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Cytotoxic and Immunomodulating Properties of Silver and Platinum Nanocomposites

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Abstract

Results of the investigation of cytotoxic and immunomodulating action of silver nanocomposites Ag15 (7.8 % Ag) and Ag13 (5 % Ag) with poly(1-vinyl-1,2,4-triazole) and platinum with arabinogalactan on primary and transformed human cells *in vitro* are presented. It is demonstrated that nanocomposites of metals with biopolymers possess more clearly pronounced cytotoxic effect in comparison with metal nanoparticles and do not induce inflammation in cell lines under investigation. It is established that silver nanocomposite Ag15 possesses selective cytotoxicity with respect to the cells of cervical adenocarcinoma HeLa.

Key words: silver and platinum nanocomposites, primary and transformed cells, cytotoxicity, proinflammatory action

INTRODUCTION

Metal nanoparticles and their nanocomposites are considered as promising pharmaceuticals of the new generation intended for therapy of cancerous tumours [1]. These preparations are to possess selective cytotoxic action on transformed cells, to cause no stimulating effect on vascularisation and metastasis, to have no proinflammatory action or, quite contrary, to enhance antitumour immunity. Metal-polymer nanocomposites for biomedical application exhibit a number of advantages over metal nanoparticles. The polymer acts as a nanostabilizing component, prevents aggregation of metal particles, increases the solubility of nanomaterials in water, allows correcting cytotoxic and proinflammatory properties of the metal core, prolongs biological action and simplifies bioavailability [2]. The introduction of nanomaterials into the production of medical preparations requires detailed investigation of the effect of nanoparticles not only on transformed cells but also on healthy cells and tissues of an organism. At the early stages of development of new nanomaterials, primary and transformed cell cultures serve as a convenient model to evaluate the effects caused by nanoparticles in normal and malignant cells *in vitro*.

The data on the synthesis and properties of new composites of silver nanoparticles with synthetic water-soluble polymer poly(1-vinyl-1,2,3-triazole) (PVT) and platinum with natural polymer arabinogalactan (AG) are described in

the present paper. Comparative studies of cytotoxic and immunomodulating properties of silver and platinum nanoparticles and nanocomposites with these polymers for normal and transformed human cells *in vitro* were carried out.

MATERIALS AND METHODS

Synthesis and characterization of silver and platinum nanoparticles

Colloidal silver was obtained through silver nitrate reduction by sodium borohydride according to the procedure described in [3]. The synthesized silver nanoparticles are stable and do not aggregate during storage and in the growth medium. The average size of metal particles, determined with the help of a Malvern Zetasizer Nano ZS, was 8 nm.

A colloidal solution of platinum was prepared by means of citrate reduction of platinum hydrochloride according to the procedure described in [4]. The synthesized platinum nanoparticles are stable and do not aggregate during storage and in growth medium. The average size of the resulting platinum particles, determined with the help of nanosizer, was 3 nm.

Synthesis and characterization of silver nanocomposites

Poly(1-vinyl-1,2,4-triazole) was synthesised by means of radical polymerization of 1-vinyl-1,2,4-triazole in DMFA in the presence of azobisisobutyric acid dinitrile at a temperature of 60 °C for 6 h [5].

Nanocomposites Ag15 and Ag13 were obtained in the presence of PVT (21.0 mmol) by the reduction of the aqueous solution of silver nitrate (1.0 mmol) by sodium borohydride (1.0 mmol) or glucose (1.5 mmol), respectively. The composites were isolated by re-precipitation into a mixture of ethanol and acetone (1 : 2), dried in vacuum above $CaCl_2$. The yields of Ag15 and Ag13 nanocomposites – dark-brown water-soluble powders – were 92 and 96 %, respectively.

According to the data of electron spectroscopy (PerkinElmer's Lambda 35 UV/Vis spectrophotometer), silver is present in the nanocomposite in nanometer-sized zero valence state. The IR spectra of nanocomposites were recorded with a Bruker Vertex 70 FT-IR spectrometer coupled to a RAM II FT-Raman module in tablets with KBr; they were identical with the IR spectra of initial polymer 1-vinyl-1,2,4-triazole. This fact is the evidence that the structure of polymer matrix does not undergo any changes during the synthesis of nanocomposites; therefore, the original properties of the polymer are conserved.

According to the data of elemental analysis and atomic absorption spectroscopy (PerkinElmer's Analyst 200 spectrometer), silver content in nanocomposites is 7.8 % (Ag15) and 5.0 % (Ag13).

According to the results of transmission electron microscopy (Leo 906E transmission electron microscope), the obtained samples of nanocomposites Ag15 and Ag13 contain silver nanoparticles 2–20 and 5–30 nm in size, respectively. The average diameter of Ag15 and Ag13 nanocomposites, determined with the help of a Malvern Zetasizer Nano ZS, was 14 and 8 nm, respectively.

Synthesis of platinum nanocomposite

Arabinogalactan with the mass of 20 kDa was extracted from the wood of larch *Larix sibirica* and characterized in [6].

Platinum nanocomposite was synthesized according to the procedure described previously [7]. Isolation and purification of the product were carried out by re-precipitation in ethanol. The yield of the product was 95 %, calculated for the metal. Platinum content of the nanocomposite was determined by means of atomic absorption to be equal to 9.6 %. Identification of the metal in the zero valence state within the nanocomposite was carried out on the basis of diffraction patterns with clearly differentiated reflections of the metal component and an amorphous halo AH. The UV spectrum also confirms the presence of the metal in reduced state in the sample.

According to the data of electron microscopy (Leo 906E transmission electron microscope, scanning electron microscope SEM 525M) [8], metal nanoparticles are homogeneous particles collected in fractal clusters with the average nanoparticle diameter of 8 nm.

Cell cultures

The HeLa and Mef-7 cells were obtained from American Tissue Cell Collection (ATCC, USA). Primary endothelial cells and primary fibroblasts were obtained from umbilical vein and human gingival tissue, respectively [9, 10]. All cells used in the work were cultivated in IMDM medium (Gibco, USA, 42200-014) in the presence of 10 % embryonic calf serum (ECS) (Gibco, USA, 10 106) in CO_2 incubator at 37 °C, 5 % CO_2. To study cytotoxicity and proinflammatory action of nanomaterials, the cells were spread (with the density of 10 000 cells per one well of a 48-well plate) and cultivated for 16 h. Then the cultural medium was removed, the fresh medium with 10 % ECS containing nanomaterials under study in different concentrations was added.

Determination of cytotoxicity of nanocomposites for human cells with the help of EZ4U test

For the quantitative estimation of the cytotoxic action of nanomaterials, the cells were washed with the IMDM cultural medium and incubated with 200 mL (48-well plate) of colloid in IMDM medium with 10 % ECS for 24 h. The concentration of the corresponding nanoparticles in colloids was 0.004 to 20 mg/mL. The cytotoxic effect was measured with the help of the commercial kit EZ4U (Biomedica, Germany). The amount of vital cells was determined by means of spectrophotometry at the wavelength of 460 nm. The values were represented as plots with the help of Origin Pro 8 by reducing to a sigmoidal dose responce curve.

Effect of nanocomposites on interleukine-6 secretion by primary endotheliocytes and fibroblasts

All components contacting the cells were tested for the presence of lipopolysaccharide (LPS) with the help of reagent kit for the determination of bacterial endotoxin by means of LAL test (Associate of Cape Cod Incorporated, USA). To study immunomodulating properties of nanocomposites, the cells were incubated in the IMDM cultural medium with 10 % ECS with colloids for 24 h. Then the cultural medium was withdrawn, centrifuged at 1500*g* and stored at −20 °C. As a positive reference, the cells were incubated in the presence of 1 mg/mL polyinosinic/polytidylic acid (pIC) (Sigma, USA, P9582) or 10 μg/L LPS (Sigma, USA, L6143) under the same conditions. To determine the basic level of cytokine secretion, the cells were incubated in the IMDM cultural medium with 10 % ECS. Before determining cytokine concentration, the samples were kept at room temperature and centrifuged. The concentration of interleukine-6 in the samples was determined with the help of the commercial kit IL-6-IFA-BEST (Vektor-Best JSC, Russia) according to the procedure recommended by the manufacturer.

RESULTS AND DISCUSSION

In the present work we studied two sets of nanomaterials. The first set included the particles based on metal silver core – Ag nanoparticles (without polymer cover) and nanocomposites Ag15 and Ag13 in which the core was stabilized by the presence of PVT. The second set included platinum nanoparticles without polymer coating and the particles stabilized with AG (nanocomposite No. 130-56).

Silver and platinum used as the materials of nanostructured core are at present the subject of extensive studies in medicine and biology; they find application in practical medicine [11]. It is known that silver in the ion form possesses bactericidal, antiviral, pronounced antifungal and antiseptic action, and serve as highly efficient aseptic means against pathogenic microorganisms causing acute infections. By present, it has been demonstrated in a number of works that silver nanoparticles and their composites, possessing large surface area and actively releasing Ag^+ ions into the medium, serve as the most efficient antiseptic means during direct contact with the surfaces that are purulent and inflamed due to bacterial contamination [12, 13]. Platinum preparations (cisplatin, carboplatin and oxaliplatin) are used mainly as chemotherapeutical means to treat some kinds of malignant growth [14]. Platinum nanoparticles in combination with irradiation are also proposed for anticancer therapy [15]. Poly(1-vi-

nyl-1,2,4-triazole) used by us as the polymer coating for silver nanocomposites is a synthetic non-toxic (LD_{50} > 3000 mg/kg) water-soluble polymer which is efficiently used to stabilize silver and gold nanoparticles [16, 17]. On its basis, promising materials were developed for soft contact lenses, efficient flocculants, and monomolecular Langmuir–Blodgett layers [19]. Arabinogalactan used to stabilize the platinum core is one of the important structural components of herbaceous and wood plants. Depending on the source, the structure of this biopolymer may differ substantially in molecular mass and in the number of end aldehyde and carboxylic groups. The physicochemical properties of AG are well studied. Pharmacological properties are less thoroughly studied, however, it was established that depending on the structure, AG possesses gastroprotective, bacteriostatic and immunostimulating activity [20, 21].

As the model systems to study biological properties of nanomaterials, we used primary endotheliocytes from the umbilical vein of newborn, primary fibroblasts from human gingival tissue, the cells of cervical adenocarcinoma HeLa and the cells of breast adenocarcinoma Mcf-7/Endothelium of vascular walls is a barrier for any preparation to penetrate from blood into underlying tissues to produce therapeutic effect. In addition, these cells are characterized by the high activity of cell transport. Fibroblasts are the major cells of connective tissue, so they can also be considered as candidates for the interaction with medical preparations. The lines HeLa and Mcf-7 are widely used in practice and they are most thoroughly studied among long-term cultivated cell lines of oncological origin.

Investigation of the cytotoxic properties of silver and platinum nanocomposites

For the quantitative estimation of the cytotoxic action of nanomaterials, we used the EZ4U test (Fig. 1).

For comparative analysis of the cytotoxic properties of nanomaterials, we determined the TC_{50} values (concentrations at which the death of a half of cells is observed) relying on the plots of the cytotoxic action of nanomaterials (see Fig. 1 and Table 1).

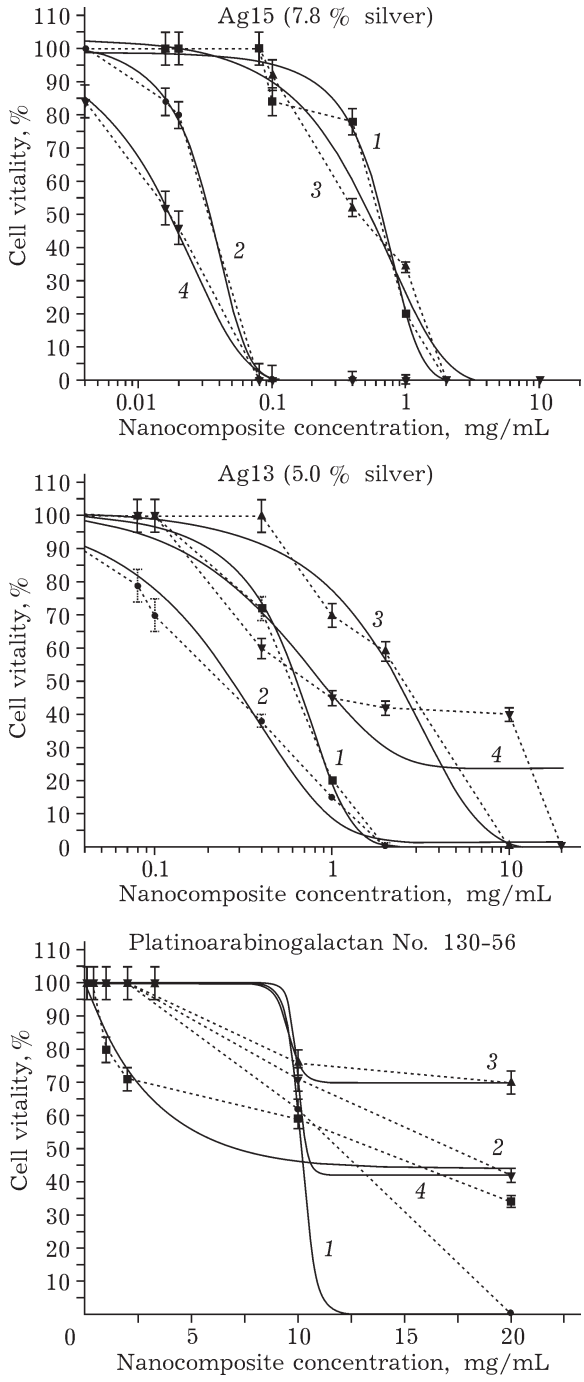

Fig. 1. Cytotoxic action of nanocomposites on primary and transformed human cells. Dependence of the amount of vital cells on nanocomposite concentration: *1* – endotheliocytes, *2* – fibroblasts, *3* – Mcf-7, *4* – HeLa.

The highest TC_{50} values are those of platinum nanocomposite No. 130-56; it is more toxic for primary cells – fibroblasts and endotheliocytes, and substantially less toxic for oncologically transformed cells HeLa and Mcf-7. The

TABLE 1

TC_{50} values for studied nanocomposites, mg/mL

Cells	Pt No. 130-56	Ag13	Ag15
HUVEC	7.18	0.63	0.66
GF	10.17	0.27	0.03
Mcf-7	–	2.34	0.53
HeLa	10.41	0.86	0.02

Note. Dash means that the maximal used concentration of platinum nanocomposite No. 130-56 induces the death of not more than 25 % cells, so TC_{50} could not be determined in this case.

behaviour of the curves of toxic response is interesting (see Fig. 1). In spite of some differences in TC_{50} values for primary fibroblasts and the cells of HeLa line (10.17 and 10.41 mg/mL, respectively), in the case of fibroblasts an increase in concentration above 10 mg/mL causes an onrush of the toxic effect, and complete death of cells is observed at the concentration of 12–13 mg/mL. At the same time, for HeLa cells, the concentration of platinum nanocomposites at which there are no vital cells left is substantially higher. Nanocomposite No. 130-56 induces cell death of endotheliocytes in lower concentration than for other cells, however, in the concentrations above 10 mg/mL the dependence of the toxicity on nanocomposite concentration reaches a plateau. The concentration of 10 mg/mL is critical for cell death induction in the rest cell lines. One can see in the data shown in Fig. 1 that near this point the concentration of vital HeLa, Mcf-7 and primary fibroblast cells reaches goes of the steady level. Decelerated cytotoxic reaction of transformed cells in the case of platinum nanocomposite may be due to less efficient transport of nanocomposites with smaller efficient surface area of these cells. Quite contrary, the reaction of endotheliocytes may be connected with the high efficiency of endocytosis in these cells [22], which causes increased intracellular concentration of the particles.

In the case of silver-containing nanocomposites (Ag13 and Ag15) TC_{50} value is substantially lower for all studied cell cultures, which provides unambiguous evidence of the increased cytotoxic properties of these materials. Nanocomposite Ag13, similar to platinum nanocomposite No. 130-56, is more toxic for primary cells in comparison with oncologically transformed cells, while nanocomposite Ag15 turned out to be more toxic for primary fibroblasts and the cells of HeLa line. The toxic action of Ag15 on the cells of HeLa line was observed even at the concentration of 0.004 mg/mL, while the concentration 0.02 mg/mL corresponded to TC_{50}. Silver nanocomposites turned out to be less toxic for endotheliocytes: TC_{50} value (0.63 and 0.66 mg/mL for Ag13 and Ag15, respectively) and the curve profile for both nanocomposites are almost identical. For other cell cultures, the toxicity of nanocomposites increased substantially with an increase in the specific fraction of silver in the nanocomposite: by a factor of 9 in the case of primary fibroblasts, by a factor of 4.4 and 43 (!) for Mcf-7 and HeLa, respectively. The behaviour of the dependence of cytotoxic action of Ag13 nanocomposite on HeLa cells is to be stressed (see Fig. 1). For concentrations above 2 mg/mL, the second steady region is observed. Relying on this fact, we may assume that substantially higher concentration of Ag13 is necessary for 100 % death of HeLa cells than for similar toxic action of this preparation on other cells.

For the purpose of determining the contribution from the polymer component into the biological activity of nanocomposites, we studied the cytotoxic action of silver and platinum particles without corresponding polymer coatings on primary and transformed cells. The concentrations of silver and platinum nanoparticles in these experiments were chosen so that metal content would be comparable with the content of corresponding metals in nanocomposites (that is, the samples contained comparable amounts of metals). The cells were incubated in the presence of the solutions of colloids; the vitality of cells was estimated with the help of the commercial kit EZ4U. The data obtained are presented in Fig. 2.

It is shown that the nanoparticles of silver (8 nm) and platinum (3 nm) are less toxic for all cell tissues than their nanocomposites with AG and PVT. For example, silver colloids in the concentration of 0.2 mg/mL (which corresponds to the concentration of 4 mg/mL Ag13 and 2.6 mg/mL Ag15) do not suppress the proliferative activity of cells but even stimulate it, while nanocomposites in these concentra-

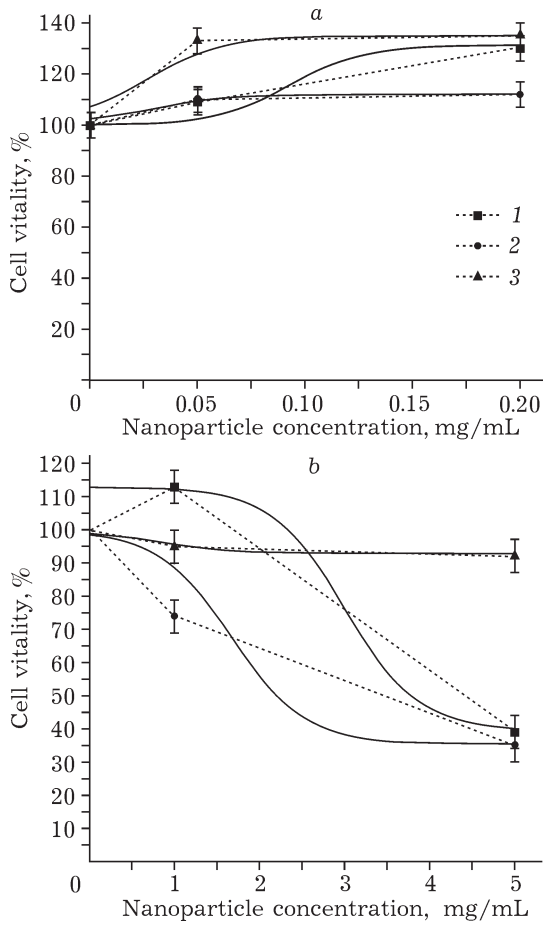

Fig. 2. Cytotoxic action of the colloids of Ag (*a*) and Pt (*b*) on primary and transformed human cells. Dependence of the amount of vital cells on the concentration of nanomaterials: *1* – endotheliocytes, *2* – fibroblasts, *3* – HeLa.

tions cause complete death of cells. After the addition of colloid platinum in the concentration of 5 mg/mL to the cells, the amount of living cells in primary cell cultures decreases to 40 %, and the amount of HeLa cells is conserved at a level of 90 %. For platinum concentration 2 mg/mL in nanocomposite with AG, vitality of all cell cultures decreases by approximately a factor of 2, which confirms the contribution of AG into the cytotoxic action of platinum nanocomposite.

It is known that increased toxicity of nanomaterials may be connected both with their small size (penetration into the karyon and mitochondria, causing mutations in DNA and structural damage of mitochondria) [23, 24] and with gradual dissolution of nanoparticles with the release of ions toxic for cells (chemical damage) [25]. Nanoparticles with smaller diameters have higher surface to volume ratio, possess larger surface activity and therefore they are characterized by higher rates the release of toxic ions and the formation of active oxygen forms [26]. The addition of a (bio)polymer coating allows governing these processes either due to the changes of the character of interaction with cells or by controlling the rate of particle dissolution. In our case, for silver nanocomposites, no correlation between particle size and an increase in the level of cytotoxicity of nanomaterials was observed, silver nanoparticles without the polymer component turned out to be less toxic than their nanocomposites with PVT. It is quite probable that nanoparticles in the presence of proteins form strong complexes with them, which changes the properties of metal colloids [27]. In the complexes with the polymers involved in our work, silver and platinum are likely to be less stable and more toxic. In general, silver-containing nanocomposites are more cytotoxic in comparison with platinum-containing ones, which agrees with literature data [28].

Investigation of immunomodulating properties of silver and platinum nanocomposites

We chose primary cells – endotheliocytes and fibroblasts – as the model to study immunomodulating action because these cells are in contact with medical preparations in the case of intravenous or any other kind of introduction. In addition, they are able to excrete a number of proinflammatory cytokines (including interleukine-6, IL-6) coordinating the local and systemic inflammation process and the production of immunoglobulins [10, 29]. To stimulate secretion of interleukine-6 by these cells, we used surely sub-lethal concentrations of nanocomposites, selected as a result of experiments on the determination of nanocomposite cytotoxicity. For platinum-containing nanocomposite, these were 1, 10, 100 and 1000 μg/mL, for silver nanocomposites Ag13 and Ag15 – 1, 10, 100 and 0.1, 1, 10 μg/mL. At these concentrations, it is possible to exclude substantial variation of cytokine concentration in connection with the change of the amount of cells, and to decrease/eliminate immune response to the products of cell death.

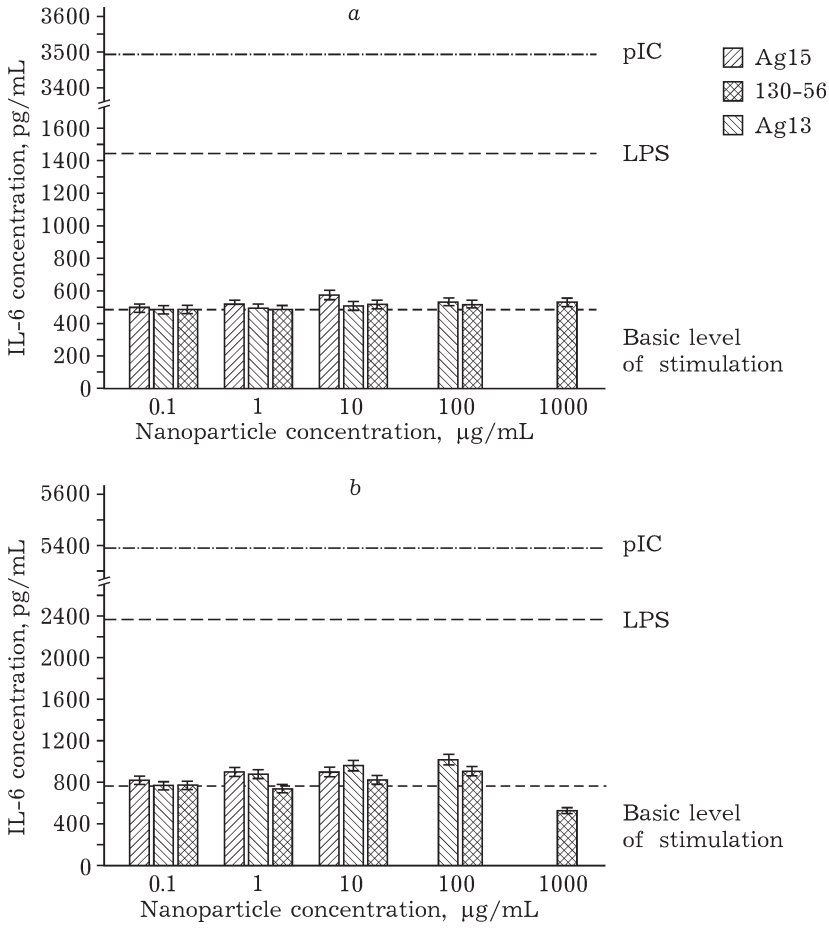

Fig. 3. Induction of proinflammatory response by primary endotheliocytes (*a*) and fibroblasts (*b*) under the action of nanocomposites.

One can see in the data presented in Fig. 3 that stimulation of primary cells by nanocomposites leads to a change in IL-6 concentration not more than by 5–10 % of the basic level, while the reference stimulation with pIC and LPS promoted at least 5-fold increase in concentration. Therefore, no one of three studied nanomaterials affects the concentration of proinflammatory cytokine IL-6 in the culture of primary cells, that is, does not induce inflammatory response.

According to literature data, AG extracted from a number of plants exhibits stimulating action both on the cells of immune system and on fibroblasts [30]. Arabinogalactan within the nanocomposite (see Fig. 3) does not stimulate the production of cytokines by primary endotheliocytes and fibroblasts of humans. Induction of proliferation of the studied cell cultures under the action of AG was not discovered, too, which may be due to the cytotoxic action of nanocomposite.

CONCLUSION

The data obtained allow us to assume that Ag13 nanocomposite may be used as an anticancer drug in combination with other cytotoxic agents. The concentration of Ag15 0.02 μg/mL, corresponding TC_{50} for HeLa cells, is not toxic for endotheliocytes and only partially toxic for fibroblasts (causes the death of 15 % of cells). In addition, this compound does not possess pronounced proinflammatory action. So, in the case of intravenous introduction this compound should not cause toxic effects on vascular endothelium or cause general inflammatory response. In addition, in view of the features of blood supply to oncologically transformed tissues, nanocomposite Ag15 can get accumulat-

ed in the lacunas of tumours. The toxic effect of nanocomposite on fibroblasts and endotheliocytes surrounding the tumour may have rather positive than negative action: its presence promotes additional increase in the zone of necrosis, prevents vascularisation and creates even less favourable conditions for the vital activity of tumour cells. Further studies of the effects of the simultaneous introduction of Ag15 and cytostatics allowed for application in medicine will allow us to evaluate the outlooks for the use of this nanocomposite as an antitumour nanopreparation.

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