The splenocyte proliferative response and cytokine secretion in mice after oral administration of commercial gold nanocolloid

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Abstract

Owing to their unique physicochemical properties, gold nanoparticles find numerous biomedical applications. Experiments on rodents prove that the main target organs of gold nanoparticles entering an organism are the liver and spleen, whose reticuloendothelial system removes foreign particles from the bloodstream. Through interactions with resident tissue macrophages, nanoparticles can evoke a systemic immunological response.

The aim of this study has been to determine the effect of oral administration of commercial gold nanocolloid, recommended by the producer inter alia as a dietary supplement, on the proliferative activity and cytokine secretion by murine splenocytes. The colloid was given to the animals in three different doses (0.25, 2.5, 25 ppm), for three different time periods (7, 14, 28 days). The influence of nanogold on splenocyte functions was time-dependent and the various doses were distinguished by distinct modes of action. The lowest dose had a pro-inflammatory or immunostimulating effect, enhancing the synthesis of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α). The effect of the highest dose can be considered as a pro-inflammatory, or immunotoxic one, because the stimulated cytokine synthesis was accompanied by a drastic decline in the proliferative activity of lymphocytes. The medium dose, while inhibiting the synthesis of pro-inflammatory cytokines of macrophages, simultaneously stimulated the proliferation of lymphocytes. All the doses also modulated the synthesis of IL-2, which may implicate their effect on the immunoregulatory mechanisms of an organism. The effect of alimentary administration of gold nanocolloid on the immune system seems to be difficult to predict, hence a risk that this type of dietary supplements might have some adverse impact on the immunity cannot be excluded, especially after their chronic administration.

Key words: gold nanocolloid, mice, splenocyte activity, cytokine response

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Introduction

Metallic gold (0) is considered the most biologically inert and biocompatible of all metals, but the main therapeutic agent is gold (I) due to its good solubility and easy stabilization by ligands. Traditional gold-based drugs are used in medicine owing to their anti-inflammatory effects, mainly in the therapy of rheumatoid arthritis. The side-effects of gold therapy, such as hypersensitivity reactions, enterocolitis and nephrotoxicity, are rarely reported. However, there is some risk of developing immunosuppression due to a long-lasting gold treatment (Sung et al. 2011, Thakor et al. 2011, Gerber et al. 2013).

Gold nanoparticles (AuNPs), structures with at least one dimension of no more than 100 nm, containing hundreds of atoms of gold, are seen as a bridge between bulk materials and molecular structures. Owing to their unique physicochemical properties, arising from their minute size and a large surface-to-volume ratio, AuNPs are used for diagnostic and therapeutic purposes, e.g. as biomarkers, bioimaging agents, biosensors, anti-angiogenic agent, photothermal agent, radiotherapy dose enhancer, in targeted delivery of anticancer drugs and gene regulation (Yen et al. 2009, Lasagna-Reeves et al. 2010, Zhang et al. 2010, Thakor et al. 2011, Gerber et al. 2013). For these applications their biocompatibility is crucial, but the small size of nanoparticles modifies their interactions with cells. Toxicity of NPs depends on their properties, route of administration, metabolism, excretion and the body’s immune response (Farkas et al. 2010, Zhang et al. 2010, Sung et al. 2011, Thakor et al. 2011).

Studies on rodents demonstrate that, irrespective of the administration route, AuNPs are broadly distributed within an organism but the main target organs are the liver and spleen, whose reticuloendothelial system quickly removes nanoparticles from the bloodstream. Their phagocytosis is affected by the composition of protein corona, which is formed in just a few seconds after nanoparticles’ contact with body fluids. Moreover, smaller nanoparticles are more easily distributed in the body and absorbed by cells (Sonavane et al. 2008, Farkas et al. 2010, Zhang et al. 2010, Thakor et al. 2011, Chen et al. 2013). Hillyer et al. (2001), who gave mice colloidal AuNPs (4-58 nm) in drinking water, found out that, depending on the particle size, they conjugated with proteins in the animals’ saliva and non-conjugated particles undergone aggregation, which diminished their absorption. On the other hand, Zhang et al. (2010), who compared different administration routes of AuNPs given to mice, concluded that the toxicity of particles administered p.o. was the highest. Their high doses increased the splenic index and decreased erythrocyte count, with gold nanoparticles being detected inside blood and bone marrow cells. The observed changes may indicate that nanogold administered orally affects the immune system. However, most research on the immunotropic properties of gold nanoparticles are still conducted under in vitro conditions with in vivo studies being really scarce. The in vitro experiments have proven that, regardless of their shape and size, AuNPs are quickly absorbed by murine macrophages. The nanoparticles uptake sometimes did not lead to any consequences, thus suggesting the lack of immunogenic effect (Shukla et al. 2005, Zhang et al. 2011, Pissuwan et al. 2013). However, there are reports implicating the anti-inflammatory (Sumbayev et al. 2012, Tsai et al. 2012) as well as pro-inflammatory effect of ingested AuNPs (Yen et al. 2009, Lee et al. 2012, Pissuwan et al. 2013), which manifests itself by the inhibition or stimulation, respectively, of the synthesis of reactive oxygen or nitrite species and pro-inflammatory cytokines (IL-1, IL-6 and TNF-α). Notwithstanding that, for some time now, there have been colloidal solutions of gold nanoparticles available on the market, which are recommended in unconventional medicine for both external and internal use, e.g. to ‘tranquillize’ an overstimulated immune system.

Since the spleen is a main target for both nanoparticles and dietary xenobiotics, the aim of this research was to determine the effect of oral administration of commercial gold nanocolloid on the proliferative activity and cytokine synthesis by mouse splenocytes. The colloid was given to animals in three different doses (0.25, 2.5, 25 ppm), for three different time periods (7, 14, 28 days). The investigated nanocolloid, although recommended by the manufacturer as a dietary supplement and claimed to be perfectly safe for both humans and animals, have not been tested before towards its toxicity and immunotropic properties.

Materials and Methods

Gold nanoparticles (AuNPs)

Colloidal (nonionic) gold solution (Nano-Tech, Poland) containing metallic gold nanoparticles (up to 5 nm, according to the manufacturer) suspended in demineralised water, at a concentration of 50 ppm was used as a source of gold nanoparticles. Colloidal gold was dissolved in distilled water to produce solutions at three concentrations: 0.25 ppm, 2.5 ppm, and 25 ppm, which were than administered to mice as drinking water ad libitum for consecutive 28 days. The drinking water of the control animals was also distilled.
The experiment was performed on 120 BALB/c mice, aged 8-10 weeks, with body weight of 20-24 g. The animals were maintained on a 12-h light/dark cycle in a controlled temperature (20 ± 1°C) and supplied with rodent chow and water *ad libitum* throughout the experiment. Mice were divided randomly into four equal groups: control group (0 ppm) not receiving the gold solution, and three experimental groups administered the gold solution at the concentrations of 0.25 ppm, 2.5 ppm or 25 ppm. After 7, 14 and 28 days of administration of the colloidal gold solution, 10 animals from each group were sacrificed. The animals were anaesthetised by inhalation of Aerrane (isoflurane, Baxter Poland), bled by heart puncture and their spleens were sampled for further analyses. At each time point the body and spleen weights of the sacrificed individuals were measured and the splenic indices were calculated by dividing the spleen weight (in mg) by the body weight (in g) of the individual. The experiment has been approved by the Local Ethics Committee.

**Isolation of splenocytes**

Splenocytes were isolated using routine procedure. Aseptically-removed spleens were pressed through a 60-μm nylon mesh in RPMI-1640 medium with L-glutamin and sodium bicarbonate (Sigma-Aldrich). The cell suspension was placed on density gradient Histopaque 1077 (Sigma-Aldrich) – in order to isolate mononuclear cells, and then centrifuged at 400 g for 30 min at 20°C. The interface cells were collected and washed three times with the RPMI-1640 medium and centrifuged at 250 g for 10 min. Viability of the isolated cells was evaluated by trypan blue exclusion (Sigma-Aldrich) and was determined to be greater than 95% in each case. The cells were suspended in RPMI-1640 medium containing 10% fetal calf serum (Sigma-Aldrich) and 1% antibiotic-antimycotic solution (Sigma-Aldrich), and dispensed into 96-well plates at a concentration of 2.5x10^5 cells ml⁻¹. Then, the cells were cultured at 37°C under a humidified air atmosphere with 5% CO₂ and used for the assays. Splenocytes isolated from five individuals of each group were used for the MTT test, and from the next five for the determination of cytokine levels.

**Proliferative response of splenocytes (MTT test)**

Mitogenic response of splenocytes was determined using the MTT colorimetric assay (Mosmann 1983). Cells were suspended in RPMI 1640 growth medium containing mitogens – concanavalin A (ConA, Sigma-Aldrich) in concentration of 5 μg ml⁻¹ as a T-cell mitogen or lipopolysaccharide from *Escherichia coli* (LPS, Sigma-Aldrich) in concentration of 10 μg ml⁻¹ as a B-cell mitogen and 100 μl of the suspension was added to each well of microtiter plates. The mixture was cultured for 72 h. After incubation, 10 μl of solution containing 7 mg ml⁻¹ of MTT (3-[4, 5 dimethylthiazoly-2-yl]-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) in PBS were added and the plate was incubated for the next 4 h. The supernatant was removed and 100 μl of DMSO was added to each well. The optical density was measured at a wavelength of 570 nm with 640 nm as a reference wavelength. All samples were tested in triplicate. The results of the proliferation assay were expressed as a stimulation index (SI), which was calculated by dividing the mean O.D. of stimulated cells by the O.D. of the non-stimulated (control) cells.

**Determination of cytokine levels**

Splenocytes isolated from animals were plated in 24 well plates in the absence or presence of of mitogens – ConA (5 μg ml⁻¹) or LPS from *Escherichia coli* (10 μg ml⁻¹). In the preliminary studies, LPS turned out to be better stimulant than ConA for IL-1β, IL-6, IL-10 and TNF-α production in the culture of mice splenocytes, so ConA was used as a stimulant in case of IL-2 and IFN-γ production only, and LPS for other cytokines. After 72 h of incubation the plates were centrifuged at 250 g for 10 min, the supernatants were collected and tested in duplicates. Cytokine levels (IL-1β, IL-2, IL-6, IL-10, IFN-γ and TNF-α) in the culture media were determined using immunoassay (ELISA) kits (R&D Systems, United Kingdom), according to manufacturer’s protocol.

**Statistical analysis**

Data were analyzed statistically by the one-way analysis of variance (ANOVA). Bonferroni’s post-test was used to determine differences between groups. Statistical evaluation of results was performed using GraphPadPrism software package.

**Results**

The animals willingly drank water with added nanocolloid. Throughout the whole experiment no...
Table 1. The body weight, spleen weight and splenic index in mice after oral administration of gold nanocolloid.

<table>
<thead>
<tr>
<th>Time</th>
<th>7 days</th>
<th>14 days</th>
<th>28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group/AuNPs (ppm)</td>
<td>control/0</td>
<td>0.25</td>
<td>2.5</td>
</tr>
<tr>
<td>Spleen weight (mg)</td>
<td>104.5±24.16</td>
<td>95.65±15.21</td>
<td>95.1±17.63</td>
</tr>
<tr>
<td>Splenic index</td>
<td>4.29±0.94</td>
<td>3.83±0.81</td>
<td>3.53±0.50</td>
</tr>
</tbody>
</table>

Fig. 1. Proliferative response of mice splenocytes (SI) after oral administration of gold nanocolloid: (A) T lymphocytes (ConA as a mitogen), (B) B lymphocytes (LPS as a mitogen)

Explanations:
* – difference statistically significant in comparison to control group (0) at p<0.05
** – difference statistically significant in comparison to control group (0) at p<0.01
*** – difference statistically significant in comparison to control group (0) at p<0.001

Cytokine levels in non-stimulated cells

During the entire experiment, regardless of the group, IL-10 and IFN-γ were undetectable in the supernatant obtained from the culture of splenocytes not stimulated by mitogens. Cells from the control group secreted small, albeit measurable, quantities of IL-1β, IL-2, IL-6 and TNF-α. In all the groups receiving gold nanocolloid, distinct changes in the cytokines production by non-stimulated cells were observed versus the control group (Fig. 2).

As soon as on day 7 of the gold nanocolloid administration, splenocytes of the animals given the highest colloid dose began to produce significantly higher amounts of pro-inflammatory cytokines IL-1β, IL-6, TNF-α (p<0.001) and IL-2 (p<0.01) than splenocytes from the control animals. This effect persisted until day 14 of the experiment. However, after 28 days, the level of IL-2 decreased significantly (p<0.001), while the concentrations of the other cytokines continued to be elevated. From day 14 on,
splenocytes of the mice administered the lowest dose of colloid responded similarly to cells of the mice given the highest dose, i.e. by raised levels of IL-1β, IL-6 (p<0.001), TNF-α (p<0.01) and IL-2 (p<0.05) after 14 days, and a significant decrease in the level of IL-2 (p<0.001) accompanied by more intensive production of the remaining cytokines (p<0.001) after 28 days (Fig. 2).

Cells of the mice given the medium AuNPs dose responded differently – with decreased synthesis of all cytokines in comparison to the control group. From day 14, level of IL-1β in this group dropped below detectability, and after 28 days only the level of IL-6 slightly surpassed the detection limit, while the other cytokines were undetectable (Fig. 2).

**Cytokine levels after stimulation with mitogens**

Following the stimulation with mitogens, the levels of all cytokines in the supernatant from the control group splenocytes were measurable. The highest concentrations were achieved by IL-2, IL-6 and IFN-γ, while IL-1β, IL-10 and TNF-α appeared in slightly lower concentrations. Cells of the animals receiving AuNPs differed from the control group cells in the cytokine response to mitogens (Fig. 3).

Splenocytes of the group given 0.25 ppm AuNPs dose, after 7-day administration, secreted lower amounts of IL-6 (p<0.001) and IL-10 (p<0.01) than the control ones. However, after 14 days the levels of pro-inflammatory IL-1β and IL-6 increased significantly. This effect lasted until day 28, when, additionally, an increase in the level of TNF-α (p<0.05) and a decrease in IL-2 (p<0.05) were noticed (Fig. 3).

In the group supplied with the highest AuNPs dose, despite the increase in the pro-inflammatory cytokine synthesis by non-stimulated cells, no pro-inflammatory effect was noticed until day 28 after the mitogenic stimulation of splenocytes. After 7 days the levels of all the cytokines were not different from the control ones, while after 14 days, a significant increase in the level of anti-inflammatory IL-10 (p<0.01) was observed. However, after another fortnight, cells from this group responded by more intensive production of pro-inflammatory IL-1β, IL-6 (p<0.001) and TNF-α (p<0.01) as well as by a decrease in the level of IL-2 (p<0.001), in which they resembled the group given the lowest AuNPs dose (Fig. 3).

The cytokine response of the medium AuNPs dose group differed from the other two groups. After the initial decline in the levels of IL-6 (p<0.01) and IL-10 (p<0.05) on day 7, a significant decrease in the level of IL-1β (p<0.05) and an increase in IL-2 (p<0.001) were noticed in the subsequent week, compared to the control. This trend continued until the end of the experiment – an elevated concentration of IL-2 (p<0.002) was accompanied by a decrease in the levels of all the other cytokines, although it was statistically significant only with respect to IL-10 (p<0.05) and IFN-γ (p<0.001) (Fig. 3).
Discussion

Xenobiotics can affect the immune organs, leading to decreased immune responses or to an over-stimulation of the immune system. Possible consequences include a higher susceptibility to infectious diseases and certain neoplasms, or immune deregulation evoking allergies and autoimmune disorders. Research on direct immunotoxicity of xenobiotics involves weight measurements and morphological evaluations of lymphoid organs. Another important aspect is the determination of the xenobiotic’s impact on the immune functions, e.g. antibody response, proliferation of lymphocytes, production of cytokines, etc. (De Jong et al. 2007). The experiment discussed here, conducted on mice given orally gold nanocolloid, did not reveal changes in the spleen weight or the splenic index of experimental mice. However, a significant, time-dependent effect of AuNPs on the activity of murine splenocytes was noticed. The results are not unambiguous and the most surprising finding is a different effect of the medium NPs dose, while the lowest and highest ones produced somewhat similar results. The main reason for that could be that each next dose of gold nanocolloid was ten-fold higher than the preceding one, thus nanoparticles’ fate in the organism could be different, inducing a different immune response.

Prolonged administration of the lowest nanogold dose (0.25 ppm) caused an obvious pro-inflammatory effect in both non-stimulated and mitogen-activated cells (increased levels of pro-inflammatory...
cytokines). The highest dose of AuNPs (25 ppm) had also a pro-inflammatory effect on non-stimulated cells during the whole experiment; however, with respect to mitogen-activated cells, a significant increase in the level of pro-inflammatory cytokines and a strong decrease in the proliferative activity of splenocytes did not occur until 28 days of administration. This result may suggest that initially, in order to maintain homeostasis, the immune system activated its reserves, which were exhausted by the prolonged administration of a high dose of colloid, whose effect can be deemed toxic. The in vitro studies on murine macrophages performed by other authors showed that high, toxic concentrations of nano-gold depressed cell viability and proliferation, while stimulating the synthesis of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α); in contrast, small, non-toxic doses activated cells, increasing the proliferation, release of Ca, NO and H2O2 as well as the synthesis of many cytokines, including IL-1β, IL-6, IL-10 and TNF-α, in which the cited reports resemble the effects obtained in the present experiment with the lowest and highest dose of AuNPs (Yen et al. 2009, Lee et al. 2012, Pissuwan et al. 2013).

As mentioned above, the effect of the medium colloid dose (2.5 ppm) was different. It can be described, on the one hand, as anti-inflammatory (decreased synthesis of the typical pro-inflammatory cytokines of macrophages), but, on the other hand, as stimulating for lymphocytes (increased IL-2 production and lymphocyte proliferation). Some of the available references confirm the suppression of macrophage functions in vitro by AuNPs. They retarded the response of human macrophages to IL-1β, and blocked the functions of TLR9 receptor in murine macrophages, decreasing the synthesis of TNF-α, IL-6 and IL-12p40 (Sumbayev et al. 2012, Tsai et al. 2012). In the in vivo studies, conducted on the animal models of inflammatory diseases, the suppression of macrophage functions by nano-gold resulted in decreased levels of pro-inflammatory cytokines and protection from oxidative tissue damage (Tsai et al. 2007, Pedersen et al. 2009, Pereira et al. 2012, Sumbayev et al. 2012). The anti-inflammatory effect of the medium AuNPs dose, demonstrated in the present experiment, may have also been a result of the inhibition of inflammatory pathways in resident macrophages, which were certainly present, albeit in small numbers, in cultures of murine splenocytes.

In turn, investigations carried out on cellular models other than macrophages demonstrate the stimulating effect of gold nanocolloid. Dokić et al. (2012), who analyzed the influence of different fractions of AuNPs obtained by ultrasonic spray pyrolysis from gold scrap on ConA stimulated rats’ splenocytes, observed that smaller and less agglomerated particles increased the synthesis of IL-2 while inhibiting the synthesis of IL-10, which resembles the effect obtained in our experiment at the medium dose of AuNPs. What is more, in studies on mouse B lymphocytes, AuNPs passed through the cytoplasmic membrane of cells, activating the cells and elevating the expression of antibodies (Sharma et al. 2013). A similar outcome was observed in vivo by Dykman et al. (2004), who analyzed the influence of AuNPs on the immunological response of laboratory animals (rabbits, rats and mice). Gold nanoparticles had a better adjuvant effect than CFA (complete Freund’s adjuvant), allowing obtaining higher antibody titre, demonstrating higher activity with a smaller amount of the antigen. The stimulating effect of AuNPs for B lymphocytes may explain the proliferative effect of the medium dose of colloid found in our experiment.

What seems particularly interesting, all the tested doses of gold nanocolloid had a significant effect on the level of IL-2. The medium dose stimulated its production via the activated T lymphocytes, whereas the two extreme doses, after the initial stimulation, significantly depressed its synthesis. In the author’s earlier study on mouse splenocytes under in vitro conditions, the same gold nanocolloid in a rather broad range of non-toxic concentrations (0.15-1.25 ppm) likewise depressed the synthesis of IL-2 following the stimulation by ConA (Malaczeska 2014), which seems to verify its impact on T lymphocytes. Interesting is also the fact that changes in the level of IL-2 were unaccompanied by an analogous effect on IFN-γ. Cells which demonstrate a limited expression of cytokines, producing only IL-2 but not IFN-γ, are CD4+ T memory cells in lymphatic glands and spleen, naive T cells, pre-Th1 cells and primed but uncommitted Thpp precursor cells (Sad and Mosmann 1994, Yang and Mosmann 2004). It is difficult to state which of the mentioned populations were stimulated or inhibited by the nanocolloid, but that probably have not been the Th1 effector cells. The influence of gold nanoparticles on the synthesis of IL-2 could be fundamental to the organism, since, besides being a pro-inflammatory cytokine and a T cell growth factor, IL-2 is also known to play important immunoregulatory role. The shortage or lack of IL-2 can be responsible for some lymphoproliferative and autoimmune disorders (Hoyer et al. 2008, Lan et al. 2008). The elevated synthesis of IL-2 by splenocytes of the mice given the middle dose of gold nanocolloid explains increase in the T lymphocyte proliferation in these animals, but also a higher proliferation of B lymphocytes, since ac-
tivated B cells show the expression of IL-2 receptor and IL-2 is one of the co-factors stimulating their proliferation, differentiation and antibody secretion (Muraguchi et al. 1985).

Recapitulating, irrespectively of the applied dose, gold nanocolloid produced a significant effect on functions performed by mouse splenocytes, increasing over time of its administration. The three tested doses demonstrated, however, different modes of action. The lowest dose effect was pro-inflammatory or immunostimulating, the medium dose acted as an anti-inflammatory one while the highest one had a pro-inflammatory or immunotoxic influence. All the doses had also a significant impact on the synthesis of IL-2, which may implicate their effect on the immunoregulatory mechanisms. Because the examined colloid is a commercial product, recommended also for internal use, it is impossible to exclude a certain risk of its adverse effect on the immune system during prolonged administration.

**References**


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