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Original article

Effect of oral administration of commercial gold nanocolloid on peripheral blood leukocytes in mice

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Abstract

During the last few decades, owing to their unique properties, gold nanoparticles (AuNPs) have found numerous biomedical applications. Studies on rodents prove that AuNPs entering an organism easily reach the bloodstream and undergo wide tissue distribution. The presence of nanoparticles inside blood and bone marrow cells of exposed animals may implicate its influence on hematopoiesis and the functions of peripheral blood leukocytes.

The aim of this study was to determine the effect of oral administration of commercial gold nanocolloid, recommended by the producer as a dietary supplement, on the percentage of lymphocyte populations and proliferative response, as well as the activity of phagocytes in the peripheral blood of mice. 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). Mice given nanoparticles showed increased activity of phagocytes and some changes in the lymphocyte phenotypes. The elevated activity of granulocytes and monocytes, in terms of both phagocytic and respiratory burst activity, was transient and noticed only after a short time of administration, which may indicate some adaptability of blood phagocytes to prolonged presence of gold nanoparticles in the body. However, phenotypic modifications among lymphocytes in the group of animals given the middle dose of colloid (i.e. increased percentage of B and CD4+CD8+ DP T cells) did not occur until after the 28-day administration, which in turn seems indicative of some immune dysregulation due to the prolonged contact with nanogold.

Key words: gold nanocolloid, immunophenotyping, phagocytic and respiratory burst activity

Introduction

Gold nanoparticles (AuNPs), structures composed of hundreds of gold atoms, but less than 100 nm in size, are a bridge between bulk materials and atomic or molecular structures. They come in different shapes and sizes, and their surface is often modi-

fied to achieve better stability, prevent aggregation and increase biocompatibility. A large surface to volume ratio gives rise to unique properties of nanoparticles, compared to bulk gold, which is why AuNPs find numerous biomedical applications. It is commonly believed that gold nanoparticles, like metallic gold, are non-toxic and non-immunogenic, but their small

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size enables them to permeate natural barriers, such as cell membranes, and affects their cellular processing. The higher reactivity of nanoparticles than bulk gold can be a cause of increased ROS (reactive oxygen species) formation inside cells and oxidative stress development, especially in phagocytes, which are particularly predisposed to remove foreign particles from an organism (Brown et al. 2008, Yen et al. 2009, Lasagna-Reeves et al. 2010, Zhang et al. 2010, Thakor et al. 2011, Gerber et al. 2013, Chueh et al. 2014).

Studies on rodents have proven that, regardless of administration routes, gold nanoparticles are broadly distributed in an organism and persist in tissues even months after their administration. AuNPs accumulate mostly in the liver and spleen, organs whose reticuloendothelial system removes them from circulation. However, gold nanoparticles have also been found in other tissues, mainly inside phagocytes. Macrophage stimulation can contribute to the development of a systemic immune response, but before they reach target organs, AuNPs enter the bloodstream, where they can react with blood cells (Hillyer et al. 2001, Sadauskas et al. 2007, Sonavane et al. 2008, Balasubramanian et al. 2010, Lasagna-Reeves et al. 2010, Arnida et al. 2011, Hirn et al. 2011, Thakor et al. 2011, Chen et al. 2013). Having administered AuNPs to mice *p.o.*, Zhang et al. (2010) observed a decrease in the erythrocyte count as well as the presence of nanoparticles on the surface of cellular membranes and in vesicles inside blood and marrow cells. Also, after intravenous application, gold nanoparticles have been detected in the cellular fraction of the peripheral blood of rats and in the bone marrow of mice (Arnida et al. 2011, Hirn et al. 2011). These observations may suggest that AuNPs affect hematopoiesis and the functions of peripheral blood leukocytes. However, there is a serious lack of studies dedicated to the evaluation of their potential impact on both the lymphocytes and phagocytes of affected animals. In the available literature only two articles deal with this issue – one describing the adjuvant properties of colloidal gold in laboratory animals immunized with various antigens (higher antibody titers, lysozyme and complement concentrations in animal sera and phagocytic activity of peripheral blood) and the second, describing the immunomodulatory effects of gold nanoparticles conjugated with herbal PST polysaccharide after its intraperitoneal injection in mice (increased lymphocyte proliferation, percentages of CD3+, CD4+, CD8+ cells and bone marrow cellularity) (Dykman et al. 2004, Joseph and al. 2013).

The purpose of this study was to determine the effect of oral administration of commercial gold nanocolloid, recommended by the producer as a dietary supplement, on lymphocyte phenotypes and pro-

liferative response, and on the activity of phagocytes in the peripheral blood of mice. The colloid was given to the animals in three different doses (0.25, 2.5 and 25 ppm), for three different time periods (7, 14, 28 days). To the best of the author's knowledge, this is the first report describing the influence of commercial gold nanocolloid after its alimentary administration on the functions of peripheral blood leukocytes in mice.

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 then administered to mice as drinking water *ad libitum* for the following 28 days. The drinking water of the control animals was also distilled.

Mice

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 at a controlled temperature ($20 \pm 1^\circ\text{C}$) and supplied with rodent chow and water *ad libitum* throughout the experiment. The mice were divided randomly into four equal groups: control group (0) not receiving the gold solution, and three experimental groups administered the gold solution at 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). Blood was collected by heart puncture. Samples from five individuals from each group were used for the MTT assay, and from the next five for flow cytometry analysis. The experiment was approved by the Local Ethics Committee.

Immunophenotyping

100 μl of whole heparinised blood samples were transferred into individual tubes and incubated with fluorochrome-conjugated monoclonal antibodies for

30 min at 4°C in the dark. The following flow cytometry antibodies were used: FITC anti-mouse CD3 (clone 17A2), PE-Cy7 anti-mouse CD19 (clone 1D3), PE anti-mouse CD4 (clone H129.19), PE-Cy5 anti-mouse CD8 (clone 53-6.7) and APC anti-mouse CD49b (clone DX5). After incubation, erythrocytes were removed using OptiLyse C Lysing Solution (Beckman Coulter) according to the manufacturer's instruction. The samples were then washed twice with FACS buffer (FB, 1x Dulbecco's PBS without Ca²⁺ and Mg²⁺, supplemented with 2% fetal calf serum; both reagents purchased from Sigma-Aldrich), re-suspended in FB and flow cytometry analysis was then performed using a FACSCanto II cytometer (Becton Dickinson Biosciences). The data were acquired using FACSDiva version 6.1.3. software (BD Biosciences) and analyzed using FlowJo software (Tree Star Inc., Stanford, CA, USA). Percentages of individual lymphocyte phenotypes were determined after setting the gate on lymphocytes. In the case of CD4 and CD8 markers an additional analysis was made within CD3+ lymphocytes.

Isolation of lymphocytes

The whole heparinised blood was placed on a His-topaque 1077 (Sigma-Aldrich) density gradient and centrifuged at 400 g for 30 min at 20°C. The interface cells were collected and washed three times with RPMI-1640 medium. The viability of isolated cells was evaluated using 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) and 1% antibiotic-antimycotic solution (Sigma-Aldrich), and dispensed into 96-well plates at a concentration of 1x10⁶ cells ml⁻¹. The cells were then cultured at 37°C under a humidified atmosphere of 5% CO₂ and 95% air atmosphere and used for the MTT assay.

Proliferative response of blood lymphocytes (MTT assay)

Mitogenic response of lymphocytes 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 cultured for 72 h. After incu-

bation, a 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 was 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.

Activity of blood phagocytes

The blood granulocyte and monocyte phagocytic activity was measured in whole heparinised blood using a commercially available kit (Phagotest kit, Orpegen Pharma, Germany) according to manufacturer's instructions. Briefly, FITC-labelled opsonised *E.coli* bacteria were added to whole blood and incubated for 10 min at 37°C (experimental tube) or 0°C (negative control tube). After incubation, the reaction was stopped, the erythrocytes were lysed and DNA staining solution was added. Fluorescence of samples was measured using a cytometer as described above. The test determines the percentage of phagocytizing cells, granulocytes and monocytes separately, and their phagocytic activity, i.e. the number of bacteria absorbed by a single cell in terms of mean fluorescence intensity (MFI).

Respiratory burst assay was performed using a commercially available kit (Phagoburst kit, Orpegen Pharma, Germany) according to the manufacturer's instructions. Briefly, opsonised *E.coli* bacteria (experimental tube) or washing solution (negative control tube) were added to whole blood and incubated for 10 min at 37°C. Following incubation, dihydrorhodamine (DHR 123) was added for 10 min, the erythrocytes were lysed and DNA staining solution was added. DHR 123 becomes fluorescent when oxidized by reactive oxygen species, and its fluorescence was measured by flow cytometry as described above. This test determines the percentage of active cells, as well as the respiratory burst intensity within a single cell in terms of mean fluorescence intensity (MFI).

Statistical analysis

Data were analysed statistically by one-way analysis of variance (ANOVA). Bonferroni's posttest was used to determine differences between groups. Statistical evaluation of results was performed using a GraphPadPrism software package.

Table 1. Analysis of peripheral blood lymphocyte phenotypes (%) in mice after oral administration of gold nanocolloid (gate set on lymphocytes).

| Cell marker | 7 days | | | | 14 days | | | | 28 days | | | |
|-------------------|--------------|--------------|--------------|--------------|--------------|-------------|-------------|-------------|--------------|-------------|--------------|--------------|
| | 0 (K) | 0.25 | 2.5 | 25 | 0 (K) | 0.25 | 2.5 | 25 | 0 (K) | 0.25 | 2.5 | 25 |
| CD3+ | 68.78±4.221 | 66.94±1.487 | 67.86±6.05 | 65±3.325 | 65.76±7.102 | 65.56±5.987 | 64.36±3.541 | 63.52±3.502 | 67.94±3.281 | 63.76±9.731 | 64.2±8.059 | 65.78±6.234 |
| CD4+ SP cells | 50.12±3.743 | 52.72±2.622 | 53.44±6.086 | 49.08±2.558 | 49.02±5.183 | 49.86±8.72 | 48.52±3.135 | 48.14±2.325 | 52.04±4.736 | 51.9±9.882 | 44.26±5.675 | 52.24±5.246 |
| CD8+ SP cells | 12.808±3.451 | 10.916±2.269 | 11.656±3.082 | 11.778±1.543 | 10.546±2.553 | 12.06±3.411 | 12.24±1.146 | 11.58±2.442 | 13.038±3.378 | 10.116±1.45 | 15.84±3.546 | 11.206±2.081 |
| CD4+CD8+ DP cells | 0.589±0.293 | 0.67±0.291 | 0.445±0.195 | 0.481±0.226 | 0.717±0.357 | 0.639±0.267 | 0.844±0.262 | 0.643±0.321 | 0.541±0.377 | 0.914±0.363 | 1.526*±0.403 | 0.529±0.308 |
| CD19+ | 20.48 ±4.073 | 18.2±3.1 | 18.5±4.088 | 20.22±1.013 | 17.96±5.08 | 21.56±3.798 | 23.5±5.189 | 23.38±4.806 | 16.04±2.871 | 18.02±3.949 | 23.44*±3.783 | 13.986±3.599 |
| CD3+CD49b+ | 1.12±0.455 | 1.476±0.425 | 0.856±0.308 | 0.86±0.354 | 0.763±0.323 | 1.284±0.568 | 0.947±0.281 | 0.938±0.212 | 1.427±0.598 | 0.668±0.278 | 1.377±0.758 | 0.897±0.554 |
| CD3-CD49b+ | 1.354±0.197 | 0.963±0.19 | 1.31±0.12 | 1.491±0.552 | 1.119±0.517 | 1.994±0.914 | 2.098±0.476 | 1.299±0.67 | 2.126±1.289 | 1.219±0.82 | 2.102±0.674 | 1.302±0.41 |
| CD4:CD8 ratio | 4.229±1.586 | 5.052±1.361 | 4.873±1.411 | 4.233±0.664 | 4.86±1.253 | 4.668±1.616 | 3.994±0.465 | 4.368±1.286 | 4.269±1.454 | 5.214±1.369 | 2.864±0.481 | 4.762±0.785 |

Table 2. Analysis of peripheral blood CD3+ lymphocyte subpopulations (%) in mice after oral administration of gold nanocolloid (gate set on CD3+ cells).

| Cell type | 7 days | | | | 14 days | | | | 28 days | | | |
|-------------------|-------------|-------------|-------------|--------------|-------------|--------------|-------------|-------------|-------------|-------------|---------------|-------------|
| | 0 ppm | 0.25 ppm | 2.5 ppm | 25 ppm | 0 ppm | 0.25 ppm | 2.5 ppm | 25 ppm | 0 ppm | 0.25 ppm | 2.5 ppm | 25 ppm |
| CD4+ SP cells | 72.36±1.315 | 78.2±3.611 | 78.3±4.263 | 75.22±3.9264 | 72.92±3.028 | 74.68±6.927 | 74.3±1.62 | 74.64±4.089 | 75.76±5.758 | 79.7±4.609 | 67.36±3.813 | 78.42±3.343 |
| CD8+ SP cells | 18.298±5.14 | 15.86±3.41 | 16.92±4.766 | 17.74±1.853 | 14.82±2.942 | 17.616±5.242 | 18±1.892 | 17.16±3.205 | 18.52±4.262 | 15.44±2.5 | 23.86±3.6 | 16.38±2.285 |
| CD4+CD8+ DP cells | 0.819±0.425 | 0.973±0.283 | 0.626±0.251 | 0.717±0.344 | 0.864±0.364 | 0.773±0.366 | 0.972±0.249 | 0.729±0.418 | 0.597±0.41 | 1.176±0.401 | 1.836***±0.42 | 0.576±0.332 |

Explanations (for Table 1 and 2):

SP cells – single positive cells

DP cells – double positive cells

* – difference statistically significant in comparison to control group (0 ppm) at p<0.05

** – difference statistically significant in comparison to control group (0 ppm) at p<0.01

*** – difference statistically significant in comparison to control group (0 ppm) at p<0.001

Results

Immunophenotyping

After 28 days of gold nanocolloid administration, the group given the middle dose (2.5 ppm) was noticed as presenting a significant increase in percentages of B lymphocytes ($p < 0.05$) and CD4+CD8+ double positive T cells (DP T cells) ($p < 0.01$) in the whole population of peripheral blood lymphocytes (Table 1, Fig. 1). Having analyzed CD4 and CD8 subpopulations of lymphocytes within CD3+ cells of this group, the increase in the percentage of CD4+CD8+ DP T cells was even more pronounced ($p < 0.001$; Table 2, Fig. 1). No significant changes in the lymphocyte phenotypes occurred during the whole experiment in the groups given the highest and the lowest doses of AuNPs (Tables 1 and 2).

served in the group given the lowest dose of the preparation (0.25 ppm) and only after a short period of administration. In this group, after 7 days of the experiment, an increase was observed in the percentage of phagocytizing cells and phagocytic capacity (MFI) of individual cells, in both granulocytes and monocytes ($p < 0.01$). Additionally, in both populations of phagocytes in this group of mice, there was a significant increase in the percentage of cells undergoing oxidative burst (granulocyte at $p < 0.001$, monocyte at $p < 0.05$), whilst its intensity (MFI) was more pronounced only in granulocytes ($p < 0.05$) (Tables 4 and 5, Figs. 2 and 3). After 14 days, the only change detected in this group was an elevated percentage of phagocytizing granulocytes ($p < 0.01$), and after 28 days, none of the analyzed parameters were different from the control (Tables 4 and 5).

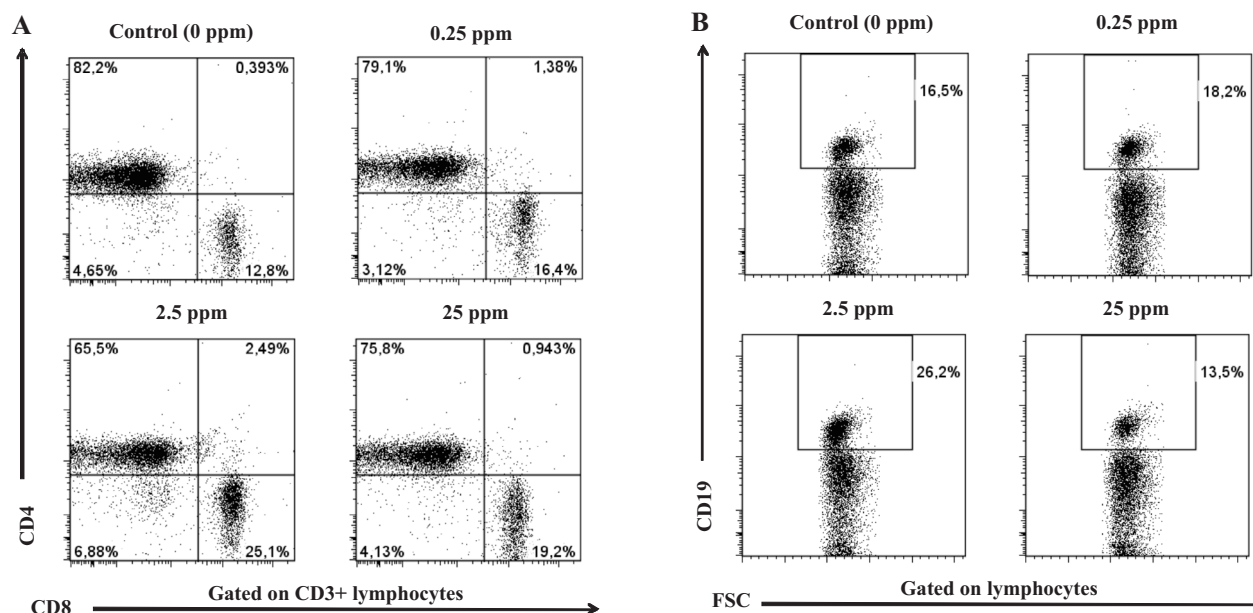


Fig. 1. Representative dot plot cytograms showing the percentage of CD4+CD8+ DP T cells (A) and CD19+ B cells (B) in mice after 28-day oral administration of gold nanocolloid.

Proliferative response of blood lymphocytes

Throughout the whole experiment, no significant differences were observed in the proliferative response of peripheral blood lymphocytes to mitogens between the experimental groups and the control animals (Table 3).

Activity of blood phagocytes

A significant effect of AuNPs on the activity of peripheral blood phagocytes in mice was mostly ob-

Regarding the groups given the two higher doses of gold nanocolloid, there was only one change noticed, namely an increased percentage of granulocytes undergoing oxidative burst after 7 days of administration (2.5 ppm at $p < 0.001$, 25 ppm at $p < 0.01$). The other parameters did not change significantly, compared to the control, during the whole experiment (Tables 4 and 5, Figs 2 and 3).

Discussion

In the current study, both peripheral blood leukocytes and splenocytes were obtained from mice

Table 3. Proliferative response of mice peripheral blood lymphocytes (SI) after oral administration of gold nanocolloid.

| Mitrogen | 7 days | | | | | 14 days | | | | | 28 days | | | | | | | | | | | | | |
|----------|-------------|------------|-------------|-------------|-------------|------------|-------------|-------------|-------------|------------|-------------|-------------|-------------|------------|-------------|-------------|-------------|------------|-------------|-------------|-------------|-------------|------------|-------------|
| | 0 ppm | 0.25 ppm | 2.5 ppm | 25 ppm | 0 ppm | 0.25 ppm | 2.5 ppm | 25 ppm | 0 ppm | 0.25 ppm | 2.5 ppm | 25 ppm | 0 ppm | 0.25 ppm | 2.5 ppm | 25 ppm | | | | | | | | |
| ConA | 1.304±0.151 | 1.269±0.14 | 1.189±0.163 | 1.258±0.205 | 1.365±0.196 | 1.51±0.143 | 1.331±0.104 | 1.413±0.154 | 1.334±0.145 | 1.447±0.16 | 1.457±0.128 | 1.383±0.163 | 1.459±0.165 | 1.415±0.17 | 1.239±0.114 | 1.325±0.184 | 1.456±0.342 | 1.508±0.19 | 1.416±0.119 | 1.644±0.285 | 1.465±0.171 | 1.429±0.155 | 1.417±0.17 | 1.475±0.164 |

Table 4. Phagocytic activity of mice peripheral blood granulocytes and monocytes after oral administration of gold nanocolloid (% of active cells and MFI) – Phagotest.

| Parameter | 7 days | | | | | 14 days | | | | | 28 days | | | | | | | | | | | | | |
|-----------------|-------------|-------------|-------------|-------------|-----------|-------------|-------------|-------------|--------------|--------------|-------------|--------------|-------------|-------------|-----------|------------|-------------|-------------|-------------|------------|-------------|-------------|------------|------------|
| | 0 ppm | 0.25 ppm | 2.5 ppm | 25 ppm | 0 ppm | 0.25 ppm | 2.5 ppm | 25 ppm | 0 ppm | 0.25 ppm | 2.5 ppm | 25 ppm | 0 ppm | 0.25 ppm | 2.5 ppm | 25 ppm | | | | | | | | |
| Granulocyte % | 58.1±13.073 | 88.82±3.955 | 76.36±6.949 | 62.42±13.82 | 56±12.017 | 80.76±6.803 | 72.62±11.13 | 62.56±5.167 | 70.86±15.263 | 73.48±10.383 | 60.3±10.935 | 63.34±14.533 | 25.56±2.371 | 40.76±0.498 | 31.6±3.86 | 23.4±4.919 | 30.32±9.727 | 20.84±5.489 | 31.78±5.012 | 18.3±4.251 | 22.32±5.627 | 22.28±4.657 | 28.2±5.784 | 18.32±8.44 |
| Monocyte % | 12302.4 | 18060** | 15220 | 11888 | 12997.2 | 17620 | 15580 | 14740 | 11962.4 | 13960 | 12000 | 12940 | ±3072.04 | ±702.139 | ±1316.055 | ±2366.373 | ±2834.385 | ±2438.647 | ±2912.387 | ±2052.559 | ±2137.117 | ±907.193 | ±1097.725 | ±2237.856 |
| Granulocyte MFI | 5722.2 | 9823** | 7294.8 | 7256 | 8229.2 | 8330.2 | 8691 | 8295 | 7917.2 | 8803.6 | 6858.2 | 8925.6 | ±862.057 | ±958.296 | ±1221.976 | ±1976.951 | ±1407.11 | ±1623.263 | ±1689.32 | ±1626.665 | ±916.833 | ±980.712 | ±947.281 | ±1836.585 |

Table 5. Respiratory burst activity of mice peripheral blood granulocytes and monocytes after oral administration of gold nanocolloid (% of active cells and MFI) – Phagoburst.

| Parameter | 7 days | | | | | 14 days | | | | | 28 days | | | | | | | | | | | | | |
|-----------------|---------|----------|----------|--------|--------|----------|---------|--------|--------|----------|---------|--------|----------|-----------|----------|-----------|----------|----------|----------|---------|-----------|-----------|----------|-----------|
| | 0 ppm | 0.25 ppm | 2.5 ppm | 25 ppm | 0 ppm | 0.25 ppm | 2.5 ppm | 25 ppm | 0 ppm | 0.25 ppm | 2.5 ppm | 25 ppm | 0 ppm | 0.25 ppm | 2.5 ppm | 25 ppm | | | | | | | | |
| Granulocyte % | 39.78 | 93.1*** | 85.84*** | 76** | 54.66 | 57.44 | 61.62 | 61.66 | 52.6 | 66.98 | 59.18 | 64.36 | ±9.873 | ±2.186 | ±5.084 | ±22.179 | ±6.21 | ±7.061 | ±1.807 | ±3.147 | ±14.636 | ±7.681 | ±8.438 | ±7.355 |
| Monocyte % | 12.1898 | 47* | 20.774 | 19.756 | 31.68 | 30.98 | 34.76 | 26.72 | 22.38 | 33.24 | 18.98± | 24.22± | ±9.008 | ±28.546 | ±12.907 | ±10.256 | ±13.056 | ±5.489 | ±16.466 | ±8.208 | ±12.07 | ±12.642 | 11.559 | 14.027 |
| Granulocyte MFI | 3079.8 | 5512.6* | 4546.2 | 3942.8 | 3457.6 | 3909.4 | 3650.4 | 4045.6 | 4221 | 5072.6 | 3108.6 | 4538.2 | ±788.971 | ±1253.106 | ±997.7 | ±1199.344 | ±946.749 | ±866.813 | ±477.898 | ±487.78 | ±1278.396 | ±1906.406 | ±168.594 | ±1516.701 |
| Monocyte MFI | 2709.4 | 2604.2 | 2504.2 | 2450.4 | 1750.4 | 1830.8 | 1809.6 | 1798.4 | 2011.4 | 1968.8 | 2337.4 | 2024.4 | ±687.083 | ±178.585 | ±308.405 | ±240.501 | ±72.099 | ±91.898 | ±136.436 | ±57.116 | ±219.135 | ±144.072 | ±568.491 | ±145.742 |

Explanations (for Table 2 and 3):

* – difference statistically significant in comparison to control group (0 ppm) at p<0.05

** – difference statistically significant in comparison to control group (0 ppm) at p<0.01

*** – difference statistically significant in comparison to control group (0 ppm) at p<0.001

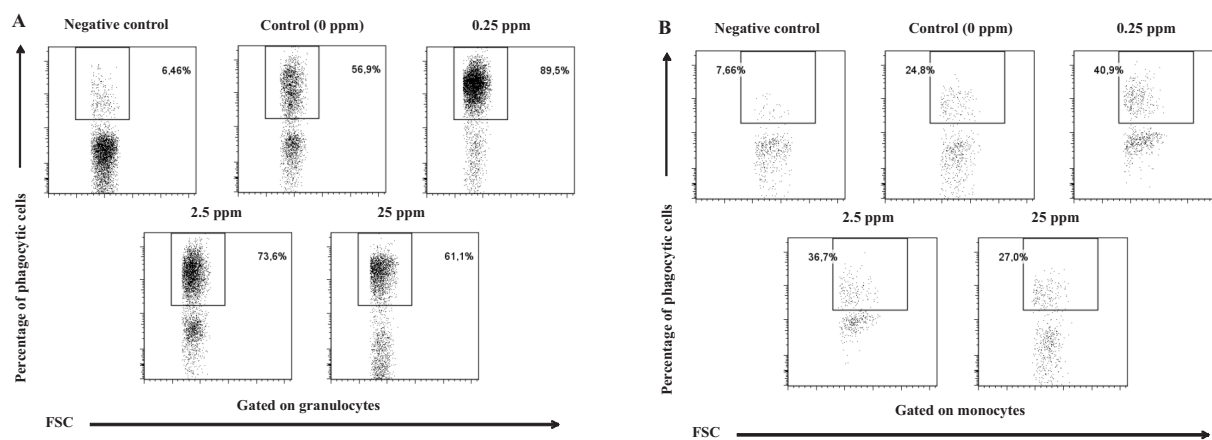


Fig. 2. Representative dot plot cytograms showing the percentage of phagocytic granulocytes (A) and monocytes (B) in mice after 7-day oral administration of gold nanocolloid

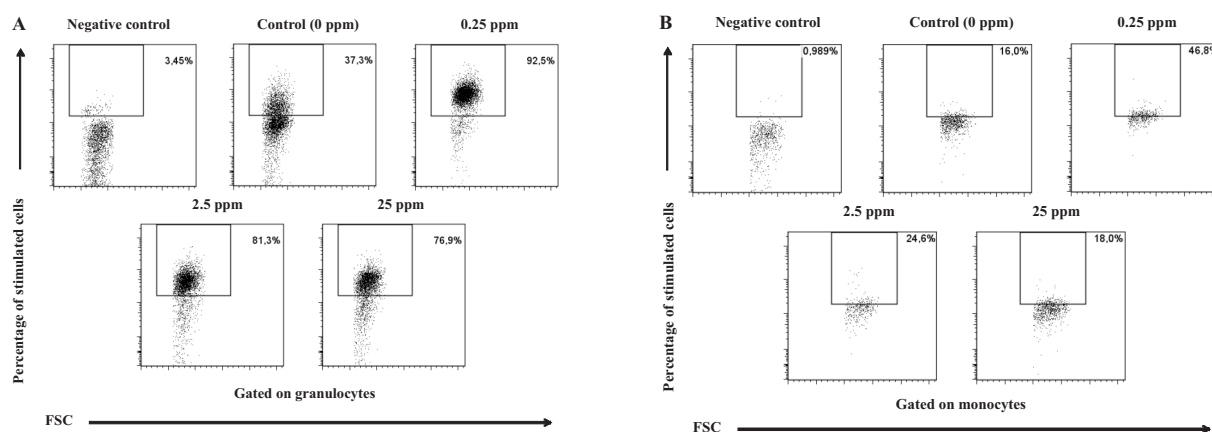


Fig. 3. Representative dot plot cytograms showing the percentage of granulocytes (A) and monocytes (B) undergoing respiratory burst following *E.coli* stimulation in mice after 7-day oral administration of gold nanocolloid

administered gold nanocolloid. As expected, the effect of the nanoparticles on splenocytes was more strongly manifested, which is quite common in the case of substances introduced through the alimentary tract. However, the results obtained were presented in another article (Małaczewska 2015). With respect to peripheral blood leukocytes, the sole effect was an increased activity of phagocytes in the early administration of gold nanocolloid and some modifications in the lymphocyte phenotypes after 28 days.

The available literature lacks any reports on the effect of oral administration of nanogold on lymphocyte phenotypes in mouse peripheral blood. Joseph et al. (2013) only reported an increase in the percentage of CD3+, CD4+ and CD8+ lymphocytes in mice given, intraperitoneally, AuNPs coated with PST polysaccharide. It is unknown, however, to what extent this was caused by the nanogold itself and to what extent the reason was the plant PST polysaccharide, as the latter possesses verified anticancer and immunomodulating properties.

In the present experiment, it was only the middle dose of gold nanocolloid, and after the longest administration period, that caused changes in the lymphocyte phenotypes in the peripheral blood. The rise observed in the percentage of B cells can easily be explained by their stimulation by nanoparticles, which has been confirmed by other research. In a line of murine B lymphocytes (CH12.LX), AuNPs passed through the cytoplasmic membrane, thus activating the cells and stimulating antibody production (Sharma et al. 2013). A similar effect was achieved *in vivo* by Dykman et al. (2004) in rabbits, rats and mice immunized with haptens or complete antigens. In the presence of AuNPs, the animals produced higher titres of more reactive antibodies, at a smaller quantity of antigen needed to immunization. It can be suspected that the stimulation of B lymphocytes by nanoparticles was accompanied by an increase in their amount in the tested animals, but this was not a question examined by the authors cited.

It is more difficult to explain the rise in the

CD4+CD8+ DP T cell percentage in the peripheral blood of mice administered the middle dose of gold nanocolloid. In the blood of healthy individuals, both humans and mice, there are very few mature double positive T cells, but their counts increase in the course of some autoimmune disorders, neoplasms or viral infections. Thus, an increase in their number can be due to some dysregulation of the immune system, or it could be a mechanism of compensation for the immunosuppression that develops in the course of some chronic diseases. However, in physiological conditions, the percentage of extrathymic DP T cells can increase as well, e.g. after the exposure of an organism to mitogens and antigens, and also with age. It is therefore suspected that DP T cells are part of the memory T cell pool, although they play helper and, probably, immunoregulatory functions as well (Zuckerman 1999, Parel and Chizzolini 2004, Perez et al. 2012). However, because the functions of DP T cells have not been fully elucidated, it is difficult to state firmly what caused their increase in the blood of mice given gold nanocolloid. One plausible explanation is the direct stimulation of lymphocytes by nanoparticles, and the newly created cells may have just played helper, memory or tolerogenic functions. Another likely cause of the increased count of DP T cells is the dysregulation of the immune system by nanogold, and the multiplied DP T cells were to restore the immune balance.

Despite changes in the percentages of lymphocyte populations in peripheral blood caused by gold nanocolloid, the preparation was not observed to have affected the proliferative activity of cells. The available literature contains few reports on this subject. Joseph et al. (2013) only demonstrated increased proliferation of peripheral blood lymphocytes in mice caused by AuNPs; however, the gold nanoparticles they used were coated with immunomodulatory PST polysaccharide. In turn, Dokić et al. (2012) observed a decrease in the proliferative response of immune cells in rats due to AuNPs, but their experiment was conducted *in vitro* on ConA stimulated splenocytes and not on peripheral blood mononuclear cells.

In the present study, gold nanocolloid had the strongest effect on the peripheral blood phagocytes, especially during the early phase of administration. Relatively numerous studies by other authors, completed on murine macrophages *in vitro*, confirm rapid scavenging of gold nanoparticles by cells (Shukla et al. 2005, Yen et al. 2009, Zhang et al. 2011, Tsai et al. 2012, Pissuwan et al. 2013). Presumably, nanoparticles were also caught from the circulatory system by granulocytes and monocytes present in blood, whose activity was detected in this study. Internalization of foreign particles by phagocytes can provide a signal of

inflammation. However, results reported by various authors are highly divergent in this respect. Some have demonstrated the non-immunogenic character of gold nanoparticles or have even proven their anti-inflammatory effect, consisting of the inhibition of the production of reactive oxygen and nitrite species as well as pro-inflammatory cytokines in macrophages (Shukla et al. 2005, Zhang et al. 2011, Sumbayev et al. 2012, Tsai et al. 2012, Pissuwan et al. 2013). Also, *in vivo* experiments conducted on several animal models of inflammatory conditions have confirmed the anti-inflammatory and antioxidant properties of gold nanoparticles, manifested by a decrease in levels of pro-inflammatory cytokines (IL-1 β , TNF- α) and markers of oxidative tissue damage (Tsai et al. 2007, Pedersen et al. 2009, Dohnert et al. 2012, Pereira et al. 2012, Sumbayev et al. 2012, Victor et al. 2012). Such effects of gold nanoparticles, contrary to what has been found in this study, can be explained by the fact that the cited observations were conducted on ill animals, with highly activated tissue macrophages, whereas in this study the effect of gold nanocolloid was tested on healthy animals, presenting no clinical signs of disease.

Moreover, some references describe the activation of phagocytes by nanoparticles. Yen et al. (2009) reported that gold nanoparticles caused morphological changes in macrophages, indicating their activation, and increased the expression of pro-inflammatory cytokines: IL-1, IL-6 and TNF- α . In a study by Lee et al. (2012), gold nanorods activated cells, inducing a proliferative effect and increasing the synthesis of IL-1 α , IL-1 β , IL-6, IL-10, IP-10, MCP-3, TNF- α , G-CSF, LIF, MIP-2, VEGF, MCP-1, GM-CSF, PGE2 and RANTES as well as the release of Ca, NO and H₂O₂. Also Dykman et al. (2004), in their *in vivo* experiments on rabbits, mice and rats, in addition to strong adjuvant properties of gold nanoparticles, observed increased phagocytic and bactericidal activities of peripheral blood phagocytes in animals. The quoted research implicates immunostimulatory properties of AuNPs towards phagocytes, including the activation of oxidative mechanisms involved in the killing of microorganisms, which were also noticed in the current study. This effect of nanogold can be undesirable in chronic inflammatory conditions, when enhanced production of inflammatory mediators leads to the development of oxidative stress and tissue damage. However, the elevated activity of peripheral blood phagocytes caused by gold nanocolloid, in the experiment reported herein, occurred only in the initial phase of AuNP administration, returning after a longer time lapse to the level determined in the control animals, which may suggest gradual adaptation of peripheral blood phagocytes to a longer lasting presence of nanoparticles in the organism.

In summary, the effect of gold nanocolloid administered orally on the peripheral blood leukocytes in mice was limited to such developments as the increased activity of phagocytes and changes in percentages of lymphocyte populations. Enhanced activity of granulocytes and monocytes was a transient phenomenon, noticed only after a short time of nanogold administration, which seems indicative of the adaptability of the organism to the presence of nanoparticles. However, the phenotypic changes among lymphocytes did not occur until 28 days of the administration of nanoparticles, which in turn might indicate exhaustion of compensatory mechanisms and certain immune dysregulation due to long-lasting contact with nanoparticles. Drawing any further-reaching conclusions will require more detailed investigations in this area.

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