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Title: Cross-reactivity among some metals in a murine metal-allergy model

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Authors: M. Kinbara^{1,2}, Y. Nagai¹, T. Takano-Yamamoto², S. Sugawara¹, Y. Endo¹

Affiliations: ¹Department of Molecular Regulation, Graduate School of Dentistry,

Tohoku University, 4-1 Seiryō-machi, Aoba-ku, Sendai 980-8575, Japan

²Department of Orthodontics and Dentofacial Orthopedics, Graduate School of

Dentistry, Tohoku University, 4-1 Seiryō-machi, Aoba-ku, Sendai 980-8575, Japan

Correspondence: Masayuki Kinbara

E-mail: kinbara@dent.tohoku.ac.jp

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What's already known about this topic?

- Lipopolysaccharide (LPS) markedly reduces the minimum allergy-inducing concentration of metals at both the sensitization and elicitation steps (especially at the latter).

What does this study add?

- Among Ni, Pd, Co, Cr, Cu, and Au, only Ni and Pd cross-react.
- In the presence of LPS or infection, some trace contaminant metals included in metal reagents may be sufficient to evoke allergy, and thus high-purity reagents should be used for clinical patch-testing.

Summary

Background Information concerning cross-reactivity among metal-allergens is scarce. We previously devised a murine metal-allergy model using lipopolysaccharide (LPS) as an adjuvant. LPS reduces the minimum allergy-inducing concentration (MAIC) of metals at both the sensitization and elicitation steps.

Objectives Here, we examined allergic cross-reactivity among some metals in this murine model, and compared the effects of ultra-pure (99.99% or more) and low-purity (93-99%) metal salts.

Methods A mixture of a metal salt and *E. coli* LPS was injected intraperitoneally into BALB/c mice (0.25 mL/mouse). Ten days later, metal salts (with or without LPS) were challenged to ear-pinnas (20 μ L/ear), and ear-swelling was measured.

Results Among the ultra-pure metals tested (Ni, Pd, Co, Cr, Cu, and Au), only Ni and Pd cross-reacted. In this cross-reaction, their MAICs were at the same level. Combined challenge with Ni and Pd at sub-MAICs (but not at higher concentrations) produced an additive effect. Surprisingly, mice sensitized with low-purity Ni reacted to all the tested low-purity metals (Ni, Pd, Co, and Cr), and the low-purity metals were shown to contain contaminant metals.

Conclusions In our model: (i) Ni and Pd (members of the same group in the periodic table of elements) cross-react with each other, (ii) this cross-reaction may depend on true and false antigens forming metal-protein complexes with similar spatial geometries, (iii) Co, Cr, Cu, and Au do not cross-react with each other, (iv) in low-purity materials, trace contaminant metals may be sufficient to evoke allergy, and thus (v) high-purity metal salts should be considered for use in clinical patch-testing.

Introduction

Metals (or alloys, such as are used in accessories, buttons, medical devices, mobile phones, leather goods, etc.) frequently induce allergic reactions, and metal allergy remains a prevalent problem. Traditionally, Ni, Co, and Cr have been seen as important contact allergens, but recently Au and Pd have attracted much attention because of the high prevalence of contact allergy involving these metals¹. Because of the historical paucity of adequate animal models, most studies of metal-allergies have been performed

in vitro using sensitized human lymphocytes, and it has been believed that T-cells are essential for metal-allergies, as well as contact hypersensitivities to classical haptens²⁻⁴. However, the detailed mechanisms underlying metal-allergy remain obscure. Unlike classical haptens, metal ions are thought to form geometrically highly defined, but reversible, coordination complexes with partner molecules within the body, thereby becoming antigens⁵. Consequently, hosts may recognize metal ions in complicated ways⁵⁻⁸.

Lipopolysaccharide (LPS, a cell-wall component of Gram negative bacteria) stimulates innate immunity via toll-like receptor 4 (TLR4). LPS stimulates dendritic cells expressing TLRs, causing them to mature⁹⁻¹¹, and LPS also modifies adaptive immunity^{12,13}. When we examined the effect of LPS on Ni-allergy in mice, we found that LPS strongly promoted metal-allergy at both the sensitization and elicitation steps, and we thereby established a reproducible murine metal-allergy model¹⁴. Surprisingly, LPS-promoted metal-allergy occurs even in T cell-deficient nude mice, and the allergic response is minimal or weak in mice that lack TLR4, IL-1, or histidine decarboxylase¹⁴. Recently, we found (i) that at the sensitization step, LPS reduces the minimum allergy-inducing concentration of Ni (Ni-MAIC) from 1×10^{-2} M to 1×10^{-6} M, and (ii) that at the elicitation step, LPS reduces Ni-MAIC from 1×10^{-5} M to 1×10^{-12} M¹⁵.

Many patients with metal-allergy exhibit positive responses to several metals, such as Ni, Pd, and Co^{1,6}. Because the details of cross-reactions remain unclear, it is difficult to judge whether such plural reactions reflect sensitizations to individual metals or cross-reactions between metals^{1,6}. Using T-cell clones or T-cells, Moulon *et al.*¹⁶, Pistor *et al.*¹⁷, and Rustemeyer *et al.*¹⁸ showed that Ni-reactive T-cells cross-react with Pd and/or Cu, but not with metals that are situated more remotely in the periodic table of elements. Those authors suggested that the Cu- and Pd-allergies occasionally

observed in Ni-allergic patients may be due to cross-reaction at the T-cell clonal level rather than to concomitant sensitization¹⁶⁻¹⁸. However, information concerning cross-reactivity among metals is still inadequate, with *in vivo* studies being especially lacking.

In the present study, we examined cross-reactivity among metal-allergens using our LPS-promoted murine model, and we compared effects between ultra-pure (99.99% or more) and low-purity (93-99%) metal salts.

Materials and methods

Animals

BALB/c mice were bred in our laboratory. They were kept under standard conditions [a light (07:00-19:00) - dark (19:00-07:00) cycle at a controlled temperature (23 ± 1 °C)]. The mice used for the present study were female (6-8 weeks old). All experiments complied with the Guidelines for Care and Use of Laboratory Animals in Tohoku University. Detailed experimental protocols are described in the section relating to each experiment.

Reagents

LPS from *Escherichia coli* O55:B5 prepared by the method of Westphal and Jann (1965) (phenol-water extraction) and ultra-pure (UP) metal salts [NiCl_2 (> 99.999%), PdCl_2 (> 99.999%), CoCl_2 (> 99.999%), CrCl_2 (> 99.99%), CuCl_2 (> 99.999%), and AuCl_3 (> 99.99%)] were purchased from Sigma (St. Louis, MO, USA). Low-purity (LP) metal salts [NiCl_2 (> 95.0%), PdCl_2 (> 99.0%), CoCl_2 (> 99%), and CrCl_2 (> 93.0%)]

were purchased from Wako Pure Chemical Ind. (Osaka, Japan). Although we cannot show all the data relating to the contaminants present in these products, the manufacture's data for CoCl_2 are presented here as an example. (a) A 100 g sample of the low-purity CoCl_2 product (> 99%) contains Cu (< 1 mg), Mg (< 2 mg), Ca (< 5 mg), Zn (< 2 mg), Pb (< 3 mg), Mn (< 5 mg), Fe (< 5 mg), and Ni (< 50 mg). It should be noted that the amounts of other possible contaminant metals are not analyzed. (b) A 100 g sample of the ultra-pure CoCl_2 product (> 99.999%) contains Cu (90 μg), Mg (< 4 μg), Ca (< 4 μg), Zn (< 4 μg), Pb (97 μg), Mn (< 4 μg), Fe (28 μg), and Ni (< 4 μg). It should be noted that other data are not shown, although various other metals are analyzed in this product. Metal salts and LPS were dissolved in sterile saline. Unless otherwise noted, we used ultra-pure materials. In this paper, for convenience, each metal salt is usually expressed simply as the symbol for the metal.

Sensitization, challenge, and measurement of allergic ear-swelling

Mice were sensitized to a given metal by an intraperitoneal (i.p.) injection of a solution containing 0.5 mM of a given metal(s) and 0.5 $\mu\text{g}/\text{mL}$ of LPS (0.25 mL/mouse). Ten days after such a sensitization injection, a solution containing a given metal(s) and/or LPS was challenged intradermally (i.d.) to the left and right ear-pinnas near the root of the ear (20 $\mu\text{L}/\text{ear}$; under ethyl ether anesthesia), their concentrations being indicated in each experiment. Ear-thickness was measured at a site 2-3 mm distant from the challenge site at the times indicated in each experiment using a Peacock dial thickness gauge (Ozaki MFG Co. Ltd., Tokyo, Japan). The net ear-swelling (i.e., the difference in thickness between before and after the challenge) was recorded.

Statistical analysis

Experimental values are given as the mean $\pm$ standard deviation (SD). The statistical significance of differences was assessed using a Bonferroni multiple-comparison test after ANOVA with the aid of InStat software. *P* values less than 0.01 were considered to indicate significance.

Results

Challenges with various metals, without LPS, to Ni-sensitized mice

First, we examined possible cross-reactions among Pd, Co, Cr, Cu, and Au in Ni-sensitized mice. As shown in Fig. 1, allergic responses were induced by challenge with Ni and by challenge with Pd if they were given at 0.01 mM or more (but not at 0.001 mM or less), and the magnitude of the response was similar between these two metals. In contrast, Co, Cr, Cu, and Au did not induce any detectable allergic swelling. These results suggest that in Ni-sensitized mice, Pd (but not Co, Cr, Cu, and Au) is a cross-reactant, and that the MAICs of the true antigen (Ni) and this false antigen (Pd) are almost at the same level. As usual in such experiments, a small amount of ear-swelling occurred within 24 h after each injection (e.g., Figs. 1 and 2). This was due to the injection itself, regardless of which solution was injected.

Challenges with various metals, together with LPS, to Ni-sensitized mice

As described in Introduction, LPS reduces Ni-MAIC at both the sensitization and elicitation steps¹⁵. We therefore examined the effects of LPS on the observed cross-reactions. Ni-sensitized mice were challenged with a mixture containing 1 nM of

Ni or Pd and/or 0.5 µg/mL LPS (20 µL/ear). It should be noted that in this experiment, a low concentration of Ni or Pd (viz. 1 nM = 1×10^{-9} M) was used. At this concentration, neither metal by itself induced an allergic response (Figs. 2a and 2b). However, both Ni (true antigen) (Fig. 2a) and Pd (false antigen) (Fig. 2b) induced allergic swelling in the presence of LPS.

We also examined the effects induced when Co, Cr, Cu, or Au, none of which cross-reacted with Ni (Fig. 1), were injected together with LPS in Ni-sensitized mice. None of these metals induced allergic swelling, even though they were injected at a high concentration (0.1 mM), in the presence of LPS (Figs. 2c-2f), confirming that Ni does not cross-react with these metals.

Cross-reactions in mice sensitized to a metal other than Ni

In this experiment, mice were sensitized with a metal other than Ni (viz. Pd, Co, Cr, Cu, or Au), and challenged with a solution including 0.1 mM of Pd, Co, Cr, Cu, or Au plus 0.5 µg/mL LPS. Under those conditions, Pd (true antigen this time) and Ni (false antigen this time) induced allergic swelling only in Pd-sensitized mice (Fig. 3a), providing further confirmation of the cross-reaction between Pd and Ni. In mice sensitized with Co, Cr, Cu, or Au, only the true antigenic metal induced allergic swelling (Figs. 3b-3e), indicating that in these mice, there were no cross-reactions among the metals tested.

Concomitant challenge with Ni and Pd (cross-reactants) to Ni-sensitized mice

The results described above strongly suggest that Ni and Pd are cross-reactants. To try to confirm this, we examined the effect of a concomitant challenge with Ni and Pd (at two different concentrations) to Ni-sensitized mice. First, the mice were challenged with

a solution containing 0.1 mM each of Ni and Pd plus LPS. This challenge induced ear-swelling of a magnitude very similar to the responses induced when either of the metals was delivered separately (with LPS) (Fig. 4a). Next, mice were challenged with a solution containing 5 μ M each of Ni and Pd (a sub-MAIC), but without LPS (cf. Figs. 1a and 1b). The ear-swelling induced by this dual challenge appeared to be (i) additive when compared to the responses to each metal alone (Fig. 4b) and (ii) similar to that induced by 10 μ M of either Ni or Pd (see Figs 1a and 1b). Taking into consideration the purity (99.999 %) of the reagents, these results suggest that the effects of both Ni and Pd are maximal at 0.1 mM in the presence of LPS, and support the inference that they are indeed cross-reactants.

Concomitant challenge with Ni and Co (non-cross-reactant metals) to

Ni+Co-sensitized mice

As described above, Ni and Co did not cross-react with each other (Figs. 1c, 2c, and 3b). Thus, it was of interest whether Ni and Co would additively induce ear-swelling in mice sensitized concomitantly to these two metals. Mice were sensitized with a solution containing 0.5 mM each of Ni and Co plus 0.5 μ g/mL LPS, and challenged with a solution containing 0.1 mM Ni and/or Co in the presence of LPS (0.5 μ g/mL). As shown in Fig. 5, in magnitude the ear-swelling responses were almost the same among the three groups (challenged with Ni alone, Co alone, or both Ni and Co). Our interpretation of this result will be found in Discussion.

Effects of low-purity (LP)-metal salts

In all the experiments described above, ultra-pure (UP) metal salts (99.99% or more) were used. As described in Introduction, we found previously that LPS reduces Ni-MAIC from 1×10^{-2} M to 1×10^{-6} M at the sensitization step, and from 1×10^{-5} M to 1×10^{-12} M at the elicitation step¹⁵. This suggests that if a preparation of a metal salt includes contaminant metal salt(s), the contaminant(s) might also be a cause of metal allergies resulting in *apparent* cross-reactions between the main (known) constituent metals. To examine this possibility: (i) mice were sensitized with LP-Ni (> 95.0%), and challenged with a solution containing 0.1 mM of a given LP-metal salt (93-99%) plus 0.5 µg/mL LPS (Fig. 6a), and (ii) other mice were sensitized with UP-Ni (> 99.999%), and challenged with a solution containing 0.1 mM of either UP-Ni or LP-Co (> 99%) plus 0.5 µg/mL LPS (Fig. 6b). In experiment (i), in stark contrast to the results obtained using UP-metal salts, all the tested LP-metal salts (Ni, Pd, Co, and Cr) induced significant allergic swelling (Fig. 6a). Incidentally, in non-sensitized mice, none of the tested LP-materials induced any significant ear-swelling (data not shown). In experiment (ii), interestingly, LP-Co induced allergic ear-swelling of a magnitude almost the same as that induced by UP-Ni (Fig. 6b). These results suggest that trace contaminants present in the tested LP materials are sufficient to evoke metal-allergies in mice, resulting in *apparent* cross-reactions between the main constituents.

Discussion

Summary of findings

Patients with metal-allergy frequently exhibit positive responses to several metals. However, it is difficult to judge whether such plural or multiple reactions reflect sensitizations to individual metals or cross-reactions. Arguably, experimental studies in

animals may be the only way of clarifying this issue¹⁹, but because of the paucity of adequate animal models of metal-allergies there is insufficient information concerning cross-reactivity among metal-allergens. Here, using a mouse model devised earlier by us, we examined allergic cross-reactivity among some metals. The findings may be summarized as follows. (i) Of the UP-materials tested (Ni, Pd, Co, Cr, Cu, and Au), only Ni and Pd cross-reacted. (ii) In this cross-reaction, their MAICs were at the same level as each other. (iii) Combined challenge with Ni and Pd, with each below its MAIC (but not at higher concentrations), produced an additive effect. (iv) Strikingly, LP-Ni-sensitized mice reacted to all the tested LP-metals (Ni, Pd, Co, and Cr), and LP-metals were shown to contain contaminant metals. We discuss these findings in the following paragraphs.

Cross-reactivity between Ni and Pd

In patch-testing, most Pd-sensitive patients also react to Ni^{1,20-23}. Indeed, Uter *et al.*²⁴ and Hosoki *et al.*²⁵ reported a significant correlation between Pd and Ni reactions in patch-tested patients. In the main part of the present study (using ultra-pure materials and mice sensitized with Ni or Pd), we confirmed that among the tested metals (Ni, Pd, Co, Cu, Cr, and Au), only Ni and Pd cross-reacted. The use of UP-metal salts (99.99% or more) virtually excludes contributions by contaminants, and allows us to draw the above conclusion. The clinical results mentioned earlier suggest that the cross-reactivity between Ni and Pd observed in our mouse model may also be present in humans. Recently, however, Faurschou *et al.* - - who reviewed the exposure, epidemiology, and clinical manifestation of Pd allergy - - suggested that in addition to cross-reactivity, there is Pd mono-sensitization, which is slightly increasing in prevalence²⁶. Hence, they

proposed that Pd salts should be included in dental screening patch-test series²⁶. In our studies in mice using LPS as an adjuvant, we could not detect such Pd mono-sensitization, leading us to speculate that there might be plural or multiple mechanisms underlying the establishment of metal allergies. For example, there might be plural partner proteins forming complexes with Ni and/or Pd. Identifying such partner proteins may provide us with more information concerning these issues.

Similarities and discrepancies between the present and previous studies

In several previous studies performed using T-cell clones or T-cells, Ni-reactive T-cells cross-reacted with Pd and/or Cu, but with none of the other tested metals (Co, Cr, Pt, Zn, or Fe)¹⁶⁻¹⁸. Hindsen *et al.*²⁷ reported that patients with Ni- and Pd-allergies exhibited flare-up reactions at sites previously tested with Ni and Pd following oral Ni ingestion. In addition, many (but not all) patients with sensitivity to Co or Cu are also allergic to Ni^{1,20-23,28-30}, although concomitant allergy to Ni and Co is generally explained by co-sensitization rather than by cross-reactivity^{1,21,31-33}. Our findings are consistent with the existence of (i) cross-reaction between Ni and Pd [but not between Ni and Cu (or Co)] and (ii) co-sensitization with Ni and Co. The major cause of the discrepancy as regards cross-reaction between Ni and Cu (or Co) might be that: (i) the purity of the metal materials differed between our study and those described above, and/or (ii) Ni-allergy in mice does not necessarily depend on T-cells (see Introduction).

Possible mechanisms underlying cross-reactions

It has often been argued that cross-reactivity between elements is determined by their positions in the periodic table²¹. In fact, Ni and Pd do belong to the same group within the periodic table. However, Cu, which belongs to the same group as Au, did not

cross-react with it. Thus, cross-reactivity cannot simply be predicted by relative positions in the periodic table, but may depend strictly on the spatial geometry of metal-protein complexes ^{16,19}. In the present study, we found that the MAICs of the cross-reactants Ni and Pd were very similar to each other, and that this similarity existed regardless of which one was the true antigen and which one the false. Moreover, combined challenge with Ni and Pd at sub-MAICs (but not at higher concentrations) produced an additive effect (Figs. 4a and 4b). On the other hand, in mice sensitized to both Ni and Co (non-cross-reactants), a concomitant challenge with Ni and Co produced no additive ear-swelling (Fig. 5). These results lead us to speculate that: (i) the partner protein(s) forming a complex(es) with Ni and Pd may be common, or very similar, between Ni and Pd, and (ii) the mediator(s) of allergic inflammation [e.g., IL-1, histidine decarboxylase, or histamine ¹⁴] might also be common among various metal allergies, and their productions might be regulated by common mechanisms. However, to clarify these points will require many experiments in future studies.

Clinical implications of the present findings

Unexpectedly, and intriguingly, LP-Ni-sensitized mice reacted to all the tested LP-metals (Ni, Pd, Co, and Cr), and UP-Ni-sensitized mice reacted to LP-Co. These results suggest that the trace amounts of contaminant metals present in LP-metal samples could be sufficient, at least in mice, to evoke allergy. In addition, our previous finding ¹⁵ in mice that LPS markedly reduces the MAICs of metals at both the sensitization and elicitation steps may suggest that if a metal salt preparation used for patch-testing contains contaminant metals, the presence of a bacterial milieu or infection may promote the establishment and elicitation of metal-allergies not only by the main metal, but also by the contaminant metals. This, of course, is valid only if our

results hold true in humans. If that is indeed the case, then we would argue that when identifying metal-allergens by patch-testing: (i) ultra-pure metal salts should be used, and (ii) it is also necessary to pay careful attention to possible infection in the patient (including infections of the skin and other sites). In this context, a bacterial milieu may be very important. Although there have been very few human studies on the relationship between metal-allergies, some reports have emphasized the importance of infection in the development and/or manifestation of metal-allergies^{34,35}. As described in Introduction, LPS interact with TLR4 to stimulate innate immunity. Interestingly, Schmidt *et al.* recently demonstrated that the human TLR4 interacts with Ni²⁺, too, and promotes Ni-allergy³⁶. This finding may suggest that in humans, as in mice, stimulation of innate immunity may be involved in promoting the establishment of Ni-allergy.

Conclusion

In conclusion, in metal-allergies in mice: (i) Ni and Pd (members of the same group in the periodic table of elements) cross-react with each other, (ii) in this-cross reaction, true and false antigens may have similar spatial geometry in the structures of their metal-protein complexes, (iii) Co, Cr, Cu, and Au do not cross-react with each other, and (iv) in LP-materials, the trace contaminant metals may be sufficient to evoke metal-allergies. Thus, if these results can be extrapolated to humans (which has not been established yet), it may be advisable to use ultra-pure metal salts for clinical patch-testing, and these findings may provide an insight into the mechanism underlying metal-allergies in humans.

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Legends to figures

Fig. 1. Challenges, without LPS, to Ni-sensitized mice. The indicated concentrations of metal salts, without LPS, were challenged to Ni-sensitized or non-sensitized mice (see Methods). Each value is the mean $\pm$ SD from three mice (i.e., six ears). $*P < 0.01$ vs. control (non-sensitized) group at same time-point.

Fig. 2. Challenges, with LPS, to Ni-sensitized mice. Solutions containing the indicated concentrations of each metal salt and/or LPS were challenged to Ni-sensitized or non-sensitized mice. It should be noted that the metal concentrations are 1 nM in **a** and **b**, but 0.1 mM in **c** to **f**. Each value is the mean $\pm$ SD from three mice (i.e., six ears). $*P < 0.01$ vs. control group (metal alone in **a** and **b**; non-sensitized in **c-f**) at same time-point.

Fig. 3. Cross-reactions in the presence of LPS in mice sensitized with a metal other than Ni. Solutions containing 0.1 mM of a given metal salt and/or 0.5 μ g/mL LPS were challenged to mice sensitized with a metal other than Ni. Each value is the mean $\pm$ SD from three mice (i.e., six ears). $*P < 0.01$ vs. control (LPS alone) group at same time-point.

Fig. 4. Concomitant challenge with Ni and Pd (cross-reactants) to Ni-sensitized mice.

(a) Solutions containing 0.1 mM Ni and/or 0.1 mM Pd, in each case with 0.5 µg/mL LPS, were challenged to Ni-sensitized mice. (b) Solutions containing 5 µM Ni and/or 5 µM Pd, without LPS, were challenged to Ni-sensitized mice. Each value is the mean ± SD from three mice (i.e., six ears). * $P < 0.01$ vs. Ni alone and Pd alone at same time-point.

Fig. 5. Concomitant challenge with Ni and Co (non-cross-reactant metals) to mice sensitized to both Ni and Co. Mice were sensitized with a solution containing 0.5 mM Ni, 0.5 mM Co, and 0.5 µg/mL LPS, and challenged with a solution containing 0.1 mM Ni and/or 0.1 mM Co, in each case with 0.5 µg/mL LPS. Each value is the mean ± SD from three mice (i.e., six ears).

Fig. 6. Effects of low-purity (LP) or ultra-pure (UP) metals. (a) A solution containing 0.1 mM of a LP-metal and/or 0.5 µg/mL LPS was challenged to LP-Ni-sensitized mice. (b) A solution containing 0.1 mM UP-Ni or LP-Co, and/or 0.5 µg/mL LPS was challenged to UP-Ni-sensitized mice. Each value is the mean ± SD from three mice (i.e., six ears). * $P < 0.01$ vs. control (LPS alone) group at same time-point.

Fig. 1

Challenge to Ni-sensitized mice

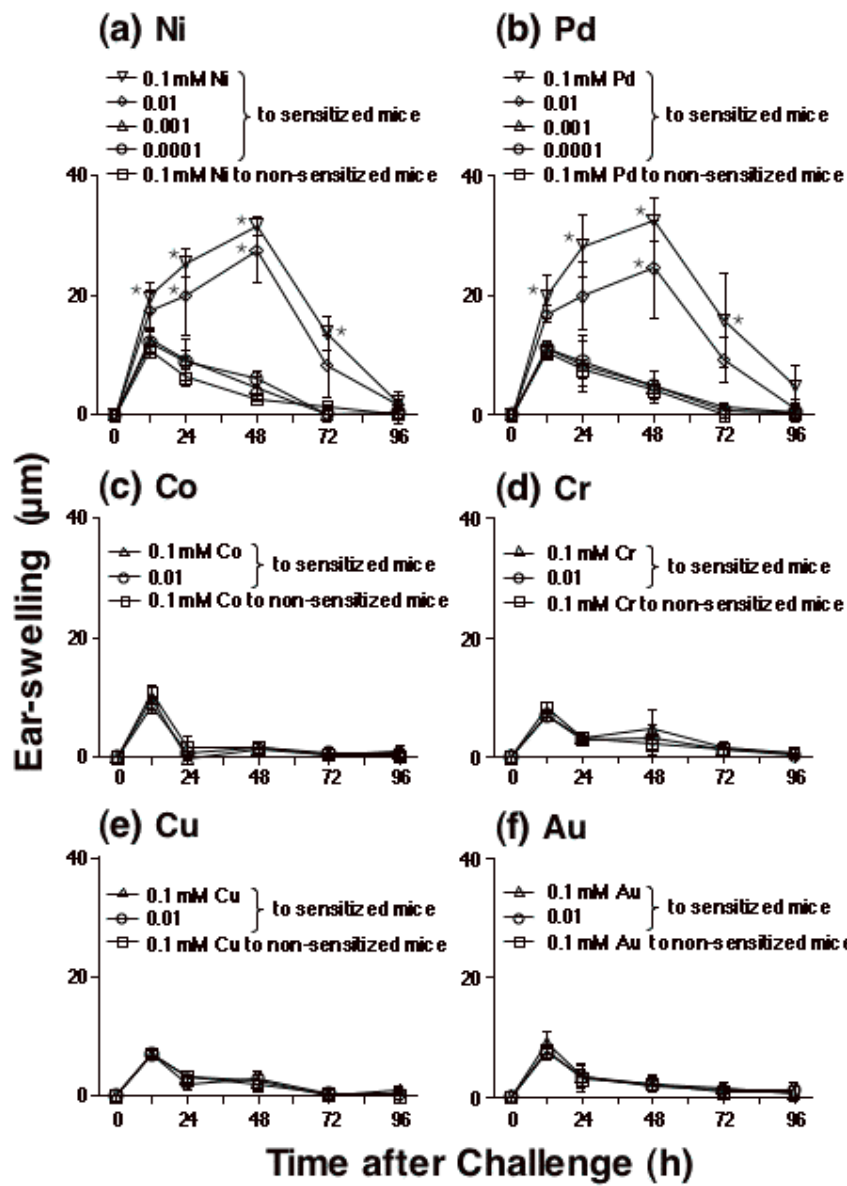


Fig. 2

Challenge to Ni-sensitized mice

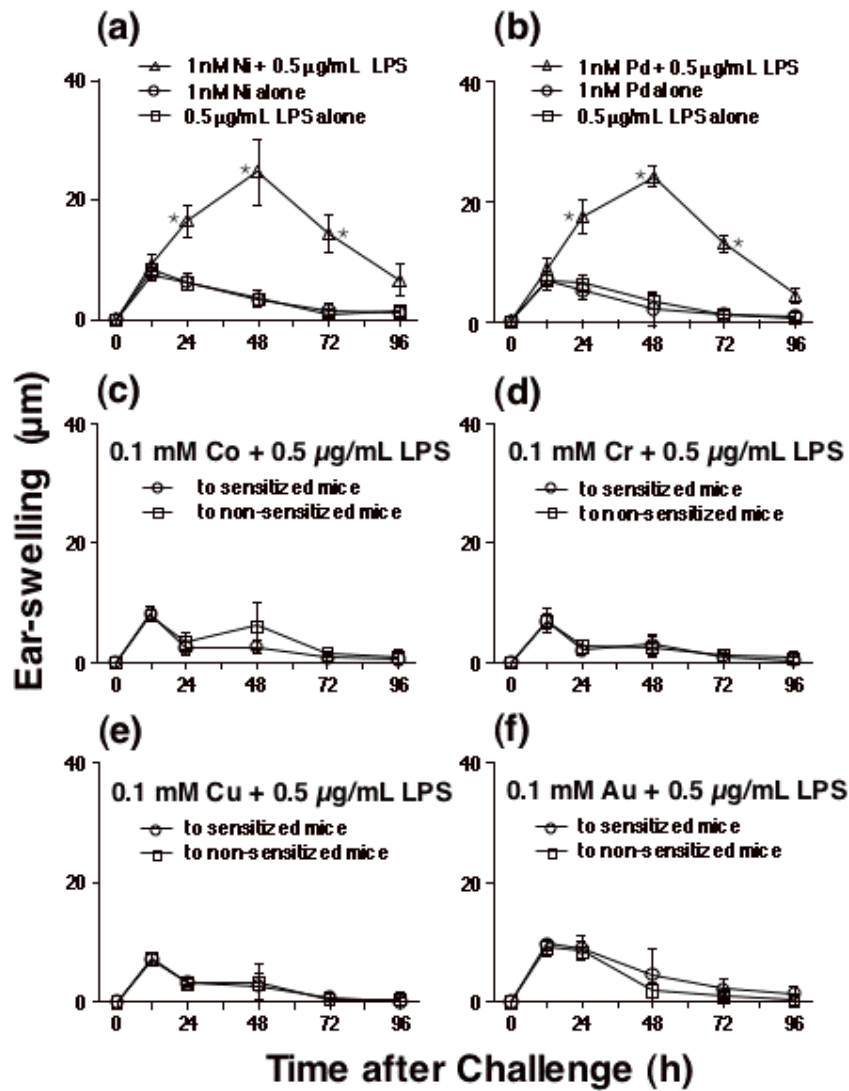


Fig. 3

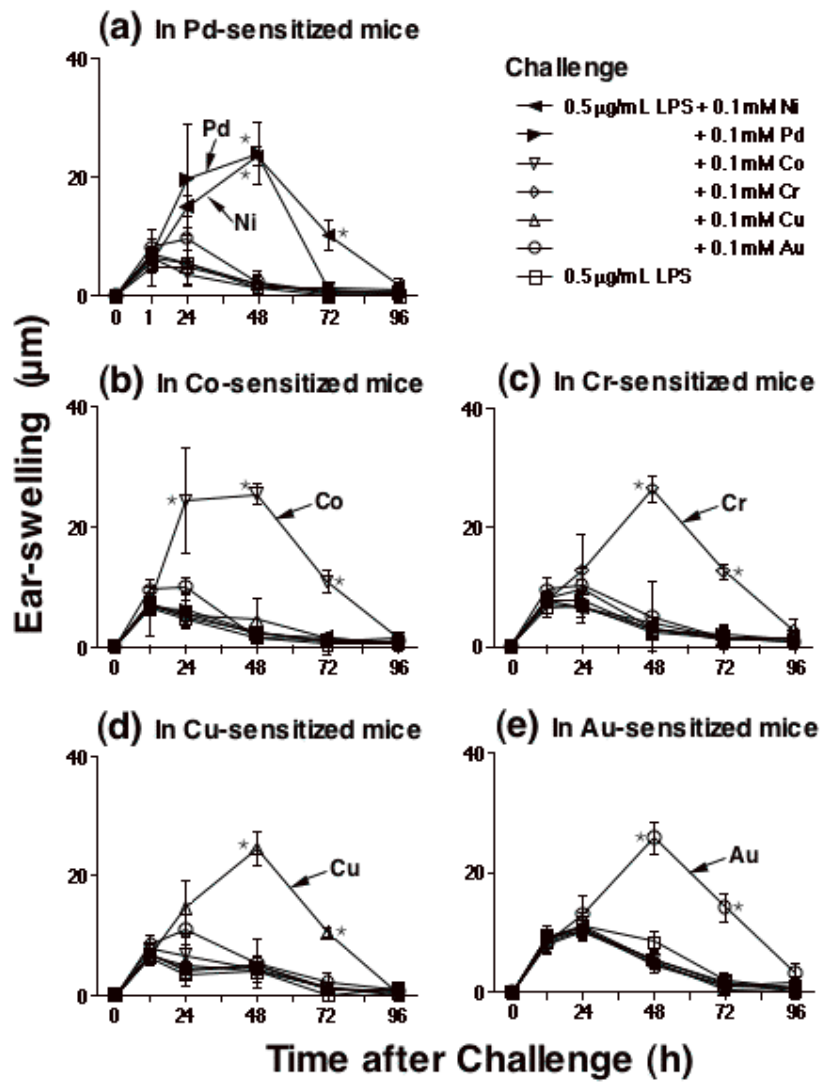


Fig. 4

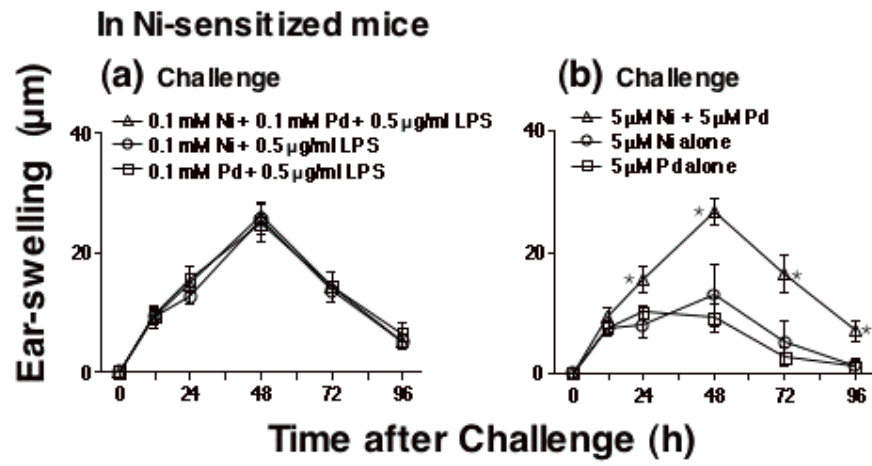


Fig. 5

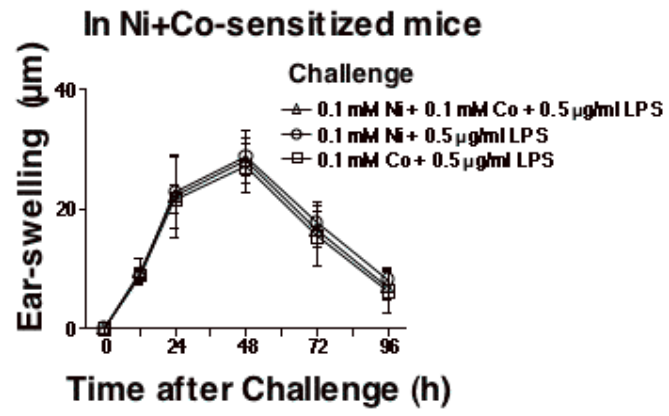


Fig. 6

