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Nickel allergy-promoting effects of microbial or inflammatory substances at the sensitization step in mice

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ABSTRACT

Microbial components stimulate innate immunity via Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and/or IL-1. We recently reported that in mice, *Escherichia coli* lipopolysaccharide (LPS, TLR4-ligand) promotes allergic responses to nickel (Ni) at both the sensitization and elicitation steps. Here, we examined in mice the effects of administering other microbial or inflammatory materials at the Ni-sensitization step. A mixture of 1 mM NiCl₂ and a test solution was injected intradermally into the ear pinnae of the mice (20 µl/ear). The following preparations or substances exhibited adjuvant activities: *Prevotella intermedia* LPS, *Saccharomyces cerevisiae* mannan, a synthetic muramyl dipeptide (NOD2-stimulating cell-wall component of bacteria), Pam₃Cys-SKKKK (TLR2-stimulating synthetic peptide), poly I:C (TLR3-stimulating double-stranded RNA), concanavalin A (a typical T-cell mitogen and T-cell-mediated hepatitis-inducer), heat-killed *Propionibacterium acnes* (Gram-positive bacterium that causes pimples and induces macrophage-mediated experimental hepatitis), and nitrogen-containing bisphosphonates (chemicals stimulating IL-1 production). Unexpectedly, *P. intermedia* LPS, which displayed the most potent adjuvant activity among the tested preparations, was effective in TLR4-dysfunctional mutant mice, but not in TLR2-deficient mice, whereas the reverse was true for *S. cerevisiae* mannan. These results suggest that (i) for the establishment of Ni-allergy in mice, stimulation of innate immunity (including TLRs, NLRs, IL-1 production, and/or other factors) may be important at the sensitization step, and (ii) *P. intermedia* may produce a substance(s) that potentially promotes Ni-allergy via stimulation of TLR2.

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1. Introduction

Metals are thought to cause various types of allergic reactions (including dermatitis, lichen planus, palmoplantar pustulosis, asthma, and rhinitis) [1–6], and even to cause carcinomas [4]. Although allergic mechanisms are known to be implicated in these diseases, the mechanism underlying metal allergy is obscure. The major reasons for this lack of knowledge seem to be that (i) unlike the classical haptens (or organic contact allergens), metal ions become allergens by forming reversible coordination complexes with certain of the host's protein molecules [7], and (ii) *in vivo* studies have been limited by the paucity of adequate adjuvants for the induction of Ni-allergy in mice.

We recently found that a lipopolysaccharide (LPS) preparation from *Escherichia coli* displayed potent adjuvant activity during the induction of metal allergies in mice [8]. In this murine model, elements of the innate immune system such as Toll-like receptor 4 (TLR4) and IL-1 (but not TNF) may play important roles in the

establishment of the metal allergy [8]. It has been accepted that T-cells play central roles in the pathology of Ni-allergy [9–11]. However, LPS promoted metal allergies even in nude mice with a severe lack of CD4⁺ and CD8⁺ T-cells [8], suggesting that in the absence of T-cells and/or in the presence of LPS, another type(s) of cells have the potential to induce Ni-allergy. It is also likely that Ni-allergy in mice involves a mechanism different from that involved in the contact hypersensitivity induced by organic haptens that form irreversible complexes via covalent bonds with the host's protein molecules.

Metals are widespread in our environment (in kitchen goods, coins, jewellery, etc.), and they are widely used in dentistry and medicine (including stent surgery) too. Nickel (Ni) is the most frequent contact allergen among the metals [2,12]. In addition, it should be noted that we are exposed to various microbes through a variety of routes, including the mouth (or oral cavity) and skin. Thus, we may be exposed concomitantly to nickel and microbes. In the innate immune system, various components of microorganisms are recognized by receptors such as TLRs [13,14] or nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) [15,16]. In view of the above background, we examined, in mice, whether (i) microbial materials, including LPS from oral bacteria, which are recognized by

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the above receptors in innate immunity, and/or (ii) inflammatory agents, including chemicals and inducers of experimental hepatitis, might exhibit adjuvant activities that cause them to act as promoters of Ni-allergy.

2. Materials and methods

2.1. Animals

Female BALB/c and C57BL/6 mice (6–7 weeks old), bred in our laboratory under conventional conditions, were used. C3H/HeJ and C3H/HeN mice were purchased from SLC (Shizuoka, Japan). TLR2-knockout (KO) mice (C57BL/6 background) were provided by Prof. Akira Shizuo [17] and raised in our laboratory. IL-1-KO mice (deficient in both IL-1 α and IL-1 β) (BALB/c background) were established from original IL-1 α -KO and IL-1 β -KO mice [18] and raised in our laboratory. Animal care and all experiments were in accordance with both the guidelines laid down by this journal (viz. the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purpose) and by our institution (viz. the *Guidelines for Care and Use of Laboratory Animals in Tohoku University*).

2.2. Reagents

E. coli O55:B5 LPS (prepared by phenol extraction), a mannan from *S. cerevisiae*, a muramyl dipeptide (MDP) (namely *N*-acetylmuramyl-L-alanyl-D-isoglutamine, a synthetic version of a cell-wall component of Gram-negative bacteria), poly I:C (a synthetic double-stranded RNA), and concanavalin A (Con A) were purchased from Sigma (St Louis, MO, USA). Pam₃Cys-SKKKK-HCl was from Calbiochem (San Diego, USA). Alendronate and zoledronate were purchased from LKT Laboratories Inc. (St. Paul, MN, USA) and Toronto Research Chemicals Inc. (North York, ON, Canada), respectively. *P. intermedia* ATCC 25611 LPS was prepared by the method of Galanos et al. [19] (phenol-chloroform-petroleum ether extraction) [20,21]. Heat-killed, lyophilized *Propionibacterium acnes* was prepared as previously described [22]. NiCl₂ (first grade: >95%) was purchased from Wako Pure Chemical Ind. (Osaka, Japan). These reagents were either suspended (*P. acnes* and LPS preparations) or dissolved in sterile saline. LPS-free saline purchased from Ohtuka Pharmaceutical Co. (Tokushima, Japan) was used throughout the present study.

2.3. Sensitization to Ni

A mixture of NiCl₂ and LPS, when it is injected either intraperitoneally or intradermally into flanks, easily sensitizes mice to Ni [8]. Intradermal injection of LPS into flanks together with intraperitoneal injection of LPS is also effective [8]. In these methods, LPS is effective when it is injected 3 h before NiCl₂, but not when it is injected after NiCl₂ [23]. In the present study, therefore, a 1:1 mixture of 1 mM NiCl₂ and a test solution (containing a microbial or inflammatory substance at the indicated concentration) was injected intraperitoneally (0.1 ml/10 g body weight) [8].

2.4. Provocation of Ni-allergy

We previously reported that when BALB/c mice were challenged with 5 mM NiCl₂ at 4, 7, 10, or 14 days after sensitization with a solution containing LPS and NiCl₂, both the magnitude and the time course of the ear-swelling were similar among the 7-, 10-, and 14-day groups, whereas the 4-day group exhibited no detectable ear-swelling [8]. Thus, for most of the experiments in the present study, we selected 10 days after the sensitizing injection as the time-point at which 5 mM NiCl₂ was challenged [via intradermal injection into the left and right pinnae near the root of the ear (20 μ l/ear)]. Mice were anesthetized with ethyl ether just before the challenge injection. Ear swelling was measured at a site 2–3 mm distant from the challenge site at the indicated times [using a

Peacock dial thickness gauge (Ozaki MFG Co. Ltd., Tokyo, Japan)], and the induced difference in thickness (after versus before the challenge) was recorded.

2.5. Statistical analysis

Experimental values are each given as the mean $\pm$ standard deviation (SD) for 3 mice (i.e., 6 ears). The statistical significance of differences was assessed either by a Student's unpaired *t*-test (for comparing two means) or by a Bonferroni multiple comparison test after ANOVA. For this, InStat software was used. *P* values less than 0.05 were considered to indicate significance.

3. Results

3.1. Effects of *P. intermedia* LPS

Black-pigmented bacteria, such as *P. intermedia*, are the dominant Gram-negative bacteria within the periodontal pockets of patients with periodontitis, and they are considered to be the pathogens responsible for periodontal diseases [24]. As shown in Fig. 1A, *P. intermedia* LPS, even at 0.01 μ g/ml (1.25 ng/mouse or 50 ng/kg), was effective as an adjuvant for the promotion of Ni-allergy in BALB/c mice. Our data suggest that its potency is similar to that of *E. coli* LPS [8] (see Table 1). Surprisingly, however, the effectiveness of *P. intermedia* LPS was about equal between C3H/HeJ mice (with mutated, inactive TLR4) and their counterpart control C3H/HeN mice (Fig. 1B), a finding that contrasts with that reported for *E. coli* LPS by Sato et al. [8]. Next, we compared the effect of *P. intermedia* LPS between TLR2-KO mice and their control wild-type (WT) C57BL/6 mice. As shown in Fig. 1C, the effect of *P. intermedia* LPS was significantly weaker in TLR2-KO than in WT mice, although the effect of *E. coli* LPS did not differ between these two types of mice (Fig. 1D).

3.2. Effects of mannan

Mannan is a component of the fungal cell surface. As shown in Fig. 2A, mannan exhibited Ni allergy-promoting adjuvant activity in BALB/c mice, although the effective concentrations were about 1000-fold higher (≥ 10 μ g/ml) than those of the above LPS preparations (≥ 0.01 μ g/ml). The allergic response displayed by C3H/HeJ mice sensitized with mannan + Ni was significantly weaker than that displayed by similarly treated C3H/HeN mice (Fig. 2B), whereas the allergic responses seen in mice sensitized with mannan + Ni were similar between TLR2-KO mice and their control C57BL/6 mice (Fig. 2C).

3.3. Effects of synthetic ligands recognized by the innate immune system

We next tested some synthetic agonists that stimulate innate immune responses via TLRs or NLRs. Pam₃Cys-SKKKK and Poly I:C are agonists of TLR2 and TLR3, respectively [13]. MDP, a cell-wall component that is abundant in Gram-positive bacteria, is an immunologically active unit of peptidoglycan and is recognized by NOD2 [15,25]. As shown in Fig. 3, all three of these synthetic agonists exhibited Ni allergy-promoting adjuvant activities, although poly I:C and MDP needed to be at much higher concentrations (mg/ml levels) than Pam₃Cys-SKKKK (μ g/ml levels).

3.4. Effects of nitrogen-containing bisphosphonates (NBPs)

NBPs have powerful anti-bone-resorptive effects and are used worldwide in patients with conditions in which there is enhanced bone resorption, including osteoporosis. However, these chemical reagents have inflammatory side effects, such as fever and jawbone necrosis [26,27]. NBPs induce a variety of inflammatory reactions in mice, too [28], although their inflammatory effects are remarkably weak in IL-1-KO mice

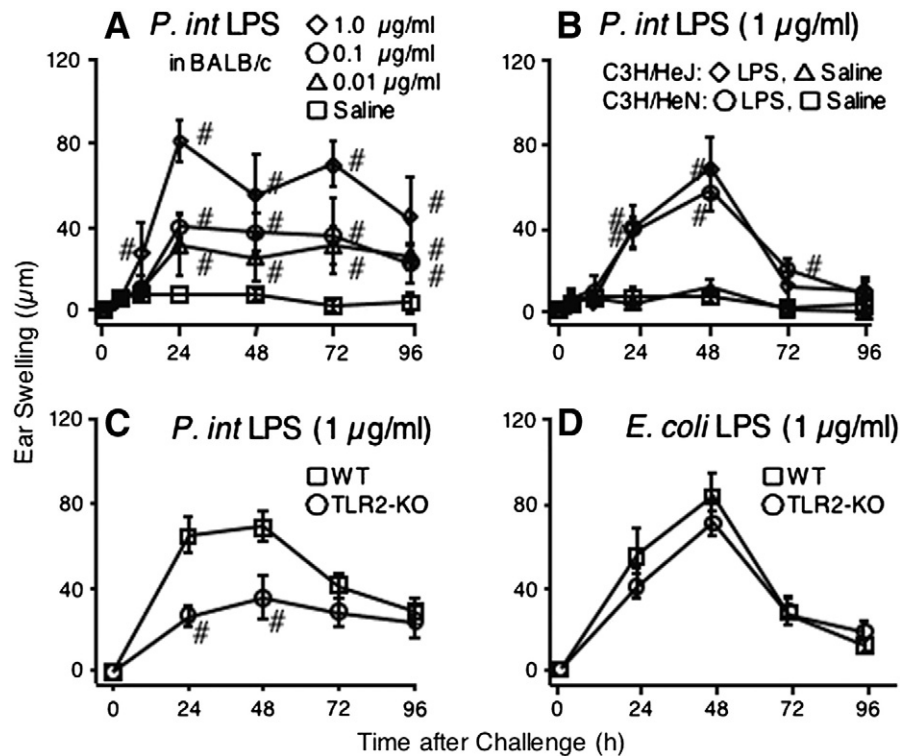


Fig. 1. Effects of *P. intermedia* LPS. (A) Effects of various concentrations in BALB/c mice. A 1:1 mixture of *P. intermedia* LPS (at the indicated concentration) and 1 mM NiCl₂ was injected intraperitoneally (0.1 ml/10 g body weight), and 10 days later 5 mM NiCl₂ was challenged intradermally into the ear pinnae of the mice. (B) Effects in TLR4 mutant mice. A 1:1 mixture of saline or *P. intermedia* LPS (1 μg/ml) and 1 mM NiCl₂ was injected intraperitoneally into C3H/HeJ (TLR4 mutant) and control C3H/HeN mice, and 10 days later the mice were challenged via the ear skin with 5 mM NiCl₂. (C and D) Effects of *P. intermedia* and *E. coli* LPS in TLR2-KO mice. A 1:1 mixture of LPS (1 μg/ml) and 1 mM NiCl₂ was injected intraperitoneally into TLR2-KO or control wild-type (WT) C57BL/6 mice, and 10 days later the mice were challenged via the ear skin with 5 mM NiCl₂. #*P* < 0.01 vs the respective control group.

[29]. As shown in Fig. 4A and B, the two NBPs tested here [alendronate (mol wt 249) and zoledronate (mol wt 272)], each exhibited Ni allergy-promoting adjuvant activity at doses reported elsewhere to induce inflammatory reactions in mice [30]. The allergic response was weak in IL-1-KO mice sensitized with alendronate + Ni (Fig. 4C).

3.5. Effects of inducers of T-cell-mediated or macrophage-mediated hepatitis

Con A, a typical T-cell mitogen, reportedly induces T-cell-mediated liver injury when injected into mice [31]. Intravenous injection of LPS

(2–3 μg/mouse) several days after intraperitoneal injection of heat-killed *P. acnes* (1 mg/mouse) (a Gram-positive bacterium that causes pimples) has been reported to induce macrophage-mediated hepatitis via the production of various cytokines, including IL-18 [32]. As shown in Fig. 5A, Con A promoted Ni allergy at 1 or 2 mg/ml, doses much higher than the required dose of LPS. We found that Con A, at 2 mg/ml, induced mild hepatitis in BALB/c mice (data not shown). *P. acnes* also promoted Ni allergy, although for this effect a concentration as high as 10 mg/ml was required (Fig. 5B), which is several times higher than that used to induce hepatitis.

4. Discussion

In the present study, we found that various microbial and inflammatory substances (including hepatitis inducers and synthetic chemical substances) exhibit adjuvant activities in the sensitization of mice to Ni. These substances, together with their approximate minimum effective concentrations, are listed in Table 1. We discuss these findings in the following paragraphs.

The present *P. intermedia* LPS preparation exhibited a potent Ni allergy-promoting adjuvant activity that was comparable to that of *E. coli* LPS. Unexpectedly, this activity was exhibited even in TLR4-mutant mice (C3H/HeJ), even though it is generally believed that the effects of LPS are exerted via TLR4 [13]. Some reports have suggested that LPS preparations from *Porphyromonas gingivalis* (a *P. intermedia*-like oral *Bacteroides*-related bacterium) and *Leptospira interrogans* possess TLR2-agonistic activities [33,34]. However, Hashimoto et al. [35] showed that *P. gingivalis* contains a lipoprotein that is a potent stimulator of TLR2, and moreover Ogawa et al. [36] recently demonstrated that the activity of a *P. gingivalis* LPS preparation was largely due to contamination with a lipoprotein that stimulates TLR2. The *P. intermedia* LPS used in our study

Table 1
Approximate minimum effective concentrations (MEC) of substances or preparations tested for their promotion of Ni-allergy in mice.

Tested substances or preparations and their properties MEC (μg/ml) ^a			
<i>E. coli</i> LPS	TLR4 ligand		0.01 ^b
<i>P. intermedia</i> LPS	Contaminated with a powerful TLR2 ligand(s)?		0.01
Pam ₃ Cys-SKKKK	Synthetic TLR2 ligand		40
Fungal mannan	TLR4 and/or TLR2 ligand		10
Alendronate	Nitrogen-containing bisphosphonate; inflammatory drug		200
Zoledronate	Nitrogen-containing bisphosphonate; inflammatory drug		100
Poly I:C	Synthetic double-stranded RNA; TLR3 ligand		5000
MDP	Synthetic NOD2 ligand		2000
Con A	T-cell mitogen; hepatitis inducer; IL-1 producer		1000
<i>P. acnes</i>	Heat-killed Gram-positive bacterium; hepatitis inducer		10,000

^a Concentrations of test samples in the solutions used for sensitization, each solution being used in an equivolume mixture with 1 mM NiCl₂ (see Section 2.3. Sensitization to Ni).

^b From Sato et al. [8].

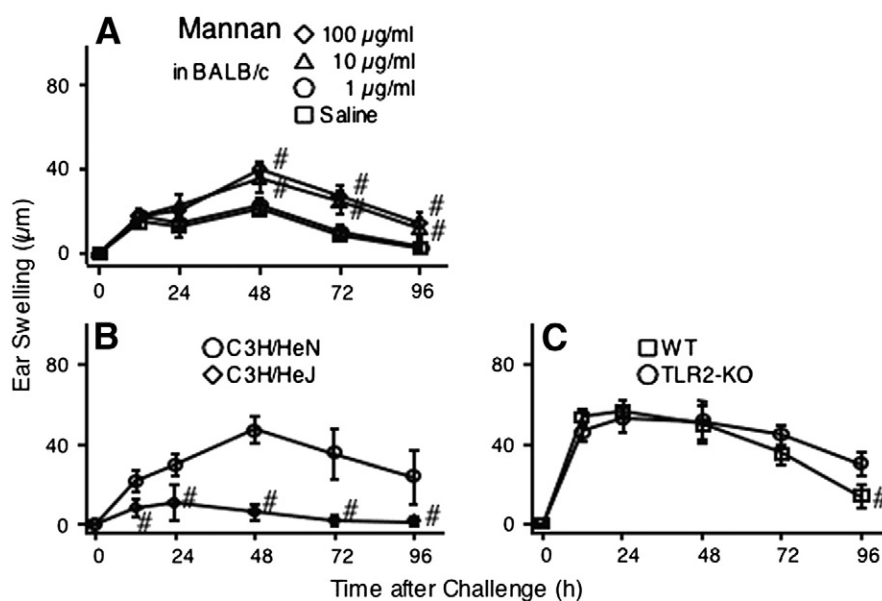


Fig. 2. Effects of mannan. (A) Effects of various concentrations in BALB/c mice. A 1:1 mixture of mannan (at the indicated concentration) and 1 mM NiCl₂ was injected intraperitoneally (0.1 ml/10 g body weight), and 10 days later 5 mM NiCl₂ was challenged intradermally into the ear pinnae of the mice. (B) Effects in TLR4-mutant mice. A 1:1 mixture of saline or mannan (0.2 mg/ml) and 1 mM NiCl₂ was injected intraperitoneally into C3H/HeJ (TLR4-mutant) and control C3H/HeN mice, and 10 days later the mice were challenged via the ear skin with 5 mM NiCl₂. (C) Effects in TLR2-KO mice. A 1:1 mixture of mannan (0.2 mg/ml) and 1 mM NiCl₂ was injected intraperitoneally into TLR2-KO or control wild-type (WT) C57BL/6 mice, and 10 days later the mice were challenged via the ear skin with 5 mM NiCl₂. #*P* < 0.01 vs the respective control group.

was prepared by the phenol-chloroform-petroleum ether extraction method [19], and when prepared in that way *P. intermedia* LPS is reportedly almost devoid of mitogenic and cytokine-producing activities *in vitro* in cells isolated from C3H/HeJ mice [21]. Darveau et al. [37] proposed a lipid A structure of *P. gingivalis*, which is capable of activating TLR2 as well as TLR4. In the present study, a synthetic TLR2-ligand (Pam₃Cys-SKKKK) was found to promote Ni-allergy, indicating that TLR2 can indeed mediate this reaction. Future research will need to determine

whether it is the LPS itself in preparations of *P. intermedia* LPS that stimulates TLR2 or whether a small amount of a contaminating substance(s) is responsible. In any event, *P. intermedia* may produce a substance(s) that potently promotes Ni-allergy via stimulation of TLR2. *P. intermedia* is the dominant Gram-negative bacterium in the periodontal pockets of patients with periodontitis [24], and it is also prevalent in endodontal and periapical infections [37,38]. Through daily oral hygiene procedures or dental surgical procedures, systemic

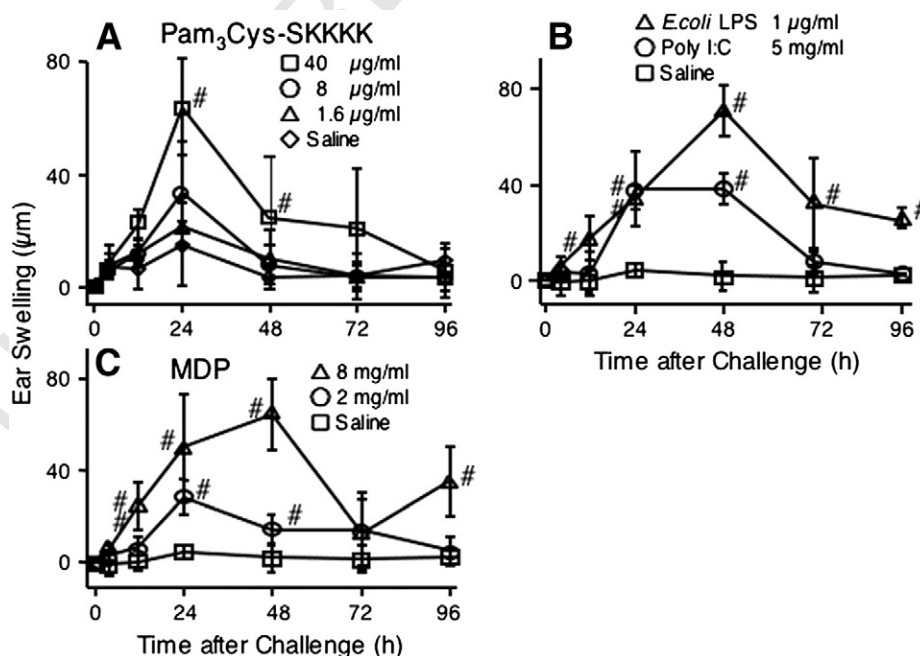


Fig. 3. Effects of synthetic ligands recognized by the innate immune system in BALB/c mice. A 1:1 mixture of a test solution (at the indicated concentration) and 1 mM NiCl₂ was injected intraperitoneally (0.1 ml/10 g body weight), and 10 days later 5 mM NiCl₂ was challenged intradermally into the ear pinnae of the mice. (A) Pam₃Cys-SKKKK (a TLR2 ligand), (B) PolyI:C (a TLR3 ligand), (C) MDP (a NOD2 ligand). #*P* < 0.01 vs the respective control group.

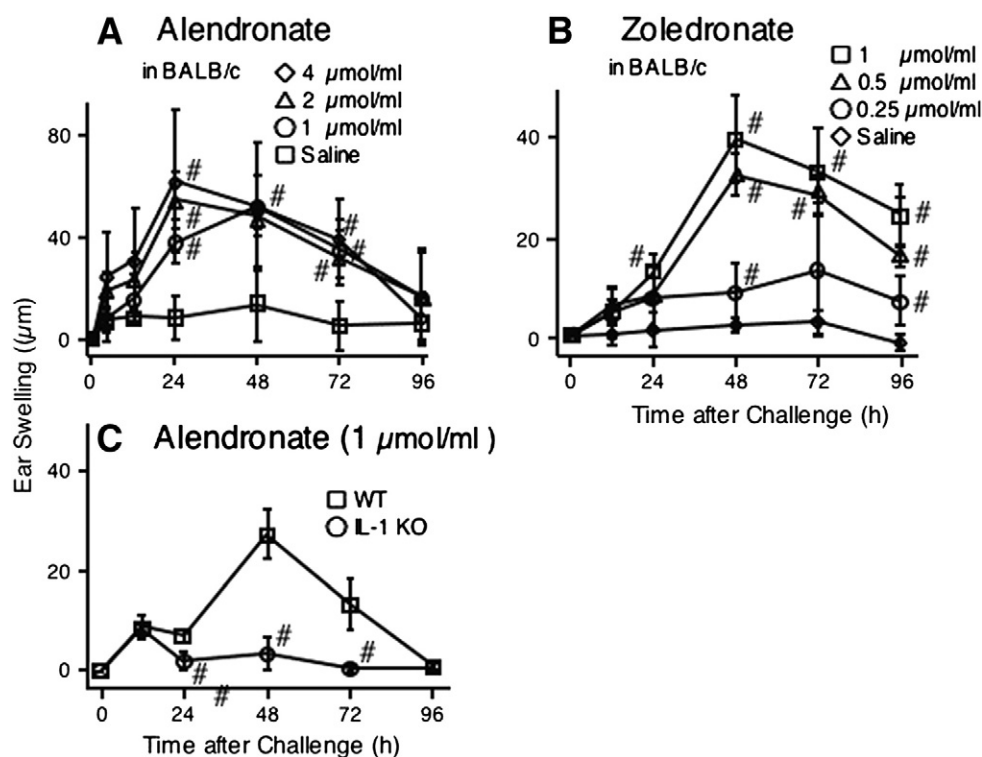


Fig. 4. Effects of nitrogen-containing bisphosphonates (NBPs). A 1:1 mixture of a test solution (at the indicated concentration) and 1 mM NiCl_2 was injected intraperitoneally (0.1 ml/10 g body weight), and 10 days later 5 mM NiCl_2 was challenged intradermally into the ear pinnae of the mice. Effects, in wild-type BALB/c mice, induced by various concentrations of (A) alendronate and (B) zoledronate. (C) Effects of alendronate in wild-type BALB/c and IL-1-KO mice. # $P < 0.01$ vs the respective control group.

exposure to oral bacteria via the blood circulation may occur repeatedly and/or frequently in patients with periodontitis [39]. It has also been reported that an intragingival injection into mice of a small dose of *P. intermedia* LPS can induce inflammatory reactions in extra-oral tissues, as well as in oral tissues [40]. Various alloys used in dentistry may come into contact with oral tissues, and the present findings suggest that oral bacteria have the potential to promote sensitization to the Ni (and possibly other metals) included in such alloys.

Fungal species not only cause mycoses, including endocarditis [41], but also modulate the immune system [42–44]. Among the various

components of the fungal cell surface (glucan, chitin, and mannan), mannan is the one that constitutes the major antigen of fungi, and host cells are thought to recognize mannan via TLR2 and/or TLR4 [13,42,44–46]. The present results suggest that the preparation of mannan we used promotes Ni-allergy via TLR4. However, we cannot exclude the possibility that the active component might be a contaminant, such as LPS or endotoxin, of the mannan preparation.

In addition to the above TLR4 and/or TLR2 ligands, poly I:C (a TLR3 ligand) and MDP (an NOD2 ligand) were also found here to promote Ni-allergy, although admittedly high concentrations were needed. These

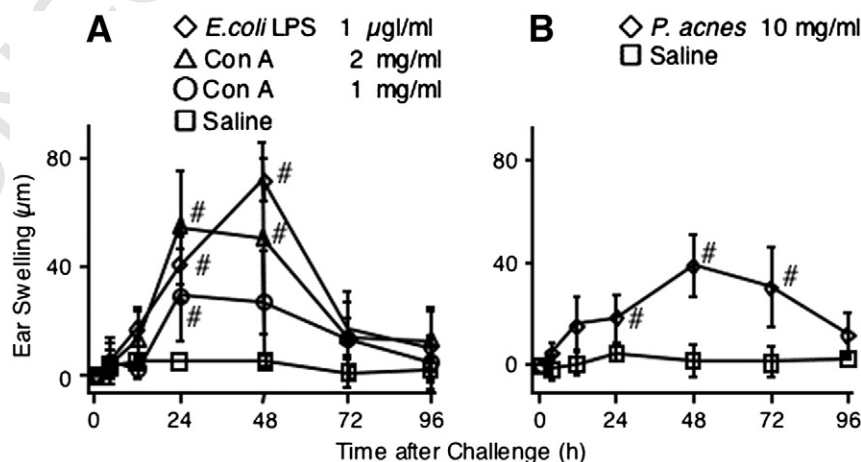


Fig. 5. Effects of hepatitis-inducers in BALB/c mice. A 1:1 mixture of a test solution (at the indicated concentration) and 1 mM NiCl_2 was injected intraperitoneally (0.1 ml/10 g body weight), and 10 days later 5 mM NiCl_2 was challenged intradermally into the ear pinnae of the mice. (A) Con A, (B) heat-killed *P. acnes*. # $P < 0.01$ vs the respective control group.

results suggest that viral and bacterial components may also promote Ni-allergy via these intracellular receptors in some situations. Thus, a broad spectrum of microbial components may be causally involved in the establishment of Ni-allergy.

The adjuvant activity of LPS + Ni is absent in IL-1-KO mice [8]. The signaling pathways downstream of TLRs are homologous to that downstream of the IL-1 receptor, and lead to activation of NF- κ B [13], which in turn results in the production of various inflammatory cytokines, including IL-1 [47,48]. Thus, it is likely that the adjuvant effects of microbial components are mediated by IL-1. We have previously shown that nitrogen-containing bisphosphonates (NBPs), such as alendronate, augment the LPS-induced production of IL-1 [49], and that alendronate itself can stimulate the production of IL-1 [50]. We have also shown that a number of NBPs induce only very weak inflammatory reactions in IL-1-KO mice [28]. In view of that background, we tested the effects of two NBPs (alendronate and zoledronate) on Ni-allergy. As expected, these NBPs promoted Ni-allergy, and moreover IL-1-KO mice were resistant to the adjuvant activity of alendronate. These results support the idea that IL-1 or its related signaling pathways may play an important role in the establishment of Ni-allergy.

Con A induces the production of various cytokines, including IL-1, both *in vivo* and *in vitro* [51,52]. It is well known (a) that an interaction between macrophages (or macrophage-related cells) and T-cells is necessary for T-cell-mediated immune reactions to occur [53,54], and (b) that IL-1 augments the stimulation of T-cells by Con A [53]. IL-18 (a member of the IL-1 family) is, like IL-1, produced via activation of caspase-1, and IL-18 is known to be a potent Th1-inducing cytokine [55,56]. IL-18 is reportedly produced in large amounts by *P. acnes*-treated mice when they are exposed to LPS [57]. We therefore tested the effects of these hepatitis-inducers (Con A and *P. acnes*), and found that they do indeed promote Ni-allergy, supporting the idea that IL-1 and/or IL-18 may be involved in the establishment of this allergy.

Finally, it is important to discuss whether the present findings in mice might be applicable to humans. Regarding this issue, it should be noted that (i) human studies on metal-allergies have largely involved *in vitro* experiments using T-cells from already sensitized patients, so that there have been few studies on the sensitization step, and (ii) as pointed out by Büdinger and Hertl [12] and Johansen et al. [58], *in vivo* studies on the sensitization step have been limited by the paucity of adequate murine models. Therefore, as yet we have too few available data to allow us rigorously consider whether our mouse model reflects human metal allergies, and it is impossible to tell whether the present findings can be extrapolated with any accuracy to humans. As mentioned in the Introduction, LPS promotes metal allergies even in nude mice with a severe lack of T-cells [8], suggesting that another type(s) of cells have the potential to induce Ni-allergy. Although it is quite possible that Ni-allergy in mice involves a mechanism different from that in humans, Schmidt et al. [59] recently demonstrated that the human TLR4 (or innate immune system) interacts not only with LPS, but also with Ni²⁺, and promotes Ni-allergy. With the above proviso about the paucity of relevant data, this suggests that in humans, as in mice, stimulation of innate immunity may indeed be involved in promoting the establishment of Ni-allergy.

In conclusion, the results described above suggest that (i) for the establishment of Ni-allergy in mice, a microbial and/or inflammatory milieu that stimulates innate immunity (including TLRs, NLRs, IL-1 production, and/or other factors) may be important at the sensitization step, and (ii) *P. intermedia* may produce a substance(s) that potentially promotes such Ni-allergy via stimulation of TLR2.

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