COBALT NANO-PARTICLES MODULATE CYTOKINE *IN VITRO* RELEASE BY HUMAN MONONUCLEAR CELLS MIMICKING AUTOIMMUNE DISEASE

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The use of particles from micro to nanoscale provides benefits to diverse scientific fields, but because a large percentage of their atoms lie on the surface, nanomaterials could be highly reactive and pose potential risks to humans. Due to their wide range of application, Cobalt nano-particles are of great interest both in industry and in life-science. To date, there are few studies on Co nano-particle toxicology. In this respect, this study aims at evaluating *in vitro* the potential interference of Co nano-particles on the production of several cytokines (IL-2, IL-4, IL-6, IL-10, IFNγ and TNFα) by PBMCs, comparing their effects to those of Co micro-particles and Co solution ($CoCl_2$). Cells were cultured in Opticell flasks with escalating concentrations (10^{-5} , 10^{-6} and 10^{-7} M), of Co nano- and micro-particles and $CoCl_2$ or without metal. Cytokines were quantified in the supernatants using a human Th1/Th2 cytokine cytometric bead array. Co micro-particles showed a greater inhibitory effect compared to other Co forms. Its inhibitory activity was detected at all concentrations and towards all cytokines, whereas Co solutions selectively inhibited IL-2, IL-10 and TNF-α at maximal concentration. Co nano-particles induced an increase of TNF-α and IFN-γ release and an inhibition of IL-10 and IL-2: a cytokine pattern similar to that detected in the experimental and clinical autoimmunity. On the basis of the obtained data, immune endpoints should be sought in the next series of studies both *in vitro* and *in vivo* in subjects exposed to cobalt nano-particles.

Following the rapid development of nanotechnology, micro- to nano-scale materials are being increasingly used in science and industry. Among them, Cobalt (Co) nano-particles are of great interest in biomedicine for applications in drug development, *in vivo* detection of biological molecules and structures, and gene delivery. However, nano-materials are in a size range that allows uptake by cells and interaction with subcellular structures, and are highly reactive with biological macromolecules because a large percentage of their atoms lies on exposed surfaces. Thus, their uncontrolled diffusion poses still poorly understood risks to humans and to the environment. The few studies on Co-nano-particles toxicology available to date have revealed *in vitro* toxic effects on endothelial cells, histiocytes and fibroblasts (1-2). Remarkably, there are no data on their effects on the immune system.

In this respect, the present study aims at

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evaluating the potential immune interference of cobalt nanoparticles on PBMCs from healthy subjects, comparing their effects to those of Cobalt forms of different size as microparticles and Co solution, ($CoCl_2$). For this purpose the production of several cytokines, characteristic of Th1, Th2 and T regulatory pattern, by peripheral blood lymphocytes exposed to escalating concentration of the three cobalt species, were evaluated *in vitro*.

MATERIALS AND METHODS

$CoCl_2$ [7791-13-1] was supplied by Alfa (Karlsruhe, Germany); Co-nano-particles (Co-nano<50nm) were supplied by Laboratory of Biomaterials, University of Modena and Reggio Emilia (Italy), and Co-micro-particles (Co-micro<2µm) [7440-48-4] by Sigma Aldrich, (Milan, Italy). The degree of purity of the compounds, suitable to avoid possible artefacts, was assessed by HPLC-inductively coupled plasma mass spectrometry (HPLC-ICPMS, Perkin-Elmer SCIEX, Ontario, Canada). Co-nano- and -micro-particles formed, in fresh prepared stock solution, aggregates with size distribution from 200 to 1000 nm and 400-700 µm respectively, as characterised by Dynamic-Light-Scattering and Scanning-Electron-Microscopy techniques

Exposure of PBLs to Cobalt nano- and micro particles and $CoCl_2$

Whole blood (50 ml) was collected by aphaeresis from 3 different healthy donors, diluted 1:1 with phosphate-buffered saline (PBS) without Ca++ and Mg++, pH 7.4 (Sigma, Milano, Italy) and immediately processed as described. The mononuclear cells were isolated by Ficoll density gradient (1.077 g/mL) centrifugation (25 minutes, 600 x g, 20°C). The light-density cells were washed twice in RPMI medium supplemented with 10% FCS, 1% L-Glutamine and 1% penicillin-streptomycin (10 minutes, 400 x g). Cell density was adjusted to 200,000 cells/mL with complete RPMI medium and incubated in 5 ml culture flasks overnight at 37°C, 5% CO_2. Cells were seeded in Opticell flasks (Tema Ricerche, Bologna Italy) and on day one were cultured under the following conditions:

(a) no other reagent added (control sample),
(b) with escalating concentrations, 10^{-5}, 10^{-6} and 10^{-7} M, of Co nano-particles
(c) with escalating concentrations, 10^{-5}, 10^{-6} and 10^{-7} M, of Co micro-particles
(d) with escalating concentrations, 10^{-5}, 10^{-6} and 10^{-7} M, of three $CoCl_2$

The specific dilutions were obtained by diluting the appropriate 100x concentrated stocks in deionised water. 5 x 10^6 cells were used for each experimental point (i.e. metal species and concentration) in 10 mL medium. The exposure concentrations, suitable for cell function studies which require a viable cell population, were selected on the basis of previous cytotoxicity studies (3). As a control, cells without Co were cultured in parallel. Opticell flasks were incubated for 72 hours at 37°C , 5% CO_2, under continuous stirring.

At the end of the incubation the cells were aspirated and centrifuged.

Culture supernatants (8 ml each sample) were stored at -80°C for cytokine quantification.

All experiments were made in duplicate.

The dilution of metal extracts was based on the experience of ECVAM, and represents a "non-toxic concentration' suitable for cell function studies which require a viable cell population.

Cytokine quantification

Th1 and Th2 cytokines including IL-2, IL-4, IL-6, IL-10, IFNγ and TNFα were quantified simultaneously using a human Th1/Th2 cytokine cytometric bead array (CBA) kit (BD, San Diego CA, USA). These assay kits provide a mixture of six microbead populations with distinct fluorescent intensities (FL-3) and were precoated with capture antibodies specific for each cytokine. Fifty µl of plasma or the provided standard cytokines were added to the premixed microbeads in 12 mm x 75 mm Falcon tubes. After the addition of 50 µl of a mixture of PE conjugated antibodies against the cytokines, the mixture was incubated for 3 h in the dark at room temperature. This mixture was washed and centrifuged at 500 g for 5 min and the pellet resuspended in 300 µl of wash buffer. The FACSCalibur flow cytometer (BD San Diego CA, USA) was calibrated with setup beads and 3000 events were acquired for each sample. Individual cytokine concentrations were indicated by their fluorescent intensities (Fl-2) and were computed using the standard reference curve of CELLQUEST and CBA software (BD San Diego CA, USA).

Statistical analysis

All data were plotted and analyzed for statistic significance in parametric (t-test) and non- parametric evaluations (Wilcoxon signed ranks test).

RESULTS

The three different forms of Cobalt showed different interference in the production of cytokine (Table I). In particular, Co microparticles at all applied concentrations induced a significant decrease

Table I. *Changes in cytokine in supernatants of PBMCs cultured with escalating concentration of different Co form. *: p<0,,05; ** p<0,01 (Wilcoxon signed ranks test).*

	IFN γ ng/ml	TNF α ng/ml	IL-10 ng/ml	IL-6 ng/ml	IL-4 ng/ml	IL-2 ng/ml
control cells	159.5 ± 144.7	6.4 ± 3	5.1 ± 2.9	711.7 ± 587.1	27.6 ± 17.7	22.8 ± 13.2
Co nano 10^{-5}	73.2 ± 32	6.4 ± 2.5	3 ± 2.3 **	597 ± 374	23.6 ± 12.2	15.3 ± 5.6**
Co nano 10^{-6}	110 ± 75.4	11.3 ± 6.6*	3.1 ± 2.6**	623.9 ± 475.5	23.1 ± 15.2	15.8 ± 8.7**
Co nano10^{-7}	199.7 ± 192*	20.3 ± 17.2*	3.9 ± 2.4**	743.3 ± 497.7	28.4 ± 15.9	20 ± 13**
Co micro10^{-5}	50.4 ± 11.1*	3.7 ± 0.6*	3.3 ± 2.9**	678.3 ± 550.3*	24.7 ± 17.2**	13.1 ± 4.4*
Co micro 10^{-6}	43.4 ± 14.6*	3.3 ± 0.4*	1.9 ± 2.5**	663.7 ± 520.3	24.6 ± 17.1**	12.3 ± 3.9*
Co micro 10^{-7}	55.7 ± 25.1*	4.1 ± 0.9*	2.9 ± 2.4**	623.1 ± 470.1	23.1 ± 14.4*	14.3 ± 7.4*
$CoCl_2$ 10^{-5}	59.8 ± 10.3	3.8 ± 0.5*	3.5 ± 2.3**	972 ± 614.4	33.9 ± 19.7	8.3 ±2.1**
$CoCl_2$ 10^{-6}	146.7 ± 127.9	4.9 ± 1.5	16.3 ±18.1	1978.5 ± 1956.5	51.1 ± 42.8	15.8 ± 14.1
$CoCl_2$ 10^{-7}	105.5 ± 43.8	4.9 ± 1.5	3.9 ± 1.9	719.7 ± 501.7	27.5 ± 16.6	15.1 ± 4.2

in the production of all studied cytokines (Table I, Fig. 1) in respect to the control cultures. Only IL 6 showed negligible changes in the supernatants of cultures exposed to 10^{-6} (p=0,02) and 10^{-7} M concentrations. On the other hand, Co nanoparticles inhibited the production of IL10 and IL2 at all concentrations (in all cases p<0,01) and significantly stimulated the production of TNF α at 10^{-6} (p=0,02) and 10^{-7} (p=0,03) M concentrations and of IFNγ at 10^{-7} (p=0,03) M concentration (Fig. 2). Finally, cobalt solutions induced a selective inhibition of cytokine production only at high concentration: 10^{-5} M $CoCL_2$ significantly inhibited the production of IL10 (p=0,004), IL2 (p=0,01) and TNFα (p=0,03), whereas 10^{-6} and 10^{-7} M $CoCL_2$ did not induce significant cytokine changes (Table I).

DISCUSSION

The present work demonstrated that the three forms of Co differently interfere with the production of cytokines by PBMCs. The Co microparticles showed a greater inhibitory effect as compared to the other Co forms. Its inhibitory activity was detected at all concentrations and towards all studied cytokines, whereas Co solutions inhibited selected cytokines, i.e. IL2, IL10 and TNF-α at maximal concentration. There are no comparative data in literature on the immune-toxicity of micro-scaled particles of Cobalt, while many authors have studied the effects of Cobalt solution. The results of various experiments were quite different. In a murine model of lung toxicity the intratracheal instillation of Cobal ions did not induce any consistent effect on TNF-α, IL1, fibronectin and cystatin-c production (4); on the contrary, the *in vitro* incubation of macrophages and leucocytes with Co solutions led to release of TNF-α, IL-6, and PGE2 without changes in cell count (5-7); finally, in a human monocyte/macrophage culture various Co solutions did not affect the release of TNF and IL-6 (8). These different results can be explained both by the different target cells used in the various experimental models, and by the different exposure conditions in terms of the amount of the metal solutions and the length of the stimulations.

In our experimental model Co nanoparticles,

Fig. 1. *Changes from controls of cytokine release in PBMCs cultured with different concentrations of Co nanoparticles. Significant increase of IFN γ and TNF α and significant decrease of IL2 and IL10 were found, mimicking the cytokine pattern of autoimmune diseases.*
** p<0,03.*

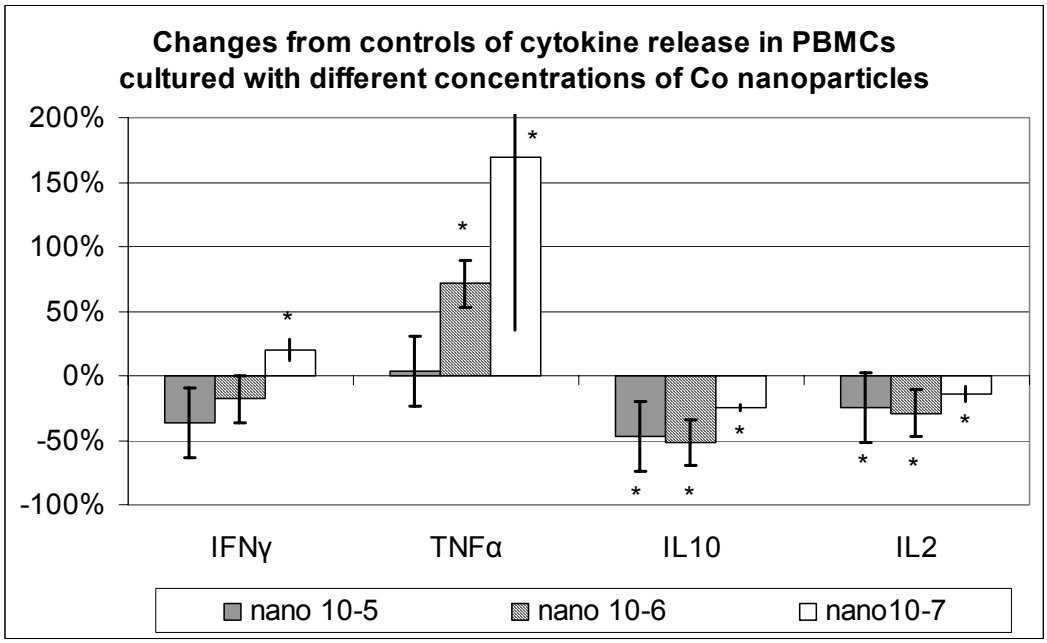
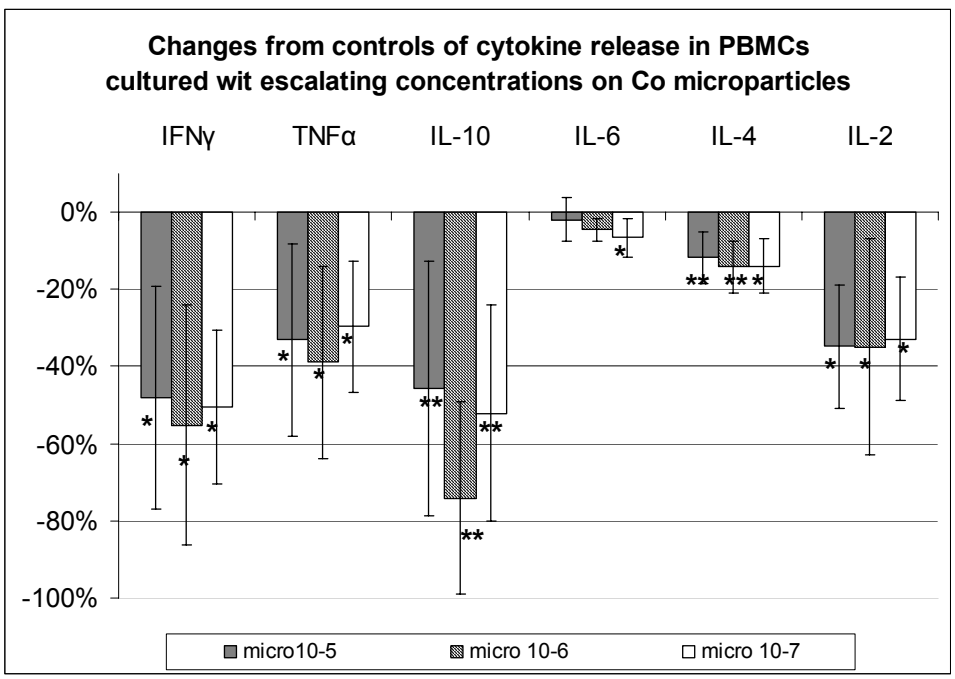


Fig. 2. *Changes from controls of cytokine release in PBMCs cultured with different concentrations of Co microparticles. Significant decrease of all cytokines, except for IL6, was found at all Co microparticles concentration, showing a intense immunetoxicity.*
**<0,05, **p<0,01.*



differently from the microparticles and solutions, modulated the production of studied cytokines, with a stimulation of TNF α and IFN γ release and a contemporary inhibition of IL10 and IL2.

There are no comparative data on the immune effects of Co nanoparticles in literature, however, cobalt-chromium nanoparticles reduced in a dose-dependent manner the viability of U937 histiocytes and L929 fibroblasts (1); an impairment of the proliferative activity and a pro-inflammatory stimulation (increase of IL 8 release) of endothelial cells by exposure to cobalt nanoparticles have also been reported (2).

The cytokine pattern induced by cobalt nanoparticles in our study is characterized by an increase in pro-inflammatory cytokines, i.e. IFNγ and TNFα in cultures exposed at low concentration of metal. It is possible that the toxicity of such Cobalt form, at higher concentration, overcomes the stimulatory effect on cytokine production.

It has been demonstrated that excess levels of TNF-α are associated with certain autoimmune diseases (9); key features of Hg- and Ag-induced autoimmunity are the up-regulation of IFN-γ and the down-regulation of IL-10 expression (10); in heavy-metal induced systemic autoimmunity, genetically susceptible mice show a decrease in IL-10 RNA expression, whereas a strong increase has been observed in resistant mice (11).

A reduction of IL 10 levels has been also observed in our experiments. Therefore, the cytokine pattern induced by Co nanoparticles *in vitro* is similar to that detected in the experimental and clinical autoimmune diseases.

On the basis of the obtained data, immune endpoints should be sought in the next series of studies both *in vitro* and *in vivo* in subjects exposed to cobalt nanoparticles.

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