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Article

Effects of sub-chronic exposure to palladium (as potassium hexachloropalladate) on cytokines in male Wistar rats

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Palladium (Pd) is a heavy metal belonging to the platinum group elements. It has been shown that Pd and its compounds can cause sensitization, asthma and dermatologic disorders. In this study, Wistar rats were exposed for 3 months to 0, 1, 10, 100, and 250 ng/ml of Pd (as potassium hexachloropalladate) in drinking water. At the end of exposure, possible changes in two type-1 cytokines [interleukin (IL)-2, interferon (INF)- γ] and one type-2 cytokine (IL-4) in the serum were measured. After the sub-chronic exposure, analysis of variance of IL-2 and INF- γ response shows that the difference between mean values in the groups of animals exposed reaches statistical significance for IL-2 ($P = 0.001$), showing a J-shaped dose-response relationship. At the higher dose of 250 ng/ml Pd, it was observed a significant increase in

IL-2 production when compared with the controls. Furthermore at 1 and 10 ng/ml of Pd we observed an increase of INF- γ production that was significant at 100 ng/ml of Pd, while at the higher dose of 250 ng/ml of Pd the response was indistinguishable from the control. At the doses investigated, Pd has been shown not to determine a modification of IL-4 response ($P = 0.10$). These preliminary findings indicate the need to carry out further investigations regarding the effect of other Pd compounds and the measurement of other types of cytokines both in the animal model used in our study and other models.

Key words: cytokines; nonlinear dose-response relationship; palladium; sub-chronic exposure

Introduction

Palladium (Pd) is a heavy metal belonging to the platinum group elements (PGEs). The general population may be exposed to Pd through food, dental alloys, jewellery, and through emissions from automotive catalytic converters. Although Pd and the other PGEs are among the rarest elements in the Earth's crust ($<1 \mu\text{g/kg}$), their excellent catalytic properties have led to an increment in their use to reduce the emission of pollutants like carbon monoxide, hydrocarbons, and nitrogen oxides by converting them into carbon dioxide, water and nitrogen, respectively.¹

Occupational exposure occurs mainly in the mining and processing of PGEs, mostly during refining and catalyst manufacturing and, to a lesser extent, in subjects exposed to automotive traffic.¹

Palladium and its compounds can cause sensitization and dermatologic disorders particularly in manufacturing, recycling and refinery workers. Furthermore, after other medical or experimental uses of Pd preparations, side-effects are noted such as fever, hemolysis, discoloration or necrosis at injection sites after subcutaneous injections and erythema and edema following topical application.^{2–7} No information is available in the literature regarding the effects on the general population of Pd emitted from automobile catalytic converters.

In vitro studies have shown that Pd led to increased T helper (Th)2-type cytokine interleukin (IL)-6 levels (fourfold) in cultures of human

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fibroblast-keratinocytes, while PdCl_2 increased Th2-type cytokine IL-6 levels (25-fold) in a three-dimensional human tissue model based on TR146 cells isolated from a squamous cell carcinoma of the buccal mucosa.^{8,9} In another *in vitro* study,¹⁰ Pd compounds were found to be more immunotoxic than Platinum (Pt) and Rhodium (Rh) salts. In particular $(\text{NH}_4)_2 [\text{PdCl}_6]$, and to a lesser extent $(\text{NH}_4)_2 [\text{PdCl}_4]$ and PdCl_2 , inhibited the release of IL-5, interferon (INF)- γ , and Tumor necrosis factor (TNF)- α from human Peripheral blood mononuclear cells (PBMC).

In a recent study,¹¹ we exposed Wistar rats for 2 weeks to 0, 1, 10, 100, and 250 ng/ml of Pd as potassium hexachloro-palladate in drinking water administered *ad libitum*. At the end of exposure, we measured possible changes in two type-1 cytokines (IL-2, INF- γ) and one type-2 cytokine (IL-4). Our findings suggested that, after a sub-acute exposure, a moderate level of Pd causes a strong Th2 response. This also occurs at higher concentrations. Although no effect on Th1 cells is observed up to a concentration of 250 ng/ml, a significant increase in IL-2 production is reported thereafter. These results, therefore, suggest that in sub-acute exposure, Pd exerts a significant immuno-modulating effect, altering the Th1/Th2 cytokine balance, even at low exposure doses.

The aim of the present study is to assess the effects on the immune system of Wistar rats exposed for 3 months to 0, 1, 10, 100, and 250 ng/ml of Pd as potassium hexachloro-palladate in drinking water by evaluating possible quantitative changes in two type-1 cytokines (IL-2, INF- γ) and one type-2 cytokine (IL-4).

Materials and methods

Animals and husbandry

Fifty male Wistar rats were obtained from the Experimental Animal Production Plant of the Catholic University of Sacred Heart, Rome, Italy. The animals were acclimated for about 2 weeks prior to initiation of dosing. At initiation of treatment, animals were approximately 12 weeks old. The mean weight of the animals was 270 g. Individual weights of animals placed on test were within $\pm 20\%$ of the mean weight. All animals were examined during the acclimation period to confirm suitability for the study. Animals were individually housed during the experiment in Macrolon® cages (Tecniplast SpA., Buguggiate, Italy).

A 12-h light/dark cycle, controlled via an automatic timer, was provided. Temperature was monitored and maintained within the specified range of 19.9–25.1 °C. Relative humidity was monitored and maintained within the range of 50–60%. Solid certified maintenance diet R (Altromin Rieper A. SpA, Vandoies, Italy) for rats was available without restriction. Drinking water was administered *ad libitum*.

Experimental design

Sub-chronic exposure Potassium hexachloro-palladate (i.v.) (Aldrich Chemical Co., Milwaukee, Wisconsin, USA) 99% (16919-73-6) was used for the Pd administration. Fifty male Wistar rats were randomly divided into five groups of 10 rats. Water was given *ad libitum* to each group for 90 days at different Pd levels: 0 (control), 1, 10, 100, and 250 ng/ml potassium hexachloro-palladate. All animals were killed at the end of the exposure time.

The protocol study was approved by the ethical committee of the Catholic University of Rome.

Blood collection Ninety days after the beginning of the experiment, rats were anesthetized with 0.5 mg medetomidine/kg body weight and 75 mg ketamine/kg body weight. Blood from each of the 50 animals was drawn by cardiac puncture and collected in a 1.5-ml vial (Eppendorf srl, Milan, Italy). Sera were separated after centrifugation at 2500 rpm/700 g per 15 min for the immunological assays. Samples were frozen and stored at –20 °C until analysis.

Immunological determinations We used the enzyme-linked immunosorbent assays for quantitative detection of IL-2, IL-4, and INF- γ in rat sera [(r)IL-2, (r)IL-4, and (r)IFN- γ], respectively, purchased from Amersham Biosciences (Little Chalfont, England, UK), according to manufacturer instructions. The absorbance was measured at 450 nm with the multilabel counter Wallac Victor³ 1420 (PerkinElmer, Turku, Finland). Cytokine concentrations are given in pg/ml after proper calibration.

Statistical methods

Statistical analysis was carried out by SPSS statistical software (Statistical Package for the Social Sciences, Inc. Chicago, Illinois, USA). For each of the three cytokines taken into consideration (IL-2, IL-4, INF- γ), we assessed the distributions of the response to potassium hexachloro-palladate exposure. The Kolmogorov–Smirnov Z-test was per-

formed to check the normal distribution of observed values of immunologic parameters.

One-way analysis of variance (ANOVA) was used to evaluate the significance of differences between the mean values of immunologic parameters in the groups defined according to different exposure levels (1, 10, 100, 250 ng/ml and controls). Dunnett *post hoc* multiple comparison test was used to test the significance of differences between the values for each parameter of exposure against the control group. The significance level established for all the tests was 95%.

Results

The Kolmogorov–Smirnov normality test is not significant for all observed variables and Levene's test for homogeneity of variance is not significant. Table 1 shows descriptive statistics for each cytokine in relation to different concentrations of exposure to potassium hexachloro-palladate in the two groups investigated. The significance of the difference between the mean values in the two groups (*P*-value ANOVA) and the significance of the Dunnett *post hoc* multiple comparison test are also reported in the Table 1. Analysis of variance shows that the difference between mean values in the groups reaches statistical significance for IL-2 (*P* = 0.001; Figure 1). Moreover, the INF- γ value after exposure to 100 ng/ml is significantly higher than that of the control animals (Figure 2).

Discussion

The findings suggest that Pd ingested from drinking water following a 90-day exposure may have affected serum levels of IL-2 (Figures 1 and 3) and INF- γ (Figures 2 and 4) across the entire range of concentrations tested. In the case of IL-2 the dose-response was J-shaped (Figures 1 and 3), whereas the INF- γ response was enhanced by treatment across all doses achieving statistical significance at 100 ng/ml (Figures 2 and 4). The lack of effect on IL-4 (90-day

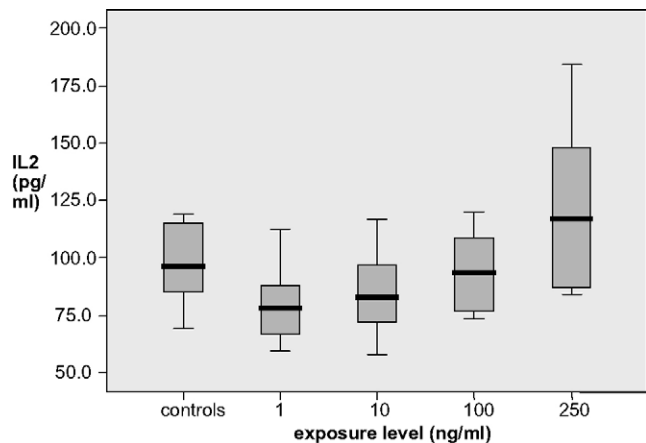


Figure 1 Serum levels of IL-2 of control rats and of four groups of Wistar rats exposed for 90 days to different levels of potassium hexachloro-palladate (1, 10, 100, and 250 ng/ml).

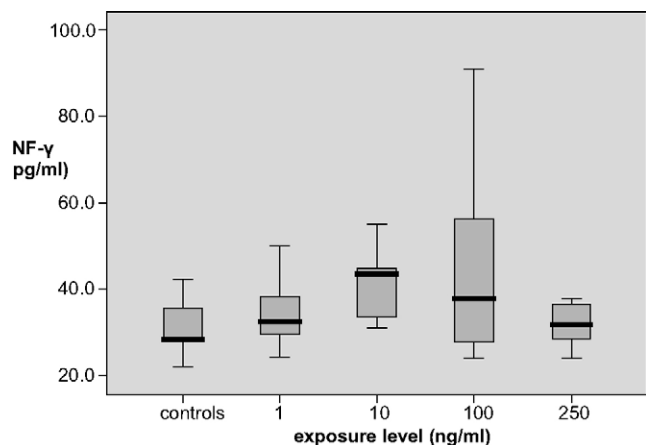


Figure 2 Serum levels of INF- γ of control rats and of four groups of Wistar rats exposed for 90 days to different levels of potassium hexachloro-palladate (1, 10, 100, and 250 ng/ml).

study) differed with that reported in an earlier 14-day study at 10 and 250 ng/ml with the same animal model and exposure conditions (Figure 5). In the case of IL-2, the J-shaped curve was unexpected because the results of the 14-day study showed a progressive increase in concentration-related responses (Figure 3). Nonetheless, various agents, such as bombesin¹² and Substance P¹³ induced

Table 1 Mean levels of serum IL-2 (pg/ml), INF- γ (pg/ml) and IL-4 (pg/ml) in rats after sub-chronic exposure to different concentrations of Pd (ng/ml) as potassium hexachloro-palladate in drinking water

Cytokines	Number of animals	1 ng Pd/ml	10 ng Pd/ml	100 ng Pd/ml	250 ng Pd/ml	Controls	P-value ANOVA
IL-2 (pg/ml)	10	79.1	85.8	92.6	122*	97.3	0.001
INF- γ (pg/ml)	10	35	41	46.2*	31.7	31.6	0.05
IL-4 (pg/ml)	10	27.4	29.7	27.8	27.3	29.3	0.10

Statistical significance of ANOVA (*P*-value ANOVA) and Dunnett *post hoc* multiple test significance (*) (*P* < 0.05).

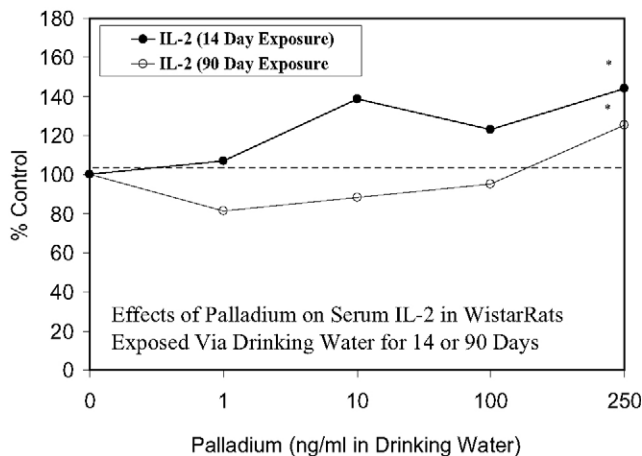


Figure 3 Effects on serum IL-2 (pg/ml) of control rats and four groups of Wistar rats exposed to different concentration of potassium hexachloro-palladate (1, 10, 100, and 250 ng/ml as Pd) in drinking water.

J-shaped dose responses with respect to IL-2 production in CRL-2 cells and in mouse T cells, respectively. These findings suggest the possibility that the J-shaped responses may be mediated by Pd-induced alterations in one or more endogenous agonists. The biological implications of the above alterations in serum cytokine concentrations are uncertain.

The rats were exposed to the Pd via drinking water. The estimated amount of Pd ingested by the average rat at the lowest dose was approximately 0.03 µg/rat/day or about 1.0 µg/10 kg. In the case of children, consumption of Pd would most likely occur via soil/dust ingestion.¹⁴ Let us assume that some children aged 1–2 years ingest very large amounts of soil each day – say 1–10 g. If this soil is along a heavily traveled road, there could be up to

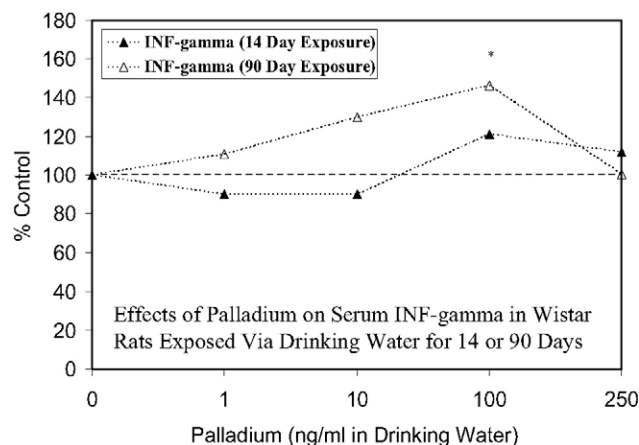


Figure 4 Effect of palladium on serum INF-γ (pg/ml) in Wistar rats exposed via drinking water for 14 or 90 days.

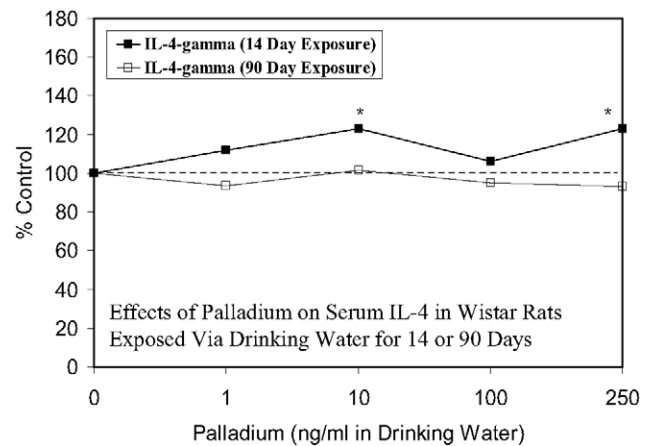


Figure 5 Effects of palladium on serum IL-4 (pg/ml) in Wistar rats exposed via drinking water for 14 or 90 days.

0.1 µg Pd/g soil.¹⁵ Thus, these children could ingest about 0.1–1.0 µg of Pd/day. If these children ingested soil at the 10 g/day rate it would be in the approximate range of the lowest rat exposure group. The US Agency for Toxic Substances and Disease Registry¹⁶ and the EPA¹⁷ assumes that children displaying soil pica behavior may ingest up to 5–10 g of soil per day. Such exposures are likely to be approached and exceeded by animals,¹⁸ including domesticated dogs.¹⁹ Consequently, these preliminary findings indicate the need to carry out further investigations regarding the effect of other Pd compounds and the measurement of other types of cytokines both in the animal model used in our study and other models.

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