REVIEW ARTICLE

IMMUNOTOXICITY AND SENSITIZING CAPACITY OF METAL COMPOUNDS DEPEND ON SPECIATION

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Immunotoxicity of metal compounds is an issue of great importance due to the recent industrial application of metals with unknown toxicity on the immune system and the discovery of metal intermediary compounds not sufficiently studied yet. In this report we show results of our study on the immunotoxicity of the following metals: the Platinum group elements (Platinum, Palladium, Rhodium), Titanium and Arsenic. We applied functional and non functional assays and investigated both innate and adaptive immune systems, in particular, cell proliferation, cytokine production by PBMCs and O^-_2 production by neutrophils. We obtained the following results: only some Ti compounds (Titanocene, Ti ascorbate and Ti oxalate) show immunotoxicity. Trivalent As compounds (Sodium arsenite and tetraphenyl arsonium chloride) are more immunotoxic than the other investigated As compounds. Genotoxicity of Pt group compounds is in the following order: Pt > Rh > Pd. Immunotoxicity of Pt group compounds is in the following order: Pd > Pt > Rh. Lymphocytes and macrophages show a different reaction of neutrophils to metal toxicity. We can conclude that these studies show that metal immunotoxicity depends on speciation. In general speciation provides additional and often essential information in evaluating metal toxicity. However, there are many difficulties in applying speciation in investigating toxico-kinetic aspects to many metals, mainly due to the lack of information about the existence and significance of species and to the lack of analytical methods for measuring species in biological samples.

Immunotoxicity of metals compounds is an issue of a great importance due to the recent industrial application of metals with unknown toxicity on the immune system and the discovery of metal intermediary compounds not sufficiently studied yet. Metals have various interactions with the immune system: essential elements, toxic or sensitizing (Table I).

Some metals, like chromium, have multiple interactions: trivalent chromium is an essential element (1), hexavalent has a strong toxicity (2) and chromium salt (potassium dichromate) may sensitize humans. Vanadium (V) is an element with wide industrial applications and environmental release, and 10^{-4} M NaVO3 exerts inhibitory effects on PBMCs, while at 10^{-7} M it exerts a stimulatory action with a slight shift of the immune response towards a Th2-type response (3). Inhibitory effects of cadmium on peripheral blood mononuclear cell proliferation and cytokine release are reversed by zinc and selenium salts (4). Arsenic (As) also shows multiple activity on the immune system depending on speciation and dose (5). In fact, 10^{-7} M trivalent As and DMAs increase *in vitro* proliferation of Peripheral Blood Mononuclear Cells (PBMC), showing a stimulatory metabolic effect, so considered to be an essential element (6-7). Results of experimental studies indicate the beneficial effect of As in drinking water with a content ranging from 12 to 50 μg/l (7), while higher levels, exceeding 200 μg/l are reported to exert toxic effects in humans (8).

Platinum (Pt) salts may induce allergic asthma in exposed workers, whereas halogenated Pt compounds [$(NH_4)_2PtCl_4$; $(NH_4)_2PtCl_6$] strongly inhibit lymphocyte proliferation and cytokine release in vitro (9-10). In previous studies, Pt allergy seemed to be confined to a small group of charged compounds containing reactive ligand systems (the most effective of which being chloride ligand systems). While metallic Pt in the metallic, oxide or other stable forms showed lower sensitizing effects (11). In the mouse popliteal lymph-node assay, finely dispersed Pt, and PtO_2, $PtCl_2$ and $[Pt(NH_2)_4]Cl_2$ did not induce an immune response, while Na_2PtCl_6 and Na_2PtCl_4 induced an increase of cells (mainly $CD4^+$) which express proliferating cell nuclear antigens (12-13). These different *in vivo* immune effects of Pt compounds were related to their capacity to modulate *in vitro* mechanisms of receptor-mediated endocytosis in murine Langerhans cells (12). Na_2PtCl_6 and Na_2PtCl_4 exerted a more

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potent contact sensitizing effect than $[Pt(NH_3)_4]Cl_2$ in endocytosis assay. The results of this experimental study are in agreement with those of an investigation on workers in a plant producing auto-catalysts: workers exposed to chloroplatinate (IV) compounds showed a cumulative probability of 51% of being sensitized after 5 years of exposure, while those exposed to the tetra-ammine Pt(II) dichloride alone were not sensitized (14).

Among the sensitizing metals, nickel may cause different immune reactions and symptoms depending on the type of exposure (15). Inhaled Ni can cause allergic asthma (IgE mediated 1st type of immune reactions). In a large number of subjects skin contact with Ni results in an allergic contact dermatitis mediated by a 4th type cell-mediated immune reaction. Food ingested Ni may have clinically relevant immune effects: gastrointestinal symptoms and a flare up of skin lesions. Studies have shown a decrease of NK cell activity (16-17) and apoptosis of $CD8^+$ cells in the intestinal epithelium, where $CD4^+45RO^+$ cells proliferate (18).

The *in vitro* effects of tellurium compounds were also determined: Te(IV) inhibited PBMC proliferation and IFN-gamma, IL-5 and TNF-alpha release from PBMC more than Te (VI) (19). In 1998, we started, in collaboration with the "European Center for the Validation of Alternative Methods", a study in order to determine the potential immunotoxicity of metal compounds. It was a mechanistic study that compared the effects of a large number of metals and their compounds. In this report we will discuss the main results obtained by the experiments on titanium, platinum, palladium, rhodium and arsenic.

For each metal, we chose the compounds present in the environment, forms employed in the industry of prevalent occupational importance, present in the food chain or utilized in the treatment of many pathologies like cancer. The oxalate of titanium has wide industrial applications, like the aero-spatial industry, the ascorbate is mainly used in agriculture, the titanocene is employed as chemotherapic agent and the dioxide is a biocompatible component of sunscreens. The Platinum group elements (platinum, palladium, rhodium), used for jewellery and for making catalytic converters, are released into the environment both by vehicular traffic and from industries. Many As compounds are part of the food chain and are ingested with food or metabolized in our body. Some metabolites of arsenic, recently detected, are synthesized after the absorption of food containing As. They seem to be responsible for the genotoxicity and cancerogenicity of As. Table II shows the complete list of utilized metal compounds.

MATERIALS AND METHODS

Various methods are employed in the study of immunotoxicity, and can be divided in non-functional, investigating the amount of specific cells or substances of immune system, and functional, studying the activity of cells in response to various stimuli and the experimental models of immune diseases. In this study the following methods were utilized: Lymphocyte proliferation, NK cell function, O_2^- production by neutrophils, lymphocyte subpopulations and cytokine production in cultured peripheral mononuclear cells (IL5, INF γ and TNFα). Cell proliferation and cytokine production will be discussed in this report.

Cell proliferation . 100 ml aliquots of PBMC suspension were placed in each well on 96-microtiter plate. Cells were then incubated for 78 hours at 37°C in humidified atmosphere with 5% CO2 and in the following conditions:

a) without PHA (control samples);
b) with 20 mg/ml PHA (control samples);
c) containing 10^{-4} or 10^{-7} M metal compounds without PHA;
d) containing 10^{-4} or 10^{-7} M metal compounds with PHA.

Sodium arsenite (III), Sodium arsenate (V) and DMAs concentrations were 10^{-4}, 10^{-5}, $5X10^{-5}$, 10^{-6}, $5X10^{-6}$, 10^{-7}, $5X10^{-7}$, 10^{-8}, $5X10^{-8}$, 10^{-9} M;

Pt, Pd, Rh, Ti compounds were added from the 10^{-3} and 10^{-7} M stock solutions and As compounds were added from the 10^{-3}, 10^{-4}, $5X10^{-4}$, 10^{-5}, $5X10^{-5}$, 10^{-6}, $5X10^{-6}$, 10^{-7}, $5X10^{-7}$, 10^{-8} M stock solutions previously prepared.

BrdU cell proliferation assay was used (Oncogene, Darmstadt, Germany) for the evaluation of cell proliferation. During the last 24 hours of culture BrdU was added to wells of the microtiter plate. Cells were fixed and permeabilized, while the DNA was denaturated by a 30-min-treatment with fixative/denaturing solution at room temperature. Anti-BrdU monoclonal antibody was pipetted into the wells and incubated for one hour. Unbound antibodies were washed away and horseradish peroxidase-coniugate goat anti-mouse was added for 30' at room temperature. Chromogenic substrate solution tetramethylbenzidine was then added to each well and incubated in the dark at room temperature for 15 min. Stop solution was added to each well in the same order as the previously added substrate solution. Absorbance in each well was measured using a spectrophotometric plate reader at dual wavelengths of 450-540 nm. Color intensity was proportional to the amount of incorporated BrdU and to the degree of cell proliferation.

Cultures for measurement of cytokines. Human PBMC were purified from EDTA-treated whole blood by Ficoll-Hypaque density gradient centrifugation. Cultures were set up in 1 ml/well 24-wells costar plastic plates, using 0.8 ml of PBMC containing 1 million cells/well in complete medium, at 37°C, in humidified atmosphere with 5% CO_2 for 48 hours and the previously described conditions:

a) without PHA (control samples)
b) with 20 mg/ml PHA (control samples)
c) containing 10^{-4} or 10^{-7} M metal compounds without PHA
d) containing 10^{-4} or 10^{-7} M metal compounds with PHA

Supernatants of cultures were stored at –70 °C in aliquots until analysis. IFN-γ and TNF-α levels in supernatants were determined by Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions.

RESULTS

Titanium Compounds. As shown in a previous study (20), Ti dioxide (biocompatible material) did not show

Table I. *Possible interactions between metals and Immune system.*

	Examples
Essential Metals	Copper, Iron, Manganese, Iodine, Zinc, Selenium, Trivalent Chromium, Arsenic
Toxic Metals	
At high dose	Zinc, Copper, Cobalt, Cadmium, Mercury
At low dose	Lead, Hexavalent Chromium
Sensitizing Metals	
Allergic Contact dermatitis	Nickel, Chromium, Cobalt, Platinum, Mercury, Tungsten, Vanadium Copper, Zinc, Palladium, Tin, Iodine, Lithium, Gold
Asthma and rhinitis	Nickel, Chromium, Cobalt, Platinum, Mercury, Vanadium

Table II. *Metal compounds utilized in the study.*

Titanium	TiO_2 (Ti dioxide), $(C_2H_5)_2TiCL_2$ (Ti ascorbate), $TiO(C_2O_4)_2$ (Ti oxalate), Titanocene
Arsenic	Sodium Arsenite (III), Sodium Arsenate (V), potassium-esa-fluorum-arsenate (V), Sodium-esa-fluorum-arsenate (V), MMAs (V), DMAs (V), Tetra-phenil-arsonium chloride (III)
Platinum	halogenated compounds as $(NH_4)_2PtCl_6$ and $(NH_4)_2PtCl_4$; chlorinated compounds as $PtCl_2$ and $PtCl_4$; NH_2PtI_6; CisPt
Palladium	halogenated compounds as $(NH_4)_2PdCl_6$ and $(NH_4)_2PdCl_4$; chlorinated compound as $PdCl_2$
Rhodium	halogenated $(NH_4)_2RhCl_6$; chlorinated $RhCl_3$

Table III. *Proliferation of PHA stimulated PBMC in presence of different concentration (10^{-4} M and 10^{-7} M) of As compounds (Mann Whitney U test: *p=0.05; **p<0,01; ***p<0.001).*

Without As	1433 ± 158	
Sodium Arsenate	904 ± 181 *	1389 ± 240
Sodium Arsenite	379 ± 202 ***	1592 ± 146 *
MMAs	1477 ± 312	1351 ± 339
DMAs	1309 ± 377	1847 ± 343 **
Tetra-phenyl-arsonium-chloride	176 ± 10 ***	1454 ± 393
Sodium-esa-fluorum-arsenate	1312 ± 315	1412 ± 310
Potassium-esa-fluorum-arsenate	1398 ± 141	1371 ± 168
	10^{-4} M	**10^{-7} M**

any immunotoxicity, while titanocene (anticancer therapy) inhibited cytokine release and Ti oxalate and ascorbate have a marked immunotoxicity (21). In particular, the Titanium compound employed in industry (oxalate) and the one used in agriculture (ascorbate), significantly inhibited the cell proliferation in PHA stimulated cultures, when added at maximal concentration. On the contrary, they do not seem to have any effect in PHA unstimulated cultures. In fact, Ti ascorbate and oxalate stimulation index was significantly reduced from the control without metals (Fig. 1).

Ti oxalate and ascorbate and Titanocene (used as chemotherapic agent) reduced the release of TNF, when used at their maximal concentration. Ascorbate showed a highly significant effect on TNF release at lower concentration also, whereas the activity of oxalate and titanocene was near the limits of statistical significance. The sensibility of INF was quite different: ascorbate did not show any activity, whereas titanocene and Ti oxalate at maximal concentration strongly inhibited INF release. The inhibitory activity of titanocene on cytokine release was so important, that in some experiments both INF and TNF were undetectable.

Arsenic Compounds. The studied immune functions, cell proliferation and cytokine release, showed that the toxicity of 10^{-4} M As compounds was in the following rank order: Sodium Arsenite (III) > Tetra-phenyl Arsonium Chloride (III) > DMAs (III) and Sodium Arsenate (V) >

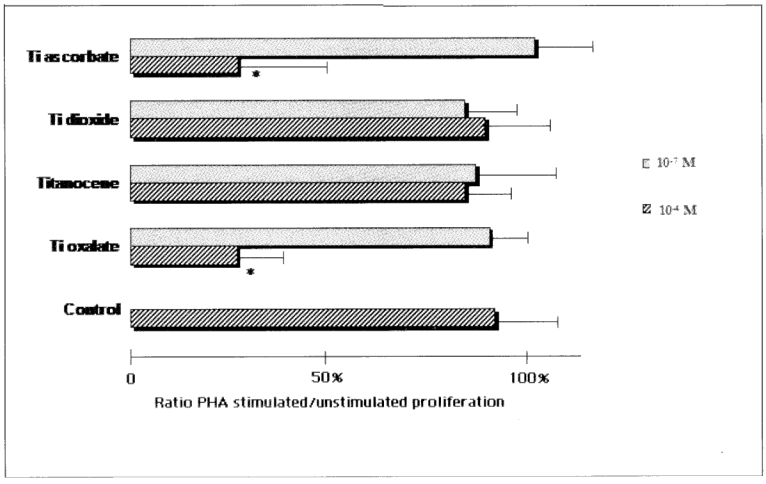

Fig. 1. *PBMC proliferation in cultures with Ti compounds: Stimulation Index (Mann Whitney U test: * $p<0.001$).*

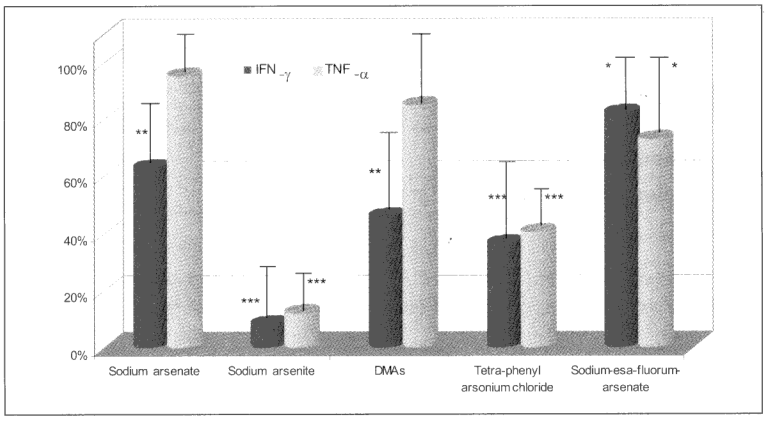

Fig. 2. *Percentage, related to the control, of IFN-g and TNF-a release from PHA stimulated PBMC in presence of 10^{-4} M As compounds (Mann Whitney U test: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.)*

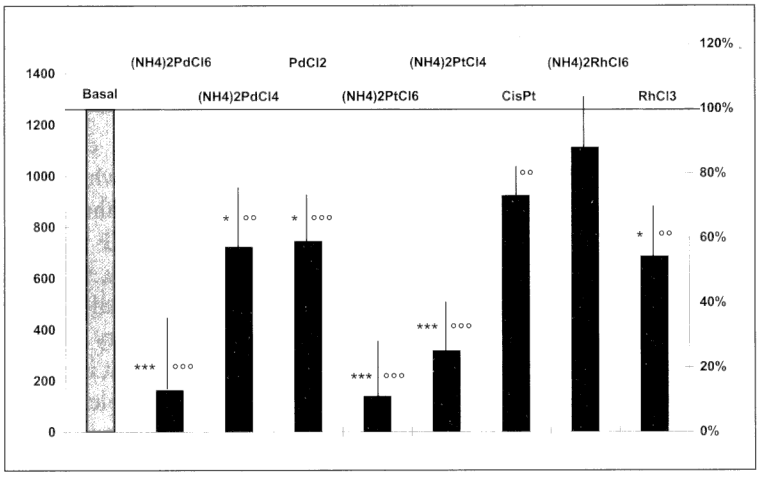

Fig. 3. *PHA stimulated PBMC proliferation without and with 10^{-4} M chemical compounds (Mann Whitney U test: * ° $p<0.05$; ** °° $p<0.01$; *** °°° $p<0.001$).*

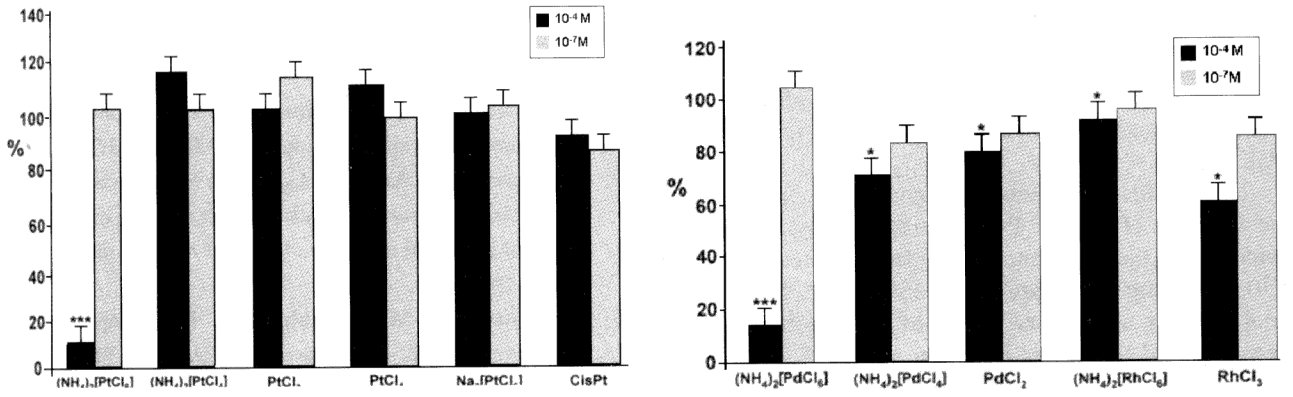

Fig. 4. *Percentage (related to the control cultures) of PHA-stimulated TNF-α release from PBMC in presence of 10^{-4} and 10^{-7} M Pd, Pt and Rh compounds. Values are mean ± S.E.M. (n = 9). TNF-α release in the control cultures is 1319 ± 133 pg/ml (Man Whitney U test. Significant difference in relation to the control cultures without metal compounds : *p<0.05; ***p<0.001).*

other compounds.

Sodium arsenite and tetraphenyl arsonium chloride are more immunotoxic than the other As compounds investigated (Fig. 2). Sodium arsenite was more efficient in inhibiting lymphocyte proliferation than tetraphenyl arsonium cloride, which was more active in inhibiting cytokine production by PBMC. Sodium arsenate inhibited PBMC proliferation also at 10^{-5} M concentration but did not inhibit TNF-α production at 10^{-4} M. Sodium-esa-fluorum-arsenate at 10^{-4} M did not inhibit PBMC proliferation but slightly inhibited TNF-α release. On the whole, each inorganic or organic As compound exerts a specific immunotoxic effect.

Trivalent compounds showed the greatest toxicity on cell proliferation at maximal concentration of 10^{-4} M. On the contrary, 10^{-7} M of arsenite and DMAs induced a stimulation (Table III). Studying a dose-response curve it was observed that the stimulation was not obtained by concentrations below 10^{-8} and 10^{-9} M. Many authors have described this phenomenon of "hormesis" and all of them sustain that 10^{-6} -10^{-7} are the stimulatory concentrations (22-23). The increased proliferation of PBMC in presence of 10^{-7} M trivalent As and DMAs could be explained by a stimulatory metabolic effect of As (6-7). Arsenic compounds at 10^{-4} M (in particular sodium arsenite > tetraphenyl arsonium chloride) inhibited cytokine release by PBMC, whereas sodium arsenate and potassium- and sodium-esa-fluorum arsenate showed lower activity.

Recent investigations, showing that genotoxic and cytotoxic effects of As may be produced by trivalent intermediary organic compounds (produced during metabolism of MMAs and DMAs), can explain the immunotoxicity of pentavalent As compounds (24-25). A careful investigation on the uptake and the metabolism of metallic compounds is necessary in order to fully explain the activity of substances produced during the metabolism of metal compounds.

Platinum group elements – PGE- (Platinum, Palladium and Rhodium). Palladium compounds at 10^{-4} M and $(NH_4)_2PtCl_6$ inhibited spontaneous and PHA-stimulated PBMC proliferation, as well as TNF-α, INF-γ and IL-5 release by PHA-stimulated PBMC. Other Pt or Rh salts did not have the same effects (Fig. 3). The effects of the ammonium hexa- and tetra-chloro-platinate and palladate compounds (IV and II oxidation state, respectively) were similar when PBMC proliferation and INF-γ or IL-5 release were considered; however, TNF-α release was reduced by all the chlorinated Pd compounds but only by 10^{-4} M $(NH_4)_2[PtCl_6]$ among the six tested Pt derivatives.

Pt(IV)ACC and Pd(IV)ACC were generally more active than Pt(II)ACC and Pd(II)ACC. Moreover, these Pt and Pd salts were more active in inhibiting PBMC proliferation and TNF-α release than ammonium hexachororhodate (RhIV). Ammonium hexachororhodate was in turn less effective than Rh(III) trichloride in reducing both PBMC proliferation and cytokine release.

$PtCl_2$ did not exert any effect on PBMC, while the only immune effect of $PtCl_4$ was to enhance the release of IL-5, known marker of the Th-2 immune response (26).

Na_2PtI_6 (a tetravalent iodide Pt compound) inhibited IFN-γ and IL-5 release. This demonstrates that iodide Pt compounds, which may be synthesized in the environment (11), can be immunotoxic.

Our study demonstrates that Pd (II, IV) and Rh (III, IV) inhibit TNF-α release and that Pt exerts a similar effect only as ammonium hexachloroplatinate (IV) salt (Fig. 4). TNF-α is involved in the pathogenesis of septic

shock and rheumatoid arthritis, and represents a cytotoxic factor for many tumour cells also by inducing apoptosis (24). In this regard, the substantial lack of effects of Pt salts on TNF-α may account for the more elevated cancer-related genotoxicity of Pt compounds among those of the PGE (28).

The results obtained may contribute to explain the immune effects of PGE compounds by providing evidences on the importance of speciation in metal toxicity (29). The same results are in agreement with the present ones showing no immune effects of Pt dichloride. It has been reported that atopy is not an important risk factor for the development of sensitization and allergy to Pt salts in exposed workers (30). However, prick test reactions to Pt, Pd and Rh were found in about 15 % of workers in an Italian Pt refinery, who also showed positive reactions to common allergens (a marker of atopy) (31). Moreover, a HLA-DR3 phenotype was found more common among Pt-sensitized symptomatic workers exposed to Pt salts, with a significant association varying with the intensity of the exposure to the sensitizing agent (32).

A study performed by three centers of Dermatology in Germany reported a surprisingly high number of positive patch test to Pd compounds (33). It is not clear whether sensitization to Pd occurs because of exposure to the metal or because of cross-reactions with other elements. Ni-reactive T lymphocyte clones from donors reacted with Pd and Cu salts (34). This cross-reactivity could have been favored by the location of Ni, Pd and Cu in the periodic table and by their similarity in binding to histidine residues of surface peptides of major histocompatibility complex class II molecules. Positive patch test reactions to both Pd and Ni were found in patients with dental restorations (35-36) and cross- reactions among Pd, Pt, Rh and iridium were shown on prick testing in workers exposed to these metals (31). Cross-reactivity between Pd and Ni sulphate was also found using patch test techniques in laboratory animals, guinea pigs (37-38).

DISCUSSION

We can conclude that these studies show that metal immunotoxicity depends on speciation. These studies indicate that chemical speciation of Titanium, Arsenic and PGE salts is important for determining their immunotoxic effects. Moreover, Pd compounds have to be taken in greater consideration also in order to prevent a possible risk of cross-reactions in subjects sensitized to other metals. In general speciation provides additional and often essential information in evaluating metal toxicity. However, there are many difficulties to apply speciation in investigating toxicokinetic aspects to many metals mainly due to the lack of information about the existence and significance of species and to the lack of analytical methods for measuring species in biological samples.

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