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Use of a synthetic foot-and-mouth disease virus peptide conjugated to gold nanoparticles for enhancing immunological response

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Abstract Foot-and-mouth disease is an acute, highly contagious infection of domestic and wild cloven-hoofed animals, which can be transmitted to humans. In many cases, the existing vaccines are not quite effective. The purpose of this study was to test the possibility of using gold nanoparticles as an antigen carrier and an adjuvant. The immunogenic properties of gold nanoparticles were assessed by conjugating the particles to a synthetic peptide of the VP_1 capsid protein of the foot-and-mouth disease virus. The resulting conjugate (with or without the use of complete Freund's adjuvant), a commercial vaccine, and the native peptide served to immunize guinea pigs. The titer and sensitivity of the raised antibodies were maximal for the combination comprising the nanoparticle–peptide conjugate and complete Freund's adjuvant. Antibody biosynthesis was accompanied by increased production of proinflammatory cytokines (especially IFN-γ) and by stimulation of the respiratory activity of peritoneal macrophages. The use of gold nanoparticles as a hapten carrier enhanced the immune response even when complete Freund's adjuvant was not used.

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Introduction

Foot-and-mouth disease (FMD) is an acute, highly contagious infection of domestic and wild cloven-hoofed animals, which can be transmitted from animals to humans. It is caused by a filterable RNA-containing aphthovirus of the family *Picornaviridae*. On the basis of antigenic structure, seven serotypes of the FMD virus (FMDV) have been identified, each of which includes several variants (about 80 in total) [1]. The number of recorded FMD outbreaks has been increasing significantly since the early 2010, and in Russia, there were 14 outbreaks in five regions in the first half of 2013 alone.

Vaccination is a major means of preventing FMD. For prophylactic vaccinations, various monovalent and polyvalent inactivated FMD vaccines are employed [2], but no live vaccines have so far been developed. Although existing vaccines are in many cases effective against the disease, they have several essential disadvantages: The immune response develops slowly; vaccinated animals can become infected before an immune response is generated; and animals can become virus carriers even after being successfully vaccinated [3, 4]. Furthermore, it is known that during the recent systematic FMD vaccination in Europe, up to half the disease outbreaks recorded annually have been due to "underinactivated" formulations and to their residual infectivity [5]. Therefore, the search for new vaccines is continuing, examples being the recently proposed recombinant peptide vaccines [6] and experimental procapsid and synthetic peptide vaccines [7, 8]. A search is also in progress for the most effective adjuvants [9].

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Much work on synthetic peptide vaccines has been done with the FMDV. This became possible once it was established that the virus protein VP_1, in particular a major neutralizing immunogenic site (amino acids 135–159) and an additional site near the C terminus of this polypeptide [10, 11], is responsible for the induction of FMDV-neutralizing antibodies. In Volpina et al. [12], a synthetic peptide with amino acids 135–159 of VP_1 was made, which was linked to keyhole limpet hemocyanin and supplemented with Freund's adjuvant. The vaccine induced neutralizing antibodies in guinea pigs, pigs, and cattle and protected against infection with a virulent FMDV strain. However, the antibody response after immunization with the peptide was 10 times lower than that observed after immunization with whole virions. Synthetic peptides corresponding to the neutralizing epitopes of VP_1 of FMDVs of three serotypes had different antigenic and immunogenic activities. Peptides specific for serotypes A and O induced protective immunity in guinea pigs, whereas the peptide specific for serotype C proved less active. Peptides with the amino acid sequences of VP_1 of all seven FMDV serotypes elicited neutralizing antibodies in guinea pigs. The antibodies were mostly serotype-specific [12]. The possibility of preparing a bivalent peptide vaccine against FMD was next established by Volpina et al. [13], who synthesized two peptides for different serotypes in tandem. The dipeptide vaccines induced synthesis of neutralizing antibodies to two FMDV serotypes. A synthetic peptide corresponding to the 135–159 region of VP_1 of FMDV type A was administered to guinea pigs, sheep, and cattle together with Freund's adjuvant, resulting in a high level of neutralizing antibody and resistance to infection with a homologous virulent FMDV strain. Immunity was found to depend on the affinity of the antibodies induced by the synthetic peptide. Immunization with the synthetic peptide construct ensured that experimentally infected pigs were protected against the disease [14].

There is a great current interest in using nanoparticles of various natures as platforms for vaccines [15–19]. Gold nanoparticles (AuNPs) are some of the most promising antigen carriers for immunization [20–22] owing to their adjuvant properties [23, 24]. AuNPs were first used as part of an antiviral vaccine to carry the protein antigen of the tickborne encephalitis virus capsid [25]. Although not containing adjuvants, the proposed experimental vaccine offered better protection than did its commercial counterparts. Subsequently, AuNPs have been used to generate antibodies and design experimental vaccines (both peptide and carbohydrate) against influenza A virus [26, 27], transmissible gastroenteritis virus [28], West Nile virus [29], the respiratory syncytial virus [30], as well as against tuberculosis [31, 32] and other bacterial infections [33–37]. In addition, AuNPs are being used in the development of experimental vaccines against tumors [38, 39].

The purpose of this study was to investigate the possibility that AuNPs could serve as a carrier of an FMDV synthetic peptide and as an adjuvant in the immunization of animals. AuNPs were chosen because they have been employed successfully in designing prototypes of peptide vaccines against several viral and bacterial infections. In Volpina et al. [12, 13], the synthetic peptides were not coupled to a nanosized carrier, and the vaccination efficacies were insufficient. We now propose the use of peptide antigen–AuNP conjugates to improve the efficacy of vaccination.

Materials and methods

Peptide antigen

The antigen used for immunization was a lyophilized commercial synthetic peptide spanning the 135–159 region of the FMDV capsid protein VP_1 (Cytokine Co., Russia). The peptide contained 25 amino acid residues [amino acid sequence, KYSAGGMGRRGDLEPLAARVAAQLP; brutto formula, $C_{111}H_{186}N_{36}O_{33}S_1$; molecular mass, 2584.96 Da (with account taken of the natural isotope content); and isoelectric point, 10.32]. The peptide was dissolved in 0.01 M phosphate-buffered saline (PBS), pH 7.4, to 1 mg ml^{-1}.

The control was a commercial inactivated tissue culture FMD vaccine (Federal Centre for Animal Health "ARRIAH," Russia) that contained an oil adjuvant Montanide ISA 206. The amount of protein present in the commercial vaccine was estimated by the colorimetric method of Lowry [40] with a Genesys 10S UV–vis spectrophotometer (Thermo Scientific, USA).

Preparation of antigen–AuNP conjugates

Spherical AuNPs (15 nm mean diameter) were prepared according to Frens [41] by the reduction of tetrachloroauric acid (Sigma-Aldrich, USA) with sodium citrate (Fluka, Switzerland). A 242.5 ml portion of 0.01 % aqueous tetrachloroauric acid was heated to 100 °C on a magnetic stirrer in an Erlenmeyer flask fitted with a water-cooled reflux tube. This was followed by the addition of 7.5 ml of 1 % aqueous sodium citrate to the flask. The total preparation time was 45 min.

The particle diameter was determined with a Specord S 250 UV–vis spectrophotometer (Analytik Jena, Germany), a Libra 120 transmission electron microscope (Carl Zeiss, Germany), and a Zetasizer Nano-ZS particle size and zeta potential analyzer (Malvern, UK), as described by Khlebtsov and Dykman [42].

To prepare antigen–AuNP conjugates, we determined the "gold number" (minimal amount of antigen that protects the sol against salt aggregation) for the peptide solution. To this

end, 20 μl of an antigen solution in PBS (initial concentration, 1 mg ml^{-1}) was titrated twofold on a 96-well microtiter plate. Each well received 200 μl of AuNPs and 20 μl of 1.7 M NaCl. The minimal stabilizing concentration was determined visually by the change in the color of the AuNP solution from red to blue in the wells of a microtiter plate. The minimal stabilizing concentration for the antigen was 12.5 μg ml^{-1}. Conjugation was done by simple mixing (without the use of coupling agents) [43].

Determination of the gold number presupposes that the antigen molecules, when being adsorbed onto the AuNP surface, protect the Au sol against salt aggregation. If there are not enough antigen molecules to cover the particle surface, the addition of an electrolyte results in particle aggregation, giving a blue color to the solution. In our case, such an effect was observed when the antigen concentration was 6.25 μg ml^{-1}. Thus, at the 12.5 μg ml^{-1} concentration, either all added peptide was adsorbed on the AuNPs or there was a slight excess of it. It should be noted that a slight excess of soluble antigen not only does not interfere with immunization but also facilitates an increase in antibody elaboration [44].

The use of a minimal stabilizing concentration presupposes that all of the peptide is conjugated to AuNPs and is almost absent from solution. Nevertheless, to remove all traces of unbound antigen, we centrifuged the conjugate at 13,000×g for 15 min, decanted the supernatant liquid, and redissolved the pellet in PBS.

Animals

Fifteen male albino guinea pigs, weighing 300–350 g by the time the experiment started, were used in this study. Animal care and handling were in accordance with the Guide for the Care and Use of Laboratory Animals, the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, and the legislation of the Russian Federation.

Immunization procedure

For immunobiological studies, the guinea pigs were divided into five groups of three in each on the basis of their body weight (deviation of the body weight within a group, ±10 %).

The animals were immunized subcutaneously at 10 points along the spinal column by two injections with an interval of 10 days between. The antigen doses used are given in Table 1.

The guinea pigs in group 1 were injected with 0.5 ml of the commercial vaccine according to the instructions supplied by the manufacturer. Group 2 received 1 ml of AuNP–peptide conjugates mixed 1:1 with complete Freund's adjuvant (CFA; Becton Dickinson, USA); group 3, 1 ml of AuNP–peptide conjugates without CFA; group 4, 1 ml of a peptide solution in PBS mixed 1:1 with CFA; and group 5 (control), a 1.7 M NaCl solution. Thus, group 2 received 29 μg Au, and group 3 received 58 μg Au. The animals were killed 10 days after the last injection.

Isolation of peritoneal macrophages and splenic lymphocytes

For peritoneal macrophages, the animals were killed and then fixed on their backs. An incision was made along the midline of the anterior abdominal wall, and the skin flap was carefully separated, with care taken to keep the peritoneum intact. After a puncture had been made with a needle connected to a syringe, 50 ml of PBS, pH 7.2, was injected into the peritoneal cavity. The anterior abdominal wall was then gently massaged, and after 5–7 min, peritoneal fluid was collected with a Pasteur pipet through a cut made in the peritoneum and was filtered into a test tube through a nylon filter. The cells were washed three times by centrifugation in PBS at 750×g, after which they were redissolved in 1 ml of PBS and counted in a Goryaev chamber. Peritoneal macrophages were cultured by standard procedures [45].

For splenic lymphocytes, an incision was made along the white line of the peritoneum after peritoneal macrophages had been isolated, and the spleen was removed. The spleen was minced with scissors, and the tissue pieces were mashed through a fine sieve into a petri plate containing sterile PBS. The resulting suspension was subjected to Ficoll–Urografin density-gradient centrifugation. The lymphocyte ring was collected into a new test tube. The lymphocytes were washed three times by centrifugation in PBS, pH 7.4, at 750×g for 10 min, and the cell pellet was redissolved in 1 ml of PBS. The lymphocytic cells were counted with a HaemaScreenvet hematology analyzer (Hospitex Diagnostics, Italy).

Table 1 Immunization parameters	No.	Animals	Antigen construction administered	Amount (ml)	Dose of protein/peptide administered (μg)
	1.	Guinea pigs (n=3)	Commercial FMD vaccine	0.5	25
	2.	Guinea pigs (n=3)	Au NP–peptide conjugate + CFA (1:1)	1	6.25
	3.	Guinea pigs (n=3)	Au NP–peptide conjugate	1	12.5
	4.	Guinea pigs (n=3)	Peptide solution + CFA (1:1)	1	6.25
	5.	Guinea pigs (n=3)	1.7 M NaCl solution	1	–

Analysis of biochemical and immunological parameters

Sera were biochemically examined with the use of a BA-88A analyzer (Mindray, China) and a standard reagent kit (Diakon-DS, Russia). The interleukin concentration in the sera was measured with the use of a Plate Screen analyzer (Hospitex Diagnostics, Italy) and reagent kits for the immunoenzymatic determination of IL-1β, IL-6, and IFN-γ (Vector-Best, Russia).

The titer of antibodies in the sera was estimated by enzyme-linked immunosorbent assay (ELISA) with horseradish peroxidase-labeled antibodies against guinea pig IgG (Jackson ImmunoResearch, UK). The synthetic peptide was used as the immobilized antigen. The reaction results were recorded on a Plate Screen microplate spectrophotometer. Briefly, the antigen was diluted 1:100 in 0.01 M PBS and incubated overnight at 4 °C. The free binding sites were blocked with 200 μl of 2 % fat-free powdered milk in 0.01 M PBS on a shaker for 1 h. Each well then received 100 μl of serum from the immunized animals, which was diluted 1:10 in PBS and then serially twofold in PBS. Incubation was done on a thermoshaker at 37 °C for 1 h. The wells were washed three times for 10 min each with 200 μl of PBS on the shaker, and then, each well received 100 μl of horseradish peroxidase-labeled antibodies (Jackson ImmunoResearch, USA) diluted 1:500 in PBS. Incubation was done on the thermoshaker at 37 °C for 1 h, after which the wells were washed three times for 10 min each with 200 μl of PBS on the shaker. The antigen–antibody reaction was developed with 100 μl of 0.006 % o-phenylenediamine in 0.1 M sodium citrate buffer containing 0.01 % hydrogen peroxide. The reaction was stopped with 100 μl of 0.1 M sulfuric acid.

Respiratory activity was determined conventionally [46] by the ability of cells to reduce nitrotetrazolium blue [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich)] to formazan. Briefly, suspensions of known concentrations of isolated animal cells (macrophages and lymphocytes) were centrifuged at $1000 \times g$ for 10 min, and the pellet was resuspended in 1 ml of 0.05 % MTT and incubated at 37 °C for 1 h. After incubation, the cells were centrifuged at $4000 \times g$ and the pellet was resuspended in 0.5 ml of dimethyl sulfoxide (Fluka, Switzerland). The amount of reduced formazan was measured with a Genesys 10S UV–vis spectrophotometer at 490 nm. To construct the calibration curve, we used commercial formazan (Sigma-Aldrich) at 0.002, 0.02, 0.2, and 2 mg ml^{-1}. The concentration of reduced formazan was converted to that per cell.

Dot immunoassay

The peptide (double dilutions; initial concentration, 1 mg ml^{-1}) was applied as a series of spots onto a Western S polyvinylidene fluoride membrane (Millipore, USA). The membrane was blocked for 1 h with 2 % fat-free powdered milk diluted in 0.01 M PBS, pH 7.2, dipped into a solution of animal blood serum diluted 100-fold in 1 ml of 0.01 M PBS, pH 7.2, and incubated at room temperature for 1 h. When there was a biospecific interaction, the serum antibodies bound to the antigen adsorbed on the membrane. The membrane was then washed free of nonspecifically bound antibodies and was immersed in a solution of AuNPs conjugated to staphylococcal protein A ($A_{520}=0.5$) (IBPPM, Russia) [43]. After 5–60 min, the binding of the conjugate to the antigen–antibody complex could be observed visually as a series of red spots.

Statistics

The obtained results were statistically processed by the standard procedures integrated in Excel 2007 software (Microsoft Corp., USA). After the arithmetic mean and the standard deviation for a given data sample had been found, we determined the standard error of the arithmetic mean and its confidence limits with account of Student's t coefficient (n, p) [number of measurements $n=3$, significance level = 95 % ($p=0.05$)]. These results are presented as histograms. The significance of differences between individual samples was evaluated by a two-sample unpaired Student's t test with unequal variances. Differences were considered significant when the experimentally found p_{exp} value was ≤0.05.

The reliability of the changes recorded in the results of each of the experiments described above for the entire set of antigen formulations examined was assessed by one-way analysis of variance (ANOVA) by using Fisher's ratio test. The dependences found were considered significant at $F>F_{crit}$, where the critical value F_{crit} at $n=3$ and $m=4$–5 (number of data sets) corresponded to $p=0.05$ (with the number of degrees of freedom (df) lying between 4 and 14) and the effective value p_{eff} was <0.05.

Results and discussion

Characterization of AuNPs

The diameter of the synthesized AuNPs was determined by spectrophotometry (Fig. 1), transmission electron microscopy (TEM; Fig. 2), and dynamic light scattering (DLS; Fig. 2). The absorption spectrum peak of the obtained sol was $\lambda_{max}=519.1$ nm, with the absorbance being $A_{520}=1.2$. As found by TEM and DLS, the average diameter of the obtained nanoparticles was 15.7 nm. The protocol used to prepare AuNPs presupposes that the mass concentration of Au is constant (58 μg per ml of sol, corresponding to $A_{520}=1$). Therefore, the number of particles per milliliter at $A_{520}=1$ was 1.6×10^{12}.
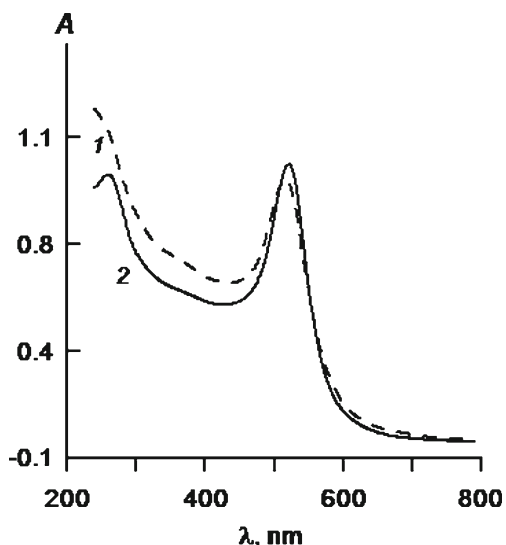
Fig. 1 Absorption spectrum of AuNPs with an average diameter of 15 nm before (*1, broken line*) and after (*2, solid line*) conjugation to the peptide

Evaluation of the humoral response

For each experiment, the legends to Figs. 3, 5, 6, 7, 8, and 9 give the *p* value obtained from a pairwise comparison of the corresponding arithmetic mean with the mean characterizing the effect of the commercial FMD vaccine (histogram 1). The bars represent confidence intervals for the arithmetic means. In addition, the legends contain the experimental values of F and p_{eff} (plus the values of F_{crit}) for the sets of experimental data being described.
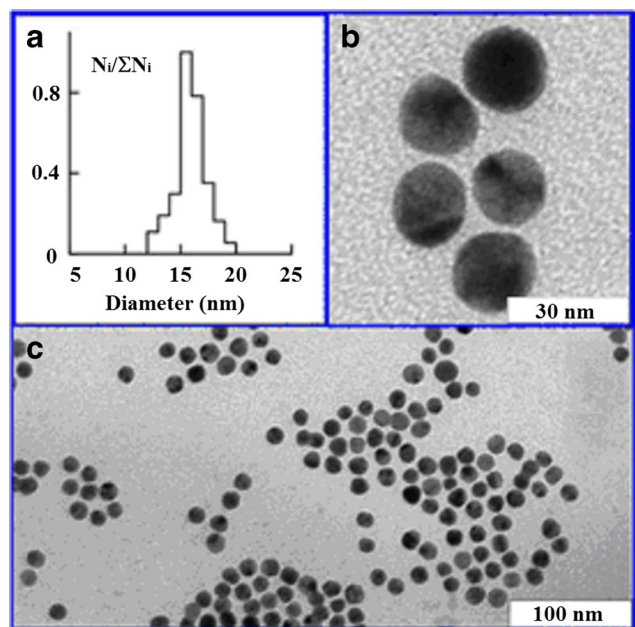
Fig. 2 The number size distribution determined by DLS (**a**) and TEM images (**b, c**) of 15 nm AuNPs
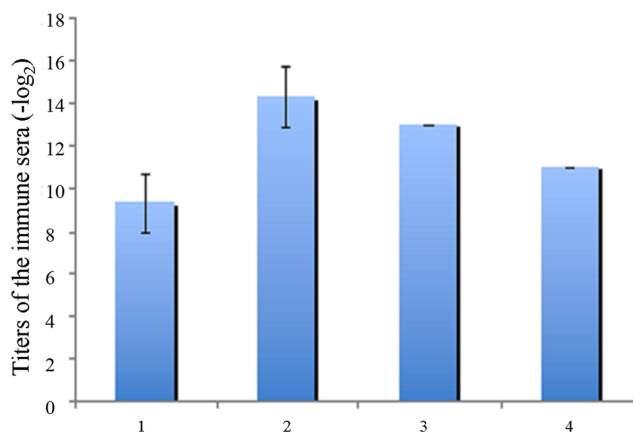
Fig. 3 Effect of the composition of the antigen constructions on the logarithm of the antibody titer: commercial FMD vaccine (*1*), peptide + AuNPs + CFA (*2*) ($p=0.00045$), peptide + AuNPs (*3*) ($p=0.0081$), peptide + CFA (*4*) ($p=0.038$); $F=57.2$ ($F_{crit}=4.12$), $p_{eff}=2\times10^{-5}$

Ten days after the twofold immunization, the titer of the antibodies formed in the blood sera of guinea pigs was determined by ELISA (Fig. 3). The titer of sera from the animals immunized with the AuNP–peptide conjugate plus CFA proved to be the highest (1/32,768). It was much higher, with statistical significance, than the titer found for the animals immunized with the commercial vaccine (1/512). In sera from the animals injected with physiological saline, no specific antibodies were detected. The titers of sera from the immunization with the AuNP–peptide conjugate proved to be significantly higher than those found with the native peptide plus CFA.

The immunization of mice with AuNP–FMDV conjugates was described by Chen et al. [26]. In their study, the mice were immunized every week for 9 weeks and throughout the immunization period; only increases in the antibody titers were determined. In this study, by contrast, immunization was conducted twice, and apart from the antibody titer, the production of cytokines and the biochemical parameters of blood serum were examined.

The results of the dot immunoassay of the peptide are shown in Fig. 4. As can be seen, the highest sensitivity was demonstrated by sera from the animals injected with the AuNP–peptide conjugate plus CFA (minimum detectable amount of peptide, ~120 pg). The sensitivity of sera from the immunization with the AuNP–peptide conjugate was ~31 ng, that from the immunization with the peptide solution plus CFA was ~62 ng, and that from the immunization with the commercial FMD vaccine was 125 ng.

Cytokine production in the sera of immunized animals

In the process of immunization, we determined the level of production of the proinflammatory cytokines IL-1β, IL-6, and IFN-γ in sera from the immunized animals (Figs. 5, 6, and 7). It was found that the animals immunized with the AuNP–
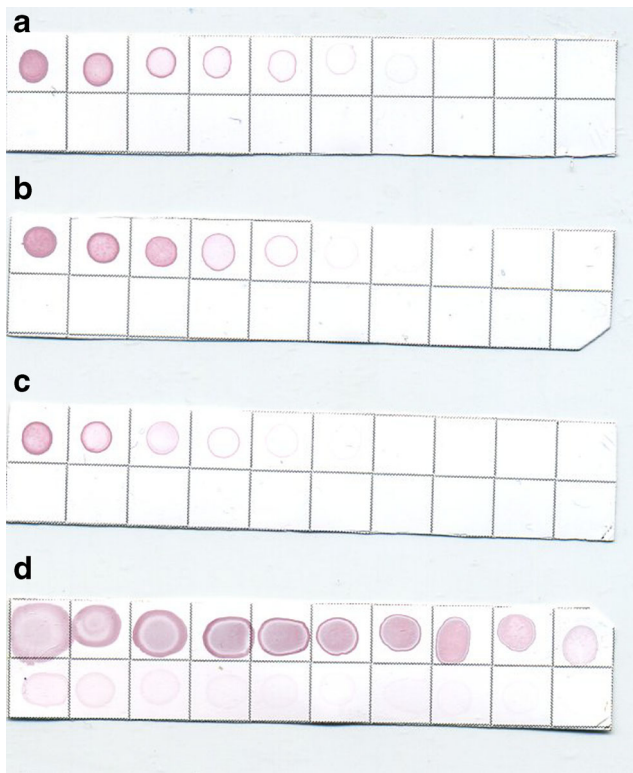
Fig. 4 Dot immunoassay of the peptide (double dilutions; initial concentration, 1 mg ml^{-1}; initial amount, 1 µg) by using sera from immunizations with **a** AuNP–peptide conjugate, **b** peptide solution + CFA, **c** commercial FMD vaccine, and **d** AuNP–peptide conjugate + CFA. The specific antibodies bound to the antigen were detected with AuNPs conjugated to staphylococcal protein A

peptide conjugate plus CFA produced the largest amounts of the proinflammatory cytokines. The highest increase in production, as compared to the effect obtained from the use of the commercial FMD vaccine (+62 %, $p=0.0028$), was observed for IFN-γ. This finding may indicate that AuNPs conjugated to the viral antigen are able to induce T lymphocytes and NK
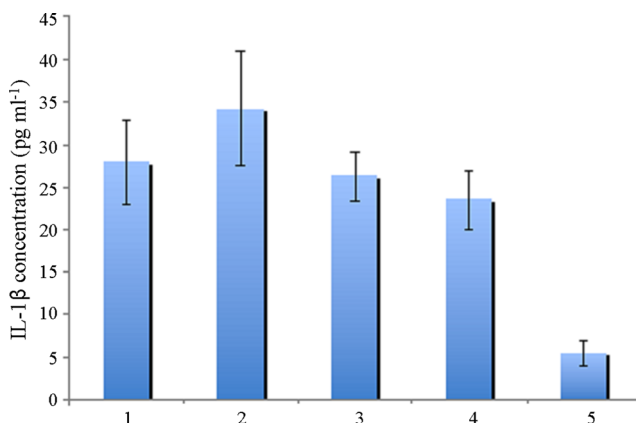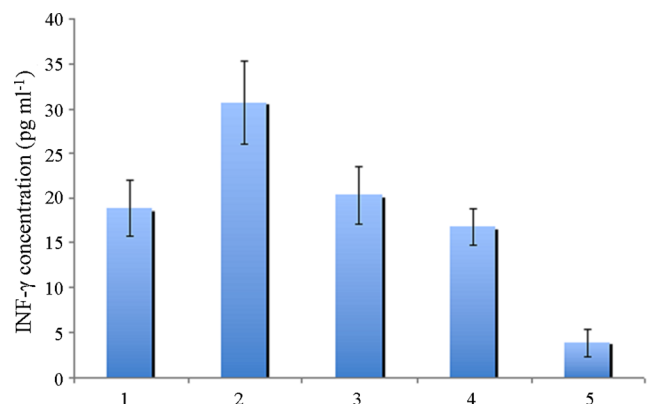

Fig. 5 Effect of the composition of the antigen constructions on the concentration of IL-1β in sera from immunizations with (1) commercial FMD vaccine, (2) peptide + AuNPs + CFA ($p=0.031$), (3) peptide + AuNPs ($p=0.31$), and (4) peptide + CFA ($p=0.034$). Control (5) ($p=0.0028$); $F=117$ ($F_{crit}=3.48$), $p_{eff}=2\times10^{-8}$
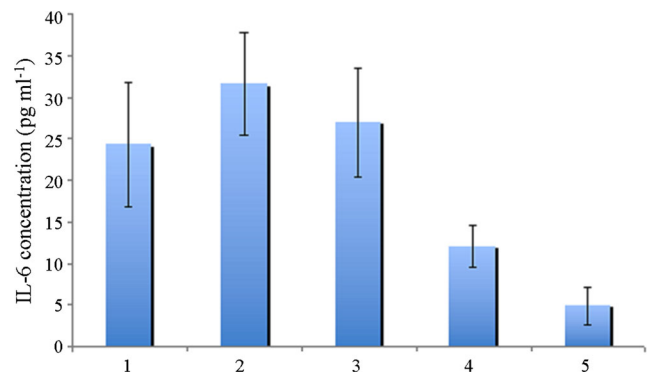
Fig. 6 Effect of the composition of the antigen constructions on the concentration of IL-6 in sera from immunizations with (1) commercial FMD vaccine, (2) peptide + AuNPs + CFA ($p=0.033$), (3) peptide + AuNPs ($p=0.32$), and (4) peptide + CFA ($p=0.022$). Control (5) ($p=0.0088$); $F=76.3$ ($F_{crit}=3.48$), $p_{eff}=2\times10^{-7}$

cells to elaborate IFN-γ and trigger a cascade of reactions directed to the immunological regulation of the antiviral immune response. A similar response of the immune system to AuNPs conjugated to a viral antigen was demonstrated earlier [47, 48]. IFN-γ, which is a major mediator of inflammation in viral infections, inhibits virus replication in cells, enhances the specific immune response by promoting an increased expression of major histocompatibility complex II, and activates macrophages and NK cells. It is, therefore, probable that AuNPs conjugated to a viral antigen promote a coordinated antiviral immune response.

It should also be noted that all the immunized animals exhibited increased cytokinetic activity. Thus, a significant increase in the concentration of interleukins 1β and 6 relative to the control group (1.7 M NaCl solution, $p<0.05$) was observed in all animals that had received the peptide in combination with various immunogens. However, the largest such increase was observed in the animals injected with the AuNP–peptide conjugate plus CFA and the smallest in those injected with the native peptide plus CFA. Because IL-6 is a factor of B

Fig. 7 Effect of the composition of the antigen constructions on the concentration of INF-γ in sera from immunizations with (1) commercial FMD vaccine, (2) peptide + AuNPs + CFA ($p=0.0028$), (3) peptide + AuNPs ($p=0.22$), and (4) peptide + CFA ($p=0.092$). Control (5) ($p=0.00032$); $F=180$ ($F_{crit}=3.48$), $p_{eff}=3\times10^{-9}$

cell differentiation and promotes the maturation of B lymphocytes into antibody-producing cells, the increase in its concentration may indicate that vaccination with the AuNP–peptide conjugate activated the polyclonal production of Ig.
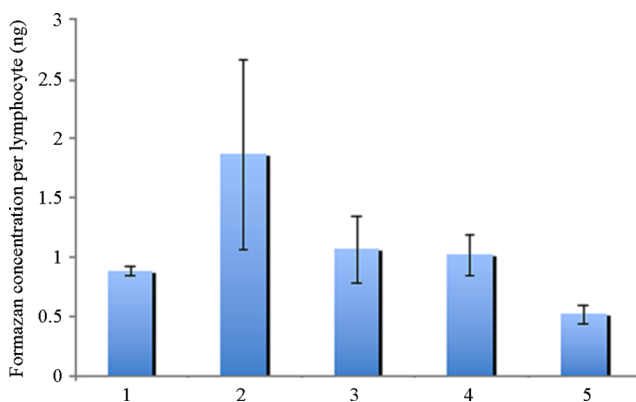
Respiratory activity of splenic lymphocytes

Compared with the control (1.7 M NaCl solution), the respiratory activity of splenic lymphocytes increased 3.7-fold ($p=0.018$) in the animals immunized with the AuNP–peptide conjugate plus CFA, 2.1-fold ($p=0.015$) in those immunized with AuNP–peptide conjugate, 2-fold ($p=0.0016$) in those immunized with the native antigen plus CFA, and 1.7-fold ($p=0.00037$) in those immunized with the commercial vaccine (Fig. 8). This fact may indicate that the antigen constructions being injected create a "depot" at the point of injection, thereby facilitating the extension of the effector phase of the immune response to the administered antigen.
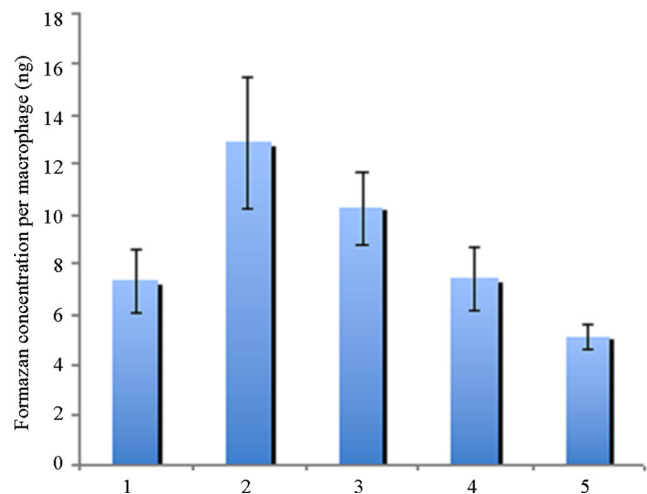
Along with this, the respiratory activity of peritoneal macrophages augmented 2.5-fold ($p=0.0064$) in the animals immunized with the AuNP–peptide conjugate plus CFA, 2-fold ($p=0.0045$) in those immunized with the AuNP–peptide conjugate, 1.5-fold ($p=0.005$) in those immunized with the native antigen plus CFA, and 1.4-fold ($p=0.0059$) in those immunized with the commercial vaccine (Fig. 9). This is consistent with the previous findings that AuNPs in complex with high- and low-molecular-weight antigens stimulate the respiratory activity of peritoneal macrophages [48].

General remarks

Thus, for all the experiments depicted in Figs. 3, 5, 6, 7, 8, and 9, comparison of the effect of the AuNP–peptide conjugate plus CFA and that of the commercial FMD vaccine shows that

Fig. 8 Effect of the composition of the antigen constructions on the respiratory activity of splenic lymphocytes: (*1*) commercial FMD vaccine, (*2*) peptide + AuNPs + CFA ($p=0.034$), (*3*) peptide + AuNPs ($p=0.11$), and (*4*) peptide + CFA ($p=0.084$). Control (*5*) ($p=0.00037$); $F=30$ ($F_{crit}=3.48$), $p_{eff}=1.5\times10^{-5}$

Fig. 9 Effect of the composition of the antigen constructions on the respiratory activity of peritoneal macrophages: (*1*) commercial FMD vaccine, (*2*) peptide + AuNPs + CFA ($p=0.0039$), (*3*) peptide + AuNPs ($p=0.0027$), and (*4*) peptide + CFA ($p=0.82$). Control (*5*) ($p=0.0059$); $F=67.8$ ($F_{crit}=3.48$), $p_{eff}=3\times10^{-7}$

the corresponding parameters increased from +23 to +110 % with statistical significance ($p<0.05$). In all the experiments, the use of the AuNP–peptide conjugate without CFA resulted in an effect comparable to (Figs. 3, 5, 6, 7, 8, and 9) or significantly greater than (by about 40 %; Figs. 3 and 9) that of the commercial FMD vaccine.

The ANOVA results (the values of F, F_{crit}, and p_{eff} given in the legends to Figs. 3, 5, 6, 7, 8, and 9) also attest that the observed relationship between the immunological parameters and the composition of the antigen constructions had high statistical significance.

The data available on the safety of AuNPs in vivo (especially when these are administered subcutaneously) are few and conflicting. Nevertheless, it can be considered proven that a rapid decrease in the concentration of nanoparticles in blood and their long-term retaining in the animal body is related to the functioning of the hepatobiliar system. As the excretion of accumulated particles from the liver and spleen can take up to 3 to 4 months, the question as to the injected doses and possible inflammation processes is still of great importance. At present, one can only assume that if AuNPs are administered for a brief time (about a week) at a daily dose not exceeding 0.5 mg kg^{-1}, no appreciable toxicity results [49]. In our experiments, the injected dose and the period of administration were inherently smaller than those.

In a biochemical study of guinea pig blood serum, we found no significant differences between the corresponding parameters for the antigen constructions used, as compared with the effect of the commercial FMD vaccine and with the control. This result indirectly indicates that the administered Au-containing antigen constructions were not toxic to the basic physiological functions of the animals.

Conclusions

The data generated by this study characterize the effect of AuNPs on the immune response of the guinea pigs immunized with a synthetic peptide of the VP_1 capsid protein of the FMDV, as compared to the responses obtained from the animals immunized with a commercial FMD vaccine and with the native peptide. It has been established that the AuNP–peptide conjugate mixed with CFA induced a much more pronounced immune response than did the other antigen constructions. This was manifested by a significantly higher titer of antibodies produced and by their higher sensitivity shown in an immunoassay of the antigen. Antibody biosynthesis was accompanied by considerably increased production of proinflammatory cytokines (especially IFN-γ) and by stimulation of the respiratory activity of peritoneal macrophages. That there were almost no significant differences between the effects of the Au-containing antigen constructions and the commercial FMD vaccine on one hand and the results of the control experiments in the biochemical study of blood serum on the other indirectly indicates that the administered antigen constructions were not toxic to the basic physiological functions of the guinea pigs.

In all the experiments, the use of the AuNP–peptide conjugate without CFA resulted in an effect comparable to or significantly greater than that of the commercial FMD vaccine. This finding suggests that AuNPs might serve as a single adjuvant in the future work on designing peptide vaccines. In addition, we believe that the use of a synthetic peptide in vaccination will cause no residual infectivity.

In summary, our present results create a basis for further research on the resistance of vaccinated animals to FMD, with a view to developing a single-use nanovaccine against FMD.

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