the matured or activated effector T cells to the site of Ni challenge, cytokine production by these T cells, and the induction of apoptosis in cells such as keratinocytes. During this sequence of events, two types of regulatory $CD4^+$ T cells have been suggested to exert inhibitory effects: (a) $CD4^+$ Tr1 cells, via the production of IL-10, and (b) $CD4^+CD25^+$ T cells, via cell-to-cell contact with either effector or naïve T cells (see the review by Cavani [9]). However, we should remember that this hypothesis is based largely on *in vitro* experiments, and thus, as pointed out by Büdinger and Hertl [7], *in vivo* studies are urgently needed to test it. Unfortunately, *in vivo* studies have been limited by the paucity of adequate murine models of Ni allergy. Indeed, it has been said that it is difficult to induce Ni allergy in mice [12]. However, Artik et al. [13] showed that it was possible to sensitize C57BL/6 mice to Ni (20 mM) by using 0.6 M H_2O_2 as an adjuvant [in contrast to the use of complete Freund's adjuvant (CFA) in current guinea-pig models].

Infections, or such bacterial components as lipopolysaccharide (LPS), reportedly either confer protection against allergic disorders or exacerbate them [14–16]. LPS, IL-1, TNF, and CD40 ligands all activate DCs to mature them [17–20], while LPS stimulates innate immunity via Toll-like receptor 4 (TLR4), and TLRs may modify adaptive immunity [21]. It has also been reported that LPS-stimulated DCs act via TLR4 to reduce the suppressive effect of regulatory T cells [22]. In this regard, it should be noted that LPS is common in our environment.

Histamine regulates immune responses associated with T-helper type 1 (Th1) and Th2 cells [23, 24] and modifies allergic reactions [25–27]. We have reported previously that a low dose of LPS or IL-1 induces the histamine-forming enzyme, histidine decarboxylase (HDC), in various tissues in mice [28, 29]. Although HDC induction by LPS is poor in those mice with a TLR4 mutation [30], HDC is fully induced in nude mice (deficient in matured T cells) [30] and even in mast-cell-deficient mice [31, 32].

Against this background, we designed the present study to examine (i) the *in vivo* effects of LPS on the establishment and development of the allergic responses to Ni and other metal, and (ii) the contributions made to these allergic responses by T cells, macrophages, IL-1, TNF, and HDC.

Materials and methods

Animals

BALB/c IL-1 KO mice (deficient in both IL-1α and IL-1β) and BALB/c TNF-α KO mice (deficient in TNF-α) were established from original IL-1α KO, IL-1β KO, and TNF-α KO mice [33, 34]. C57BL/6 HDC KO mice were established by Ohtsu et al. [35]. Mast-cell-deficient W/W^v mice, their normal litter mate +/+ mice, and BALB/c nude mice were purchased from SLC (Shizuoka, Japan). Females (6–7 weeks old) were used in the present study. Starting at least 1 week before use, mice were kept in plastic cages (with a top made of stainless-steel wires), with water available *ad libitum* from a glass bottle through a glass tube. All experiments complied with the Guidelines for Care and Use of Laboratory Animals in Tohoku University.

Reagents

Escherichia coli 055:B5 LPS (prepared by phenol extraction), clodronate (dichloromethylene diphosphonate), and CFA were purchased from Sigma (St. Louis, MO, USA), while $CrCl_2$, $CoCl_2$, $NiCl_2$, $PdCl_2$, and $AgNO_3$ were from Wako Pure Chemical Ind. (Osaka, Japan). LPS and metal salts (except $AgNO_3$) were dissolved in sterile saline. $AgNO_3$ was dissolved in sterile 0.1 M phosphate buffer (pH 7).

Sensitization to Ni

An adjuvant, $NiCl_2$, or a mixture thereof was injected intradermally (i.d.) or intraperitoneally (i.p.) as follows: (1) i.d. route: an equivolume mixture of $NiCl_2$ and LPS solutions, or of a $NiCl_2$ solution and CFA, was injected i.d. into both the right and left flanks (50 μL each) of mice that had been anaesthetized with ethyl ether just before the injections. The concentrations of $NiCl_2$ and LPS are indicated in the relevant experiments. (2) i.p.–i.d. route: LPS was injected i.p. (0.25 mL/mouse), and 5 min later $NiCl_2$ was injected i.d. into both the right and left flanks (50 μL each). (3) i.p. route: an equivolume mixture of a metal salt solution and an LPS solution was injected i.p. (0.25 mL/mouse), the concentrations being indicated in the relevant experiments. In some parts of this paper, we used an equivolume mixture of 1 mM $NiCl_2$ (or 1 mM of another metal salt) and 1 μg/mL LPS (0.25 mL/mouse, i.p.) as a standard sensitizing solution, abbreviated as SSS-Ni (or SSS-metal).

Provocation of ear swelling

At various intervals (days) after a sensitizing injection, a metal salt solution or an equivolume mixture of a metal salt solution and an LPS solution was given as a challenge by i.d. injection into the left and right pinnas near the root of the ear (20 μL each ear). The concentrations used are indicated in the relevant experiments. Saline was injected (20 μL, i.d.) as a control for the challenging injection. However, this saline injection produced no significant ear swelling (so, these data are not shown in some figures). Mice were anaesthetized with ethyl ether just before the challenging 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

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Co. Ltd, Tokyo, Japan), and the induced difference (after vs. before the challenge) was recorded.

Depletion and detection of macrophages

Clodronate-encapsulated liposomes (Clo-lip) selectively deplete phagocytic macrophages [36]. As described previously [37, 38], a suspension of Clo-lip was intravenously injected into mice, and depletion of macrophages was confirmed by immunostaining of liver using an F4/80 antibody.

Assay of histidine decarboxylase activity in ears

The ears were removed after decapitation, and HDC activity was assayed using our previously described method [32, 39]. HDC activity is expressed as nanomoles of histamine formed during a 1-h period of incubation by the enzyme contained in 1 g (wet weight) of tissue (nmol/h/g).

Flow cytometry

Flow cytometric analysis was performed using a FACScalibur flow cytometer and CELLQuest software (BD Biosciences, San Diego, CA, USA). Murine spleen cells were incubated on ice for 30 min with fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD8a monoclonal antibody (clone 53-6.7, rat IgG2a; BD Biosciences) and r-phycoerythrin (R-PE)-conjugated anti-mouse CD4 monoclonal antibody (clone H129.19, rat IgG2a; BD Sciences).

Statistical analysis

Experimental values are given as the mean ± standard deviation (SD). The statistical significance of differences was analysed using an analysis of variance (ANOVA), with a Bonferroni's multiple-comparison test performed *post hoc* (InStat software). *P*-values less than 0.01 were considered to indicate significance.

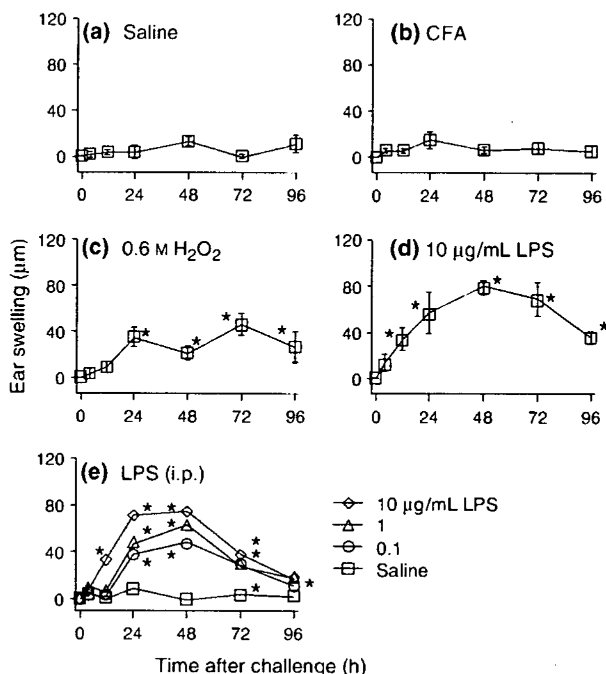

Fig. 1. Effects of intradermally (i.d.)-injected lipopolysaccharide (LPS), complete Freund's adjuvant (CFA), and H_2O_2 on sensitization to Ni in BALB/c mice. An equivolume mixture of $NiCl_2$ (4 mM) and an adjuvant [(a) saline, (b) CFA, (c) H_2O_2 (0.6 M), or (d) LPS (10 μg/mL)] was injected i.d. into both the right and left flanks (50 μL each) of BALB/c mice. In the experiment shown in (e), $NiCl_2$ (2 mM) was injected i.d. into right and left flanks of BALB/c mice 5 min after i.p. injection of various concentrations of LPS. In all experiments (a–e), each ear was challenged with 5 mM $NiCl_2$ (20 μL) at 10 days after sensitization. Each value is the mean ± standard deviation (SD) from three mice (i.e. $n = 6$ ears). In (e), to reduce complexity, mean values are shown without SD. *$P < 0.01$ vs. time 0. Each experiment (a–e) was repeated a further two times, and the results obtained were similar to those shown.

Results

Comparison of adjuvant effects of complete Freund's adjuvant, H_2O_2, and lipopolysaccharide

In these experiments, a 5 mM $NiCl_2$ challenge was delivered at 10 days after a sensitizing injection (i.d. or i.p.). (i) i.d. injection of a mixture of an adjuvant and $NiCl_2$. An equivolume mixture of an adjuvant (saline, CFA, 0.6 M H_2O_2, or 10 μg/mL LPS) and 4 mM $NiCl_2$ was injected i.d. into both the right and left flanks in BALB/c mice. Saline+$NiCl_2$ was ineffective in inducing ear swelling (Fig. 1a), and it produced no detectable lesions at the injection sites. The adjuvant effect of CFA was negligible (Fig. 1b), although it produced keloidal lesions at the injection sites in all mice. Incidentally, when CFA was mixed with 40 mM $NiCl_2$, marked ear swelling occurred in C57BL/6 mice too (Figs 7b and c). The adjuvant effect of H_2O_2 was significant (Fig. 1c), but variable, and it too produced keloidal lesions at the injection sites. In contrast, LPS displayed powerful adjuvant activity (Fig. 1d) without producing detectable lesions at the injection sites. (ii) i.p. injection of LPS and i.d. injection of $NiCl_2$. Next, we examined the adjuvant effect of i.p.-injected LPS. Soon (5 min) after i.p. injection of LPS (at various concentrations), $NiCl_2$ (2 mM) was injected i.d. into both the right and left flanks in BALB/c mice (Fig. 1e). LPS displayed dose-dependent adjuvant activity, and it was effective even at 0.1 μg/mL. (iii) i.p. injection of a mixture of LPS and $NiCl_2$. i.p. injection of $NiCl_2$ alone at 20 mM made mice moribund within a few hours, and some died within 24 h, while others recovered after 48 h. At 10 mM, $NiCl_2$ reduced the movement of mice within 30 min, and although some fell into a moribund state within a few hours, they recovered after 24 h. The sensitizing effect of $NiCl_2$ alone was significant at 10 mM, but not at 5 mM (Fig. 2a),

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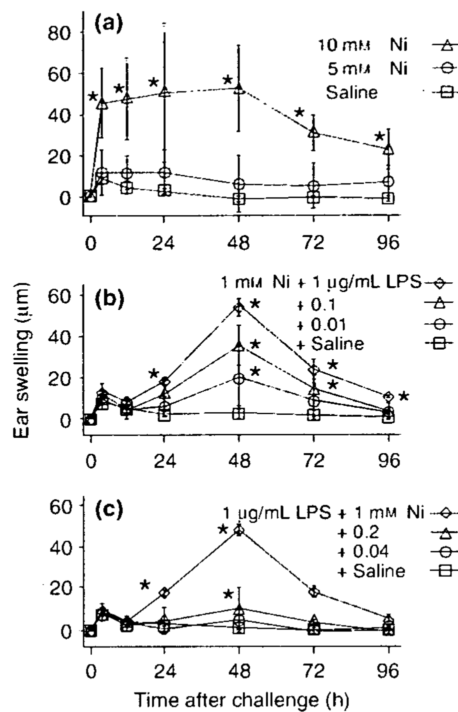

Fig. 2. Effects of intraperitoneal (i.p.) injection of lipopolysaccharide (LPS)+$NiCl_2$ on sensitization to Ni in BALB/c mice. (a) Saline or $NiCl_2$ (5 or 10 mM) was injected (0.25 mL, i.p.). (b) An equivolume mixture of 1 mM $NiCl_2$ and LPS (0.01–1 μg/mL) was injected (0.25 mL, i.p.). (c) An equivolume mixture of $NiCl_2$ (0.04–1 mM) and LPS (1 μg/mL) was injected (0.25 mL, i.p.). In (a–c), each ear was challenged 10 days later with 5 mM $NiCl_2$ (20 μL). Each value is the mean ± SD from four mice (i.e. $n = 8$). $^*P < 0.01$ vs. time 0. Each experiment was repeated a further two times, and the results obtained were similar to those shown.

although the ear-swelling responses varied markedly from mouse to mouse. Injection of an equivolume mixture of LPS (0.01–1 μg/mL) and 1 mM $NiCl_2$ induced ear swelling in a manner that depended on the concentration of LPS (Fig. 2b). An equivolume mixture of 1 μg/mL LPS and $NiCl_2$ induced slight, but significant, ear swelling at 48 h at 0.2 mM $NiCl_2$ (Fig. 2c). Under these conditions, the activity of the mice was normal and they seemed healthy. In the following experiments, we sensitized mice by an i.p. injection of SSS-Ni (see 'Materials and methods'). Incidentally, when BALB/c mice were challenged with 5 mM $NiCl_2$ at 4, 7, 10, or 14 days after sensitization with SSS-Ni, both the magnitude and the time course of the ear swelling were similar among 7, 10, and 14 days, whereas the challenge at 4 days induced no detectable ear swelling (data not shown).

Effects of lipopolysaccharide at the time of Ni challenge

In mice sensitized with SSS-Ni (i.p.), the magnitude of the ear-swelling response depended on the concentration of the challenging $NiCl_2$ (Fig. 3a), with 0.08 mM or more of

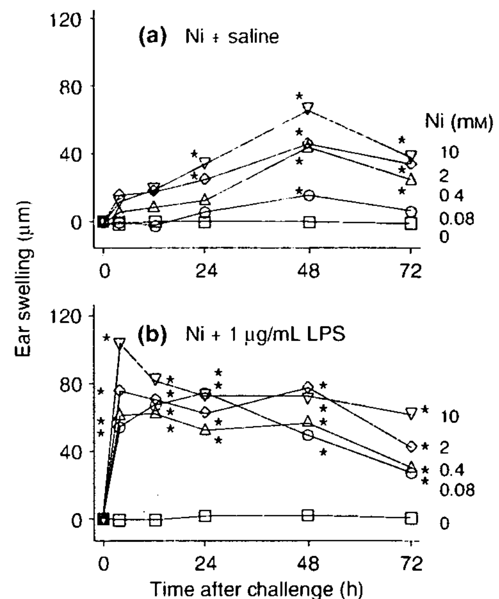

Fig. 3. Effects of lipopolysaccharide (LPS) at the time of Ni challenge in BALB/c mice. At 10 days after SSS-Ni injection (see text), ears were challenged with 20 μL of (a) an equivolume mixture of $NiCl_2$ (0.08–10 mM) and saline or (b) an equivolume mixture of $NiCl_2$ (0.08–10 mM) and LPS (1 μg/mL). To reduce complexity, mean values (from four mice, i.e. $n = 8$) are shown without standard deviation. $^*P < 0.01$ vs. time 0. Each experiment was repeated a further two times, and the results obtained were similar to those shown.

$NiCl_2$ inducing significant ear swelling. Incidentally, in the H_2O_2 model employed by Artik et al. [13], mice are challenged with a much higher concentration (10 mM) of $NiCl_2$. Surprisingly, when we combined LPS with the challenging $NiCl_2$ (i.e. when we used an equivolume mixture of $NiCl_2$ and 1 μg/mL LPS), maximal ear swelling (even at 0.08 mM $NiCl_2$) occurred at as early as 4–12 h, and the swelling lasted for a long time (Fig. 3b).

Involvement of innate immunity in Ni allergy in mice

In the following experiments, mice were treated with SSS-Ni (i.p.) and challenged with 5 mM $NiCl_2$. This Ni allergy is hereafter termed 'Ni(+LPS) allergy'. We obtained the following results. (i) Unexpectedly, Ni(+LPS) allergy was fully induced in nude mice (Fig. 4a). We confirmed that nude mice mostly lack $CD4^+$ T cells and $CD8^+$ T cells (Fig. 5). (ii) Unlike in control C3H/HeN mice, Ni(+LPS) allergy was negligible in C3H/HeJ mice with a mutant TLR4 (Fig. 4b). (iii) Ni(+LPS) allergy was very weak in macrophage-depleted mice (− Mϕ) (Fig. 4c). (iv) Interestingly, IL-1 KO mice were resistant, but TNF-α KO mice were sensitive to Ni(+LPS) allergy (Figs 4d and e). (v) In C57BL/6 mice, ear swelling developed more rapidly (reaching peak at 24 h) and was of a greater magnitude than in the other mice (Fig. 4f).

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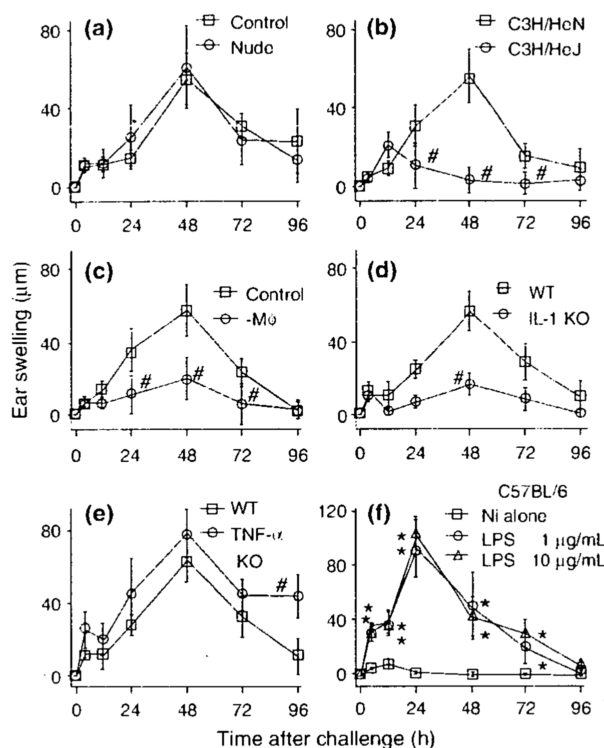

Fig. 4. Induction of Ni[+lipopolysaccharide (LPS)] allergy in (a) athymic (nude) BALB/c and control BALB/c mice, (b) TLR4-mutant C3H/HeJ and control C3H/HeN mice, (c) macrophage-depleted BALB/c and control BALB mice, (d) IL-1 KO BALB/c and control wild-type (WT) BALB/mice, (e) TNF-α KO BALB/c and control WT BALB/c mice, and (f) C57BL6 mice. Note different ordinate scale in (f). Mice were treated with SSS-Ni (in f, an SSS-Ni containing 10 μg/mL LPS was also used), and 10 days later their ears were challenged with 20 μL of 5 mM $NiCl_2$. In (c), SSS-Ni was given 24 h after Clo-lip injection. Each value is the mean ± standard deviation from four mice (i.e. $n = 8$). $^{\prime}P < 0.01$ and vs. control or WT mice. Each experiment was repeated a further two times, and the results obtained were similar to those shown.

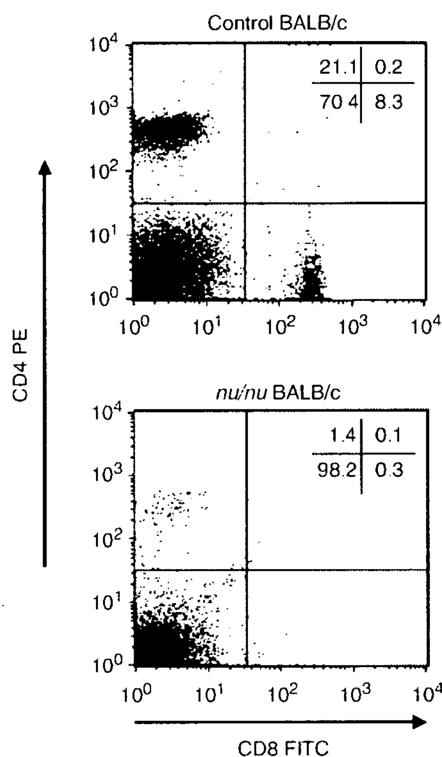

Fig. 5. Flow cytometric analysis of T cells in the spleen in control and nude BALB/c mice (see 'Materials and methods'). Similar results were obtained in one additional experiment.

Involvement of histamine in Ni(+LPS) allergy

(i) Ni(+LPS) allergy in mast-cell-deficient mice. W/W^v and +/+ mice were treated with SSS-Ni (i.p.), and 10 days later their ears were challenged with $NiCl_2$. However, as ear swelling was very slight in both +/+ and W/W^v mice (data not shown), they were again treated with SSS-Ni (at 12 days after the first SSS-Ni injection), and 10 days later again challenged with $NiCl_2$. This time, both +/+ and W/W^v mice exhibited marked ear swelling (Fig. 6a). Unexpectedly, the ear swelling lasted much longer in mast-cell-deficient W/W^v mice.

(ii) Contribution of HDC to Ni(+LPS) allergy. In contrast to our finding in W/W^v mice, the Ni(+LPS) allergy was markedly weaker in HDC KO mice than that in their control (wild-type C57BL/6 mice) (Fig. 6b). In BALB/c mice, HDC activity in the ears increased roughly in parallel with the development of ear swelling (Fig. 7a), as it did in C57BL/6 mice sensitized (i.d.) with CFA+40 mM $NiCl_2$ (Fig. 7b). Moreover, ear swelling and HDC activity each showed dependence on the concentration of $NiCl_2$ used for the challenge (Fig. 7c). These results indicate that HDC activity in the ear correlates with ear swelling, irrespective of differences in adjuvant or strain of mice.

Sensitization to other metals

BALB/c mice were treated (i.p.) with an equivolume mixture of 1 μg/mL LPS (or saline) and 1 mM $CrCl_2$, $CoCl_2$, $PdCl_2$, or $AgNO_3$, and 10 days later their ears were challenged with 5 mM of the same metal salt. LPS was effective at sensitizing the mice to each of these metals (Figs 8a–d), with Pd inducing the greatest ear-swelling response. Like Ni(+LPS) allergy, Pd(+LPS) allergy was weak in IL-1 KO and HDC KO mice, but was fully induced in TNF-α KO mice (Figs 8e and f).

Discussion

Adjuvant effects of lipopolysaccharide

CFA is used as an adjuvant to sensitize guinea-pigs to Ni [40], while H_2O_2 (0.6 M) is used as an adjuvant to sensitize C57BL/6 mice to Ni [13]. In these models, CFA and H_2O_2

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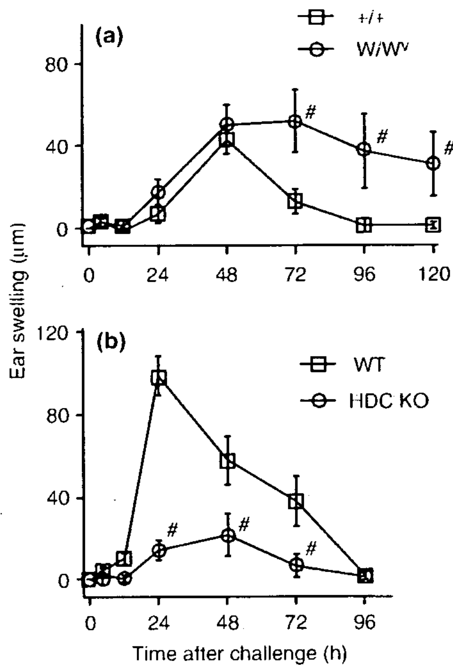

Fig. 6. Induction of Ni allergy in histamine-deficient mice: (a) mast-cell-deficient W/W^v and their normal litter mate +/+ mice, and (b) HDC KO C57BL/6 and control wild-type (WT) C57BL/6 mice. Mice were treated with SSS-Ni, and 10 days later their ears were challenged with 20 μL of 5 mM $NiCl_2$. Although a challenge at 10 days induced only minor ear swelling in both +/+ and W/W^v mice (data not shown), marked ear swelling was observed following a second sensitization and challenge, as shown in this figure (see 'Results' for details). Each value is the mean ± standard deviation from four mice (i.e. $n = 8$). #$P < 0.01$ vs. +/+ or WT. Each experiment was repeated a further two times, and the results obtained were similar to those shown.

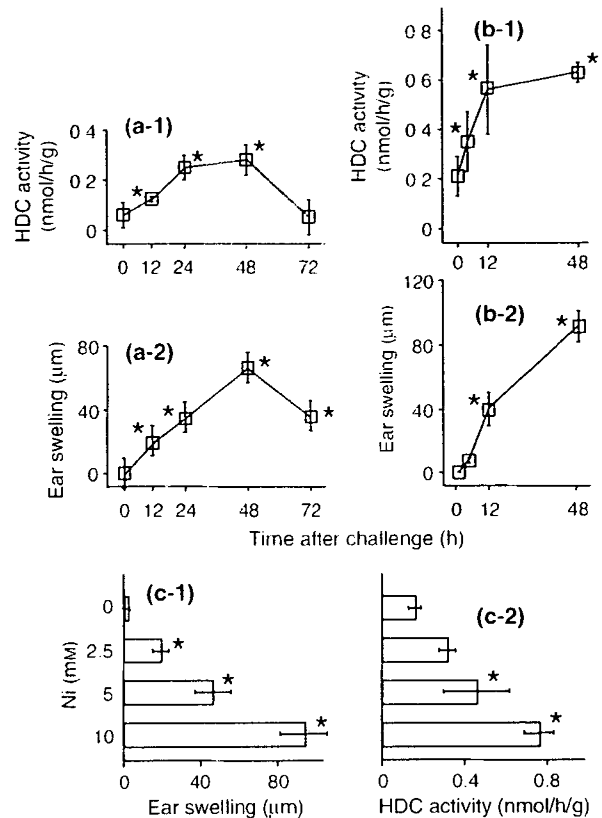

Fig. 7. Elevation of histidine decarboxylase (HDC) activity in the ears following Ni challenge in BALB and C57BL/6 mice. (a) BALB/c mice were divided into five groups (four mice per group), and then treated with SSS-Ni (i.p.). (b) C57BL/6 mice were divided into four groups and sensitized with an equivolume mixture of complete Freund's adjuvant (CFA) and $NiCl_2$ (40 mM), which was injected i.d. into both right and left flanks (50 μL each). (c) C57BL/6 mice were divided into four groups and sensitized with an equivolume mixture of CFA and $NiCl_2$ (40 mM), which was injected i.d. into both the right and left flanks (50 μL each). Ears were challenged 10 days later with 20 μL of 5 mM (a), 10 mM (b), or various concentrations (c) of $NiCl_2$, and ear swelling was measured at the indicated times (a and b) or at 48 h (c) after the Ni challenge. Just after measurement of ear swelling, the mice were decapitated and their ears removed for the assay of HDC activity. Each value is the mean ± standard deviation from four mice (i.e. $n = 8$ for ear swelling and $n = 4$ for HDC activity). *$P < 0.01$ vs. time 0 or dose 0. Each experiment was repeated a further two times, and the results obtained were similar to those shown.

are applied topically to the skin, and the range of Ni concentrations employed for sensitization is 20–200 mM. However, we noted that both CFA and H_2O_2 (0.6 M) are very injurious to mice. The present study indicates that LPS is a potent adjuvant for the sensitization of mice to Ni (even when a $NiCl_2$ concentration of 1 mM or less is employed) without producing any detectable lesions at the injection sites. Further, we found that i.d., i.p., or i.p.-i.d. injection of LPS+Ni easily sensitizes mice to Ni. In preliminary experiments, we observed that LPS obtained from *Prevotella intermedia* (an oral bacterium) (1 μg/mL) was also active as an adjuvant (data not shown). This is potentially important as surgical dental procedures and daily oral hygiene procedures can expose us systemically to oral bacteria [41], and the LPS produced in oral tissues may be transported easily to extraoral tissues [42]. Interestingly, LPS (from *Escherichia coli*) promoted sensitization to all the metals tested (Cr, Co, Pd, and Ag). In addition, it augmented ear swelling when injected at the time of the Ni challenge. These results suggest that in real life, LPS may be a factor promoting metal allergy via various routes.

Contribution of T cells

As described in 'Introduction', T cells are believed to play critical roles in the establishment of Ni allergy. However, Ni(+LPS) allergy occurred fully in nude mice (mostly lacking mature T cells), suggesting that T cells may not be essential (or possibly that very low levels of T cells are sufficient) for the establishment of Ni allergy. However, we cannot exclude the possibility that in mice, Ni(+LPS) allergy is a different type of allergy from the so-called Ni allergy. C57BL/6 mice are Th1-dominant mice, whereas BALB/c mice are Th2 dominant [43]. Ear swelling was

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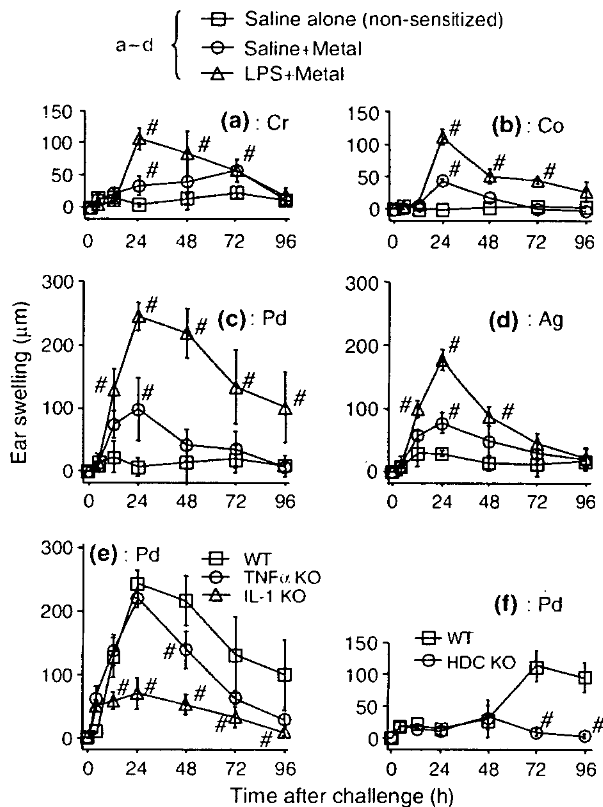

Fig. 8. Induction of allergies to metals other than Ni. (a–d) Lipopolysaccharide (LPS)-induced sensitization of BALB/c mice to other metals ($CrCl_2$, $CoCl_2$, $PdCl_2$, or $AgNO_3$). Saline alone, an equivolume mixture of saline and 1 mM of a metal salt, or an equivolume mixture of 1 μg/mL of LPS and 1 mM of a metal salt was injected into BALB/c mice [0.25 mL/mouse, intrapritoneal (i.p.)]. Ten days later, ears were challenged with 5 mM of the same metal salt (20 μL/ear). Each value is the mean ± standard deviation (SD) from four mice (i.e. $n = 8$). #$P < 0.01$ vs. saline+metal. (e and f) Pd(+LPS) allergy in IL-1 KO mice, TNF-α KO mice, and HDC KO mice. These mice and their control wild-type (WT) mice were injected i.p. with an equivolume mixture of 1 μg/mL LPS and 1 mM $PdCl_2$. Ten days later, ears were challenged with 5 mM $PdCl_2$ (20 μL/ear). Each value is the mean ± SD from four mice (i.e. $n = 8$). #$P < 0.01$ vs. WT. Each experiment was repeated a further two times, and the results obtained were similar to those shown.

more rapid and of greater magnitude in C57BL/6 than in BALB/c mice, although the difference was not marked, suggesting that Th1 immune responses may contribute more to Ni(+LPS) allergy. Be that as it may, careful studies will be required to evaluate the contribution made by T cells to Ni(+LPS) allergy in mice.

Contribution of innate immunity

In contrast to our finding in nude mice, Ni(+LPS) allergy was very weak both in C3H/HeJ mice and in macrophage-depleted mice, suggesting that TLR4 and macrophages are important in the establishment of Ni allergy. IL-1 and TNF are produced by various immune-competent cells and share many biological activities [44]. The major difference between them is that IL-1 shares TLR4-signal cascades, whereas TNF stimulates apoptotic cascades. Interestingly, IL-1 KO mice (but not TNF-α KO mice) were resistant to the establishment of Ni(+LPS) allergy. Pd(+LPS) allergy was also very weak in IL-1 KO mice. These results support the idea that innate immunity is essential for the establishment of metal allergies, and also that TLR- or IL-1-related innate signals are more important than TNF-related signals for metal allergies.

Roles of histamine

Although histamine levels in the tissues (such as skin) are lower in W/W^v mice than in either +/+ or BALB/c mice [31, 32], HDC is normally induced in various tissues in W/W^v mice in response to LPS or IL-1 [32]. In W/W^v mice, the ear-swelling response to Ni was not only fully induced, but lasted much longer than that in control +/+ mice. Histamine levels in HDC KO mice are very low [45], and we confirmed that we could not detect HDC activity in their tissues even after LPS injection (data not shown). In contrast to the situation in W/W^v mice, Ni(+LPS) allergy was markedly lower in HDC KO mice than in their control mice. Further, HDC activity increased in the ears of both BALB/c and C57BL/6 mice in parallel with the swelling (Fig. 7). Pd(+LPS) allergy was also very weak in IL-1 KO mice. NC/Nga mice develop dermatitis in conventional (but not in specific pathogen-free) environments [46], and we previously reported that HDC activity increases in their skin in parallel with the development of the dermatitis [47]. As described in Introduction, histamine modifies Th1 and Th2 responses. Although, as is well known, mast cells store histamine and release it upon stimulation by allergens, cells other than mast cells also release histamine through HDC induction in response to inflammatory stimuli [including LPS and inflammatory cytokines (such as IL-1, TNF, IL-12, and IL-18)] [29, 48–50]. These results suggest that the histamine newly formed following HDC induction in non-mast cells (via TLRs or cytokines) may be involved in the establishment of Ni(+LPS) allergy. However, it remains to be clarified whether the newly formed histamine is involved in the course of sensitization or in the elicitation of inflammation, and whether histamine acts on blood vessels (inducing plasma extravasation) [45] or on immune-competent cells [23, 24].

Comparison with classical contact hypersensitivity models

Unlike the classical haptens (such as dinitro- or trinitrophenyl derivatives), which covalently modify self-proteins, metal ions produce reversible complexes with unidentified self-proteins [8]. Hence, it is possible that the mechanism underlying contact hypersensitivity (CHS) may differ between that induced by metals and that

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induced by the classical haptens [8]. The present findings highlight some similarities and dissimilarities between these two types of CHS responses as follows: our results suggest that TLRs are important in metal allergies. Similar promoting effects of TLR ligands (such as TLR7 and TLR9 ligands) have been reported in models entailing CHS induction by the classical haptens, and in these models TLR ligands enhance the antigen-presenting functions of dendritic cells (via up-regulation of HMC class II, CD80, and CD86 molecules or an enhanced production of IL-12) [51-53]. In addition, IL-1β has been implicated as an important player in the classical hapten-induced CHS responses (as both mice treated with a neutralizing antibody to IL-1β and mice deficient in IL-1β display impaired CHS responses) [54, 55]. Moreover, it has been reported (a) that activation of caspase-1 (the enzyme converting pro-IL-1β to IL-1) via NALP3 (a member of the Nod family) is essential for the classical CHS responses [56, 57], and (b) that NALP3 is required for caspase-1 activation in TLR-stimulated macrophages [57]. However, in contrast to metal(+LPS) allergies, the CHS induced by the classical haptens is reported to be similar or more severe in HDC KO mice than in WT mice [45, 58]. Although at present we have no data to help explain this difference, it is likely that the CHS models induced by the classical haptens involve a mechanism different from that involved in metal(+LPS) allergies.

Conclusion

In conclusion, we found LPS to be a potent adjuvant in mice for the establishment of allergic responses to Ni and to some other metals. LPS also promoted the development of allergic responses when given at the time of challenge. Metal(+LPS) allergies may depend both on innate immunity and on the *de novo* formation of histamine (via HDC induction) in non-mast cells. We need further careful studies, using *in vivo* models, to assess the contribution made by T cells to the establishment of allergies to Ni and other metals. Interestingly, O'Leary et al. [59] have recently reported that natural killer cells can mediate allergies induced by classical haptens.

Acknowledgements

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Inductions of Histidine Decarboxylase in Mouse Tissues following Systemic Antigen Challenge: Contributions Made by Mast Cells, Non-Mast Cells and IL-1

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Key Words
Anaphylaxis · Allergy · Histidine decarboxylase · Histamine · Mast cells · Non-mast cells · Interleukin-1

Abstract
Background: Previous findings suggest that antigen challenge (AC) may induce histidine decarboxylase (HDC) in cells other than mast cells (MCs) via MC-derived IL-1. We examined this hypothesis. ***Methods:*** Mice were sensitized to ovalbumin. After the sensitization, an AC was delivered intravenously. ***Results:*** In control mice, AC markedly induced HDC at a postanaphylactic time in the liver, lung, spleen, and ears. In MC-deficient W/W^v mice, AC also induced HDC, although the effect was weaker than in control mice. AC increased IL-1 in the tissues, the pattern being similar in W/W^v and control mice. AC induced HDC similarly in IL-1-deficient and control mice. In control mice, AC decreased histamine in the tissues (except the liver) for several hours. ***Conclusion:*** (1) AC induces HDC in both MC-dependent and MC-independent ways. (2) AC induces IL-1 mostly in non-MCs, but this IL-1 is not a prerequisite for the induction of HDC by AC. (3) HDC induction may contribute to the replenishment of the reduced pool of MC histamine in the anaphylactic period. (4) In the case of MC-dependent HDC induction, AC may stimulate MCs in such a way as to induce HDC within the MCs themselves, and/or AC-stimulated MCs may stimulate HDC induction in other cells, which will need to be directly identified in future studies.

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Introduction

In 1966, Kahlson et al. [1] found that in guinea pigs and rats, a protein antigen challenge (AC) accelerates the rate of histamine formation in a variety of tissues. This histamine formation occurs slowly after the phase of anaphylaxis. On the basis of these findings, they proposed that in hypersensitivity reactions, the tissues are exposed to histamine generated by two different mechanisms with different time courses, that is an initial brief phase of histamine release from mast cells (MCs) and a subsequent prolonged phase following its de novo formation in unidentified cells. Later, Hirasawa et al. [2] supported this interesting hypothesis in a study using their air pouch model of allergic inflammation.

The histamine-forming enzyme, histidine decarboxylase (HDC), is induced in response to a number of inflammatory stimuli [3–5]. For example, lipopolysaccha-

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ride (LPS) and inflammatory cytokines (IL-1α, IL-1β, TNFα, IL-18, and IL-12) induce HDC in various tissues in mice [6–9]. In contrast, hematopoietic cytokines (IL-3, G-CSF, and GM-CSF) induce HDC only in the hematopoietic organs (i.e., spleen and bone marrow) [10, 11]. The induction of HDC by LPS or IL-1 occurs through the de novo formation of HDC-mRNA [12]. It has also been reported that HDC is induced in various cell types other than MCs. For example, HDC is reportedly induced in macrophages [13–15] and T lymphocytes in vitro [16]. In MC-deficient mice, HDC is induced in the skin by phorbol 12-myristate 13-acetate [17] and in various tissues by staphylococcal enterotoxin A [18], carbon tetrachloride (CCl_4) [19], or LPS [20, 21]. The major cells in which HDC is induced by enterotoxin A and CCl_4 are reported to be macrophages [18], while those in which it is induced by LPS have been suggested to be vascular endothelial cells and granulocytic precursor cells [6, 22, 23].

Kahlson et al. [1] conjectured, without direct evidence, that the acceleration of histamine formation that occurs following an AC may take place largely in non-MCs, while Shiraishi et al. [24] suggested that HDC is induced in neutrophils in the late phase of local allergic inflammation. Interestingly, recent in vitro studies have demonstrated that MCs produce not only Th2 cytokines, but also inflammatory cytokines (IL-1β and TNFα) [25–28]. IL-1 induces HDC fully in MC-deficient mice, which indicates that it induces HDC in non-MCs [29]. In addition, Fcε receptors have recently been found on various cell types, including non-MCs such as monocytes, eosinophils, Langerhans cells, and neutrophils [30]. Thus, it is likely that AC induces HDC in non-MCs (1) via the IL-1 and/or TNF produced by MCs and/or (2) via Fcε receptors on the non-MCs themselves. In the present study, we examined this hypothesis using MC-deficient mice (W/W^v), their normal littermates (+/+), IL-1-deficient BALB/c mice, and control BALB/c mice. These mice were sensitized to ovalbumin (OVA) and intravenously challenged with OVA.

Materials and Methods

Animals and Materials

MC-deficient W/W^v mice and their normal littermates (+/+ mice) were obtained from SLC Japan (Shizuoka, Japan), while BALB/c mice were from the animal facility of our university. IL-1-KO BALB/c mice (deficient in both IL-1α and IL-1β) were established from original IL-1α-KO and IL-1β-KO mice [31]. Female mice (6–10 weeks old) were used for the present study. Chicken OVA (5× crystallized) was purchased from Seikagaku Kogyo (Tokyo, Japan). All experiments conformed to national requirements (Japanese law No. 105, notification No. 6) and complied with the Guidelines for the Care and Use of Laboratory Animals in Tohoku University. Experimental protocols, doses of reagents, and the numbers of mice are described in the text or in the legend to the figure relating to each experiment.

Sensitization of Mice and Assessment of Anaphylaxis

A suspension (0.5 ml) containing OVA (50 μg) and alum (3 mg) was injected intraperitoneally on day 0 and again on day 10. Experiments involving the induction of anaphylaxis were performed on days 20–23. The AC was delivered by intravenous injection (via a tail vein) of OVA dissolved in saline (at the dose indicated for each experiment). Scores were allocated for shock signs (piloerection, dyspnea, sluggish gait, paresis, prostration, and convulsions) at 3–10 min after OVA challenge. Scoring was as follows: none (0), weak (1), medium (2), strong (3), and death (4).

Assay of HDC Activity

Briefly, mice were decapitated and tissues rapidly removed at the indicated times after OVA challenge. Ears were cut off at their root. HDC activities in the tissues were assayed using our previously described method [32] with a slight modification [33]. HDC activity was expressed as nanomoles of histamine formed during a 1-hour period of incubation by the enzyme contained in 1 g (wet weight) of each tissue (nmol/h/g).

Determination of Histamine

Several drops of blood from each decapitated mouse were directly collected into a test tube containing 3 ml of 0.4 M $HClO_4$. The weight of the blood collected was determined by weighing the test tube before and after the blood collection. After the collection of blood, tissues were rapidly removed and kept on dry ice until needed. The histamine in the blood and tissues was determined as described previously [33]. The amounts of histamine in the blood and tissues are expressed as nanomoles of histamine in 1 g of blood or wet tissue (nmol/g).

Determination of Cytokines in Serum

Blood was collected directly into test tubes following decapitation. Serum was recovered by centrifugation at 2,000 *g* at 4°C, then stored at −80°C until used. IL-1α and IL-1β in the serum were assayed using ELISA kits from Endogen (Cambridge, Mass., USA), while TNFα was measured using an ELISA kit from Biosource (Camarillo, Calif., USA). The assay procedures were performed exactly as described by the manufacturer.

Determination of Cytokines in Tissues

Frozen tissues were homogenized in RPMI 1640 solution containing Triton X-100, HEPES, bovine serum albumin, gentamicin sulfate, and proteinase inhibitor cocktail containing 4-(2-amino ethyl)benzenesulfonyl fluoride, aprotinin, leupeptin, bestatin, pepstatin A, and E-64 [34]. The supernatant obtained by centrifugation (10,000 *g* for 10 min at 4°C) of the homogenate was then assayed for each cytokine (IL-1α, IL-1β, and TNFα) as described above.

Data Analysis

Experimental values are given as mean ± standard deviation (SD). The statistical significance of the difference between two

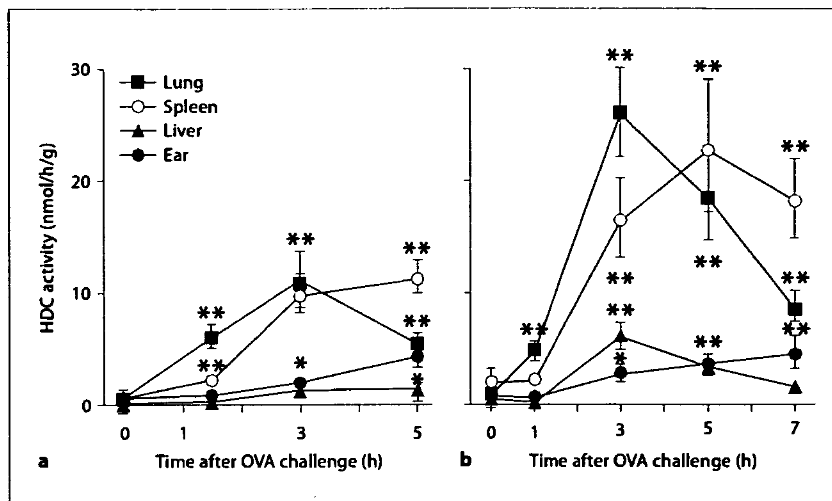

Fig. 1. Changes in HDC activity after OVA challenge to sensitized BALB/c mice. OVA, at 1 mg/kg (**a**) or 2 mg/kg (**b**), was injected intravenously, and tissues were taken at the indicated times. Each value is the mean ± SD of 4 mice. * $p < 0.05$; ** $p < 0.01$ vs. time 0.

means was evaluated using Student's unpaired t test or Dunnett's multiple comparison test after an analysis of variance (ANOVA). p values less than 0.05 were considered to be significant.

Results

HDC Activities following OVA Challenge in BALB/c Mice

HDC activities in the blood, liver, lung, spleen, and ear were very low in both sensitized and nonsensitized BALB/c mice, and there was no detectable difference between these two groups (data not shown). An OVA challenge at 1 mg/kg of sensitized BALB/c mice induced anaphylactic shock within 10 min of the challenge (score 1–2). HDC activity increased in all the tissues tested, and it peaked at 3–5 h after a 1 mg/kg OVA challenge (fig. 1a). An OVA challenge at 2 mg/kg induced a more severe anaphylactic shock (score 2–3 within 10 min of the challenge) and produced higher HDC activities (fig. 1b). There was no detectable HDC activity in the blood at these experimental times (data not shown). We confirmed that OVA (2 mg/kg) did not induce HDC activity in any of the tissues tested in nonsensitized mice (data not shown).

HDC Activities following OVA Challenge in MC-Deficient W/W^v Mice

AC at 1 mg/kg induced HDC activity in the tissues in all three types of mice at 4 h after OVA challenge (fig. 2). A similar magnitude of shock occurred among BALB/c, +/+, and W/W^v mice, as observed previously [35]. The HDC elevations in +/+ mice were similar to those seen in BALB/c mice. The HDC elevations were weaker in W/W^v mice, although the levels of HDC induced in the tissues were still quite substantial, especially in the liver and lung.

IL-1α, IL-1β, and TNFα Levels following OVA Challenge

First, we measured IL-1β and TNFα levels in the blood and tissues of BALB/c mice following an OVA challenge (fig. 3). There were marked increases in IL-1β in the liver, lung, and spleen, the increases being nearly maximal at 90 min, but there were no changes in TNFα levels in these tissues. We could detect no significant increases in either of these cytokines in the blood (levels of IL-1β and TNFα were 7–9 and 20–25 pg/ml, respectively). Next, we compared the induced increases in IL-1α and IL-1β in BALB/c and W/W^v mice at 90 min after challenges with different doses of OVA (fig. 4). In the ears in this experiment, both of these cytokines increased (in BALB/c mice) or showed a (nonsignificant) tendency to increase (in W/W^v mice). In other tissues, the increases in these cytokines followed a similar overall pattern in the two types of mice, although the IL-1β levels in the lung in W/W^v mice were lower than those in BALB/c mice. Sensitization itself did not affect the basal levels of these cytokines in the tissues (except in the spleen of W/W^v mice, in which IL-1α increased slightly but significantly). Incidentally, we confirmed the reliability of the method used to measure IL-1 levels in the tissues, and also confirmed that the

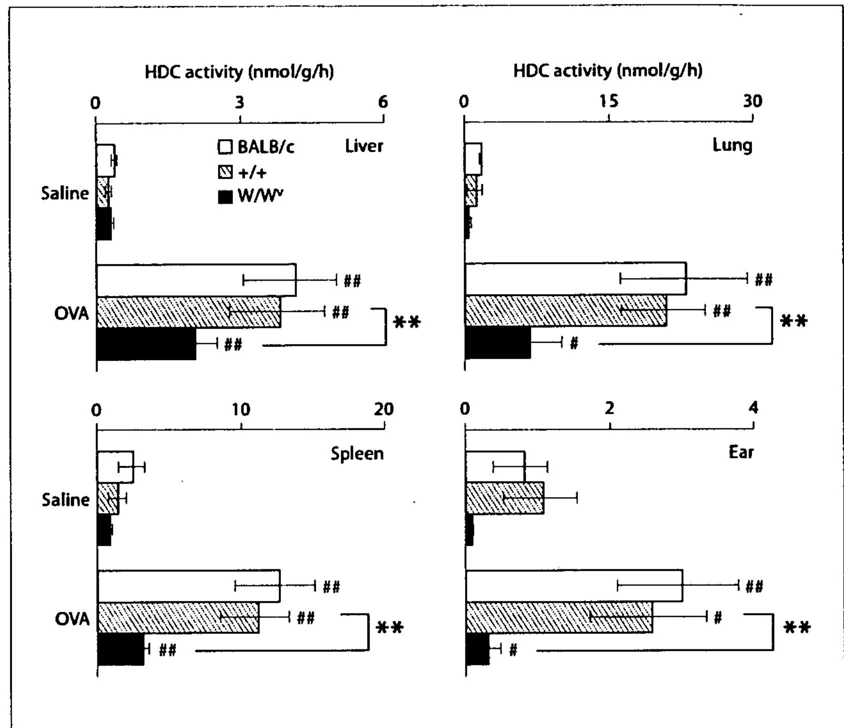

Fig. 2. HDC activities induced in sensitized BALB/c, +/+, and W/W^v mice by an OVA challenge. Saline or OVA (1 mg/kg) was injected intravenously, and tissues were taken 4 h later. Each value is the mean ± SD of 4 mice. # $p < 0.05$, ## $p < 0.01$ vs. saline group in same mouse strain; ** $p < 0.01$ between the indicated groups.

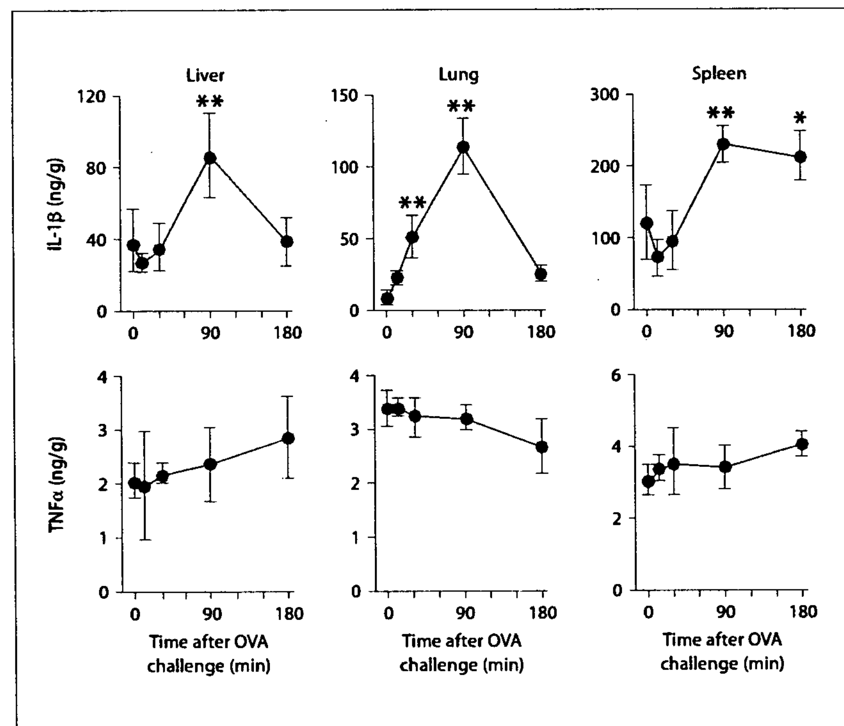

Fig. 3. Effects of OVA challenge on the levels of IL-1β and TNFα. OVA (1 mg/kg) was injected intravenously in sensitized BALB/c mice, and tissues were taken at the indicated times. Each value is the mean ± SD of 4 mice. * $p < 0.05$; ** $p < 0.01$ vs. time 0.

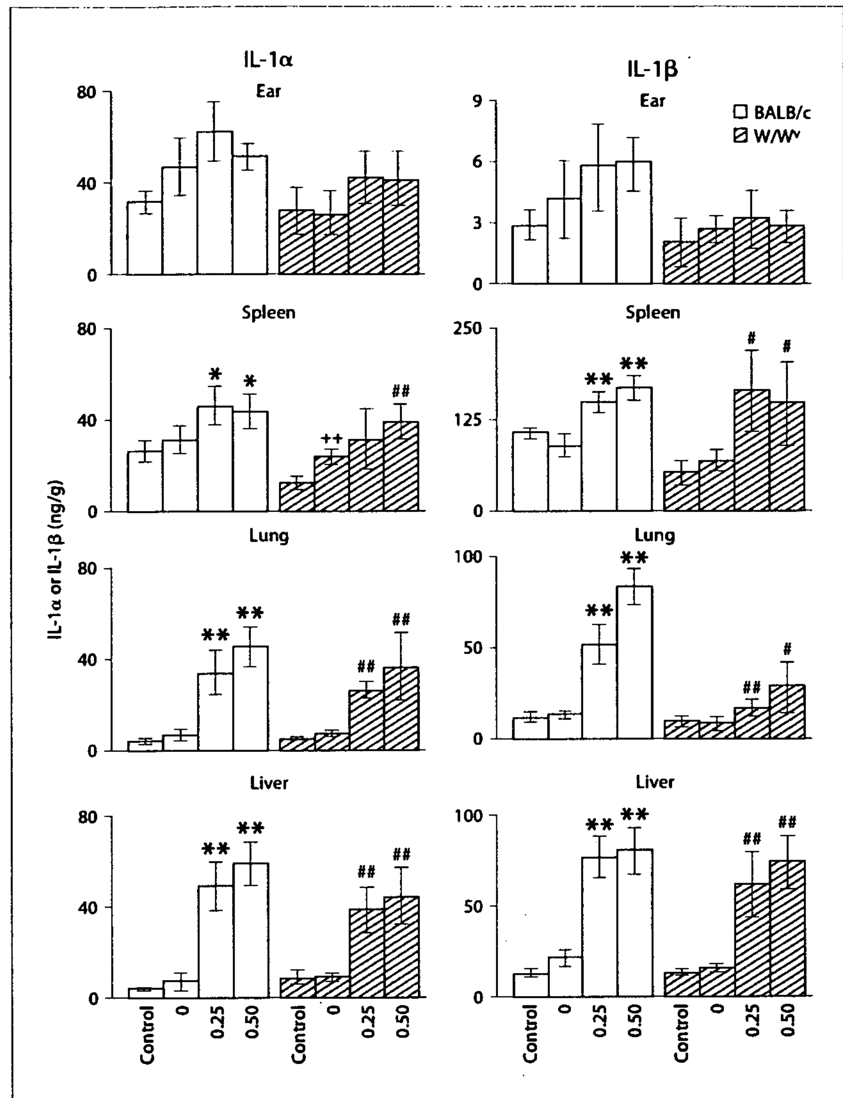

Fig. 4. Comparison of IL-1 increase between BALB/c and W/Wᵛ mice. Saline or OVA (0.25 or 0.5 mg/kg) was injected intravenously into sensitized mice, and tissues were taken 90 min later. Each value is the mean ± SD of 4 mice. 'Dose 0' indicates sensitized mice injected with saline. Control indicates nonsensitized, nonchallenged mice. * $p < 0.05$, ** $p < 0.01$ versus dose 0 in BALB/c mice; # $p < 0.05$, ## $p < 0.01$ vs. dose 0 in W/Wᵛ mice; ++ $p < 0.01$ versus control.

basal levels of IL-1α and IL-1β were much lower in IL-1-KO mice than in control BALB/c mice [36].

HDC Activities following OVA Challenge in IL-1-KO Mice

An OVA challenge at 1 mg/kg induced both anaphylactic shock (score 1–2 within 10 min of the challenge) and HDC elevations in the tissues in IL-1-KO BALB/c mice, too (fig. 5). In IL-1-KO mice, the HDC elevations were similar to those seen in control BALB/c mice.

Histamine Levels following OVA Challenge in Control BALB/c Mice

First, we compared histamine levels between nonsensitized and sensitized BALB/c mice. A significant increase in histamine was detected in some tissues (liver, lung, and ear) in sensitized mice (fig. 6). It should be noted that the histamine levels in the blood and liver were markedly lower than those in the other tissues, and that MCs are very scarce in the liver (data not shown). It should also be noted that there was a particularly high level of histamine in the ear, and that the skin of the ears is very rich in MCs [33].

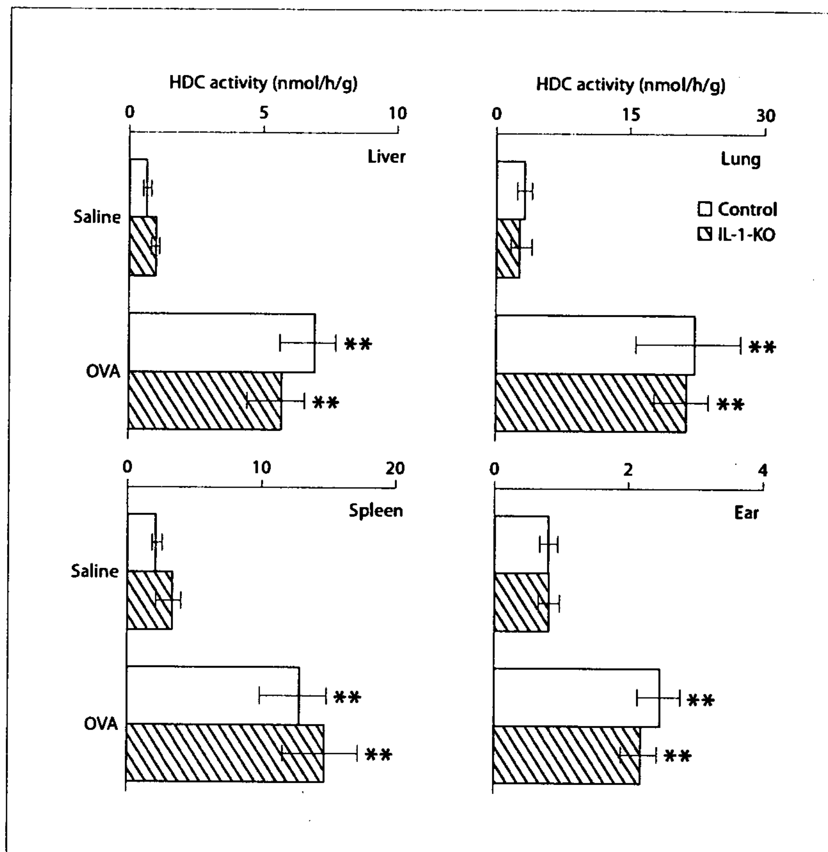

Fig. 5. HDC induction by OVA challenge in IL-1-KO mice. Saline or OVA (1 mg/kg) was injected intravenously, and tissues were taken 3 h later. Each value is the mean ± SD of 4 mice. ** $p<0.01$ vs. corresponding saline group.

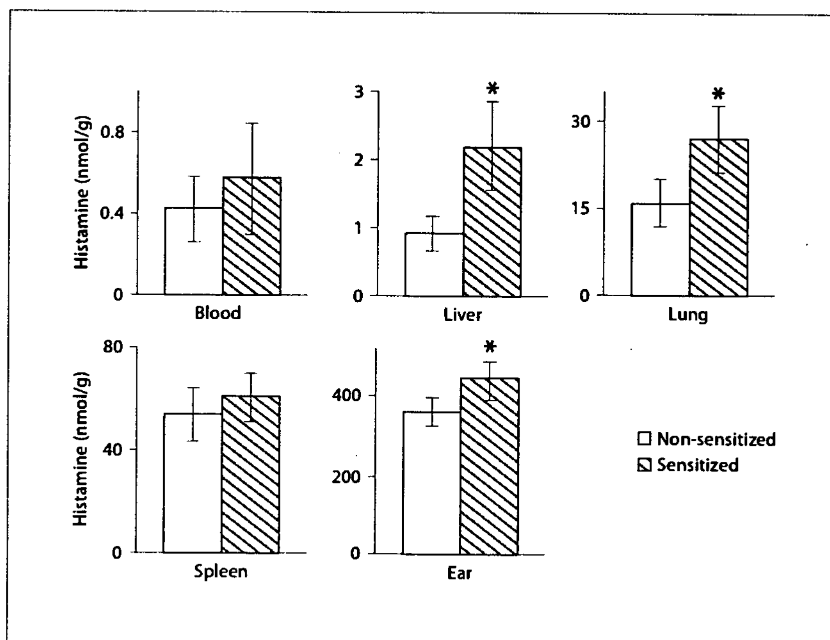

Fig. 6. Histamine levels in nonsensitized and sensitized BALB/c mice. Each value is the mean ± SD of 4 mice. * $p < 0.05$ vs. nonsensitized mice.

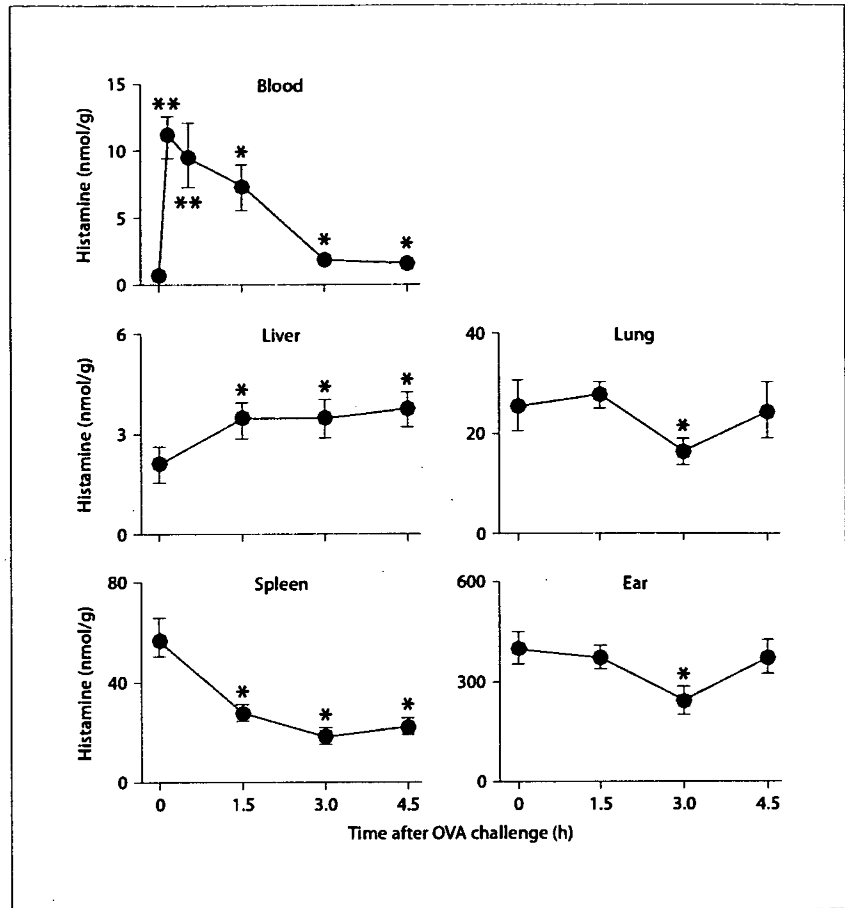

Fig. 7. Time course of changes in histamine following an OVA challenge to sensitized BALB/c mice. OVA (2 mg/kg) was injected intravenously, and blood or tissues were taken at the indicated times. Each value is the mean ± SD of 4 mice. * $p < 0.05$; ** $p < 0.01$ vs. time 0. The data at 10 and 30 min (histamine levels in the blood) were obtained in an additional experiment.

An OVA challenge at 2 mg/kg induced an explosive elevation of histamine in the blood within 10 min of the OVA challenge (fig. 7). This elevation was largely lost within 3 h, although at 3 and 4.5 h the level was still slightly, but significantly, higher than at time 0. In addition, the OVA challenge increased histamine in the liver, while it reduced histamine in the other tissues (fig. 7). The reduced histamine levels in the lung and ear recovered within 4.5 h of the OVA challenge. In another experiment, the reduced histamine level in the spleen recovered at 7 h after OVA challenge (data not shown). An OVA challenge at 1 mg/kg had effects on histamine levels similar to those induced by the 2 mg/kg challenge (i.e., a marked increase in the blood, a marked decrease in the spleen, a tendency to increase in the liver, and tendencies to decrease in the lung and ear; data not shown).

Discussion

As described in the introduction, we expected that OVA challenge might induce HDC in non-MCs (such as vascular endothelial cells, neutrophils, and macrophages). In the present study, we found that although the HDC inductions observed following OVA challenge were weaker in W/W^v mice than in BALB/c mice, the levels of HDC induced in the tissues (especially in the liver and lung) in W/W^v mice were quite substantial (fig. 2). Our results suggest that OVA challenge induces HDC in MCs and/or non-MCs, and that MCs are responsible for a large proportion of the HDC induction observed in the spleen and ear, while in the liver and lung non-MCs contribute nearly 50% of the induced HDC. The finding of HDC induction in MC-deficient mice is also important, indicating that OVA challenge is capable of inducing HDC in an MC-independent manner.

As described in the introduction, both IL-1 and TNFα are released in vitro from MCs upon their stimulation. In the present in vivo study, OVA challenge induced marked increases in both IL-1α and IL-1β, but not in TNFα, in the tissues examined (fig. 3). We previously reported that following administration of IL-1α or IL-1β, the induced HDC levels in various tissues are similar in normal and W/W^v mice [11, 29]. These results seem to support our hypothesis that MC-derived IL-1 may mediate the HDC induction observed following OVA challenge. However, despite the comparatively weak HDC induction in W/W^v mice, the pattern of the production of IL-1α and IL-1β in various tissues was, overall, much the same in BALB/c and W/W^v mice (the only difference being smaller IL-1β productions in the ear and lung in W/W^v mice). These results indicate that IL-1α and IL-1β are both largely produced in vivo from non-MCs in response to OVA challenge, although MCs in the lung may produce a significant amount of IL-1β. Moreover, an OVA challenge fully induced HDC in IL-1-KO mice (fig. 5). Hence, these results suggest that contrary to our expectations, the contribution, if any, made by IL-1 to HDC induction following an OVA challenge is small. It is possible that non-MCs as well as MCs may directly respond to OVA with an HDC elevation via cross-linking of IgE on Fcε receptors, because these receptors are expressed on non-MCs, such as monocytes, eosinophils, Langerhans cells, and neutrophils [30]. Moreover, it is likely that IgE-independent mechanism(s) are also involved, because anaphylaxis can occur in IgE-deficient mice [37]. Further studies will therefore be needed to clarify this issue.

The number of MCs has been shown to increase during the development of allergy [38, 39]. Thus, the increase in tissue histamine we observed in sensitized BALB/c mice (fig. 6) may reflect an increase in the number of MCs. In addition, it has been shown that the number of Fcε-receptor-positive basophils in the spleen and bone marrow increases in mice immunized with foreign proteins [40, 41], and that monomeric or oligomeric IgE itself is capable of inducing HDC in MCs in vitro [42, 43]. Thus, it is also likely that histamine synthesis is enhanced in MCs or basophils during the course of sensitization, although this remains to be clarified in future studies.

In BALB/c mice, OVA challenge induced (1) an explosive elevation of histamine in the blood (possibly via a release of histamine from the tissues), and (2) decreases in histamine levels in the tissues (except the liver) (fig. 7). The reduced histamine levels in the tissues recovered within a few hours (i.e., over the period during which HDC is induced). These results contrast with our previous finding that LPS injection, which induces HDC in non-MCs, elevates histamine levels in tissues such as liver, lung, and spleen [29]. Thus, the present results indicate that (1) the level of histamine present in a given tissue after OVA challenge is determined by the rates of release and formation of histamine and (2) the HDC induction that occurs following OVA challenge may contribute to the replenishment of the reduced histamine pools in MCs. The increase in histamine in the liver (fig. 7) may be explained by histamine production in non-MCs being greater than its release from MCs (because MCs are scarce in this organ). It is conceivable that in tissues not examined by us in the present study, MCs may respond rapidly to OVA by releasing histamine (as observed in the spleen), contributing to the explosive histamine increase in the blood during the anaphylactic phase. It would be interesting to try to identify such tissues in future studies. We also noticed that although the fall in the amount of histamine in the ear was very large following an OVA challenge (fig. 7) and the HDC activity induced in the ear was low (fig. 1), there was a fast recovery of histamine in the ear. Although we have as yet no data capable of explaining this result, it is interesting to note that Ohtsu et al. [44] found that the MCs in the skin of HDC-deficient mice actively take up histamine from their environment. Hence, we speculate that the fast recovery in the ear in OVA-challenged mice may be due not only to the induction of HDC in the ear skin, but also to the uptake from the circulation of histamine that had been produced by non-MCs at other sites and then released into the circulation.

The histamine newly produced by non-MCs is released upon its formation, without being stored [45–47]. Thus, the present findings support the hypothesis of Kahlson et al. [1] that in hypersensitivity reactions, the tissues are exposed to histamine generated by two different mechanisms with different time courses, namely an initial anaphylactic phase of histamine release from MCs and a subsequent prolonged phase following its de novo formation in non-MCs. We observed that after the initial explosive elevation of histamine in the blood (fig. 7), its level in the blood was still somewhat higher at 3 and 4.5 h than at time 0. This may be explained (at least in part) by the entry into the blood of the histamine newly formed by non-MCs. The histamine released explosively from MCs acts as a mediator of inflammation in the anaphylactic phase. On the other hand, the newly formed histamine may be involved in immune responses (or delayed inflammation) and/or in the replenishment of the reduced histamine pool in MCs in the anaphylactic phase, although we have not examined this idea.

In conclusion, contrary to our expectations the present findings suggest that (1) AC induces HDC in both MC-dependent and MC-independent ways, (2) AC induces IL-1 mostly in non-MCs, but this IL-1 is not a prerequisite for the induction of HDC by AC, (3) HDC induction may contribute to the replenishment of the reduced pool of MC histamine in the anaphylactic period, and (4) in the case of MC-dependent HDC induction, AC may stimulate MCs in such a way as to induce HDC within the MCs themselves, and/or AC-stimulated MCs may stimulate HDC induction in other cells, which will need to be directly identified in future studies.

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Induction of serum IL-18 with *Propionibacterium acnes* and lipopolysaccharide in phagocytic macrophage-inactivated mice

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Abstract: IL-18, an important regulator of immune responses, is expressed in activated macrophages and also in nonimmune cells, such as keratinocytes and epithelial cells. Increased levels of serum IL-18 are reported in patients with a wide variety of diseases, but it is unclear which type of cell is the major source of serum IL-18. Here, we showed that the administration of liposomes encapsulating clodronate (Clo-lip) in mice selectively depleted F4/80$^+$ phagocytic macrophages in the liver and spleen. Serum levels of mature IL-18 with 18 kDa were increased markedly in mice treated with *Propionibacterium acnes* and LPS, whereas administration of Clo-lip and gadolinium chloride, another widely used macrophage inactivator, showed no obvious effect on serum IL-18 levels, which were marginal in the liver, lung, and spleen and more pronounced in the intestines, especially in the duodenum. Treatment with *P. acnes* alone induced IL-18 more than twofold in each organ, and *P. acnes* and LPS induced a marked increase in IL-18 levels in the liver and spleen but decreased in the intestines. The administration of Clo-lip showed only a marginal effect on the IL-18 levels in these organs. Furthermore, serum levels of liver enzymes and TNF-α and liver injury (necrotic change and granuloma formation) induced by *P. acnes* and LPS were reduced moderately by Clo-lip. These results suggest that phagocytic macrophages do not actively contribute to the induction of serum IL-18 and liver injury in mice treated with *P. acnes* and LPS. *J. Leukoc. Biol.* 82: 327–334; 2007.

***Key Words:** cytokine · inflammation · macrophages · mucosal cells*

INTRODUCTION

IL-18 was identified originally as an IFN-γ-inducing factor from a murine liver cell cDNA library generated from mice primed with heat-killed *Propionibacterium acnes* and subsequently challenged with LPS [1]. IL-18 is produced intracellularly as an inactive, 24-kDa precursor form (proIL-18) and secreted as an 18-kDa mature form after cleavage by caspase-1, originally designated IL-1β-converting enzyme [2–4]. IL-18 is now recognized as a multifunctional regulator of innate and acquired immune responses through its activation of Th1 and Th2 responses [2–5]. IL-18 has also been suggested to be a potent, proinflammatory cytokine, which regulates autoimmune and inflammatory diseases [2–4].

Recent studies showed that IL-18 is identified, not only in activated macrophages, including dendritic cells (DC) and Kupffer cells, but also in nonimmune cells, such as keratinocytes, osteoblasts, adrenal cortex cells, epithelial cells of various organs and tissues, microglial cells, and synovial fibroblasts [2–4]. This wide range of distribution implies that IL-18 plays physiological roles and acts as a component of immune regulation.

Increased levels of IL-18 have been reported in the sera from patients with a wide variety of diseases, including autoimmune and inflammatory disorders [6–11], allergy [12], allograft rejection [13], and infectious diseases [14–16], and the elevated serum IL-18 levels are considered to be a parameter for the disease severity and a diagnostic marker. We have shown recently that human oral epithelial cells constitutively express a precursor form of IL-18, stimulation of the cells with neutrophil proteinase 3 (PR3) and LPS induces the secretion of an active form of IL-18 after IFN-γ priming [17], and PR3 activates the cells through a G protein-coupled protease-activated receptor 2 (PAR2) on the cell surface in vitro [18, 19]. Subsequently, we also revealed that neutrophil recruitment and PAR2 activation are critically involved in the induction of serum IL-18 in mice treated with heat-killed *P. acnes* and LPS in vivo [20].

It is still unclear whether the major source of serum IL-18 is activated macrophages or nonimmune cells, such as those of epithelial origin in vivo. A macrophage "suicide" technique, using liposomes encapsulating dichloromethylene bisphosphonate (clodronate), specifically depletes phagocytic macrophages but not neutophils and DC within 1 day or 2 of the i.v. injection of such liposomes into mice or rats [21–23]. It is also reported that i.v. injection of gadolinium chloride ($GdCl_3$) not only blocks phagocytosis of Kupffer cells but also eliminates

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these cells [24, 25]. These techniques have allowed us to investigate whether the major source of serum IL-18 is activated macrophages in mice treated with *P. acnes* and LPS. We also examined the effect of macrophage inactivation by the liposomes on liver injury in the mice.

MATERIALS AND METHODS

Mice

Female C57BL/6 mice (6–9 weeks old), obtained from the Institute for Experimental Animals of the Tohoku University Graduate School of Medicine (Sendai, Japan), were used for the experiments.

Bacteria and reagents

P. acnes was grown in brain-heart infusion medium (Difco Laboratories, Detroit, MI, USA) with L-cysteine and Tween-80, as described previously [20]. The harvested bacteria were washed with sterile, distilled water, killed by heating at 60°C for 1 h, and then lyophilized. The lyophilized bacteria were next suspended in PBS (5 mg/ml) and used to prime the mice. LPS from *Escherichia coli* 055:B5 and clodronate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Mouse recombinant (mr)IL-18 was obtained from Medical and Biological Laboratories (Okayama, Japan). Rabbit anti-mouse IL-18 polyclonal antibody (pAb) was provided by Tomaki Hoshino (Kurume University, Kurume, Japan). All other reagents were obtained from Sigma-Aldrich, unless otherwise indicated.

Treatment of mice with clodronate-liposomes (Clo-lip) or $GdCl_3$

A suspension of liposomes encapsulating clodronate was prepared according to a method described previously [22, 23]. Briefly, 75 mg phosphatidylcholine and 11 mg cholesterol were dissolved in chloroform (20 ml) in a round-bottomed flask (1000 ml). The thin film, which formed on the walls of the flask after rotary evaporation at 37°C, was dispersed by gentle shaking for 10 min in 10 ml clodronate solution (200 mg/ml) in PBS. This suspension was kept for 2 h at room temperature, then sonicated for 3 min (50 Hz), and kept for another 2 h. The resulting liposomes floating on the aqueous phase were collected using a Pasteur pipette, suspended in 10 ml PBS, and centrifuged at 5000 *g* for 30 min. The precipitated liposomes were finally suspended in 4 ml PBS; this preparation is referred to as the "original" suspension of Clo-lip. This original suspension was diluted (as described in the text) using PBS, and the diluted suspension was injected i.v. at 0.2 ml/mouse.

The mice were also injected i.v. with $GdCl_3$ (10 mg/kg) or saline [25].

Histological analysis

Immunohistochemistry was conducted as follows. Tissues were fixed in periodate-lysine-4% paraformaldehyde for 6 h at 4°C. After washing in PBS containing sucrose, fixed tissues were embedded in OCT compound (Sakura, Tokyo, Japan) and frozen immediately. Frozen tissue sections (6 μm) were incubated with rat anti-mouse F4/80 mAb (Serotec, Oxford, UK) overnight at 4°C. After that, the sections were treated with peroxidase-blocking reagent (Dako Cytomation, Tokyo, Japan) for 20 min and secondary antibodies, such as the goat anti-rat simple-stain mouse MAX-PO (Nichirei, Tokyo, Japan). The chromogen used was 3',3-diaminobenzidine tetrahydrochloride (Dako Cytomation). The sections were counterstained with hematoxylin. As a negative control, rat isotype-matched control Ig G2b (BD Biosciences, San Diego, CA, USA) was used.

For histopathological analysis, formalin-fixed samples were embedded in paraffin and stained with H&E.

Treatment of mice and preparation of serum and tissue extracts

The mice were injected i.p. with heat-killed *P. acnes* (1 mg dry weight/mouse), and 7 days later, they were challenged i.v. with LPS (1 μg/mouse). As *P. acnes*-primed mice started to die of endotoxin shock 4 h after the LPS challenge [20], blood and tissues were taken from the mice 2 h after the LPS challenge in this study. Blood was collected directly into test tubes following their decapitation, and the serum was recovered by centrifugation at 2000 *g* at 4°C, after which it was stored at −80°C until use. Frozen tissues or organs were homogenized in RPMI 1640 containing Triton X-100 (5 μl/ml), HEPES (10 μmol/ml), BSA (100 μg/ml), gentamicin sulfate (50 μg/ml), and proteinase inhibitor cocktail (10 μl/ml) [26]. The cocktail contains 4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin, leupeptin, bestatin, pepstatin A, and E-64. The supernatants obtained by centrifugation at 10,000 *g* for 10 min at 4°C of the homogenates were then stored at −80°C until use. The experimental procedure followed in this study was approved by the Ethical Board for Non-Human Species of the Tohoku University Graduate School of Medicine (Sendai, Japan).

Measurement of cytokines and liver enzymes

The levels of IL-18 and TNF-α in the samples were determined using a mouse IL-18 ELISA kit (Medical and Biological Laboratories, Woburn, MA, USA) and a mouse TNF-α OptEIA ELISA kit (BD PharMingen, San Diego, CA, USA), respectively. According to the manufacturer, the IL-18 ELISA kit mainly detects an 18-kDa, mature form, and the sensitivity to the precursor form was less than 10% compared with the mature form. The amount of IL-18 in each tissue was expressed as μg/g wet tissue.

Serum asparate aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured photometrically using commercial kits (Wako Pure Chemical Industries, Osaka, Japan).

Western blotting

All samples were solubilized with Laemmli sample buffer [27]. SDS-PAGE was performed in a 15% polyacrylamide slab gel under reducing conditions, according to the method of Laemmli [27]. Proteins were transferred to a polyvinylidene difluoride membrane using a semidry transblot system (Atto Instruments, Tokyo, Japan). The blot was blocked for 2 h with 3% w/v nonfat dry milk and 0.05% Tween 20 in PBS (Blotto/Tween) and incubated with anti-mouse IL-18 pAb at 6 μg/ml in Blotto/Tween overnight at 4°C. The blot was washed four times with Blotto/Tween and then incubated for 90 min with HRP-conjugated, affinity-purified goat anti-rabbit IgG at 1:3000 (Pierce Biotechnology, Rockford, IL, USA) in Blotto/Tween. After being washed, IL-18 was visualized with West Femto maximum sensitivity substrate (Pierce Biotechnology). The molecular weight of the proteins was estimated by comparison with the position of a standard (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Experimental values were expressed as the mean ± SD, and the statistical significance of differences between two means was evaluated by one-way ANOVA using the Bonferroni or Dunnett method, for which values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Effect of macrophage inactivation on the induction of serum IL-18 in mice

As it is suggested that activated macrophages, such as Kupffer cells, are the major source of serum IL-18 in vivo [1, 2], we examined the effect of macrophage inactivation by Clo-lip or $GdCl_3$ on the induction of serum IL-18 in mice after treatment with *P. acnes* and LPS. Clo-lip was administered in mice on Days 0, 3, and 5, and the livers and spleens were taken on Day 8. $GdCl_3$ was administered in mice on Days 0, 4, and 7, and the livers and spleens were taken on Day 9. The results from immunohistochemistry showed that the administration of Clo-lip eliminated $F4/80^+$ macrophages (Kupffer cells) in the liver (**Fig. 1A**). $F4/80^+$ macrophages were abundant in the red pulp of the spleen, and the Clo-lip treatment also eliminated $F4/80^+$ macrophages in the spleen (Fig. 1B). In contrast, $GdCl_3$ did not deplete $F4/80^+$ macrophages in the liver and spleen. These