

Cetirizine Decreases Interleukin-4, Interleukin-5, and Interferon- γ Gene Expressions in Nasal-Associated Lymphoid Tissue of Sensitized Mice

Hong-Ryul Jin, M.D.,* Yoshitaka Okamoto, M.D.,# Zensei Matsuzaki, M.D.,# Shuichiro Endo, M.D.,# and Eiko Ito, Ph.D.§ (Korea) (Japan)

ABSTRACT

Although the action of cetirizine dihydrochloride (cetirizine), a potent histamine H₁ receptor antagonist, has been well known, its effect on the cytokine profiles in the nasal immune inductive site has not been elucidated yet. We studied the effect of cetirizine on the cytokine profiles in the nasal-associated lymphoid tissue (NALT), which is a principal mucosal lymphoid tissue of the respiratory tract in rodents. Two different doses of cetirizine were given intraorally for 5 days before the nasal challenge of ovalbumin in sensitized mice. The sensitized group was given normal saline instead of cetirizine, and the nonsensitized group had no sensitization or medication. The cytokine gene expressions in the NALT taken from the mice were investigated with real-time quantitative reverse-trans-

cription polymerase chain reaction. The effect of cetirizine on the allergic symptom score, histamine threshold, and the eosinophil count in the nasal septal mucosa were examined also. Compared with the normal mice, the sensitized mice showed significantly increased levels of interleukin (IL)-4 and IL-5 gene expression although the increase of interferon (INF)- γ gene expression was not significant. In the cetirizine groups, the levels of expression of IL-4, IL-5, and INF- γ in the NALT were significantly decreased compared with the sensitized group. The cetirizine groups also showed decreased allergic symptom score, histamine threshold, and eosinophil count in the nasal septal mucosa compared with the sensitized group. In conclusion, cetirizine reduced the levels of expression of IL-4, IL-5, and INF- γ in the NALT of ovalbumin-sensitized mice. Cetirizine also reduced the acute allergic symptom, histamine sensitivity, and eosinophil count in the nasal septal mucosa. (American Journal of Rhinology 16, 43–48, 2002)

From the *Department of Otolaryngology, College of Medicine, Chungbuk National University, Korea, #Department of Otolaryngology, Yamanashi Medical University, Japan, and §Department of Otolaryngology, School of Medicine, Akita University, Japan

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Address correspondence and reprint requests to Hong-Ryul Jin, M.D., Department of Otolaryngology, Chungbuk National University Hospital, 62 San Gaeshin-dong, Heungduk-gu, Cheongju, 361–711 Korea

Cetirizine, a well-known second-generation antihistamine, inhibits the effect of histamine, which is a major mediator of the allergic reaction. Cetirizine also inhibits inflammatory mediator release from basophils and mast cells and reduces intercellular adhesion molecule 1 expression.^{1–4} Despite these well-known effects, the action of cetirizine on cytokine secretion in allergic inflammation is somewhat controversial. A number of *in vitro* studies have shown that cetirizine inhibits cytokine secretion from mast cells, basophils, and nasal epithelial cells.^{5–7} But stud-

ies using stimulated bronchial epithelial cells⁸ or skin biopsy specimens of allergic patients⁹ have shown that antihistamines including cetirizine had no significant effect on cytokine production. Thus, the action of cetirizine on cytokine production in allergic inflammation needs further elucidation.

Murine nasal-associated lymphoid tissue (NALT), found as bilateral strips of subepithelial, nonencapsulated lymphoid tissue on the ventral aspect of the posterior nasal tract, is a principal mucosal lymphoid tissue of the respiratory tract in rodents and is considered to be the equivalent of Waldeyer's ring in human.¹⁰ NALT also is an effective site of inducing nasal mucosal immunity against intranasal allergen.^{11,12} This study investigated the effect of cetirizine on Interleukin (IL)-4, IL-5, and Interferon γ (INF)- γ gene expression profiles of mucosal lymphocytes of NALT in ovalbumin (OA)-sensitized mice. The effects of cetirizine on the acute allergic symptom, histamine threshold, and eosinophil infiltration of the nasal mucosa were studied also.

MATERIALS AND METHODS

Animal Sensitization, Challenge, and Cetirizine

Healthy young male C57BL/6 mice 6–8 weeks of age and weighing ~20 g were obtained from Nippon SLC (Shizuoka, Japan). Mice were maintained in barrier-protected animal facilities under pathogen-free conditions in the experimental animal facilities of the Yamanashi Medical University. The animal protocol used in this study was reviewed and approved by the Animal Care Advisory Committee of the Yamanashi Medical University.

General sensitization was induced with intraperitoneal injection of 1 μ g of OA (chicken egg albumin, grade V; Sigma, Saint Louis, MO) conjugated with 2 mg of alum (Pierce, Rockford, IL). This was repeated on the 5th, 12th, and 18th day with four total injections. *Bordetella pertussis* toxin (0.02 mL) was injected intradermally on the 1st and 18th days. One week after the last intraperitoneal injection, the animals were sensitized locally daily for 5 days by applying 400 μ g of OA dissolved in 2 μ L of normal saline intranasally. Allergy was confirmed with percutaneous anaphylaxis reaction using guinea pigs and sera of the sensitized mice.

Cetirizine, dissolved in normal saline, was given orally to 14 mice (3.5 and 35 μ mol/kg, 7 mice each) 2 hours before the nasal sensitization for 5 days (low-dose and high-dose cetirizine groups). Normal saline instead of cetirizine was given orally to seven mice (sensitized group). Five normal mice with no sensitization or treatment (nonsensitized group) were used also for comparison.

Allergic Symptom Score and Histamine Threshold Test

One day after the final local sensitization, the number of nasal scrubbing movements was counted for 10 minutes after intranasal OA application. One point was given to

every five consecutive scrubbing movements, which are very fast, or to one isolated, relatively slow scrubbing movement. The next day, histamine threshold was measured by scoring the scrubbing movements after intranasally applying various concentrations of histamine solution. If the symptom score was under 20, the mice were tested with histamine solution of a higher dose. The histamine threshold was determined by averaging the histamine concentration of the mice in which the symptom scores were over 20.

Preparation of NALT Lymphocytes

The following day after evaluation of allergic symptom score and histamine threshold, mice were killed with cervical dislocation and then intracardiac blood was extracted with a syringe. After collecting blood for allergy confirmation, the head of the mouse was severed along the line between the upper and lower jaws. The tip of the nose containing the foreteeth was cut off. The facial skin and cheek muscles were removed, and the nasal part was separated from the rest of the head along the line of eyeballs. After peeling away the palatal mucosa, NALT, which is localized bilaterally on the posterior side of the palate, was teased gently with sharp forceps and collected in a 1.5-mL Eppendorf tube. The NALT fragment, which accumulated and formed a nodule between a columnar epithelium and the palate wall, was liberated easily. After removing NALT, the remaining tissue was immersed in 10% formaldehyde solution for histology.

Number of Eosinophils in the Nasal Mucosa

The fixed nasal mucosa was stained with hematoxylin and eosin after sectioning with thickness of 4–5 μ m. The number of eosinophils was counted in 10 high-power fields (400 $\times$) and the mean was obtained.

RNA Extraction, Reverse Transcription, and Real-Time Quantitative Polymerase Chain Reaction

Total RNA was isolated from NALT using an RNA extracting kit (RNeasy Mini Kit; Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. The quality of RNA was assessed by conventional denaturing gel electrophoresis. Approximately 1000 ng of total RNA from each sample was reverse transcribed (RT) in a 100- μ L volume reaction using 125 U of MultiScribe reverse transcriptase (Perkin-Elmer Corp./PE Applied Biosystems, Foster City, CA), 40 U of ribonuclease inhibitor (Perkin-Elmer Corp./PE Applied Biosystems), 2.5 μ M of random hexamer (Perkin-Elmer Corp./PE Applied Biosystems), 500 μ M of deoxynucleoside triphosphate mixture, and 5.5 mM of magnesium chloride.

Predeveloped primers and probes for mouse IL-4, IL-5, and INF- γ complementary DNA (cDNA) were used for polymerase chain reaction (PCR). The amplicons produced by PCR were subsequently analyzed by gel electrophoresis. Real-time quantitative PCR was achieved in 96-well reaction plate using a cDNA equivalent of 80 ng/total of RNA.

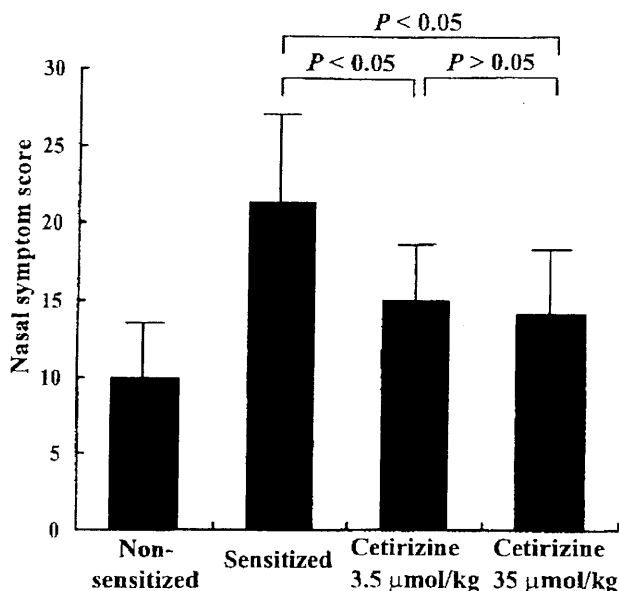


Figure 1. Allergic symptom score. Nasal scrubbing movements were counted for 10 minutes after OA challenge. One point was given to every five consecutive scrubbing movements or one isolated scrubbing movement.

50 µL/tube with the TaqMan Universal Master Mix including AmpliTaq Gold DNA polymerase, AmpErase uracil-N-glycosylase, deoxynucleoside triphosphate, deoxyuridine triphosphate, and optimized buffer (Perkin-Elmer Corp./PE Applied Biosystems) according to the manufacturer's instructions. PCR was developed on a GeneAmp 5700 Sequence Detector (Perkin-Elmer Corp./PE Applied Biosystems). To normalize for differences in the amount of total RNA added to the reaction, amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was performed as an endogenous control. Primers and probes for cytokines and GAPDH gene were obtained from the Perkin-Elmer Corp./PE Applied Biosystems. The mRNA content of each target gene was determined in 14 samples of the two cetirizine-treated groups, 7 control samples, and 5 normal samples.

Statistical Method

The symptom score, histamine threshold, eosinophil count, and mRNA expression profiles were compared between each group using analysis of variance with Duncan's multiple comparison tests. Significant difference was accepted when $p < 0.05$.

RESULTS

Allergic Symptom Score, Histamine Threshold, and Eosinophil Count

The sensitized group showed a significantly increased symptom score ($p < 0.05$) and eosinophil number ($p < 0.05$), and a significantly decreased histamine thresh-

old ($p < 0.05$) compared with the nonsensitized group. The cetirizine groups showed a significantly decreased symptom score ($p < 0.05$) and eosinophil number ($p < 0.05$) and a significantly increased histamine threshold ($p < 0.05$) compared with the sensitized group (Figs. 1–3). No differences were found between the two different cetirizine groups in symptom score, histamine threshold, and eosinophil numbers ($p > 0.05$).

Level of Cytokine Gene Expression

To validate the real-time PCR method for comparative quantitation of messenger RNA (mRNA) levels, the amplification efficiency of the target gene and GAPDH was measured by serially diluting the cDNA samples. The efficiency of the amplification, as determined by the slope of the plot curve, allowed us to quantify the cytokine gene expression by using the comparative threshold cycle method according to the manufacturer's instructions. The calibrator was constituted from one sample of normal mice; it was used as the 1× sample, and all other levels were expressed as an n -fold difference relative to the calibrator (Table I). Fig. 4 shows one example of the amplification plots for mRNA of cytokines and GAPDH.

Compared with the nonsensitized group, the IL-4 and IL-5 gene expressions were significantly higher in the sensitized group ($p < 0.01$). In contrast, the mean INF- γ gene expression showed a trend toward increase compared with the nonsensitized group, but failed to achieve significance ($p > 0.05$).

In both cetirizine groups, profound alterations were detected in the expression of the three target genes. Compared with the sensitized group, the cetirizine groups showed significantly decreased levels of all three cytokine gene expressions. In the low-dose cetirizine group, the IL-4 mRNA level was 4- to 20-fold (mean, 10-fold), the IL-5

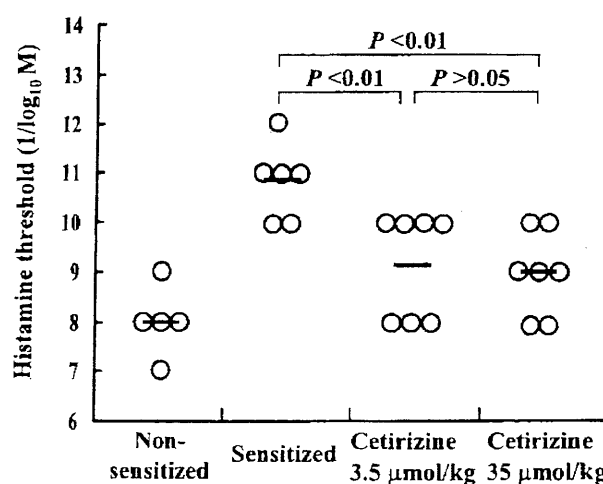


Figure 2. Level of histamine threshold. Concentrations of histamine causing an allergic symptom score of >20 during 10 minutes were indicated as thresholds.

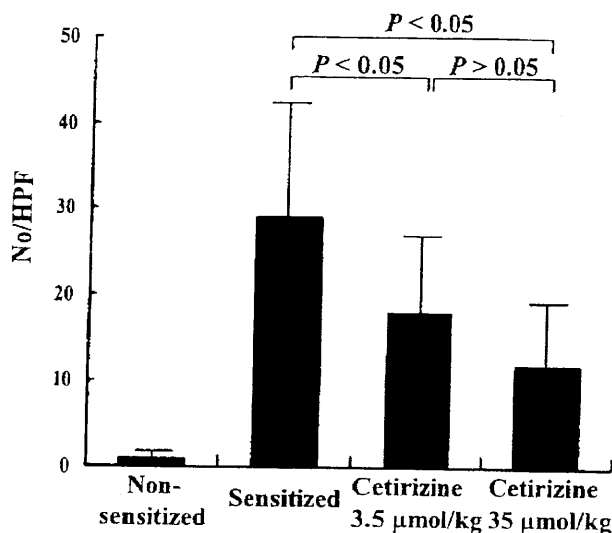


Figure 3. The number of eosinophils in the nasal septal mucosa. The number of eosinophils was counted in 10 high-power fields (magnification $\times 400$) and averaged.

mRNA level was 4- to 18-fold (mean, 11-fold), and the INF- γ mRNA level was 2- to 31-fold (mean, 11-fold) lower than that in the sensitized group ($p < 0.01$). In the high-dose cetirizine group, the IL-4 mRNA level was 20- to 9-fold (mean, 47-fold), the IL-5 mRNA level was 7- to 27-fold (mean, 17-fold), and the INF- γ mRNA level was 2- to 23-fold (mean, 8-fold) lower than that in the sensitized group ($p < 0.01$).

Comparing the levels of cytokine gene expressions between the cetirizine groups and the nonsensitized group, the INF- γ in both cetirizine groups and the IL-4 in the high-dose cetirizine group showed lower levels than that in the nonsensitized group ($p < 0.05$). Although the levels of IL-5 gene expressions were not significantly different in normal and in both cetirizine groups ($p > 0.05$), the level of IL-4

gene expression in the low-dose cetirizine group was higher than that in the nonsensitized group ($p < 0.05$).

DISCUSSION

Applying various methods of measuring cytokine expression in NALT is very limited because the small numbers of cells recovered prohibit the analysis of cytokine secretion directly. In most studies, the cytokine expression was measured by enzyme-linked immunospot assay¹³ or by conventional RT-PCR^{11,14} from the cultured T cells isolated from NALT or by ELISA from supernatant of cultured cells after antigen stimulation.¹⁵ In this study, using real-time quantitative RT-PCR from NALT, we could measure cytokine gene expression more sensitively and accurately than enzyme-linked immunospot assay or conventional RT-PCR and also more directly than previous methods by excluding the variations evolving from culture of cells.

The dosage of cetirizine we used in this study was higher than the usual dosage in humans. Higher dosage was needed to compensate for the relatively short period of cetirizine administration. Because the cetirizine powder dissolved in normal saline was very acidic (pH was almost 2–3), we adjusted the pH into neutral to make the solution more acceptable to the mice.

The clinical actions of cetirizine in patients who were allergic are summarized as a decrease of acute allergic symptoms and histamine sensitivity.¹⁶ These actions were confirmed in this study by decreased allergic symptom score and increased histamine threshold in the both cetirizine groups compared with the sensitized group.

It has been revealed that T cells in NALT are CD4⁺ in 75% and such CD4⁺ T cells have Th0 environment, which can gain a Th1 and/or Th2 phenotype depending on the stimulus.^{11,15} The allergic inflammation induced by OA sensitization may have directed the Th0 environment of NALT to the Th2 environment and thus increasing the levels of Th2 cytokines like IL-4 and IL-5 as shown in this

TABLE I

Distribution of mRNA Values According to the Group [Mean (SD), Median (min–max)]

Groups	No.	IL-4	IL-5	INF- γ
Nonsensitized	5	2.2 (2.3) 1.0 [0.6–6.3]	1.3 (0.6) 1.2 [0.6–2.2]	2.2 (2.5) 1.0 [0.6–6.3]
Sensitized	7	51.7 (31.8) 52.0 [22.5–109]	16.5 (7.4) 15.9 [6.5–26.7]	3.2 (3.0) 2.5 [0.7–9.2]
Low-dose cetirizine (3.5 μ mol/kg)	7	5.4 (3.6) 3.7 [2.0–10.7]	1.5 (0.7) 1.2 [0.9–2.8]	0.3 (0.2) 0.3 [0.0*–0.5]
High-dose cetirizine (35 μ mol/kg)	7	1.1 (0.5) 1.2 [0.2–1.7]	1.0 (1.1) 0.5 [0.2–3.4]	0.4 (0.3) 0.3 [0.0*–0.9]

Results are expressed as an n-fold difference relative to the calibrator.

* < 0.1 .

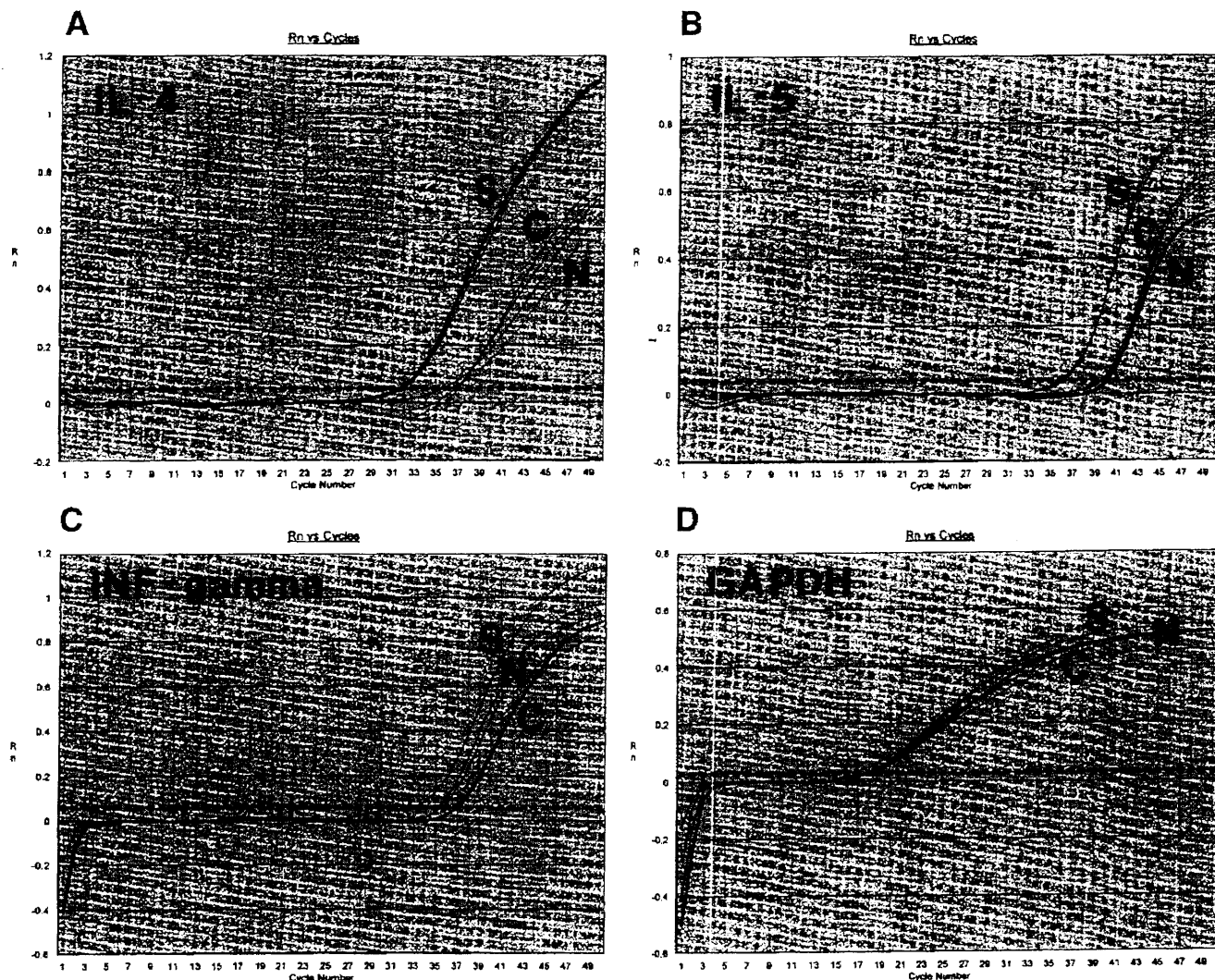


Figure 4. Amplification plots of the (A) IL-4, (B) IL-5, and (C) INF- γ mRNAs plus (D) GAPDH genes. Eighty nanograms of total RNA extracted from a mouse of each group were used in the Taqman PCR assay. Rn indicates the magnitude of fluorescence generated by the PCR reaction. N, nonsensitized mouse; S, sensitized mouse; C, cetirizine (35 μ mol/kg)-treated mouse.

study. Although the levels of Th2 cytokines were significantly increased in sensitized mice compared with normal mice, the increase of INF- γ was not significant. Despite controversial results about the level of INF- γ in patients who were allergic,¹⁷⁻¹⁹ the current hypothesis is that imbalances between Th2 and Th1 cytokines seem to be responsible for allergic conditions.¹⁷ Our study clearly showed an increased ratio of Th2 (IL-4 and IL-5)/Th1 (INF- γ) cytokines in sensitized mice compared with normal mice.

This study showed that high Th2/Th1 cytokine ratio in mice that were allergic changed to a low Th2/Th1 ratio with cetirizine treatment, indicating the role of cetirizine in normalizing cytokine balance. This action was more effective with higher doses (35 μ mol/kg) of cetirizine. This was primarily caused by the more dramatic decrease in the Th2 cytokine expression compared with the decrease of Th1

cytokine expression. This effect probably is because cetirizine interfered with the cytokine cascade through its antiallergic actions such as blocking inflammatory mediator release from basophils and mast cells, as well as reducing intracellular adhesion molecule 1 expression in epithelial cells,^{2,16} thus limiting the incursion of inflammatory cells to the site of allergic reaction. The reduced nasal inflammation may have influenced the NALT, which is a well-known site of effectively inducing nasal mucosal immunity against intranasal allergen.^{11,12,15} Although *in vivo* studies are rare, some *in vitro* studies have shown the same inhibitory action of cetirizine on the cytokine production from cultured mast cells, basophils, or respiratory epithelial cells.⁵⁻⁷

Reduced expression levels of IL-4 and IL-5, which promote eosinophil recruitment and activation, may be one explanation for our finding of significantly decreased eo-

sinophils in septal mucosa in the cetirizine groups compared with the sensitized group. Another possible mechanism for decreased eosinophils is the blocking of inflammatory mediator release from the mast cell leading to decreased inflammatory cell recruitment including eosinophils. These results are in line with other literature, which indicates decreased eosinophils in the site of allergic inflammation by cetirizine treatment.²⁰⁻²²

CONCLUSION

Cetirizine treatment reduced the levels of IL-4, IL-5, and INF- γ gene expressions in NALT of OA-sensitized mice. Cetirizine treatment also reduced the acute allergic symptom and eosinophil count in the nasal septal mucosa and increased the histamine threshold in OA-sensitized mice.

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XYZAL												
Nature	Country	Investigator	Proposal short title	Date in	LRC	GRC	UEG - Sample	NSSL	ample se	Status + FU	Start	End
C	CDN	Day	Environmental Exposure	02-mag-01	NA	questions	NA	NA	Bulle	CANCELLED; => R&D st	NA	NA
C	CDN	Simons	PK-PD levo in children	10-set-01	NA	11-ott-01	NA	NA	Bulle	CANCELLED; CAG	NA	NA
C	CDN	Wilson	AMP challenge	12-ott-01	NA	26-ott-01	NA	NA	Bulle	CANCELLED; CAG	NA	NA
C	CH	Müller	Levo + immunotherapy	22-ott-01	yes	13-nov-01	64 K Sfr, table	#####	Bulle	Ongoing; IRO	mag-02	ago-03
C	CH	Spertini	Nasal provocation	23-ott-01	yes	07-nov-01	levo, deslorat, p	#####	Bulle	Ongoing; IRO	Mar-02	set-02
C	CH	Wüthrich	Wheal & Flare, levo, de	06-lug-01	yes	24-ott-01	levo + deslorat.	?	Bulle	Ongoing; IRO	mag-02	set-02
C	F	Michel	Eosinophils in skin	30-nov-01	yes	17-dic-01	tablets + placebo	#####	Bulle	Ongoing; MME	apr-02	giu-03
C	D	Gutges	Physical urticaria	12-mar-02	yes		tablets + placebo	?		Pending; BSC	giu-02	dic-02
C	I	Marcucc	Nasal ECP, children	09-mag-01	yes	07-ago-01	drops + placebo + CRF + i		Bulle	Ongoing; BFE	nov-01	apr-02
C	UK	Church	Wheal & Flare	04-apr-01	yes	30-mag-01	active + placebo	NA	Bulle	Ongoing; NBR	?	?
C	UK	Shakir (	Prescription Event Mon	19-apr-02	yes	02-mag-02	80 K Pound, UK	?	NA	Pending; JRO	lug-02	gen-03
NC	B	Bachert	Human macrophages	24-mar-01	yes	08-nov-01	25 K Euro, sampl	NA	Bulle	Ongoing; JKS, ACA	mar-02	?
NC	D	Kapp	Human lymphos. & eosin	13-feb-01	yes	22-feb-01	sample	#####	Bulle	Completed: report 09-J	NA	NA
NC	UK	Hill	Human smooth muscle	26-ott-01	yes	08-nov-01	sample	NA	Bulle	Ongoing; NBR, ACA	?	?
NC	UK	Warner	Proposal?				sample			Pending; Proposal? ACA, NBR		