

Role of Adrenocorticotrophic Hormone in the Modulation of Pollinosis Induced by Pollen Antigens

Maki Hashimoto^a Eisuke F. Sato^c Keiichi Hiramoto^c Emiko Kasahara^a
Masayasu Inoue^b Seiichi Kitagawa^a

^aDepartment of Physiology, Osaka City University Medical School, and ^bDepartment of Biochemistry, Health Science Laboratory Osaka, and ^cDepartment of Pharmacology, Suzuka University Medical Science School, Suzuka, Japan

Key Words

Immunoglobulin E · Interferon- γ · Interleukin-10 · Adrenocorticotrophic hormone · T cell differentiation

Abstract

Background: A mild restraint stressor suppressed an increase in the levels of Th2-dependent cytokines and IgE, thereby reducing the symptoms of pollinosis. In the present study, to clarify the mechanism of action of adrenocorticotrophic hormone (ACTH) in improving the symptoms of pollinosis, we studied the effects of ACTH on the plasma level of histamine, mast cell number in nasal-associated lymphoid tissue (NALT) and the T cell differentiation in splenocytes. **Methods:** The role of ACTH in the development of pollen antigen-induced pollinosis was studied in mice. Allergic symptoms and parameters were measured on day 17 after sensitization. To investigate the effects of ACTH on T cell differentiation, we stimulated splenocytes obtained from control mice with ACTH and CD3/CD28 in vitro, and measured the cytokine production in the culture supernatant. **Results:** The plasma levels of IL-10, IgE and histamine and mast cell number in NALT were increased in the sensitized animals in association with a concomitant increase in the incidence of sneezing and nasal rubbing. The intraperitoneal administration of ACTH decreased the IL-10, IgE and histamine levels in the plasma and mast cell number in NALT, while increasing

the IFN- γ level and suppressing the incidence of nasal rubbing. Furthermore, the production of IFN- γ increased, while the IL-4 level was suppressed after 2 days in culture. **Conclusions:** The present findings showed that ACTH directly affects T cell differentiation and promotes Th1-type reactions. The regulation of the Th1/Th2 balance by ACTH may result in a decrease in the pathological features of pollinosis.

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Introduction

Japanese cedar pollinosis, which is caused by the pollen of the Japanese cedar tree (*Cryptomeria japonica*), is one of the most common allergic respiratory diseases in Japan [1]. The prevalence of Japanese cedar pollinosis increased 2.6-fold between 1980 and 2000, and the disease has currently been reported to occur at a prevalence of more than 16% in the Japanese population [2, 3]. Factors associated with air pollution, farming and a Western lifestyle, such as a Western diet and insulated housing conditions, have been proposed to be risk factors increasing the prevalence of allergic diseases [4–7]. Furthermore, stress has been reported to be a risk factor for allergic disease.

Various stressors have been reported to affect the symptoms of allergic diseases, such as asthma, atopic dermatitis and allergic rhinitis, by activating the hypothala-

mo-pituitary-adrenal axis (HPA axis), which modulates the balance between Th1- and Th2-type immune responses. Previously, we showed that a mild restraint stressor suppressed an increase in the levels of Th2-dependent cytokines and IgE, thereby reducing the symptoms of pollinosis, while both the HPA axis and adrenocorticotrophic hormone (ACTH) play important roles in the regulation of these cytokines, thus modulating symptoms [8].

ACTH is released from the pituitary in response to stressors, including pathogens, bacterial endotoxins and cytokines [9–11]. Classically, ACTH acts via melanocortin receptor 2 to stimulate the production of corticosteroids in the adrenal glands. Recently, it has been reported that ACTH (1–39) and its metabolites, such as α -MSH (α -melanocyte stimulating hormone), also directly act through corticosteroid-independent mechanisms to inhibit inflammatory responses in peripheral immune cells via other melanocortin receptors (MCRs). Five subtypes of this melanocortin receptor family have so far been recognized [9, 10, 12, 13]. MCRs are widely distributed in various brain regions and peripheral cells. The activation of MCRs by natural and/or synthetic ligands exerts both anti-inflammatory and immunomodulatory effects. ACTH is able to bind to all MCRs, albeit with differences in binding affinity. In addition, ACTH has been found to affect IgE synthesis [14] and is known to increase or inhibit the production of IFN- γ by T lymphocytes [15, 16]. Furthermore, ACTH-related peptides, such as α -MSH, have been shown to be effective in treating allergic diseases, such as atopic dermatitis [17]. However, the effects and mechanism of action of ACTH with respect to the symptoms of pollinosis are unclear.

Therefore, in the present study, in order to clarify the mechanism of action of ACTH in modulating the symptoms of pollinosis, we studied the effects of ACTH on the symptoms of rhinitis, plasma levels of histamine and various cytokines, and the number of mast cells in nasal-associated lymphoid tissue (NALT) in mice sensitized with pollen antigens. We also focused on the effects of ACTH on T cell differentiation and measured the production of cytokines in the supernatant of T cell cultures treated with ACTH and CD3/CD28 costimulation.

Materials and Methods

Animals

Seven- to nine-week-old male BDF-1 and C57BL/6 mice were purchased from SLC Co. (Shizuoka, Japan). The mice were housed under standard environmental conditions (3–6 mice per cage) at 22–24°C under a 12:12-hour light-dark cycle and fed laboratory

chow and water ad libitum. The animal experiments were carried out according to the Animal Care and Use Committee of Osaka City University Medical School.

Sensitization of Mice

The mice were intraperitoneally injected with 1 μ g of pollen allergen (Cry j 1; Hayashibara Biochemical Laboratories Inc., Okayama, Japan) mixed with 0.3 ml of aluminum hydroxide gel (Sigma Chemical Co., St. Louis, Mo., USA) on days 0 (first day) and 7. Next, 10 μ g of pollen antigen was administered into the bilateral nasal cavity once per day on days 15 and 16.

Administration of ACTH

From days 0 to 16, the mice were injected intraperitoneally with 0.3 ml of physiological saline containing 57 ng of ACTH 1–24 (Wako Pure Chemical Industries, Osaka, Japan) three times a week, as previously described [18].

Analysis of Symptoms

Nasal rubbing and sneezing behaviors were observed on day 17, as previously described [19]. Five microliters of antigen (0.1 μ g/mice) was bilaterally instilled into the nasal cavities.

Following antigen challenge, the animals were immediately placed into the observation cage (1 animal/cage, 11 $\times$ 6 cm), and the incidence of nasal rubbing and sneezing in a 5-min period was counted. For the evaluation of nasal rubbing, each time the animal rubbed or touched the area near their nose with their forepaws was counted as one event. Touching around the eyes and mouth was disregarded.

Blood Samples

On day 17, the mice were anesthetized with ether (Wako Pure Chemical Industries Ltd., Osaka, Japan), and blood samples were collected from the heart in heparinized tubes. The blood samples were centrifuged at 15,000 g for 10 min, and the plasma samples were stored at –20°C until use for the analysis.

Analysis of the Levels of Cytokines, IgE and Histamine

The plasma levels of IFN- γ , IL-10, IgE and histamine were determined using IFN- γ and IL-10 ELISA kits (Pierce Biotechnology Inc., Rockford, Ill., USA), an IgE EIA kit (Yamasa Co., Ciba, Japan) and a histamine EIA kit (Bertin Pharma, Montigny-le Bretonneux, France), respectively, according to the manufacturer's instructions.

NALT Preparation

Pooled NALT cell suspensions were prepared using a dissection procedure. On day 17, the mice were killed via cervical dislocation and decapitated, and the facial skin, lower jaw, tongue and cheek muscles were removed. After rinsing with ice-cold Hanks' balanced salt solution (HBSS) to remove blood, the heads were immobilized with pins on a wax dissection slab in order to reveal the upper palate. The palate was excised, gripped behind the incisor teeth with fine forceps and gently pulled toward the molar teeth while using the scalpel to gently free the tissue between the palate, jawbone and nasal septum. The palates were immediately placed into a 24-mm Petri dish containing 1.0 ml of ice-cold HBSS, and the NALT was teased gently into the medium in order to release the cells. The NALT cell suspensions obtained from the individual animals were pooled, and the cells were washed twice with HBSS via centrifugation (200 g for 10 min at 4°C) in HBSS and resuspended in RPMI-1640 medium

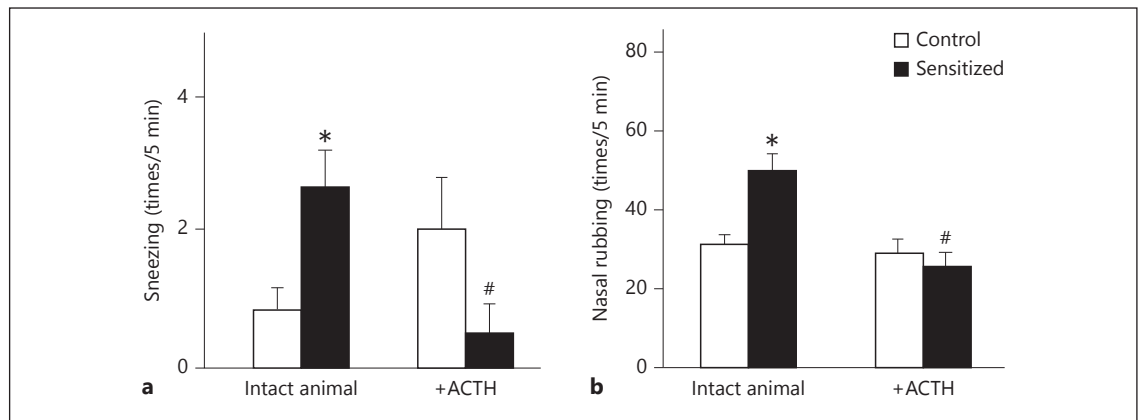


Fig. 1. Effects of ACTH on symptoms of pollinosis. The mice were intraperitoneally injected with 1 µg of pollen allergen mixed with 0.3 ml of aluminum hydroxide gel on days 0 and 7. Then, 10 µg of pollen antigen was administered into the bilateral nasal cavity once per day on days 15 and 16. A total of 57 ng of ACTH was subsequently administered intraperitoneally three times a week from days 0 to 16. On day 17, the number of sneezing and nasal rubbing episodes was counted for 5 min, as described in the text.

a Sneezing: the data are expressed as the mean ± SE (intact animal/control, n = 17; intact animal/sensitized, n = 18; +ACTH/control, n = 13; +ACTH/sensitized, n = 9). **b** Nasal rubbing: the data are expressed as the mean ± SE (intact animal/control, n = 20; intact animal/sensitized, n = 20; +ACTH/control, n = 8; +ACTH/sensitized, n = 7). * p < 0.05 vs. control, # p < 0.05 vs. intact animal/sensitized.

supplemented with 5% heat-inactivated fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine and 1% 4-(2-hydroxyethyl)-1-piperazine-1-ethanesulfonic acid (HEPES) at a pH of 7.4 [20].

Flow Cytometric Analysis

The pooled NALT cells obtained from the treated and untreated animals were phenotypically characterized using the FACSCalibur and CellQuest software programs (BD Biosciences, San Jose, Calif., USA). A minimum of 5,000 cells were analyzed for each phenotype characteristic dataset.

Mast cells were identified using a lymphocyte gate defined by forward and side light scatter and two-color staining with PE-anti-mouse CD117 and FITC-anti-mouse IgE or FcεR1 (BD Pharmingen, San Jose, Calif., USA). Equivalent gates were used in the analyses of cells derived from different tissues for comparison. The results represent the percentage of positively stained cells among the total cell population exceeding the background staining signal.

Preparation of the Cell Suspension

C57BL/6 mice splenocytes (5×10^6 /well/200 µl) were isolated and resuspended in medium containing different concentrations of ACTH with anti-CD28 (0.5 µg/ml; BioLegend, San Diego, Calif., USA) and incubated in anti-CD3 T cell activation plates (BD Pharmingen) for 2 days. The supernatant was assayed for the IFN-γ, IL-4 and IL-10 concentrations using ELISA according to the manufacturer's protocol.

Statistical Analysis

The data are expressed as the mean ± SE derived from 3–20 animals. A p value <0.05 was considered to be statistically significant. Differences between the groups were analyzed using a one-way ANOVA (for multiple-group comparisons), followed by the Newman-Keuls post hoc test.

Results

Effects of ACTH on Clinical Symptoms

The number of sneezing and nasal rubbing episodes increased markedly following sensitization of the animals with pollen allergen. When the mice were injected intraperitoneally with ACTH during sensitization, the incidence of sneezing and nasal rubbing decreased (fig. 1).

Effects of ACTH on the Plasma Cytokine, IgE and Histamine Levels

In a preliminary study, we measured the number of sneezes and the plasma levels of IgE as indices of allergic symptoms, and compared the nontreatment group and vehicle (saline) treatment group. The mean ± SD number of sneezes was 1.0 ± 1.5 (nontreatment group) and 1.5 ± 1.8 [vehicle (saline) treatment group]. The plasma levels of IgE were 166.2 ± 57.3 ng/ml (nontreatment group) and 180.4 ± 58.2 [vehicle (saline) treatment group]. There were no significant differences in the results between the nontreatment group and the vehicle (saline) treatment group. We did not find any nonspecific influence of vehicle (saline) treatment compared to the control group. Thus, we used nontreatment animals as the control group in our study.

Sensitization with pollen antigen significantly increased the plasma levels of IL-10 without affecting the levels of IFN-γ, thus shifting the Th1/Th2 balance to the

Fig. 2. Effects of ACTH on plasma cytokine levels. The experimental conditions were the same as in figure 1. On day 17, the levels of plasma IFN- γ and IL-10 were measured as described in the text. **a** Plasma IFN- γ : the data are expressed as the mean $\pm$ SE (intact animal/control, n = 6; intact animal/sensitized, n = 5; +ACTH/control, n = 5; +ACTH/sensitized, n = 6). **b** Plasma IL-10: the data are expressed as the mean $\pm$ SE (intact animal/control, n = 7; intact animal/sensitized, n = 6; +ACTH/control, n = 3; +ACTH/sensitized, n = 3). ** p < 0.01 vs. intact animal/control, # p < 0.05 vs. intact animal/sensitized.

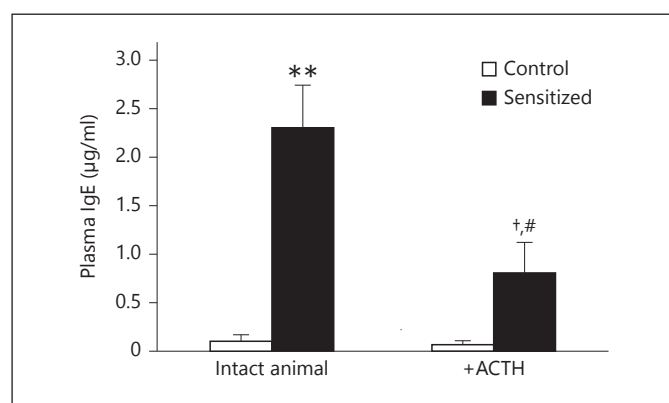
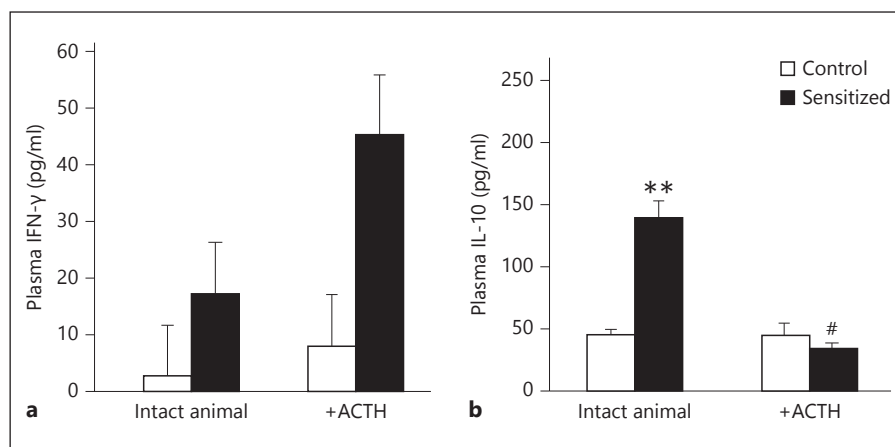


Fig. 3. Effects of ACTH on plasma IgE levels. The experimental conditions were the same as in figure 1. On day 17, the levels of plasma IgE were measured as described in the text. The data are expressed as the mean $\pm$ SE (intact animal/control, n = 6; intact animal/sensitized, n = 5; +ACTH/control, n = 5; +ACTH/sensitized, n = 5). ** p < 0.01 vs. intact animal/control, # p < 0.05 vs. intact animal/sensitized, † p < 0.05 vs. +ACTH/control.

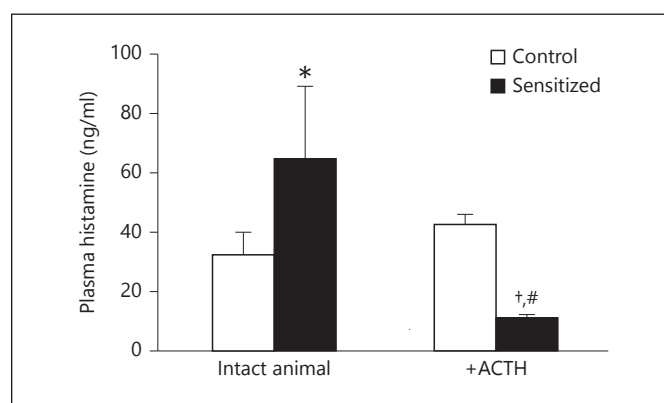


Fig. 4. Effects of ACTH on plasma histamine levels. The experimental conditions were the same as in figure 1. On day 17, the levels of plasma histamine were measured as described in the text. The data are expressed as the mean $\pm$ SE (intact animal/control, n = 4; intact animal/sensitized, n = 4; +ACTH/control, n = 4; +ACTH/sensitized, n = 3). * p < 0.05 vs. intact animal/control, # p < 0.05 vs. intact animal/sensitized, † p < 0.05 vs. +ACTH/control.

Th2-dominant-type reaction (fig. 2) and enhancing the secretion of IgE from B cells (fig. 3) and histamine from mast cells (fig. 4). The increased IL-10, IgE and histamine levels in the sensitized animals were decreased by the administration of ACTH (fig. 2b, 3, 4). In contrast, the plasma levels of IFN- γ tended to increase following the administration of ACTH (fig. 2a).

Effects of ACTH on the Number of Mast Cells

The number of Fc ϵ R1/CD117 and IgE/CD117 mast cells in NALT increased markedly following sensitization of the animals with pollen allergen. When the mice were

injected intraperitoneally with ACTH during sensitization, the number of Fc ϵ R1/CD117 and IgE/CD117 mast cells decreased (fig. 5).

Effects of ACTH on T Cell Differentiation in vitro

ACTH was added to the culture, and the culture supernatants were harvested on day 2. The production of IFN- γ , IL-4 and IL-10 in the culture supernatants was measured using an ELISA kit. Treatment of T cells with ACTH at a concentration of 1,000 nM resulted in a significant increase in the production of IFN- γ compared to that observed in the control cells (fig. 6a). The production

Fig. 5. Effects of ACTH on the number of mast cells in NALT. The experimental conditions were the same as in figure 1. On day 17, the number of mast cells in NALT was measured as described in the text. **a** FcεR1/CD117 cells: the data are expressed as the mean ± SE (intact animal/control, n = 4; intact animal/sensitized, n = 16; +ACTH/control, n = 4; +ACTH/sensitized, n = 4). **b** IgE/CD117 cells: the data are expressed as the mean ± SE (intact animal/control, n = 4; intact animal/sensitized, n = 4; +ACTH/control, n = 4; +ACTH/sensitized, n = 4). ** p < 0.01 vs. intact animal/control, # p < 0.05 vs. intact animal/sensitized.

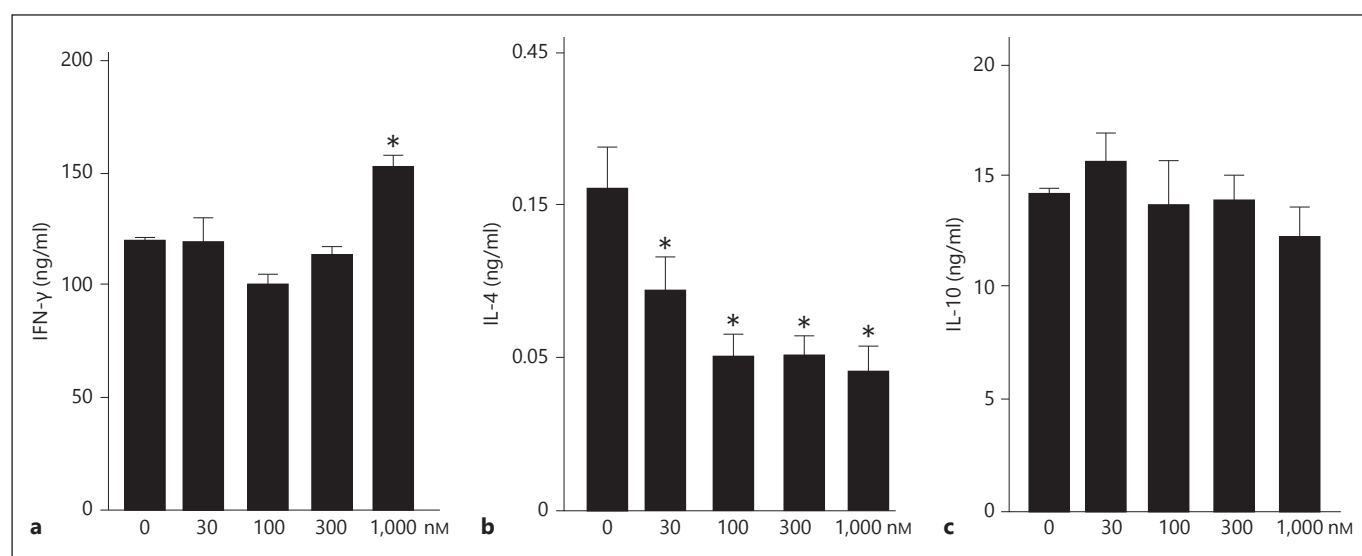
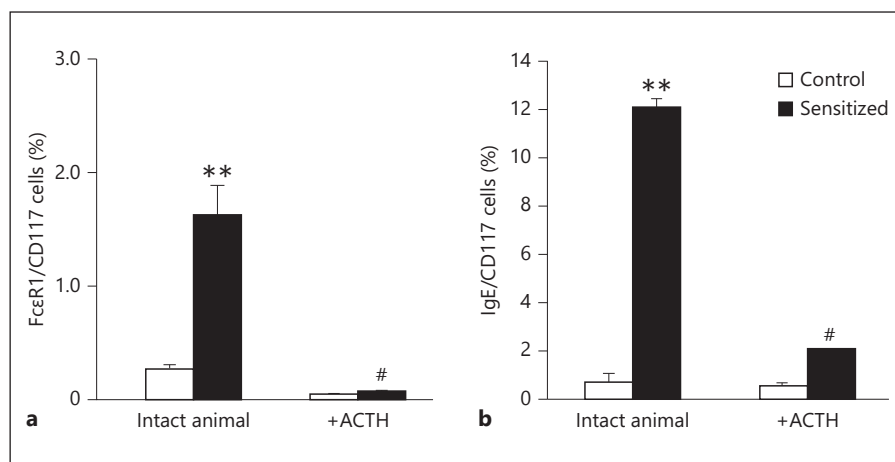


Fig. 6. Effects of ACTH on T cell differentiation in vitro. C57BL/6 mice splenocytes (5×10^6 /well/200 μ l) were resuspended in medium containing different concentrations of ACTH (0, 30, 100, 300 and 1,000 nM) with anti-CD28 (0.5 mg/ml) and incubated in anti-

CD3 T cell activation plates for 2 days. The supernatant was assayed for the IFN- γ , IL-4 and IL-10 concentrations, as described in the text. **a** IFN- γ . **b** IL-4. **c** IL-10. The data are expressed as the mean ± SE (n = 4). * p < 0.05 vs. control (0 nM).

of IL-4 secreted into the culture medium by T cells was decreased by ACTH at a concentration of more than 100 nM (fig. 6b). Meanwhile, the levels of IL-10 were not affected by ACTH (fig. 6c).

Discussion

Previously, we showed that a mild restraint stressor suppressed an increase in the levels of Th2-dependent cytokines and IgE, thereby reducing the symptoms of pol-

linosis, and that both the HPA axis and ACTH play important roles in the regulation of these cytokines, thus modulating symptoms [8]. However, most of the evidence implicating the HPA axis as an endogenous regulator of allergic diseases is indirect, including that observed in our previous study. Therefore, the purpose of the present report was to investigate how ACTH suppresses the symptoms of pollinosis and affects the Th1/Th2 balance.

In addition to analyzing the plasma levels of cytokines and IgE, we investigated the effects of ACTH administration on the plasma levels of histamine and FcεR1/CD117,

as well as the number of IgE/CD117 mast cells in NALT. In the present study, we showed that ACTH administration in pollen antigen-sensitized mice enhances the plasma levels of IFN- γ , while decreasing the levels of IL-10 and IgE, thus suppressing the symptoms of pollinosis, as determined according to the incidence of sneezing and nasal rubbing.

In our previous study, the symptoms of pollinosis were suppressed by a mild restraint stressor in association with increasing plasma levels of both glucocorticoids and ACTH via HPA axis activation. The symptoms of pollinosis in mice treated with hypophysectomy or adrenalectomy were also analyzed. In that study, the symptoms of pollinosis were aggravated by hypophysectomy (low plasma levels of both ACTH and glucocorticoids), but suppressed by adrenalectomy (high plasma levels of ACTH and low plasma levels of glucocorticoids). Based on these results, we considered that the changes observed in the animals' symptoms and inflammatory reactions were primarily modulated by the balance of ACTH and glucocorticoids via the HPA axis system, although more strongly affected by ACTH than glucocorticoids. Therefore, we studied the effects of the physiological level of ACTH [18] without changes in the plasma levels of ACTH (1.7 ± 2.22 ng/ml in ACTH-treated mice vs. 0.7 ± 1.33 ng/ml in control mice; $p = 0.39$) and glucocorticoids (2.0 ± 0.39 ng/ml in ACTH-treated mice vs. 1.7 ± 0.71 ng/ml in control mice; $p = 0.5$) on pollen antigen-sensitized mice.

Recently, in rodents, paired lymphoid cell aggregates, collectively termed NALT, the equivalent of Waldeyer's rings in humans, have been recognized to be the only well-organized mucosal-associated lymphoid tissue in the upper respiratory tract. NALT contains all lymphoid cells, including T cells, B cells and antigen-presenting cells, necessary for the induction and regulation of inhaled antigen-specific mucosal immune responses. Therefore, the role of NALT in the local immune response in the upper respiratory tract is very important [21–23]. Generally, mast cells are relatively abundant in the skin, thymus, lymphoid tissue, lungs, nasal mucosa, conjunctiva, uterus, urinary bladder, tongue, synovia and mesentery, as well as the subserosal and submucosal layers of the digestive tract and areas surrounding large and small blood vessels [24].

It is well known that mast cells act as the initial effector cell in patients with allergic diseases by virtue of their resident tissue location. Binding of multivalent antigens to receptor-bound IgE aggregates the complexes on the cell surface and induces degranulation of the cell, with the consequent release and generation of mediators of the al-

lergic reaction, such as histamine and substance P. Through the release and production of these proinflammatory molecules, mast cells set in motion a series of events that result in both immediate and late-phase allergic responses. The consequences of mediator release are usually observed within minutes, hence the term 'immediate hypersensitivity.' These mediators interact with the surrounding tissue and elicit allergic inflammation, the manifestations of which are determined by the local environment [25].

Histamine is one of the major ligands released from mast cells, thus inducing the symptoms of allergies, such as nasal rubbing and sneezing. CD117 and Fc ϵ R1 are consistent biologic markers for mast cell identification.

The present results showed that the administration of ACTH in pollen antigen-sensitized mice decreases the plasma levels of histamine and Fc ϵ R1/CD117, as well as the number of IgE/CD117 mast cells in NALT, as compared with that observed in pollen antigen-sensitized mice. We therefore suggest that the decreased number of mast cells and plasma level of histamine are related to suppression of the symptoms of pollinosis.

Among T lymphocytes, cytokines are secreted by two major types of Th cells, Th1- and Th2-type lymphocytes [26]. Th2 cell subsets are characterized by cytokines, such as IL-4 and IL-5, which promote the production of IgE. In constant, Th1 cell subsets inhibit IgE production by secreting IFN- γ [27–29]. It has been reported that a Th1/Th2 imbalance (Th2 response dominance) is involved in the onset and/or clinical progression of allergic diseases.

We hypothesized that regulation of the Th1/Th2 balance by ACTH administration ultimately leads to suppression of the symptoms of pollinosis. Therefore, we investigated whether ACTH affects naïve T cell (intact splenocyte) differentiation into Th1 or Th2 cells when isolated naïve T cells are stimulated with combinations of CD3 and CD28 in order to induce T cell activation. We subsequently found that ACTH increases the production of IFN- γ (Th1-type cytokine) while decreasing the production of IL-4 (Th2-type cytokine), whereas the production of IL-10 (Th2-type cytokine) did not change significantly as compared to that observed in the control cells. In addition, there were no differences in cell death or proliferation (data not shown). These data suggest that our results are not due to differences in the levels of proliferation or the cell count. Rather, our findings indicate that ACTH administration prevents a shift in the balance between Th1/Th2 towards Th2 by affecting naïve T cells.

ACTH, a melanocortin peptide shown to bind to MCRs on leukocytes, can directly modulate immune re-

sponses. For example, Johnson et al. [15] reported that ACTH enhances the production of IFN- γ in lymphocytes. This observation is consistent with the results of our in vivo and in vitro studies. In contrast, Johnson et al. [16] reported that ACTH suppresses the production of IFN- γ in lymphocytes, while Aebischer et al. [14] and Takubo et al. [19] reported that ACTH accentuates the production of IL-4, thus inducing an IgE response in vitro. This discrepancy may be partly explained by differences in culture conditions, such as the presence or absence of costimulation (CD3/CD28 or concanavalin A) to induce T cell activation, the exposure time and/or the concentration of ACTH. Indeed, preliminary studies have shown that the production of IFN- γ is increased at 300 nM, whereas the production of both IL-4 and IL-10 is not changed by ACTH treatment without CD3/CD28 co-

stimulation. Moreover, the production of these cytokines is not detected at 1 or 3 h after treatment with ACTH and CD3/CD28 costimulation. Therefore, we suggest that the concentration of ACTH used in our in vivo study was well suited to suppress the symptoms of pollinosis.

In conclusion, we herein demonstrated that ACTH directly affects T cell differentiation and promotes Th1-type reactions. The regulation of the Th1/Th2 balance by ACTH may decrease the pathological features of pollinosis that lead to increased plasma levels of IgE and histamine, as well as an elevated number of mast cells in NALT. Further studies focusing on the HPA axis-dependent and independent roles of stress hormones, including ACTH, in the pathogenesis of allergic diseases, such as pollinosis, are required to elucidate the effects of stress on allergic diseases.

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