in vivo *28*: 657-666 (2014)

Inhibitory Action of Levocetirizine on the Production of Eosinophil Chemoattractants RANTES and Eotaxin *In Vitro* and *In Vivo*

AYAKO KANEI[1], KAZUHITO ASANO[2], KEN-ICHI KANAI[1], ATSUKO FURUTA[1], KEIGO SASAKI[3] and HARUMI SUZAKI[1]

[1]*Department of Otolaryngology, School of Medicine, Showa University, Tokyo, Japan;*
[2]*Division of Physiology, School of Nursing and Rehabilitation Sciences, Showa University, Yokohama, Japan;*
[3]*SASAKI Hospital, Yokohama, Japan*

Abstract. *Eosinophils are well known to play essential roles in the development and maintenance of allergic diseases. However, the influence of histamine H_1 receptor antagonists on eosinophil functions, especially chemokine production, are not well-defined. Therefore, in the present study, we examined the influence of histamine H_1 receptor antagonist on chemokine production by eosinophils through the use of levocetirizine in vitro and in vivo. Eosinophils prepared from mice were stimulated with specific antigens in the presence of different concentrations of levocetirizine. After 24 h, regulated on activation normal T cell expressed and secreted (RANTES) and eotaxin levels in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA). Patients with Japanese cedar pollinosis were treated with 5 mg levocetirizine once a day for four weeks during the pollen season (February 2012 to April 2012). RANTES and eotaxin levels in nasal secretions were also examined by ELISA. The addition of levocetirizine to eosinophil cultures caused a dose-dependent decrease in the ability of cells to produce RANTES and eotaxin in response to antigen stimulation, and the minimum concentration that caused a significant decrease was 0.05 μM. Although cetirizine also exerted suppressive effects on the production of RANTES and eotaxin by eosinophils, the minimum concentration that caused significant suppression was 0.15 μM, which was three-times higher than that of levocetirizine. Oral administration of levocetirizine for four weeks also reduced RANTES and eotaxin levels in nasal secretions from patients with pollinosis, along with attenuation of clinical symptoms. The ability of levocetirizine to reduce RANTES and eotaxin levels may account, at least in part, for the clinical efficacy of the agent for allergic disorders, including allergic rhinitis.*

Correspondence to: Harumi Suzaki, Department of Otolaryngology, School of Medicine, Showa University, Tokyo 142-8666, Japan. Tel: +81 337848676, Fax: +81 337843757, e-mail: h-suzaki@med.showa-u.ac.jp

Key Words: RANTES, Eotaxin, allergic rhinitis, eosinophil, nasal secretion, levocetirizine, production, suppression.

Allergic diseases, such as allergic asthma, allergic rhinitis and atopic dermatitis, are characterized by an increased number of eosinophils in the blood and at the sites of disease. By releasing their cytotoxic granule contents (*e.g.* major basic protein, eosinophil cationic protein and eosinophil-derived neurotoxin), eosinophils are thought to play a key role in causing tissue damage, leading to the exacerbation of inflammatory responses (1, 2). This was confirmed by ultrastructual studies which demonstrated extensively degranulated eosinophils in tissues during active disease (1, 3, 4). Accordingly, high levels of extracellularly-deposited protein granules have been demonstrated by histochemical analysis of diseased tissues (5). Levels of extracellular granular proteins reflect both the severity of allergic symptoms and disease activity (6, 7). In addition to the secretion of granule contents, eosinophils have been reported to produce several types of cytokines and chemokines that are responsible for inflammatory cell recruitment (including eosinophils, macrophages and type-2 helper T-cells), their activation, and the prolongation of inflammatory cell survival at the sites of allergic diseases (2). Furthermore, eosinophils can also produce fibrogenic factors, such as transforming growth factor and matrix metalloproteinases, which are crucial for tissue remodeling at the sites of allergic diseases (2, 8). Judging from these reports, eosinophils may be thought as an important therapeutic target in the management of allergic diseases. Anti-histamines, such as fexofenadine hydrochloride (FEX) and epinastine hydrochloride (EP), have been used in the treatment of allergic diseases, and successful results have

0258-851X/2014 $2.00+.40

been reported (9, 10). These agents are recognized to block the H_1 receptor, and inhibit the release of both pre-formed mediators, such as histamine, and *de novo* synthesized mediators, including leukotrienes and prostaglandins from mast cells and eosinophils. In addition, it has been also reported that anti-histamines suppress the production of inflammatory cytokines and chemokines from peripheral blood T-cells after immunological and non-immunological stimulation (11). However, the influence of anti-histamines on eosinophil activation, especially chemokine production, is poorly understood.

Levocetirizine is a third-generation non-sedative anti-histamine developed from the second-generation anti-histamine cetirizine (12-14). Levocetirizine is the R-enantiomer of cetirizine, and, like cetirizine, is a long lasting anti-histamine against allergic diseases, relieving discomfort and promoting recovery (14). The same data suggest that levocetirizine has a 2-fold increased affinity for histamine H_1 receptor over that of cetirizine alone (15, 16). It is also reported that levocetirizine possesses higher receptor occupancy at 24 h than other well-established second-generation anti-histamines, including FEX and loratadine (14). Furthermore, levocetirizine inhibits eotaxin-induced eosinophil migration and improves airway hyper-responsiveness induced by adenosine monophosphate (16). In pharmacological studies, levocetirizine modulates the ability of eosinophils to produce growth factor, matrix metalloproteinases, and tissue inhibitors of matrix proteinases *in vitro* (16). However, the influence of levocetirizine on chemokine production by eosinophils is not clear at present. In the present study, therefore, we examined the influence of levocetirizine on chemokine production by eosinophils after immunological stimulation.

Materials and Methods

Materials. Levocetirizine and cetirizine were purchased from Toronto Research Chem., Inc. (North York, ON, Canada) as preservative-free pure powders. BAY11-7085 (BAY), a nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) inhibitor, and SP600125 (SP), an activator protein 1 (AP-1) inhibitor, were purchased from Sigma-Aldrich Co., Ltd. (St. Louis, MO, USA). These agents and chemicals were dissolved in RPMI-1640 medium (Sigma Chemicals, Co., Ltd., St Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (RPMI-FCS; Nihon Bio-Supply Center, Tokyo, Japan) at a concentration of 1.0 mM, sterilized by passing these through 0.2 μm filters, and stored at 4˚C until used.

Mice. Specific pathogen-free male BALB/c mice were purchased from Charles River Japan Inc. (Atsugi, Japan). They were maintained in our animal facilities under a controlled environment (25±5˚C, 55±5% humidity and a 12-h light/dark cycle). All animal experimental procedures were approved by the Animal Care and Use Committee of Showa University and were carried-out in accordance with the guidelines of the Physiological Society of Japan.

Patients and treatment. The study included 12 patients (seven female and five male) with Japanese cedar pollen-sensitized rhinitis, who were recruited at the Otolaryngology Outpatient Clinic of the Sasaki Hospital (Yokohama, Japan) under written informed consent, which was approved by the Ethics Committee of Showa University. Pollinosis was diagnosed by otorhinolaryngologists in accordance with the established criteria on the basis of patient history and rhinoscopic examination. To confirm the diagnosis and demonstrate allergen-caused pollinosis, skin prick testing (mean wheal diameter at least 4 mm greater than that of the negative control) and a nasal provocation test were performed with commercial crude extracts used *in vitro* and *in vivo* (Torii Pharmaceutical Co., Ltd., Tokyo, Japan). We also recruited healthy individuals (three female and four male) from members of the Sasaki Hospital (Yokohama, Japan) under written informed consent, which was also approved by the Ethics Committee of Showa University. The characteristics of participants in the study are shown in Table I. Patients with pollinosis were orally treated with 5 mg levocetirizine (GlaxoSmithKline, Tokyo, Japan) once a day for four weeks during the Japanese cedar pollen season (February 2012 to April 2012).

Preparation of eosinophils. Mouse peritoneal exudate eosinophils were prepared from *Mesocestoides corti*-infected BALB/c mice according to the method described previously (17). In brief, BALB/c mice (Charles River Japan Inc.) were infected intraperitoneally with 500 *M. corti* larvae, which were kindly donated by Dr. A. Miwa (School of Medicine, Kinki University, Osaka, Japan). These mice were killed under ether anesthesia 21 days after infection. Peritoneal exudate cells were obtained by washing the mouse peritoneal cavity with 10 ml sterile phosphate-buffered saline (PBS). The cells were washed three times with RPMI-FCS, and incubated in plastic tissue culture plates to remove plastic adherent cells in a humidified atmosphere with 5% CO_2 at 37˚C. After two hours, non-adherent cells were collected and suspended in RPMI-FCS at a density of 5×10^6 cells/ml. The purity of eosinophils was >98%.

Preparation of mouse IgE. BALB/c mice were infected intraperitoneally with 500 *M. corti*. After 21 days, blood was obtained from cardiac puncture and *M. corti*-sepcific IgE was purified with KAPTIV-AE (Tecnogen S.C.p.A., Monte Verna, Italy) according to the manufacturer's instructions. The protein concentration of the extracted solution was measured with a protein assay kit from Bio-Rad Laboratories (Berkeley, CA, USA), adjusted to 1.0 mg/ml with RPMI-FCS and stored at −80˚C until used.

Preparation of M. corti excretory/secretory antigens. *M. corti* larvae obtained from BALB/c mice 21 days after infection were washed five times with sterile PBS. These worms were then suspended in PBS at a density of 1×10^4 worms/ml and incubated for 12 h at 37˚C in a humidified atmosphere with 5% CO_2. Supernatants were obtained and the protein concentration was measured with a protein assay kit from Bio-Rad Laboratories, adjusted to 10 ng/ml with RPMI-FCS and stored at –80˚C until used.

Sensitization and culture of eosinophils. To sensitize eosinophils with IgE, eosinophils were incubated with 500 ng/ml IgE for two hours at 37˚C, washed three times with RPMI-FCS and resuspended at a density of 5×10^5 cells/ml in RPMI-FCS. The sensitized eosinophils were then stimulated with 1.0 ng/ml specific antigens

Table I. *Patients' baseline characteristics*.

	Healthy controls		Patients	
	Female	Male	Female	Male
Number of subjects	3	4	7	5
Median age, years (range)	46 (32-65)	55 (44-61)	53 (26-70)	61 (34-71)
Disease severity	None	None	Mild	Mild
Serum IgE (U/ml)	24.4±10.6	45.6±10.9	146.4±11.6	135.6±12.9
Blood eosinophil counts (%)	2.2±0.2	3.4±0.6	11.2±0.5	13.5±0.8
IgE RAST score				
Cryptomeria japonica	0	0	41.3±7.4	48.0±12.2
Ambrosia artemisiifolia	0	0	0	0
Artemisia princeps	0	0	0	0
Dermatophagoides farinae	0	0	0	0
Aspergillus fumigatus	0	0	0	0
Cat dander	0	0	0	0
Dog dander	0	0	0	0

in the presence of different concentrations of the agents (18). After 24 hours, culture supernatants were obtained and stored at –80˚C until use.

Recovery of nasal secretions. Nasal secretions were obtained as previously described (19). Briefly, filter papers (Whatman No. 42) were cut into 7×30 mm strips. A filter strip was placed on the anterior portion of the inferior turbinates of the right and left nasal cavity and left for 5 minutes. They were then cut into small pieces and suspended in PBS and rocked for 12 h at 4˚C to prepare the extract of nasal secretions. After measuring IgA concentration in the extract with enzyme-linked immunosorbent assay (ELISA) (Bethyl Laboratories, Inc., Montogomery, TX, USA) according to the manufacturer's recommendations, samples were stored at –80˚C until used.

Assay for chemoattractants. Eosinophil chemoattractants, RANTES and eotaxin, in both culture supernatants and nasal secretions were measured with commercially available ELISA test kits (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's recommendations. The minimum detectable level of these ELISA kits was 5 pg/ml for human eotaxin, 2 pg/ml for human RANTES, 1.5 pg/ml for murine eotaxin, and 2 pg/ml for marine RANTES. The levels of RANTES and eotaxin in culture supernatants were expressed as the mean pg/ml±SE of five individual mice and those of nasal secretions were expressed as the mean pg/ng IgA±SE.

Nasal symptom scores. Nasal discharge was scored from 0 to 3 (0=none, 1=mild, 2=moderate, 3=severe symptoms). Nasal congestion was also scored in the same way. The number of sneezes during one hour were counted and transformed into a score (0=0 sneezes, 1=1-4 sneezes, 2=5-9 sneezes, and 3=10 or more sneezes), and a total symptom score was calculated by adding the three scores.

Assay for mRNA expression. Poly A$^+$ mRNA was separated from cultured cells with oligo(dT)-coated magnetic micro beads (Milteny Biotec, Bergisch Gladbach, Germany). The first-strand cDNA was synthesized from 1.0 mg of PolyA$^+$ mRNA using a Superscript cDNA synthesis kit (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was then carried out using a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The PCR mixture consisted of 2.0 μl of sample cDNA solution (100 ng/μl), 25.0 μl of SYBR-Green Mastermix (Applied Biosystems), 0.3 μl of both sense and antisense primers, and distilled water to give a final volume of 50.0 μl. The reaction was conducted as follows: 4 min at 94˚C, followed by 40 cycles of 15 s at 95˚C and 60 s at 60˚C. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified as an internal control. mRNA levels were calculated by using the comparative parameter threshold cycle and normalized for GAPDH. The nucleotide sequences of the primers were as follows: for *RANTES*, 5'-CCTCACCATCATCCTCACTGCA-3' (sense) and 5'-TCTTCTCTGGGTTGGCACACAC-3' (antisense), for eotaxin, 5'-CCCTTTTCTGTTCTGCTGACAAG-3' (sense) and 5'-GAAGAGTCCCTCGATGTGGCTA-3' (antisense), and for *GAPDH*, 5'-GTCTTCTGGGTGGCAGTGAT-3' (sense) and 5'-CCCTTT TCTGTTCTGCTGACAAG-3' (antisense) (20).

Assay for transcription factor activation. NF-κB activity in cultured eosinophils was analyzed by commercially available NF-κB ELISA test kits (Active Motif, Co., Ltd., Carlsbad, CA, USA) that contained sufficient reagents and a monoclonal antibody against p65, according to the manufacturer's recommendations. In brief, nuclear extract (5.0 mg of protein) from eosinophils was introduced into each well of 96-well microplates precoated with oligonucleotide containing NF-κB consensus site (5'-GGGACTTTCC-3') in a volume of 20.0 μl, followed by incubation for one hour at 25˚C. After washing three times, 100 μl of monoclonal antibody against p65 was added to the appropriate wells and incubated for a further one hour at 25˚C. Anti-IgE horseradish peroxidase (HRP) conjugate in a volume of 100 μl was then added and the plates incubated for a further one hour at 25˚C. The absorbance at 450 nm was measured after the addition of tetramethylbenzidine (TMB) solution. AP-1 activity was also measured with commercially available AP-1 ELISA test kit (Active Motif, Co., Ltd.) in a similar manner.

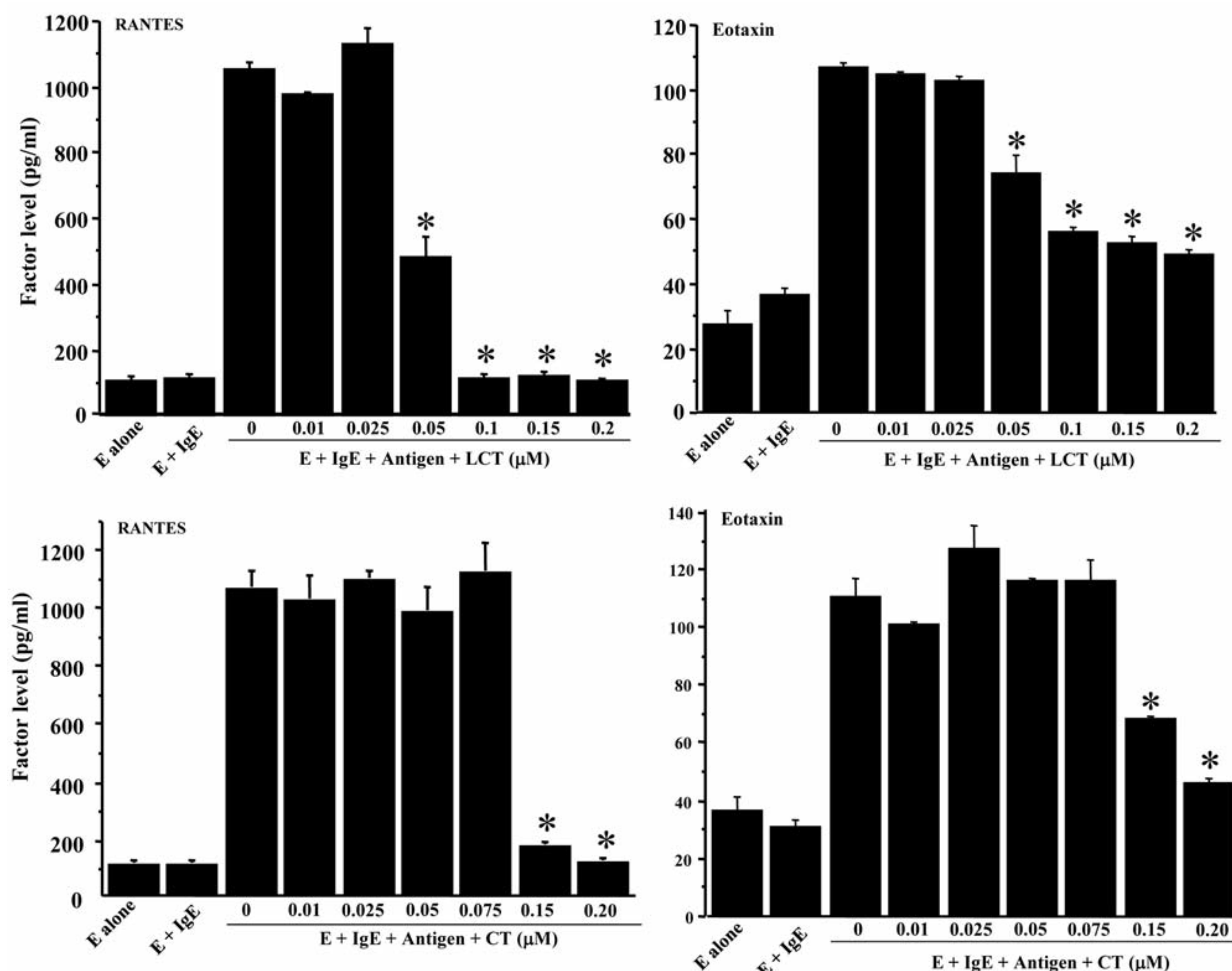

Figure 1. *Suppressive effect of levocetirizine (LCT) and cetirizine (CT) on the production of regulated on activation, normal T-cell expressed and secreted (RANTES) and eotaxin by eosinophils (E) in vitro. Eosinophils (5×10^5 cells/ml) obtained from five mice were individually stimulated with 1.0 ng/ml of excretory/secretory antigens of Mesocestoides corti in the presence of either LCT (upper two panels) or CT (lower two panels) for 24 h. RANTES and eotaxin levels in culture supernatants were examined by enzyme-linked immunosorbent assay (ELISA). Data are means±SE. *$p<0.05$ vs. 0 μM LCT or CT; Antigen: excretory/secretory antigens of M. corti.*

Statistical analysis. Statistical significance of the differences between control and experimental animal groups was examined by Dunnett's multiple comparison test. The paired *t*-test was used to examine the statistical significance of differences before and after treatment with levocetirizine. The level of statistical significance was set at $p<0.05$.

Results

Influence of levocetirizine and cetirizine on eosinophil activation induced by antigenic stimulation. The first experiments were performed to examine the influence of levocetirizine on eosinophil activation. IgE-sensitized eosinophils (5×10^5 cells/ml) were stimulated with 1.0 ng/ml antigens in the presence of different concentrations of levocetirizine. After 24 h, RANTES and eotaxin levels in culture supernatants were examined by ELISA. As shown in Figure 1 (upper panels), the addition of levocetirizine to cell cultures caused a dose-dependent suppression of eosinophil activation: as the concentration of levocetirizine in cell cultures was increased, the levels of both RANTES and eotaxin gradually decreased, and the minimum concentration of the agent that caused significant suppression of factor production was 0.05 μM. We further examined whether cetirizine also inhibited antigen-induced eosinophil activation. As shown in Figure 1 (lower panels), the addition of cetirizine to IgE-sensitized eosinophil cultures also caused eosinophil activation. However, the minimum concentration of the agent that caused significant suppression of the production of both RANTES and eotaxin was 0.15 μM, which is three-times that compared to levocetirizine.

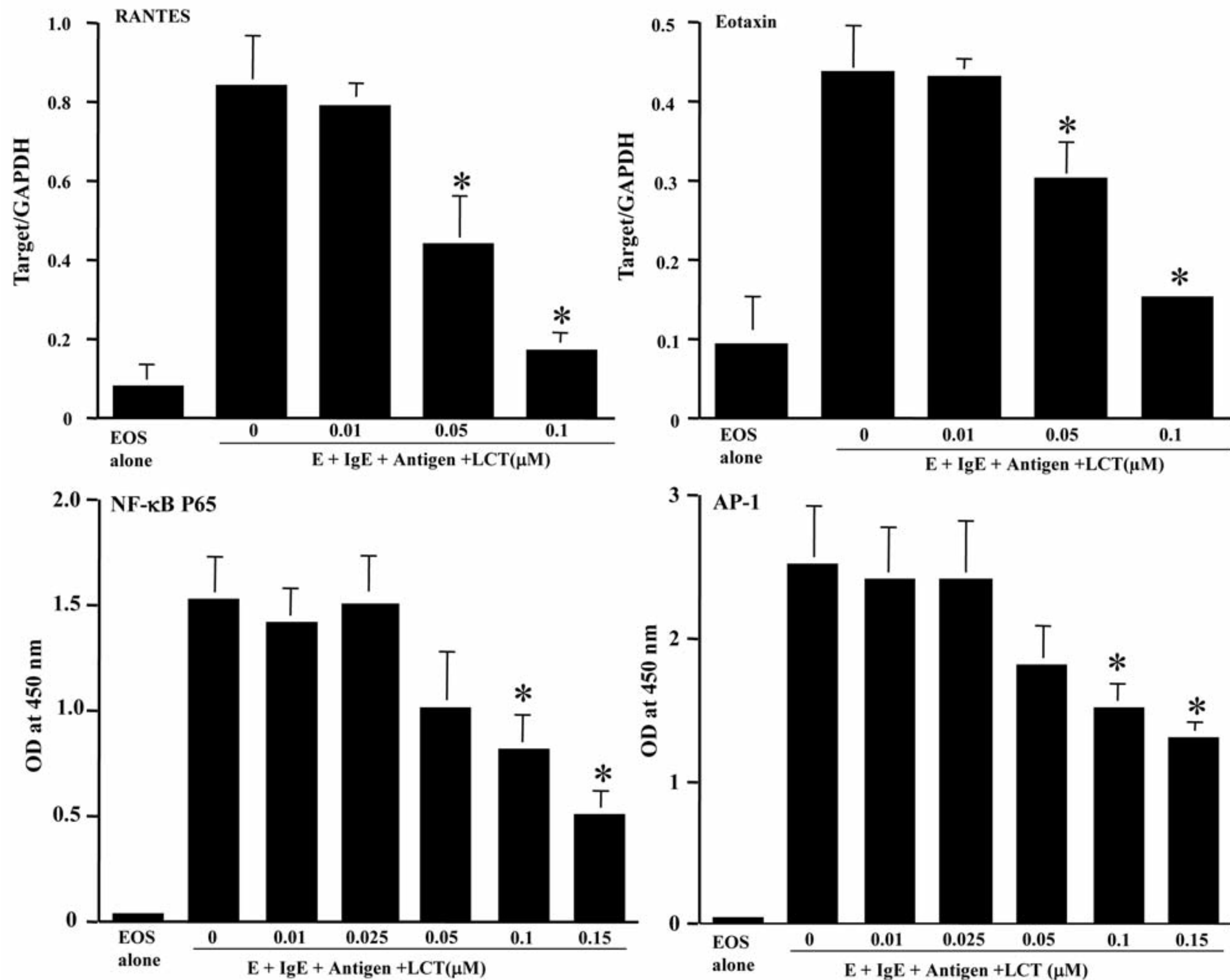

Figure 2. *Suppressive effect of levocetirizine (LCT) on mRNA expression and transcription factor activation in eosinophils. Eosinophils (5×10^5 cells/ml) obtained from five mice were individually stimulated with 1.0 ng/ml of excretory/secretory antigens of Mesocestoides corti in the presence of LCT for 12 h. The levels of mRNA for regulated on activation, normal T cell expressed and secreted (RANTES) and eotaxin shown in upper panels were examined by real-time real-time reverse transcription polymerase chain reaction (RT-PCR) The activation of transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65 and activator protein 1 (AP-1) shown in lower panels were examined by enzyme-linked immunosorbent assay (ELISA). Data are means±SE. *p<0.05 vs. 0 μM LCT or CT; EOS: eosinophils; antigen: excretory/secretory antigens of M. corti.*

Influence of levocetirizine on mRNA expression and transcription factor activation in eosinophils after antigenic stimulation. The next experiments were carried-out to examine the influence of levocetirizine on mRNA expression for RANTES and eotaxin in eosinophils after antigenic stimulation. IgE-sensitized eosinophils (5×10^5 cells/ml) were stimulated with 1.0 ng/ml antigens in the presence of either 0.01, 0.05 or 0.1 μM levocetirizine for 12 h and mRNA expression was examined by real-time reverse transcription polymerase chain reaction (RT-PCR). As shown in Figure 2 (upper panels), treatment of eosinophils at more than 0.05 μM levocetirizine caused significant inhibition of mRNA expression for RANTES and eotaxin, which were increased by antigenic stimulation. Experiments were then undertaken to examine whether levocetirizine treatment of eosinophils could suppress transcription factor activation that results in the inhibition of both mRNA expression and protein production. IgE-sensitized eosinophils (5×10^5 cells/ml) were stimulated with 1.0 ng/ml antigens in the presence of different concentrations of levocetirizine. After 12 h, transcription factor activation was examined by ELISA. As shown in Figure 2 (lower panels), treatment of eosinophils with levocetirizine caused a dose-dependent suppression of NF-κB p65 activation, and the

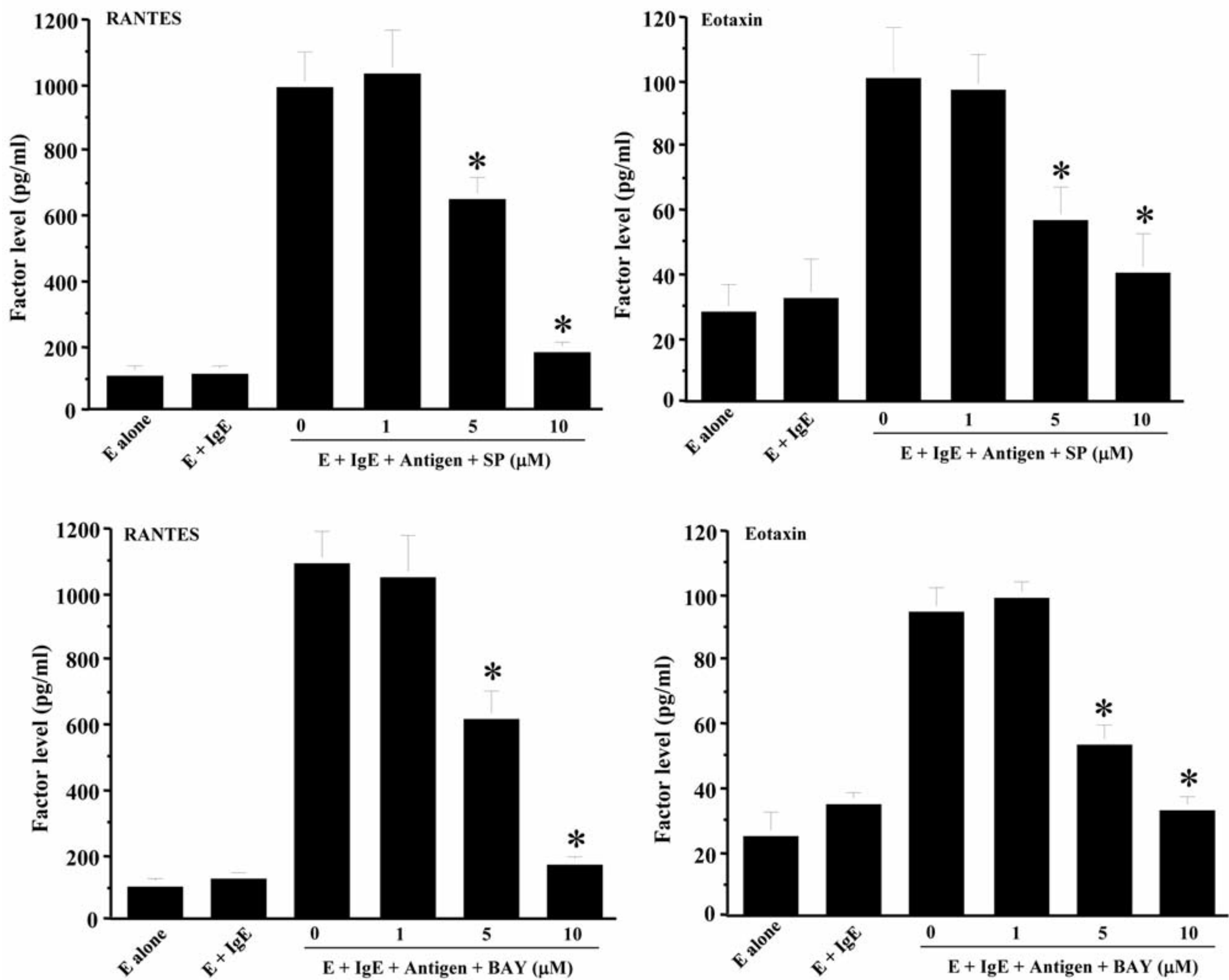

Figure 3. *Influence of inhibition of transcription factor activation on the production of regulated on activation, normal T cell expressed and secreted (RANTES) and eotaxin by eosinophils (E) in vitro. Eosinophils (5×10^5 cells/ml) obtained from five mice were individually stimulated with 1.0 ng/ml of excretory/secretory antigens of Mesocestoides corti in the presence of either SP600125 (SP), an activator protein 1 (AP-1) inhibitor (upper two panels), BAY11-7085 (BAY), an nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) inhibitor (lower two panels), for 24 h. RANTES and eotaxin levels in culture supernatants were examined by enzyme-linked immunosorbent assay (ELISA). Data are means±SE. *$p<0.05$ vs. 0 μM SP or BAY; antigen: excretory/secretory antigens of M. corti.*

minimum concentration of the agent that cause significant suppression was 0.1 μM. The data in Figure 2 (lower panels) also clearly show the suppressive effect of levocetirizine on AP-1 activation that was increased by antigenic stimulation as in the case of NF-κB.

Influence of inhibition of transcription factor activation on chemokine production by eosinophils after antigenic stimulation. A third set of experiments was carried-out to examine the influence of transcription factor activation on eosinophil chemoattractant production by eosinophils in response to antigenic stimulation. IgE-sensitized eosinophils (5×10^5 cells/ml) were stimulated with 1.0 ng/ml antigens in the presence of different concentrations of either BAY or SP. After 24 h, RANTES and eotaxin levels in culture supernatants were examined by ELISA. As shown in Figure 3 (upper panels), treatment of eosinophils with SP at more than 5 μM caused significant suppression of both RANTES and eotaxin production by eosinophils after antigenic stimulation. The data in Figure 3 (lower panels) also show the suppressive effects of BAY on the production of RANTES and eotaxin by eosinophils in response to antigenic stimulation. The minimum concentration that caused significant suppression was 5 μM (Figure 3, lower panels).

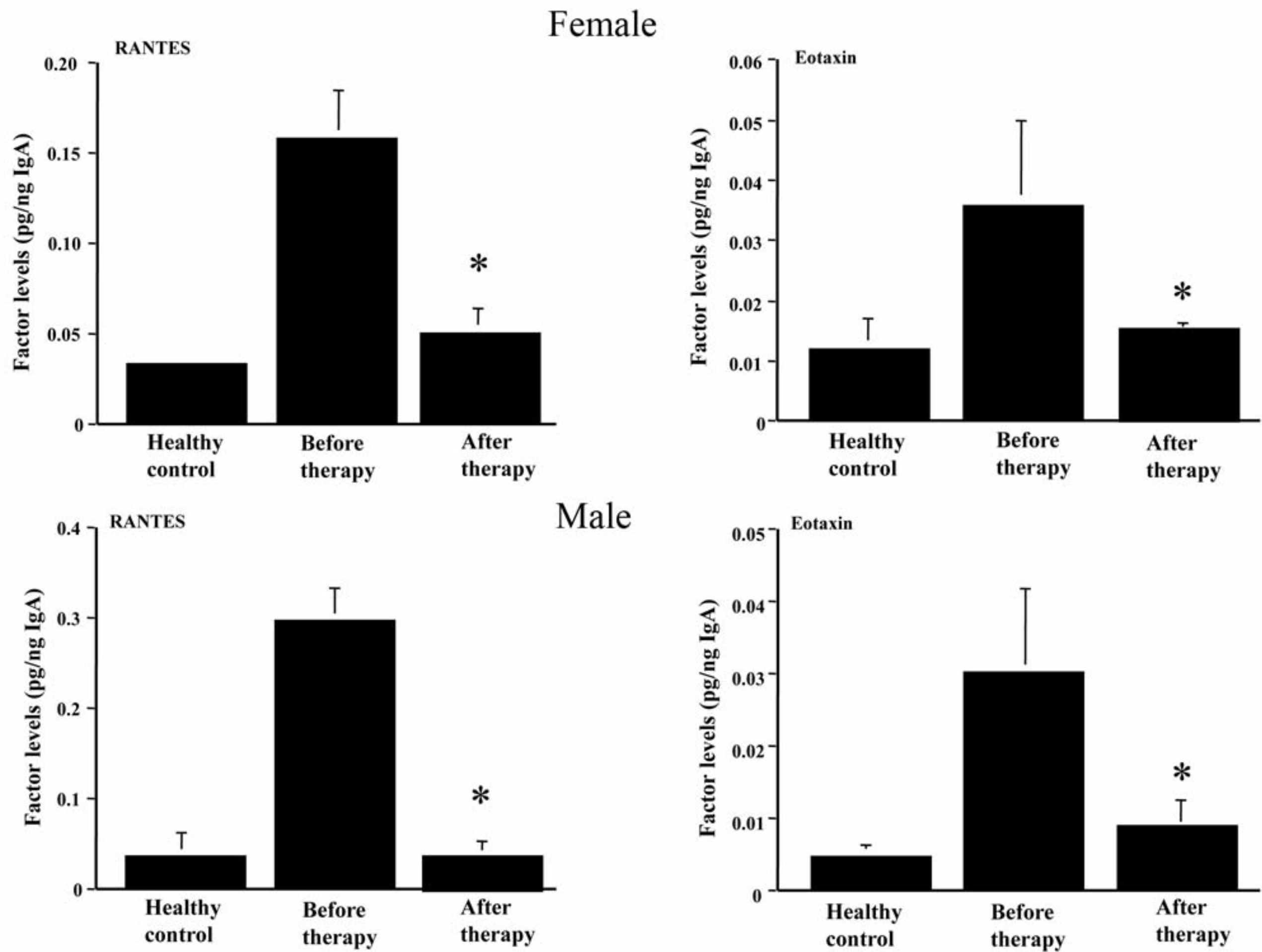

Figure 4. *Influence of levocetirizine (LCT) treatment on regulated on activation, normal T cell expressed and secreted (RANTES) and eotaxin in nasal secretion. Patients with pollinosis were treated with 5 mg LCT once a day for four weeks. Nasal secretions were obtained from patients before and after treatment. RANTES and eotaxin levels were examined by enzyme-linked immunosorbent assay (ELISA). Data are means±SE. *p<0.05 compared with levels before treatment.*

Influence of levocetirizine treatment on eosinophil activation in patients with pollinosis. The fourth set of experiments was designed to examine the influence of levocetirizine on eosinophil chemoattractant production *in vivo*. To do this, patients with Japanese cedar pollinosis were orally administered 5 mg levocetirizine once a day for 28 days during the Japanese cedar pollen season, and RANTES and eotaxin levels in nasal secretions were examined by ELISA. As shown in Figure 4 (upper panels), oral administration of levocetirizine to female patients caused significant suppression of RANTES and eotaxin in nasal secretions. This suppressive activity of levocetirizine on both RANTES and eotaxin was also observed in male patients (Figure 4, lower panels).

Influence of levocetirizine treatment on clinical symptoms in patients with pollinosis. The final experiments were performed to examine whether oral administration of levocetirizine could favorably modify the clinical conditions of patients. As shown in Table II, the clinical symptom scores decreased after treatment.

Discussion

The present results clearly show that levocetirizine inhibits the production of eosinophil chemoattractants, RANTES and eotaxin by eosinophils after antigenic stimulation in a dose-dependent manner, through the suppression of NF-κB, of AP-1 activation and of chemokine mRNA expression. The minimum concentration of levocetirizine that caused significant suppression of factor production was 0.05 μM, which is much lower than therapeutic blood levels (0.348 μM) (21). The data also show that levocetirizine was more potent at

Table II. *The effects of levocetirizine on total nasal symptoms scores in patients with pollinosis.*

Symptoms		Before (Mean±SE)	After* (Mean±SE)
Female	Sneezing	2.0±1.0	0.7±0.5
	Nasal discharge	1.9±0.9	1.1±0.9
	Congestion	2.7±1.3	0.8±0.4
Male	Sneezing	2.3±0.6	0.5±0.5
	Nasal discharge	2.4±1.1	1.6±1.1
	Congestion	2.3±1.2	0.8±0.5

*p<0.05 compared with levels before treatment.

inhibiting chemokine production by eosinophils after antigenic stimulation as compared with cetirizine: The suppressive effects of levocetirizine on chemokine production by eosinophils occurred at a three-times lower concentration than that of the parental drug, cetirizine, which caused significant suppression at 0.15 μM.

Eosinophils are well-accepted as secreting a wide variety of proinflammatory mediators and immunoregulatory molecules, such as major basic protein, leukotrienes, chemokines and cytokines, among others (2, 22). They normally reside in mucosal tissues, and, during Th2-type immune responses, are recruited from the bone marrow and blood to sites of inflammatory responses (22). The trafficking of eosinophils to inflammatory sites is dependent on the coordinated actions of several types of cytokines, chemokines and adhesion molecules (21). Among these, Interleukin-5 (IL5) and the eotaxin family of chemokines selectively regulate eosinophil trafficking (23, 24). It is also reported that RANTES is crucial for eosinophil migration (21). In addition to the chemotactic activity of RANTES and eotaxin, these two chemokines function in inflammatory cells to activate the secretion of harmful mediators, cytokines and chemokines (23-25). RANTES directly stimulates basophils to secret histamines, which occurs much more rapidly when compared to an antigen–IgE interaction (26). It is also reported that intradermal injection of RANTES provokes an increase in both histidine decarboxylase mRNA expression, the sole enzyme responsible for the production of histamine (27), and the secretion of prostaglandin D_2 (27) and E_2 (28), which are important mediators in the development of inflammatory responses, from basophils, mast cells and eosinophils. Furthermore, eotaxin is reported to participate directly in tissue inflammation through the release of reactive oxygen species and the induction of histamine and leukotriene C_4 de-granulation in mast cells, basophils and eosinophils (29). RANTES and eotaxin exert their biological effects after binding to specific receptors, especially C-C chemokine receptor type 3 (CCR3), expressed on inflammatory cell surfaces (22, 29). CCR3 is expressed not only on inflammatory cells, but also on fibroblasts, keratinocytes and epithelial cells, among others (22, 29). The complexes of chemokines and CCR3 cause an increase in cell migration to and proliferation at the sites of inflammation (22, 29). It is also recognized that chemokine signaling through CCR3 leads to an increase in the production of matrix proteins and transforming growth factor-β (TGF-β), which are responsible for the development of tissue remodeling at sites of inflammation. Taken together, the present results strongly suggest that the suppressive effects of levocetirizine on the production of RANTES and eotaxin by eosinophils underlie, in part, the therapeutic mode of action of this agent on allergic diseases. To further confirm this speculation, we then examined the influence of levocetirizine on RANTES and eotaxin in nasal secretions obtained from patients with Japanese cedar pollinosis treated with levocetirizine for four weeks during the pollen season.

The present results clearly show that nasal secretions obtained from patients before treatment contained higher levels of RANTES and eotaxin compared with those from healthy controls, and that oral administration of levocetirizine in patients reduced the chemokine levels in nasal secretions, accompanied by attenuation of clinical conditions.

Although the present results strongly suggest that the suppressive effect of levocetirizine on chemokine production may be due, in part, to its inhibitory action on transcription factor activation, the precise mechanisms of levocetirizine suppression of transcription factor activation are not clear at present. It has been reported that the activation of eosinophils by antigen and IgE *via* the high-affinity receptor, namely Fc epsilon RI causes the activation of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK) (39), which are essential kinases for NF-κB and AP-1 activation (31), indicating that levocetirizine likely suppressed the activation of MAPKs and this resulted in the inhibition of chemokine production by eosinophils. This speculation may be supported by our previous observation showing the suppressive activity of antihistamine on the activation of MAPKs, such as ERK and p38 MAPK, induced by antigenic stimulation *in vitro* (11).

There is an established concept that RANTES and eotaxin are produced by several types of cells, such as epithelial cells and fibroblasts, in response to immunological and non-immunological stimulation (22, 27-29). The present data clearly show the suppressive effect of levocetirizine on chemokines in nasal secretions, indicating that levocetirizine inhibits not only the ability of eosinophils, but also the functions of epithelial cells and fibroblasts of producing chemokines, and results in the decrease of chemokine levels in nasal secretions. This speculation may be supported by the observation that H_1 receptor antagonists such as FEX, and EP suppressed the ability of human nasal cells, including

epithelial cells and fibroblasts, to produce eosinophil chemoattractants and inflammatory cytokines *in vitro* (10, 32). Further experiments are required to clarify this point.

Our previous experiments clearly showed that EP, a second-generation H_1 receptor antagonist, suppressed eosinophil activation by stem cell factor stimulation *in vitro* (10) and results in the inhibition of RANTES production (17) when eosinophils were treated with EP at 25 ng/ml, which is a similar concentration to therapeutic blood levels. It was also observed that FEX inhibited the production of RANTES and eotaxin by nasal fibroblasts, when the cells were stimulated with lipopolysaccharide or tumor necrosis factor-α in the presence of FEX at 250 ng/ml (32), which is similar to the concentration in plasma after oral administration of the therapeutic dose in humans. Another H_1 receptor antagonist, carebastine, has been reported to inhibit mRNA expression of CC chemokines, including RANTES and eotaxin in human nasal epithelial cells when the cells were stimulated with mite antigen and histamine when treated at more than 10^{-7} M (33). Furthermore, *ex vivo* experiments using peripheral blood mononuclear cells obtained from patients allergic to *Parietaria judai* treated with desloratadine or deflazacort for three weeks clearly showed the suppressive effects of these agents on mRNA expression for eosinophil chemoattractants, such as RANTES, with improvement of clinical conditions (34). Together with these reports, the present results strongly suggest that second-generation antihistamines, such as carebastine, FEX, and cetirizine, among others, may act as down-regulators of eosinophilic inflammatory responses observed in allergic diseases through the suppression of eosinophil chemoattractant production and result in the attenuation of clinical symptoms in allergic diseases. Furthermore, the present results may be interpreted to mean that third-generation antihistamines are more effective at inhibiting eosinophil production of chemoattractant than are second-generation antihistamines. Further experimentations are required to clarify this point.

In conclusion, the present results strongly suggest the suppressive activity of levocetirizine on chemokine production by eosinophil constitutes, in part, the therapeutic mode of action of the agent against allergic diseases.

Conflicts of Interest

The Authors declare that there is no conflict of interest regarding the publication of this article.

References

1 Malm-Erjefalt M, Persson CGA and Erjefalt JS: Degranulation status of airway tissue eosinophils in mouse models of allergic airway inflammation. Am J Respir Cell Mol Biol *24*: 352-359, 2001.

2 Kay AB: The role of eosinophils in the pathogenesis of asthma. Trends Mol Med *11*: 148-152, 2006.

3 Overbeek SE, O'Sullivan S, Leman K, Mulder PG, Hooqsteden HC and Prins JB: Effect of montelkast compared with inhaled fluticasone on airway inflammation. Clin Exp Allergy *34*: 1388-1394, 2004.

4 Widegren H, Korsgren M, Andersson M and Grieff L: Effects of TNF-alpha on the human nasal mucosa *in vivo*. Respir Med *101*: 1982-2087, 2007.

5 Davoine F, Ferland C, Chakir J, Lee JE, Adamko DJ, Moqbel R and Laviolette M: Interleukin-12 inhibits eosinophil degranulation and migration but does not promote eosinophil apoptosis. Int Arch Allergy Immunol *140*: 277-284, 2006.

6 Rosenberg ME and Hogan SP: The eosinophil. Ann Rev Immunol *24*: 147-174, 2006.

7 Matsumoto K, Tamari M and Saito H: Involvement of eosinophils in the onset of asthma. J Allergy Clin Immunol *121*: 26-27, 2008.

8 Kita H: Eosinophils: Multifunctional and distinct properties. Int Arch Allergy Immunol *161*: 3-9, 2013.

9 Casale TB, Andrade C and Qu R: Safety and efficacy of once-daily fexofenadine HCl in the treatment of autumn seasonal allergic rhinitis. Allergy Asthma Proc *20*: 193-198, 1999.

10 Watase F, Watanabe S, Kanai K, Yamada N, Sakaue S, Asano K, Hisamitsu T and Suzaki H: Modulation of eosinophil survival by epinastine hydrochloride, an H1 receptor antagonist *in vitro*. In Vivo *22*: 687-692, 2008.

11 Shoji N, Asano K, Furuta A, Hirano K and Suzaki H. Effect of histamine H1 receptor antagonists on TARC/CCL17 and MDC/CCL22 production from $CD44^+$ cells induced by antigenic stimulation *in vitro*. Int Arch Allergy Immunol *155*: 38-51, 2011.

12 Mahmoud F, Arifhodzic N, Haines D and Novotney L: Levocetirizine modulates lymphocyte activation in patients with allergic rhinitis. J Pharmacol *108*: 149-56, 2008.

13 Lee CF, Sun HL, Lu KH, Ku MS and Leu KH: The comparison of cetirizine, levocetirizine and placebo for the treatment of childfood perennial allergic rhinitis. Pediatr Allergy Immunol *20*: 493-499, 2009.

14 Tzanetos DB, Fahrenholz JM, Scott T and Buchholz K: Comparison of the sedating effects of levocetirizine and cetirizine: A randomized, double-blind, placebo-controlled trail. Ann Allergy Asthma Immunol *107*: 517-522, 2011.

15 Gillard M, Benedetti MS, Chatelain P and Baltes E: Histamine H_1 receptor occupancy and pharmacodynamics of second generation H_1-antihistamines. Inflamm Res *54*: 367-369, 2005.

16 Thomson L, Blaylock MG, Sexton DW, Campbell A and Walsh GM: Cetirizine and levocetirizine inhibit eotaxin-induced eosinophil transendothelial migration through human dermal or lung microvascular endothelial cells. Clin Exp Allergy *32*: 1187-1192, 2002.

17 Mochizuki Y, Furuta A, Furuya A, Kanai K, Asano K and Suzaki H: Suppressive activity of epinastine hydrochloride on eosinophil activation *in vitro*. In Vivo *22*: 13-20, 2008.

18 Kanei A, Asano K, Suzaki I, Kanai K and Suzaki H: Suppressive activity of levocetirizine on eosinophil degranulation *in vitro* and *in vivo*. Jpn Pharmacol Ther *41*: 597-604, 2013.

19 Nogaki T, Asano K, Furuta A, Kanai K, Suzaki I, Kanei A and Suzaki H: Enhancement of Clara cell 10 kDa protein (CC10) production from nasal epithelial cells by fexofenadine hydrochloride. Asian Pacific Allergy Imunol *30*: 139-145, 2012.

20 Dixon H, Blanchard C, deSchoolmeester ML, Yuill NC, Christie JW, Rothenberg ME and Else KJ: The role of Th2 cytokines, chemokines and parasite products in eosinophil recruitment to the gastrointestinal mucosa during helminth infection. Eur J Immunol *36*: 1753-1763, 2006.
21 Schoepke N, Church MK and Maurer M: The inhibition by levocetrizine and fexofenadine of the histamine-induced wheal and flare response in healthy Caucasian and Japanese volunteers. Acta Derm Venereol *93*: 286-293, 2013.
22 Trivedi SG and Lloyd CM: Biomedicine and diseases: Eosinophils in the pathogenesis of allergic airways disease. Cell Mol Life Scie *64*: 1269-1289, 2007.
23 Conroy DM and Williams TJ. Eotaxin and the attraction of eosinophils to the asthmatic lung. Respir Res *2*: 150-156, 2001.
24 Pease JE: Asthma, allergy and chemokine. Current Drug Targets *7*: 3-12, 2006.
25 Romagnani S: Cytokines and chemoattractants in allergic inflammation. Mol Immunol *38*: 881-885, 2001.
26 Kaplan AP: Chemokines, chemokine receptors and allergy. Int Arch Allergy Immunol *124*: 423-431, 2001.
27 Conti P, Pang X, Boucher W, Letourneau R, Reale M, Barbacane RC,Thibault J and Theoharides TC: Impact of RANTES and MCP-1 chemokines on *in vivo* basophilic cell recruitment in rat skin injection model and their role in modifying the protein and mRNA levels for histidine decarboxylase. Blood *89*: 4120-4127, 1997.
28 Conti P and DiGioacchino M: MCP-1 and RANTES are mediators of acute and chronic inflammation. Allergy Asthma Proc *22*: 133-137, 2001.
29 Amerio P, Frezzolini A, Feliciani C, Verdolini R, Teofoli P, Pita O and Puddu P: Eotaxins and CCR3 receptor in inflammatory and allergic skin diseases: Therapeutic implications. Curr Drug Targets Inflamm Allergy *2*: 81-94, 2003.
30 Shakoory B, Fitzgerald SM, Lee SA, Chi DS and Krishnaswamy G: The role of human mast cell-derived cytokines in eosinophil biology. J Interferon Cytokine Res *24*: 271-81, 2004.
31 Kanai K, Asano K, Hisamitsu T and Suzaki H: Suppression of matrix metalloproteinase production from nasal fibroblasts by macrolide antibiotics *in vitro*. Eur Respir J *23*: 671-678, 2004.
32 Asano K, Kanai K and Suzaki H: Suppressive activity of fexofenadine hydrochloride on the production of eosinophil chemoattractants from human nasal fibroblasts *in vitro*. Arzneim Forsch/ Drug Res *54*: 436-43, 2004.
33 Fujikura T, Shimosawa T and Yakuo I: Regulatory effect of histamine H1 receptor antagonist on the expression of messenger RNA encoding CC chemokines in the human nasal mucosa. J Allergy Clin Immunol *107*: 123-128, 2001.
34 Di Sciascio MB, Vianale G, Verna N, Petrarca C, Perrone A, Toniato E, Muraro R, Conti P and Di Gioacchino M: Eosinophil recruiting chemokines are down-regulated in peripheral blood mononuclear cells of allergic patients treated with deflazacort or desloratadine. Int J Immunophathol Pharmacol *20*: 745-751, 2007.

Received February 2, 2014
Revised February 25, 2014
Accepted February 26, 2014