

Full Paper

Levocetirizine Modulates Lymphocyte Activation in Patients With Allergic RhinitisFadia Mahmoud^{1,*}, Nirmina Arifhodzic², David Haines³, and Ladislav Novotney⁴¹Department of Medical Laboratory Sciences, Faculty of Allied Health Sciences, ⁴Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Kuwait University, Sulaibikhat 90805, Kuwait²Al-Rashed Allergy Center, Sulaibikhat 90806, Kuwait³Department of Molecular and Cell Biology, University of Connecticut, Storrs, CT 06269, USA

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Abstract. Levocetirizine, a second generation non-sedating antihistamine that blocks the H₁ histamine receptor, may exhibit immunoregulatory properties that augment its primary pharmacological mechanism. To investigate this possibility, 13 Kuwaiti seasonal allergic rhinitis (SAR) patients were treated with levocetirizine for four weeks in comparison with a 7-member placebo-treated control group, followed by clinical evaluation and flow cytometric analysis of peripheral venous blood for inflammatory cell and lymphocyte subpopulation profiles. Relative to the controls, levocetirizine-treated patients exhibited an expected reduction in early phase allergic symptoms, including sneezing ($P<0.001$), nasal itching ($P<0.01$), nasal congestion, and running nose ($P<0.001$); reduced percentages of eosinophils ($P<0.05$); and three subpopulations of activated T lymphocytes: CD4+CD29+, CD4+CD212+, and CD4+CD54+ ($P<0.05$). Levocetirizine treatment also correlated with a significant increase in the percentage of CD4+CD25+ T cells ($P<0.001$). The ability of levocetirizine to reduce percentage representation of cell phenotypes known to contribute to inflammatory tissue damage (eosinophils, CD4+CD29+, CD4+CD212+, and CD4+CD54+) and expand percentages of CD4+CD25+, which may include protective immunoregulatory (Treg) cells, indicates that the drug has pharmacological potential beyond the immediate effects of H₁ histamine–receptor inhibition. Although the present data does not define a therapeutic mechanism, the results reported here establish important trends that may be used to guide future mechanistic examination of immunoregulatory capacity of H₁ inhibitors.

Keywords: T cell, peripheral blood, rhinitis, regulatory T cell (Treg), interleukin-12 receptor β_1

Introduction

Seasonal allergic rhinitis (SAR) is a global health condition affecting an estimated 20% of the population worldwide (1) and is closely associated with a variety of other conditions including asthma due to common underlying inflammatory processes (2). SAR is a typical atopic disease caused by immunoglobulin-E (IgE) antibody–mediated inflammation of nasal mucosa in genetically susceptible people who produce high levels of IgE in response to environmental antigens such as those found in pollen grains, dust mites, and other

immunogenic sources (allergens). Binding of allergen-IgE complexes to high affinity IgE receptors on mast cells and basophils triggers release by these cells of histamine, a leading mediator of the initial (early phase) allergic response. In patients with allergic rhinitis, histamine is responsible for early phase clinical symptoms such as nasal itching, sneezing, and running nose that are induced by histamine binding to H₁ histamine receptors (3, 4). The pathogenesis of allergic rhinitis is also characterized by nasal obstruction, a symptom caused by influx of activated inflammatory cells, predominantly eosinophils, allowing the disorder to be characterized as a chronic inflammatory syndrome (5). Antihistamines have demonstrated clinical efficacy against symptoms of SAR and consequently remain a first-line therapy for many allergic conditions (6, 7).

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These drugs have been accepted as highly effective therapy for allergic rhinitis based on their ability to specifically counteract major symptoms of the disorder, sneezing, nasal itching, and rhinorrhea (8, 9). A new generation of antihistamines capable of modulating inflammatory immune processes at various stages may optimize treatment of allergic disease. Several second-generation antihistamines have proven anti-inflammatory properties, including suppression of nasal congestion (9–12), down-regulation of cell adhesion molecule expression, and inhibition of cytokine production (13). The combined anti-allergic/anti-inflammatory properties of H₁-inhibitory antihistamines have been confirmed by mechanistic studies of a number of agents in this drug class, which may partly explain the high efficacy of these drugs in the treatment of allergic disease with an underlying inflammatory pathology (14). Some antihistamines can modulate immunological mechanisms involved in the pathogenesis of allergic inflammation by reducing inflammatory mediator release and the expression of adhesion molecules, and by regulating the release of cytokines and chemokines and consequently inflammatory cell recruitment in vitro (15). However, with some exceptions, the majority of anti-allergic/anti-inflammatory effects observed in vitro are noted at concentrations that are several hundred-fold higher than therapeutic levels and are therefore clinically irrelevant. Additionally, it is clear that the H₁ antihistamines differ with regard to their anti-allergic/anti-inflammatory potencies in vitro and in vivo (14). Levocetirizine (Xyzal[®]) is an oral histamine H₁-receptor antagonist of the latest generation that is licensed for the symptomatic treatment of allergic rhinitis (16). It is the active enantiomer of cetirizine, with twice the affinity for the H₁ receptor compared with cetirizine and is a potent antihistamine as demonstrated by inhibition of histamine-induced wheal and flare reactions (17) and in clinical studies (18). The compound is effective and generally well tolerated in the treatment of SAR (19). Levocetirizine affects several major pro-inflammatory

processes. The drug inhibits eotaxin-induced eosinophil transendothelial migration (20) and improves airway hyper-responsiveness to adenosine monophosphate (AMP) (21, 22), each of which are potent pro-inflammatory mechanisms known to contribute to the pathogenesis of allergies and asthma. Studies of its pharmacology suggest that it modulates the profile of inflammatory mediators, including cytokines, growth factors, proteinases, and antiproteinases produced by eosinophils in vitro (23); however, the effect of therapeutic levels in vivo is still under investigation. As the secretion of cytokines from lymphocytes, particularly the Th2 subset of lymphocytes, appears to be central to the establishment and maintenance of allergic inflammation, it would seem pertinent to examine the effects of levocetirizine on lymphocyte regulation. The aim of this study was to investigate the effectiveness of levocetirizine in exerting anti-inflammatory effects at therapeutic concentrations in allergic rhinitis.

Materials and Methods

Patients

Table 1 shows the description of patients included in this study. Subjects included twenty SAR patients (11 males and 9 females, mean 31.1 ± 2.2 years). The diagnosis of SAR was made, according to international guidelines (24), on the basis of typical clinical history and clinical features for at least 3 consecutive pollen seasons in Kuwait, confirmed by a positive skin prick test (SPT) to one or more of local pollen allergen (e.g., *Salsola kali*, Bermuda grass). SPT evaluations were accomplished using a standardized test (Stallerpoint) incorporating a battery of allergens (Stellergens SA, Antony Cedex, France). Wheal-and-flare reactions were recorded 15 min after application of the test substances. All the patients were recruited from the outpatient clinic of Al-Rashid Allergy Center, Kuwait in the second half of September, just at the beginning of the main pollen season in Kuwait, caused by pollination of local weeds,

Table 1. Description of patients receiving levocetirizine and placebo

Patients	Levocetirizine group	Placebo group
Number	13	7
Female	8	4
Male	5	3
Age	31.8 ± 2.8	29.4 ± 3.3
Duration of disease (years)	5 ± 3	4 ± 3
Positive SPT or sIgE >class 2		
Weed pollen sensitivity	9	4
Grass pollen sensitivity	5	2

known to be the main offending pollen allergen (4, 25). The following symptoms were assessed before and after treatment: sneezing, running and itchy nose, and nasal congestion. Each symptom was evaluated on the following scale: 0 = no symptoms, 1 = mild, 2 = moderate, 3 = severe. The total symptom score (TSS) being the sum of each individual symptom was considered. Only patients with history of SAR due to seasonal allergen exposure for the previous 2 years, rhinitis symptoms for the 2 previous weeks, and TSS minimum value >6 at baseline were included. Patients with acute and chronic upper respiratory infections within 30 days before the study, anatomic nasal disorders (i.e., septum deviation), nasal polyps, and those using antibiotics, nasal, or oral corticosteroids within the previous 4 weeks or antihistamines within the previous week were excluded. The blood samples were collected before treatment. Thirteen patients received 5 mg/day levocetirizine (UCB Pharma S.A., Brussels, Belgium) for four weeks and 7 patients received a placebo; this was followed by collection of another blood sample from each patient. Informed consent was obtained from every patient.

Lymphocyte analysis

A 5-ml sample of peripheral venous blood was collected from each subject in Vacutainer Brand, Evacuated Blood Collection sodium EDTA collection tubes (Vacutainer Systems; Becton Dickinson, Rutherford, NJ, USA) and analyzed within 4–6 h. A 50- μ l aliquot of blood was incubated for 30 min at room temperature with 5 μ l of fluorescein-isothiocyanate (FITC), phycoerythrin (RD1), or PerCP (peridinin chlorophyll protein) conjugated monoclonal antibodies (mAb), to surface markers of interest. The cells were then treated with Q-prep (Coulter Corporation, Hialeah, FL, USA) for hemolysis, stabilization, and amplification of the antigen-antibody reaction and fixation with paraformaldehyde. Two and three color fluorescence analysis using an automated flow cytometer (Coulter Epics Altra) was performed. Positive analysis regions for cells expressing specific surface antigens were compared with isotypic controls, and the specific binding of fluorophore-conjugated monoclonal antibodies was analyzed according to standard methods recommended by the manufacturer. Monoclonal antibodies specific for human T lymphocytes (CD3+) and subpopulations were used that included the following cell types within the CD3+ compartment: CD4+CD25+ (activated T cells – some with a regulatory phenotype), CD4+CD45RO+ (memory T cells), CD4+CD45RA+ (Naive T cells), CD4+CD29+ (helper/inducer T cells), CD4+CD54+ (ICAM1+ T cells), CD4+CD62+ (L-Selectin+ T cells), and CD4+CD212 [interleukin-12

receptor β_1 (IL-12R β_1)]. All fluorophores were purchased from Dakopatts, A/S, Copenhagen, Denmark and from Immunotech, Coulter Corporation, Hialeah, FL, USA.

Statistical analyses

Statistical analysis was performed using the independent *t*-test. All analyses were performed using the SPSS for Windows statistical package (Norusis/SPSS, Inc., Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant.

Results

The analysis of single symptoms showed that levocetirizine treatment of SAR patients reduced clinical symptoms typical for the early phase of the allergic response: reduced sneezing ($P < 0.001$), nasal itching ($P < 0.01$), nasal congestion and running nose ($P < 0.001$); while placebo treatment was not associated with a significant reduction in symptoms (Fig. 1). As shown in Fig. 2. Levocetirizine treatment significantly reduced the percentages of eosinophils ($P < 0.05$) but not neutrophils, whereas placebo treatment did not show any change in these cell populations.

No change was observed in total numbers or percentages of lymphocyte major populations (CD3+, CD4+, CD8+, CD19+, and CD16+CD56+ cells) at baseline and following levocetirizine or placebo treatment (data not shown). There was a significant decrease in the percentage of the effector helper/inducers cells (CD4+CD29+) and activated T cells expressing IL-12R β_1 (CD4+CD212+), as well as (CD4+CD54+: ICAM-1), ($P < 0.05$) following levocetirizine treatment, whereas a significant increase in frequency of regulatory T cells (Tregs) (CD4+CD25+) ($P < 0.001$) was observed. No significant change was observed in the placebo-treated group in these cell populations (Fig. 3). Furthermore, CD4+CD45RO+ (memory T cells) and CD4+CD45RA+ (Naive T cells) showed no significant difference at baseline versus levocetirizine or placebo treatment (data not shown).

Correlations were observed between lymphocyte subpopulation frequency and scores of single SAR symptoms in all patients at baseline. The percentage of CD4+CD25+ cells exhibited a positive correlation with nasal obstruction ($r = 0.771$) (Fig. 4), whereas the percentage of CD4+CD212+ cells correlated inversely both with nasal congestion ($r = -0.723$) (Fig. 5) and sneezing ($r = -0.768$) (Fig. 6). TSS was not associated with frequency of any lymphocyte subpopulation.

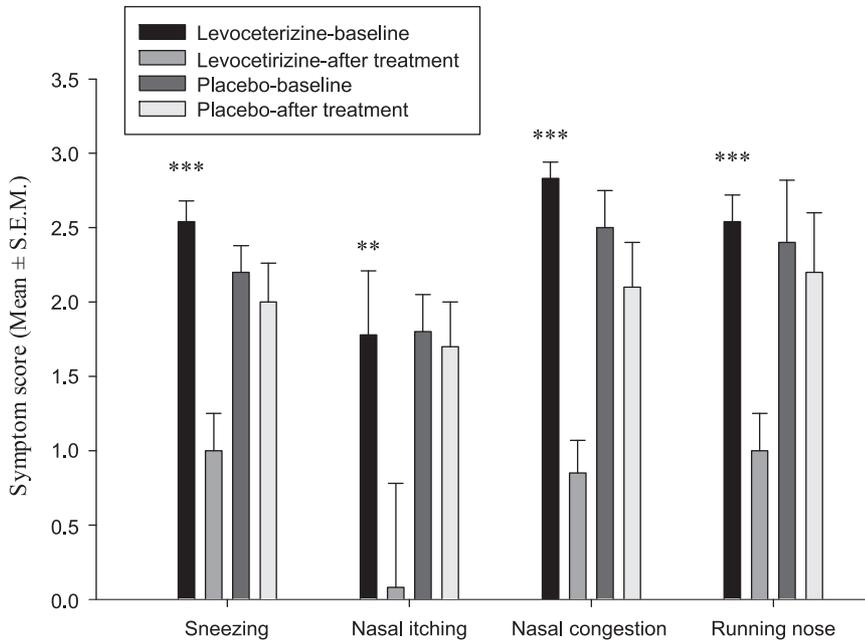


Fig. 1. Single nasal symptoms evaluation at baseline and after treatment in seasonal allergic rhinitis patients (SAR). Thirteen SAR patients treated with 5 mg/day levocetirizine and 7 receiving placebo are evaluated for major symptoms of seasonal allergic rhinitis (SAR) at baseline and following 4 weeks of treatment with levocetirizine or placebo. Each symptom was evaluated on the following scale: 0 = no symptoms, 1 = mild, 2 = moderate, 3 = severe. The total symptom score (TSS) being the sum of each individual symptom. No statistically significant change was observed in the placebo-treated group. Statistically significant differences in levocetirizine-treated group at baseline versus after treatment are shown, ** $P < 0.01$, *** $P < 0.001$.

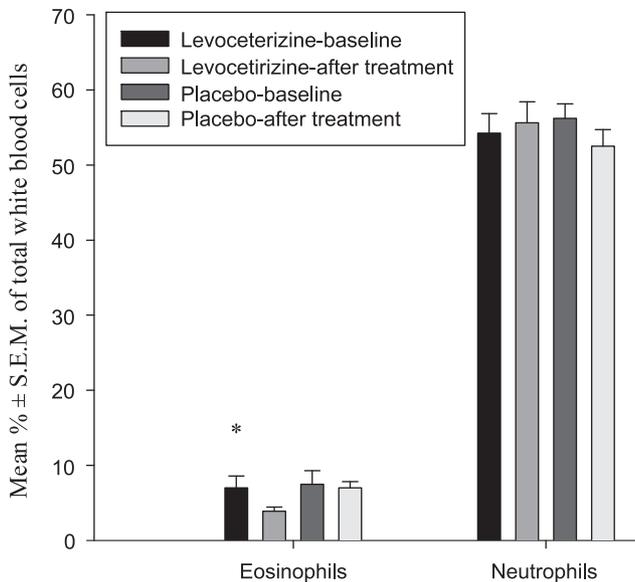


Fig. 2. Eosinophils and neutrophils in peripheral blood of seasonal allergic rhinitis patients (SAR) at baseline and after treatment. Percentage of eosinophils and neutrophils in total peripheral blood white blood cells were determined for 13 SAR patients treated with 5 mg/day levocetirizine and 7 receiving placebo. Measurements were made at baseline and following 4 weeks of treatment with levocetirizine or placebo. No statistically significant change was observed in the placebo-treated group. Statistically significant differences in levocetirizine-treated group at baseline versus after treatment are shown, * $P < 0.05$.

Discussion

The present study evaluates the capacity of levocetirizine to influence activation of lymphocytes and inflammatory cells in ways that may affect the pathogenesis of inflammation-associated features of SAR. Data in Fig. 1 shows levocetirizine to be effective in controlling the major symptoms of SAR, an expected result based on its known clinical effect. Treatment with the drug resulted in a significant decrease in peripheral blood eosinophils (Fig. 2) and three categories of activated T cells: CD4+CD29+, CD4+CD54+, and CD4+CD212+ (Fig. 3). Interestingly, one activated T cell population, CD4+CD25+, was observed to be significantly increased in the peripheral blood of persons receiving drug treatment (Fig 3). This effect might be accounted for by an increase in regulatory CD4+ T cells (Tregs) that express the CD25+ activation antigen and are known to increase representation in peripheral blood as a result of successful allergen-specific immunotherapy in allergic disease (26). Nevertheless, presence of Tregs among the CD4+CD25+ populations described in this study may only be inferred, since definitive proof would require demonstration of expression of the Treg-specific surface antigen FoxP3 (27). Figure 4 shows a strong positive correlation between size of the CD4+CD25+ population and nasal congestion SAR patients taken at baseline and therefore unrelated to treatment. Since nasal congestion is an indicator of inflammatory infiltrate within the nasal mucosa, increased levels of peripheral blood CD4+CD25+ may

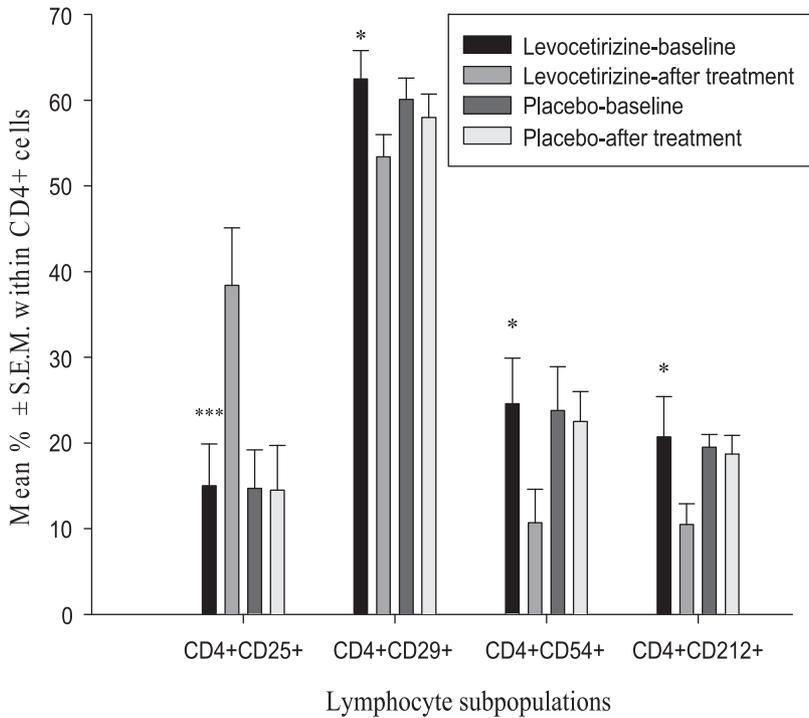


Fig. 3. T lymphocyte subpopulations in peripheral blood of seasonal allergic rhinitis (SAR) at baseline and after treatment. Percentages of 4 categories of activated T lymphocytes are determined by 3-color flow cytometry within the peripheral blood CD4+ subpopulation from 13 SAR patients treated with 5 mg/day levocetirizine and 7 receiving placebo. Measurements were made at baseline and following 4 weeks of treatment with levocetirizine or placebo. No statistically significant change was observed in the placebo-treated group. Statistically significant differences in levocetirizine-treated group at baseline versus after treatment are shown, * $P < 0.05$, *** $P < 0.001$.

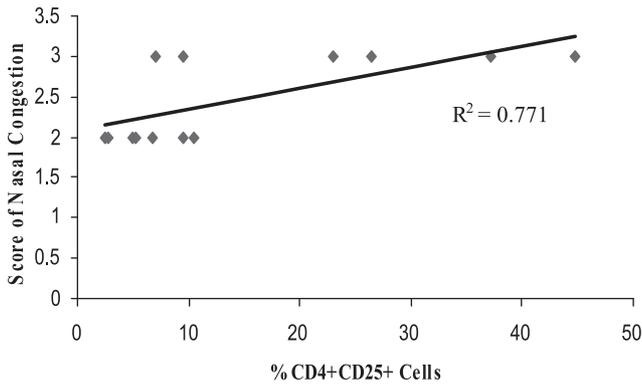


Fig. 4. Pearson correlation was used to measure the association between the score of nasal congestion and % CD4+CD25+. The correlation coefficient is given.

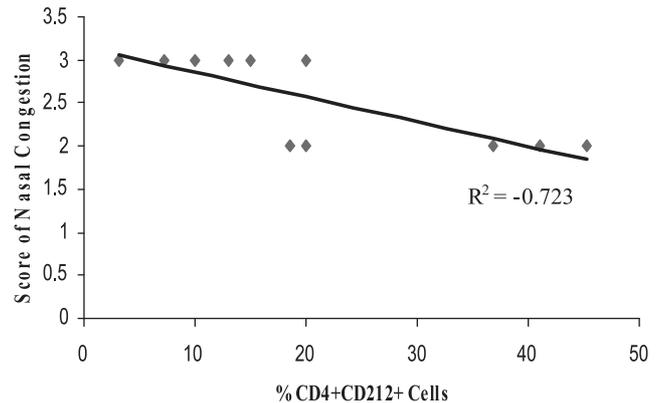


Fig. 5. Pearson correlation was used to measure the association between the score of nasal congestion and % CD4+CD212+. The correlation coefficient is given.

represent a naturally occurring host defense in which Treg activity is increased in order to downregulate the inflammatory process. Although this conclusion also will remain speculative until confirmation of the extent of Treg activity among the CD4+CD25+ population can be assessed, its occurrence would provide additional incentive for clinical use of levocetirizine in treatment of SAR, since the drug might augment Treg-mediated host immune defenses. These results are significant in optimizing clinical use of levocetirizine based on the known contribution to SAR pathogenesis of each of the aforementioned cell species exhibiting sensitivity to

the drug. Eosinophils and activated T cells are major mediators of inflammatory tissue damage in allergic disease (20, 23, 28–30). Relevance of this effect to SAR pathogenesis may be seen by consideration of the known biological roles of these cell species: CD4+CD29+ T cells (a helper/inducer subset) are increased in peripheral blood of patients with T cell-mediated diseases such as Guillain-Barre syndrome (28), in lymphocyte proliferation response tests to specific allergens in allergic contact dermatitis (29), in tissue infiltrate in atopic dermatitis (31), and nasal allergy (30). ICAM-1/CD54, a transmembrane glyco-

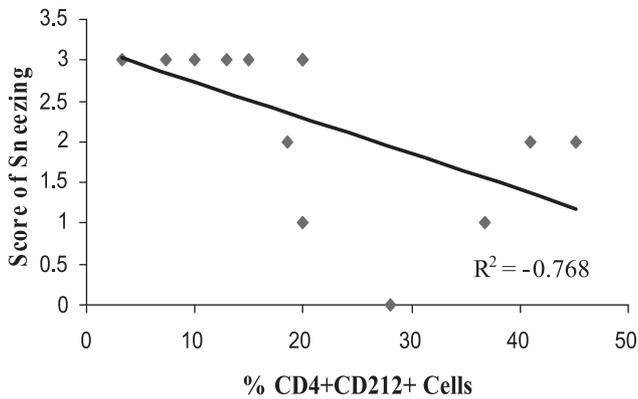


Fig. 6. Pearson correlation was used to measure the association between the score of sneezing and % CD4+CD212+. The correlation coefficient is given.

protein promoting adhesion in immunologic and inflammatory reactions, was found to be increased on nasal epithelial cells of patients with allergic rhinitis. Treatment with cetirizine, the isomer of levocetirizine, was shown to reduce expression of ICAM-1 on nasal epithelial cells in pollen allergy during the pollen season (32). Furthermore, the IFN γ -mediated upregulation of ICAM-1/CD54 on primary cultured normal cells was inhibited by cetirizine (33). Our study shows a reduction in the peripheral blood CD4+CD54+ population following levocetirizine treatment in patients with allergic rhinitis during the pollen season. This suggests that levocetirizine may down-regulate the expression of ICAM-I on peripheral blood CD4+ T cells in addition to nasal epithelial cells. This is particularly interesting because ICAM-1/CD54 is the main rhinovirus receptor, and rhinoviruses are the principal cause of asthma exacerbation in children (33). Thus, levocetirizine may have an important role in the reduction of infectious exacerbation of asthma in atopic patients. CD4+CD212+ cells, which are T-helper subpopulations expressing the high-affinity IL-12R β_1 , respond to stimulation with this cytokine by promoting Th1 responses (34) We have previously observed a significant increase in CD4+CD212+T cells in SAR patients during allergen pollen season compared to healthy controls (data not shown). In the present study, the negative relationship between major SAR symptoms and CD212 (Figs. 5 and 6) suggests that the pathogenesis is associated with prevalence of Th2 character in overall trends in immune reactivity exhibited by participating SAR patients. Downregulation of CD4+CD212+ promoted by levocetirizine therapy (Fig. 3) reflects the anti-inflammatory capacity of the drug. Moreover, based on previous work, levocetirizine may also modulate NF- κ B-dependent processes (35). This however is speculative and will

require additional investigation. The ability of levocetirizine to suppress activity of the above-described cell species strongly suggest that the drug has substantial potential to downregulate pathogenic immune processes through its effect on cells other than those expressing the H₁ receptor. Nevertheless since antihistamine interaction with lymphocytes or inflammatory cells is not considered to be a major contributor to their therapeutic effects, the degree to which such interactions occur has not been extensively explored. Results of the present study suggest that levocetirizine facilitates a complementary cell-mediated regulatory mechanism for mast cell and basophil proliferation as well as IgE synthesis. This could occur via direct interaction between the drug and eosinophils and T cells, through some intermediate cell species, or as a result of feedback due to H₁-receptor inhibition. However, a distinction between these alternatives cannot be made on the basis of data presented in this report. It is likely that feedback processes resulting from H₁ inhibition by levocetirizine play a significant, perhaps dominant role. Lymphocytes, in particular T cells, often act as master switches for extensive cascades of immunological processes. As an example, during the pathogenesis of atopic asthma, Th2 cells in the lung express cytokines (principally IL-5) that interact with eosinophils to cause a massive release of inflammatory mediators, resulting in events that include mast-cell sensitization, further eosinophil and lymphocyte recruitment, and mucus secretion (36). Hence, agents that have relatively minor effects on lymphocyte activation may ultimately cause major changes in multiple processes coordinated by T cells. Therefore, the suppression of T cell activation by antihistamines observed in this study may be accounted for by the fact that cellular signaling pathways that promote tissue damage in SAR exert positive feedback and increase T cell activation (37, 38). By this analysis, drugs that inhibit release of inflammatory metabolites or their biological activity are also expected to exhibit immunosuppressive properties. Indeed, the H₁-receptor inhibitor terfenadine is observed to inhibit proliferation and expression of IL-4 and IL-5 production by anti-CD3/-CD28 and PMA-activated human T cells in vitro (39). Since both of these Th2 cytokines are implicated as major factors in asthma pathogenesis, it is probable that therapeutic effects of this drug are mediated at least in part by suppression of T cell activity. A particularly interesting outcome of treatment with levocetirizine is the apparent induction by the drug of an expanded CD4+CD25+ T cell subpopulation in the peripheral blood of patients (Fig. 3) – an effect opposite that of levocetirizine treatment on the three other activated T cell populations. Data presented here do not allow characterization of this effect;

however an intriguing possibility is that it may represent drug-mediated amplification of a major host protective mechanism: specifically, the induction of Treg populations. Tregs are typically CD4+CD25+ cells that suppress excessive immune responses (32, 40). They play a key role in the maintenance of self-tolerance, thus preventing autoimmune disease, as well as inhibiting harmful chronic inflammatory mechanisms in patients with asthma and some other similar inflammatory diseases (41). In the present study, a positive correlation was observed between severity of a major SAR symptom and CD4+CD25+ (Table 1). If a substantial fraction of this CD4+CD25+ population were in fact of the Treg phenotype, the observed correlation might represent a pathogenic process inducing increased activity of its negative regulator. This conclusion is nevertheless speculative since the presence of Tregs among the CD4+CD25+ population was not confirmed. The positive correlation between the presence of inflammatory infiltrate (as evidenced by nasal obstruction) and presumptive Treg subpopulations (Fig. 4) is a host response expected based on work of other investigators (26). Moreover, if increased peripheral blood representation of CD4+CD25+ cells can be accounted for by changes in Tregs, then the present study provides increased insight into the outcome of histamine interaction with its cognate receptors: H₁–H₄. For example, it has been shown that histamine stimulation of H₁ receptor promotes pathogenesis of allergic disease, an effect that is counteracted by antihistamines (42), and that stimulation of H₄ receptor with histamine suppresses pathogenic processes and promotes expansion of peripheral blood Treg subpopulations (42). These observations raise the possibility that levocetirizine may have additional sites of action beyond H₁-receptor blockade or that signaling events downstream of the drug – H₁ interaction may converge or reinforce histamine stimulation of H₄ receptors. The present study has established a direction for future elucidation of these processes and development of improved therapies. Future studies will evaluate the presence of Tregs by screening for CD4+CD25+ cells expressing the (Treg-specific) FoxP3 antigen (27).

This study demonstrates that levocetirizine induces changes in eosinophil and T cell subpopulations of SAR patients in vivo that are expected to contribute to improved clinical prognosis. These observations may indicate important immunomodulatory effects of this drug. The entire anti-inflammatory mechanism by which the new generation of antihistamines, including levocetirizine, blocks key immune effector molecules is still elusive. The present study establishes directions for future investigations of the cellular and molecular mechanisms of antihistamine-mediated immunoregulation.

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