EXPERIMENTAL ARTICLES

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ADJUVANT PROPERTIES OF NANOPARTICLES IMMOBILIZED RECOMBINANT DIPHTHERIA TOXOID FRAGMENT

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The aim of the research was to compare the characteristics of nanoparticles with different chemical structure and size (colloidal gold Gold 1 and Gold 2, calcium phosphate CaP and poly(D,L-lactide-co-glycolide) PLGA 1 and 2) to find the most efficient carriers of antigen — recombinant diphtheria toxoid for *per os* immunization. According to the MTT test, all studied particles show no significant cytotoxic impact on the studied cells *in vitro*, with the exception of CaP nanoparticles, which in high concentrations have cytotoxic effect on the U937 cells, and Gold nanoparticles 1 and 2, that are able to inhibit growth of the L929 cells. The most effective phagocytosis by macrophage-like cells J774 is observed for PLGA nanoparticles 1 and 2 with the immobilized antigen, while Gold nanoparticles 1 and 2 with antigen can interact with the surface of these cells without being phagocytated by them. In BALB/c mice immunized *per os* with antigen immobilized on PLGA 1 and 2 as well as Gold 2 carriers, the concentration of specific IgA antibodies in blood significantly increases after the second immunization, compared with controls. In the group of mice treated with PLGA 2 conjugated antigen, the concentration of specific IgG in blood after the third immunization also increases. These results show the promise of nanoparticles PLGA 1 and 2 as adjuvant for immunization *per os*.

Key words: nanoparticles, calcium phosphate, colloidal gold, poly(D,L-lactide-co-glycolide), diphtheria toxoid, oral immunization.

The immune system of the mucous membranes is represented by the specialized MALT lymphoid structures (mucosa-associated lymphoid tissues) [1]. It protects the epithelium against the infection and colonization by potentially dangerous microorganisms. The mucous membranes also absorbs and processes the foreign antigens from food, air or own microflora [2]. Of various mucous membrane cells, M-cells play an important role selectively absorbing the antigens and transporting them through mucosal barriers to subepithelial space, where subepithelial macrophages and dendritic cells actively capture, process and present transmitted antigens [3]. Human and rat M-cells also express MHC II [4], probably acting as antigen-presenting cells (APC). The barrier function of the mucous membrane is enhanced by the production of class A antigen-specific secretory antibodies (SIgA) which prevent the penetration of pathogens and toxins [5]. A deeper knowledge about the peculiarities of development of the immune response in mucous membranes opens up new avenues for the development of protective remedies. That is why the interest in mucosal vaccination is growing.

Parenteral vaccine promotes induction of systemic immunity, but is not intended for immune protection of mucous membranes which are on the frontline of neutralizing most pathogenic microorganisms [6]. The advantage of mucosal vaccination is that it can trigger the humoral and cellular immunity both systemically and locally on the mucous membranes. In this case, the antigens

administered through the mucous membranes induce the long-term immune memory by the formation of the corresponding T- and B-cells [7, 8].

Of the various ways to vaccinate mucous membranes, *per os* is the most promising method of administering preparations. Despite the benefits of oral delivery, the gastrointestinal tract (GIT) is characterized by low permeability of the membranes, highly active proteolytic enzymes, and the formation of mucosal tolerance for antigens introduced *per os* [9], which creates some difficulties in using this method. That's why it should be noted that injectable vaccines can be "ignored" by the mucous membrane protection system in case of oral administration [10].

The nanoparticles are promising for the *per os* delivery of protein antigens [9]. Due to their unique properties, nanoparticles can penetrate the mucous membranes barrier, transfer the immobilized antigen to MALT, protecting the protein from the damaging factors of the gastrointestinal tract, and enhancing the antigenic immunogenicity [8]. Various nanoparticles based on organic polymers, metal colloids, lipids, lipopolysaccharides, etc. are used as antigen carriers [8, 11–13]. However, the physicochemical properties of all these carriers are quite different, which may affect their adjuvant properties and interaction with APC and M-cells.

Thus, the purpose of this work was to determine the immuno-adjuvant properties of nanoparticles with different chemical structures with antigen immobilized on them for *per os* introduction. To do this, it was necessary to synthesize the nanoconjugates with a recombinant diphtheria toxoid, to investigate the cytotoxic effect of these conjugates, to determine the effectiveness of their interaction with APC, and to demonstrate the immunogenic properties of *per os* administration.

Materials and Methods

Preparation of nanoparticles based on colloidal gold (Gold 1 and Gold 2). The modification of the citrate method was used as described previously for synthesis of the desired amount of colloidal gold particles and their conjugation with antigen-protein [14]. In different test tubes, the mixtures ($HAuCl_4$ + H_2O) and ($Na_3C_6H_5O_7 + K_2CO_3 + C_{76}H_{52}O_{46}$) were pre-heated to 60 °C, than mixed and incubated (20 min, 60 °C). After that, the test tubes were transferred to ice and completely cooled.

For protein conjugation, EGFP solution (green fluorescent protein) or EGFP-SubB (recombinant subunit B of diphtheria toxin fused to EGFP) was added to the synthesized colloidal gold solutions in 0.2% sodium citrate solution and incubated for 40 min, 0 °C. EGFP and EGFP-SubB proteins were obtained as described previously [15]. The conjugate was further stabilized by dissolving the precipitate in 1 ml 0.2% sodium citrate containing 129 mM cellobiose (Sigma, USA) and incubation for 24 h, 4 °C. The resulting conjugates were then stored in 0.2% sodium citrate solution.

Obtaining nanoparticles based on calcium phosphate (CaP). For the nanoparticle synthesis, 12.5 mmol $CaCl_2$ (Alfarus, Ukraine), 12.5 mM Na_2HPO_4 and 15.6 mM sodium citrate solutions were added one by one to the protein solution in the respective ratios 7.5:7.5:1.5. The resulting mixture was incubated constantly mixing for 48 h, 4 °C. To stabilize the conjugate, the formed particles were re-suspended in 2 ml of 129 mmol cellobiose solution in PBS (0.14 M NaCl, 0.03 M KCl, 0.011 M Na_2HPO_4, 0.002 M KH_2PO_4, pH 7.4) (Miranda C, Ukraine) and incubated for 24 h, 4 °C. The resulting conjugates were then stored in PBS.

Preparation of nanoparticles based on PLGA by solvent replacement method followed by organic phase evaporation (PLGA 1). Synthesis of PLGA particles was carried out as described earlier [16] with certain modifications. 3 ml 0.5% PLGA solution (Sigma, USA) in acetone (organic phase) was added in drops to 30 ml PBS solution with simultaneous stirring on a magnetic stirrer. After evaporation of organic phase, the particle solution was filtered through a filter with 1 μm pores. The prepared solution of PLGA 1 particles was used for antigen sorption.

Preparation of nanoparticles based on PLGA by double emulsification with subsequent evaporation of the organic phase (PLGA 2). Synthesis of PLGA 2 particles was carried out as described earlier [16], with several modifications. 1 ml of PBS was added to 5 ml of 6% PLGA solution in methylene chloride, and mixed using MPW-309 homogenizer (*Universal Laboratory Aid, Poland,* for 3 min, 14 000 rpm). The obtained emulsion was mixed with 30 ml of dioctyl sulfosuccinate (Sigma, USA) (0.5% of the weight of added PLGA) in PBS and homogenized in an ice bath for 10 min, 7 500 rpm. For evaporation of the organic solvent, the solution was constantly stirred at room temperature (~12 hours). The prepared solution of PLGA 2 particles was used for antigen sorption.

Antigen sorption on PLGA particles. The resulting PLGA 1 and PLGA 2 particles were precipitated (12.000 g, 3 min), and the precipitate was diluted in 1 ml of EGFP protein or EGFP-SubB in PBS. Sorption of the antigen was carried out by stirring at a magnetic stirrer for 12 hours at 4 °C. To remove the unbound antigen, the particles were precipitated for 3 min at 12.000 g. In order to increase the stability of the conjugate, the precipitate was dissolved in 1 ml of 129 mM cellobiose in PBS and incubated for 24 hours at 4 °C. The resulting conjugates were precipitated and stored in PBS buffer.

Quantitative evaluation of antigen immobilization. The effectiveness of antigen immobilization on nanoparticles was determined by densitometric electrophoregram method using TotalLab TL120 software. The electrophoretic separation of the resulting particle-antigen complexes was performed in polyacrylamide gel with sodium dodecyl sulfate (Sigma, USA) using the modified Schagger technique [17].

Electron microscopy of obtained particles. The studies were carried out using Hitachi H-600 transmission electron microscope. ImageJ v1.43u software (NIH, USA) was used to measure the diameter of the synthesized particles according to the electron microscopy data. There were at least 15 analyzed objects per sample.

Cultivation of L929, U937 and J774 cell line cultures. Cells were cultured on RPMI-1640 nutrient medium with L-glutamine (Sigma, USA) containing 5% fetal bovine serum (Sigma, USA), streptomycin (100 mg/l), penicillin (10.000 U), and amphotericin B (250 μg/ l), 37 °C, 5% CO_2.

Determination of cell viability with MTT test. Cytotoxic affect of nanoparticles was studied as follows. Cells of L929 and U937 lines were inoculated in 96 well plates in 50 000 cells per well and added control protein or nanoparticles in concentrations of 0.1 mg/ml, 1 mg/ml, 10 mg/ml and 25 mg/ml. Semi-lethal doses (IC_{50}) for cytotoxic nanoparticles were calculated by incubating cell cultures of L929 and U937 lines in the medium with Gold 1 and Gold 2 particles in concentrations of 0.1–7 mg/ml. Also, cell culture of the line U937 were incubated with CaP nanoparticles in concentrations of 0.1–27 mg/ml. Samples were incubated for 12 h, 37 °C, 5% CO_2.

Cell viability was determined using MTT test as described [18] in own modification. Medium in the wells was refreshed with added MTT reagent (Sigma, USA) according to manufacturer's recommendations and incubated for 4 h. After that, MTT-reagent solution was substituted with the therapeutic solution: 10% SDS, 0.6% acetic acid in dimethyl sulfoxide (DMSO). Then, its optical absorption was determined at StatFax 2100 microplate reader (Awareness Technology, USA) at 650 nm, against 545 nm. The testing was done in triplicate.

To find out the semi-lethal nanoparticle doses, experimental data was analyzed using SigmaPlot software. The iterative modeling of the optimal equation describing the experimental data resulted in the three-parameter sigmoid dose-depending curve:

$$y = \min + \frac{\max - \min}{1 + 10^{(\log IC_{50} - x)}},$$

where x is the concentration of particles which inhibit cell growth, y is the number of viable cells at x particle concentration, *min* is cell viability in the absence of inhibiting agents, *max* is cell viability at the maximum concentration of inhibiting agent, IC_{50} is semi-lethal dose.

FITC (Fluorescein Isothyocyanate) particle marking. Per 10 μg of protein immobilized on particles, 25 μg FITC were added and incubated with constant stirring for 2 h at room temperature. Unbound FITC was washed off from the resulting complexes and they were placed into PBS for further storage.

Effectiveness of phagocytosis of obtained particles by flow cytometry. J774 cells were dropped to 24-well plate and incubated for 12 h (37 °C, 5% CO_2). To analyze the phagocytosis effectiveness, particle solution in 1% BSA in PBS was added to adherent cells and incubated for 5 min, 30 min, 60 min and 120 min (37 °C, 5% CO_2). The interaction of particles with the adherent cell surface was analyzed adding particle solution in 1% BSA in PBS and 0.02% NaN_3 and incubated for 15 min, 4 °C. The particles were added in amounts of 3 μg of immobilized protein per sample. After incubation, the cells were removed with 0.03 M EDTA, and the unbound particles were washed off. Then, intensity of cell fluorescence was measured using Coulter Epics XL flow cytometer.

Analyzing the cell-particle interaction using confocal microscopy. Cells of the line J774 were cultured in 24-well plates on special slides (12 h, 37 °C, 5% CO_2). Then, cells were washed with 1% BSA in PBS solution and added 1 ml of particle solution in 1% BSA in PBS, introducing 2.5 μg of antigen immobilized

on particles per sample. Nuclei were stained with Hoechst 33342, added simultaneously with particles to the concentration of 10 μM in medium. The incubation was performed for 60 min at 37 °C and 5% CO_2. Cells were washed off and fixed with 4% paraformaldehyde solution (Sigma, USA) in 0.1 M phosphate buffer 40 min at 4 °C. Cover glass with cells was fixed at the microscope slide in the polyvinyl alcohol based medium with DABCO (1,4-diazabicyclo[2.2.2]octane, Sigma, USA). The preparations were analyzed under Carl Zeiss LSM 510 Meta confocal microscope.

Mouse immunization with the particles and sampling. Female BALB/c mice, identical in age and weight, were immunized *per os* by the particles conjugated with antigen at a rate of 250 micrograms of protein per kg of weight three times at 2 weeks intervals. Blood was sampled from the tail vein 1 week after each immunization. Blood serum samples were stored in a freezing chamber (−20 °C) for further analysis.

Determination of SubB antibody levels in serum of immunized mice. The level of specific antibodies was evaluated in an indirect enzyme-linked immunosorbent assay (ELISA) using recombinant derivative diphtheria toxin (DT) as antigen. The antigen was sorted in 96 well plates at a rate of 1 μg per well (18 hours at 4 °C) then 1% dry skim milk (Sigma, USA) in PBS was added and incubated for 1 hour at 37 °C. Blood sera of mice were introduced in dilutions from 1: 100 to 1: 3200 in PBST (0.04% Tween 20, Sigma, USA) in PBS and incubated for 1 hour at 37 °C. The antigens which bound IgG or IgA antibodies were identified with appropriate secondary antibodies (Sigma, USA) labeled with horseradish peroxidase. After each incubation, the plates were washed three times with PBST. The peroxidase activity was evaluated with the chromogenic TMB (3,3′, 5,5′-tetramethylbenzidine) substrate at a wavelength of 450 nm on BioTek ELx800 microplate reader (BioTek Instruments, USA).

Results and Discussion

To use antigen-nanoparticle complexes for oral immunization, they should be stable: unbreakable under the influence of GIT damaging factors, and indestructible for a long time. In addition, it is necessary that they should be able to penetrate mucosal barriers, interact with immune cells and induce long-term immune memory. The particles should not have a cytotoxic effect on the cells of the body and should not affect the antigenic structure of the immobilized antigen.

With different approaches and methods of synthesis we obtained five types of particles with different chemical nature and size, and with an immobilized recombinant fragment of diphtheria toxin. The particle types differ in physical and chemical properties and the ability to biodegradate the resulting complexes. All conjugates were tested for their cytotoxic effect, immunogenic properties, and ability to interact with antigen presenting cells.

Characterization of particles with immobilized recombinant subunit B of diphtheria toxin.

The synthesized particles carrying SubB antigen first of all were analyzed for the effectiveness of the subunit's inclusion, since this is key in calculating the preparation dose, and may have an effect on its immunogenicity (Table 1). Calculations were made based on the ratio of protein concentrations in the solution before and after conjugation. Gold 1 and Gold 2 particles (63.52 μg/mg and 83 μg/mg respectively) demonstrated the highest loading efficiency. The efficiency of antigen inclusion for PLGA 1 and PLGA 2 particles was significantly lower (1.52 μg/mg and 4.96 μg/mg respectively).

Another important factor in oral vaccine production is the shape and size of the particles, because these properties affect their absorption by immune cells. As can be seen from the electronic microphotographs (Fig. 1), all the synthesized particles were symmetrical and round. The colloidal gold-based particles had the smallest proportions compared with the CaP and PLGA particles (Table). The sizes of the colloidal gold nanoparticles depend on the concentrations of the reaction mixture components, which is why we obtained particles of two sizes: 9 nm (Gold 1) and 16 nm (Gold 2). The CaP particles were somewhat larger, 37 nm in average. Two different approaches were used to obtain PLGA-based particles. As can be seen from the microphotographs, replacing the solvent with subsequent evaporation of the organic phase (PLGA 1) gave more homogeneous particles, 35 nm in average. The average size of a particle synthesized by double emulsification with subsequent evaporation of the organic phase (PLGA 2) was 100 nm.

Investigation of cytotoxic properties of received particles in vitro. Using the synthesized particles as adjuvants for oral immunization requires testing their toxicity to various cells. Determination of cytotoxic effect was

Table. **Properties of the obtained nanoparticles**

Particle type	Gold 1	Gold 2	CAP	PLGA 1	PLGA 2
Mean size (nm)	9	16	37	35	100
Mean effectiveness of antigen inclusion (μg/mg of particles)	63.52	83	14.85	1.52	4.96

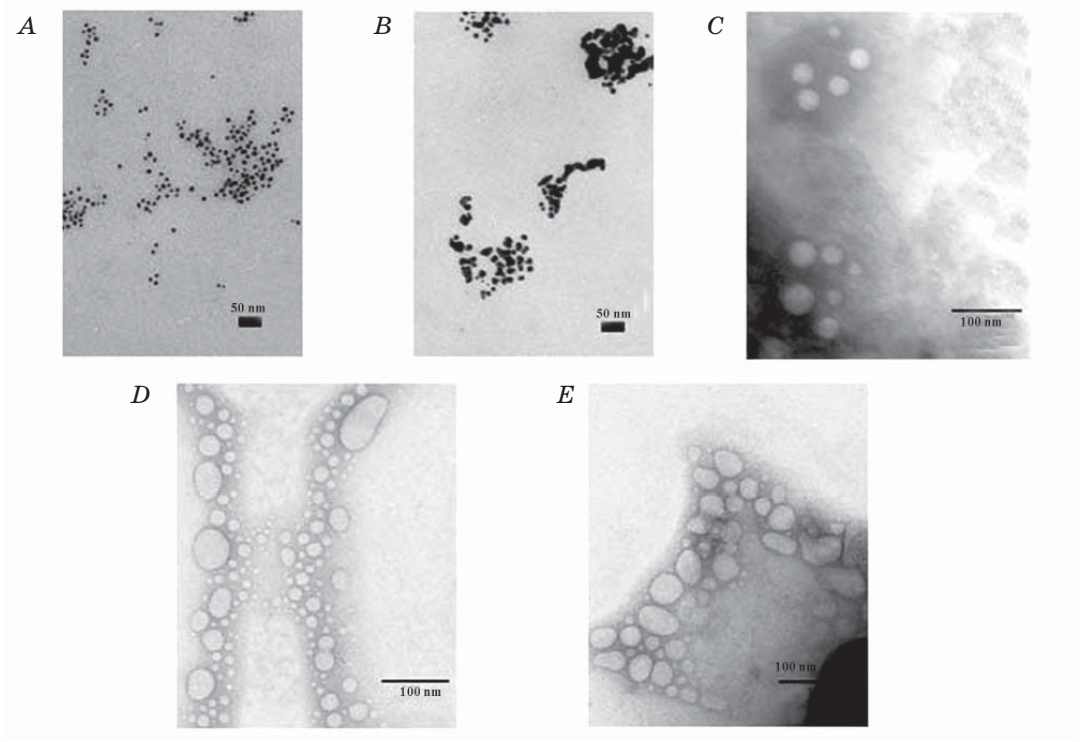

Fig. 1. **Electron microphotographs of particles based on:** colloidal gold Gold 1 (*A*) and Gold 2 (*B*); Calcium phospate CaP (*C*), and poly(D,L lactide-co-glycolide): PLGA 1 (*D*) and PLGA 2 (*E*)

performed with the MTT test. L929 and U937 cell lines were used because of their properties. For example, the U937 cells have such properties of monocytes as the increased phagocytic ability, and L929 have properties of fibroblast cells.

The results demonstrate the dose-dependent cytotoxic effect of the Gold 1 and Gold 2 nanoparticles on both cell types, as well as the toxicity of CaP particles to the cells of the U937 line (Fig. 2). PLGA particles had no cytotoxic effect in the studied concentrations.

Subsequently, IC_{50} inhibitory doses for cytotoxic nanoparticles were calculated by analyzing the inhibition pattern of cell growth (Fig. 3). The results of this simulation are shown in Fig. 3.

The IC_{50} values for both types of Gold 1 and Gold 2 particles are similar. They have a significant cytotoxic effect on the phagocytic U937 cells, which was almost 5 times greater than that of the CaP. The influence of gold particles on the L929 cells was somewhat different. The toxicity of the Gold 2 particles was almost thrice the influence of the Gold 1 particles.

Cytometry of the nanoparticle phagocytosis. To study the nanoparticle adjuvant properties as antigen carriers, their interaction with antigen-presentative cells were investigated *in vitro*. The study used macrophage-like J774 cells derived from histiocytic sarcoma of mice. *Escherichia coli* containing fluorescent EGFP were used as a positive control for the phagocytic activity of the J774 cells.

It is evident from the results of the cytometric study (Fig. 4) that almost all nanoparticles were capable of sorption on the cell surface, and the results for $PLGA_{EGFP\text{-}SubB}$ particles of both types significantly exceeded the values for the interaction of cells with *E. coli* bacteria. $Gold_{EGFP\text{-}SubB}$ 1 and $Gold_{EGFP\text{-}SubB}$ 2 particles showed the lowest results.

The cellular phagocytic activity showed a certain dependence on their sorption ability (Fig. 4). The strongest phagocytosis was

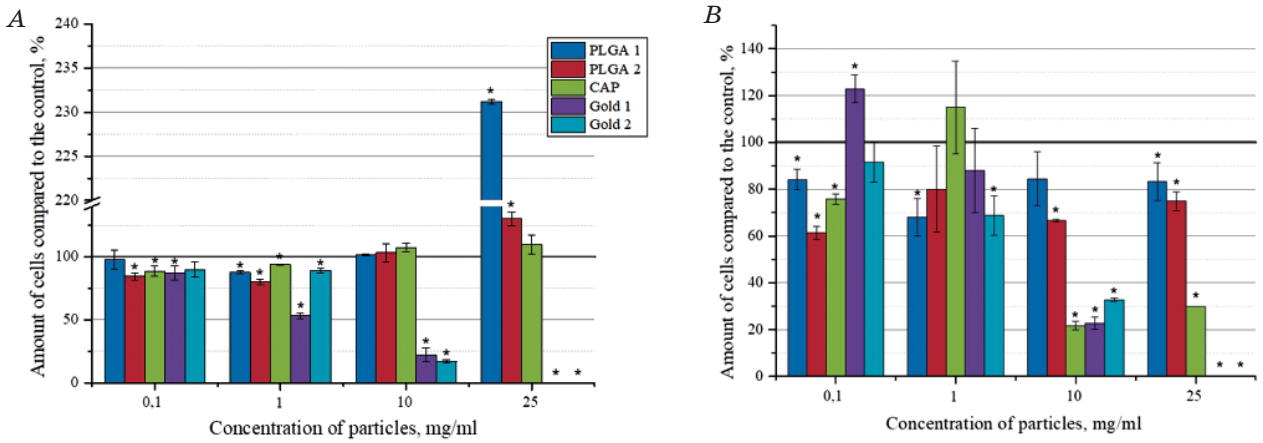

Fig. 2. **L929 (*A*) and U937 (*B*) cell viability after incubation for 12 hr with CAP, PLGA 1, PlGA 2, Gold 1 and Gold 2 particles added in concentrations of 0.1, 1, 10 and 25 mg/ml:**
100% — the number of control cells incubated without nanoparticles $M \pm S$, $n = 3$);
here and after: * $P \leq 0.05$ compared to control (100%)

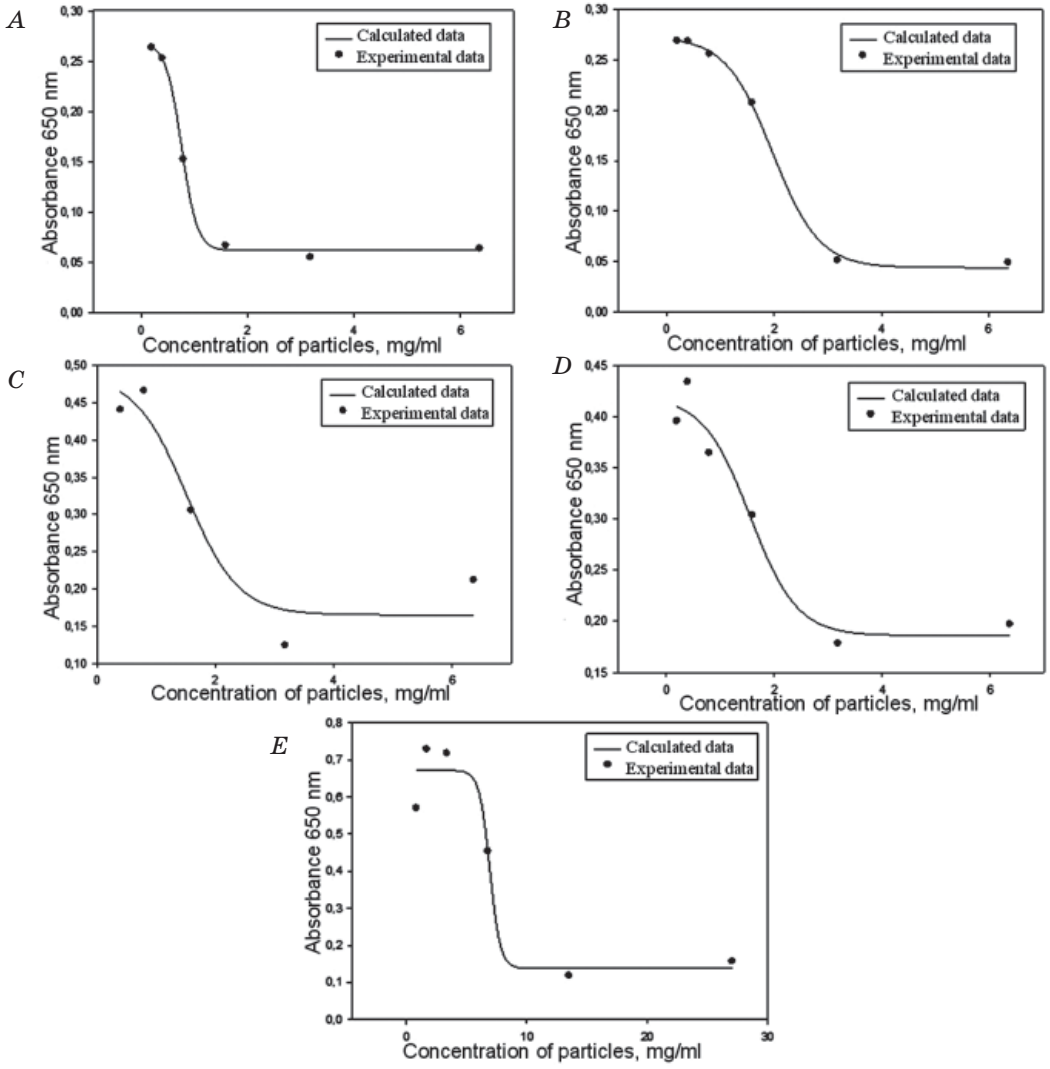

Fig. 3. **Relationships of L929 cell viability after incubation for 12 h with Gold 1 (*A*) and Gold 2 (*B*) particles; and U937 cell viability after incubation for 12 h with CAP (*C*), Gold 1 (*D*) and Gold 2 (*E*) particles added in different concentrations:** diagram points represent the experimental data of MTT test, lines are the theoretical approximations of points on sigmoid viability curves, calculated in SigmaPlot software

observed for $PLGA_{EGFP\text{-}SubB}$ particles, 1.5 times the *E. coli* sorption rates, and twice the $PLGA_{EGFP\text{-}SubB}$ 2 rates. The lowest values were recorded for $Gold_{EGFP\text{-}SubB}$ 1 and $Gold_{EGFP\text{-}SubB}$ 2 particles.

To increase the sensitivity of particle detection, they were conjugated to a FITS fluorescent label, which has a nearly 10-times higher quantum yield compared to EGFP. This modification allowed determining temporal peculiarities of the particle absorption by J774 cells. Notably, the absorption efficiencies were quite similar for FITC- and EGFP- labeled particles (results are not presented).

According to the diagram reflecting the dynamics of particle absorption (Fig. 5), 1 hour incubation was sufficient for almost all samples to reach maximum absorption by cells. The strongest absorption was recorded for $PLGA_{EGFP\text{-}SubB}$ 2, and the weakest for $Gold_{EGFP\text{-}SubB}$ 2, which confirms the data presented earlier.

Confocal microscopy of the interaction between the received particles and cells.

To confirm data on the absorption of particles by macrophage cells we used confocal microscopy to locate the complexes of particles with a fluorescent label.

As can be seen, the J774 cells most actively phagocyted $PLGA_{EGFP\text{-}SubB}$ 2 and $PLGA_{EGFP\text{-}SubB}$ 1 particles, followed by bacteria and $Gold_{EGFP\text{-}SubB}$ 1 particles (Fig. 6). The obtained results demonstrate the immunotropic properties of these particle types. In addition, they coincide with the results of cytometric research.

It should be noted that phagocytosis of the $CaP_{EGFP\text{-}SubB}$ and $Gold_{EGFP\text{-}SubB}$ 2 particles was not noted. This is probably due to the short incubation of cells with these particles.

Investigation in vivo of the immunogenic properties of the obtained particles.

The ability of antigen-particle conjugates to induce a specific immune response against the administered antigen after oral immunization was studied with *per os* immunization of experimental animals. Their serum was studied for the content of antigens-specific G antibodies providing long-term humoral immunity and of specific antibodies of class A, which form the local immunity of the mucous membranes.

Determination of class G antibody titers against antigen immobilized on particles in serum of immunized animals. GST-SubB protein (SubB cross-linked with an alternative GST tag and without an EGFP fluorescence tag) was used as an antigen in the indirect ELISA to study serum content of the specific antibodies against SubB. Serum from animals that were immunized *per os* with EGFP-conjugated particles or with a free EGFP-SubB or EGFP antigen was used as controls.

After the first immunization, the level of specific G antibodies to the antigen was high only in animals immunized with particles of PLGA 1, PLGA 2 and Gold 2, conjugated with EGFP-SubB (Fig. 7, *A*).

After the second immunization, serums of animal immunized with $PLGA_{EGFP\text{-}SubB}$ 1 and $Gold_{EGFP\text{-}SubB}$ 2 particles had high levels of specific G antibodies, but it decreased compared to the first immunization. Significantly, a relatively low content of specific IgG was detected in animals that have been immunized with $PLGA_{EGFP\text{-}SubB}$ 2. It is likely that re-immunization with such particles resulted in tolerance of the immune system to the introduced antigen.

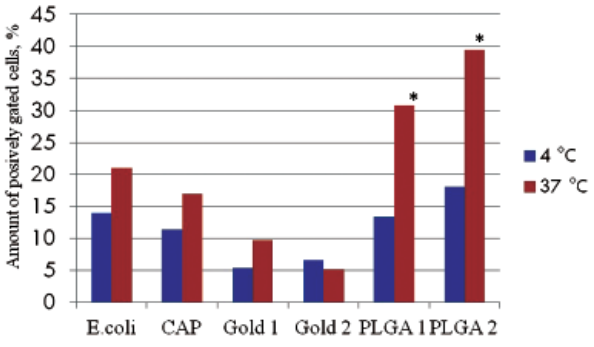

Fig. 4. **Interaction of J774 cells with CAP, PLGA 1, PLGA 2, Gold 1 and Gold 2 particles, and *E. coli* bacteria used as positive control at 4 °C and 37 °C;** $P \leq 0.05$ compared to control values of J774 cell interaction with *E. coli*

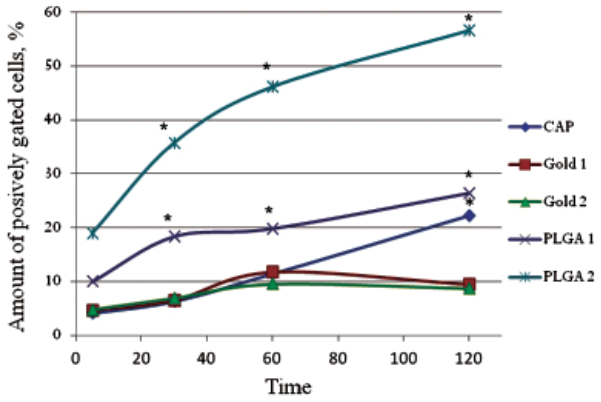

Fig. 5. **Absorption of CAP, PLGA 1, PLGA 2, Gold 1 and Gold 2 particles by J774 cells depending on the incubation time:**
control — Gold 1; $*P \leq 0.05$ compared to control values before incubation with particles

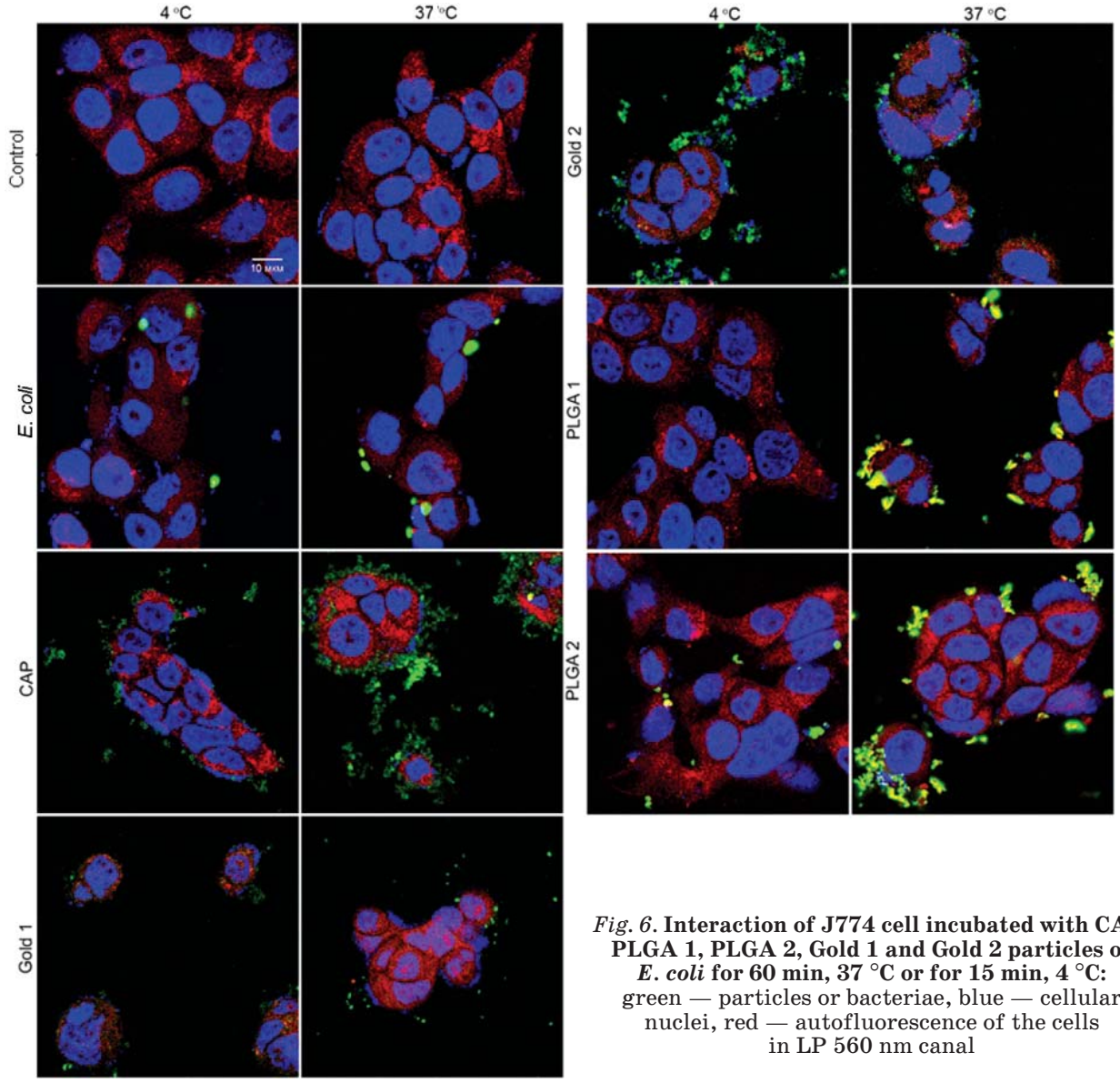

Fig. 6. **Interaction of J774 cell incubated with CAP, PLGA 1, PLGA 2, Gold 1 and Gold 2 particles or *E. coli* for 60 min, 37 °C or for 15 min, 4 °C:** green — particles or bacteriae, blue — cellular nuclei, red — autofluorescence of the cells in LP 560 nm canal

Based on the results obtained after the third immunization, almost all immunized animals had low levels of SubB-specific IgG. A high level of specific IgG was maintained only in animals immunized with $PLGA_{EGFP\text{-}SubB}$ 1 particles.

The specificity of the humoral immune response against the investigated antigen in experimental animals was confirmed by an additional indirect ELISA performed using BSA as a control antigen. Thus, GST-SubB serum recognition indicated the level of IgG capable of recognizing SubB, while BSA detection by serum indicated the level of nonspecific, polyreactive antibodies capable of recognizing a wide range of antigens.

The level of antibodies detecting BSA in almost all samples ranges from one third to one-half from a similar experiment with GST-SubB recognition. In blood serum of mice immunized with PLGA 1 and PLGA 2 particles conjugated to SubB, more non-specific antibodies were revealed. This may indicate the ability of such particles to stimulate production not only of antibodies specific to the introduced antigen, but also the nonspecific. In general, these findings confirm that preliminary data on the level of IgG antibodies in blood serum of animals immunized with nanoparticles provide mainly specific recognition of the test antigen.

Determination of IgA antibody titres against antigen immobilized on received particles in blood serum of immunized animals. Blood sera from animals immunized with different types of immobilized nanoparticles were also analyzed for the IgA antibodies specific to SubB (Fig. 7).

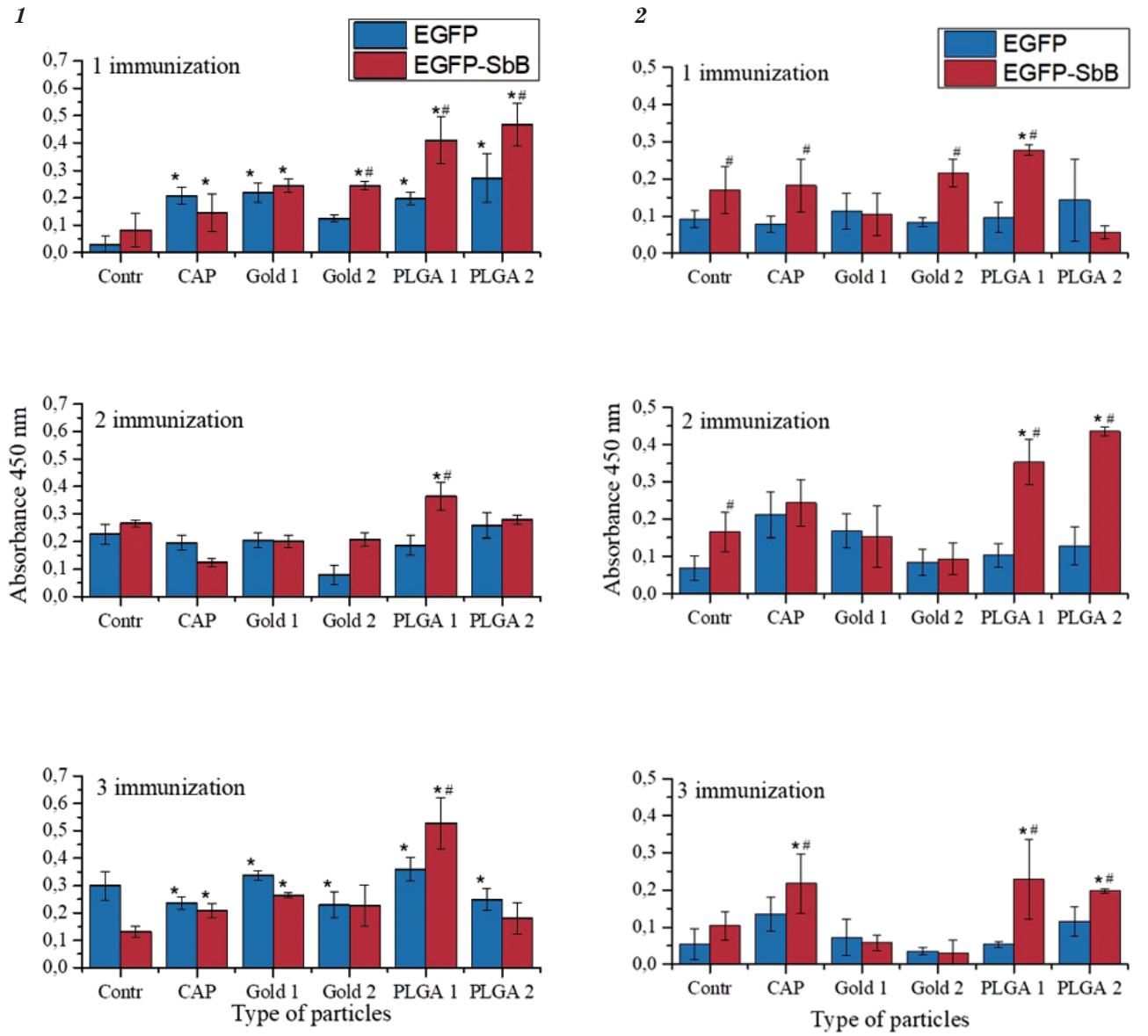

Fig. 7. **Histograms presenting the specific IgG (*1*) and IgA (*2*) levels against subunit B DT in blood sera of mice immunized with particles with immobilized EGFP-SubB antigen or the control EGFP antigen:** Histograms present the results of the 1st, 2nd or 3rd immunization. Statistical data are given as $M \pm SE$, $n = 4–6$; $*P \leq 0.05$ compared to control group immunized with EGFP-SubB antigen, $\#P \leq 0.05$ compared to control group immunized with EGFP immobilized antigen

After the first immunization, SubB-specific IgA antibody levels were elevated in blood serum from mice immunized with $CaP_{EGFP\text{-}SubB}$, $Gold_{EGFP\text{-}SubB}$ 2, $PLGA_{EGFP\text{-}SubB}$ 1 particles and in mice immunized with free EGFP-SubB compared to groups immunized with particles conjugated with EGFP.

As a result of the second immunization, levels of specific IgA antibodies in animals immunized with free EGFP-SubB remained unchanged. However, IgA levels increased in blood serum of animals immunized with $PLGA_{EGFP\text{-}SubB}$ 1 and $PLGA_{EGFP\text{-}SubB}$ 2. This suggests that, though a free antigen can trigger the activation of the immune response of the mucous membranes, its conjugation with PLGA particles increases the antigen immunogenicity by nearly 1.5-times. Levels of IgA antibodies in animals immunized with $Gold_{EGFP\text{-}SubB}$ 2 particles decreased, which probably indicates the tolerance to the antigen of animals immunized with these particles.

After the third immunization, there was a general decrease in the IgA levels. The

elevated IgA level, as compared with control, was maintained in groups immunized with $CAP_{EGFP\text{-}SubB}$, $PLGA_{EGFP\text{-}SubB}$ 1 and $PLGA_{EGFP\text{-}SubB}$ 2. However, samples of blood serum from mice immunized with CAP contained a high level of nonspecific antibodies. This decline suggests a possible tolerance to the introduced antigen.

Thus, the obtained data show the promise for the implementation of PLGA particles as carriers of antigen for oral immunization. This particle type does not have cytotoxic effects on cells, even in ultrahigh concentrations. The results of flow cytometry and confocal microscopy showed their ability to be absorbed and penetrate into phagocytic cells. Also, PLGA particles showed the best results as *in vivo* adjuvants and influenced the synthesis of specific IgA and IgG antibodies to the immobilized antigen.

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REFERENCES

1. *Montilla N. A., Blas M. P., Santalla M. L., Villa J. M.* Mucosal immune system: A brief review. *Immunologia*. 2004, 23 (2), 207–216.
2. *Holmgren J., Czerkinsky C.* Mucosal immunity and vaccines. *Nat. Med*. 2005, 11 (4), 45–53. doi: 10.1038/nm1213
3. *Torché A.M., Jouan H., Le Corre P., Albina E., Primault R., Jestin A., Le Verge R.* Ex vivo and in situ PLGA microspheres uptake by pig ileal Peyer's patch segment. *Int. J. Pharmaceut*. 2000, 201 (1), 15–27. doi: 10.1016/S0378-5173(00)00364-1
4. *Finzi G., Cornaggia M., Capella C., Fiocca R., Bosi F., Solcia E., Samloff I.M.* Cathepsin E in follicle associated epithelium of intestine and tonsils: localization to M cells and possible role in antigen processing. *Histochemistry*. 1993, 99 (3), 201–211. doi: 10.1007/BF00269138
5. *Schenk M., Mueller C.* The mucosal immune system at the gastrointestinal barrier. *Best practice & Research. Clin. Gastroenterol*. 2008, 2 2(3), 391–409. doi: 10.1016/j.bpg.2007.11.002
6. *Yuki Y., Kiyono H.* Mucosal vaccines: novel advances in technology and delivery. *Expert review of vaccines*. 2009, 8 (8), 1083–1097. doi: 10.1586/erv.09.61
7. *Lycke N.* Recent progress in mucosal vaccine development: potential and limitations. *Nat. Rev. Immunol*. 2012, 12 (8), 592–605. doi: 10.1038/nri3251
8. *Csaba N., Garcia-Fuentes M., Alonso M.J.* Nanoparticles for nasal vaccination. *Adv. Drug Deliv. Rev*. 2008, 61 (2), 140–157. doi: 10.1016/j.addr.2008.09.005
9. *Yun Y., Cho Y. W., Park K.* Nanoparticles for oral delivery: targeted nanoparticles with peptidic ligands for oral protein delivery. *Adv. Drug Deliv. Rev*. 2012, 65 (6), 822–832. doi: 10.1016/j.addr.2012.10.007
10. *Neutra M. R., Kozlowski P. A.* Mucosal vaccines: the promise and the challenge. *Nat. Revi. Immunol*. 2006, 6 (2), 148–158. doi: 10.1038/nri1777.
11. *He Q., Mitchell A.R., Johnson S.L., Wagner-Bartak C., Morcol T., Bell S.J.* Calcium phosphate nanoparticle adjuvant. *Clin. Diagnost. Lab. Immunol*. 2000, 7 (6), 899–903. doi: 10.1128/CDLI.7.6.899-903.2000
12. *Chadwick S., Kriegel C., Amiji M.* Delivery strategies to enhance mucosal vaccination. *Expert Opin. Biol*. 2009, 9 (4): 427–440. doi: 10.1517/14712590902849224
13. *Chadwick S., Kriegel C., Amiji M.* Nanotechnology solutions for mucosal immunization. *Adv. Drug Deliv. Rev*. 2009, 62 (4–5): 394–407. doi: 10.1016/j.addr. 2009.11.012
14. *Labyntsev A. Yu., Oliinyk O. S., Kaberniuket A. A., Chunihin O. J., Gorchev V. F., Kyrchenko T. O., Chernushov V. I., Kolibo D. V.* Optimizing protein A — colloidal gold conjugates synthesis conditions and developing approaches for their characterization. *Biotekhnolohiia*. 2009, 2 (2), 76–83. (In Ukrainian).
15. *Kaberniuk A. A., Labyntsev A. J., Kolibo D. V., Oliinyk O. S., Redchuk T. A., Korotkevich N. V., Gorchev V. F., Karahim S. O., Komisarenko S. V.* Fluorescent derivatives of diphtheria toxin's subunit b and their interaction with vero cells. *Ukr. Biochem. J*. 2009, 81 (1), 67–77. (In Ukrainian).
16. *Malyala P., Singh M.* Micro/nanoparticle adjuvants: preparation and formulation with antigens. *Meth. Mol. Biol. Clifton N.J. Springer protocols*. 2010, 91–101.
17. *Schägger H., von Jagow G.* Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem*. 1987, 166 (2), 368–379.
18. *Ohno M., Abe T.* Rapid colorimetric assay for the quantification of leukemia inhibitory factor (LIF) and interleukin–6 (IL–6). *J. Immunol. Meth*. 1991, 145 (1–2), 199–203.

АД'ЮВАНТНІ ВЛАСТИВОСТІ НАНОЧАСТИНОК З ІММОБІЛІЗОВАНИМ ФРАГМЕНТОМ ДИФТЕРІЙНОГО ТОКСОЇДУ

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Метою роботи було порівняння характеристик частинок з різною хімічною будовою та розміром (колоїдного золота Gold 1 та Gold 2, фосфату кальцію CaP та полі(лактид-ко-глюколіду) PLGA 1 і PLGA 2) для пошуку найбільш ефективних носіїв антигену (рекомбінантного дифтерійного токсоїду) за імунізації *per os*. За даними МТТ-тесту всі досліджувані частинки не виявляли помітного цитотоксичного впливу стосовно досліджуваних клітин *in vitro*, за винятком частинок CaP, що у високих концентраціях чинили цитотоксичний вплив на клітини лінії U937, та частинок Gold 1 і Gold 2, які також пригнічували ріст клітин лінії L929. Найбільш виражене фагоцитування макрофагоподібними клітинами лінії J774 було зафіксовано для частинок PLGA 1 і PLGA 2 з іммобілізованим антигеном, у той час як кон'югати частинок Gold 1 і Gold 2 з антигеном виявили здатність до сорбції на поверхні цих клітин, але не поглиналися ними. У мишей BALB/c, що отримували перорально антиген, іммобілізований на носіях PLGA 1 та PLGA 2, а також Gold 2, концентрація у крові антитіл IgA, специфічних до субодиниці B (SubB), порівняно з контролем, помітно збільшувалася вже після другої імунізації. У групи мишей, що отримували кон'югати антигену з PLGA 2, відначено також збільшення концентрації специфічних IgG у крові після третьої імунізації. Наведені результати свідчать про перспективність дослідження частинок PLGA 1 та PLGA 2 як ад'ювантів для імунізації *per os*.

Ключові слова: наночастинки, фосфат кальцію, колоїдне золото, полі(лактид-ко-гліколід), дифтерійний токсоїд, пероральна імунізація.

АДЪЮВАНТНЫЕ СВОЙСТВА НАНОЧАСТИЦ С ИММОБИЛИЗОВАННЫМ ФРАГМЕНТОМ ДИФТЕРИЙНОГО ТОКСОИДА

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Целью работы было сравнение характеристик частиц с разным химическим строением и размерами (коллоидного золота Gold 1 и Gold 2, фосфата кальция CaP и поли(лактид-ко-глюколида) PLGA 1 и PLGA 2 для поиска наиболее эффективных носителей антигена — рекомбинантного дифтерийного токсоида — при иммунизации *per os*. По данным МТТ-теста все исследуемые частицы не проявляли заметного цитотоксического воздействия по отношению к рассматриваемым клеткам *in vitro*, за исключением частиц CAP, которые в высоких концентрациях оказывали цитотоксическое влияние на клетки линии U937, и частиц Gold 1 и Gold 2, также подавивших рост клеток линии L929. Наиболее выраженное фагоцитирование макрофагоподобными клетками линии J774 было зафиксировано для частиц PLGA 1 и PLGA 2 с иммобилизованным антигеном, в то время как конъюгаты частиц Gold 1 и Gold 2 с антигеном оказались способными к сорбции на поверхности этих клеток, но не поглощались ими. У мышей BALB/c, получавших перорально антиген, иммобилизованный на носителях PLGA 1 и PLGA 2, а также Gold 2, концентрация в крови антител IgA, специфичных к субъединице B — SubB, заметно увеличивалась по сравнению с контролем уже после второй иммунизации. У группы мышей, получавших конъюгаты антигена с PLGA 2, отмечено также увеличение концентрации специфических IgG в крови после третьей иммунизации. Приведенные результаты свидетельствуют о перспективности исследования частиц PLGA 1 и PLGA 2 в качестве адъювантов для иммунизации *per os*.

Ключевые слова: наночастицы, фосфат кальция, коллоидное золото, поли(лактид-ко-гликолид), дифтерийный токсоид, пероральная иммунизация.