

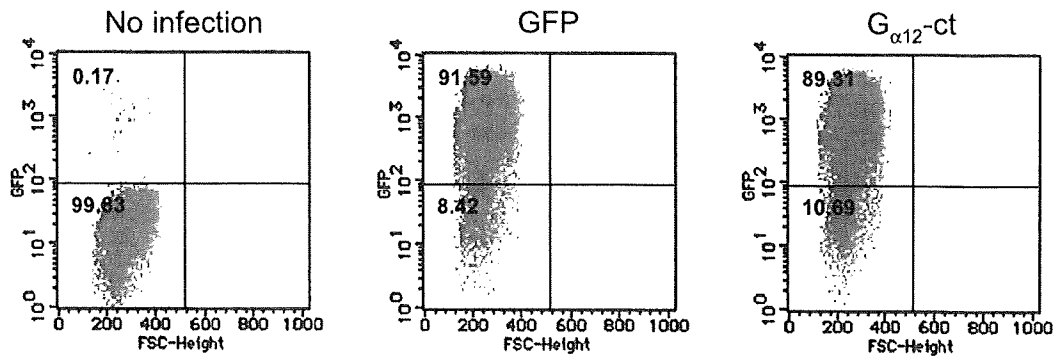
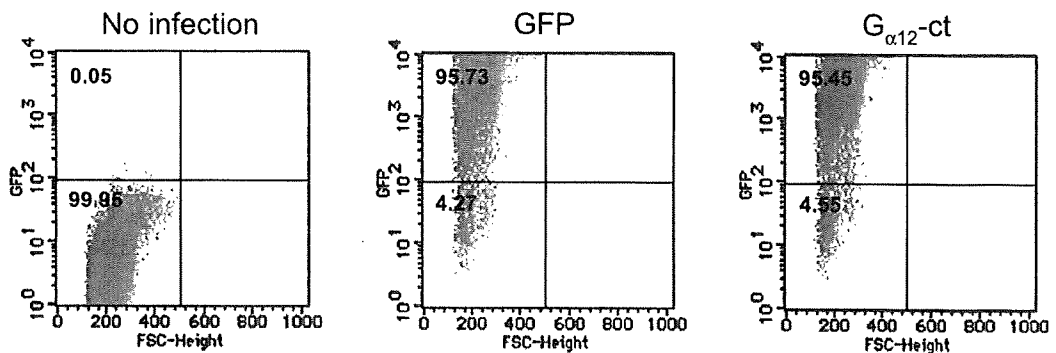
A. ET_AR-high-CHOB. ET_AR-low-CHO

Fig. 7. The efficiency of adenoviral infection at 300 MOI in ET_AR-high-CHO (A) and ET_AR-low-CHO (B) determined by detecting fluorescence of GFP with flow cytometry. Numbers in the left lower and upper quadrants indicate the percentages of GFP-negative and -positive cells, respectively.

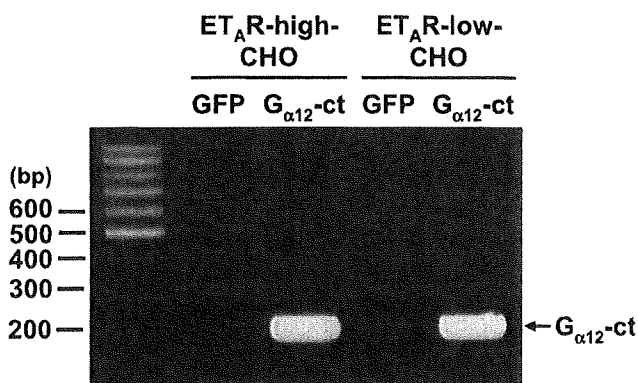


Fig. 8. Detection by RT-PCR of G_{α12}-ct mRNA in ET_AR-high-CHO and ET_AR-low-CHO infected by adenovirus encoding GFP or G_{α12}-ct. RT-PCR experiments were carried out using total RNA prepared from 3 individual cell populations. Left lane shows a 100-bp DNA ladder (Promega, Madison, WI, USA).

influx in cooperation with G_{q/11}-mediated signaling pathways. Furthermore, the ET-1-induced sustained increases in [Ca²⁺]_i in ET_AR-high-CHO was attenuated by inhibitors of either NHE (EIPA) or p38MAPK (SB203580) (Fig. 3). p38MAPK is reported to play a central role in the activation of NHE (9). These data implicate the involvement of the p38MAPK/NHE pathway in the sustained Ca²⁺ response to ET-1 in ET_AR-high-CHO. Therefore, in the present study, we attempted to clarify specific ET_AR signaling cascades, which were activated in an expression-level dependent manner, with special attention to G₁₂ protein, NHE, and p38MAPK.

It has been reported that stimulation of GPCRs coupled to G_q (31), G_s (9), and G₁₂ (32) activate NHE. Notably, ET_AR can be coupled with these types of G proteins. To characterize intracellular mechanisms underlying NHE activation by ET_AR, we employed the CytosensorTM microphysiometer, because the machine is

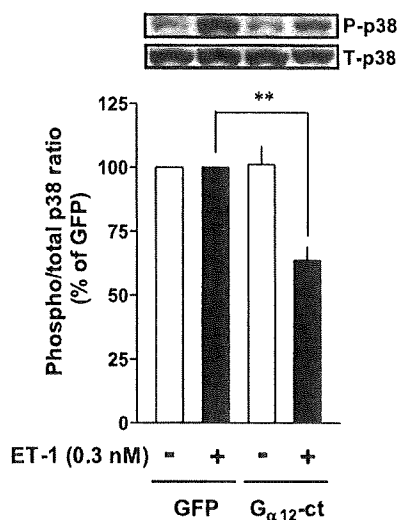
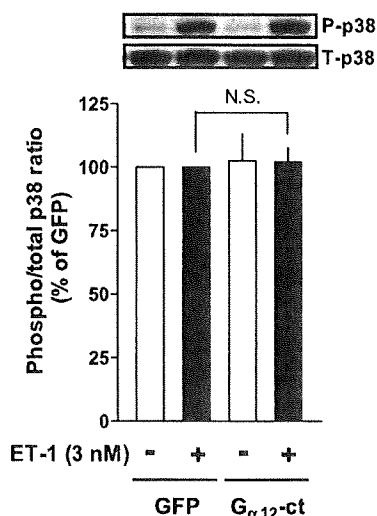
A. ET_AR-high-CHOB. ET_AR-low-CHO

Fig. 9. Effects of introducing GFP and G_{α12}-ct along with GFP by adenovirus infection on p38MAPK phosphorylation in response to 30-min exposure to 0.3 nM ET-1 in ET_AR-high-CHO (A) and 10-min exposure to 3 nM ET-1 in ET_AR-low-CHO (B). Data are presented as means ± S.E.M. of the results obtained from 6 experiments (***P* < 0.01; N.S., not significant between the indicated columns).

reported to be a valuable tool for evaluation of signaling molecules regulating NHE function (23). Functional study with this instrument showed that stimulation of ET_AR induced an increase in ECAR in both ET_AR-high-CHO and ET_AR-low-CHO. The ECAR responses to ET-1 in these cells were considered to be mediated solely by NHE1, since the responses were suppressed by EIPA that shows higher selectivity for NHE1 rather than other members of an NHE family (20).

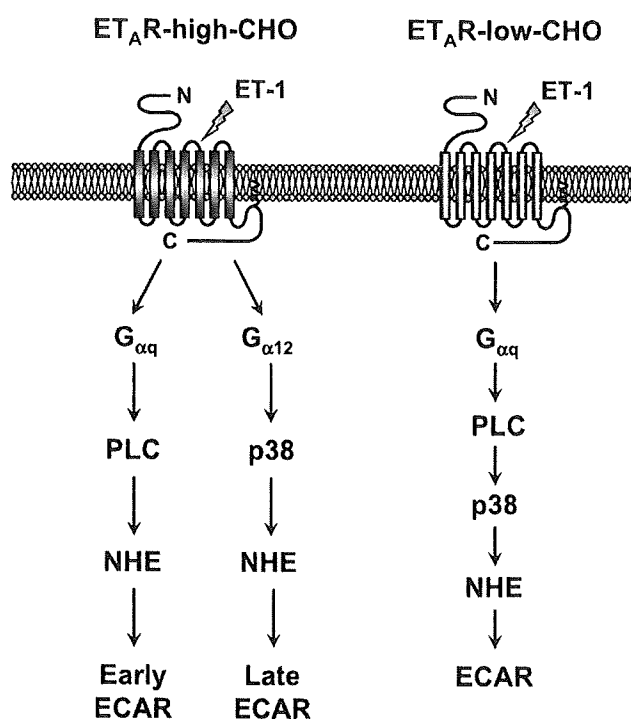


Fig. 10. Multiple signaling pathways utilized by human ET_AR. In ET_AR-high-CHO, ET-1 activates G_{αq/11}/PLC/NHE and G_{α12}/p38MAPK/NHE cascades, leading to an increase in ECAR. The involvement of these pathways in the ECAR response to ET-1 varies with time. In ET_AR-low-CHO, ET-1 increases ECAR predominantly via activation of the G_{αq/11}/PLC/p38MAPK/NHE pathway.

To clarify molecular mechanisms for ET_AR-mediated NHE activation, real-time analyses together with functional studies using inhibitors for G_{q/11}, PLC, and p38MAPK were carried out. This approach has revealed that there is a marked difference in ET_AR signaling involved in NHE activation between ET_AR-high-CHO and ET_AR-low-CHO. In ET_AR-high-CHO, both the G_{q/11}/PLC-dependent, p38MAPK-independent pathway and the G_{q/11}/PLC-independent, p38MAPK-dependent pathway were involved in the ECAR responses to ET-1, but the contribution of these pathways to the ECAR response varied with time. In contrast, in ET_AR-low-CHO, a unique pathway was found to be predominant, which is G_{q/11}/PLC-dependent and p38MAPK-dependent. The possible candidates for the G_q/PLC-independent response observed in ET_AR-high-CHO are G_s- and G₁₂-dependent pathways. However, the involvement of G_s can be ruled out by the result that the ET-1-induced increase in ECAR was unaffected by a G_s inhibitor, NF449 (24), and an AC inhibitor, 2',5'-dideoxyadenosine, which blocks the augmentation of ECAR mediated via the G_s-coupled β₃-AR (9). These results raised the possibility that ET_AR essentially couples to G_{q/11} protein but that excessive receptor expression results in further

coupling of the receptor to other G proteins, possibly G_{12} , in addition to $G_{q/11}$.

The difference in receptor density is also reported to affect the extent of p38MAPK activation by GPCRs. That is, a comparative study with CHO cells stably expressing mouse β_3 -ARs at high and low levels has shown that the maximum phosphorylation of p38MAPK mediated via β_3 -ARs in low-expressor was larger than that in high-expressor (9). In contrast to β_3 -AR, the maximal level of p38MAPK phosphorylation induced by ET-1 in ET_AR-high-CHO was approximately 2-times higher than that in ET_AR-low-CHO. These findings clearly indicate that the correlation between the degree of p38MAPK phosphorylation and receptor expression level is dependent on the types of GPCRs or G proteins specifically coupled to the receptors. Interestingly, Western blot analysis has provided evidence that activation of p38MAPK triggered by ET-1 was mainly mediated via G_{12} in ET_AR-high-CHO but via $G_{q/11}$ /PLC in ET_AR-low-CHO. This implies that G_{12} protein would be responsible for the ET-1-induced, p38MAPK-dependent increase in ECAR in ET_AR-high-CHO, while there is no evidence to suggest the involvement of G_{12} in the ECAR response to ET-1 in ET_AR-low-CHO. Unfortunately, a functional approach with combination of Cytosensor™ and adenovirus to inhibit G_{12} is unavailable for demonstrating G_{12} contribution to the ET-1-induced increase in ECAR estimated on the basis of basal ECAR, since it is very difficult to rule out the possibility that inhibition of G_{12} by the adenovirus affects basal ECAR.

In summary, the present study showed that a difference in expression level of human ET_AR results in a multiplicity of receptor signaling as shown in Fig. 10. The pharmacological evidence in the present study allows the following conclusion to be reached concerning the signaling cascades involved in the ECAR response to ET-1: the response in ET_AR-low-CHO is mediated via the $G_{q/11}$ /PLC/p38MAPK/NHE pathway, while the response in ET_AR-high-CHO is mediated via either the $G_{q/11}$ /PLC/NHE cascade or the G_{12} /p38MAPK/NHE cascade. In addition, both p38MAPK and NHE participate in a sustained increase in $[Ca^{2+}]_i$ triggered by ET-1. These results, taken together with previous reports indicating the involvement of G_{12} in the ET_AR-mediated sustained $[Ca^{2+}]_i$ increase (1, 2), suggest that G_{12} -dependent p38MAPK activation is required for the sustained Ca^{2+} response to ET-1. With regard to the pathophysiological relevance of changes in receptor expression level to diseases, evidence has accumulated to indicate that the up-regulation of ET_AR occurs in various cardiovascular disorders such as ischemic cardiomyopathy (33), hypoxia (34), diabetes mellitus

(35), and renal failure (36). Therefore, alternations in receptor signaling associated with changes in receptor density may be important as a key pathophysiological process responsible for these diseases.

Acknowledgments

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Effects of Nickel on Eosinophil Survival

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Key Words

Eosinophils · Metal · Nickel

Abstract

Background: Accessories, watches, coins and other items containing metal sometimes cause contact dermatitis and metal allergy. Among metals, nickel in alloys is ionized by sweat on the surface of the skin and exhibits particularly marked irritancy and allergenicity. Although eosinophils play important roles in allergy, the effects of nickel on eosinophils have not been elucidated. **Methods:** Eosinophils were prepared from the peritoneal cavity in rats immunized with *Ascaris suum* extract. Purified rat eosinophils were incubated in the presence of various kinds of metals including nickel. The viability of eosinophils was analyzed using a flow cytometer. **Results:** When rat eosinophils were incubated for 3 days in the presence of nickel chloride at 30–1,000 μM , the viability of eosinophils was decreased in a concentration-dependent manner. Nickel chloride at 300 μM significantly increased the percentage of annexin V⁺ PI⁻ eosinophils. The population of annexin V⁺ PI⁻ eosinophils was also increased by nickel sulfate, cobalt chloride and zinc sulfate. The binding of nickel ions to eosinophils was detected by flow cytometer. **Conclusions:** Nickel ions bind to eosinophils and decrease the viability of eosinophils through the induction of apoptosis. Nickel ions may exhibit activity which modifies the function of eosinophils in allergy.

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Introduction

Accessories, watches, coins and other items containing metals sometimes cause contact dermatitis and metal allergy [1, 2]. Among metals, nickel in alloys is ionized by sweat on the surface of the skin and is characterized by irritancy and allergenicity. It has been reported that nickel challenge in the dermis of patients with contact allergy by the skin window technique revealed the infiltration of mononuclear cells, basophils and eosinophils [2, 3].

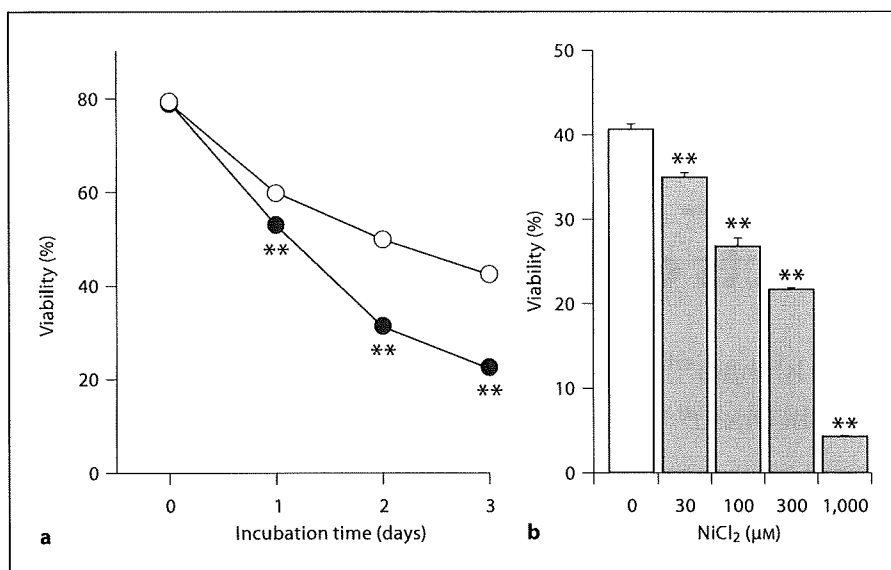
Eosinophils are suggested to play important roles in the pathogenesis of allergic inflammation such as bronchial asthma and atopic dermatitis [4, 5]. In patients suffering from these allergic diseases, the number of eosinophils in the bone marrow, peripheral blood and at inflammatory sites is increased because of their prolonged survival. Recently, it has been reported that asthmatic patients have a higher sensitivity to nickel than normal [6]. However, the effects of nickel on eosinophils have not been elucidated. In this study, therefore, we analyzed the effects of nickel on the viability of eosinophils.

Materials and Methods

Preparation and Culture of Rat Eosinophils

Preparation of rat peritoneal eosinophils was carried out as previously reported [7]. The purity of eosinophils was more than 95%, and the viability was more than 98%. Eosinophils were seed-

Fig. 1. Effects of nickel chloride on the viability of eosinophils. Eosinophils were incubated at 37°C (a) for the periods indicated in medium in the absence (○) or presence of nickel chloride (300 μM; ●) and (b) for 3 days in the presence of the indicated concentrations of nickel chloride. After the incubation, to determine the viability of eosinophils, the cells were stained with 7-AAD and analyzed by flow cytometer. Values are the means from 4 samples with SEM. SEM is within each symbol. Statistical significance: ** p < 0.01 vs. corresponding control.



ed in each well of 24-well plates at a density of 1×10^6 cells in 1 ml of 10% FBS-RPMI-1640 medium containing various kinds of metals. The metals compounds used in this experiment were nickel chloride, cobalt chloride, manganese chloride, nickel sulfate, zinc sulfate, and aluminum sulfate (Wako, Osaka, Japan). These metals were dissolved in water and added to the medium. The concentration of the vehicle in the medium was adjusted to 0.1% (v/v).

Flow Cytometry

After the incubation of eosinophils for various periods in the presence of metal compounds, the cells were stained with 7-aminoactinomycin D (Sigma, St. Louis, Mo., USA) for analysis of viability, or with FITC-labeled annexin V and propidium iodide (PI) (Sigma) for analysis of apoptosis for 5 min at room temperature. To detect binding of nickel ions to eosinophils, the cells were incubated in medium containing nickel chloride for 5 min at room temperature, washed with PBS three times, and further incubated with Newport Green DCF (Invitrogen, Carlsbad, Calif., USA) for 5 min at room temperature. After washing with PBS, the fluorescence of Newport Green binding on nickel ions and eosinophils was analyzed using a flow cytometer (FACScan; Beckton Dickinson, Franklin Lakes, N.J., USA).

Statistical Analysis

The statistical significance of the results was analyzed using Dunnett's test for multiple comparisons.

Results and Discussion

When rat eosinophils were incubated for 1, 2 and 3 days at 37°C, their viability was decreased time-dependently (fig. 1a). Nickel chloride further decreased the via-

bility of cells at each day (fig. 1a). Upon treatment with nickel chloride at 30–1,000 μM, the viability of cells at 3 days was decreased by nickel chloride in a concentration-dependent manner (fig. 1b). To clarify whether the decrease in the viability of eosinophils by nickel chloride was due to the induction of apoptosis, we examined the annexin V⁺ PI⁻ population in eosinophils incubated for 1 day with medium containing 300 μM of nickel chloride. As shown in figure 2, nickel chloride increased the percentage of annexin V⁺ PI⁻ eosinophils. These findings suggest that nickel chloride decreases the viability of eosinophils through the induction of apoptosis. Next, we analyzed whether other metal compounds at 300 μM induce eosinophil apoptosis. Nickel sulfate induced apoptosis at almost the same level as nickel chloride, while that induced by cobalt chloride was moderate and that induced by zinc sulfate was weak compared with induction by nickel chloride and nickel sulfate (fig. 2). These results suggest that nickel ions, cobalt ions and zinc ions induce the apoptosis of eosinophils. It has been reported that nickel, cobalt and zinc are allergenic, and it is well known that nickel and cobalt cause allergy [1]. In contrast, manganese chloride inhibited apoptosis (fig. 2). Manganese is an essential element for all types of tissue, and plays important roles in the free radical defense system as manganese superoxide dismutase (MnSOD), which protects mitochondria from damage by superoxide radicals [8, 9]. Eosinophils have been reported to undergo apoptosis by reactive oxygen species (ROS) [10], and MnSOD is involved in the inhibition of eosinophil apoptosis [11].

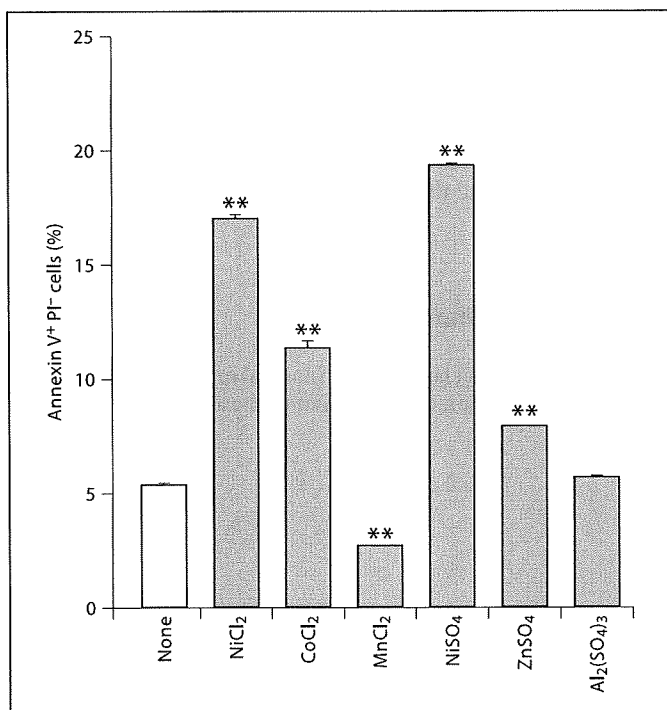


Fig. 2. Effects of various metal compounds on eosinophil apoptosis. Eosinophils were incubated at 37°C for 1 day in medium in the presence of 300 μM of the indicated metal compounds. After the incubation, the cells were stained with FITC-labeled annexin V and PI and analyzed by flow cytometer. Values are the means from 4 samples with SEM. Statistical significance: ** $p < 0.01$ vs. corresponding control.

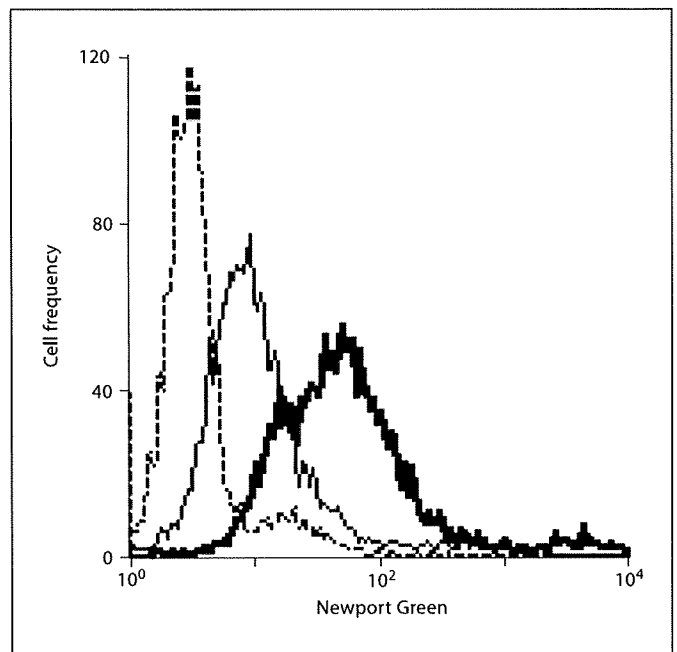


Fig. 3. Binding of nickel ions to eosinophils. Eosinophils were incubated in the presence (bold line) or absence (thin line) of 300 μM of nickel chloride for 5 min and then with 1 μM of Newport Green for 5 min at room temperature. After washing with PBS, the binding of nickel ions to eosinophils was analyzed by flow cytometer. The dashed line represents the fluorescence of cells incubated in the absence of nickel chloride and Newport Green. The experiment shown is representative of 4 independent experiments.

Therefore, it is possible that manganese inhibits eosinophil apoptosis via MnSOD. Taken together, our findings suggest that the metal specificity of the induction of eosinophil apoptosis is correlated with allergenicity.

We analyzed whether nickel ions bind to eosinophils, and detected an increase of fluorescence of Newport Green, which is a cell-impermeable fluoropore for nickel ions, on eosinophils by flow cytometry (fig. 3). Pretreatment of eosinophils with nickel chloride enhanced the fluorescence intensity, suggesting that nickel ions bind to the surface of eosinophils (fig. 3). It has been reported that nickel ions bind to numerous proteins including cell surface molecules, major histocompatibility complex (MHC) class I and MHC class II [12]. Therefore, it is possible that nickel ions bind not only to the molecules on the cell surface as shown in figure 3, but also to intracellular molecules in eosinophils.

It has been reported that nickel ions induce the production of ROS and prostaglandins, expression of Fas ligand, and various cytokines, such as IL-8, TNF- α and

TGF- β , via activation of signal transduction molecules including mitogen-activated protein kinases [13–19]. In these molecules, ROS, Fas ligand and TGF- β are reported to induce apoptosis of eosinophils. Therefore, it is suggested that eosinophils undergo apoptosis by nickel ions through the production of these molecules. In contrast, infiltration of eosinophils toward inflammatory sites is induced by nickel in patients with contact allergy [3] and bronchial asthma [6], and it is thought that eosinophils play important roles in the pathogenesis of allergies. However, this study indicated that eosinophil viability is decreased by nickel ions (fig. 1b). In patients, nickel ionized by sweat on the surface of the skin induces the expression of various cytokines at the inflammatory sites [2, 13–19]. IL-8, which is a chemotactic factor for eosinophils, produced by nickel ions from epithelial cells [16] might be involved in the infiltration of eosinophils into the sites. Eosinophils might also produce inflammatory mediators such as cytokines and lipids involved in the pathogenesis of allergies on exposure to nickel ions. Thus,

it is possible that simultaneous signaling for the expression of various inflammatory mediators from eosinophils and that for the induction of apoptosis in eosinophils might be involved in the inflammation and allergy induced by nickel *in vivo*. Therefore, eosinophils might play important roles for the nickel-induced allergy as effector cells.

In the study of metal allergy, T cells have been focused on because metal allergy is recognized as being a type IV allergy. However, nickel ions were found to act on eosinophils in the present study. Because it has been unknown whether nickel ions induce the expression of mediators from eosinophils, further investigation is necessary for the implication of eosinophils in metal allergy.

In conclusion, we suggest that nickel may exhibit a modifying activity on the function of eosinophils in allergy.

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Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of the article.

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Cutting Edge: Histamine Receptor H₄ Activation Positively Regulates In Vivo IL-4 and IFN- γ Production by Invariant NKT Cells¹

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Histamine (HA) is a biogenic amine with multiple activities in the immune system. In this study we demonstrate that histamine-free histidine decarboxylase-deficient (HDC^{-/-}) mice present a numerical and functional deficit in invariant NK T (iNKT) cells as evidenced by a drastic decrease of IL-4 and IFN- γ production. This deficiency was established both by measuring cytokine levels in the serum and intracellularly among gated iNKT cells. It resulted from the lack of HA, because a single injection of this amine into HDC^{-/-} mice sufficed to restore normal IL-4 and IFN- γ production. HA-induced functional recovery was mediated mainly through the H₄ histamine receptor (H₄R), as assessed by its abrogation after a single injection of a selective H₄R antagonist and the demonstration of a similar iNKT cell deficit in H₄R^{-/-} mice. Our findings identify a novel function of HA through its H₄R and suggest that it might become instrumental in modulating iNKT cell functions. *The Journal of Immunology*, 2009, 182: 1233–1236.

Histamine (HA)³ is one of the most versatile biogenic amines with multiple physiological functions in the CNS, the intestinal tract, and inflammatory reactions. More recently, a number of studies have established that besides its most obvious contribution to allergic reactions, HA also exerts more subtle regulatory functions influencing the orientation of the immune response, thus rekindling interest in this field of investigation (1–4). It has been assumed until lately that these immunomodulatory effects were mediated mainly through classical HA receptors of the H₁ and H₂ subtypes (5, 6). However, this explanation has since been complicated by

the identification of organic cation transporter 3, OCT3, as a means through which HA can be taken up by murine basophils and exert a negative feedback on their HA, IL-4, IL-6, and IL-13 production (7), as well as by the identification of an additional HA receptor, HA receptor subtype 4 (H₄R) expressed mainly in hematopoietic and immunocompetent cells (8, 9). The most clearly established activities of H₄R consist in the recruitment and activation of cells involved in inflammatory responses such as eosinophils, mast cells, neutrophils, conventional T lymphocytes, and dendritic cells (10–13). However, its functional expression in the immunoregulatory invariant NK T (iNKT) cells has not been investigated so far.

iNKT cells constitute a distinctive population of mature T lymphocytes positively selected by the nonpolymorphic MHC class-I-like molecule CD1d. They coexpress a highly restricted TCR repertoire composed of a single invariant V α 14J α 18 chain in mice and a V α 24J α 18 chain in humans, preferentially paired with a limited TCR V β -chain repertoire that specifically recognizes glycolipids (14–16). iNKT cells are implicated in the control of several immune responses, most likely because of their capacity to promptly produce several cytokines (14–20) such as IL-4 and IFN- γ . In the present study we demonstrate that HA participates in this functional tuning to ensure optimal IL-4- and IFN- γ production by iNKT cells.

Materials and Methods

Animals

Male C57BL/6J mice (7–9 wk old) were purchased from Janvier. Histidine decarboxylase (HDC)-deficient (HDC^{-/-}) and H₄R^{-/-} mice, backcrossed 12 and 10 times to C57BL/6J mice, respectively (21, 22), were bred in our own facilities. HDC^{-/-} mice received a histamine-low diet (SAFE Scientific Animal Food and Engineering) to avoid exogenous uptake. Animal experiments were performed according to the French institutional committee.

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³ Abbreviations used in this paper: HA, histamine; α -GalCer, α -galactosylceramide; H₄R, HA receptor subtype 4; HDC, histidine decarboxylase; iNKT, invariant NK T (cell); MNC, mononuclear cell.

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In vivo treatment

Mice received a single i.p. administration of 2 μ g of α -galactosylceramide (α -GalCer; Alexis) 90 min before sacrifice. In some experiments, mice were injected i.p. 1 h before α -GalCer administration with a single dose of HA (Sigma-Aldrich) (20 mg/kg) with or without the H4R antagonist [JN] 777120 (23) (20 mg/kg) administered i.p. 1 h before HA injection.

Cell preparation

Lymphocytes were isolated from the spleen using a homogenizer. Mononuclear cells (MNC) were separated from hepatocytes and cellular debris by way of a 35% isotonic Percoll density gradient (Amersham Biosciences). Liver and spleen MNC were depleted of RBC using red cell lysis buffer (8.3 mg/ml NH_4Cl , 1 mg/ml KHCO_3 , and 3.72 μ g/ml EDTA).

Flow cytometry

Splenocytes and liver MNC were preincubated with mAbs against Fc γ R (clone 2.4G2 culture supernatant), washed, and incubated with CD1d/ α -GalCer tetramer-allophycocyanin or control tetramers, anti-CD4 PerCP-Cy-5.5, anti-TCR β -FITC, anti-IL-4-PE, anti-IFN- γ -PE, or isotype control (BD Pharmingen) as described (20). In some experiments an anti-H4R (clone Y-19; Santa Cruz Biotechnology) was used according to the manufacturer's instructions. Cells were analyzed on a FACSCanto II (BD Biosciences) flow cytometer using FACSDiva software.

Determination of cytokines

IL-4 and IFN- γ were measured by ELISA as described (20).

Statistical analysis

The nonparametric test *t* was used to calculate significance levels for all measurements. Values of *p* < 0.05 were considered statistically significant.

Results and Discussion

IL-4 and IFN- γ production by iNKT cells is decreased in HA-free HDC $^{-/-}$ mice

Prompt production of IL-4 and IFN- γ in response to TCR cross-linking constitutes a typical feature of iNKT cells. We measured these cytokines to establish whether exogenous HA participated in their modulation. To this end, we injected wild-type and histamine-free HDC $^{-/-}$ mice (deficient for the HA-forming enzyme HDC) with α -GalCer, a glycolipid widely used as a specific activator of iNKT cells, to determine its capacity to specifically activate and promptly induce these cytokines. We found that both IL-4 and IFN- γ levels generated after a single injection were significantly lower in the serum of HDC $^{-/-}$ mice than in wild-type controls (Fig. 1, *A* and *B*). This decrease could result either from a lower incidence or a functional defect of iNKT cells. Indeed, we found that CD1d/ α -GalCer tetramer $^+$ cells were effectively reduced in spleen and liver of HDC $^{-/-}$ mice, both in terms of cell counts and percentage (Fig. 1, *C-E*).

The lower iNKT cell counts in histamine-free mice do not exclude the presence of functional deficiencies in the remaining cells, promoting us to analyze cytokine production in single cells by intracellular staining. It turned out that among gated iNKT cells the percentage that was actually positive for IL-4 and IFN- γ cells after injection of α -GalCer was strikingly reduced in HDC $^{-/-}$ mice compared with controls (Fig. 2, *A* and *B*). These data clearly show that iNKT cells are both numerically and functionally impaired in histamine-deficient mice.

HA injection restores the IL-4- and IFN- γ -producing capacity of iNKT cells

To confirm the implication of HA in the cytokine-producing capacity of iNKT cells, HDC $^{-/-}$ mice were treated with HA 1 h before α -GalCer stimulation. Remarkably, a single injection of HA was sufficient to restore the seric levels of IL-4 and

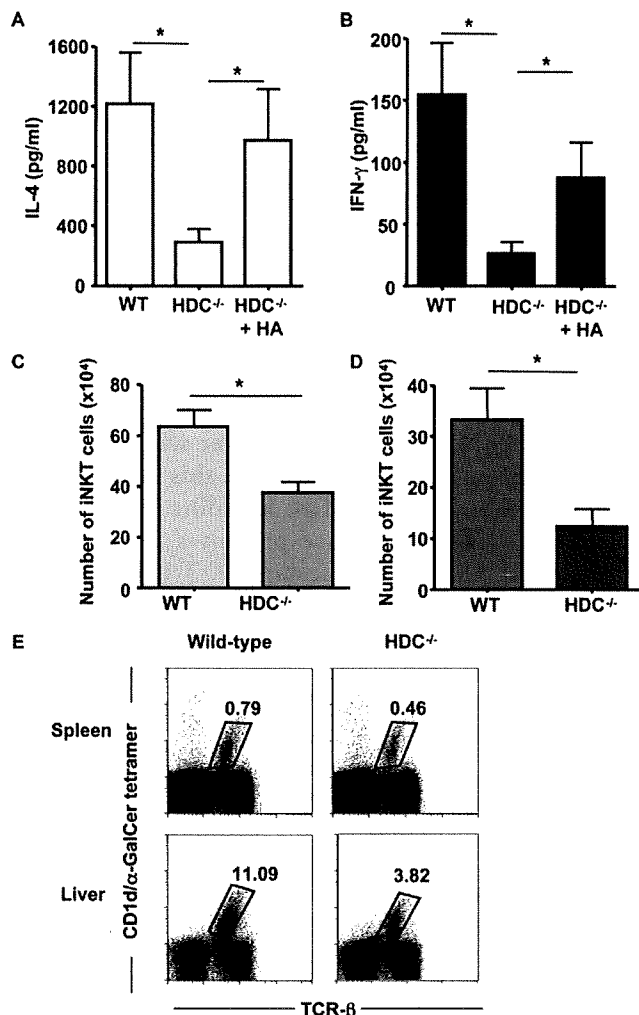


FIGURE 1. Marked decrease in cytokine production by iNKT cells in HDC $^{-/-}$ mice. Wild-type (WT) and HDC $^{-/-}$ mice were injected with α -GalCer. A group of HDC $^{-/-}$ mice were treated with HA 1 h before α -GalCer injection (HDC $^{-/-}$ + HA). Ninety minutes later, IL-4 (*A*) and IFN- γ (*B*) were measured in the serum. No cytokine was detected without α -GalCer stimulation (data not shown). The total number (*C* and *D*) and the percentage (*E*) of iNKT cells were analyzed in both spleen (*C* and *E*) and liver (*D* and *E*) from untreated wild-type or HDC $^{-/-}$ mice. Data represent the mean \pm SEM from 8–20 mice (*A* to *D*). *, *p* < 0.05. Representative FACS profiles showing the percentage of CD1d/ α -GalCer $^+$ TCR β^+ cells (*E*) in wild-type vs HDC $^{-/-}$ mice.

IFN- γ in HDC $^{-/-}$ mice (Fig. 1, *A* and *B*). Even though this treatment did not enhance the percentage or the absolute number of iNKT cells significantly (data not shown), it did increase the proportion of IL-4 $^+$ and IFN- γ $^+$ cells among gated iNKT lymphocytes (Fig. 2), consistent with the restored seric cytokine levels. It can therefore be concluded that HA is capable of up-regulating both IL-4 and IFN- γ production by iNKT cells activated in vivo.

Cytokine production by iNKT cells is impaired in H4 receptor-deficient mice

Knowing that histamine exerts its biological effect through four specific receptors and that the most recently discovered H4 subtype is preferentially expressed in hematopoietic cells, we used mice in which the corresponding gene had been disrupted (H4R $^{-/-}$) to assess their IL-4 and IFN- γ production following

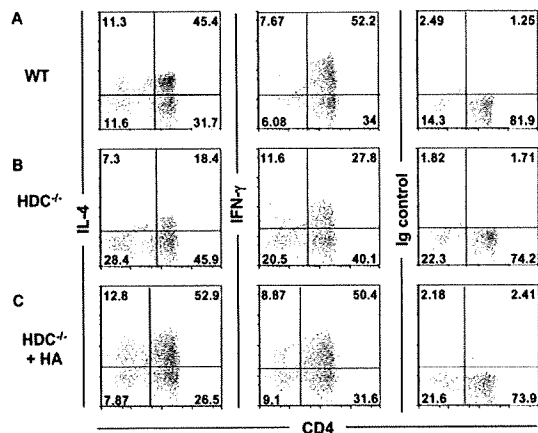


FIGURE 2. Impaired IL-4 and IFN- γ production by in vivo α -GalCer-stimulated iNKT cells from HDC^{-/-} mice. Representative FACS profiles showing the percentage of IL-4, IFN- γ , and isotype control (Ig control) positive cells among gated CD1d/ α -GalCer tetramer⁺TCR β ⁺ iNKT splenocytes from α -GalCer treated wild-type (WT) (A) or HDC^{-/-} mice (B). In the later group, HDC^{-/-} mice were treated with HA (HDC^{-/-} + HA) before α -GalCer injection (C).

α -GalCer injection. We found that H4R^{-/-} mice, which presented no significant modification in the absolute number of iNKT cells ($5.2 \times 10^5 \pm 0.9 \times 10^5$ vs $6.7 \times 10^5 \pm 1.2 \times 10^5$ splenic iNKT cells from wild-type and H4R^{-/-} mice, respectively), generated significantly fewer circulating cytokines than wild-type controls (Fig. 3, A and B), suggesting that the positive effect of HA on these biological activities was mediated through H4R activation.

In vivo treatment of HDC^{-/-} mice with a H4R antagonist abrogates the restoration of iNKT cell functions in response to HA

To prove that H4Rs were required for the up-regulation of IL-4 and IFN- γ production by iNKT cells, we blocked their binding sites with the highly selective H4 antagonist JNJ7777120 injected 1 h before HA into HDC^{-/-} mice. In this case HA failed to restore a normal IL-4 and IFN- γ production, as shown in Fig. 3, C and D. In further support of this result, we show that iNKT cells express H4R (Fig. 3E), leading us to conclude that HA modulates iNKT cell functions through this receptor subtype.

The contribution of iNKT cells to immune responses is complex because of their capacity to produce both IFN- γ and IL-4, thereby supporting Th1 or Th2 responses, respectively. We and others have reported that iNKT cells can aggravate asthma through their Th2 cytokine profile (18, 24). Similarly, HA plays a major role in atopic diseases, namely in allergic asthma, because its release in the airways is one of the typical features of this pathology that triggers a cascade of events, including airway constriction, mucus secretion, vascular leak, and recruitment of immune cells. Our present data suggest an additional means for HA to enhance the severity of asthma by promoting optimal IL-4 production by iNKT cells. Consistent with this assumption, it has recently been reported that asthmatic mice treated with the JNJ7777120 antagonist used herein develop less airway inflammation than untreated controls (11).

Taken together, our data reveal a new role of histamine through H4R activation in the functional modulation of the immunoregulatory iNKT cell population and provide addi-

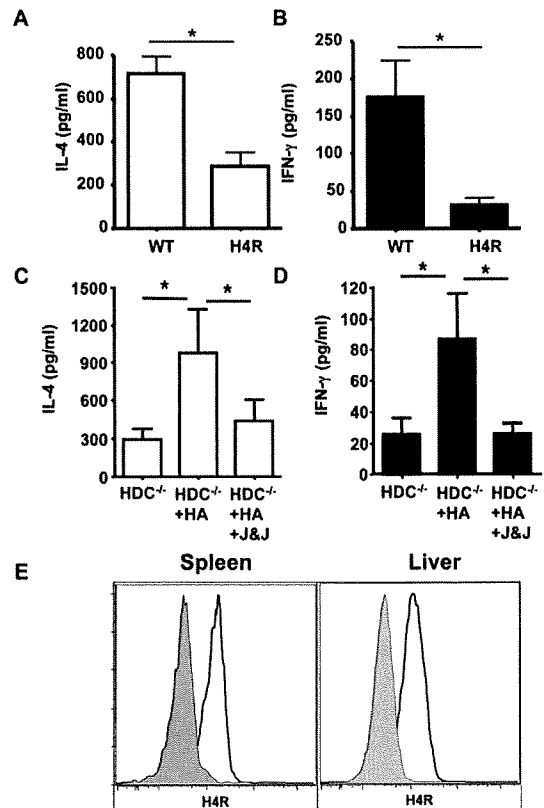


FIGURE 3. HA can modulate iNKT cell functions through H4R. A and B, Wild-type (WT) and H4R^{-/-} mice were injected with α -GalCer. Ninety minutes later, IL-4 (A) and IFN- γ (B) were measured in the serum. C and D, HDC^{-/-} mice were treated or not with the selective H4R antagonist JNJ7777120 (J&J), followed by HA and then α -GalCer injection as described in *Materials and Methods*. Ninety minutes after the last injection, IL-4 (C) and IFN- γ (D) were measured in the serum. Data represent the mean \pm SEM from 6–10 mice; *, $p < 0.05$. E, Representative FACS profiles showing the expression of H4R (empty histogram) compared with control (filled histogram) among gated CD1d/ α -GalCer⁺TCR β ⁺ splenocytes (left panel) or liver MNC (right panel) recovered from wild-type mice.

tional evidence for the complex influence of the microenvironment on iNKT cell functions.

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Disclosures

The authors have no financial conflict of interest.

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Enhanced Goblet Cell Hyperplasia in HDC Knockout Mice with Allergic Airway Inflammation

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ABSTRACT

Background: Histamine is known to have immunoregulatory roles in allergic reactions through histamine receptor 1 (H1R), H2R, H3R and H4R. However, its role in goblet cell hyperplasia in the airways of asthma patients is yet to be clarified.

Objective: This study was designed to examine the role of histamine in goblet cell hyperplasia using histamine-deficient mice (*Hdc*^{-/-} mice) with allergic airway inflammation.

Methods: Wild-type and *Hdc*^{-/-} C57BL/6 mice were sensitized with ovalbumin (OVA). After a 2-week exposure to OVA, goblet cell hyperplasia was evaluated. Cell differentials and cytokines in BALF were analyzed. The mRNA levels of MUC5AC and Gob-5 gene were determined quantitatively.

Results: The number of eosinophils in BALF increased in both the sensitized wild-type mice and *Hdc*^{-/-} mice with OVA inhalation. In addition, the numbers of alveolar macrophages and lymphocytes in BALF increased significantly in the sensitized *Hdc*^{-/-} mice with OVA inhalation compared to the wild-type mice under the same conditions. The concentrations of Interleukin-4 (IL-4), IL-5, IL-13, Interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α) and IL-2 in the BALF all increased significantly in both groups compared to those exposed to saline. In particular, the concentration of TNF- α in the *Hdc*^{-/-} mice exposed to OVA was significantly higher than that in the wild-type mice under the same conditions. The mRNA levels of Gob-5 and MUC5AC, and the ratio of the goblet cells in the airway epithelium significantly increased in *Hdc*^{-/-} mice exposed to OVA compared to wild-type mice.

Conclusions: These results suggested that histamine may play a regulatory role in goblet cell hyperplasia in allergic airway inflammation.

KEY WORDS

airway remodeling, asthma, Gob-5, MUC5AC, TNF- α

INTRODUCTION

Goblet cell hyperplasia and mucus overproduction are important features of bronchial asthma.^{1,2} Although goblet cell hyperplasia with mucus hypersecretion has been reported to be associated with the development of airway hyperresponsiveness and the increase of severity and mortality in bronchial asthma¹⁻⁴, the mechanisms responsible for goblet cell

hyperplasia are not completely understood.

Histamine is known to be a strong chemical mediator that stimulates secretion from goblet cells. Bryce *et al.* recently reported that the airway inflammatory response and goblet cell hyperplasia to allergic stimuli diminished in H1 receptor (H1R) knock-out mice, suggesting that histamine may be involved in the immunomodulation in airway allergic reaction including Th1 and Th2 cytokine production and goblet cell hy-

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perplasia.⁵

It has been reported that histamine can modulate T-cell-mediated immune responses.⁶ Mast cells and basophils, which have been considered mainly to be effector cells for IgE-mediated reactions, can modulate the immune response through secreted histamine. Jutel *et al.* demonstrated, using H1R and histamine H2 receptor (H2R) knock-out mice, that histamine augmented TH1 response T lymphocytes through H1R and suppressed both TH1 and TH2 responses through H2R.⁷ In addition, histamine H4 receptor (H4R) has recently been identified and an H4R inhibitor has been demonstrated to have a suppressive effect on allergic airway inflammation, including eosinophil and mast cell infiltration in murine asthma models.⁸⁻¹¹ In this context, histamine may have various roles in allergic airway inflammation through H1R, H2R and H4R in immune cells including T lymphocytes and dendritic cells.

To date, goblet cell hyperplasia has been thought to be induced after epithelial damage by numerous stimuli, such as endogenous oxidants induced by neutrophil elastase,¹² or exogenous oxidants produced by cigarette smoke¹³ or diesel engine emissions.¹⁴ On the other hand, it has been demonstrated that the TH2 lymphocyte-derived cytokines interleukin-4 (IL-4), IL-5, IL-9, and IL-13 induce goblet cell hyperplasia in animal models and *in vitro* studies.¹⁵⁻¹⁹

According to previous reports, several genes are up-regulated in association with goblet cell hyperplasia. Among them, MUC genes encode various mucin glycoproteins which are primary secretory proteins consisting of a family of genes (ie, MUC-1, MUC-2 and MUC4).^{20,21} MUC5AC is the predominant MUC gene expressed in the airway.²¹ It has been demonstrated that the level of MUC5AC gene expression increases in both animal models of asthma and tissue samples from patients with asthma.²²⁻²⁴

In addition, the overexpression of the Gob-5 gene has been reported in airway epithelia with exacerbated AHR, goblet cell hyperplasia, mucus overproduction, and eosinophil infiltration in a murine asthma model.²⁵ Gob-5 gene in the mouse corresponds to CLCA1 gene in humans, which regulates calcium activated chloride conductance. It has been shown to induce mucin gene expression (MUC5AC) in the human mucoepidermoid cell line NCI-H292 and its expression was demonstrated to increase in patients with asthma.²⁵⁻²⁷

Histamine-deficient mice are now available by disrupting *L*-histidine decarboxylase gene (HDC knock-out mice: *Hdc*^{-/-}).²⁸ Histamine exerts its various biological actions via H1, H2, H3 and H4. Due to the overlapping, and sometimes antagonistic function of the receptors in the presence of endogenous histamine, receptor blocking alone cannot achieve complete elimination of the histamine system. It is, there-

fore, reasonable to evaluate the total role of histamine with *Hdc*^{-/-} mice.

An analysis of cytokine production and cell differentials in BALF in sensitized *Hdc*^{-/-} mice with allergic airway inflammation has been previously reported, however, these investigated only the acute airway responses in murine asthma models with a short exposure to allergens.^{29,30} There have been few data about airway remodeling that included goblet cell hyperplasia in histamine-deficient mice.

In this study, we designed an experimental asthma model with a relatively long duration of allergen exposure to *Hdc*^{-/-} mice to elucidate the roles of histamine in airway remodeling, focusing especially on goblet cell hyperplasia.

METHODS

ANIMALS

HDC knockout (*Hdc*^{-/-}) mice were generated by Ohtsu *et al.*²⁸ In the present study, we used 6 to 8-week-old female *Hdc*^{-/-} mice backcrossed for six generations with C57BL/6 mice, and used age/gender-matched C57BL/6N mice (Japan SLC, Shizuoka, Japan) as controls. The mice used for this study were generated by breeding homozygous *Hdc*^{-/-} mice in the facilities of Tohoku University (Sendai, Japan). Both *HDC*^{-/-} and wild-type mice were kept on a normal diet containing <0.3 mg of histamine/g of food. All experiments described in this study were performed according to the guidelines for the care and use of experimental animals as determined by the Japanese Association for Laboratory Animals Science in 1987.

IMMUNIZATION AND AEROSOLIZATION PROTOCOL

The mice were sensitized according to the methods described in a previous study.³¹ In brief, mice were sensitized on days 0 and 5 of the protocol by an intraperitoneal injection of 0.5 ml aluminum hydroxide-precipitated antigen containing 8 µg OVA (Sigma Chemical Co., St. Louis, MO) adsorbed overnight at 4°C to 4 mg of aluminium hydroxide (Wako Chemical Co., Tokyo, Japan) in phosphate-buffered saline (PBS). Twelve days after the second immunization, the wild-type mice and *Hdc*^{-/-} mice were divided into 3 groups respectively, each consisting of 6 animals. Each of the wild-type mice groups and one of the *Hdc*^{-/-} mice groups were killed for analysis as controls without inhalation. To perform chronic exposure of OVA, 2 groups of each of the wild mice and *Hdc*^{-/-} mice were placed in a plastic chamber (10 cm × 15 cm × 25 cm) and exposed to aerosolized OVA (5 mg/ml in 0.9% saline) for 1 hour every other day for 14 days. The other 2 groups of the wild-type mice and *Hdc*^{-/-} mice were exposed only to 0.9% saline every other day for 14 days. The aerosolized OVA was produced by a Pulmo-Aide Compressor/Nebulizer

(Devilbiss) (Sunrise Medical HHG, Inc., Somerset, PA, USA) at a flow rate of 5–7 liter/minute.

COLLECTION AND MEASUREMENT OF SPECIMENS

After being exposed to aerosolized saline or OVA every other day for 2 weeks, each group of mice was killed on the 14th day, 24 hours after final inhalation, and bronchoalveolar lavage fluid (BALF) and lung tissues were collected. To collect BALF, the lungs were dissected and the trachea was cannulated with a polyethylene tube (Becton Dickinson, Sparks, MD, USA). The lungs were lavaged twice with 0.5 ml PBS, and up to 0.8 ml of the instilled fluid was consistently recovered. The recovered fluid was centrifuged (300 × *g* for 6 minutes) and the cells were resuspended in 0.5 ml PBS. The total number of cells was counted using an improved Neubauer hemocytometer chamber. An air-dried slide preparation was made from each sample containing 10,000 cells by cyto-spin (Cytocentrifuge, Sakura Seiki, Tokyo, Japan) and stained with May-Grunwald-Giemsa stain. Differential counts of at least 500 cells were made according to standard morphologic criteria. The numbers of cells recovered per mouse were then expressed as the mean and standard error of the mean (SEM) for each treated group.

After centrifugation, supernatants were stored at –80°C for the measurement of cytokines. After harvesting BALF, lungs were fixed with formaldehyde and were embedded in paraffin. These 3- μ m-thick sections were stained with hematoxylin eosin (HE) and periodic acid-Schiff (PAS). In addition, other parts of the left lungs were stored at –80°C for RNA extraction.

MEASUREMENT OF SERUM CONCENTRATION OF IgE

Serum IgE levels were determined using the commercially available ELISA kit (Yamasa, Chiba, Japan).

HISTOLOGICAL MEASUREMENT OF GOBLET CELL NUMBER

The 3 mm paraffin sections of the lungs described above were stained with PAS for evaluation of goblet cells. Goblet cell hyperplasia was determined by counting the number of PAS positive cells in more than 5 large (diameter >200 μ m) and more than 10 small bronchi (diameter <150 μ m) per lung under microscopy. The results were expressed as percentages of PAS positive cells per total epithelial cells. In addition, lung sections were stained with HE.

ISOLATION OF TOTAL RNA AND REAL-TIME QUANTITATIVE PCR

Total RNA from the whole lobes of the left lungs was obtained using ISOGEN (Wako Pure Chemicals, Osaka, Japan), quantified by spectrophotometry. The quality of the obtained RNA was confirmed by aga-

rose gel electrophoresis.

To quantify the mRNA of Gob-5, MUC5AC, and glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) expression in the murine lungs, quantitative PCR was carried out using an ABI Prism 7700 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) as previously described.³²

Oligonucleotide PCR primer pairs and fluorogenic probes for murine MUC5AC and Gob-5 were designed from the published sequences using Primer Express software (Perkin-Elmer) (sense primer: 5'-ACTGTTACTATGCGATGTGTAGCCA-3', antisense primer; 5'-GAGGAAACACATTGCACCGA-3'; Taqman probe: 5'-[FAM] ACTGCCACCTGTCACTGGGCGG [TAMRA]-3') for MUC5AC and (sense primer: 5'-AGGGCATCGTCATCGCC-3'; antisense primer: 5'-TCCTTTATGTGTTGAATGAGGGC-3'; Taqman probe: 5'-[FAM] AGACCACGACGTGCCGGAAGATG [TAMRA]-3') for Gob-5.³³ Primers and the labeled probe (VIC) for rodent GAPDH were purchased from Perkin-Elmer Applied Biosystems. 100 ng of RNA dissolved in 10 μ l of water from each aliquot of murine lung tissue was denatured at 90°C for 90 seconds. Each RNA sample (100 ng/10 μ l of water) was mixed in 40 μ l of buffer containing the following reagents for the one-step RT-PCR reaction: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.01 mM EDTA, 60 nM Passive Reference 1 (Applied Biosystems), 5 mM MgCl₂, 100 nM sense primer, 100 nM antisense primer, 0.3 mM deoxynucleoside triphosphate (Boehringer), 0.4 U/ μ l RNase inhibitor (Promega), 0.4 U/ μ l Moloney murine leukemia virus RT (Perkin Elmer), 0.0025 U/ μ l Taq Gold Polymerase (Perkin Elmer), and 100 nM Taqman probe, as described above. The fragment of mRNA for Gob-5, MUC5AC and GAPDH was reversely transcribed into cDNA (30 minutes at 48°C) and amplified by PCR for 40 cycles (15 seconds at 95°C and 1 minute at 60°C). Whole reactions of the RT-PCR and detection of the fluorescence emission signal for every PCR cycle were performed at the same time in a single tube in a sequence detector (ABI 7700). The minimum PCR cycle to detect the fluorescent signal was defined as the cycle threshold (C), which is predictive of the quantity of an input target fragment.³⁴ The standard curve was obtained between the fluorescence emission signals and C by means of duplicated serial dilutions of each quantified cDNA fragment which contained the targeted sequences. The expression of MUC5AC and Gob-5 mRNA was normalized to the constitutive expression of GAPDH mRNA.

CYTOKINE ASSAY

Concentrations of IL-4, IL-5, Interferon-g (IFN-g), tumor necrosis factor- α (TNF- α) and IL-2 in BALF were determined with cytokine Bead Array inflammatory kits using flow cytometry according to the manufacturer's instructions (BD PharMingen, San Diego, CA,

Table 1 Total cell number and cell differential in BALF of the wild-type and *Hdc*^{-/-} mice after saline and OVA inhalation

	C57BL/6: wild saline, 14 th day	C57BL/6: wild OVA, 14 th day	C57BL/6: <i>Hdc</i> ^{-/-} saline, 14 th day	C57BL/6: <i>Hdc</i> ^{-/-} OVA, 14 th day
Total cells ($\times 10^4$ /ml)	2.29 \pm 0.64 (n = 6)	28.2 \pm 5.1 (n = 6)	3.60 \pm 0.21 (n = 6)	62.1 \pm 7.0* (n = 6)
Alveolar macrophages	2.16 \pm 0.06 (94.2 \pm 2.5%)	2.09 \pm 0.56 (7.40 \pm 2.0%)	3.43 \pm 0.11 (95.2 \pm 3.0%)	23.5 \pm 3.73* (37.9 \pm 6.0%)
Lymphocytes	0.13 \pm 0.06 (5.75 \pm 2.5%)	0.45 \pm 0.25 (1.6 \pm 0.9%)	0.15 \pm 0.09 (4.20 \pm 2.6%)	10.8 \pm 4.04* (17.4 \pm 6.5%)
Eosinophils	< 0.001 (< 0.1%)	25.7 \pm 0.50 (91.0 \pm 1.8%)	0.22 \pm 0.01 (0.6 \pm 0.4%)	27.8 \pm 6.40 (44.8 \pm 10.3%)
Neutrophils	< 0.01 (< 0.1%)	< 0.01 (< 0.1%)	< 0.01 (< 0.1%)	< 0.01 (< 0.1%)
Epithelial cells	< 0.01 (< 0.1%)	< 0.01 (< 0.1%)	< 0.01 (< 0.1%)	< 0.01 (< 0.1%)

The absolute number of cells in BALF on 14th day after saline and OVA inhalation in mice sensitized with OVA was calculated. The numbers in parentheses indicated % in the total cells in BALF. BALF was collected as described in "Methods". Values were expressed as the means \pm SEM of 6 mice. * $p < 0.01$ relative to the respective value of wild-type mice with OVA inhalation.

USA). The concentration of IL-13 in BALF was measured using ELISA (RayBio, Norcross, GA, USA).

STATISTICAL ANALYSIS

Data were expressed as mean \pm SEM. Multiple comparisons of mean data among the groups were analyzed by the Mann-Whitney U test. Probability values of less than 0.05 were considered to indicate a statistically significant difference.

RESULTS

CELL DIFFERENTIALS IN BALF OF *Hdc*^{-/-} MICE

Repetitive exposure to OVA for 2 weeks induced a marked increase of the total cell numbers in BALF in both wild-type and *Hdc*^{-/-} mice sensitized with OVA, compared with those from the wild-type mice group and mice sensitized with OVA and exposed to saline (Table 1). The total cell numbers in BALF from the *Hdc*^{-/-} mice sensitized with OVA and then exposed to OVA increased significantly more than in wild-type mice under the same conditions. The absolute numbers of alveolar macrophages, lymphocytes and eosinophils in *Hdc*^{-/-} mice sensitized with and exposed to OVA increased significantly compared to mice sensitized with OVA and exposed to saline. In particular, the absolute numbers of alveolar macrophages and lymphocytes in *Hdc*^{-/-} mice sensitized with and exposed to OVA were significantly higher than those of wild-type mice under the same condition. Eosinophils were the predominant cells in BALF from the wild-type mice exposed to OVA and the ratio of eosinophils in BALF was higher compared with that of *Hdc*^{-/-} mice. However, there was no significant difference in the absolute cell numbers of eosinophils in these 2 groups.

CYTOKINES IN BALF OF *Hdc*^{-/-} MICE

TNF- α , IL-4, IL-5, IL-13, INF- γ and IL-2 in BALF were

measured. The concentrations of TNF- α in BALF of both wild (15.08 \pm 0.58 pg/ml; mean \pm SEM) and *Hdc*^{-/-} mice (25.30 \pm 4.23 pg/ml) exposed to OVA increased markedly compared to those of wild-type (2.28 \pm 0.40 pg/ml) and *Hdc*^{-/-} mice (2.27 \pm 0.40 pg/ml) exposed to saline (Fig. 1A). The concentration of TNF- α in the *Hdc*^{-/-} mice exposed to OVA was significantly higher than that of the wild-type mice under the same conditions.

The concentrations of IL-4 in BALF from both wild-type and *Hdc*^{-/-} mice sensitized with and exposed to OVA increased significantly compared to wild and *Hdc*^{-/-} mice sensitized with OVA and exposed to saline. The concentration of IL-4 in the *Hdc*^{-/-} mice exposed to OVA was significantly lower than that of wild mice under the same conditions (Fig. 1B).

The concentrations of IL-2, IL-5, IL-13 and INF- γ in BALF from both wild-type and *Hdc*^{-/-} mice sensitized with and exposed to OVA increased significantly compared with that of wild-type and *Hdc*^{-/-} mice sensitized with OVA and exposed to saline. No statistically significant difference in these cytokines between the wild-type and *Hdc*^{-/-} mice exposed to OVA (Fig. 1C, D, E, F).

SERUM IgE OF *Hdc*^{-/-} MICE

The concentrations of serum IgE in both wild-type (2133.6 \pm 370.3 ng/ml) and *Hdc*^{-/-} mice (998.9 \pm 254.0 ng/ml) exposed to OVA were markedly higher than those exposed to saline (wild-type exposed to saline: 416.1 \pm 25.1 ng/ml, *Hdc*^{-/-} mice exposed to saline: 371.7 \pm 17.2 ng/ml) and without exposure (wild-type: 390.1 \pm 32.5; *Hdc*^{-/-}: 328.1 \pm 14.8) (Fig. 2). The concentration of serum IgE in the *Hdc*^{-/-} mice exposed to OVA was significantly lower than that of the wild-type mice under the same conditions.

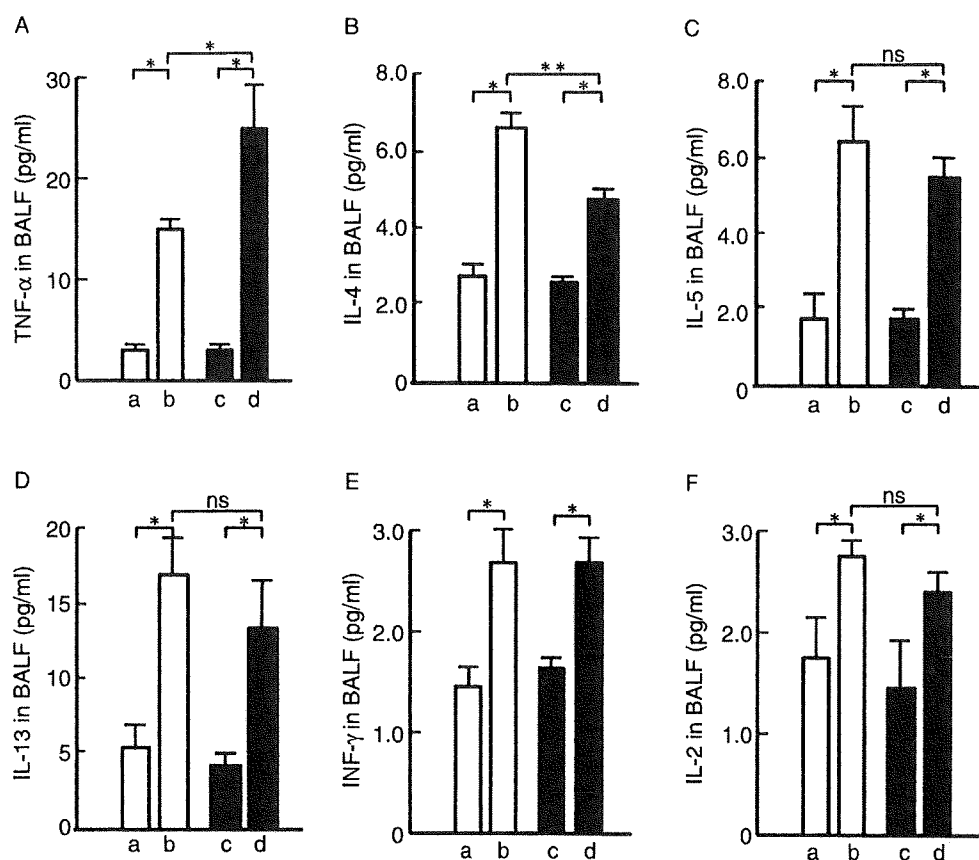


Fig. 1 A, B, C, D, E, F: Outlined column: wild-type mice; Solid column: *Hdc*^{-/-} mice; Column a and c: saline inhalation; Column b and d: OVA inhalation, **A**: TNF- α , **B**: IL-4, **C**: IL-5, **D**: IL-13, **E**: INF- γ , **F**: IL-2 concentration in BALF. * $p < 0.01$, ** $p < 0.05$.

Gob-5 GENE EXPRESSION IN THE LUNG OF *Hdc*^{-/-} MICE

Levels of Gob-5 mRNA in lung tissue of both wild-type and *Hdc*^{-/-} mice sensitized with and exposed to OVA increased significantly compared with those of wild-type and *Hdc*^{-/-} mice sensitized with OVA and exposed to saline. The level of Gob-5 mRNA in the *Hdc*^{-/-} mice exposed to OVA was significantly higher than that of wild-type mice under the same conditions (Fig. 3).

MUC5AC GENE EXPRESSION IN THE LUNG OF *Hdc*^{-/-} MICE

Levels of MUC5AC mRNA in lung tissue of both wild-type and *Hdc*^{-/-} mice sensitized with OVA and exposed to OVA increased markedly compared with those of wild and *Hdc*^{-/-} mice sensitized with OVA exposed to saline. The level of MUC5AC mRNA in the *Hdc*^{-/-} mice exposed to OVA was significantly higher than that of wild-type mice under the same conditions (Fig. 4).

GOBLET CELLS OF LARGE AND SMALL AIRWAYS

PAS staining demonstrated an increase in the number of goblet cells in the epithelium in large and small airways of both wild-type and *Hdc*^{-/-} mice sensitized with OVA (Fig. 5A a-f, B a-f). Goblet cell hyperplasia was more prominent in the *Hdc*^{-/-} mice exposed to OVA than in the wild-type mice under the same conditions (Fig. 5A c, A f, B c, B f). Repetitive saline inhalation induced a mild increase in the number of goblet cells in large and small airways of both wild-type and *Hdc*^{-/-} mice (Fig. 5A b, A e, B b, B e).

QUANTIFICATION OF GOBLET CELLS IN LARGE AND SMALL AIRWAYS

The ratios of goblet cells in the epithelium in large and small airways, of both wild-type and *Hdc*^{-/-} mice sensitized with and exposed to OVA, increased markedly compared with those of the wild-type and *Hdc*^{-/-} mice sensitized with OVA and exposed to saline (Fig. 6A, B). The ratios of goblet cells in the epithelium in large and small airways of the *Hdc*^{-/-} mice exposed to OVA were significantly higher than that of wild-type mice under the same condition.

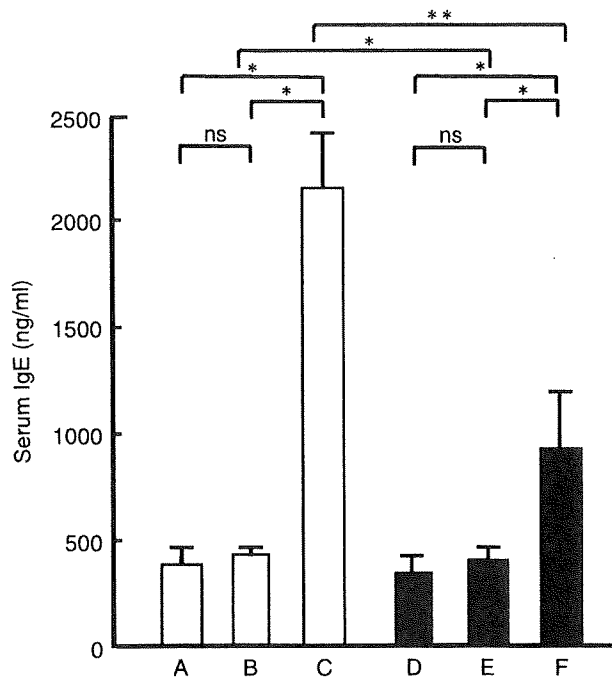


Fig. 2 IgE concentration in serum. Outlined column: wild-type mice; Solid column: *Hdc*^{-/-} mice; Column A and D: before inhalation; Column B and E: saline inhalation; Column C and F: OVA inhalation. **p* < 0.01, ***p* < 0.05, ns: not significant.

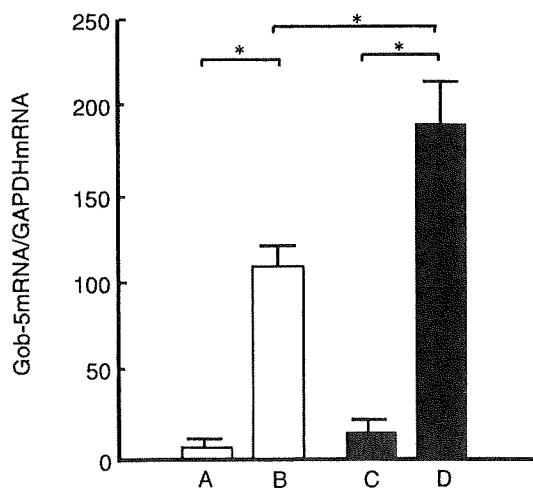


Fig. 3 Gob-5 gene expression in the lung. Outlined column: wild-type mice; Solid column: *Hdc*^{-/-} mice; Column A and C: saline inhalation; Column B and D: OVA inhalation. **p* < 0.01.

DISCUSSION

In this study, we demonstrated that goblet cell hyperplasia was enhanced in *Hdc*^{-/-} mice sensitized with OVA after repetitive OVA exposure for 14 days. We

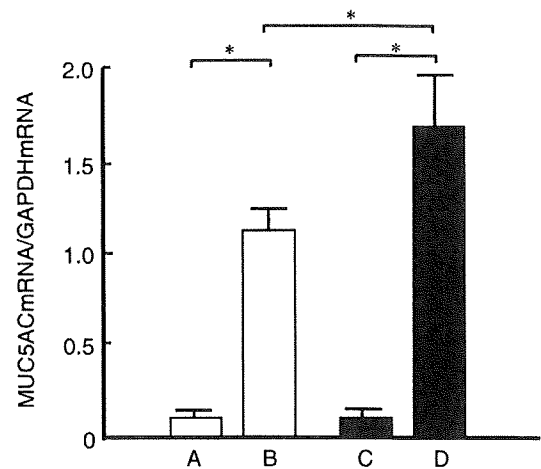


Fig. 4 MUC5AC gene expression in the lung. Outlined column: wild-type mice; Solid column: *Hdc*^{-/-} mice; Column A and C: saline inhalation; Column B and D: OVA inhalation. **p* < 0.01.

also demonstrated a striking difference in differential cytological features in BALF between 2 murine asthma models with *Hdc*^{-/-} mice, representing the acute allergic airway response with a short period of OVA exposure previously reported^{29,30} and a relatively chronic allergic airway response shown in the present study. We found significant increases of alveolar macrophages and lymphocytes in BALF of *Hdc*^{-/-} mice with repetitive exposure to OVA for 2 weeks compared with the wild-type mice under the same conditions. The concentration of TNF-α in BALF in the *Hdc*^{-/-} mice after repetitive OVA exposure was significantly higher than that of the wild-type mice under the same conditions.

Histamine has been recognized as a chemical mediator playing a central role in allergic reactions including mucosal edema, mucous gland secretion, smooth muscle contraction, etc. Recent studies have demonstrated that histamine plays a critical role in immunomodulation by acting on different types of histamine receptors such as H1R, H2R and H4R on the surface of immune cells and inflammatory cells.⁶⁻¹¹ Using mutant mice lacking H1R and H2R, Jutel *et al.* demonstrated that histamine enhances TH1-type responses by triggering H1R, whereas both TH1- and TH2-type responses were negatively regulated by H2R. Indeed, the deletion of H1R resulted in suppression of IFN-γ, IL-4 and IL-13, and the deletion of H2R showed an up-regulation of both TH1 and TH2 cytokines (IL-4 and IL-13).⁷ Bryce *et al.* recently demonstrated the interesting opposite effects of H1R inhibition on cultured T cells *in vitro* and the airway response with allergic inflammation *in vivo* using a murine asthma model.⁵ In their report, allergen-stimulated splenic T cells from sensitized H1R knock-

The Role of Histamine in Goblet Cell Hyperplasia

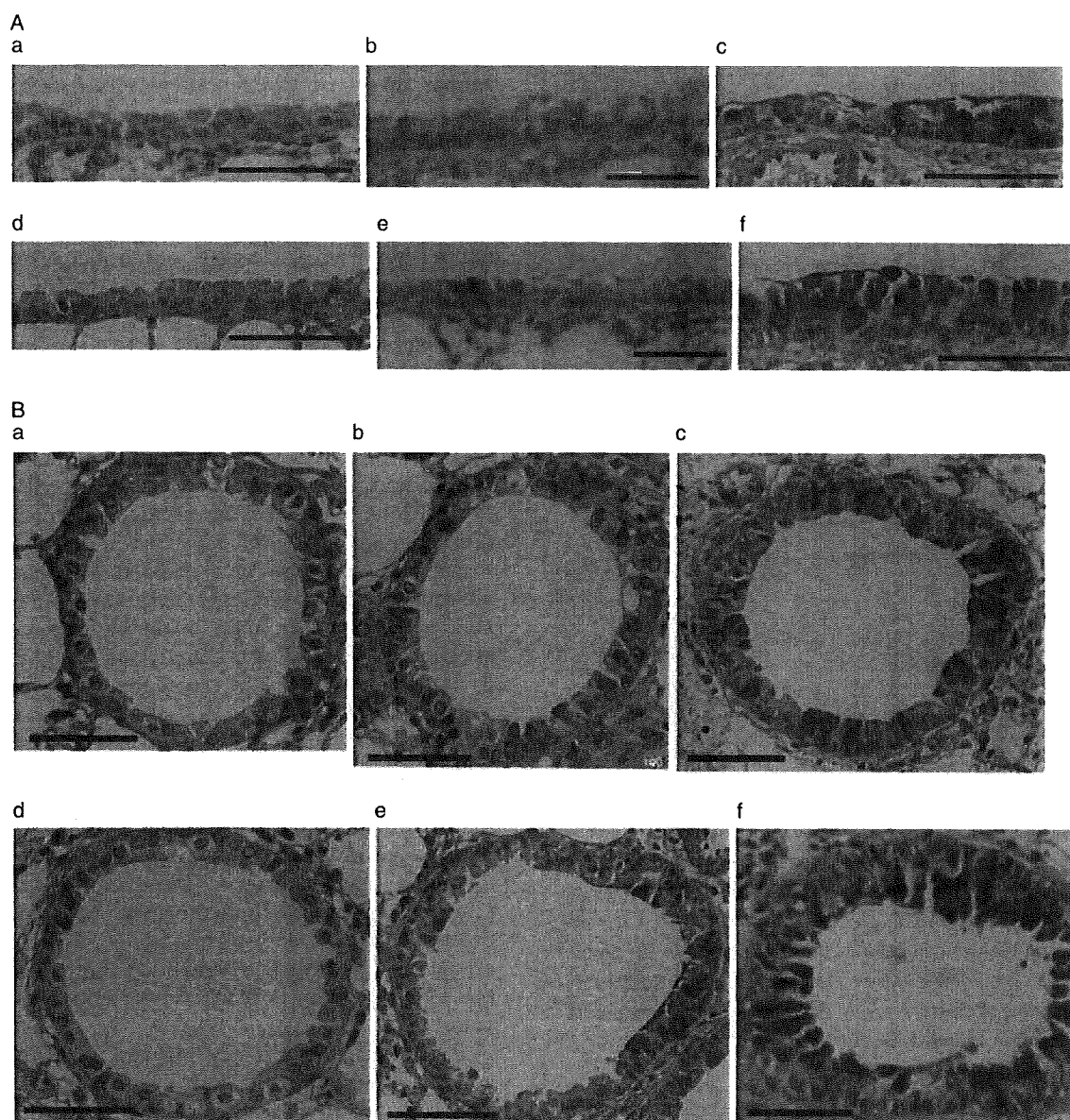


Fig. 5 **A:** Goblet cells in the large airways, **B:** Goblet cells in the small airways. The goblet cells in the epithelium were stained with PAS. **a, b** and **c:** wild-type mice; **d, e** and **f:** *Hdc*^{-/-} mice; **a** and **d:** before inhalation; **b** and **e:** saline inhalation; **c** and **f:** OVA inhalation, black bars indicate 50 μ m.

out mice exhibited enhanced TH2 cytokine production. However, in contrast, allergen-challenged H1R knockout mice exhibited diminished lung TH2 cytokine mRNA levels, airway inflammation, goblet cell metaplasia, and airway hyperresponsiveness (AHR).

The present study demonstrated that the concentration of IL-4 in BALF was significantly lower in the *Hdc*^{-/-} mice than in the wild-type mice after exposure to OVA, which was consistent with the experiment of the *Hdc*^{-/-} mice by Kozma *et al.*,³⁰ despite differences in the duration of OVA exposure. The concentrations of IL-2, IL-5, IL-13 and INF- γ showed no significant differences between the *Hdc*^{-/-} mice and the wild-

type mice. The lower level of IL-4 in the *Hdc*^{-/-} mice was consistent with the lower level of serum IgE in the *Hdc*^{-/-} mice after the exposure to OVA. These changes may be explained at least in part by the suppressed recruitment of activated TH2 cells into the lung due to the blockade of H1R in T lymphocytes, as suggested by Bryce *et al.*⁵

H1R, H2R and H4R are expressed in monocytes, macrophages and dendritic cells, and histamine plays a regulatory role in expression of cytokines and adhesion molecules in these cells.³⁵ Mazzoni *et al.* demonstrated that histamine inhibited INF- α and released TNF- α from activated plasmacytoid dendritic cells

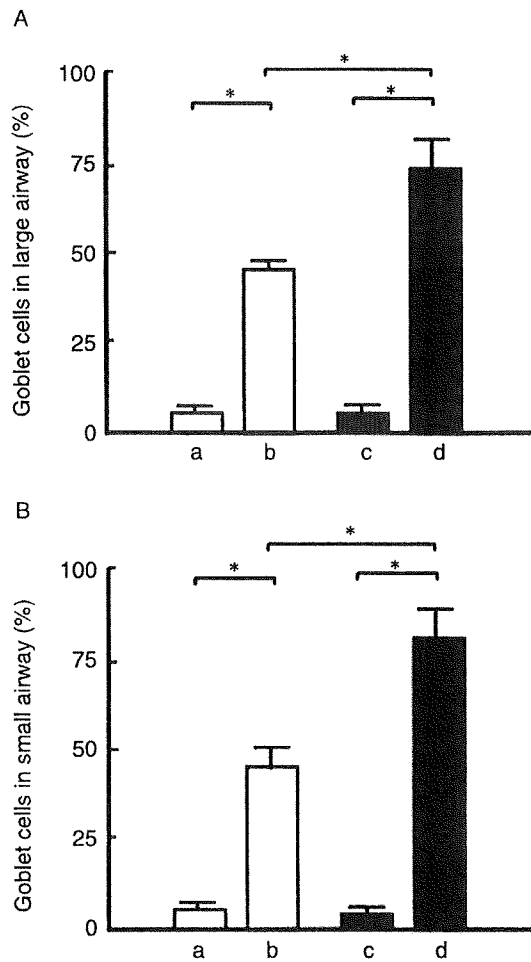


Fig. 6 A: Goblet cell ratio in the large airways, B: Goblet cell ratio in the small airways. Outlined column: wild-type mice; Solid column: *Hdc*^{-/-} mice; Column a and c: saline inhalation, Column b and d: OVA inhalation. **p* < 0.01.

through H2R.³⁶ Histamine also inhibited lipopolysaccharide-induced TNF- α production by down-regulating intercellular adhesion molecules (ICAM)-1 in human monocytes through H2R.³⁷ Alveolar macrophages are known to be a major source of TNF- α in the lung, and the increased production of TNF- α by alveolar macrophages in patients with asthma has been reported.^{38,39} The present study demonstrated an increase in the TNF- α concentration in BALF in the *Hdc*^{-/-} mice exposed to OVA compared to the wild-type mice under the same condition. This result suggested that histamine may play a role in suppressing TNF- α production through H2R in allergic airway inflammation in mice.

Busse *et al.* have recently demonstrated that chronic exposure of TNF- α to the airway induced goblet cell hyperplasia.⁴⁰ Taking these findings into consideration, we speculate that the depletion of histamine in the *Hdc*^{-/-} mice induced the up-regulation of TNF- α production, resulting in the increase of gob-

let cells in the airways with allergic inflammation.

In association with the increase of goblet cells, higher levels of Gob-5 and MUC5ac mRNA were found in the *Hdc*^{-/-} mice exposed to OVA. Previous reports have suggested that the expression of the Gob-5 gene, which corresponds to hCLCA1 in human beings, would be one of the first steps in mucus cell metaplasia and hyperplasia by inducing mucin gene expression.²⁵ MUC5ac gene, a member of the mucin gene family, has been shown to be expressed exclusively in the airway and associated with the hyperplasia of goblet cells. In addition to IL-4, IL-5 and IL-13, TNF- α was reported to increase the expression of the Gob-5 and MUC5ac genes.⁴¹ These findings suggested that the increased production of TNF- α in the BALF of the *Hdc*^{-/-} mice exposed to OVA may be, at least in part, involved in the increase of mRNA levels of the Gob-5 and MUC5ac genes, leading to the increased number of goblet cells in the airway.

Antigen challenge of sensitized mice or human asthmatic subjects results in increased TNF- α expression in BALF, peripheral blood, and tissue biopsy specimens.⁴²⁻⁴⁶ TNF- α production is partly regulated by histamine via H2R in macrophages and dendritic cells, as described above. Although we had no direct evidence to evaluate the role of TNF- α in goblet cell hyperplasia, the results of our study suggested that the increased level of TNF- α might contribute to enhance goblet cell hyperplasia and that histamine may be involved in the negative regulation of TNF- α production in allergic airway inflammation *via* the histamine receptors of inflammatory cells, including macrophages. To confirm the above, further studies are needed, such as experiments using mice in which the TNF- α activity is blocked with antibody or gene disruption.

There was a discrepancy between the TNF- α levels and the macrophage counts. The concentration of TNF- α in the knock-out mice was twice that in the wild-type mice, and the number of alveolar macrophages in *Hdc*^{-/-} mice was ten times that in the wild-type mice. We have no data to explain this, however, to answer this question, it would be necessary to evaluate the capacity of TNF- α production by alveolar macrophages in *Hdc*^{-/-} mice and wild mice. It would also be necessary to evaluate the time course of the concentration of TNF- α and the number of alveolar macrophages to understand their relationship.

We demonstrated the increased number of lymphocytes in BALF of *Hdc*^{-/-} mice. It has been reported by Jutel *et al.* that histamine stimulated the Th1 response through the H1 receptor. On the other hand, the activities of both Th1 and Th2 lymphocytes were inhibited by histamine through the H2 receptor.⁷ Elimination of the inhibitory actions on lymphocytes by histamine through the H2 receptor in *Hdc*^{-/-} mice may contribute to the increased number of lymphocytes in BALF.