLR (types 2, 4, and 6) gene sequences. The results of PCR with the primers to the household gene encoding β-actin were used as a positive control. The addition of EA1 to rPA (without adjuvants) was shown to increase the expression of the studied types of TLR genes (Fig. 3, lanes *2*, *4*, *6*, *8*, *11*, and *13*).

The effect of the antigens obtained from the recombinant producer strain on the biomodel immune system was evaluated by the proliferative activity of lymphocytes of the organs of central and peripheral immunogenesis. The BALB/c mice (six in each group) were immunized once with subcutaneous preparations of rPA or rPA with the addition of EA1 (10 μg). Intact animals were used as a control. Thymus and spleen

Table 1. Ratio of thymocytes and splenocytes at the stages of proliferation and apoptosis in the mice immunized with rPA

Immunizing preparation	Time after immunization	Proportion of proliferating cells	Proportion of apoptotic cells	Ratio of the number of cells at the stage of apoptosis and growth
	Thymus			
Control	–	28.7 ± 1.5	3.6 ± 0.3	0.12
PA (10 μg)	4 h	32.4 ± 0.8	3.2 ± 0.3	0.10
	1 day	32.8 ± 0.9	2.8 ± 0.2	0.09
	3 day	30.1 ± 1.3	2.3 ± 0.5	0.08
	7 day	30.9 ± 2.7	3.1 ± 0.5	0.10
	14 day	26.6 ± 0.7	2.8 ± 0.2	0.10
	Spleen			
Control	–	27.7 ± 1.6	2.8 ± 0.3	0.10
PA (10 μg)	4 h	25.5 ± 2.5	4.5 ± 0.8	0.18
	1 day	24.2 ± 0.8	4.4 ± 1.2	0.18
	3 day	22.7 ± 1.9	5.8 ± 1.4*	0.25
	7 day	33.7 ± 1.6	5.2 ± 1.3	0.15
	14 day	33.7 ± 2.4	2.4 ± 0.4	0.07

* Reliability of differences between experimental and control data $p < 0.05$.

samples were collected after 4 h on days 1, 3, 7, and 14 after immunization. The ratio between thymocytes and splenocytes at the phases of cell division was registered with a flow cytometer.

When mice were immunized with rPA, the apoptotic and proliferative activity of thymus cells did not differ significantly from the similar indicators of the control group at all intervals of the study (Table 1). In spleen samples collected after 4 h and on the days 1 and 3, the number of proliferating splenocytes also approximately corresponded to the control values. However, a slight increase in the proliferative activity of the spleen cells up to 33.7 ± 1.6% and 33.7 ± 2.4% (the control samples had a value of 27.7 ± 1.6%) was registered on days 7 and 14. This increase could be a result of the proliferation of B cells and the production of antibodies. The injection of rPA to mice slightly increased the apoptotic activity of the splenocytes to a maximal value of 5.8 ± 1.4 on the third day as well. However, the ratio of spleen cells at the phase of apoptosis and proliferation did not exceed 1 (from 0.08 to 0.25) in all cases, which indicated no damaging effect of rPA on immunocompetent cells.

When mice were immunized with EA1 protein, the number of thymocytes at the phase of apoptosis and proliferation also did not differ significantly from those of the control group at all studied time intervals (Table 2). At the same time, the injection of EA1 in laboratory animals was accompanied by an increase in the number of apoptotic spleen cells to 9.5 ± 2.8% (the control samples had a value of 2.8 ± 0.3%) on the seventh day after immunization. Nevertheless, by the end of the observation period, this indicator decreased to a value of 3.7 ± 0.5%. Over the entire experiment, the ratio of the thymus and spleen cells at the phase of apoptosis and proliferation was significantly less than 1, ranging from 0.09 to 0.29.

Assessment of the damaging effects of rPA and EA1 in experiments in vitro was performed by flow cytometry on the basis of monitoring of the apoptosis state of the induced leukocytes of human blood. After the incubation of antigenic preparations with defibrinated blood, leukocytes were isolated and stained with a solution containing mithramycin and ethidium bromide. According to the study, the tested antigens were not shown to have any damaging effects on human blood cells (data not shown).

Thus, rPA and EA1 were shown to have no damaging effect on the cells of immunocompetent organs of the laboratory animals. At the same time, rPA and EA1 significantly increase the expression of the genes encoding TLRs (types 2, 4, and 6) of innate immunity.

Study of the Toxicological Safety of the Antigens Obtained from the Recombinant Strain 55ΔTPA-1Spo⁻

BALB/c mice (ten individuals in each group) were subcutaneously immunized with rPA or EA1 once at a dose of 50 μg to assess the toxicity of antigenic preparations. Guinea pigs were injected with a 100-μg dose of rPA or EA1 for the same purpose. These amounts were 4–10 times higher than the single doses of antigens used to immunize these laboratory animals (25.5 μg of rPA

Table 2. Ratio of thymocytes and splenocytes at the stages of proliferation and apoptosis in the mice immunized with EA1

Immunizing preparation	Time after immunization	Proportion of proliferating cells	Proportion of apoptotic cells	Ratio of the number of cells at the stage of apoptosis and growth
	Thymus			
Control	–	28.7 ± 1.5	3.6 ± 0.3	0.12
EA1 (10 μg)	4 h	31.4 ± 1.5	3.1 ± 0.4	0.10
	1 day	30.5 ± 0.4	2.8 ± 0.6	0.09
	3 day	28.1 ± 2.2	3.2 ± 0.8	0.11
	7 day	30.3 ± 0.6	3.2 ± 0.4	0.10
	14 day	29.7 ± 1.1	2.6 ± 0.1	0.09
	Spleen			
Control	–	27.7 ± 1.6	2.8 ± 0.3	0.10
EA1 (10 μg)	4 h	31.0 ± 1.5	6.6 ± 0.6*	0.21
	1 day	21.4 ± 2.0	2.8 ± 0.3	0.13
	3 day	18.6 ± 0.5	5.3 ± 0.6	0.29
	7 day	32.5 ± 1.0	9.5 ± 2.8*	0.29
	14 day	26.7 ± 2.1	3.7 ± 0.5	0.14

* Reliability of differences between experimental and control data $p < 0.05$.

and 12.75 μg of EA1 for guinea pigs; 10 μg of rPA and 5 μg of EA1 for mice). In two weeks of observation, no animal deaths, reductions in body weight, or disorders of the animals' general state were registered.

A pathomorphological study was carried out to assess the effect of antigens on the organs and tissues of the macroorganism. Guinea pigs (six individuals in each group) were immunized subcutaneously once with 50 μg of rPA (group 1) or 50 μg of rPA and 25.5 μg of EA1 (group 2). Intact guinea pigs were used as a control. Histological changes were analyzed on days 1, 3, 7, 21, and 27 after immunization.

The injection of antigenic preparations in guinea pigs did not affect their general state. During the time of observation, their body mass slightly increased in a natural way.

On the first day of observation, a slight plethora of the subcutaneous tissue and focal injection of the vessels were observed at the place of injection of the preparations. Apart from moderate edema and focal plethora of the vessels of the subcutaneous tissue, the histological study revealed small segments of the lymphocytic infiltration of the derma, which was more pronounced in animals immunized only with rPA. No changes at the place of injection were observed in the material taken from the animals of both groups after immunization in all time intervals; histological examination indicated a complete lack of the exudative-infiltrative component (data not shown).

There were no significant changes found by macroscopic examination in the mass and size, nor any gross dystrophic changes, infiltrative processes, or pronounced changes in the blood filling of the vessels, in parenchymatous organs for the entire period of observation. Signs of moderate functional stress of the parenchyma cells of the liver, kidneys, and cardiomyocytes, apart from the focal plethora of vessels, were recorded in the first 7 days of histological examination (data not shown).

The described changes were more pronounced in the kidneys of laboratory animals immunized only with rPA (Fig. 4a). Individual cells of the epithelium of convoluted tubules were found to be in a state of hydropic dystrophy; focal lymphocytic infiltration of the stroma in individual animals was observed. In guinea pigs of this group, changes in the glomerular apparatus were more often characterized by a moderate plethora of the capillary loops of the vascular glomeruli of the renal corpuscles. Thus, on the first day of observation, the number of renal corpuscles with the plethora of capillaries reached 52% (control values varied from 4 to 6%) in guinea pigs immunized with rPA. The number then gradually decreased and did not exceed 20% on the seventh day. The corresponding values were significantly lower in the group of laboratory animals immunized with rPA + EA1 (Fig. 4b): 32% on the first day and 7% on the seventh day. The described changes in the glomerular apparatus were not accompanied by a decrease in the number of functionally active renal corpuscles in the field of view of the section. This indicator was even slightly higher in both groups than in the control intact animals. Over the whole period of observation, it ranged from 7.9 ± 3.2 to 6.4 ± 2.38 in the group of guinea pigs

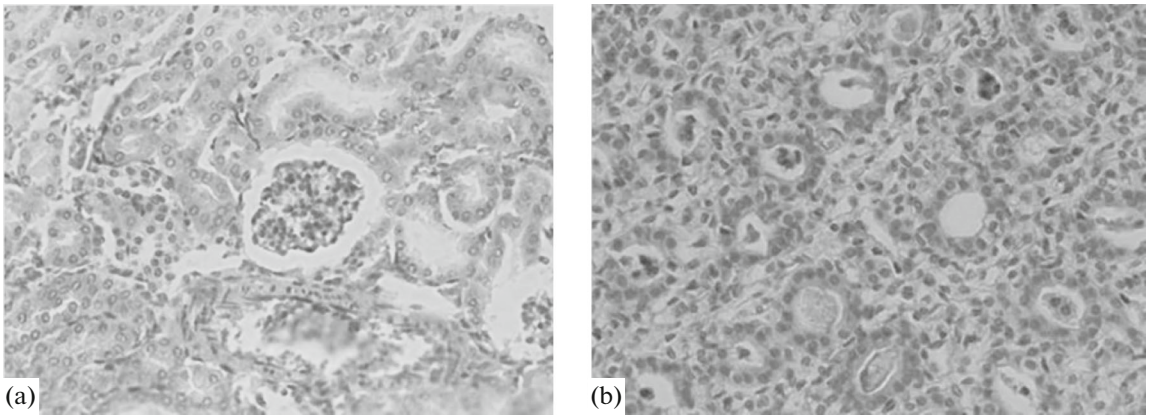

Fig. 4. Sections of guinea pig kidney (hematoxylin and eosin staining) at various time intervals after subcutaneous immunization of test animals with the obtained antigens: rPA, the first day (a). Focal distrophic changes of kidney convoluted tubules and moderate plethora of capillary loops of vascular glomerulus of renal corpuscle are seen (magnification ×100); rPA + EA1, the third day (b). Squamous epithelial cells in lumen of tubules are seen (magnification ×200).

immunized with rPA and from 9.8 ± 1.82 to 6.6 ± 3.16 in another group of animals immunized with a combination of rPA and EA1. The control value was 6.3 ± 1.17.

For the first 3 days, moderate stagnation in the blood circulation system (plethora of intralobular sinusoidal hemocapillaries) and in the outflow system (plethora of central veins) were observed in the liver of the immunized animals of both groups. The described changes were combined with the functional stress of the light hepatocytes of the center of hepatic lobules. However, the morphological characteristics of the functional structures of the organ in immunized and intact guinea pigs did not differ significantly by the seventh day (data not shown).

When both immunization preparations were used, an increase in the number of Kupffer cells was observed in the liver, which indicated the functional activation of the reticuloendothelial system. In guinea pigs immunized with rPA, the number of these elements reached a value of 21.5 ± 2.1 after the first 7 days, which was three times higher than that in the intact control group (6.7 ± 0.94). In subsequent periods, the number of Kupffer cells decreased slightly to 13.0 ± 1.29. For the entire period of observation, the number of Kupffer cells was two times higher (than the control values) in the samples obtained from guinea pigs injected with both rPA and EA1.

Changes in the heart and lungs of both groups were within acceptable limits throughout the entire period of observation. Visible functional stress of cardiomyocytes (focal granular dystrophy) and moderate plethora of the capillary system were observed in the heart. A histological study of lungs revealed alternation of the areas with uneven airiness of the lung tissue, as well as the presence of full blood vessels. Peribronchial lymphohistiocyte reaction was observed in single animals (Fig. 5).

The general stress response of the organism to the introduction of rPA and rPA + EA1 was a slight decrease in the adrenal gland mass during the first 3 days of observation, which was more pronounced in the guinea pigs of the first group. The mass of the right adrenal gland decreased to a value of 0.06 ± 0.005 g (the control value was 0.12 ± 0.03 g, and the mass of the left adrenal gland decreased to a value of 0.035 ± 0.005 g (the control value was 0.07 ± 0.05 g) (Table 3).

A histological study of the adrenal glands of the test animals revealed moderate expansion of the beam zone with changes in the nuclear-cytoplasmic ratio of individual cells, focal destruction of the cells of the glomerular zone, a slight decrease in the pheochromia of the medulla substance, and a normal ratio of the cortex and medulla substances of the organ. The described changes were also more pronounced in laboratory animals immunized only with rPA. Moderate stress of cells providing the synthesis of corticosteroids and mineralocorticoids and a slight background "depletion" of the chromaffin system agreed with the stress response of the biomodel to the injected preparation by the size, nature, periods of development, and attenuation.

No inhibition of the function of lymphoid organs was observed. On the 21st day, immunized guinea pigs of both groups had an increased spleen mass by more than 1.5 times in comparison with the weight of organs of the control animals (1.39 ± 0.015 g and 1.28 ± 0.1 g against 0.8 ± 0.09 g). Within the same time intervals, the thymus mass increased by almost 2 times only in guinea pigs immunized with rPA + EA1 (to a value of 0.75 ± 0.05 g a control value was 0.42 ± 0.15 g). Histological examination showed moderate hyperplasia of follicular structures. On the 27th day, the mass of the spleen and thymus of experimental and control animals differed insignificantly.

Histological examination of the thymus of animals with a normal ratio of cortex and medulla substances revealed moderate hyperplastic processes in lymphoid

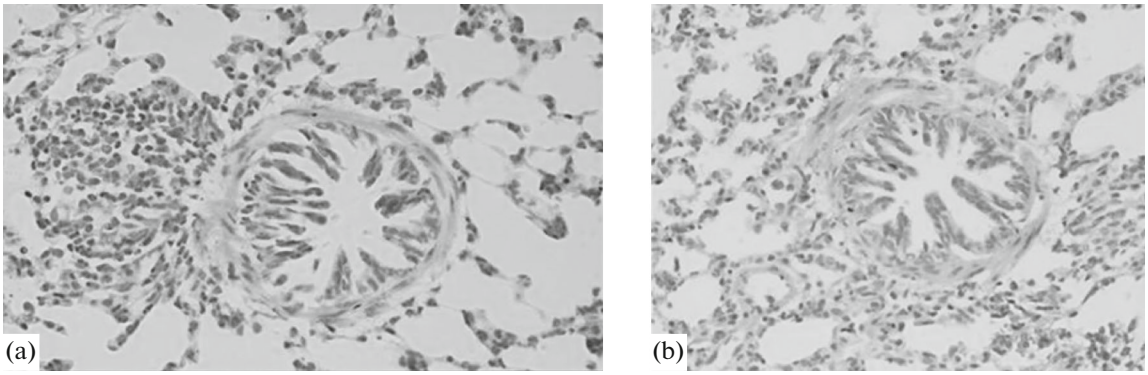

Fig. 5. Sections of guinea pig lungs (hematoxylin and eosin staining) on the 21st day after subcutaneous immunization of animals with the obtained antigens: rPA, peribronchial lymphohistiocyte reaction is seen (a); rPA + EA1, no visible changes are seen (b). Magnification ×100.

elements and relative activation of mitotic activity in the medulla in both groups starting after 7 days and a decrease in these processes by the 27th day (data not shown).

The changes in local lymph nodes of animals immunized with rPA were characterized by a systematic increase in the weight of the organ in the period from the first day (0.035 ± 0.015 g) to the seventh day (0.06 ± 0.01 g) with a return to the original value on the 27th day. In control guinea pigs, the mass of regional lymph nodes was 0.01 ± 0.00 g, which is six times lower than the maximal value for immunized animals. The mass of distant lymph nodes also increased from the first day (0.025 ± 0.005 g) to the seventh day (0.06 ± 0.005 g) and decreased by the 27th day (0.045 ± 0.005 g). Thus, the maximal value in the experiment differed two times as compared to the control value (0.03 ± 0.01 g).

The weight of local lymph nodes in the animals immunized with rPA + EA1 increased twice, starting from the first day (0.04 ± 0.001 g) to the 21st day (0.08 ± 0.001 g), and it almost returned to the initial value on the 27th day (0.045 ± 0.005 g). The maximal indicator exceeded the control value by eight times. A similar tendency was observed for distant lymph nodes; nevertheless, the organ weight on the 21st day

Table 3. Weight (g) of organs of guinea pigs immunized with rPA or rPA + EA1

Time after immunization	Adrenal gland		Spleen	Thymus	LLN	DLN
	right	left				
Control	0.12 ± 0.03	0.07 ± 0.05	0.8 ± 0.09	0.42 ± 0.15	0.01 ± 0	0.03 ± 0.01
Immunization rPA						
1 day	0.060 ± 0.005*	0.035 ± 0.005*	0.36 ± 0.02*	0.35 ± 0.01	0.035 ± 0.015*	0.025 ± 0.005
3 day	0.070 ± 0.001*	0.055 ± 0.001	0.36 ± 0.025*	0.18 ± 0.04*	0.060 ± 0.01*	0.035 ± 0.005
7 day	0.060 ± 0.005*	0.060 ± 0.005	0.37 ± 0.03*	0.12 ± 0.005*	0.075 ± 0.015*	0.060 ± 0.005*
21 day	0.065 ± 0.005	0.070 ± 0.005	1.39 ± 0.015	0.11 ± 0*	0.050 ± 0.01*	0.050 ± 0.01
27 day	0.090 ± 0.03	0.090 ± 0.03	0.49 ± 0.02	–	0.035 ± 0.005*	0.045 ± 0.005
Immunization rPA + EA1						
1 day	0.060 ± 0.025*	0.060 ± 0.001	0.42 ± 0.13	0.14 ± 0.01*	0.040 ± 0.001*	0.050 ± 0.005
3 day	0.055 ± 0.005*	0.060 ± 0.001	0.44 ± 0.03	0.18 ± 0.08*	0.040 ± 0.005*	0.035 ± 0.015
7 day	0.090 ± 0.005	0.070 ± 0.02	0.37 ± 0.03*	1.29 ± 0.12	0.035 ± 0.002*	0.011 ± 0.009*
21 day	0.085 ± 0.005	0.075 ± 0.005	1.28 ± 0.1	0.75 ± 0.05	0.080 ± 0.001*	0.900 ± 0.001*
27 day	0.075 ± 0.005	0.085 ± 0.005	0.51 ± 0.025	0.17 ± 0.02*	0.045 ± 0.005*	0.040 ± 0

LLN, local lymph nodes; DLN, distant lymph nodes.
* Reliability of differences between experimental and control data $p < 0.05$).

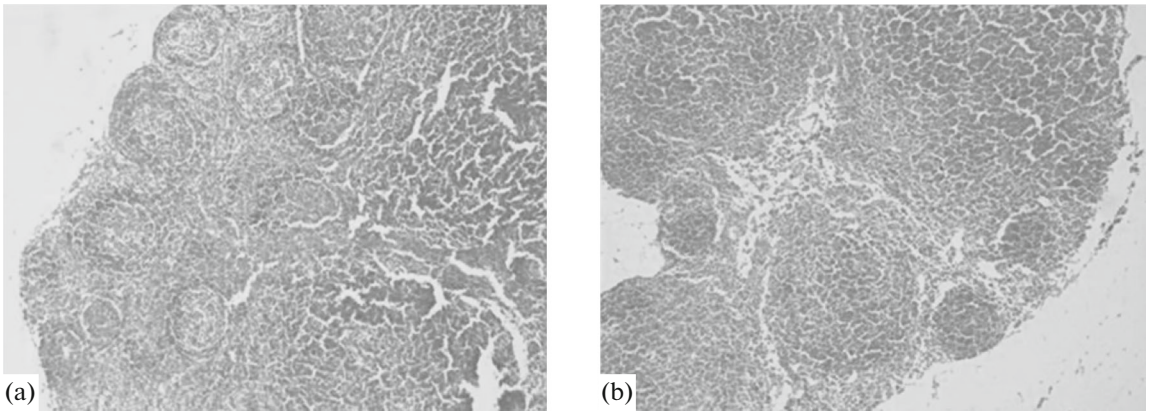

Fig. 6. Sections (stained with hematoxylin and eosin) of local lymph nodes in guinea pigs immunized with the obtained antigens at various time intervals after immunization: rPA, 21st day (a); rPA + EA1, 27th day (b). Signs of hyperplastic processes in follicles and paracortical zones are seen in both cases. Magnification ×40.

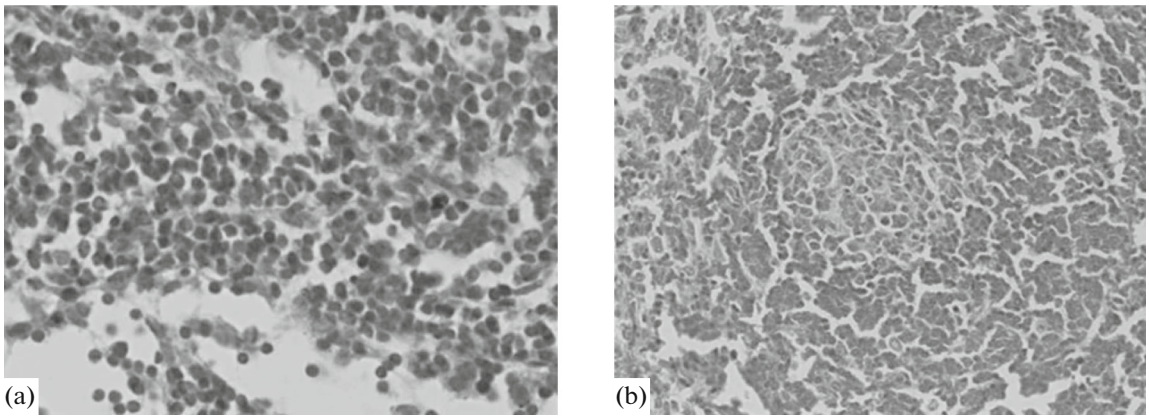

Fig. 7. Sections (stained with hematoxylin and eosin) of spleen of guinea pigs immunized with the obtained antigens at various time intervals after immunization: rPA, 21st day (magnigfication × 400) (a). Eosinophils are visible in splenic cords; rPA + EA1, 27th day (magnigfication ×200) (b). Signs of hyperplastic processes in follicles are seen.

(0.9 ± 0.001 g) was 18 times higher than the initial value (0.05 ± 0.005 g) and 30 times higher than the control value.

Changes in the organs of the peripheral immune system caused by immunogenetic processes were observed in the form of successive activation of the T and B zones. In the spleen and lymph nodes of experimental animals, a moderate increase in proliferative activity from 1 to 3 points, as well as changes in the cellular composition of organs in the period from day 3 to 27, were observed. No significant differences in the severity of these processes and at the time of their emergence in animals immunized with rPA and rPA + EA1 were observed. The fluctuations of the parameters were within the statistical error.

In the lymph nodes (Fig. 6), various degrees of activation of individual follicles (B zones), follicle emergence (in the center of which blast cell forms were present), an increase in the number of mitoses, the presence of single macrophages containing fragments of nuclei, and moderate hyperplasia of paracortical (T) zones were observed. A mixed cellular composition (moderate number of blasts, unactivated lymphocytes, and single mature plasma cells) in the paracortical zones or a predominance of blast cell forms (2 points), starting from day 21 in the first group and already after 7 days in the second group, were observed. After 7 days, the appearance of an insignificant number of eosinophilic leukocytes was registered in the medulla (medullary cords) of the lymph nodes in animals immunized with rPA.

An increase in hyperplastic activity in the T and B zones of the spleen was registered in both groups after 7 days. The number of functionally active follicles in the organ section increased to 20 by the 27th day in the first group and to 17 in the second group, while up to eight inactive follicles were detected in the control section. Starting from the 21st day, the appearance of a moderate amount of eosinophilic leukocytes was registered in the spleen cords in animals immunized only with rPA (Fig. 7). Their presence in some cases

may indirectly indicate the initial stages of the development of hypersensitivity reactions with delayed action.

Thus, the strain *B. anthracis* 55ΔTPA-1Spo$^-$ is an effective source of anthrax antigens, which are components of a prototype anthrax vaccine. A common technological scheme to obtain rPA and EA1 from the recombinant producer has been tested in the course of the present work. The proposed approach will increase the profitability of the production of the vaccine preparation. The absence of selective antibiotic at the main stage of cultivation rules out a negative influence on the biological characteristics of the final product. Realization of the method provides for a high yield of both the rPA and the S-layer EA1 protein. Concentration and diafiltration of rPA and EA1 with an ultrafiltration unit, as well as a two-step chromatographic purification on various carriers, makes it possible to obtain a high degree of antigen purification. Purified antigens isolated from the recombinant *B. anthracis* strain have no toxic effect on the organs and tissues of immunized animals. The small changes in the organs of guinea pigs immunized with rPA or with a combination of rPA and EA1 that were revealed during pathomorphological examination reflect the adaptive-compensatory reactions of the macroorganism and tend to normalize. A twofold excess of the number of Kupffer cells in the liver tissue confirms the functional activation of the reticuloendothelial system. The reaction of immunocompetent organs corresponds to moderate immunogenesis activation. The addition of the EA1 antigen to rPA does not result in an increased toxic effect on the macroorganism. Conversely, it is accompanied by a lower permissibility of pathomorphological changes in the organs and tissues of immunized animals and a more pronounced reaction of the lymphoid organs.

REFERENCES

1. Galkin, V.V., Loktionova, M.N., Simonova, E.G., and Khadartsev, O.S., The problems of safety of burial grounds of anthrax-infected cattle, *Epidemiol. Infekts. Bolezni*, 2007, no. 6, pp. 54–57.
2. Onishchenko, G.G., Kozhukhov, V.V., Vasil'ev, N.T., Bondarev, V.P., Borisevich, I.V., Darmov, I.V., Gorin, O.V., Vorob'ev, A.A., Pyatkov, V.A., Shevtsov, A.N., Seroglazov, V.V., Kryuchkov, A.V., and Lub, M.Yu., *Sibirskaya yazva: aktual'nye problemy razrabotki i vnedreniya meditsinskikh sredstv zashchity (Anthrax: Actual Problems of Development and Introduction of Medical Protection Means)*, Onishchenko, G.G. and Kozhukhov, V.V., Eds., Moscow: Meditsina, 2010, pp. 5–424.
3. Selyaninov, Yu.O., Egorova, I.Yu., Kolbasov, D.V., and Listishenko, A.A., Anthrax on Yamal: causes and problems of diagnostics, *Veterinariya*, 2016, no. 10, pp. 3–7.
4. Popova, A.Yu., Demina, Yu.V., Ezhlova, E.B., et al., The outbreak of anthrax in the Yamalo-Nenets Autonomous District in 2016: epidemiological features, *Probl. Osobo Opasnykh Infekts.*, 2016, no. 4, pp. 42–46. doi 10.21055/0370-1069-2016-4-42-46
5. Brachman, P., Bioterrorism: an update with a focus on anthrax, *J. Epidemiol.*, 2002, vol. 155, pp. 981–987.
6. Sayapina, L.V., Bondarev, V.P., and Olefir, Yu.V., Current status of vaccine prevention of especially dangerous infections, *Probl. Osobo Opasnykh Infekts.*, 2016, no. 2, pp. 107–110. doi 10.21055/0370-1069-2016-2-107-110
7. Mikshis, N.I., Semakova, A.P., Popova, P.Yu., et al., The prospects of using various adjuvants in combination with the recombinant protective antigen of the anthrax microbe, *Biotekhnologiya*, 2014, no. 6, pp. 36–42.
8. Scopes, R.K., *Protein Purification. Principles and Practice*, New York: Springer?Verlag, 1982.
9. Mikshis, N.I., Korsakova, A.Yu., Bolotnikova, M.F., et al., Production of S-layer proteins by *Bacillus anthracis* strains, *Biotekhnologiya*, 2004, no. 5, pp. 22–32.
10. Farchaus, J., Ribot, W., Downs, M., and Little, S., Purification and characterization of the major surface array protein; from the avirulent *Bacillus anthracis* delta Sterne-1, *J. Bacteriol.*, 1995, vol. 177, pp. 2481–2489.
11. *Lymphocytes: A Practical Approach*, Klaus, G.G.B., Ed., Oxford: IRL Press, 1987.
12. Liu, T., Matsuguchi, T., Tsuboi, N., et al., Differences in expression of toll-like receptors and their reactivities in dendritic cells in BALB/c and C57BL/6 mice, *Infect. Immun.*, 2002, vol. 70, no. 12, pp. 6638–6645.
13. Korzhevskii, D.E. and Gilyarov, A.V., *Osnovy gistologicheskoi tekhniki* (Basics of Hydrological Techniques), Moscow: Spetslit, 2010.
14. Mikshis, N.I., Kudryavtseva, O.M., Goncharova, A.Yu., et al., Evaluation of the efficiency of the protective antigen production by the asporogenous recombinant *Bacillus anthracis* strain, *Biotekhnologiya*, 2011, no. 5, pp. 38–43.

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