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# Immunogenicity of Conjugates of Protective Antigen Complexes of Tularemia Microbe with Gold Nanoparticles

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**Abstract**—Conjugates of gold nanoparticles with two isolated tularemia microbe antigens, a protective antigen complex and a glycosylated protein complex, are used to obtain anti-tularemia sera and to vaccinate animals. A conjugate of gold nanoparticles with the glycosylated protein complex during the subcutaneous immunization of mice is more effective than the unconjugated antigen, which is evident from the increase in protectiveness and antibody titers. The use of conjugates of both antigens with gold nanoparticles during the immunization of rabbits makes it possible to obtain sera with a high titer of specific antibodies (1/64–1/128 titer in the diffusion precipitation reaction and 1/5120–1/10240 in the reaction of indirect hemagglutination) during a relatively short period of time and with minimal antigen consumption (1.8–10 mg). The use of immunoglobulins extracted from sera during the enzyme-linked immunosorbent assay makes it possible to detect *Francisella tularensis* cells of different subspecies in the amount of $(5.2 \pm 0.5) \times 10^5$ MC/mL with 100% specificity for heterologous strains at a concentration of $10^8$ MC/mL, which enables their subsequent application in the production of preparations for the diagnostics of tularemia.

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## INTRODUCTION

Tularemia is an especially dangerous zoonotic infection with natural foci. It is characterized by intoxication; fever; and damage to the lymph nodes and skin, as well as sometimes to the mucous membrane of the eyes, throat, and lungs. *Francisella tularensis* is the causative agent of this disease [1]. Nowadays, territories in the Russian Federation where the activity of natural foci of tularemia and morbidity among the population are recorded are expanding [2]. Vaccination of the population with a live attenuated tularemia vaccine in the endemic areas is a specific way to prevent tularemia [3]. Less commonly, an inactivated whole-cell vaccine is used; live genetic, subunit, vector, targeted, and DNA vaccines are being developed [4]. Molecular genetic (PCR), allergic (skin test with tularin and leukocytolysis reaction), bacteriological, and biological methods are actively used to diagnose tularemia. The specific immunodiagnostics of tularemia is carried out using enzyme-linked immunosorbent assay (ELISA), agglutination reactions, indirect hemagglutination assay (IHA) and neutralization of antibodies, and immunofluorescent and immunochromatographic analyses [5, 6]. The main step in the development of modern effective methods of immunodiagnostics of tularemia is to obtain highly active specific antibodies. The search for *F. tularensis* antigens, promising for the development of new methods for preventing and diagnosing tularemia is being actively conducted [7, 8].

At the present time, the use of nanoparticles of different nature as carriers of antigens for immunization and vaccination is of great interest [9–12]. Gold nanoparticles (GNPs) are among the most promising carriers of antigens [13, 14]. This is due to the fact that GNPs can serve not only as carriers of antigens, but they can also possess adjuvant properties [15]. GNPs were used to produce antibodies and develop experimental vaccines against bacteria such as *Yersinia pestis*, *Y. pseudotuberculosis*, *Salmonella typhimurium*, *Brucella abortus*, *Mycobacterium tuberculosis*, *Streptococcus pneumoniae*, *Neisseria meningitides*, *Burkholderia mallei*, *Escherichia coli*, *Listeria monocytogenes*, *Clostridium tetani*, and *Pseudomonas aeruginosa*, as well as some viral and tumor antigens [16].

The goal of this research was to study the efficiency of the use of conjugates of antigenic preparations from *F. tularensis* cells with GNPs to obtain anti-tularemia sera with high activity and specificity, as well as to evaluate their protective activity for laboratory animals with experimental tularemia.

## MATERIALS AND METHODS

*Subject of research.* Preparations of the protective antigenic complex (author's name PAK-15; mol wt. 280 kDa) and glycosylated protein complex (author's name PAK-M; mol. wt. 56 kDa) of the vaccine strain *F. tularensis* 15 NIIEG were used as antigens [17, 18]. Both preparations had immunogenicity and pronounced protective activity for white mice with experimental infection caused by the infecting test strain of the *F. tularensis* 503/840 holarctic subspecies and did not have a damaging effect on immunocompetent cells [7, 18].

*Nanoparticle synthesis.* Spherical gold nanoparticles with an average diameter of 15 nm were obtained by the Frens method [19] using the reduction reaction of aurichlorohydric acid (Sigma-Aldrich, United States) with sodium citrate (Fluka, Switzerland). The reduction was carried out by heating of a 0.01% aqueous solution of aurichlorohydric acid (242.5 mL) to 100°C in an Erlenmeyer flask on a magnetic stirrer with a reverse water cooler. After that, a 1% aqueous solution of sodium citrate (7.5 mL) was added.

The diameter of the synthesized GNP was determined using a Specord S 250 UV-Vis spectrophotometer (Analytik Jena, Germany), a Libra 120 transmission electron microscope (Carl Zeiss, Germany), and a Zetasizer Nano-ZS zeta potential analyzer (Malvern, United Kingdom) in the Simbios Center for Collective Use of the Institute of Biochemistry and Physiology of Plants and Microorganisms (Russian Academy of Sciences) as previously described [20].

The golden number (the minimum amount of antigen protecting the sol from salt aggregation) for antigenic preparations was determined in order to obtain the conjugates of GNP with antigens. To accomplish this, a solution (20 μL) was titrated twice with an antigen solution with an initial concentration of 1 mg/mL in phosphate buffered saline (PBS), pH 7.2, in the 96-well microtiter plate. GNP (200 μL) and 1.7 M NaCl (20 μL) were added to each well. The minimum stabilizing concentration was ~20 μg/mL. Conjugation was conducted by the simple mixing of the reagents without using crosslinking agents [20].

*Laboratory animals.* Laboratory albino mice weighing 18–20 g at the beginning of the experiment were used in the vaccination experiments. Chinchilla rabbits weighing 2.5–3 kg were used in immunization experiments. Care and work with laboratory animals were carried out in accordance with the requirements of the Guidelines for the Laboratory Animal Management and Use, regulations of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, and legislation of the Russian Federation.

*Vaccination of animals.* To determine the immunogenicity of the conjugates, mice were immunized once in different ways: subcutaneously and intraperitoneally. The number of animals for each variant of the experiment was 24 white mice. One immunizing dose (10 μg for PAK-15–GNP and 14 μg for PAK-M–GNP) was used to determine the dynamics of antibody production; the animal blood was collected on day 7, 14, 21, and 28 by decapitation and were examined for the presence of specific antibodies in the enzyme-linked immunosorbent assay (ELISA) and the reaction of indirect hemagglutination (RIHA). The indicators of cellular immunity in the reaction of leukocytolysis (RL) were also determined. Five mice were used for each period of time and in each group.

*Immunological studies.* The commercial diagnosticum erythrocyte tularemia antigenic liquid reagent kit (RNGA-Tul-Ag-StavNIPCHI, Russia) ser. 2–12 was used to carry out RIHA by the macromethod. ELISA was performed by the indirect method. PAK (at a concentration of 100 μg/mL) was a sensitin; peroxidase-labeled antibodies against mouse immunoglobulins (Gamaleya Scientific Research Institute of Epidemiology and Microbiology, Russia) were used as a conjugate, while ABTS (Sigma-Aldrich, United States) was a substrate solution. The ELISA results were recorded and evaluated using an iMark plate photometer (Bio-Rad, United States) at an operating wavelength of 405 nm.

RL based on the registration of the destruction of leukocytes of the sensitized organism under the effect of a specific allergen (antigen) was used for the detection and evaluation of in vitro hypersensitivity. The reaction of leukocytolysis was carried out and registered according to the method developed for the evaluation of the acquired anti-tularemia immunity (leukocytolysis test with tularin) [6]. The commercial preparation of cutaneous tularin containing $1 \times 10^{10}$ bacteria per 1 mL (Mikrogen, Russia) was used as an allergen during immunization with preparations of the prototype tularemia chemical vaccine. Saline was used as a control. The following scale of leukocytolysis index was used to assess the results: 15%, negative or doubtful result; 16–20%, weakly positive result; 21–30%, positive result; and ≥31%, sharply positive result.

*Evaluation of the protective effect.* The protectiveness of the PAK-15–GNP and PAK-M–GNP conjugates was evaluated in the acute experiment with experimental tularemia caused by the test strain *F. tularensis* subsp. *holarctica* 503/840. The selection of the infecting strain and dose was determined by the requirements for the type strains for controlling the vaccinating activity of the live tularemia vaccine. Mice were vaccinated once, either subcutaneously or intraperitoneally, in a dose of 1 to 1/8 with an interval of 2 (PAK-15–GNP: dose 1 = 12 μg; PAK-M–GNP: dose 1 = 14 μg). Animals vaccinated with the same doses of antigens without GNPs were the positive control. Animals injected with an equal volume of saline were the negative control. On day 21 after immunization, the laboratory animals were infected by subcutaneous administration of 100 $LD_{50}$ [a dose of 100 microbial

cells (MC), 1 $LD_{50}$ = 1 MC] of the *F. tularensis* 503/840 test strain in 0.2 mL of saline. The observation period was 21 days.

The $LD_{50}$ of the infecting strain and the $ED_{50}$ of the preparations were determined. These indicators were calculated according to the conventional Kerber method [21]. The average $ED_{50}$ immunizing dose of the tested preparation was calculated with the formula

$$\log ED_{50} = \log DN - d(\Sigma Li - 0.5),$$

where $DN$ was the maximum value among the tested doses, d was logarithm of the ratio of each subsequent dose to the previous one (logarithm of the dilution step), Li was the ratio of the number of surviving animals to the total number of animals immunized with a given dose, ΣLi was the sum of the Li values determined for all doses in the experiment, and 0.5 was constant.

The calculation of the $LD_{50}$ values and confidence intervals (for a probability of 9%) was performed according to the formula

$$\log LD_{50} = \log D_{max} - \log d(\Sigma Li - 0.5),$$

where $D_{max}$ was the maximum value of the infecting dose; $d$ was the ratio of each subsequent dose to the previous one (dilution ratio of the tested doses); Li was the ratio of the number of dead animals during infection with the $i$-th dose to the total number of animals to which this dose was administered, $i = 1, 2, ..., n$; and n was the total number of infecting doses tested.

Animals were immunized once, subcutaneously at a dose of 1 $ED_{50}$ for each preparation, to assess their level of immunity. The animals were infected on day 21 by the subcutaneous administration of 1000, 100, 10, and 1 MC (1 $LD_{50}$ = 1 MC) with the test strain *F. tularensis* 503/840. The indicator of the immunity level was the index of immunity (II), which was calculated as a ratio of $LD_{50}$ for the immune group to the $LD_{50}$ for the control group.

The survival rates of animals after immunization with conjugated and unconjugated antigens were evaluated according to the Kaplan–Meier method and were shown as survival curves [22]. Mice of each group (ten mice for immune groups and six mice for the control group) were immunized once subcutaneously with a dose of 10 μg of antigen (conjugated antigens: PAK-15–GNP and PAK-M–GNP; unconjugated antigens: PAK-15 and PAK-M) or 0.86% NaCl solution (control). Infection with a virulent strain was performed 21 days after immunization; the observation period was 21 days. Statistical processing was performed according to the Kaplan–Meier method, $P \le 0.05$.

*Immunization procedure.* For the immunization of rabbits, antigens of the tularemia microbe were used (PAK-15 and PAK-M) without GNPs (Scheme A) and using GNPs (Schemes B and C). Immunization with Scheme A was carried out according to [13], while immunization with Schemes B and C was performed according to [23].

Scheme A.

1. PAK-15 (1 mL) in the concentration of 10 mg/mL in a 1 : 1 ratio with IFA subcutaneously under the scapula.

2. After 30 days, repeated immunization with IFA at the same dose.

3. After 30 days, intravenous (into the marginal vein of the ear) immunization without IFA, 0.5 mL of the preparation in the concentration of 5 mg/mL.

4. After 1 day, three intravenous injections at the same dose every 24 h.

Scheme B.

1. PAK-15–GNPs (0.5 mL) in a 1 : 1 ratio with complete Freund's adjuvant (CFA) subcutaneously at 10 points along the spine.

2. Every 14 days, cyclic 4-fold subcutaneous immunization with PAK-15–GNP at the same dose and volume with CFA.

3. After 14 days, 1 mL of PAK-15 in the concentration of 6 mg/mL intramuscularly in the thigh and 1 mL of PAK-15–GNP subcutaneously at 4 points along the spine (boosting).

4. After 10 days, 1 mL of PAK-15 in the concentration of 4 mg/mL intravenously (into the marginal vein of the ear).

Scheme C.

1. PAK-M–GNPs (0.5 mL) in a 1 : 1 ratio with CFA subcutaneously at 10 points along the spine.

2. Every 14 days, cyclic 4-fold subcutaneous immunization with PAK-M–GNP at the same dose and volume with CFA.

3. After 14 days, 0.3 mL of PAK-M intramuscularly in the thigh and 1 mL of PAK-M–GNP subcutaneously at 4 points along the spine (boosting).

4. After 10 days, 0.3 mL of PAK-M intravenously (into the marginal vein of the ear).

*Immunochemical methods.* Reaction of diffusion precipitation (RDP) according to Ouchterlony, RIHA, and dot immunoassay (DIA) were used to evaluate the obtained antisera. When performing RDP [21], an immunizing antigen was used as an antigen. For Schemes A and B, it was PAK-15 in a concentration of 1 mg/mL, while for Scheme C it was PAK-M in the concentration of 1 mg/mL. To determine the specific activity of the sera, DIA was performed using *F. tularensis* 15 NIIEG cells; titration with an interval of 2 from $10^8$ MC/mL to $10^5$ MC/mL. To determine the specificity of the sera, DIA was performed using cells of the following strains: *Y. pestis* EV NIIEG, *Y. pseudotuberculosis* V serovar, and *B. abortus* 19VA, in the initial concentration of $10^9$ MC/mL. A nitrocellulose membrane (Vladisart, Russia) ruled with squares of 5 × 5 mm with a pore size of 0.2 μm was used as a substrate. Cell suspension was titrated twice

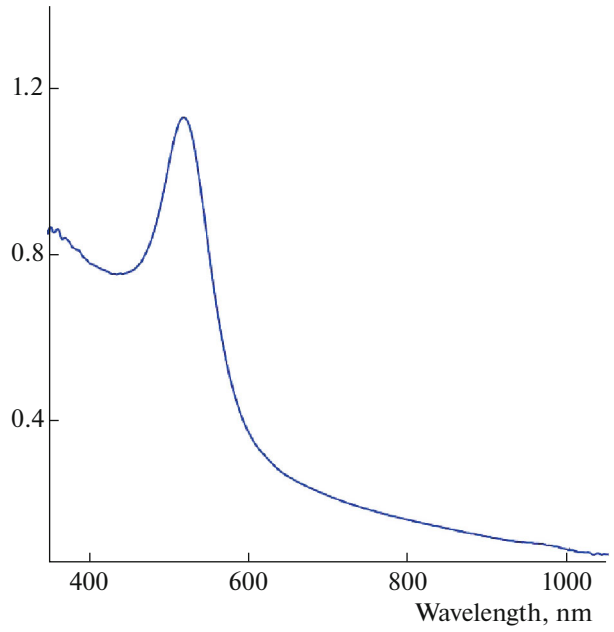


**Fig. 1.** (Color online) Absorption spectrum of GNPs with an average particle diameter of 15 nm.

in 0.01 M PBS and added to the corresponding square of the nitrocellulose membrane in a volume of 1 μL in duplicate; PBS was added as a negative control. The fixation of antigen on the membrane was carried out for 10 min at 60°C. To block nonspecific binding sites, the membrane was immersed in a 0.5% solution of bovine serum albumin (BSA) and incubated for 15 min at 37°C. After that, the membrane was washed three times for 3 min in PBS and incubated in the solution of the test serum in a dilution of 1/100 in PBS, containing 0.5% Tween 20, at 37°C for 30 min. The membrane was washed subsequently as described above and incubated for 1 h at 37°C in the solution of goat antibodies against rabbit immunoglobulins labeled with horseradish peroxidase (Gamaleya Scientific Research Institute of Epidemiology and Microbiology, Russia) in the working dilution with 0.5% BSA solution. The preliminarily washed membrane was immersed in the substrate solution of o-dianisidine (ICN Biomedicals, United States) until the reaction was observed. Analysis of the DIA results was carried out visually, taking the largest dilution of the studied sample into account, which enabled the development of the reaction in the form of a clearly colored spot.

**Table 1.** Determination of the level of antibodies to PAK-15 (average value, $n = 5$) in white mice vaccinated with the PAK-15–GNP preparation; immunizing dose of 10 μg

| Time of analysis | Method of immunization | | | | | |
|---|---|---|---|---|---|---|
| | subcutaneously | | | intraperitoneally | | |
| | K*, % | ELISA | RIHA | K*, % | ELISA | RIHA |
| Day 7 | 4 | 1/60 | 1/256 | 21 | 1/16 | 1/192 |
| Day 14 | 5 | 1/200 | 1/384 | 20 | 1/160 | 1/320 |
| Day 21 | 6 | 1/272 | 1/544 | 8 | 1/120 | 1/128 |
| Day 28 | 1.1 | 1/152 | 1/320 | 10.4 | 1/496 | 1/64 |

*Value of the leukocytolysis coefficient (K) for RL.

All results were statistically processed by standard procedures integrated into the Excel 2007 software (Microsoft Corp.). The arithmetic mean and standard deviation for each data set are given, based on the significance level $p \le 0.05$ (95%).

## RESULTS AND DISCUSSION

The diameter of the synthesized GNP was determined by spectrophotometry (Fig. 1), transmission electron microscopy (TEM), and dynamic light scattering (DLS) (Fig. 2). The absorption spectrum maximum of the obtained sol was $\lambda_{max} = 519.1$ nm, while the optical density was $A_{520} = 1.15$. On the basis of the data on TEM and DLS, the average diameter of the nanoparticles was 15.7 nm. The number of particles in 1 mL at $A_{520} = 1$ was $1.6 \times 10^{12}$. According to our data, the use of spherical GNPs with an average diameter of 15 nm is optimal for immunization [24].

The determination of the golden number suggests that antigen molecules adsorbed on the surface of GNPs protect the gold sol from salt aggregation. If there are not enough antigen molecules to cover the surface of the particles, the addition of electrolyte causes the aggregation of the particles, coloring the solution blue. In our case, this effect was observed when the concentration of antigen was 10 μg/mL. Thus, at a selected concentration of 20 μg/mL, either the entire added antigen was adsorbed on GNPs or there was a slight excess of it. It is noteworthy that a slight excess of the soluble antigen not only does not interfere with immunization, but also contributes to an increase in the production of antibodies.

When analyzing the data obtained when vaccinating mice, it was found that the administration of PAK-15–GNP reduced the level of cellular immunity and the formation of specific antibodies at early stages (Table 1). A single subcutaneous immunization of white mice with the PAK-15 preparation was previously shown to cause the formation of specific antibodies on day 3 with a maximum on day 14 [7]. The level of reaction of leukocytolysis reached sharply positive values by day 21 (53–56%).

The administration of the PAK-M–GNP preparation also resulted in an increase in the antibody titer only by day 21, although it suppressed the cellular response at the early stages less strongly (Table 2).

The protectiveness of the PAK–GNP and PAK-M–GNP conjugates via the subcutaneous and intraperitoneal ways of administration was evaluated in the acute experiment with a model of white mice

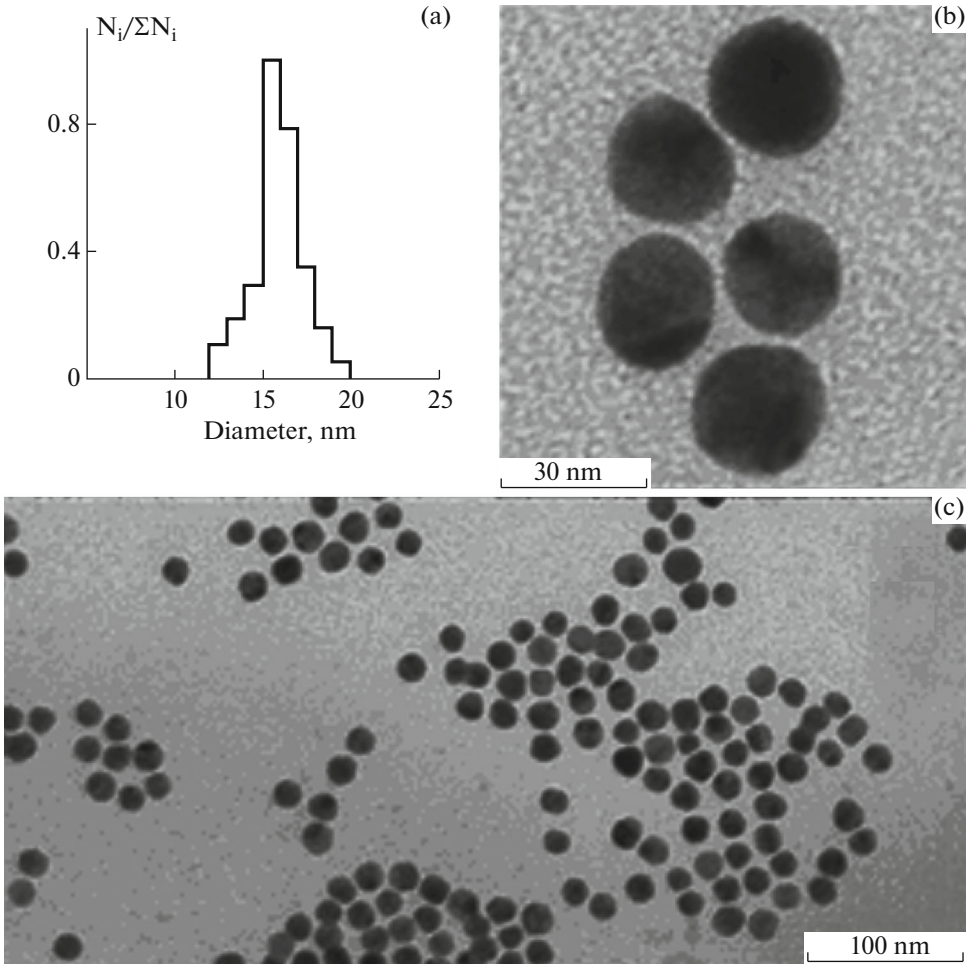


**Fig. 2.** Size distribution (a) and TEM images (b, c) of GNPs with an average particle diameter of 15 nm.

with experimental tularemia caused by the test strain *F. tularensis* 503/840. The specificity of the experimental tularemia infection was confirmed by the data from the control autopsy of the dead and surviving animals, microscopy of imprint smears, and plating on nutrient media. A pathoanatomical pattern characteristic of tularemia infection was observed in the dead animals. The culture of the tularemia microbe was registered in all organ imprints 2–3 days after plating on a selective medium. The culture of the causative agent of tularemia in surviving animals was not registered.

Table 3 shows the index of protection (IP, %), which was the ratio of the number of surviving animals to the total number of animals in this group, as well as the indicator of the effective immunizing dose ($ED_{50}$), calculated by the Kerber method.

Table 4 shows the data on the average life expectancy of mice with experimental tularemia caused by a virulent strain of the holarctic subspecies.

Thus, it was found that a single immunization of white mice with the preparations of conjugated tularemia antigens caused the production of specific antibodies with a maximum on days 14–21 during subcutaneous immunization and days 21–28 with intraperitoneal administration. All studied antigens had protective activity, regardless of the method of immunization. In the case of the PAK-M–GNP preparation, a significant increase in the average life expec-

**Table 2.** Determination of the level of antibodies to PAK-M (average value, $n = 5$) in white mice vaccinated with the PAK-M–GNP preparation; immunizing dose of 14 μg

| Time of analysis | Method of immunization | | | | | |
|---|---|---|---|---|---|---|
| | subcutaneously | | | intraperitoneally | | |
| | K*, % | ELISA | RIHA | K*, % | ELISA | RIHA |
| Day 7 | 25.8 | 1/44 | 1/352 | 15 | 1/60 | 1/480 |
| Day 14 | 5.5 | 1/172 | 1/512 | 13 | 1/128 | 1/320 |
| Day 21 | 5.3 | 1/416 | 1/48 | 1.7 | 1/144 | 1/416 |
| Day 28 | 1.4 | 1/480 | 1/16 | 3 | 1/248 | 1/128 |

*Value of the leukocytolysis coefficient (K) for RL.

**Table 3.** Immunogenicity of PAK preparations conjugated with GNPs for white mice with tularemia infection against the strain *F. tularensis* 503/840

| Preparation | Method of immunization | IP, % | $ED_{50}$, μg |
|---|---|---|---|
| PAK-15–GNP | Subcutaneously | 20.8 | 10.6 ± 1.2 |
| | Intraperitoneally | 33.3 | 6.0 ± 0.7 |
| PAK-15 | Subcutaneously | 58.3 | 5.8 ± 0.6 |
| | Intraperitoneally | 54.2 | 4.5 ± 0.8 |
| PAK-M–GNP | Subcutaneously | 62.5 | 3.5 ± 0.4 |
| | Intraperitoneally | 45.8 | 4.7 ± 0.5 |
| PAK-M | Subcutaneously | 54.2 | 7.6 ± 1.2 |
| | Intraperitoneally | 41.7 | 6.2 ± 0.4 |

tancy of immune animals was observed in comparison to the control group. Ambiguous results were obtained for the PAK-15–GNP preparation. Despite the high level of specific antibodies after subcutaneous administration on day 21 of immunogenesis, low IP and life expectancy of this group of animals were observed. The coefficient of leukocytolysis (an indicator of the specific response of the cellular immunity to the tularemia antigen) for this group of animals was also minimal.

Thus, we analyzed the effect of conjugation of tularemia antigens with GNPs on animal survival using the Kaplan–Meier method [22]. Figure 3 shows the survival curves of white mice immunized once subcutaneously with conjugated and unconjugated antigens at a dose of 10 μg, after infection with a virulent strain of tularemia microbe on day 21. The results of the previous experiment were confirmed and all the preparations used were shown to be immunogenic and to protect animals from death during the subsequent experimental tularemia infection. In the case of conjugation of the low-molecular-weight PAK-M preparation of the protein nature with GNPs, the survival rate increased in comparison to the unconjugated variant, whereas the conjugation of the high-molecular-weight PAK-15 preparation with GNPs resulted in a significant decrease in the proportion of surviving animals in the group.

In the case of the subcutaneous method of immunization, the indicator of the immunity level was also evaluated: immunity index (II), which was calculated as the ratio of the average lethal dose (infecting dose at which 50% of biomodels died) ($LD_{50}$) for the immune group to the $LD_{50}$ for the control group. II was 7 (2–27) for the PAK-15–GNP preparation and 215 (54–860) for PAK-M–GNP. For unconjugated preparations, the immunity indicator was 124 (32–476) for the PAK-15 preparation, while it was 92 (26–358) for the PAK-M preparation. According to the requirements for live tularemia vaccines (Rospotrebnadzor guidelines 3.3.1.2161-07), this index should be at least 10.

An analysis of the immunogenicity of the conjugated tularemia antigens with the model of white mice showed that they induced primarily humoral immune response in animals, while the indicators of the cellular component of the immune system decreased. The data agree with the thesis on the main role of the cellular component of anti-tularemia immunity [8, 25]. In particular, a decrease in the cellular response was observed for the PAK-15–GNP complete antigen conjugate by a decrease in protectiveness, despite the high level of antibodies. During subcutaneous administration, the PAK-M–GNP conjugate was more effective than the unconjugated control (PAK-M) according to the survival, longevity, and protection indices.

Data on the immunization of rabbits with tularemia antigens in five different schemes are shown in Table 5. When immunizing rabbits–producers with the PAK-15–GNP conjugated preparation, 50 μg of antigen was consumed. When using PAK-M–GNP, 175 μg was consumed. However, it was necessary to perform double boosting with pure antigens in the amount of 6 and 4 mg for PAK-15 and 0.95 and 0.7 mg for PAK-M to reach higher titers of specific antibodies. The duration of the immunization process was 2.5 months.

This scheme made it possible to obtain sera with a high content of anti-tularemia antibodies (RIHA titer: 1/5120–1/10240) while consuming from 1.8 to 10 mg of antigen for the entire immunization scheme. Currently, in commercial immunodiagnostic preparations for the detection of tularemia, horse hyperimmune

**Table 4.** Average life expectancy (days) of white mice (immune and control groups) after infection with the virulent strain *F. tularensis* 503/840 at the initial dose of 100 MC

| Dose | PAK-15–GNP | | PAK-M–GNP | |
|---|---|---|---|---|
| | subcutaneously | intraperitoneally | subcutaneously | intraperitoneally |
| 1 d. | 7.7 ± 1.0 | 21 (100% survival) | 21 (100% survival) | 12.3 ± 1.2 |
| 1/2 d. | 12.0 ± 2.2 | 14.0 ± 1.2 | 16.3 ± 2.2 | 18.7 ± 2.1 |
| 1/4 d. | 12.5 ± 1.5 | 13.5 ± 2.0 | 10.0 ± 1.0 | 12.8 ± 1.5 |
| 1/8 d. | 9.3 ± 1.2 | 15.0 ± 1.4 | 16.3 ± 2.2 | 12.8 ± 1.5 |
| Control | 7 | | | |

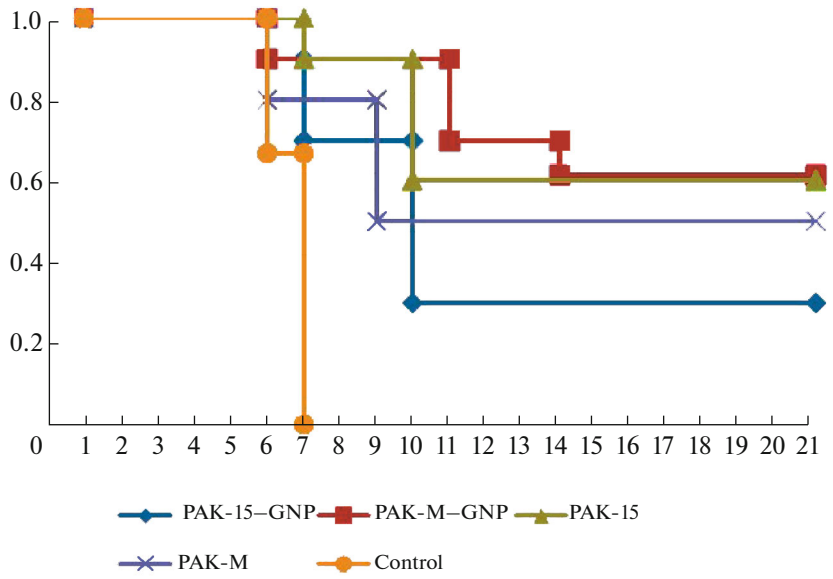


**Fig. 3.** (Color online) Survival of white mice immunized with GNP-conjugated and unconjugated tularemia microbe antigens after infection with 100 MC of the *F. tularensis* 503/840 virulent strain. Kaplan–Meier survival curve: abscissa axis, time after infection (days), observation for 21 days; ordinate axis, survival (the proportion of surviving animals in the group of the total number of animals).

serum and monoclonal antibodies to LPS are used. Horse diagnostic serum is obtained by the hyperimmunization of the animals with cells of the vaccine strain of the tularemia microbe that were killed. Water–salt cell extracts, purified protein preparations, and LPS preparations are used for experimental polyclonal rabbit sera. Doses of antigenic preparations are dozens of milligrams. To obtain monoclonal antibodies, LPS preparations in a dose of up to 1–1.5 mg are primarily used.

The sensitivity of sera in DIA (working dilution of 1/100) for isolated antigens was about 10 ng/mL, while it was $10^8$–$10^9$ MC/mL for heterologous strains of the causative agent. At the same time, the immunization of rabbits with antigen preparations conjugated with GNPs, followed by boosting with the isolated antigen, was the most effective. In contrast with the serum obtained according to Scheme A, there was no need to eliminate nonspecific antibodies. To assess the diagnostic significance of the sera obtained according to this scheme, immunoglobulin fractions were isolated from them and experimental test systems were developed to detect the tularemia microbe in the ELISA. Their activity was tested on a panel of pure *F. tularensis* cultures of various subspecies and was $5.2 \pm 0.5 \times 10^5$ MC/mL on average, with 100% specificity for heterologous strains in the concentration of $10^8$ MC/mL.

**Table 5.** Characteristics of anti-tularemia sera obtained after immunization of rabbits with tularemia antigens in three different schemes

| Serum characteristic | Immunization scheme | | |
|---|---|---|---|
| | A | B | C |
| Titer in RDP | 1/64–1/128 | 1/64–1/128 | 1/64–1/128 |
| Titer in RIHA | no | 1/5120 | 1/10240 |
| Sensitivity in DIA, MC/mL | $10^6$ | $10^6$ | $10^7$ |
| Specificity in DIA, MC/mL | $5 \times 10^7$ | $10^8$ | $10^9$ |
| Antigen consumption | 30 mg | 10 mg | 1.8 mg |
| Duration of immunization | 2 months | 2.5 months | 2.5 months |

Thus, the application of antigens conjugated with GNPs during the immunization of rabbits made it possible to obtain sera with a high titer of specific antibodies after a relatively short period of time and with the minimal antigen consumption, which makes it possible to use them in the production of tularemia diagnostics in future. The protectiveness of the PAK-M–GNP conjugate after subcutaneous administration to mice was higher than that of the unconjugated antigen, which will promote the application of the proposed approach for the development of the effective anti-tularemia vaccine.

## CONCLUSIONS

A number of recent reviews [12, 16, 26–29] report that the potential of GNPs for stimulating the immune response is very high. Nanoparticles were shown not

only to enhance humoral reactions against target antigens, but also to activate cellular immunity, as well as immunological memory. An increasing number of studies shed light on the mechanisms underlying the effect of nanovaccines, including targeted delivery to the lymph nodes, multivalence property of the antigen, and the coordinated delivery of antigens and adjuvants. However, the mechanisms of interaction between the functionalized GNPs and cells of the immune system are still far from a complete understanding and require subsequent study. These interactions correlate with the physicochemical characteristics of GNPs such as size, charge, and hydrophobicity, as well as with the characteristics of the antigen. It is no coincidence that the immunization of animals with GNP conjugates with antigens of various microorganisms leads to different results associated with the characteristics of the produced antibodies. Therefore, immunization schemes are not uniform and the optimization of the procedure is important for each specific subject. When using GNPs as a carrier and adjuvant for producing antibodies, the study of the characteristics of the immune response will make it possible to assess their potential for the development of effective vaccines.

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