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Original article

The *in vitro* effect of commercially available noble metal nanocolloids on the splenocyte proliferative response and cytokine production in mice

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Abstract

Noble metal nanoparticles, currently among the most popular types of nanomaterials, are capable of penetrating through biological barriers once they enter a living organism. There, they can permeate into organs possessing the reticuloendothelial system, such as the spleen. The objective of this study was to determine the effect of commercial nanocolloids of noble metals (silver, gold and copper), recommended by the manufacturer as dietary supplements, on the *in vitro* viability, proliferative activity and production of cytokines (IL-1β, IL-2, IL-6, IL- 10 and TNF-α) by mouse splenocytes.

All of the analyzed colloids had some effect on the activity of mouse splenocytes. Silver colloid was characterized by high toxicity – concentrations of 1.25 ppm and above substantially depressed the viability of cells as well as their proliferative activity and ability to synthesize cytokines. The other two colloids were far less toxic than nanosilver, although their non-toxic concentrations had a significant effect on the production of cytokines by mitogen activated splenocytes. The colloid of gold decreased the level of IL-2, and the colloid of copper caused an increase in IL-2, IL6 and Il-10. At the same time, copper colloid alone induced the synthesis of IL-1β in mitogen unstimulated cells. The results indicate that colloids of noble metals are capable of affecting the activity of immunocompetent cells in important peripheral organs of the immune system.

Key words: noble metal nanocolloids, mouse splenocytes, proliferative response, cytokines

Introduction

Nanoparticles (NPs) of noble metals are broadly used in various branches of industry, owing to their unique physicochemical attributes. Because of their small size, which enhances the surface to volume ratio, the number of atoms exposed on the surface of a nanoparticle is much higher and consequently the reactivity of NPs is superior to that of bulk materials. Unlike many other nanomaterials, nanoparticles of noble metals have also found a variety of biomedical applications, one of the reasons being their antibac-

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terial properties. As the range of possible applications of nanoparticles in various life areas increases, so does the risk of exposure of a living organism to their action. There are three main routes through which NPs can enter an organism: alimentary, inhalatory and cutaneous one, with the alimentary portal being particularly important in the case of disinfectants such as noble metal nanoparticles. There is a wealth of reports indicating that irrespective of the way noble metal NPs reach an organism, because of their extremely small size, they can penetrate through biological barriers, enter the circulatory system and affect internal organs. However, their distribution in mammalian tissues and body depends on several factors, e.g. route of administration, size of particles, aggregability, ability to react with the body's proteins and species-specific properties. In all studies on rodents testing noble metal NPs, independent of the route of administration, the spleen was one of the target organs eventually affected by these substances, most probably because it is one of the principal organs possessing the reticuloendothelial system, whose function is to eliminate foreign particles from the blood stream. Thus, the toxic effect of excessive quantities of nanoparticles penetrating into an organism may lead to immunosuppression due to the impact on the spleen, an essential organ of the immune system (Takenaka et al. 2000, 2001, Pelkonen et al. 2003, Chen et al. 2006, Lankveld et al. 2010, Park et al. 2010a, Zhang et al. 2010).

The objective of this study was to evaluate the effect of commercial nanocolloids of noble metals, such as silver, gold and copper, on the *in vitro* activity of mouse splenocytes. The effect of nanoparticles on the viability, proliferative activity and production of cytokines was determined experimentally. The results are extremely important in practice, as the manufacturer of the tested colloids claims they can be taken orally as a dietary supplement.

Materials and Methods

Noble metal nanocolloids

Three commercially available colloidal nonionic solutions of silver, gold and copper nanoparticles (AgNPs, AuNPs, CuNPs), called by the producer "Silver water", "Gold water" and "Copper water" (Nano-Tech Poland), containing metallic nanoparticles (Ag: 10-20 nm, Au and Cu: up to 5 nm), suspended in demineralised water, at a concentration of 50 ppm were used as a source of nanoparticles. Just before use the nanocolloids were dissolved in the cell growth medium to reach the final concentrations of: 0, 0.15, 0.3, 0.6, 1.25, 2.5, 5 and 10 ppm.

Mice

The experiment was performed on 48 male Swiss mice, aged 10-12 weeks, with body weight of 24-28 g. The animals were randomly divided into six equal groups. They were anaesthetised by inhalation of AErrane (isofluranum, Baxter Poland), and the spleens was sampled after bleeding. Splenocytes isolated from the individuals within each group were pooled before performing the assays, and tested in duplicate (ELISA) or triplicate (MTT assay). The experiment has been approved by the Local Ethics Committee.

Isolation of splenocytes

The spleens were removed aseptically, and pressed through a 60-µm nylon mesh in RPMI-1640 medium with L-glutamin and sodium bicarbonate (Sigma-Aldrich). The splenocyte cell suspensions were 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 at 250 g for 10 min. Viability of isolated cells was evaluated by trypan blue exclusion 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), 1% antibiotic-antimycotic solution (Sigma-Aldrich) and different concentrations of silver, gold and copper nanocolloids, and dispensed into 96-well plates at a concentration of $2x10^6$ cells ml^{-1}. Then the cells were cultured at 37°C under a humidified atmosphere of 5% CO_2 and 95% air atmosphere and used for the following assays.

Proliferative response of splenocytes (MTT assay)

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) at a concentration of 5 µg ml^{-1} as a T-cell mitogen or lipopolysaccharide from *Escherichia coli* (LPS, Sigma-Aldrich) at a 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 incubated for 72 h in the presence of different concentrations of noble metal nanocolloids. After incubation, 10 µl of solution containing 7 mg ml^{-1} of MTT (3-[4, 5 dimethyl-

Table 1. The *in vitro* effect of noble metal nanocolloids on the viability of mice splenocytes after 48 h of incubation (% of control cells viability).

NPs	Measures	NPs concentration (ppm), n=6							
		0 (control)	0.15	0.3	0.6	1.25	2.5	5	10
Ag	M	100	97.107	109.195	113.793	52.873**	37.913**	28.735**	27.577**
	SD	12.661	8.309	8.127	10.391	6.502	4.901	8.498	10.937
Au	M	100	98.735	100.51	98.03	107.907	104.093	107.142	84.183
	SD	9.563	8.66	7.525	9.477	8.096	13.038	6.768	15.396
Cu	M	100	104.999	98.947	94.512	93.867	94.645	90.567	74.611*
	SD	13.535	14.363	9.096	12.283	11.352	7.278	9.257	13.59

Table 2. The *in vitro* effect of noble metal nanocolloids on the proliferative response of mice splenocytes after 72 h of incubation (SI).

NPs	Mitogen	Measures	NPs concentration (ppm), n=6							
			0 (control)	0.15	0.3	0.6	1.25	2.5	5	10
Ag	ConA	M	1.364	1.419	1.509	1.321	0.993**	0.929**	0.885**	0.788**
		SD	0.158	0.119	0.114	0.276	0.071	0.059	0.092	0.084
	LPS	M	1.779	1.759	2.507**	2.124	1.146**	0.971**	0.944**	0.938**
		SD	0.209	0.147	0.327	0.214	0.187	0.259	0.164	0.194
Au	ConA	M	1.307	1.286	1.572	1.358	1.303	1.219	1.165	1.093
		SD	0.106	0.176	0.141	0.158	0.152	0.118	0.147	0.109
	LPS	M	1.705	1.837	2.37**	2.012	1.908	1.731	1.665	1.524
		SD	0.234	0.15	0.114	0.287	0.225	0.243	0.253	0.132
Cu	ConA	M	1.341	1.351	1.23	1.374	1.291	1.607	1.524	1.243
		SD	0.106	0.193	0.114	0.126	0.139	0.163	0.219	0.147
	LPS	M	1.837	2.09	1.893	1.825	1.772	1.908	1.983	1.671
		SD	0.41	0.119	0.184	0.117	0.145	0.161	0.245	0.293

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

thiazoly-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 in a Sunrise absorbance reader (Tecan, Austria) at a wavelength of 570 nm with 640 nm as a reference wavelength. Samples obtained from each group 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.

MTT assay was also used to determine the viability of splenocytes incubated for 48 h in the presence of nanocolloids, without mitogens. In this case, the results were expressed as the percent of the control cells viability.

Determination of cytokine levels

Splenocytes isolated from each group of animals were plated in 24 well plates in the absence or presence of mitogens – ConA (5 µg ml^{-1}) or LPS from *Escherichia coli* (10 µg ml^{-1}) and different concentrations of silver, gold or copper nanocolloids. 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 mixed culture of mice splenocytes, so ConA was used as a stimulant in case of IL-2 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 and TNF-α (major pro-inflammatory cytokines), IL-2 (central T cells interleukin),

IL-6 (important pleiotropic cytokine) and IL-10 (major anti-inflammatory cytokine) 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 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

Viability of splenocytes in the presence of nanocolloids

The colloid of silver proved to be most toxic among the three nanocolloids tested. Within the range of concentrations from 1.25 to 10 ppm, it significantly ($p<0.01$) depressed the viability of cells (Table 1).

The toxicity of the other two nanocolloids was much lower, hence the viability of cells was lowered only by the highest applied concentration of nanoparticles (10 ppm): by 74.6% and 84.2% versus the control, in response to CuNPs and AuNPs, respectively. However, the decrease was significant only in the case of copper ($p<0.05$) (Table 1).

Proliferative response of splenocytes

Toxic concentrations of silver nanocolloid (1.25 to 10 ppm) also significantly decreased the proliferative activity of cells exposed to mitogens ($p<0.05$). At low AgNPs concentrations, stimulation of the proliferation of cells occurred, both under the influence of ConA (0.15 and 0.3 ppm) and LPS (0.3 and 0.6 ppm). The stimulation, however, was significant ($p < 0.01$) only in the case of LPS and concentration of 0.3 ppm (Table 2).

None of the tested concentrations of CuNps, not even the toxic one (10 ppm), had a significant effect on the mitogenic response of splenocytes. Regarding the colloid of gold, proliferative activity of cells was not observed to have been depressed at none of the concentration tested. Moreover, analogously to AgNPs, the concentration of 0.3 ppm significantly stimulated the proliferation of cells exposed to LPS (Table 2).

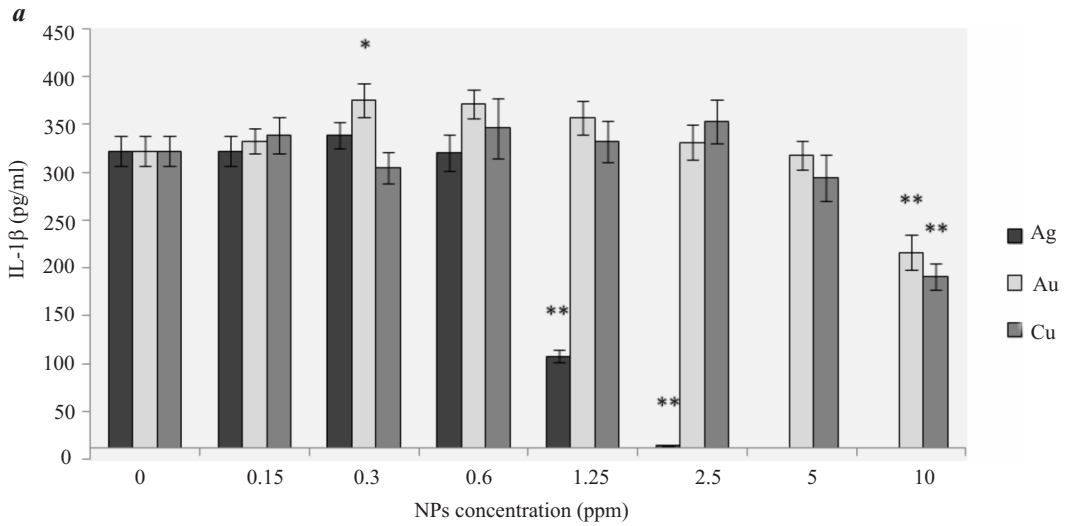
Cytokine levels in the splenocyte culture media

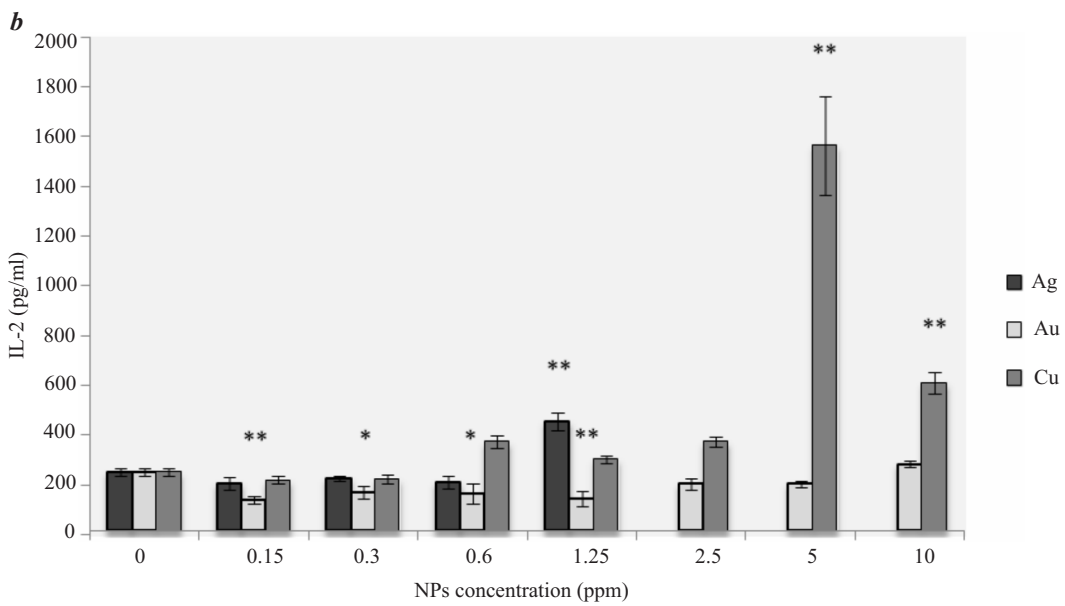
The level of cytokines (IL-1β, IL-2, IL-6, IL-10 and TNF-α) in the culture media sampled from control cells not stimulated with mitogens was very low, typically below or on the assay's sensitivity threshold. Similar results were achieved in the case of cells incubated with silver and gold nanocolloids, regardless of the concentration of nanoparticles applied. Copper nanocolloid did not affect the levels of IL-2, IL-6, IL-10 or TNF-α, either. The only substantial change concerned production of IL-1β in the presence of CuNPs at concentrations of 0.15-5 ppm. The level of this interleukin was measurable, in contrast to the control, but still very low: 35.1 do 44.5 pg/ml, independent of the applied concentration of nanoparticles (data not shown).

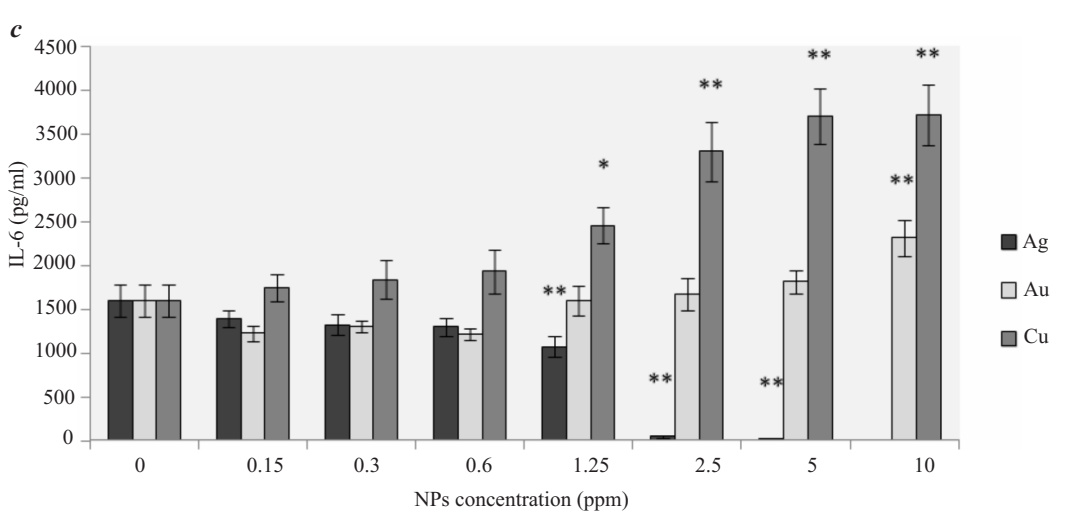
All of the nanocolloids examined, albeit to a different degree, affected production of cytokines by mouse splenocytes under the influence of mitogens (Fig. 1). In the case of silver colloid, high toxic concentrations of nanoparticles (2.5-10 ppm) considerably inhibited production of all cytokines, to the practically undetectable levels at the highest AgNPs concentration. The lowest of the toxic concentrations (1.25 ppm) demonstrated a somewhat different mode of action – it significantly depressed production of IL-1β and IL-6 ($p<0.01$), while stimulating synthesis of IL-2 and IL-10 ($p<0.01$). In turn, none of the non-toxic concentrations of AgNPs (0.15-0.6 ppm) had any effect on production of cytokines by mitogen-activated cells (Fig. 1).

With respect to gold nanoparticles, the intermediate concentrations (2.5-5 ppm) were the only ones that left production of all cytokines unaffected. The highest concentration (10 ppm) significantly decreased production of IL-1β and TNF-α ($p<0.01$), but stimulated IL-6 ($p<0.01$). On the other hand, low concentrations stimulated production of IL-1β (a significant change, $p<0.05$, was induced only by the concentration of 0.3 ppm), but decreased IL-2 (concentrations of 0.15 and 1.25 ppm at $p<0.01$; concentrations of 0.3-0.6 ppm at $p<0.05$) and TNF-α (significant decrease only at 0.3 ppm, $p<0.05$) (Fig. 1).

Regarding copper colloid, similarly to AgNPs, its low concentrations (0.15-0.6 ppm) did not affect production of cytokines by splenocytes activated by mitogens, whereas the toxic concentration (10 ppm) was the only one that had a significant effect on the level of all cytokines: it depressed production of IL-1β and TNF-α ($p<0.01$), but stimulated production of IL-2, IL-6 and IL-10 ($p<0.01$). The intermediate concentrations stimulated production of IL-2 (significant change only at the concentration of 5 ppm, $p<0.01$), IL-6 (concentrations of 1.25-5 ppm,







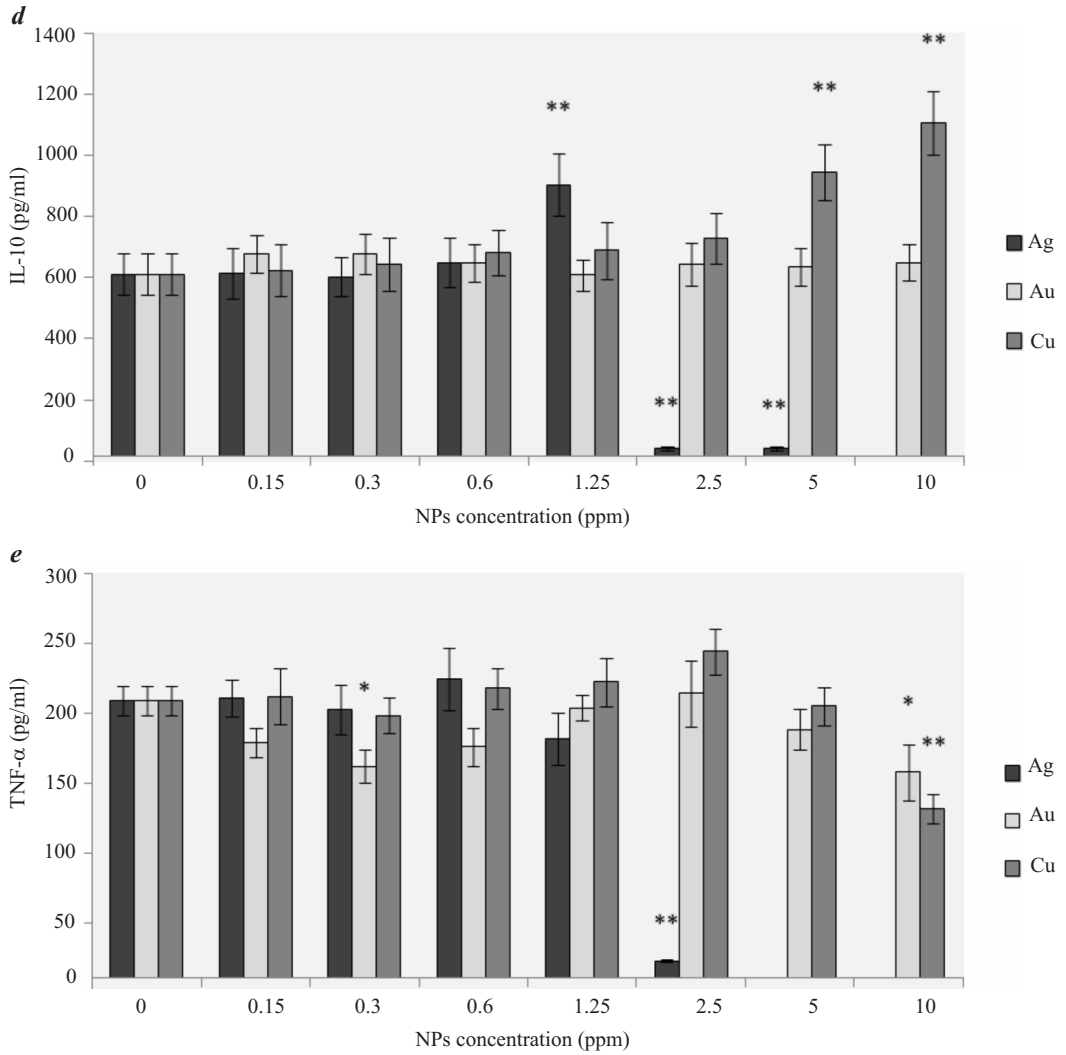


Fig. 1. The *in vitro* effect of noble metal nanocolloids on the cytokine level (pg/ml) in the culture media after 72h of splenocyte incubation in the presence of mitogens: LPS (*a, c, d, e*) or ConA (*b*); *a* – IL-1β, *b* – IL-2, *c* – IL-6, *d* – IL-10, *e* – TNF-α. Explanations: * – difference statistically significant in comparison to control (0) at $p < 0.05$
** – difference statistically significant in comparison to control (0) at $p < 0.01$

$p<0.01$) and IL-10 (significant change only at the concentration of 5 ppm, $p<0.01$) (Fig. 1).

Discussion

The research on kinetics and distribution of noble metal NPs in rodents, cited in the introduction, makes it evident that they can penetrate into organs containing large amounts of phagocytic cells. At the same time, numerous studies using monocyte/macrophage cell lines imply that metallic NPs can be toxic to phagocytic cells via induction of ROS and apoptosis or necrosis (Foldbjerg et al. 2009, Yen et al. 2009, Park et al. 2010b, Greulich et al. 2011, Park et al. 2011, Marttnez-Gutierrez et al. 2012). There are, however, very few investigations on the effect of noble metal NPs (mainly silver) on viability of primary cultures of lymphocytes. In a report of Shin et al. (2007), high concentrations of AgNPs (15-30 ppm) were toxic to the mixed culture of mononuclear cells isolated from human peripheral blood, while Greulich et al. (2011) demonstrated that the influence of AgNPs (5-30 μg/ml) mainly depended on the type of cells: T lymphocytes were not sensitive to the toxic effect of silver but monocytes became less viable (from the concentration of 25 μg/ml). In the present study, about 95% of the isolated cells represented non-adherent cells (lymphocytes), while only the remaining 5% was composed of adherent (phagocytic) cells, and silver nanoparticles proved to be highly toxic to this mixed culture of splenocytes (concentrations from 1.25 ppm). In the author's earlier study, conducted with the same silver colloid on

cells isolated from another strain of mice (NMRI), AgNPs were also shown to be toxic, but the viability of splenocytes was depressed by higher concentrations (from 5 ppm), while peripheral blood mononuclear cells were more sensitive (from 2 ppm) (Małaczewska 2010). The higher toxicity of the Ag nanocolloid on Swiss mice splenocytes may be conditioned by the strain-specific vulnerability of these mice.

Copper nanoparticles, although much less toxic, also impaired the viability of mouse splenocytes when applied at the highest concentration. Admittedly, the available literature lacks any reports on the effect of CuNPs on viability of immune cells, but it has been demonstrated on other cell types that, similarly to nanosilver, the toxicity of copper nanoparticles depends on the degree of their solubility and capacity for releasing ions when inside a cell, in the acidic pH of the lysosomes (Trojan horse-type mechanism) (Studer et al. 2010). Furthermore, excess of copper, despite it being an essential element and a constituent of several important enzymes, may generate an immunosuppressive response. Mitra et al. (2013) revealed stimulation of CD4+ cells apoptosis in the spleen and thymus of Swiss mice by sublethal doses of copper salts.

The lack of toxicity of gold colloid was an unsurprising finding because low reactivity is a well-known property of this noble metal. Analogously to the present results, Downs et al. (2010) verified that gold colloids containing nanoparticles of different size (1 and 20 nm, concentrations of 6 and 11 μg/ml) did not act toxically on cells in mouse or human peripheral blood. In a study of Connor et al. (2005), it was concluded that even very high concentrations of AuNPs (up to 250 μM) were not toxic on a line of human leukaemic cells K562.

Another parameter analyzed in this research was the effect of nanocolloids on proliferation of splenocytes after stimulation with mitogens. References dealing with this issue are also extremely scanty and discuss only silver nanoparticles. For example, Greulich et al. (2011) concluded that AgNPs (5-30 μg/ml) did not produce any effect on proliferation of human T cells exposed to ConA. In contrast, Shin et al. (2007) recorded a decline in proliferation of human PBMCs (peripheral blood mononuclear cells) stimulated with PHA in the presence of high concentrations of nanosilver (from 10 ppm), and the concentrations which inhibited proliferation were simultaneously the ones that depressed viability of cells, a finding which corresponds with the results of the present experiment. None of the researchers described stimulation of proliferation at low concentrations of NPs, a situation which occurred herein at a concentrations of 0.3 ppm of either silver or gold colloid. A similar effect had also been obtained in the author's earlier research on the same silver colloid, conducted on cells of NMRI mice, at concentrations of 0.2-0.5 ppm (Małaczewska 2010).

The last question raised during this research on the effect of noble metal NPs on the activity of splenocytes was the influence of the former on synthesis of cytokines. The significant decrease of all cytokine levels in mitogen-stimulated splenocytes observed at high concentrations of AgNPs (2.5 – 10 ppm), as well as the modulation of cytokine production by the lowest toxic concentration (1.25 ppm), combined with significant depression of cells' viability and proliferation, were the obvious aspects of their cytotoxicity. Contrary to that, non-toxic concentrations of the same nanocolloid had no effect on the given parameter, not even at the level of 0.3 ppm, which stimulated proliferation of cells under the influence of LPS. The literature contains several reports on the effect of AgNPs on production of cytokines by different cell types, but the results obtained by different authors are not always congruent. Shin et al. (2007) observed a decrease induced by AgNPs in the synthesis of cytokines by mononuclear leukocytes of human peripheral blood stimulated with PHA. In this study high concentrations of nanoparticles inhibited production of cytokines due to the cytotoxic effect, but also lower, non-toxic concentrations depressed the synthesis of IL-1β and IL-6. On the other hand, Greulich et al. (2011) demonstrated lack of any effect whatsoever of nanosilver on production of cytokines by T lymphocytes, while Yang et al. (2012) showed that it did not stimulate production of IL-1β by peripheral blood mononuclear cells. Also studies on phagocytic cells describe various effects of non-toxic concentrations of nanoparticles: from proinflammatory effect, e.g. increased expression of TNF-α (Park et al. 2010b), IL-1β (Yang et al. 2012), IL-6 and TNF-α (Martinez-Gutierrez et al. 2010), IL-1α and β, IL-6, TNF-α (Park et al. 2011) or IL-6 and IL-8 (Greulich et al. 2011), through slight stimulation of proinflammatory IL-1β and IL-6 (Santoro 2007), to lack of any effect on expression of IL-1β, IL-6 and TNF-α (Yen 2009). The researchers who had obtained more intense synthesis of proinflammatory cytokines by phagocytes attributed this effect to the ingestion of NPs by phagocytes, intracellular ionization of particles, formation of ROS and, consequently, stimulation of cells and proinflammatory action (Park 2010b, Greulich et al. 2011, Park et al. 2011, Yang et al. 2012). In the experiment reported herein, the percentage of phagocytic cells was small, hence the lack of effect produced by nanosilver on production of

cytokines seems justifiable and stems from differences in the sensitivity of various types of immune cells to the activity of these particles, as described by Greulich et al. (2011).

A different effect on production of cytokines by mouse splenocytes was exerted by the other two colloids. The highest concentration of gold colloid (10 ppm) modulated cytokine response of stimulated splenocytes. However, because this concentration depressed viability of cells and their proliferation, even though these effects were not statistically significant, it can be concluded that the influence on secretion of cytokines was a result of the toxic action of that concentration, particularly so because two slightly lower concentrations (5 and 2.5 ppm) did not affect the given parameter. Noteworthy was the effect of low concentrations of nanogold. The observed decrease in production of IL-2 by activated cells seems to be an unfavourable development because IL-2 is an important cytokine of T cells, essential for their proper growth and functioning, and in extreme cases its dysfunctions may lead to autoimmunity or immunological deficiencies (Lan et al. 2008). In the present study, however, no negative effect of nanogold on the viability or proliferative activity of T cells was observed. At the concentration of 0.3 ppm of AuNPs, the stimulation index of T cells was even higher than that found in the control (statistically a non-significant difference). The same concentration of 0.3 ppm, the most active among the analyzed ones, additionally enhanced proliferation of LPS-stimulated cells and decreased the level of TNF-α while raising IL-1β. Such a mode of action is not unambiguous and is difficult to interpret. Both IL-1β and TNF-α are proinflammatory cytokines predominantly secreted by macrophages and to a much lesser degree by other types of cells, acting synergistically; the level of both cytokines usually increases simultaneously at a site of inflammation (Dinarello 2000). Such an effect had been observed previously, when gold nanoparticles at a low concentration (1 ppm) increased the expression of proinflammatory cytokines IL-1β, IL-6 and TNF-α in a culture of mouse macrophages after just 3-6 hours of incubation (Yen et al. 2009). However, after longer incubation (24-72 h), the expression of both cytokines decreased and this effect, according to the researchers cited, proved that nanoparticles of gold were quickly ingested by macrophages, activating an inflammatory response. The raised level of IL-1β coinciding with a decrease in TNF-α, recorded in the present experiment may have been caused by the prolonged incubation of cells with nanogold (72 h), especially that the change observed in both cases was not particularly strongly pronounced, despite being statistically significant.

The colloid of copper was the most active one with respect to modifications in synthesis of cytokines. CuNPs alone, i.e. without mitogens, stimulated synthesis of IL-1β almost within the whole spectrum of concentrations. Although the level of this pro-inflammatory cytokine was not high, the above evidence almost certainly proves that splenocytes were activated by nanocopper. When cells were incubated simultaneously with non-toxic concentrations of CuNps and LPS, a considerable increase in the level of IL-6 was observed at concentrations of 1.25-5 ppm. This pleiotropic cytokine plays an important role in regulation of the inflammatory process by inhibiting synthesis of IL-1 and TNF-α, while itself being inhibited by anti-inflammatory cytokine IL-10 (Opal and DePalo 2000). What is more, the highest non-toxic concentration (5 ppm) intensified additionally the synthesis of IL-2 by ConA stimulated splenocytes and IL-10 after LPS stimulation, which supports the claim that nanocopper is able to activate immunocompetent cells. The results obtained are somewhat contradictory to those reported by Cohen et al. (2013) who demonstrated an *in vitro* pro-inflammatory effect of CuO nanoparticles on the human skin model, manifested by rising levels of IL-1α, TNFα and IL-6, decreasing level of IL-1β and declined viability of cells. In the experiment described herein, despite similar stimulation of IL-6 and depressed IL-1β at the concentration of 5 ppm, no cytotoxic or adverse effect on proliferation of splenocytes was observed, and the additional stimulation of pro-inflammatory IL-10 in conjunction to the lack of effect on TNF-α seem to imply that a different mechanism is involved in the action of CuNPs than that of CuO NPs. This effect may be associated with a different composition of particles, as in a study of Studer et al. (2010), where toxicity of CuO was extremely high compared to carbon-stabilized copper nanoparticles, which were hardly toxic at all.

Recapitulating, all the analyzed commercial nanocolloids of noble metals affected the activity of mouse splenocytes, although they acted differently. From the concentration of 1.25 ppm and above, AgNPs were characterized by high toxicity, manifested by the depressed viability and proliferative activity of cells as well as their inferior ability to synthesize cytokines following stimulation with mitogens. Despite certain expectations, the non-toxic concentrations did not have any effect on the inflammatory response of cells measured by the level of cytokines, although increased proliferation of cells was observed at the concentration of 0.3 ppm in the presence of LPS. The other two colloids were characterized by toxicity lower than that of nanosilver, but their non-toxic concentrations affected significantly production of cytokines by

activated splenocytes. Gold nanocolloid had the strongest effect on CD4+ cells decreasing the level of IL-2, while CuNPs increased both ConA (IL-2) and LPS stimulated cytokine response (IL-6 and IL-10 levels). Of these two colloids, copper had a stronger influence on mouse splenocytes, as it additionally activated the synthesis of IL-1β in mitogen unstimulated cells. In view of the proven ability of NPs to permeate through the intestinal barrier and to affect important organs of the immune system, it becomes obvious that their administration as dietary supplements does not leave the living organism unaffected. Further studies are necessary in order to recognize possible benefits and risks of dietary supplementation with nanoparticles.

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