

Effects of Palladium Nanoparticles on the Cytokine Release from Peripheral Blood Mononuclear Cells of Palladium-Sensitized Women

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Objective: To investigate the effects of palladium (Pd) nanoparticles on cytokine release from peripheral blood mononuclear cells (PBMCs) of control or Pd-sensitized nonatopic women. **Methods:** TNF- α , IL-5, IL-10 and IFN- γ release and/or expression from PBMCs incubated in presence of 5 to 10 nm Pd⁰ nanoparticles or Pd^{IV} salt (potassium hexachloropalladate) were determined by enzyme-linked immunosorbent assay and reverse transcription-polymerase chain reaction analysis. Transmission electronmicroscopy was performed. **Results:** In lipopolysaccharide-stimulated PBMCs from controls, Pd salt inhibited IFN- γ and IL-10 release, whereas Pd nanoparticles enhanced IFN- γ release and inhibited TNF- α secretion. In lipopolysaccharide-stimulated PBMCs from Pd-sensitized women showing high IFN- γ release, Pd nanoparticles inhibited TNF- α release and Pd salt IL-10 release. TNF- α and IFN- γ release and messenger RNA expression were correlated. Transmission electronmicroscopy demonstrated uptake of nanoparticles in the endocytic compartment and activation of autophagy. **Conclusions:** Palladium ions and nanoparticles exert different effects in vitro on the expression and release of cytokines.

Studies on palladium (Pd) concentrations in ancient ice and in recent snow samples reflect the increase in mining, smelting and use in the last decades.¹ The environmental exposure to Pd of the general population derives from contact with ornaments or dental alloys, or from emissions of catalytic converters.^{2,3} The sources of exposure are changing: the concentrations of Pd in sewage sludges of Germany (mainly due to the use of dental alloys containing Pd) decreased in the last decades,⁴ while Pd exposure due to the use of catalytic converters is increasing, reflecting in part changes in alloy composition.⁵

Palladium is one of the platinum (Pt) group elements (PGEs) emitted from catalytic converters along with Pt, rhodium (Rh), and iridium. Available emission data suggest that the PGEs deriving from catalytic converters are almost exclusively in metallic or oxide form. About 90% of the Pd and Pt emissions are particulated: aluminum

oxide particles in lower micrometer range carrying Pd particles of a few nanometers dispersed on their surface.⁶

Today, the content of Pt in catalytic converters is decreasing, but Pd is increasing, as it is cheaper and has extremely high catalytic activity. In 2004, the concentrations of Pd in soils along a German highway were found to be about 15 times higher than those measured in 1994, while the concentrations of Pt and Rh were only 2 and 1.6 times higher, respectively.⁷ At present, there are preliminary studies on Pd exposure in the city of Rome showing that Pd levels in urban air were 8 to 11 orders of magnitude below the values thought to cause adverse effects on adult worker health; moreover, Pd levels in the urine of the urban population and of workers exposed to traffic were determined.^{8,9}

In the air of factories producing catalytic converters PGE concentrations are in the range of 12 to 64 ng/m³ compared to less than 1 ng/m³ in the environmental air of roads with heavy traffic.^{10,11} Asthma, rhino-conjunctivitis, urticaria, and allergic contact dermatitis (ACD) induced by sensitization to Pt, Pd, Rh and/or iridium were reported in PGE-exposed workers employed in the production of catalytic converters, in chemical industries and goldsmithing,^{11,12} while the nonoccupationally-exposed population was reported to suffer only from Pd ACD.¹³⁻¹⁷

The high percentages of Pd sensitization detected in German children,¹³ and in the Austrian¹⁴ and Italian¹⁵ population may reflect not only the presence of Pd in dental alloys^{16,17} but also the introduction of catalytic converters. Today in the city of Rome, more than 1% of the population is sensitized to Pd, while, in 2000 before the use of catalytic converters in Italy, Pd sensitization was not noticeable.¹²

At present, the data on the immune effects of Pd in humans derive mainly from in vitro immunotoxicity experiments on PBMCs, which demonstrated that the ammoniacal salts of Pd, Rh, and Pt (used as intermediate industrial products) are more immunotoxic than their chlorinated compounds, and these latter more than Pt, Pd, and Rh in elementary form.^{18,19} Furthermore, the available studies were performed with Pd salts, while the immune potential of Pd nanoparticles remains to be evaluated. In fact the biological effects of metal nanoparticles could differ from those obtained with the ionic forms of the corresponding metal, as the nanoscale size allows cellular uptake and direct interactions with subcellular structures.²⁰

The present study aims at evaluating the immune potential of Pd⁰ nanoparticles in the size of those emitted from car exhaust converters,⁶ compared with that of a Pd^{IV} salt (hexachloropalladate). To this end, we determined the expression and release of cytokines from peripheral blood mononuclear cells (PBMCs) of non-sensitized control and Pd-sensitized women with ACD, and whether Pd nanoparticles exerted their effects after intracellular uptake.

MATERIALS AND METHODS

Palladium model nanoparticles (5–10 nm diameter) were provided by Prof K. Leopold, Institut für Analytische und Bioanalytische Chemie, Universität Ulm, Germany. These nanoparticles were characterized by transmission electron microscopy (TEM) for particle

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The authors declare that they have no conflict of interest.

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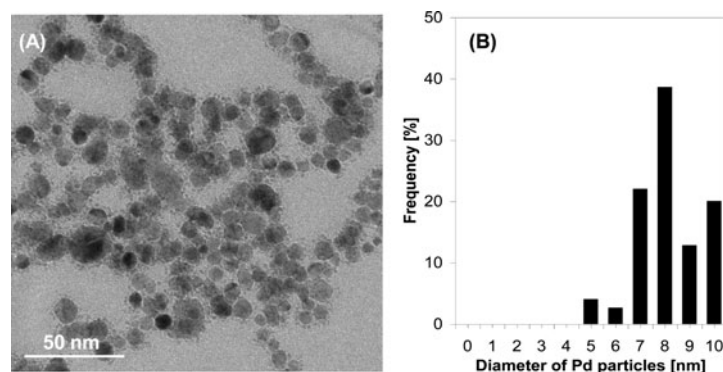


FIGURE 1. (A) TEM picture and (B) core size distribution histogram of Pd nanoparticles ($n = 100$). The particles as either isolated or aggregated in the solution.

size distribution, high resolution TEM for oxidative state, selective area diffraction for chemical structure and graphite furnace atomic absorption spectrometry for measurements of Pd concentrations.⁵ Figure 1A shows an exemplary TEM image of Pd nanoparticles and Fig. 1B gives the size distribution histogram. The suspension of agglomerated Pd nanoparticles (10^{-4} M) in ultrapure water was treated for 60 minutes in an ultrasonic bath and either used immediately for experiments or kept in a refrigerator (4°C). Potassium hexachloropalladate (IV) was supplied by Aldrich Chemical Co (Milwaukee, WI), dissolved in deionized water and stored at $+4^{\circ}\text{C}$.

Subjects

Allergic contact dermatitis is more common in women than in men. A group of 12 healthy nonatopic female volunteers (mean age: 32 years, range: 20–46 years) was recruited for this study. Among patients with a history of ACD and patch-tested with the European Standard series, eight female subjects (mean age: 33.6 years, range: 20–52 years) were selected on the basis of Pd positivity. Four out of these eight patients presented a concomitant positivity to nickel. The same patients were negative to all the other allergens of the standard series. They did not refer a clinical history of atopy (rhinoconjunctivitis and/or asthma) and all resulted negative when examined with the prick test for common allergic inhalants (for this reason, they were classified as nonatopic). Exclusion criteria were recent infections and presence of any chronic disease. None of the study subjects was taking any drug and all showed routine blood analyses (including white blood cell count) in the normal range. The study was conducted according to the declaration of Helsinki and subsequent revision, and approved by the ethical committee of the G. d'Annunzio University of Chieti-Pescara. All subjects signed a written informed consent to the study.

Procedures

Peripheral blood samples drawn between 8 AM and 10 AM were collected into 4 mL endotoxin-free ethylenediaminetetraacetate tubes (Vacutainer, Becton Dickinson, NJ). Tubes were kept at room temperature and transported to the laboratory for processing within 1 hour of collection. Isolation of PBMC by Ficoll-HyPaque density gradient centrifugation (20 minutes at $400 \times g$) resulted in enrichment of mononuclear cells with less than 3% contamination of neutrophils. After three washings with Hanks' balanced salt solution, PBMCs ($10^6/\text{mL}$) were resuspended in complete Roswell Park Memorial Institute 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 100 U/mL penicillin and 100 mg/mL streptomycin, immediately plated and incubated overnight at 37°C in a 95% humidified 5% CO_2 cell culture incubator under the following:

- No other added reagent (control sample basal).
- With bacterial lipopolysaccharide (LPS) (Sigma, Italy), $10 \mu\text{g}/\text{mL}$ (control sample with LPS).

- With 10^{-5} M Pd nanoparticles (Pd nano sample) or 10^{-5} M potassium hexachloropalladate (Aldrich Chemical Co., St. Louis, MO), (Pd salt sample).
- With $10 \mu\text{g}/\text{mL}$ of LPS plus 10^{-5} M Pd nanoparticles (LPS + Pd nano sample) or 10^{-5} M potassium hexachloropalladate (LPS + Pd salt sample).

Cell viability, assessed by Trypan blue exclusion, was not significantly influenced by differences in cell culture conditions, and more than 98% of the PBMCs were viable. At the end of the incubation, cell-free supernatants were removed and stored at -70°C until assay. Cell pellets were kept at -80°C until analysis. No solution contained detectable LPS, as determined by the Limulus amoebocyte lysate assay (sensitivity limit $12 \text{ pg}/\text{mL}$; Associates of Cape Cod, East Falmouth, MA) and mycoplasma contamination (General-probe II, General-probe Inc, San Diego, CA) was found negative. The same batch of serum and medium were used in all experiments.

Quantitation of Cytokine Levels

Cytokine concentrations (IL-10, IL-5, $\text{TNF-}\alpha$, and $\text{IFN-}\gamma$) were determined by solid phase sandwich enzyme-linked immunosorbent assay kit (Endogen, Woburn, MA) with human monoclonal antibody according to the manufacturer's instructions. All steps were performed at room temperature. Cytokine levels were then calculated plotting the optical density of each sample against the standard curve. Detection limits of the enzyme-linked immunosorbent assay were as described in the Endogen Lab manual. The sensitivity of the enzyme-linked immunosorbent assay kit has been determined to be less than $3 \text{ pg}/\text{mL}$ for IL-10, less than $2 \text{ pg}/\text{mL}$ for $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$. Intra and inter-assay reproducibility was greater than 90%. Duplicate values that differed from the mean by greater than 10% were considered suspect and were repeated.

RNA Extraction and Reverse Transcription–Polymerase Chain Reaction Analysis

These analyses were performed on 4 out of the 12 control women and on 4 out of the 8 Pd-sensitized women. Total RNA was extracted from the PBMC cultures using the TRIzol reagent (Invitrogen, Life Technologies, Paisley, UK) according to the manufacturer's protocol. The RNA concentration was estimated by measuring the absorbance at 260 nm using a BioPhotometer (Eppendorf AG, Hamburg, Germany), and RNA samples were kept frozen at -80°C until use. Purified RNA was electrophoresed on a 1% agarose gel to assess integrity. Three micrograms of RNA was reverse transcribed into complementary DNA using the High Fidelity Superscript reverse transcriptase kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. All polymerase chain reactions (PCRs) were performed in PCR-express cyclers (Hybaid, Heidelberg, Germany). The PCR amplification program was as follows: an initial period of 5 minutes at 95°C , after a variable number of cycles of 30 seconds, denaturation at 95°C , 30 seconds annealing at 60°C

and finally 30 seconds of extension at 72°C. The program was terminated with 10 minutes at 72°C. To remain within the exponential PCR phase, the appropriate number of cycles was newly established for every set of samples. Polymerase chain reactions products were separated by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining. All gels were scanned and the normalized intensities of all reverse transcription (RT)-PCR products were determined by the BioRad gel documentation system (BioRad, Hercules, CA). Mean $\pm$ SEM intensities were calculated for all experiments.

Transmission Electronmicroscopy

Cells were fixed overnight at 25°C in 2% paraformaldehyde-2% glutaraldehyde in PBS, post-fixed in 1% osmium tetroxide in veronal acetate buffer (pH 7.4) for 2 hours at 25°C, stained with 2% uranyl acetate (5 mg/mL), dehydrated in acetone and embedded in Epon 812 (Shell Chemical Co., San Francisco, CA). Thin sections were examined under a Philips CM10 TEM (Phillips, Amsterdam, Netherlands) after post-staining with uranyl acetate and lead hydroxide.

Statistical Analysis

Analysis of the data was performed with the Statistical Package for Social Science (SPSS 11.5, SPSS Inc, Chicago, IL). The Kolmogorov-Smirnov test, used to determine data distribution, showed that most of the data did not conform to the parametric distribution. The Wilcoxon ranked sum test was used to evaluate matched control and test samples (10^{-5} M Pd nanoparticles or potassium hexachloropalladate).

RESULTS

Cell viability, as assessed at the end of the incubation, was not significantly modified compared to preincubation values and was not influenced by the different exposure conditions. More than 98% of the PBMCs were viable. The amounts of cytokines released in the supernatants of the PBMC cultures from control and Pd-sensitized women, with or without LPS stimulation, are reported in Table 1.

Under basal culture conditions the release of IL-10, TNF- α , and IFN- γ was about two times higher for PBMCs from Pd-sensitized women, while IL-5 secretion was not significantly increased. Lipopolysaccharide stimulation increased IL-10, TNF- α , IFN- γ , and IL-5 release from the PBMCs of both nonatopic and Pd-sensitized women. In particular, in PBMCs from Pd-sensitized women LPS increased IFN- γ release six times more than in PBMCs from controls (Table 1).

The addition of 10^{-5} M Pd nanoparticles did not significantly modify the release of cytokines in unstimulated (without LPS) cultures from both control and Pd-sensitized women, while potassium hexachloropalladate significantly reduced IL-10 release in the cultures from both control and Pd-sensitized women (Table 2).

In LPS-stimulated PBMCs from control women, Pd nanoparticles significantly reduced TNF- α release, increased IFN- γ release, and did not affect IL-10 and IL-5 levels. In the same LPS-stimulated PBMCs, potassium hexachloropalladate reduced the release of IL-10, TNF- α , and IFN- γ , but did not affect IL-5 secretion. In the LPS-stimulated cultures from Pd-sensitized women, Pd nanoparticles significantly reduced TNF- α release, while potassium hexachloropalladate reduced IL-10 release (Table 3).

TABLE 1. Basal or LPS-Stimulated Cytokine Release From PBMCs of Nonatopic Control or Pd-Sensitized Women

	IL-10 (pg/mL)		TNF- α (pg/mL)		IFN- γ (pg/mL)		IL-5 (pg/mL)	
	Median	25%–75% range	Median	25%–75% range	Median	25%–75% range	median	25%–75% range
Control								
Basal	62	58–89	129	83–136	0.3	0.1–0.6	0.6	0.3–1.0
LPS (10 μ g/mL)	631	592–699	1173	1014–1541	6.6	4.3–14.4	2.6	0.7–4.7
Pd-sensitized								
Basal	125	35–599	211	164–518	0.6	0.2–1.0	0.9	0.4–1.5
LPS (10 μ g/mL)	717	440–1082	1327	1068–1639	46.6	28.0–85.4	2.1	1.1–2.9

LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cells; Pd, palladium.

TABLE 2. Cytokine Release From PBMCs of Nonatopic Control or Pd-Sensitized Women Cultured With Pd⁰ Nanoparticles (nano) or Pd^{IV} Hexachloropalladate (salt)

	IL-10 (pg/mL)		TNF- α (pg/mL)		IFN- γ (pg/mL)		IL-5 (pg/mL)	
	Median	25%–75% range	Median	25%–75% range	Median	25%–75% range	Median	25%–75% range
Control								
Basal	62	58–89	129	83–136	0.3	0.1–0.6	0.6	0.3–1.0
Pd nano 10^{-5} M	36	10–68	104	59–225	0.3	0.2–0.7	0.8	0.6–1.1
Pd salt 10^{-5} M	21	8–67*	142	102–373	0.5	0.3–0.9	0.6	0.4–1.9
Pd-sensitized								
Basal	125	35–599	211	164–518	0.6	0.2–1.0	0.8	0.4–1.5
Pd nano 10^{-5} M	46	26–235	130	90–195	0.7	0.5–0.9	0.8	0.6–1.2
Pd salt 10^{-5} M	38	23–144*	191	161–222	0.6	0.1–1.1	0.6	0.4–1.9

Pd, palladium.

Wilcoxon ranked sum test: statistical significant difference.

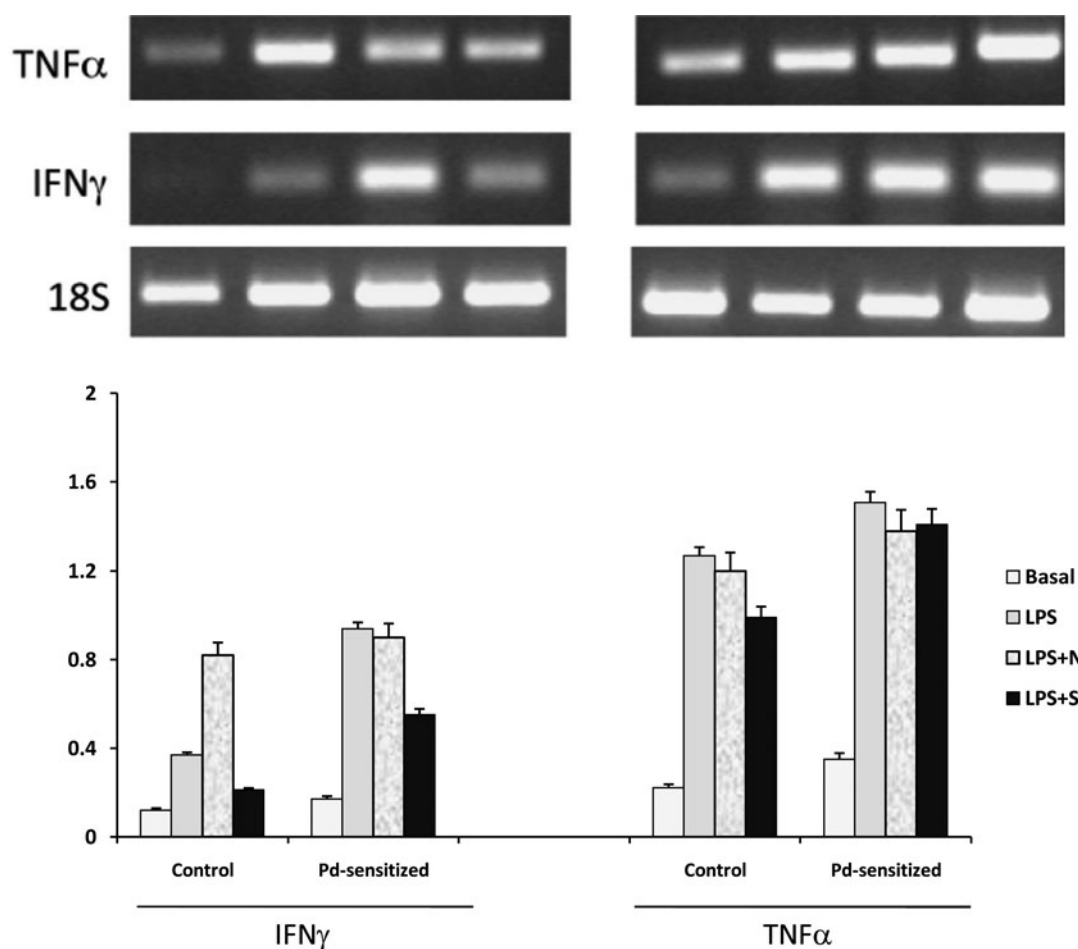
* $P < 0.05$.

TABLE 3. Cytokine Release From LPS-Stimulated PBMCs of Control or Pd-Sensitized Women Cultured With Pd⁰ Nanoparticles (nano) or Pd^{IV} Hexachloropalladate (salt)

	IL 10 (pg/mL)		TNF- α (pg/mL)		IFN- γ (pg/mL)		IL-5 (pg/mL)	
	Median	25%–75% range	Median	25%–75% range	Median	25%–75% range	Median	25%–75% range
Control								
LPS (10 μ g/mL)	631	592–699	1,173	1,014–1,541	6.6	4.3–14.4	2.6	0.7–4.7
LPS + Pd nano 10 ⁻⁵ M	649	541–771	996	686–1,324*	11.5	6.1–19.4**	1.8	1.6–2.3
LPS + Pd salt 10 ⁻⁵ M	34	16–502***	905	10–1,044**	4.2	0.1–8.8*	2.4	1.2–2.7
Pd-sensitized								
LPS (10 μ g/mL)	717	440–1,082	1,327	1,068–1,639	46.6	28.0–85.4	2.1	1.1–2.9
LPS + Pd nano 10 ⁻⁵ M	717	508–851	1,099	761–1,282*	45.8	29.8–107.4	1.9	0.7–4.6
LPS + Pd salt 10 ⁻⁵ M	319	73–603*	1,323	934–2,073	29.5	6.8–57.1	1.1	0.3–2.8

LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cells; Pd, palladium.

Wilcoxon test: statistical significant difference.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ **FIGURE 2.** Expression of TNF- α and IFN- γ determined using RT-PCR assays and representative data. The experiments were expanded to PBMC derived from all subjects and the results are reported as mean $\pm$ SD of messenger RNA levels of TNF α or IFN γ relative to the level of 18S.

We performed RT-PCR analysis to evaluate whether Pd nanoparticles or potassium hexachloropalladate could act through a transcriptional mechanism. A relation was observed between TNF- α and IFN- γ messenger RNA expression and production of the two cytokines. Figure 2 shows representative TNF- α and IFN- γ

messenger RNA expression in PBMCs from a nonatopic and a Pd-sensitized woman (similar results were obtained in all the investigated cases). Lipopolysaccharide stimulation increased IFN- γ expression about two to three times more in the PBMC cultures from Pd-sensitized women than in those from the control women

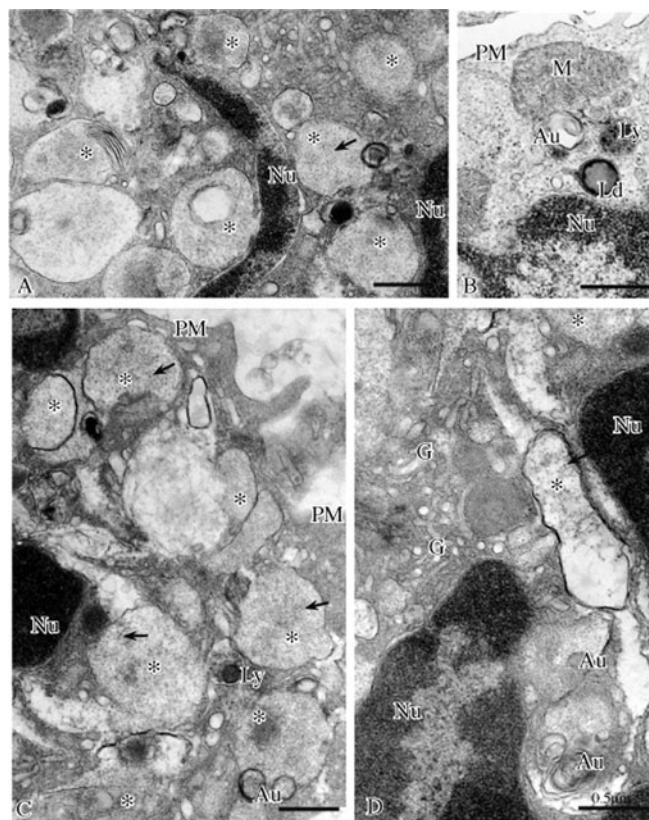


FIGURE 3. Ultrastructural identification of palladium nanoparticles and morphological alterations in in vitro-exposed peripheral blood mononuclear cells from control women. (A) A cytoplasmic area with markedly dilated endocytic vacuoles containing nanometer-scaled (arrow) particulated material (asterisks) and, often, multilamellar bodies or intravacuolar structures, suggesting autophagic processes. (B) A lysosome fused with a lipid droplet. Another electron-dense lysosome appears close to an autophagic vacuole adjacent to a mitochondrion with dilated cristae. (C and D) Large vacuolar structures (asterisks) containing nanometer (arrow) particles fusing with lysosomes or autophagosomes (PM, plasma membrane; Nu, nucleus; Au, autophagosomes; Ly, lysosome; Ld, lipid droplet, bar = 0.5 μ m).

and slightly more TNF- α gene expression. Moreover, in the PBMC cultures from the control women, Pd nanoparticles respectively increased and decreased the expression of the IFN- γ and TNF- α genes. The addition of Pd nanoparticles to the PBMCs from Pd-sensitized women did not modify IFN- γ expression, while salt addition reduced IFN- γ gene expression in PBMCs from both control and Pd-sensitized women and TNF- α gene expression in PBMCs from controls. In the PBMC cultures from Pd-sensitized women, both the nanoparticles and the salt did not exert effects on TNF- α gene expression.

The Pd nanoparticles-exposed PBMC cultures from control women were analyzed by TEM to confirm nanoparticles internalization and assess ultrastructural alterations (Fig. 3). The mononuclear cells showed numerous markedly dilated endocytic vacuoles, filled with particulated nanometer-scale material. Often these vacuoles contained also multilamellar bodies or intravacuolar membranous structures, suggesting autophagic processes. In addition, lysosomes, often in various stages of fusion with autophagosomes (autophagolysosomes), were prominent, together with lipid droplets, which, in some cases, showed evidence of fusion with lysosomes. The mitochondria revealed evidence of damage, documented by dilated cristae. These data indicate that, at the used concentrations, Pd nanoparticles are abundantly endocytosed by PBMCs and accumulate in the endocytic vacuolar compartment, exerting cell damaging effects that appear to activate autophagy.^{21,22}

DISCUSSION

Our results support the view that soluble Pd ions (from potassium hexachloropalladate) and Pd nanoparticles exert different immune effects in vitro on the expression and release of cytokines by human PBMCs. Exposure to Pd ions reduced unstimulated as well as LPS-stimulated IL-10 secretion in PBMCs from both control and Pd-sensitized women. Moreover, Pd ions inhibited LPS-stimulated IFN- γ and TNF- α release in PBMCs from controls. On the other hand, Pd nanoparticles seemed to exert modulatory effects on LPS-stimulated cytokine release, enhancing IFN- γ release in PBMCs from controls and inhibiting TNF- α secretion in PBMCs from both control and Pd-sensitized women.

The enhanced IFN- γ release by LPS-stimulated PBMCs from controls may represent a fingerprint of the immune potential of Pd nanoparticles. The ability of Pd nanoparticles to induce Th1-mediated responses in vitro correlates with the clinical observation that subjects exposed to Pd in the working or living environment are prone to develop Pd ACD,^{12–15} a typical Th1-mediated reaction.²³ In this regard, the LPS-increased release of IFN- γ was higher in cultures from women with Pd ACD than in cultures from control women; moreover Pd nanoparticles increased IFN- γ release only in LPS-stimulated cultures from nonatopic control women. The lower response to LPS and Pd nanoparticles in PBMCs from Pd-sensitized women may reflect an overload resulting in immunological over-activation. This suggests that activated PBMCs are not able to

produce an additional response to nanoparticles. We hypothesize that this could protect cells from excessive stimulation, contributing to cell homeostasis.

Th2 cytokines, IL-5 in particular, are involved in the humoral reactions against bacteria and parasites, but are also the most important inducers of the immediate type of immune-reaction, being able to induce and maintain IgE antibody responses.²³ In our experiments IL-5, a marker of the Th2 response, was not significantly modulated by Pd nanoparticles and was only weakly influenced by Pd salt in PBMCs from both control and Pd-sensitized women. This confirms that both Pd nanoparticles and Pd salt do not exert significant effects on the Th2 response.

In our experiments, Pd salt (but not Pd nanoparticles) induced a significant inhibition of basal or LPS-stimulation of IL-10 in PBMCs from both nonatopic and Pd-sensitized women. This agrees with data reported by Bordignon et al¹⁷ on patients affected with Pd ACD, where Pd decreased IL-10 levels in patients with ACD to Pd more than in nonallergic subjects. Allergic contact dermatitis is one of the best-established model diseases for T cell-mediated immune responses. Besides the important role that T cells play in the initiation and maintenance of this immune response, cytokines have been shown to be important mediators of the inflammatory process. Some of them, such as IL-10, are essential mediators in the induction of ACD. Production of IL-10 at later stages of ACD-development has been linked to counter-regulatory processes, resulting in the induction of regulatory T cells that orchestrate reaction/tolerance to environmental antigens.^{24–26}

The biological effects of nanoparticles, still poorly understood, depend on composition, size, shape, surface area and charge; surface area seems mainly to influence apoptosis, while composition and surface reactivity seem to affect the inflammatory potential.²⁷ In this regard recent reports demonstrate that several types of nanoparticles (including nanoparticulated Pt¹¹) act as free radical scavengers and antioxidants,²⁸ while other nanoparticles, such as those composed of titanium dioxide and cobalt, stimulate inflammatory mechanisms, resulting in the production of pro-inflammatory mediators and cytokines.²⁹ In agreement with the present results, studies conducted with other types of nanoparticles support the view that nanoparticles have modulatory actions on immune cells. In particular, cobalt nanoparticles, differently from cobalt salts, induce an in vitro pattern of cytokine production by PBMCs similar to that associated with experimental autoimmune diseases, ie, increase in Th1 activity and decrease in regulatory cytokines.²⁰

Most of the Pd-sensitized women investigated in this study showed cross sensitization with nickel. Regarding this, the possible role of exposure to Pd emitted from catalytic car exhaust converters in inducing ACD to Pd, or cross sensitization to Pd in nickel-sensitized subjects of the general population, has to be determined.

We could demonstrate in our study that the Pd nanoparticles exerted their effects after intracellular uptake and accumulation within the endocytic vacuolar compartment of the exposed PBMCs, a localization consistent with the internalization mechanism.^{30,31} Furthermore, the ultrastructural morphological evidence suggests that the Pd nanoparticles were able to induce damage consistent with oxidative and metabolic stress, resulting in the stimulation of autophagic activity and in the formation of lipid droplets; with regard to this, autophagy may be considered a dynamic process that allows to maintain cellular homeostasis and adaptation in response to adverse environmental stimulation.^{21,22} With regard to this, further studies are needed on the intracellular uptake of Pd nanoparticles in PBMCs of both Pd nonsensitized and Pd sensitized subjects; in particular, the role of the autophagic activity in modulating cytokine release and expression will be determined.

Finally, we must consider that we applied well-characterized Pd model nanoparticles with the same size as those emitted from

car exhaust converters,⁵ consequently our results should not be extrapolated to different nanoparticles, but only to those produced by traffic.

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