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Cytotoxic and Immunomodulating Properties of Gold Nanoparticles

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Abstract

Results of the investigation of cytotoxic and immunomodulating properties of gold nanoparticles of different sizes (12, 16, 22 and 35 nm) on primary and transformed cells *in vitro* are presented. Their localization and distribution in cells are studied. It is established that gold nanoparticles are not toxic within concentration range 0.06–6 μg/mL for all the cell cultures used, actively penetrate into the cell and get accumulated inside the vacuoles of different sizes, selectively activate the anti-inflammatory immune response depending on nanoparticle size and cell type.

Key words: gold nanoparticles, toxicity, immunomodulating properties, primary and transformed cell cultures

INTRODUCTION

The possibility to use chemically stable biocompatible gold nanoparticles in biological systems is under intense investigation at present. It is proposed to use golden nanoparticles both in the form of single-component preparations and in multicomponent systems, as inert carriers for various biomolecules (DNA, peptides, antibodies, tissue-specific markers). Due to the surface plasmon resonance (SPR), phenomenon, gold particles are very interesting for photodynamic therapy, while the particles with ligands bound on the surface are interesting for diagnostics [1], as transport systems to deliver therapeutic and cosmetic preparations [2, 3], inducers of immune response [4] *etc*. A necessary stage for the use of nanomaterials in medical practice is preliminary investigation of their safety/toxicity and inertness/proinflammatory action in biological systems *in vivo* and *in vitro*.

In the present work we study cytotoxic and immunomodulating properties of gold particles of different diameters with respect to normal and transformed human cells, as well as the localization and distribution of these particles in a cell.

MATERIALS AND METHODS

Synthesis of gold nanoparticles

Gold particles were obtained according to Frens method [5], that is, through citrate re-

duction of aurohydrochloric acid (AHCA). The 1 % aqueous solution of sodium citrate (Cit) was added to the boiling 0.01 % aqueous solution of AHCA using the molar Cit/Au ratios equal to 4/1, 3/1, 2.3/1, 1.5/1, to obtain the particles with the average diameter of 12 (Au-1), 16 (Au-2), 22 (Au-3) and 35 nm (Au-4), respectively.

Method of gold nanoparticle examination

The concentration of gold in the solutions was determined by means of atomic emission spectroscopy with inductively coupled plasma using a Perkin–Elmer Optima 4300 DV ICP spectrometer.

The size characteristics of gold were studied by means of transmission electron microscopy (TEM), atomic force microscopy (AFM) and UV-Vis spectroscopy. The TEM studies of preparations deposited on the grids with formvar supports stabilized with carbon were carried out with the help of a JEOL JEM 1400 electron microscope (Japan). For AFM, a drop of the preparation 20 μL in volume was deposited onto a fresh chip of mica with the area of 25–30 mm^2, adsorbed for 1 h at room temperature and studied in the semi-contact mode in the atomic force microscope SolverP47Bio manufactured by NT-MDT (Zelenograd city, Russia). The electron transmission spectra of solutions were recorded with a Shimadzu UV-2501PC spectrophotometer. The spectra were recorded with the compensation of absorption with respect to water within wavenumber range 11 000–54 000 cm^{-1} in a quartz cell with the optical path of 10 mm.

Cell cultures

The HeLa and Mcf-7 cells were obtained from the American Tissue Cell Collection (ATCC, USA). Primary endothelial cells and fibroblasts were obtained from umbilical vein and gingival tissue of humans according to the protocols described previously [6, 7]. All cells were cultivated in the IMDM medium (Gibco, USA, 42200-014) in the presence of 10 % fetal bovine serum (FBS) (Gibco, USA, 10106) in the atmosphere of 5 % CO_2 at 37 °C. To dissociate primary fibroblasts, HeLa and Mcf-7 cells, we used a solution of 0.05 % trypsin in a phosphate-buffered saline (PBS) (Sigma, USA, T-4799). To dissociate primary endotheliocytes, we used a 0.01 % solution of collagenase (Invitrogen, USA, 17104-019). Primary endotheliocytes were cultivated on dished coated preliminarily with a 0.5 % solution of gelatine (Sigma, USA, G-2500).

All the components in contact with the cells were tested for the presence of an admixture of bacterial endotoxin with the help of LAL test (Associate of Cape Cod Inc., USA).

Investigation of the cytotoxic action of nanocomposites

Primary fibroblasts and endotheliocytes were reseeded with a density of 7000, HeLa and Mcf-7 – with a density of 15 000 cells per one well of a 48-well plate. The cells were cultivated for 16 h, and then washed once with IMDM. Then 200 μL (for a 48-well plate) of colloid (in the concentration of 6, 0.6 and 0.06 μg/mL) in IMDM with 10 % FBS was added, the mixture was incubated for 24 h, and the number of vital cells was estimated using the EZ4U commercial kit (Biomedica, Germany).

Investigation of localization and distribution of gold nanoparticles in a cell by means of TEM

The cells were grown on plastic substrates (Agar Scientific, Great Britain) in a 24-well plate, washed twice with the cultural medium and incubated with gold nanoparticles (6 μg/mL) for 24 h. After incubation with the colloid, the cells were washed twice with the cultural medium, fixed for 1 h in 2.5 % solution of glutaraldehyde in PBS and then for 1 h in 1 % OsO_4 in PBS. Then the samples were washed with the buffer solution to remove the fixing agent, dehydrated in alcohols and acetone, enclosed in epon-812 and polymerized in thermostat. Ultrathin (50–80 nm) sections of cells were made from the resulting blocks according to the protocol described previously [8]. Cell sections were studied with the help of a JEOL 100 SX electron microscope (Japan).

Investigation of the secretion of interleukins IL-6 and IL-8 by cells

To study immunomodulating properties of nanocomposites, fibroblasts and endotheliocytes

were reseeded with the density of 7000 cells per one well of a 48-well plate; the cells were cultivated for 16 h, then washed once using IMDM. Then the cells were incubated in the cultural medium IMDM with 10 % ECS with gold colloids (in the concentration of 6, 1.2 and 0.24 μg/mL) for 24 h. The cultural medium was sampled, centrifuged for 10 min at 1500*g* and stored at −20 °C. For positive reference, the cells were incubated in the presence of the analog of double-stranded RNA polyinosinic/polycytidylic acid (pIC) (Sigma, USA, P9582) in the concentration of 0.1mg/mL under the same conditions. To determine the basic level of cytokine secretion, the cells were incubated in the cultural medium IMDM with 10 % FBS. Before determining cytokine concentrations, the samples were refrozen at room temperature and centrifuged for 5 min at 3000*g*. The concentrations of interleukins 6 and 8 (IL-6 and IL-8) in supernatants were determined with the help of commercial kits IL-6-IFA-BEST and IL-8-IFA-BEST (Vektor-Best, Russia) according to the procedure recommended by the manufacturer.

RESULTS AND DISCUSSION

Characterization of gold colloids

It was shown previously [9, 10] that the smallest gold nanoparticles (2−7 nm) possess substantial pronounced cytotoxic and immunostimulating action, while larger particles (10−15 nm and larger) are not toxic for cells. The size of nanoparticles may define their biological effects, likely because of different efficiencies of transport and the features of interaction with cell biopolymers. In the present work we studied colloid solutions containing gold nanoparticles 12 to 40 nm in size, obtained using Frens method. This method is most frequently used to prepare colloids for subsequent medical biological examination and allows obtaining gold particles within the size range 8 to 70 nm. The characteristics of resulting gold colloids are shown in Table 1.

The size of nanoparticles was studied by means of UV-Vis spectroscopy, TEM and AFM. The UV-Vis spectra of the preparations of gold nanoparticles (Fig. 1) contain absorption bands

TABLE 1

Characteristics of the studied gold nanoparticles (concentration of gold nanoparticles: 60 μg/mL)

Particles	Number of nanoparticles, 10^{12} np/mL	Size, nm
Au-1	3.40	12±1.8
Au-2	1.40	16±2.3
Au-3	0.56	22±3.2
Au-4	0.14	35±60

in the region of 518 nm for Au-1, 519 nm for Au-2, 524 nm for Au-3 and 528 nm for Au-4. The authors of [11] report a calibration curve to determine the size of gold nanoparticles relying on wavelength λ_{max} on the basis of the best smoothing polynomial approximation of spectrophotometric and electron microscopic data; the size of particles in the preparations Au-1, Au-2, Au-3 and Au-4 was 12, 15, 25 and 35 nm, respectively.

The TEM micrographs of the prepared experimental samples are shown in Fig. 2. According to these data, the preparations contain mainly spherical nanoparticles within a narrow size range: (12±1.8) nm for Au-1, (16±2.3) nm for Au-2, (22±3.2) nm for Au-3 and (35±6) nm for Au-4. The data of AFM studies for Au-1 and Au-4 samples (Fig. 3) confirm the conclusion concerning good uniformity of particle size for the preparations and correlate with the results obtained by means of TEM and UV-Vis spectroscopy on particle size.

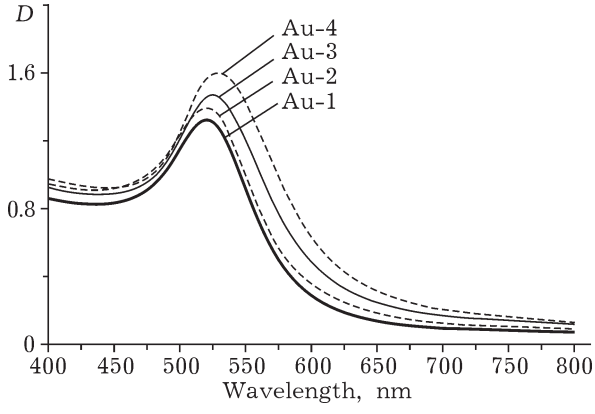

Fig. 1. UV-Vis spectra of colloid gold preparations Au-1−Au-4.

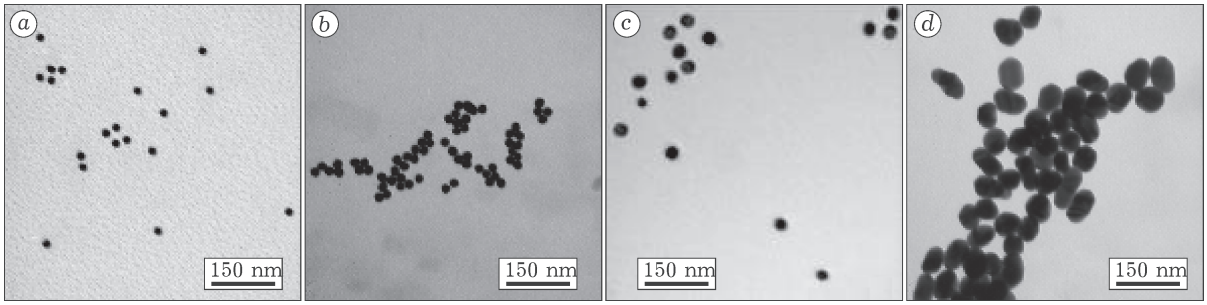

Fig. 2. TEM micrographs of colloid gold preparations Au-1 (*a*), Au-2 (*b*), Au-3 (*c*), Au-4 (*d*).

Integrating the data obtained using different analysis methods, we may state that colloids contain the particles having the diameters (12±1.8), (16±2.3), (22±3.2) and (35±6) nm.

Directly before experiments *in vitro*, the preparations of colloid gold were purified by dialysis against apyrogenic water at a temperature of 4 °C for 24 h. After dialysis, darkening of initially purple colour of colloids was observed. It is known that the surface of gold particles Au(I) in initial colloid solutions obtained through citrate reduction is doped with citrate ions in the co-ion layer [12]. Dialysis of preparations leads to the removal of excess citrate and to partial loss of charge on the surface of gold particles, which, in turn, promotes aggregation. The observed darkening of sol colour is due to the start of nanoparticle aggregation. This is confirmed by the shift of absorption bands to longer wavelengths in UV-Vis spectra observed by us. At the same time, no substantial changes of particle size after dialysis in comparison with the initial data were recorded by means of TEM and AFM.

Investigation of the cytotoxic properties of gold colloids

To study the effect of gold nanoparticles on normal human cells, we chose we chose the following model systems: primary endotheliocytes from the umbilical vein of newborn babies (HUVEC), primary fibroblasts from human gingival tissue, the cells of cervical adenocarcinoma HeLa and the cells of mammary gland adenocarcinoma Mcf-7. Endothelium of vessel walls is the major barrier for a preparation to overcome in order to pass from blood to target tissues for therapeutic effect. In addition, endotheliocytes are the cells characterized by the high activity of cell transport and by participation in the formation of immune response [13]. Connective tissue is incorporated in all parenchymatous organs; fibroblasts forming this tissue comprise one of the most widely represented cell types in human organism. In spite of the presence of specialized functions determined by the localization of connective tissue in the organism, fibroblasts are similar both in

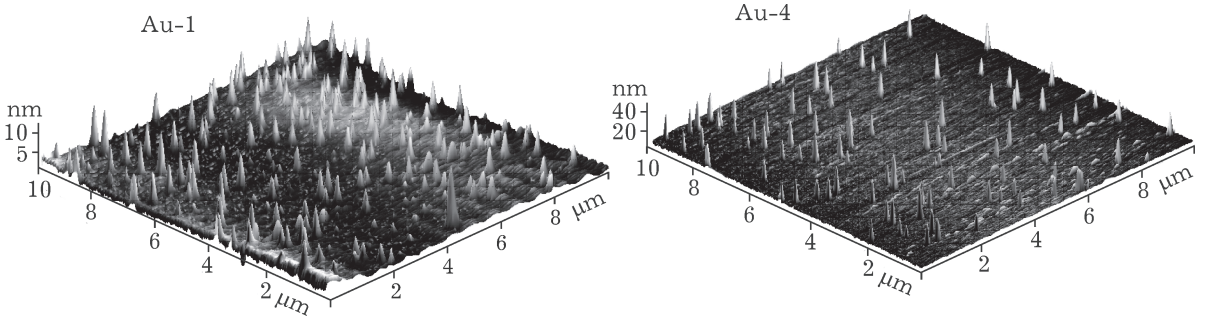

Fig. 3. AFM images (3D) of colloid gold preparations Au-1 and Au-4.

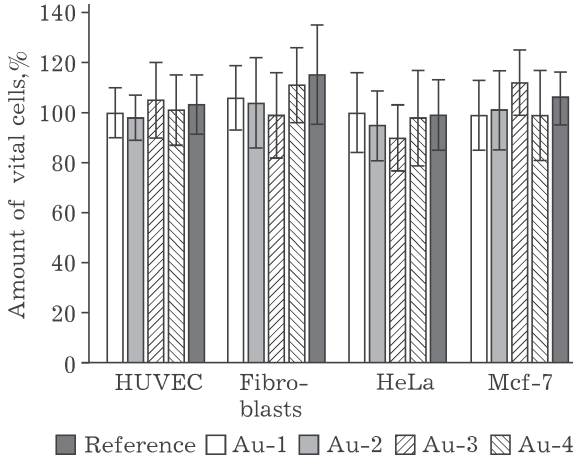

Fig. 4. Cytotoxic action of gold nanoparticles. Dependence of the amount of vital cells on the concentration of nanocomposite.

morphology and in functions; they are potential candidates for interaction with medical preparations [14]. At present, approaches to cancer diagnostics and therapy relying on the use of gold nanoparticles are under development [1]. Because of this, we chose the cell lines of cervical and mammary gland adenocarcinoma (HeLa and Mcf-7, respectively) as the models of transformed cells.

The quantitative analysis of cytotoxic /proliferative action of gold nanoparticles with the help of the E Z4U test showed that gold nanoparticles within the studied concentration range (0.06–6 μg/mL) are not toxic for all kinds of cells used in the work (Fig. 4), which corresponds to the literature data [10, 15]. Due to the absence of cytotoxic effect, the data are presented for the maximal concentration of nanoparticles (6 μg/mL) (see Fig. 4).

Investigation of localization and distribution of gold nanoparticles in cells

The distribution and localization of gold nanoparticles in cells was studied for primary (HUVEC) and transformed (HeLa) cells by means of TEM. The results are shown in Figs. 5–7. One can see (see Fig. 5) that there are long membranes of rough endoplasmic reticulum (EPR), numerous Golgi complexes and large autophagosomes in endothelial cells. The cytoplasm of HeLa cells forms numerous microvillus's, contains weakly developed ERPR and Golgi complexes, as well as lysosomes and autophagosomes of small size. So, endothelial cells and adenocarcinoma cells are similar to

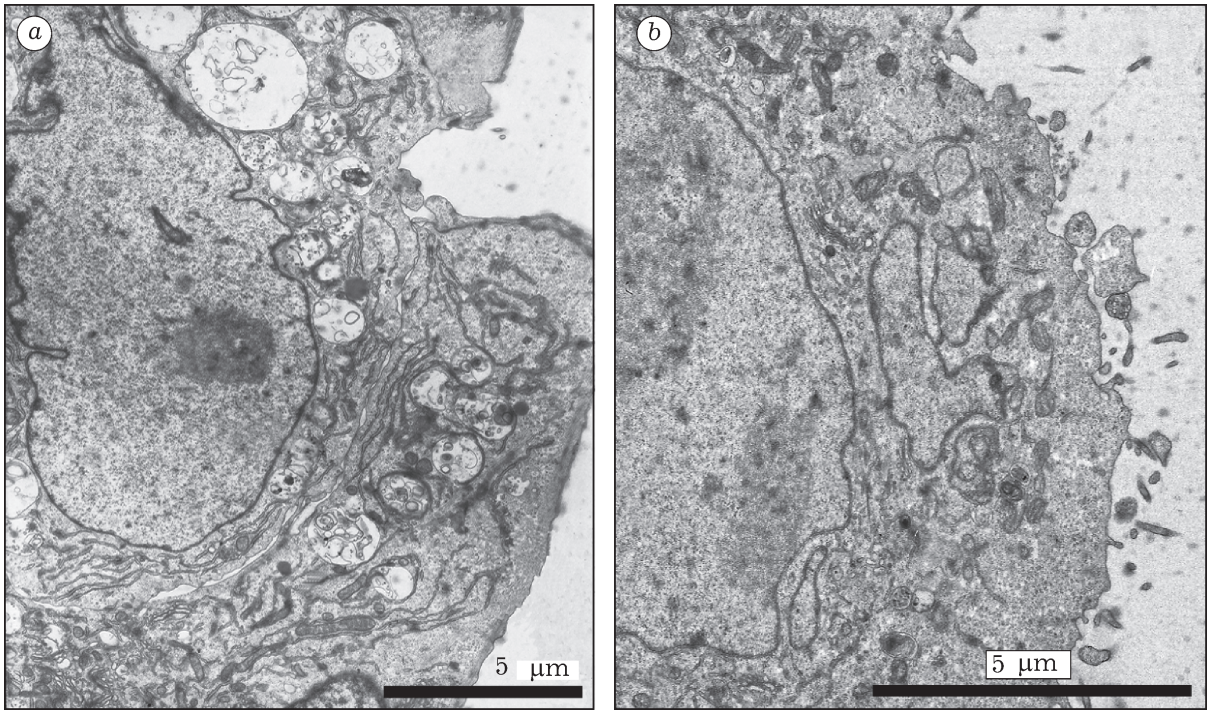

Fig. 5. Ultrastructural arrangement of endothelial cells of HUVEC line (*a*) and transformed cells of HeLa line (*b*).

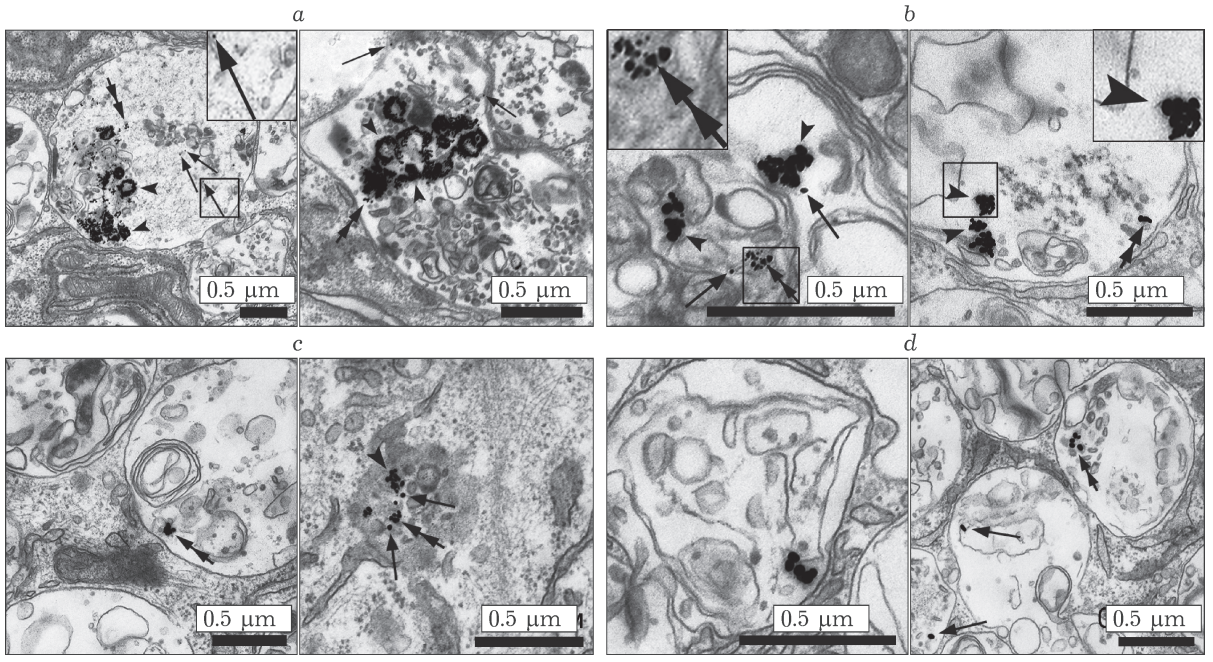

Fig. 6. Transmission electron microscopy of ultrathin sections of the cells of HUVEC line after incubation with gold nanoparticles Au-1 (*a*), Au-2 (*b*), Au-3 (*c*), Au-4 (*d*). The sites of gold particle localization are marked with arrows: black arrows – sole particles, double arrows – groups of 2 to 6 particles, heads of arrows – aggregates of tightly stuck particles.

each other in the density of cytoplasm, though the former contain better developed EPR and larger amount of autophagous vacuoles of different sizes in comparison with transformed cells (see Fig. 5). This is the evidence of active synthesis and exchange processes of endothelial cells. It should also be noted that HeLa cells form numerous villuses; villuses are almost completely absent from the surface of endothelial cells.

According to the data of electron microscopic studies, the size of gold particles revealed in cells corresponds to their size in initial colloids. One can see (see Fig. 6) that gold particles (independently of their size) are revealed in endotheliocytes exclusively inside autophagous vacuoles containing the residual fragments of destroyed membrane structures. Sole particles, groups of two-six particles and aggregates of tightly stuck particles occur (see Fig. 6). The largest amount of gold particles is observed in autophagosomes in the case of the particles 12 nm in diameter (see Fig. 6, *a*), while the minimal one corresponds to the particles 35 nm in diameter (see Fig. 6, *d*).

A smaller amount of gold nanoparticles is observed in the HeLa cells (see Fig. 7) in comparison with primary endotheliocytes (see Fig. 6). Sole and dual gold particles are located inside small autophagous vacuoles and multivesicular bodies after cell incubation with 12 nm Au-1 (see Fig. 7, panel *a*). After cell incubation with 22 nm Au-3 (see Fig. 7, panel *b*) small conglomerations are observed on the surface of microvillus's and inside multivesicular bodies, while large conglomerations occur within phagosomes and near cell surface.

So, in all experiments, gold particles are not revealed in nuclei but are localized in cell cytoplasm (see Figs. 6, 7) inside the vacuoles of different sizes containing large amounts of small smooth-wall vesicles (multivesicular bodies) or residual fragments of destroyed membrane structures (likely autophagosomes). The distribution of the particles of different sizes in primary endotheliocytes (HUVEC) and in transformed (HeLa) cells is similar. The particles of the smallest diameter occur as sole particles, as well as in small (two-six particles) and large conglomerates (up to 50–100 particles inside one vacuole). The number of particles per cell decreases with an increase in particle size. The particles of large diameter (30–40 nm) occur more rarely, either in groups (less than 10) or sole. It is interesting that the conglomerations

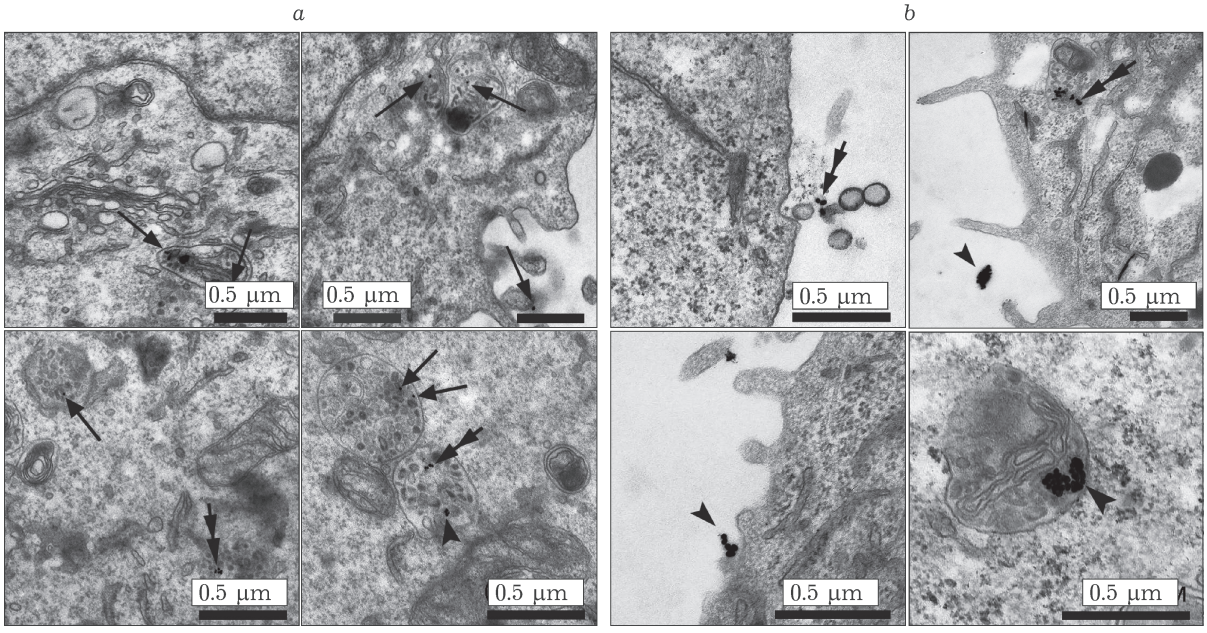

Fig. 7. Transmission electron microscopy of ultrathin sections of HeLa line cells after incubation with gold nanoparticles Au-1 (12 nm) (*a*) and Au-3 (22 nm) (*b*). For designations, see Fig. 6.

of particles in transformed cells (see Fig. 7) are observed in contact with the membrane of numerous microvilli and outside the cells. It may be assumed that these particles are at different stages of endo- or exocytosis.

The data obtained by us coincide in general with the data of other studies. The authors of [10] established that gold particles 4 to 30 nm in size get into the cells rather quickly, and after 3 h they are detected already in the cytosol. It was demonstrated by means of TEM that gold particles are localized in the cells in vesicular structures; the particles are arranged inside these structures in the form of clusters and do not get into the cell nuclei [10]. It is known that the lysosomal system responds to the presence of foreign components in cells by inducing the formation of lysosomes, adaptive synthesis of enzymes, enhancement of autophagocytosis, directed exocytosis and apoptosis. Thus arranging the mechanisms of protection from the action of xenobiotics at the cell level. We observe such activation when gold particles get into cells, so that the main pool of particles is accumulated in autophagosomes. Vesicular localization of gold nanoparticles in the endothelial cells was demonstrated in [15]: spherical nanoparticles 15 nm in diameter are localized in vesicules in the groups of 10 to 100 pieces in the amount of about 2000 pieces per cell. The penetration of other type of particles (silicon) into endothelial cells through endocytosis and accumulation in lysosomes in the form of aggregates were also demonstrated in [16]. During incubation of endotheliocytes with other (quartz) nanoparticles varying in size from 10 to 400 nm they were accumulated not only inside vesicules and vacuoles but also in cytosol [16], which may be the evidence of different transport mechanisms for the particles differing from each other in composition. Higher toxicity of the particles 10 nm in size in comparison with the toxicity of larger particles was established. A possible reason may be faster penetration and accumulation in cells.

The surface of gold nanoparticles may be represented by a monomolecular layer of bifunctional reagents with amino or carboxylic groups exposed in solution, which results in positively or negatively charged particles (Z potential from +30 to −36 mV). It was demonstrated with the tumour cylindroid model that negatively charged particles penetrate into cells more rapidly than positively charged ones [17].

It was established in [18] that for negatively charged gold particles the efficiency of binding with serum proteins and transport into cells depends on particle size, the particles are transported due to receptor-mediated endocytosis and can interact with intracellular immune mediators during transporting process. Comparative analysis of the penetration of gold particles with different size into COS-1 cells showed that after cell incubation with small (2–4 nm) nanoparticles the latter are detected not only in cytoplasm but also in nucleus. In the case when medium-sized (5.5–8.2 nm) particles are applied, they are detected in cytoplasm and in perinuclear space, while the particles with a size of 16 nm are localized in the form of aggregates exclusively in cytoplasm [19]. Similar results were obtained with cancer cells [20]. This is the evidence that the transport of gold nanoparticles into the nucleus differs from the transport into cytosol compartment and is limited by particle size, that is, larger particles are not transported through the pores of the nuclear membrane.

The data of electron microscopy provide evidence that the sites where sole and aggregated gold particles are localized in endothelial and transformed cells are identical, and the accumulation of particles depending on their size is similar in primary and transformed cells. At the same time, it was established that the colloid of identical size is accumulated in these cells with different intensity. It may be assumed that this is due both to differences in the functional activity of cells and to the features of specific response of their transporting systems to the penetration of gold particles into the cytoplasm.

Investigation of immunomodulating properties of gold colloids

According to the data obtained in a number of studies, gold nanoparticles are biologically inert, nontoxic and suitable for *in vivo* application [2]. It was demonstrated previously [10] that mouse macrophages of J774 A1 line are stimulated under the action of definite concentrations of gold colloids. However, the fact that the authors of [10] did not evaluate the presence of bacterial lipopolysaccharide in colloid preparations does not allow stating reliably that gold particles possess immunostimulating ability. In addition, the effect of gold colloids on primary cells has not been investigated yet. We studied the effect of gold nanoparticles on human primary cells – endotheliocytes of umbilical vein and fibroblasts from gingival tissue. It was demonstrated in recent studies that along with playing the major parts (barrier and structural ones) these cells take active part in regulating inherent immune response. Fibroblasts and endotheliocytes express the receptors of interleukins and pattern-recognizing receptors (PRRs), provide response to the components of bacterial and viruses and to interleukins by producing proinflammatory mediators, such as IL-6, IL-8, IL-1b, IFN of the first type [21]. The content of bacterial lipopolysaccharide (LPS) in the preparations of gold nanoparticles, determined with the help of LAL test,

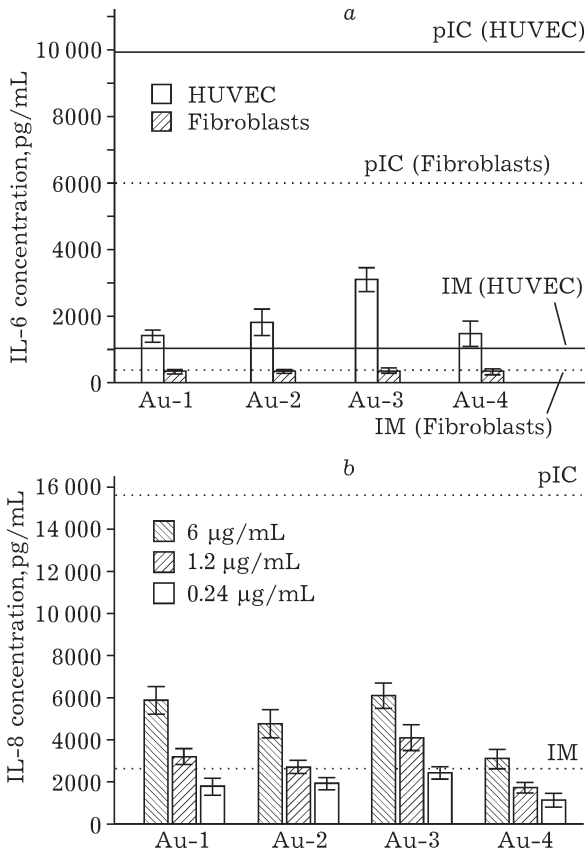

Fig. 8. Induction of proinflammatory response in primary cells under the action of gold nanoparticles: *a* – IL-6 concentration in endotheliocytes and fibroblasts, *b* – IL-8 concentration in endotheliocytes; horizontal dash and continuous lines relate to interleukin concentrations in the medium with intact cells (IM) and after their stimulation with pIC (1 mg/mL); concentration of gold nanoparticles (μg/mL): 6 (*a*), 6, 1.2 and 0.24 (*b*).

did not exceed two units of endotoxin (EU) per 1 mg of gold nanoparticles, which corresponds to 0.0125 EU/mL for the working concentration of gold nanoparticles equal to 6 μg/mL. Bacterial LPS in such low concentrations does not induce immune response in primary endotheliocytes and fibroblasts [22]. The results of the effect of nanomaterials on secretion of proinflammatory cytokines by primary cells are presented in Fig. 8.

One can see that nanoparticles did not induce any change of the concentration of proinflammatory cytokines IL-6 (see Fig. 8, *a*) and IL-8 (the data are not presented) in the culture of primary fibroblasts. Incubation of endotheliocytes in the presence of gold nanoparticles induced secretion of both IL-6 and IL-8 by these cells. The maximal increase (by a factor of 3 for IL-6 and 2.3 for IL-8 in comparison with the basic level) was observed for the case of Au-3 nanopreparation with particle size 22 nm. In endotheliocytes, a dependence of the intensity of interleukin secretion on the size and concentration of colloid particles is observed: larger particles in low concentration inhibit normal secretion of IL by resting endotheliocytes, while the particles 22 nm in diameter activate IL secretion in all the studied concentrations. The dependence of the induction of proinflammatory cell response on the size of gold nanoparticles was established in [10]: the authors of that work demonstrated that gene expression of proinflammatory cytokines IL-1, IL-6 and IFN of the 1st type increases with a decrease in the size of gold nanoparticles [10].

Differences in the activation of primary endotheliocytes and fibroblasts by gold nanoparticles require further studies. It is known that the immune response in the cells of different kinds is determined by expression and localization of PRRs. The majority of PRRs are intracellular (in the cytoplasm and endosomes); therefore, their activation is directly dependent on the transport of ligands into the cells [23, 24]. So, immunostimulating action of gold nanoparticles in primary endotheliocytes can be connected with the high activity of endocytosis (especially caveolin-dependent one), which is characteristic of this type of cells [11]. According to the data of electron microscopy, primary endotheliocytes contain a large number of vesicular structures inside which almost at gold particles are localized. The size of gold particles also has a substantial effect on their transport, intracellular distribution and immunomodulating properties. For instance, the particles 22 nm in diameter, unlike for larger particles localized as sole particles or in small groups, dominate in vesicular structures in the form of tightly stuck clusters of 20–40 pieces. It may be assumed that these aggregations interact with endosomal PRRs more actively because of larger surface, thus they define more pronounced immune response.

CONCLUSION

Investigations showed that these gold particles are nontoxic within the studied range of concentrations (0.06 to 6 μg/mL), actively penetrate in cells and selectively activate proinflammatory immune response (depending on particle size and cell type). It is necessary to take these data into account when developing nanostructured preparations for internal administration, based on gold colloids.

In addition, on the basis of our data and the data obtained by other authors [9, 25], it may be concluded that gold nanoparticles can serve not only as inert platform-type carriers for medical preparations but also as active reagents able (depending on size and possibly charge) to affect the functions of cell systems that are connected with the endosomal, exosomal and lysosomal systems.

REFERENCES

1 Lim Z. Z., Li J. E., Ng C. T., Yung L. Y., Bay B. H., *Acta Pharmacol. Sin.*, 32 (2011) 8983.
2 Vigderman L., Zubarev E. R., *Adv. Drug Deliv. Rev.*, 65, 5 (2013) 663.
3 Rippel R. A., Seifalian A. M., *J. Nanosci. Nanotechnol.*, 11, 5 (2011) 3740.
4 Bastús N. G., Sánchez-Tilly E., Pujals S., Farrera C., Kogan M. J., Giralt E., Celada A., Lloberas J., Puntes V., *Mol. Immunol.*, 46, 4 (2009) 743.
5 Frens G., *Nature*, 241 (1973) 20.
6 Jaffe E. A., Nachman R. L., Becker C. G., Minick C. R., *J. Clin. Invest.*, 52 (1973) 2745.
7 Takada H., Mihara J., Morisaki I., Hamada S., *Infection and Immunity*, 59, 1 (1991) 295.
8 Kruglova A. A., Matveeva N. M., Gridina M. M., Battulin N. R., Karpov A., Kiseleva E. V., Morozova K. N., Serov O. L., *Cell Tissue Res.*, 340, 3 (2010) 437.

9 Jiang W., Kim B. Y., Rutka J. T., and Chan W. C., *Nat. Nanotechnol.*, 3 (2008) 145.
10 Yen H.-J., Hsu S., and Tsai C.-L., *Small*, 5, 13 (2009) 1553.
11 Khlebtsov N. G., Bogatyrev V. A., Dykman L. A., Mel'nikov A. G., *Kolloid. Zh.*, 57, 3 (1995) 412.
12 Stendroff C. J., Herschbach D. R., *Langmuir*, 1 (1985) 131.
13 Simionescu M., Popov D., Sima A., *Cell Tissue Res.*, 335, 1 (2009) 27.
14 Aboussekhra A., *Int. J. Dev. Biol.*, 55, 7?9 (2011) 841.
15 Bartczak D., *Small*, 8, 1 (2012) 122.
16 Corbalan J. J., *Int. J. Nanomed.*, 6 (2011) 2821.
17 Kim B., Han G., Toley B. J., Kim C. K., Rotello V. M., Forbes N. S., *Nat. Nanotechnol.*, 5, 6 (2010) 465.
18 Chithrani B. D., Ghazani A. A., Chan W. C, *Nano Lett.*, 6, 4 (2006) 662.
19 Oh E., Delehanty J. B., Sapsford K. E., Susumu K., Goswami R., Blanco-Canosa J. B., Dawson P. E., Granek J., Shoff M., Zhang Q., Goering P. L., Huston A., Medintz I. L., *ACS Nano*, 5, 8 (2011) 6434.
20 Huang K., Ma H., Liu J., Huo S., Kumar A., Wei T., Zhang X., Jin S., Gan Y., Wang P. C., He S, Liang X. J., *ACS Nano*, 6, 5 (2012) 4483.
21 Uehara A., Takada H., *J. Dent. Res.*, 86 (2007) 249.
22 Darveau R. P., Pham T. T., Lemley K., Reife R. A., Bainbridge B. W., Coats S. R., *Infect. Immun.*, 72, 9 (2004) 5041.
23 Hornung V., Latz E., *Nat. Rev. Immunol.*, 10 (2010) 123.
24 Takeuchi O., Akira S., *Immunol. Rev.*, 227 (2009) 75.
25 Tsai C. Y., Lu S. L., Hu C. W., Yeh C. S., Lee G. B., Lei H. Y., *J. Immunol.*, 188, 1 (2012) 68.