The splenocyte proliferative response and cytokine secretion in mice after 28-day oral administration of silver nanocolloid

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Abstract

An increasing number of applications of silver nanoparticles in industry, medicine and everyday life means that the risk of exposure of the human organism to their potential harmful influence is growing. This study has sought to assess the effect of 28-day alimentary administration of different concentrations (0.25, 2.5 and 25 ppm) of a commercial silver nanocolloid on the proliferative activity and synthesis of cytokines by mouse splenocytes.

All of the analyzed doses of the colloid had a significant, albeit different, effect on the activity of splenocytes. At the lowest dose, a significant decrease in the proliferation of T cells and more intensive synthesis of pro-inflammatory cytokines, both by non-stimulated and LPS-stimulated cells, was observed. The intermediate dose, on the other hand, stimulated proliferation of B cells while producing a pro-inflammatory effect regarding the synthesis of cytokines. Finally, the highest dose decreased the synthesis of cytokines by non-stimulated cells, but after LPS stimulation, through the strong activation of the IL-10 synthesis, it raised the proliferation of B cells and decreased the synthesis of pro-inflammatory cytokines.

The results suggest that silver nanoparticles administered orally have an easy access to the peripheral organs of the immune system, such as the spleen, but the effect of long-term exposure of this organ to the effect of silver nanocolloid depends on several factors, including the dose of nanoparticles, and seems as difficult to predict.

Key words: silver nanocolloid, oral administration, splenocyte activity, cytokine level, mice

Introduction

Silver nanoparticles (AgNPs), owing to their disinfectant properties, find an increasingly broader application in households and in medicine. In recent years, preparations based on silver nanocolloids have appeared on the market, which are recommended in alternative medicine as dietary supplements, for protection against infections and for stimulation of the immune system. However, there is certain risk associated with the exposure of the human organism to the effect of silver nanoparticles, and its magnitude depends on...
the degree of absorption of the nanoparticles – when no absorption occurs, only a local effect, that is at the site of exposure (lungs, skin, the digestive tract), may occur. Research on the kinetics and distribution of silver nanoparticles in rodents indicate that nanoparticles can easily penetrate through biological barriers and permeate into the circulatory system and internal organs, irrespective of the way they are administered. Particularly vulnerable to the accumulation of AgNPs are organs which possess an extended reticuloendothelial system, such as the spleen, as these organs are the major “cleaning agents” of the body, whose function is to remove foreign, potentially harmful particles from the bloodstream (Takenaka et al. 2000, 2001, Pelkonen et al. 2003, Lankveld et al. 2010, Park et al. 2010). It is also known that once they are absorbed by phagocytic cells, AgNPs cannot be destroyed and therefore they remain inside phagosomes, chronically stimulating the cells to respiratory burst, and through the stimulation of the synthesis of reactive oxygen species they lead to the peroxidation of lipids, damage of biological membranes, disorders in the functioning of mitochondria and apoptosis or necrosis. After the death of a phagocytic cell, nanoparticles can be phagocytosed by subsequent foraging cells or can enter into interactions with other cells present in the environment (Jovanović and Palić 2012). A study by Lankveld et al. (2010), conducted on mice, confirms that once silver nanoparticles have been distributed in the whole organism, they are slowly eliminated from the organs in which they have accumulated, while a case of chronic exposure of immunological organs can contribute to an inadequate immunological response of the organism, or even to the development of immunosuppression.

The purpose of this study has been to determine the effect of a 28-day alimentary administration of different concentrations of silver nanocolloid, recommended by the producer as a dietary supplement, on the proliferative activity and synthesis of cytokines by mouse splenocytes.

**Materials and Methods**

**Silver nanoparticles (AgNPs)**

Colloidal (nonionic) silver solution (Nano-Tech, Poland) containing metallic silver nanoparticles (10-20 nm, according to the manufacturer) suspended in demineralised water, at a concentration of 50 ppm was used as a source of silver nanoparticles. Colloidal silver 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 28 days. The drinking water of the control animals was also distilled.

**Mice**

The experiment was performed on 40 male NMRI mice, aged 10-12 weeks, with body weight of 26-31 g. The animals were maintained on a 12-h light/dark cycle in a controlled temperature (20 ± 1°C) and supplied with rodent chow an water *ad libitum* throughout the experiment. Mice were divided randomly into four equal groups: control group (0) not receiving the silver solution, and three experimental groups administered the silver solution at the concentrations of 0.25 ppm, 2.5 ppm or 25 ppm. Since nanoparticles have a tendency to aggregate, water was replenished each day. After 28 days of administration of the colloidal silver solution, mice were sacrificed, and their spleens were sampled for further analyses. The animals were anaesthetised by inhalation of Aerrane (isoflurane, Baxter Poland). 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%. 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 1x10⁶ cells ml⁻¹. Then the cells were cultured at 37°C under a humidified atmosphere of 5% CO₂ and 95% air atmosphere 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 cultures by the O.D. of the non-stimulated (control) cultures.

Determination of cytokine levels

Splenocytes isolated from each group of animals were plated in 24 well plates in the absence or presence of LPS from Escherichia coli (10 μg ml⁻¹). After 24, 48 and 72 h of incubation the plates were centrifuged at 250g 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 analysed statistically by 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

Proliferative response of splenocytes

All the tested doses of silver nanocolloid affected the proliferative activity of splenocytes, although in different ways. The lowest dose (0.25 ppm) significantly inhibited the proliferation of T cells (p<0.01). It also caused a depressed response of B cells, although this effect was not statistically significant. The two higher doses did not affect T cells, but significantly stimulated the proliferation of B cells (p<0.001), and this effect was most strongly pronounced at the highest dose (25 ppm) (Fig. 1).

Cytokine levels in non-stimulated cells

Splenocytes of the animals from the control group, not stimulated with LPS, released small amounts of IL-6, IL-10 and IFN-γ after prolonged incubation: IL-6 after 48 hours of incubation and the other cytokines after as much as 72 hours. No presence of IL-1β, IL-2 or TNF-α was detected in the culture medium (Fig. 2).

The administration of silver nanocolloid affected the synthesis of cytokines by non-activated splenocytes, but the actual effect differed, depending on the dose of AgNPs. The smallest differences, relative to the control, were noticed in the group administered the highest concentration of nanosilver (25 ppm). Analogously to the control group, no detectable concentrations of IL-1β, IL-2 or TNF-α were determined, and a very low content of IL-10 was detected only following 72-h incubation. The differences appeared in the levels of IL-6 and IFN-γ. The IL-6 became detectable after 24 hours of incubation, but its level remained very low until the incubation was terminated, thus after 72 hours it was much lower (p<0.001) than in the control group, and the INF-γ was undetectable until the end of the experiment (Fig. 2).
A more profound effect was observed in the groups receiving the lower concentrations of the nanocolloid, with the results in both groups being similar. In contrast to the control and the group treated with the highest dose of AgNPs, the culture medium in the other two groups was determined to contain detectable concentrations of IL-1β, IL-2 and TNF-α. The level of IL-1β was rather low and similar in both groups (about 30 pg/ml); however, in the group administered the lowest dose of silver it was detectable already after 24 h and remained invariable until the end of the experiment, whereas in the group given the intermediate dose of nanocolloid it became detectable just after 72 h. The level of IL-2 in both groups became observable on 48 h and continued to increase, ultimately reaching a higher value in the group administered 2.5 ppm Ag. On the other hand, a detectable level of TNF-α appeared in both groups already after 24 h and increased slightly as the experiment progressed, also attaining a higher value in the intermediate group. The level of the remaining cytokines was also different from the one determined in the control group. IL-6 in cells from both groups was detected already after 24 hours, and its level continued to increase until the experiment terminated, but it was significantly higher than in the control group (p<0.001 at 0.25 ppm and p<0.01 at 0.25 ppm) only at 48 hour. The levels of IL-10 and IFN-γ in both experimental groups were significantly higher than in
the control group (p<0.01); moreover, in the group administered the lowest concentration of silver colloid, these cytokines were detectable 24 hours earlier than in the control group (Fig. 2).

**Cytokine levels after stimulation with LPS**

After LSP simulation, the levels of nearly all cytokines in the supernatant from the culture of splenocytes in the control group were detectable after 24 h incubation. IL-2 was an exception, remaining undetectable throughout the whole experiment. As the time progressed, the concentrations of all cytokines increased, except TNF-α, whose amount in the medium remained on the same level during the whole experiment. IL-6 reached the highest concentrations (4-5.5 ng/ml) although the concentrations of IL-10 and IFN-γ were also considerable (0.85 and 1 ng/ml, respectively). Typical macrophage cytokines, that is IL-1β (max. ca 90 pg/ml) and TNF-α (max. ca 300 pg/ml) stayed at a relatively low level (Fig. 3).

![Graphs and tables showing cytokine levels](image)

The 28-day administration of nanosilver had a significant effect on the inflammatory response of splenocytes, and the results induced by nanoparticles depended on their dose. Again, the two lowest doses produced similar results whereas the highest one was responsible for contrary effects.
Low doses of AgNPs (0.25 and 2.5 ppm) significantly raised the level of IL-1β: the lower dose starting from 24 h (p<0.001) and the intermediate one – from 48 h of incubation (p<0.01). Also the level of TNF-α was higher, but the difference was significant only when the intermediate dose had been administered (p<0.001). At both doses, in contrast to the control, the level of IL-2 was detectable from 48 h on, although it was lower than in cells not stimulated with LPS. Besides, both tested concentrations at first lowered the synthesis of IL-6 (p<0.001), but later the level of this cytokine was similar to that in the control cells (on 48 h at the dose of 0.25 ppm and on 72 h at the dose of 2.5 ppm). A significant decrease in the level of cytokines, compared to the control, was also noticed in the case of IL-10 and IFN-γ (p<0.001), yet – unlike IL-6 – it lasted during the whole incubation. The dose of 0.25 ppm showed a stronger effect on IL-10, whereas the dose of 2.5 ppm affected more profoundly the IFN-γ cytokine (Fig. 3).

In turn, the highest dose of nanosilver (25 ppm) caused a drastic decline in the levels of IL-1β, IL-6, IFN-γ and TNF-α versus the control throughout the whole experiment. The level of IL-2, similarly to the control group, was undetectable during the observations. At the same time, changes were noticed in response to the highest concentration of Ag in the synthesis of the pro-inflammatory cytokine IL-10, namely after the initial decline (24 h) its level increased to the one recorded in the control group (48 h) and finally (after 72 h) underwent strong stimulation (a three-fold increase versus the control, p<0.001) (Fig. 3).

Discussion

In the experiment reported herein, the animals willingly drank water with added nanoparticles, and no substantial differences were observed in the water intake, body weight or behaviour of mice from particular groups. It is difficult to assess, however, how much of the ingested dose of nanosilver actually affected the animal organisms because the absorption of NPs from the digestive tract depends on a series of factors, of which the most important ones are: tendency of NPs to aggregate, degree of solubility (release of ions) and interactions with the proteins present in systemic fluids (the corona formation) (Lankveld et al. 2010).

Irrespective of the fate of orally administered AgNPs in a mouse’s body, sometimes difficult to trace, the results of our experiment clearly show that nanosilver produces an extremely important effect on the spleen, a peripheral immune system organ. Among various aspects of such influence, noteworthy is impact on the proliferative response of splenocytes to mitogens, with the lowest dose (0.25 ppm) having a reverse effect on this parameter comparing to the higher rates. The smallest concentration of NPs significantly decreased the proliferation of T cells (and also that of B cells, although non-significantly), while those of 2.5 and 25 ppm affected only B lymphocytes, stimulating their proliferation in cells exposed to LPS.

It is not easy to discuss this finding because the only reports available in the world literature on the effect of AgNPs on proliferation of immunocompetent cells concern in vitro studies, and their results are divergent. In a study of Shin et al. (2007), concentrations of AgNPs from 10 ppm limited the proliferation of human peripheral blood mononuclear cells stimulated by PHA, whereas in an experiment reported by Greulich et al. (2011) silver nanoparticles had no impact on the proliferation of human T cells exposed to ConA, although they were tested in higher concentrations (5-30 μg/ml). On the other hand, in the authors' previous experiment conducted to test the same colloid administered into the alimentary tract of NMRI mice, in the same doses but for shorter lengths of time (7 and 14 days), silver nanoparticles were found to have produced diverse effects on the proliferation of splenocytes depending on the dose and duration of the administration. The smallest rate (0.25 ppm), having initially stimulated T lymphocytes after 7-day administration, had no stimulating effect on these cells after the subsequent 7-day application. The intermediate dose (2.5 ppm) stimulated the proliferation of lymphocytes, but only after 14 days of the administration (Małaczewska 2011a). The results obtained in the present experiment are in a way a continuation of the tendencies observed earlier, but with a shorter administration time of silver nanoparticles.

Another important aspect of the influence produced by nanosilver included the production of cytokines by both resting and lipopolysaccharide-stimulated cells. It is known that most cytokines are not produced physiologically by non-stimulated cells of healthy organisms. The expression of genes coding these proteins is typically a result of the stimulation of an organism by inflammatory or infectious agents, although in an in vitro culture some types of cells constitutionally produce small amounts of cytokines. Traditionally, cytokines are divided into pro-inflammatory (IL-1, IL-8, TNFα, IFNγ), aggravating the inflammatory process, and anti-inflammatory ones (IL-4, IL-10, IL-13), which inhibit the activity of pro-inflammatory cytokines. Some, for example IL-6, have pleiotropic properties and can demonstrate either a pro- or an anti-inflammatory effect, depending on target cells. Another popular division of cytokines distinguishes...
cytokines Th1 (IL-2, TNFα and IFNγ), associated with the cellular response, and Th2 (IL-4, IL-5, IL-6, IL-10, IL-13), connected with the humoral response to extracellular pathogens (Dinarello 2000, Opal and DePalo 2000).

The administration of silver nanocolloid to mice had a significant effect on the production of cytokines by non-stimulated splenocytes. In the culture of splenocytes originating from the control group of animals, after prolonged incubation, synthesis of small quantities of pleiotropic IL-6 and anti-inflammatory IL-10 was observed. No trace of the most important pro-inflammatory cytokines (IL-1 and TNFα) was found, and the amount of IFNγ was very small. However, it is known that the latter cytokine is produced by resting, non-stimulated macrophages (Gessani and Belardelli 1998), a small percentage of which was certainly present in the analyzed mixed culture of splenocytes. This response by splenocytes should therefore be considered as a normal and balanced one, with all the tested AgNPs doses disturbing it, although in different ways. The lower doses showed similar effects, stimulating the synthesis of all cytokines except IL-6. The effect was more pronounced in the case of IL-1β, TNFα and IFNγ than in case of the anti-inflammatory IL-10, hence it can be said that these two doses had a pro-inflammatory influence. Contrary to that, the highest dose reduced the synthesis of IL-6 and IFNγ compared to the control. Opposite effects induced by different doses of silver colloid were also observed after the stimulation of splenocytes. The response of the control group to LPS was expressed by the simultaneous, albeit different in intensity, synthesis of pro-inflammatory, anti-inflammatory and pleiotropic cytokines. Again, the two lower doses of nanosilver had a pro-inflammatory effect, stimulating the synthesis of pro-inflammatory IL-1β and TNFα while limiting the level of the anti-inflammatory L-10. In contrast, the highest dose significantly decreased levels of the pro-inflammatory cytokines and raised the level of IL-10.

The growing body of references on production of cytokines after administration of silver nanoparticles to animals yields discrepant results. In general, studies on the anti-inflammatory effect of AgNPs applied externally as wound dressing or ointment for treatment of injuries and burns have generated good clinical effects, and one of the aspects regarded as a beneficial consequence of the application of nanosilver is the depression or modulation of the expression of pro-inflammatory cytokines, such as TNF-α, IL-6, IL-8, IL-12, TGF-β (Wright et al. 2002, Bhol and Schechter 2005, Nadworny et al. 2008). However, it is known that AgNPs do not penetrate much through the skin and thus the observed effects concerned a localized action of nanosilver. Contradictory results have been achieved when analyzing other routes of administration of AgNPs into an organism. For example, Park et al. (2010), who administered AgNPs to mice orally for 2 or 4 weeks, noticed a substantial increase in the level of cytokines and IgE in the peripheral blood under higher concentrations of nanosilver (0.5-1 mg/kg b.w.). More intensive simultaneous secretion of TGF-β, cytokines Th1 (IL-12), Th2 (IL-4, IL-10) and pro-inflammatory cytokines (IL-1, IL-6) was observed, although the response of Th2 was more pronounced, which – the authors claimed – indicated potential induction of allergic response by nanoparticles of silver. A similar conclusion was drawn by Xu et al. (2013), who examined the adjuvant effect of silver nanoparticles in mice immunized peritoneally or subcutaneously with ovalbumin or bovine serum albumin. Silver nanoparticles applied together with the proteins produced an effect comparable to the influence of classical adjuvant (CFA and Alum), mainly with respect to the Th2-type response (an elevated IgG1/IgG2a ratio and an increase in the concentration of IgE), which – as the cited authors concluded – may lead to the formation of an allergic response. The same authors also observed an increase in the level of the pro-inflammatory cytokines TNFα and IFNγ following the peritoneal administration of nanosilver to mice. Also the intratracheal administration of AgNPs to rats caused a pro-inflammatory response, which was manifested as an increase in the level of pro-inflammatory cytokines (TNFα and IL-6) and altered concentrations of oxidants and antioxidants in BALF (Liu et al. 2013).

The pro-inflammatory effect of the low doses of AgNPs observed herein can be claimed, at least to some extent, as concordant with the results of the cited research. A similar effect was also obtained in our previous experiment, when the administration period of the same nanocolloid to mice was shorter (7 and 14 days) (Małczewska 2011b). None of the referred papers explains the reason why the highest dose of silver nanoparticles produced an opposite effect. In our earlier studies, after a shorter administration period, the highest rate of AgNPs had a pro-inflammatory effect, similarly to the other two tested doses. The effect of the former concentration of the nanocolloid observed after 28 days of administration on the synthesis of cytokines by cells previously stimulated with LPS could be claimed to reflect an extremely beneficial anti-inflammatory effect, but when correlated to the depressed synthesis of cytokines by resting splenocytes and an exceptionally strongly expressed proliferation of B cells by the
same dose of silver nanocolloid, the overall effect does not seem to be univocal. Obviously, most of the changes in the response of splenocytes caused by this dose of nanosilver resulted from the strong stimulation of the secretion of IL-10, which is the most important anti-inflammatory cytokine, an inhibitor of the synthesis of the Th1 cytokines (IL-2, IFN-γ) and pro-inflammatory cytokines of phagocytizing cells (TNFα, IL-1, IL-6, IL-8) as well as a stimulator of the proliferation of B cells. Like other anti-inflammatory cytokines under physiological conditions, IL-10 reduced the harmful effect of prolonged or excessively intensive inflammation. However, in a very high concentrations IL-10 can inhibit the mechanisms engaged in the removal of microorganisms from an organism, and the equilibrium in the cytokine milieu plays an essential role in the inflammatory and immune responses of the body (Dinarello 2000, Opal and DePalo 2000). In the course of some inflammations, invasive diseases or long-lasting exposure to stress, the hyporesponsiveness of the immune system may occur, due to the lack of balance in the cytokine-related response of the body. In healthy individuals, a response to an inflammatory agent involves a balanced reaction of cytokines, with a simultaneous secretion of pro- and anti-inflammatory cytokines. But in diseased or stressed patients, this response becomes unbalanced, with the dominant activity of IL-10, which may depress the host’s immune response when the level of other cytokines, including pro-inflammatory ones, is lower. The effect is the suppression of cellular resistance and stimulation of humoral response, i.e. disturbed Th1/Th2 balance rather than the general immunosuppression (Benard et al. 2001, Viveros-Paredes et al. 2006).

Recapitulating, the results obtained in the present experiment clearly indicate that silver nanoparticles administered orally can easily reach the peripheral immune organs, such as the spleen, irrespective of the applied dose of silver nanocolloid. The 28-day administration of the preparation had a significant effect on the activity of splenocytes. Contrary to the expectations, the effect achieved by the lowest dose of the colloid (retardation of the proliferation of T cells, stimulated synthesis of pro-inflammatory cytokines) seems more harmful to a healthy organism at first sight than that produced by higher rates of silver nanoparticles, although the exact effects are difficult to assess unequivocally. The effect of long-term administration of silver nanocolloid as a dietary supplement appears difficult to predict and it is obvious that prior to making any precise recommendations and establishing optimum dosage of silver nanoparticles, some further and broad-scale studies are needed.

References


