In Vitro Effects of Platinum Compounds on Lymphocyte Proliferation and Cytokine Release

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Abstract. In vitro immune effects of Pt compounds of occupational and/or environmental importance, or those used in cancer treatment were studied. Spontaneous and PHA-stimulated proliferation of peripheral blood mononuclear cells (PBMC) and in vitro release of TNF-α, IFN-γ, and IL-5 were assessed in presence of high and very low concentrations of Pt salts: 10^-4 and 10^-7 M (NH₄)₂[PtCl₆], (NH₄)₂[PtCl₄], PtCl₄, PtCl₂, Na₂PtI₆, and cis-diaminedichloroPt (CisPt). Spontaneous and PHA-stimulated PBMC proliferation were both inhibited by 10^-4 M (NH₄)₂[PtCl₆] and (NH₄)₂[PtCl₄], while only PHA-stimulated proliferation was inhibited by 10^-4 M CisPt, without significant effects of the other Pt salts. TNF-α release from PBMC was reduced by 10^-4 M (NH₄)₂[PtCl₆] and INF-γ release was reduced by 10^-4 and 10^-7 M hexa- and tetra-chloroplatinate and 10^-4 M Na₂PtI₆, but not by other Pt salts. IL-5 release (related to the Th2 immune response) was inhibited by 10^-4 M (NH₄)₂[PtCl₆], (NH₄)₂[PtCl₄] and Na₂PtI₆, but it was enhanced by both 10^-4 and 10^-7 M PtCl₄. PtCl₂ did not influence the immune effects. The study shows Pt salts have immune effects and their potency is ranked in the following order: (NH₄)₂[PtCl₆] > (NH₄)₂[PtCl₄] > Na₂PtI₆ and CisPt > PtCl₄ > PtCl₂. These results indicate that certain Pt salts affect lymphocyte proliferation and cytokine release. The intracellular mechanisms responsible for such effects have not been identified.

Keywords: platinum, lymphocyte proliferation, cytokines, immunotoxicity, allergy

Introduction

The emission of Pt group elements (PGE) from catalytic converters began around 1976 in the United States, Canada, and Japan and about 10 yr later in other countries [1]. These elements include platinum (Pt), palladium (Pd), and rhodium (Rh). Today, the troposphere of the Northern hemisphere is heavily contaminated by PGE, as shown by the amounts of these metals in recent snow in Central Greenland, which are 40-120 times higher than in ice dated 7000 yr ago [2].

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The Federal Environmental Agency of Germany observed an increase of environmental Pt concentrations in Frankfurt/Main and the adjacent city of Offenbach during the period 1988-1998 [3]. However, the Pt concentrations were less than 15 pg/m³, the maximal suggested guidance value. Available emission data suggest that Pt in airborne particles derived from catalytic converters is almost exclusively in metallic or oxidic form; about 10% of the Pt, contained in the smaller particles, is soluble in hydrochloric acid solution and forms halogenated derivatives [1,4]. Halogenated Pt compounds, which have lower chemical stability and higher reactivity than the metallic element, are secondarily formed under environmental conditions in water, sediments, and soil [1].
Despite increasing Pt environmental pollution, there is little or no epidemiological evidence of environmental health effects produced by Pt in metallic form or chlorinated Pt salts. At present, the evidence of noxious effects produced by Pt is restricted to allergic, respiratory, and cutaneous diseases (i.e., asthma, rhino-conjunctivitis, urticaria, and contact dermatitis) in workers occupationally exposed to Pt salts [1,5-7], especially miners, workers in foundries and chemical industries that produce catalytic converters, manufacturers of ornaments and electrical appliances, and dental technicians [1,5-7]. Pt compounds for prick and patch tests are available (hexachloroplatinic acid, potassium tetrachloroplatinate, sodium hexachloroplatinate). Pt positive prick tests were found in 26 of 153 Italian workers exposed to Pt in a refinery, 22 of whom had rhinitis, asthma, and urticaria, and 4 had hand dermatitis. Non-occupational dermatoses (contact stomatitis) have also been reported due to combined sensitization to Pt and Pd in dental alloys [8], whereas there are no clear reports of allergic reactions to jewellery containing Pt alone [1]. Patch test reactions to Pt-group elements were reported in two patients with hand dermatitis [9].

Catalytic converters emit Pt that is mostly coated on larger aluminum oxide carrier particles and inhalation of these particles induces maximal absorption of the metal. In a laboratory experiment on rats, 90-day inhalation of aluminum oxide particles (5 µm) loaded with Pt particles (<4 nm) demonstrated up to 30% Pt bioavailability in body fluids and tissues [10].

Excepting CisPt and other Pt compounds used in cancer chemotherapy [11,12], there are few in vitro investigations of immune responses to Pt compounds [13-16]. In this work, we evaluated the in vitro effects of very high and very low concentrations of various Pt compounds on PBMC proliferation and on the spontaneous or mitogen-induced synthesis of cytokines of the Th1 and Th2 patterns. The aim was to identify the highly immune active Pt compounds, which will later be evaluated in respect to concentration-response relationships, as well as their cellular uptake, intracellular distribution, binding, and metabolism.

**Materials and Methods**

**Pt salts.** Solutions (10⁻³ M) of 6 platinum compounds, (NH₄)₂[PtCl₆], (NH₄)₂[PtCl₄], PtCl₄, PtCl₂, Na₂PtI₆ and CisPt, were obtained from ECVAM (Ispra, Italy) and diluted to 10⁻⁴ M and 10⁻⁷ M.

**Isolation of human PBMC.** Healthy male volunteers (n = 9, mean age 34 yr, range 24-58 yr) were recruited for this study. They were not taking any drugs. Fasting EDTA-treated whole blood samples were obtained from each subject at 8 am and PBMC were purified by Ficoll-Hypaque (BioSpa, Milan, Italy) density gradient centrifugation (20 min at 400 x g). After 3 washings, PBMC were resuspended in RPMI 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, 25 mM HEPES, 100 U/mL penicillin, and 100 mg/ml streptomycin (Sigma, St Louis, MO, USA). This mixture is designated as complete medium.

**PBMC proliferation.** PBMC were suspended at 10⁶ cells/ml in complete medium. Aliquots (100 µl) of cell suspension were placed in each well of a standard 96-well microtiter plate (Falcon, Oxnard, CA, USA). The cells were incubated for 78 hr at 37°C in a humidified atmosphere with 5% CO₂ under the following conditions:

(a) no other reagent added (control sample),
(b) with 20 µg/ml PHA (Sigma),
(c) with 10⁻⁴, or 10⁻⁷ M of each of the following salts: (NH₄)₂[PtCl₆], (NH₄)₂[PtCl₄], PtCl₄, PtCl₂, Na₂PtI₆ and CisPt, in both the presence and the absence of PHA.

**Enzymatic immunoassay of PBMC proliferation.** Proliferation was evaluated by the BrdU cell proliferation assay (Oncogene Research Products, Darmstadt, Germany). BrdU was added to wells of the microtiter plate. Cells were fixed and permeabilized and DNA was denaturated by treatment for 30 min at room temperature with a fixative/enaturing solution. Anti-BrdU monoclonal antibody was pipetted into the wells and allowed to incubate for 1 hr. Unbound antibody was washed away and horseradish peroxidase-conjugated goat anti-mouse
antibody was added for 30 min at room temperature. Contents of the wells were removed by inverting over a sink and tapping on paper towels. Chromogenic substrate solution, tetramethylbenzidine (TMB), was added to each well and the plate was incubated in the dark at room temperature for 15 min. Stop solution was added to each well in the same order as the previous addition of substrate solution. All of the reagents were provided with the assay kit and were used in accordance with the manufacturer’s instructions. Experiments were performed in triplicate. The absorbance of the contents of each well was measured using a spectrophotometric microtiter plate reader at dual wave-lengths (450 and 540 nm). The color intensity was proportional to the amount of BrdU incorporated in the cells and hence to the degree of cell proliferation.

Production and measurement of cytokines. Cultures were set up in 1 ml/well 24-well Costar plastic microtiter plates using 0.8 ml of PBMC (containing 10^6 cells) in complete medium under the same conditions described for PBMC proliferation. The cultures were incubated at 37°C in humidified atmosphere with 5% CO_2 for 48 hr; afterwards, cells were checked for viability by trypan blue dye exclusion, using an inverted microscope. Supernatants were collected and stored at -70°C until analysis. Interferon (IFN)-γ, IL-5, and tumor necrosis factor (TNF)-α levels in the culture supernatants were determined by Quantikine colorimetric ELISA kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer’s instructions.

Statistics. Statistical analyses were performed with the Statistica program (release 4.5). The Kolmogorov-Smirnov test was used to evaluate data distributions.

Results

The Kolmogorov-Smirnov test showed that most of the data sets had non-parametric distributions. The values conformed more closely to a parametric distribution when they were expressed as percentages of the corresponding controls, rather than as absolute values. Therefore, data for PBMC proliferation were reported both as absorbance units and % change in relation to control cultures (Table 1 and Fig. 1, respectively), while data for cytokine release from PBMC were only reported as % change (Fig. 2).

Ammoniated Pt compounds, (NH_4)_2[PtCl_6] and (NH_4)_2[PtCl_4], at 10^-4 M significantly inhibited (p = 0.01 and 0.05, respectively) both spontaneous and PHA-stimulated PBMC proliferation (Table 1, Fig. 1). At 10^-4 M, CisPt induced a significant inhibitory effect only on PHA-stimulated PBMC proliferation, when the data were expressed as a percentage. On the contrary, the other Pt compounds at 10^-4 M and all Pt compounds at 10^-7 M showed no significant modification of spontaneous or PHA-stimulated PBMC proliferation (Table 1).

The stimulation index (SI) of blastogenesis of cultures without Pt salts was 8.32±0.99; it was significantly reduced by 10^-4 M (NH_4)_2[PtCl_6] (1.34±0.8; p < 0.001), (NH_4)_2[PtCl_4] (3.42±0.75; <0.05), and CisPt (6.01±6.9; p < 0.05), but not by the other Pt salts.

### Table 1. Spontaneous and PHA-stimulated proliferation of PBMC incubated in presence of 10^-4 and 10^-7 M Pt salts. Results are expressed as absorbance units (mean±SD, n=9).

<table>
<thead>
<tr>
<th>PBMC Proliferation</th>
<th>Concentrations of Pt compounds</th>
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<tbody>
<tr>
<td></td>
<td>10^-4 M</td>
</tr>
<tr>
<td>Spontaneous</td>
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<tr>
<td>Without Pt salts</td>
<td>163±17</td>
</tr>
<tr>
<td>(NH_4)_2[PtCl_6]</td>
<td>106±17*</td>
</tr>
<tr>
<td>(NH_4)_2[PtCl_4]</td>
<td>120±18*</td>
</tr>
<tr>
<td>PtCl_4</td>
<td>157±19</td>
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<tr>
<td>Na_2PtI_6</td>
<td>181±13</td>
</tr>
<tr>
<td>CisPt</td>
<td>161±9</td>
</tr>
<tr>
<td>PHA-stimulated</td>
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<tr>
<td>Without Pt salts</td>
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<td>(NH_4)_2[PtCl_6]</td>
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<td>(NH_4)_2[PtCl_4]</td>
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<tr>
<td>CisPt</td>
<td>924±121</td>
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</table>

*p <0.05; **p<0.01; ***p<0.001  vs the corresponding control cultures without Pt salts, by Mann-Whitney U test.
Fig. 1. Spontaneous and PHA-stimulated proliferation of human PBMC incubated in presence of $10^{-4}$ M Pt salts reported as ratio (%) with/without (control) platinum salts. Values are mean±SEM ($n = 9$). * $p <0.05$; ** $p <0.01$; *** $p <0.001$ vs control cultures without Pt salts, computed by Mann-Whitney U test.

Fig 2. TNF-α release from human PHA-stimulated PBMC in presence of $10^{-4}$ and $10^{-7}$ M Pt salts, reported as ratio (%) with/without (control) Pt salts. Values are mean±SEM ($n = 9$). *** $p <0.001$ vs control cultures without Pt salts, computed by Mann-Whitney U test.
Fig 3. IFN-γ release from human PHA-stimulated PBMC in presence of $10^{-4}$ and $10^{-7}$ M Pt salts reported as ratio (%) with/without (control) Pt salts. Values are mean±SEM (n = 9) * p < 0.05; ** p < 0.01 vs control cultures without Pt salts, computed by Mann-Whitney U test.

Fig 4. IL-5 release from human PHA-stimulated PBMC in presence of $10^{-4}$ and $10^{-7}$ M Pt salts reported as ratio (%) with/without (control) Pt salts. Values are mean±SEM (n = 9). * p < 0.05; ** p < 0.01; *** p < 0.001 vs control cultures without Pt salts, computed by Mann-Whitney U test.
Spontaneous release of TNF-α was 89±52 pg/ml, the spontaneous release was close to the detection limit (8±6 pg/ml) for IFN-γ, and below the detection limit for IL-5. However, PHA-stimulated cytokine production was: TNF-α = 1819±436 pg/ml, IFN-γ = 1125±281 pg/ml, and IL-5 = 91±9 pg/ml. At 10^-4 M, (NH₄)₂[PtCl₆] significantly (p = 0.001) inhibited the PHA-stimulated release of TNF-α from PBMC (Fig. 2); the other 10^-4 M Pt salts and all 10^-7 M Pt compounds did not cause such inhibition.

IFN-γ production after PHA stimulation was significantly inhibited by 10^-4 M (NH₄)₂[PtCl₆], (NH₄)₂[PtCl₄] and Na₂PtI₆ (p = 0.01, 0.05 and 0.01, respectively) and by 10^-7 M (NH₄)₂[PtCl₆] and (NH₄)₂[PtCl₄] (p = 0.01 and 0.05, respectively) (Fig. 3).

IL-5 production was significantly reduced in supernatants of PHA-stimulated cultures with 10^-4 M (NH₄)₂[PtCl₆], (NH₄)₂[PtCl₄] and Na₂PtI₆ (p = 0.01, 0.05, and 0.001, respectively), whereas, PtCl₄, inactive on the other immune parameters, significantly (p = 0.05) increased IL-5 production, both at low and high concentrations (Fig. 4).

**Discussion**

This study shows that most of the Pt salts are active on immune system in vitro, and that chlorinated ammonium Pt compounds are the most immunotoxic. The immune activity of the Pt salts was ranked in the following order: (NH₄)₂[PtCl₆] > (NH₄)₂[PtCl₄] > Na₂PtCl₆ and CisPt(II) > PtCl₂. Ammonium hexachloroplatinate and tetrachloroplatinate were active on all studied parameters (with the exception of TNF-γ production, which was not modified by tetrachloroplatinate). Platinum chloride (PtCl₂) showed stimulatory effects (it increased the production of Th2 cytokine) and platinum dichloride did not show any immunotoxicity. The other studied Pt salts were active on selected immune parameters.

The results demonstrate the importance of speciation in Pt immunotoxicity. In previous studies, immunotoxicity seemed to be confined to compounds that contained reactive ligand systems, the most effective being the chlorides, whereas Pt in metallic, oxidic, or other stable forms exerted minor effects [13-15]. In the mouse popliteal lymph node assay, finely dispersed elemental Pt, PtO₂, PtCl₂ and [Pt(NH₄)₄]Cl₂ did not induce immune responses, while Na₂PtCl₆ and Na₂PtCl₄ increased the number of cells (mainly CD4+ cells) expressing proliferating cell nuclear antigen [13]. Exposure to Na₂PtCl₆ was also able to enhance specific autoantibodies in a mouse strain susceptible to autoimmunity [17]. In exposed human populations, there is no evidence of autoimmune phenomena or diseases induced by Pt salts.

This study shows that tetravalent chloroplatinates exert more immune effects than the bivalent salts. This supports a report that hexa- and tetra-chloroplatinates are more capable of modulating receptor-mediated endocytosis on murine Langerhans cells than bivalent chloroplatinates [15]. This also agrees with a report that workers exposed to chloroplatinate(IV) compounds show higher risk of sensitization than those exposed to tetra-ammine Pt(II) dichloride alone [5]. Both the sensitizing capacity of Pt salts and their activity on lymphocytes and macrophages may, in part, depend on the mechanisms of intracellular uptake of the Pt compounds uptake, eg, by endocytosis [15].

At 10^-4 M, CisPt affected only PHA-stimulated PBMC proliferation and not cytokine-induced production, confirming a previous study [11] and suggesting that Pt inhibition of PBMC proliferation depends on regulatory mechanisms other than IL-2 and other cytokines [18]. A recent in vitro study, utilizing the human micronucleus assay coupled with fluorescence in situ hybridization, demonstrated that Pt salts are genotoxic, inducing oxidative DNA damage [19]. This mechanism may also explain the immune effects of Pt salts.

Ammonium hexachloroplatinate was the most toxic compound on cytokine production. At 10^-4 M concentration, it inhibits TNF-α, IFN-γ, and IL-5 release by cultured PBMCs. IFN-γ production appeared the most sensitive, since it was inhibited by a low concentration (10^-7 M) of this Pt salt. IFN-γ and IL-5 production was also reduced by ammonium tetrachloroplatinate (low statistical significance, p = 0.05) and disodium hexaiodo-
platinate (p = 0.01 and 0.001, respectively). On the contrary, platinum chloride significantly enhanced IL-5 release, known as a marker of Th-2 response [20,21]. PtCl4 may thus exert a marked sensitizing capacity. This is confirmed by the finding of in vitro stimulatory effects of different concentrations of PtCl4 on keratinocytes, which act also as antigen presenting cells (unpublished data).

On the other hand, Pt compounds are among the most potent sensitizers known. The most reactive salts (halogenated and ammine halogenated Pt compounds) are synthesized during refining and treatment in factories. Such sensitization has been reported almost exclusively from occupational exposures. Pt concentrations in samples from catalyst production areas ranged from 12 to 64 ng/m3, compared to the suggested TLV for the environmental air of <15 pg/m3 [1,6]. Sensitized workers exhibit sneezing, rhinorrhoea, chest tightness, wheezing, shortness of breath, and cyanosis, and some of them also develop scaly erythematous dermatitis with urticaria [6,22,23].

With occupational exposures, sensitization reactions have been observed for soluble Pt compounds down to the limit of detection of 0.05 µg/m3. However, these effects have occurred only in individuals who were previously sensitized by higher exposure levels. While extensive studies have been performed regarding a hypersensitive response in Pt-exposed workers who are being routinely monitored, little attention has been paid to possible effects of exposure to platinum emitted into the general environment from motor vehicle catalysts. It is unlikely that the general population exposed to ambient concentrations of soluble Pt, which are at least three orders of magnitude lower, will develop similar effects. In a small study, 3 subjects highly sensitive to Pt salts at low concentrations (as shown by a positive skin-prick test) were tested with particulate exhaust samples. The total Pt content at the highest concentration exceeded 5 µg/ml, which would normally be sufficient to elicit a response. Five extracts at different concentrations did not elicit a positive response on the skin-prick test in the 3 subjects [24]. This observation is compatible with what is known regarding speciation of emitted Pt. Since the emitted Pt is probably in metallic form [25], the sensitizing potential of Pt emissions from automotive catalysts is probably very low. No information is available on the possible environmental conversion of emitted Pt to complex halide salts.

Further studies are needed on the speciation of Pt in the environment. In this study, speciation appeared to be the most important variable in relation to Pt effects on lymphocyte proliferation and cytokine release. The mechanisms responsible for such effects require elucidation, in particular relationship to cellular uptake, intracellular distribution, binding, and metabolism of the metal. The continuation of this research program includes evaluating intermediate concentrations of the Pt compounds in order to delineate their concentration-response relationships.

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