JPP Journal of Pharmacy and Pharmacology

JPP 2005, 57: 1027–1035

Received February 4, 2005
Accepted April 18, 2005
DOI 10.1211/0022357056640
ISSN 0022-3573

Suppressive activity of epinastine hydrochloride on TARC production from human peripheral blood CD4$^+$ T cells in-vitro

Ken-ichi Kanai, Kazuhito Asano, Tadashi Hisamitsu and Harumi Suzaki

Abstract

Thymus- and activation-regulated chemokine (TARC) is an important molecule in the development and maintenance of allergic diseases. However, there is little information about the influence of anti-allergic agents on TARC production. The aim of this study is to examine the influence of epinastine hydrochloride, an H_1-receptor antagonist, on TARC production from human peripheral blood CD4$^+$ T cells using an in-vitro cell culture technique. CD4$^+$ T cells prepared from healthy subjects were cultured in wells coated with a combination of OKT3 and anti-CD28 monoclonal antibody in the presence or absence of epinastine HCl for 24 h. The cells were also stimulated with interleukin (IL)-4 in a similar manner. Levels of TARC and IL-4 in culture supernatants were examined by ELISA. The addition of epinastine HCl exerted a dose-dependent suppressive effect on the production of both TARC and IL-4 from CD4$^+$ T cells under co-stimulatory molecule stimulation. The minimum concentration of the agent showing a significant suppressive effect on TARC and IL-4 production was 5.0 μM and 2.5 μM, respectively. Epinastine HCl also suppressed the ability of cells to produce TARC in response to IL-4 stimulation, when the agent was added to cell cultures at more than 2.5 μM. It was concluded that this inhibitory action of epinastine HCl may be partially responsible for epinastine's attenuating effect on allergic diseases.

Introduction

Allergic rhinitis, including pollinosis, is defined as an allergic inflammation in the nasal membranes and is characterized by a symptom complex that consists of any combination of conditions, such as sneezing, nasal congestion and nasal itching, which are caused by lipid mediators and other toxic proteins (e.g. histamine, tryptase, leukotriens, etc.) (Fokkens et al 1997). Over several hours, these mediators, through a complex interplay of events, lead to the recruitment to the nasal mucosa of inflammatory cells, such as eosinophils, lymphocytes and macrophages. This results in continued inflammation, termed the late-phase response (Bensch et al 2002). Although allergic rhinitis is not a life-threatening condition, complications can occur and the condition can significantly impair the sufferer's quality of life, which leads to a number of indirect costs (Barnes et al 1996; Beltrani 1999).

The best treatment of allergic rhinitis is avoidance of the allergens. However, since this is not always possible, a variety of antihistamines, decongestants and intranasal steroids may be prescribed (Barnes et al 1998). Studies performed on the underlying mechanisms controlling the allergic immune response have identified new classes of therapeutic targets. The first approach is to target Th2 T cell-derived cytokines, such as interleukin (IL)-4 and IL-5. This was tentatively performed using antagonists against IL-4 and IL-5, and their attenuating effects on the clinical conditions of allergic diseases have been reported (Boushey & Fahy 2000; Chantry 2000). Immunoglobulin E (IgE), which is produced from B cells under signals derived from Th2 T cells, is believed to be the second therapeutic target (O'Garra 1998; Milgrom et al 1999). The third strategy in the disease setting would interfere with the migration of the appropriate effector cell populations, such as eosinophils and Th2 T cells, into the site of the allergic inflammation (Andrian & Mackay 2000; Chensue et al 2001). However, to the

Department of Otolaryngology, School of Medicine, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan

Ken-ichi Kanai, Harumi Suzaki

Department of Physiology, School of Medicine, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan

Kazuhito Asano, Tadashi Hisamitsu

Correspondence: K. Asano, Department of Physiology, School of Medicine, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan. E-mail: asanok@med.showa-u.ac.jp

Funding: This study was supported in part by Nippon Boehringer Ingelheim Co. Ltd (Hyougo, Japan).

best of our knowledge, there is no clear evidence showing the efficacy of antagonists against receptors in the treatment.

Recently, we examined the influence of second-generation H_1-receptor antagonists, fexofenadine hydrochloride and epinastine hydrochloride on the production of eosinophil chemo-attractants, RANTES and eotaxin, in-vitro and in-vivo (Asano et al 2004a, b). Our findings suggested that the suppressive activity of H_1-receptor antagonists on eosinophil chemo-attractants may underlie the clinical efficacy of the agents on allergic diseases, such as pollinosis.

Thymus- and activation-regulated chemokine (TARC) is a member of the CC chemokine family. TARC is produced by monocytes, dendritic cells and Th2 T cells by antigenic stimulation, and may facilitate the recruitment, activation and development of Th2 polarized cells that express CCR4 (Terada et al 2001). Plasma TARC levels in patients with allergic rhinitis and atopic dermatitis are elevated and have been correlated with disease activity (Kakinuma et al 2001; Terada et al 2001). These reports suggest that inhibition of TARC production may be useful in the treatment of allergic diseases, including pollinosis.

In this study, therefore, we examined the influence of epinastine HCl on TARC production from human peripheral blood T cells in response to co-stimulatory molecule stimulation.

Materials and Methods

Agents

Epinastine HCl, a preservative-free pure powder, was kindly donated by Nippon Boehringer Ingelheim Co. Ltd. and Co. KG (Kawanashi, Japan). This was dissolved in RPMI-1640 medium (Sigma Chemicals, St Louis, MO, USA) supplemented with 10% fetal calf serum (FCS) at $2.0\,mg\,mL^{-1}$, sterilized by passing through a 0.2-μm filter and stored at 4°C as a stock solution. All dilutions used in this study were prepared from this stock solution by diluting with RPMI-FCS just before use.

Monoclonal antibodies

The monoclonal antibody (mAb) OKT3 was obtained from the American Tissue Culture Collection (no. CRL 8001; ATCC, Rockville, MD, USA). Anti-CD28 was purchased from Genzyme/Techne Co. Ltd (Cambridge, MA, USA). These mAbs were diluted with phosphate-buffered saline (PBS) at a concentration of $20.0\,\mu g\,mL^{-1}$ and used for the experiments.

Preparation and purification of human peripheral blood $CD4^+$ T cells

Human heparinized peripheral blood was obtained from five male non-allergic healthy subjects (40.0 ± 10.3 years) under written informed consent, which was approved by the Ethics Committee of Showa University. The blood was diluted twice with PBS, layered onto lymphocyte separation medium (Organon Technica, Durham, NJ, USA) and centrifuged at 25 ± 2°C for 30 min at 1000 *g*. Peripheral blood lymphocytes (PBLs) at the plasma and medium interface were then collected, washed several times with PBS and suspended in PBS. $CD4^+$ T cells were separated from PBLs using a magnetic cell separator (Milteny Biotec GmbH, Bergisch Gladbach, Germany) as described previously (Asano et al 2001). The cells were suspended in RPMI-FCS at a concentration of 1×10^6 cells/mL. The cell purity was more than 95% as judged by a flow cytometer (FACScan; Becton Dickinson, San Jose, CA).

Cell culture

PBS (500 μL) containing OKT3, or with anti-CD28, was introduced into 24-well culture plates (IWAKI GLASS, Tokyo, Japan) in triplicate and incubated overnight at 4°C (Asano et al 2001). Before use, these plates were washed three times with PBS. $CD4^+$ T cells (1×10^6 cells/mL) were cultured in mAb-coated plates that contained various concentrations of epinastine HCl in a final volume of 2.0 mL for 24 h. PBLs were also stimulated with $10.0\,ng\,mL^{-1}$ IL-4 (R & D systems, Minneapolis, MN, USA) (Oda et al 2002) in the presence of various concentrations of epinastine HCl in a similar manner. The supernatant was then collected after pelletting cells by centrifugation at 4°C for 10 min at 2000 *g*. The supernatants were stored at −40°C until assayed for TARC. For examining transcriptional factor activation and mRNA expression, cells were cultured in a similar manner for 4 h and stored at −80°C until used. To examine the influence of epinastine HCl on cell proliferation, $CD4^+$ T cells (100 μL) were cultured in mAb-coated 96-well flat-bottomed culture plates that contained various concentrations of epinastine HCl in a final volume of 200 μL for 72 h. Cells were pulsed with 37 kBq ^{3}H-thymidine (^{3}H-TdR; specific activity 740 GBq $mmol^{-1}$; New England Nuclear, Boston, MA, USA) for 8 h and the thymidine incorporation into nucleui of cells cultured in triplicate was measured by liquid scintillation counting.

Assay for TARC and IL-4

TARC and IL-4 levels in culture supernatants were assayed using human TARC and IL-4 enzyme-linked immunosorbent assay (ELISA) kits (Genzyme/Techne) according to the manufacturer's recommendation. The sensitivity of the TARC and IL-4 ELISA kits was $7.0\,pg\,mL^{-1}$ and $3.0\,pg\,mL^{-1}$, respectively.

Real-time polymerase chain reaction (PCR)

mRNA was extracted from fibroblasts using μMACS mRNA isolation kits (Milteny Biotec GmbH) according to the manufacturer's instructions. The first-strand cDNA synthesis from 1.0 μg mRNA was performed using the SuperScript Preamplification System for cDNA synthesis (GIBCO BRL, Gaithersburg, MD). PCR was then carried

out using a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). The PCR mixture consisted of 2.0 μL of sample cDNA solution (10.0 ng μL^{-1}), 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 μL. The reaction was conducted as follows: 4 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. β-Actin was amplified as an internal control. mRNA levels were calculated by using the comparative parameter threshold cycle (Ct) and normalized to β-actin. The nucleotide sequence of the primers was as follows: for TARC, 5′-CCACTGAAGATGCTGGCCCTG-3′ and 5′-GAGGCTTCAAGACCTCTCAAG-3′; for β-actin, 5′-CGGAACCGCTCATTGCC-3′ and 5′-ACCCACAC TGTGCCCATCTA-3′ (Oda et al 2002).

Assay for NF-κB

Nuclear Factor (NF)-κB activity was analysed by a commercially available ELISA test kit (Active Motif, Co. Ltd, Carlsbad, CA) that contained sufficient reagents and monoclonal antibody against P50 according to the manufacturer's recommended procedure. In brief, nuclear extract (5.0 μg protein) from $CD4^+$ T cells was introduced into each well of 96-well microtitre plates pre-coated with oligonucleotide containing the NF-κB consensus site (5′-GGGACTTTCC-3′) in a volume of 20.0 μL, and incubated for 1 h at 25°C. After washing three times, 100 μL of monoclonal antibody against P50 was added to the appropriate wells, and incubated for a further 1 h at 25°C. Anti-IgG HRP-conjugate in a volume of 100 μL was then added and incubated for 1 h at 25°C. The absorbance at 450 nm was measured after the addition of tetramethylbenzine solution.

Statistical analysis

The statistical significance of the data between the control and experimental groups was analysed by analysis of variance followed by Fisher's PLSD test. $P < 0.05$ was accepted as statistically significant.

Results

Influence of epinastine HCl on the production of TARC and IL-4 from $CD4^+$ T cells in-vitro

This study was undertaken to examine the influence of epinastine HCl on TARC production from $CD4^+$ T cells under co-stimulatory molecule stimulation in-vitro. $CD4^+$ T cells, at a concentration of 1×10^6 cells/mL, were stimulated with immobilized mAbs OKT3 or anti-CD28 in the presence of various concentrations of epinastine HCl for 24 h. Stimulation of cells with OKT3 or anti-CD28 alone did not enhance the ability of cells to produce TARC, whereas cells stimulated with OKT3 plus anti-CD28 produced a large amount of TARC (Figure 1). The data in Figure 1 clearly show the suppressive effect of epinastine HCl on TARC production from $CD4^+$ T cells in response to co-stimulatory molecule stimulation. This suppressive activity was dose dependent, and was first observed at 5.0 μM epinastine HCl. We next examined whether epinastine HCl could also suppress IL-4 production after co-stimulatory molecule stimulation as in the case of TARC production. Addition of epinastine HCl

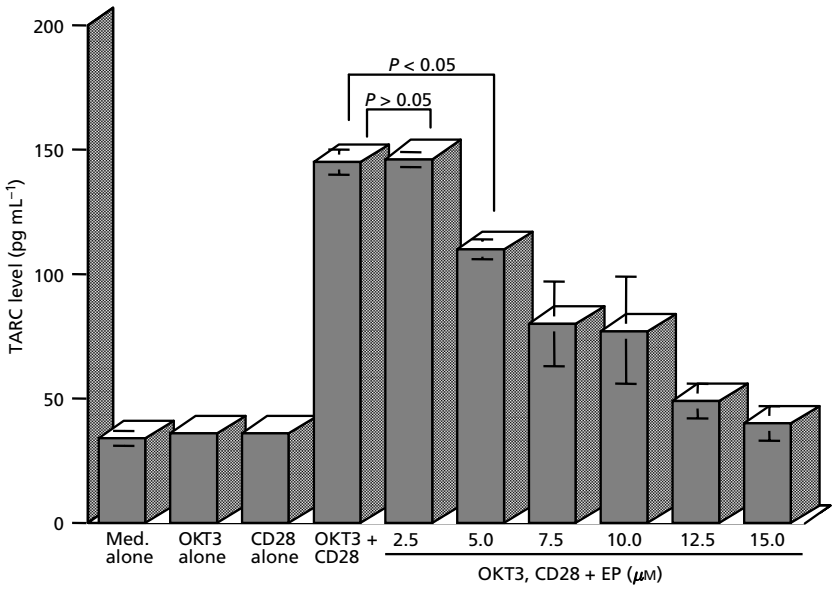

Figure 1 Influence of epinastine hydrochloride (EP) on TARC production from human $CD4^+$ T cells. $CD4^+$ T cells (1×10^6 cells/mL) were stimulated by immobilized OKT3 and anti-CD28 in the presence of various concentrations of EP. After 24 h, culture supernatants were collected and assayed for thymus- and activation-regulated chemokine (TARC) by ELISA. Data are expressed as the mean ± s.e. of five different subjects.

into cell cultures caused dose-dependent suppression of IL-4 production from $CD4^+$ T cells (Figure 2). The minimum concentration of the agent that caused significant suppression was 2.5 μM. The final experiment in this section was carried out to examine the influence of epinastine HCl on TARC production from $CD4^+$ T cells in response to IL-4 stimulation. The ability of cells to produce TARC was significantly increased by IL-4 stimulation (Figure 3). The addition of epinastine HCl into cell cultures at concentrations lower than 2.0 μM scarcely affected TARC production – TARC levels in experimental supernatants were similar and identical to those in control supernatants. However, epinastine HCl significantly suppressed TARC production from cells when the agent was added to cultures at concentrations of more than 2.5 μM (Figure 3).

Influence of epinastine HCl on $CD4^+$ T-cell proliferation through co-stimulatory pathway

This experiment was designed to examine the influence of epinastine HCl on $CD4^+$ T-cell proliferation induced by in-vitro stimulation through co-stimulatory pathways. To do this, $CD4^+$ T cells were cultured with immobilized mAbs in the presence of various concentrations of epinastine HCl. The cell proliferation was assessed by examining ^{3}H-TdR uptake into DNA. Although stimulation of $CD4^+$ T cells with OKT3 alone or anti-CD28 alone did not induce cell proliferation, extensive T-cell proliferation was observed when the stimulus to OKT3 was combined with an additional stimulus, such as anti-CD28 (Figure 4). We next examined the influence of epinastine HCl on $CD4^+$ T-cell proliferation by co-stimulatory molecule stimulation. As shown in Figure 4, epinastine HCl did not affect cell proliferation, even when added to cell cultures at a concentration of 15.0 μM.

Influence of epinastine HCl on mRNA expression for TARC in $CD4^+$ T cells

This experiment was carried out to examine whether epinastine HCl suppressed mRNA expression and resulted in the inhibition of protein production, or whether it directly inhibited protein production. $CD4^+$ T cells were stimulated with co-stimulatory molecules in the presence of various doses of epinastine HCl for 4 h. The level of mRNA expression was evaluated by real-time RT-PCR. Addition of epinastine HCl at more than 2.5 μM significantly suppressed the TARC mRNA expression in $CD4^+$ T cells (Figure 5), which had been enhanced by co-stimulatory molecule stimulation.

Suppressive activity of epinastine HCl on NF-κB activation in $CD4^+$ T cells

The final experiment was designed to examine the influence of epinastine HCl on NF-κB activation in $CD4^+$ T cells by co-stimulatory molecule stimulation. $CD4^+$ T cells were stimulated with co-stimulatory molecules in the presence of various doses of epinastine HCl for 4 h. The nuclear extracts were prepared and NF-κB activity was examined by ELISA. Addition of epinastine HCl at a dose of 2.5 μM into cell cultures suppressed NF-κB activation (Figure 6) – the optical density at 450 nm in the experimental groups was significantly lower than those in appropriate controls ($P < 0.05$).

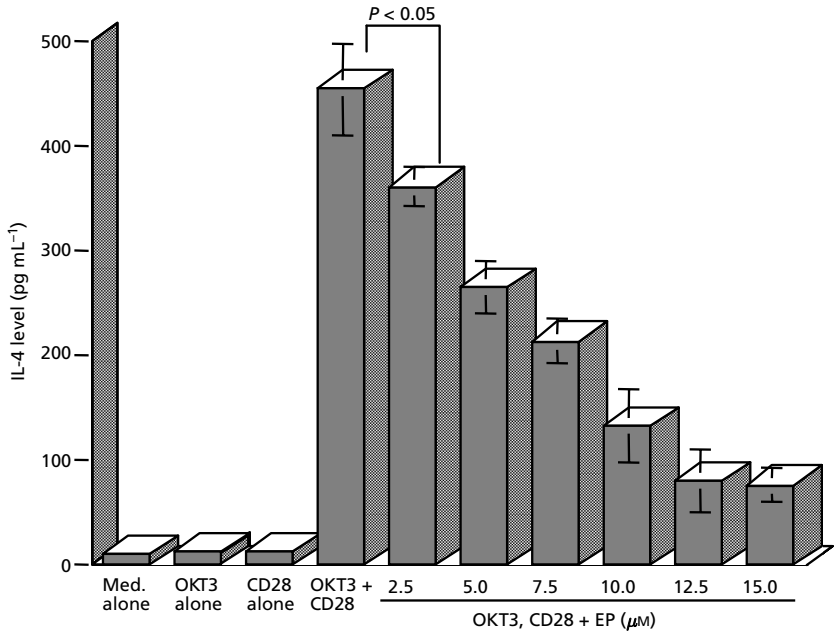

Figure 2 Influence of epinastine hydrochloride (EP) on IL-4 production from human $CD4^+$ T cells. $CD4^+$ T cells (1×10^6 cells/mL) were stimulated by immobilized OKT3 and anti-CD28 in the presence of various concentrations of EP. After 24 h, culture supernatants were collected and assayed for IL-4 by ELISA. Data are expressed as the mean ± s.e. of five different subjects.

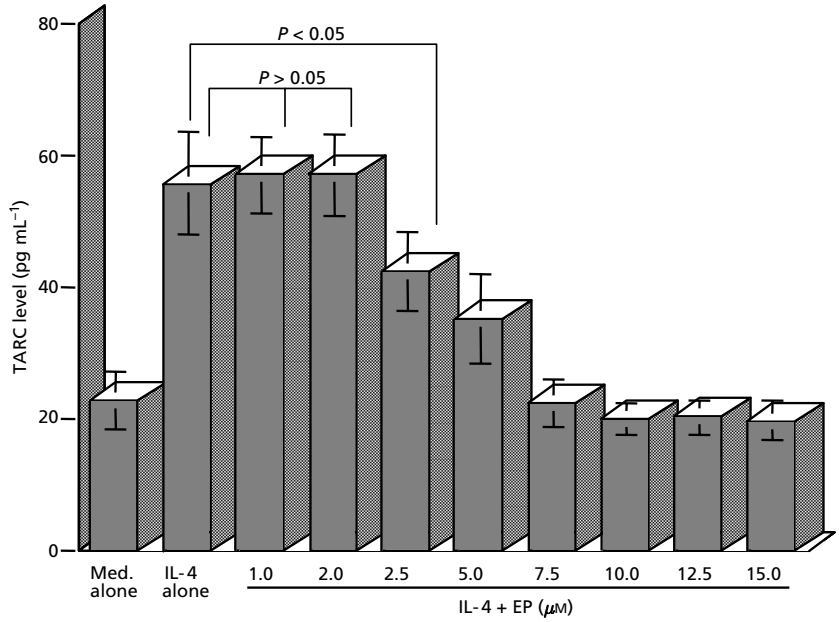

Figure 3 Influence of epinastine hydrochloride (EP) on TARC production from $CD4^+$ T cells in response to IL-4 stimulation. $CD4^+$ T cells (1×10^6 cells/mL) were stimulated with 10.0 ng mL^{-1} IL-4 in the presence of various concentrations of EP. After 24 h, culture supernatants were collected and assayed for thymus- and activation-regulated chemokine (TARC) by ELISA. Data are expressed as the mean ± s.e. of five different subjects.

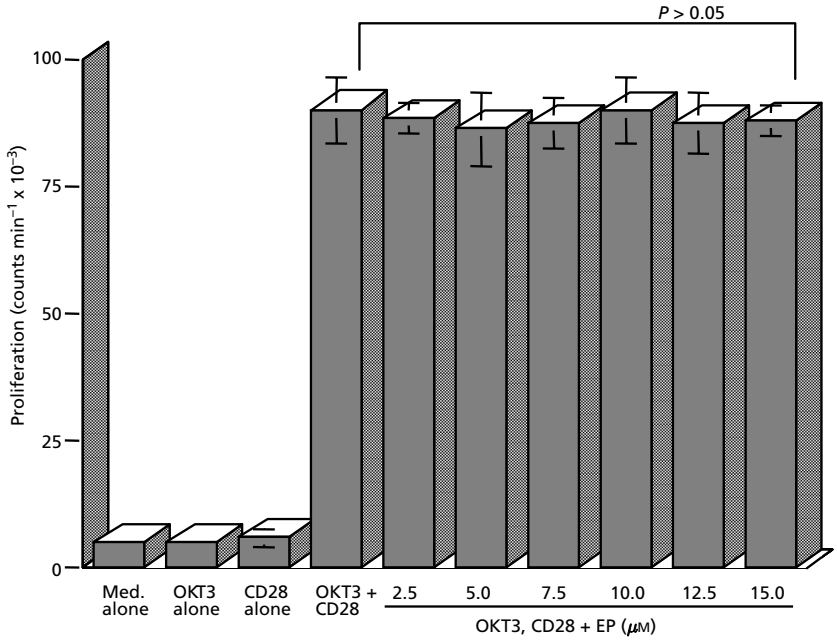

Figure 4 Influence of epinastine hydrochloride (EP) on in-vitro proliferative response of $CD4^+$ T cells induced by co-stimulatory molecule stimulation. $CD4^+$ T cells (1×10^5 cells) were stimulated by immobilized OKT3 with anti-CD28 in the presence of various concentrations of EP. The cells were pulsed with 37 kBq ^{3}H-thymidine for the last 8 h of the 72-h culture. Data are expressed as the mean ± s.e. from triplicate cultures of five different subjects.

Discussion

Epinastine HCl is a selective and potent H_1-receptor antagonist with no anticholinergic or sedative effect. Although it is frequently used for the treatment of seasonal allergic rhinitis and atopic dermatitis with remarkable success, the precise therapeutic mechanisms are not well understood.

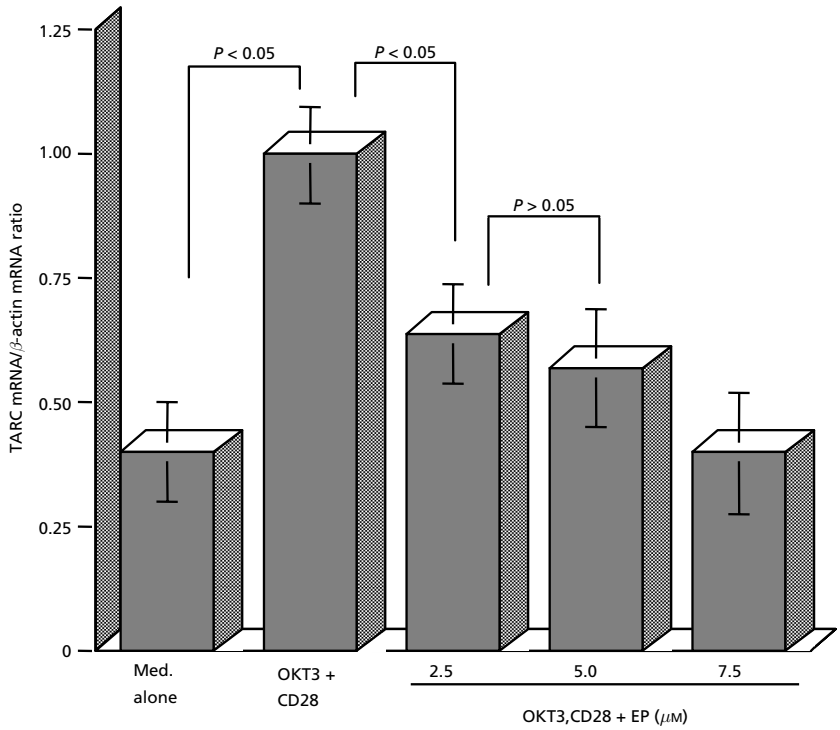

Figure 5 Suppressive activity of epinastine hydrochloride (EP) on TARC mRNA expression in $CD4^+$ T cells after co-stimulatory molecule stimulation. $CD4^+$ T cells (1×10^6 cells/mL) were stimulated by immobilized OKT3 and anti-CD28 in the presence of various concentrations of EP. After 4 h, poly A^+ RNA was obtained from cultured cells and assayed for mRNA expression for thymus- and activation-regulated chemokine (TARC) by real time RT-PCR. Data are expressed as the mean ± s.e. of five different subjects.

In a hypersensitivity response, $CD4^+$ T cells play a key role in triggering the allergic inflammatory response (Asano et al 2001). Namely, cytokines produced by Th2 T cells are indispensable for initiating and maintaining the allergic responses (Asano et al 2001). Trafficking of activated T cells into inflammatory sites is a tightly controlled process directed by multiple molecules, particularly adhesion molecules and chemokines (Kakinuma et al 2001; Terada et al 2001). TARC and macrophage-derived chemokines are CC chemokines that selectively attract Th2 T cells (Kakinuma et al 2001; Terada et al 2001; Oda et al 2002). It has been reported that nasal epithelial cells from pollinosis patients released higher levels of TARC than those derived from normal subjects after in-vitro stimulation with inflammatory cytokines (Terada et al 2001). Immunohistochemical analysis of nasal biopsies also showed TARC immuno-reactivity in nasal epithelium (Terada et al 2001). In atopic dermatitis, elevated levels of TARC were found in the serum of patients compared with normal controls, and levels of serum TARC have been correlated with the severity of atopic dermatitis as assessed by scoring atopic dermatitis system (Kakinuma et al 2001). These reports suggest that TARC may be strongly implicated in the development and maintenance of allergic diseases, especially allergic rhinitis and atopic dermatitis. Therefore, our results showing the suppressive activity of epinastine HCl on TARC production may be interpreted as demonstrating that epinastine HCl decreases the recruitment of Th2-type T cells into the site of diseases through the suppression of TARC production, and reduces disease severity after antigenic stimulation in-vivo. This suggestion is supported by the observation that a monoclonal antibody against TARC could effectively prevent airway hyperresponsiveness induced by antigenic stimulation in a murine asthma model (Kawasaki et al 2001).

IL-4 is a key cytokine in the development and maintenance of allergic inflammation. It is associated with induction of the ϵ-isotype switch and secretion of IgE by B cells (Coffman et al 1986). IgE-mediated immune responses are further enhanced by IL-4 through its ability to upregulate IgE receptors on cell surfaces, mast cell activation and the hypersecretion of mucus (Pawankar et al 1997; Dabbagh et al 1999). Il-4 also increases the production of several types of inflammatory cytokines, such as eotaxin from fibroblasts, that might contribute to inflammation and tissue remodelling in allergic diseases (Doucet et al 1998). Our results, therefore, may be interpreted as meaning that the suppressive activity of epinastine HCl on IL-4 production from $CD4^+$ T cells is also, in part, responsible for the attenuating effect of epinastine HCl on allergic diseases, including pollinosis.

Induction of allergic immune responses requires two distinct signals. The first signal is generated by the interaction between T-cell receptors and antigen peptides presented in the major histocompatibility complex class II

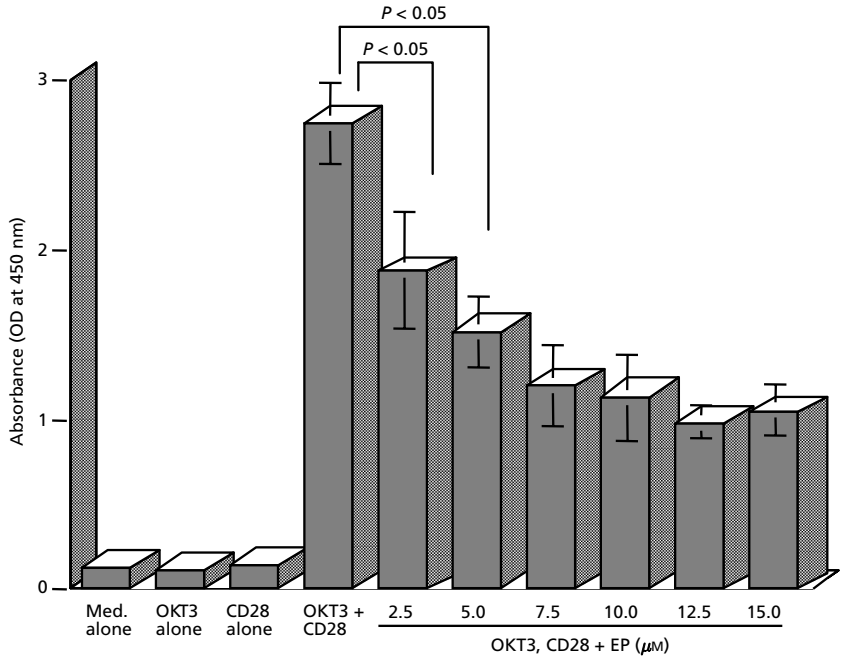

Figure 6 Influence of epinastine hydrochloride (EP) on NF-κB activation in $CD4^+$ T cells. $CD4^+$ T cells (1×10^6 cells/mL) were stimulated with immobilized OKT3 and anti-CD28 in the presence of various concentrations of EP. After 4 h, cells were collected and assayed for NF-κB (P50) activation by ELISA. Data are expressed as the mean optical density (OD) ± s.e. of five different subjects.

molecule (Lenschow et al 1996). The second, or co-stimulatory, signal is provided by direct contacts of co-stimulatory molecules on T cells with their ligands on antigen-presenting cells (Schwartz 1992). These two signals activate PI3-kinase (Ward et al 1993) and protein tyrosine kinase (PTK) (Raab et al 1995), and then cause dissociation of NF-κB from IκB (Ward et al 1993; Raab et al 1995). After translocation into the nucleus, NF-κB binds to the promoter region of the NF-κB-dependent gene and there is increase in mRNA expression for inflammatory cytokines and chemokines, such as IL-4 and TARC (Berin et al 2001; Palanki 2002). In addition to Th2 T-cell cytokines and chemokines, epinastine HCl can suppress the ability of $CD4^+$ T cells to produce Th1 T-cell cytokines, IL-2 and IFN-γ, which is controlled by NF-κB, in response to the stimulation of co-stimulatory pathway (Zhou et al 2002). The activation of PI3-kinase and PTK is known to require Ca^{2+}, which is increased by ligation of co-stimulatory molecules and their ligand CD28 (Kobarg et al 1997; Sotsios et al 2000). It has been reported that epinastine HCl at 10^{-5} M was effective in inhibiting Ca^{2+} influx and Ca^{2+} release from the intracellular calcium store of immune cells exposed to compound 48/80 and substance P (Kamei et al 1992). Epinastine HCl at 3×10^{-5} M also inhibited the ability of mast cells to produce chemical mediators depending on the increase in intracellular Ca^{2+} concentration (Columbo et al 1995; Mita & Shida 1995). From these reports, our results may be interpreted such that epinastine HCl inhibits TARC mRNA expression through the modulation of NF-κB activation by the suppression of changes in Ca^{2+} concentration in $CD4^+$ T cells after stimulation through a co-stimulatory pathway, resulting in inhibition of TARC production. This interpretation may be supported, at least in part, by our observation that epinastine HCl suppressed both TARC mRNA expression and NF-κB, P50, activation.

These results clearly show that epinastine HCl could suppress the production of Th2 type cytokines, such as IL-4 and, CC chemokines, such as TARC. However, the minimum concentration of epinastine HCl required to suppress TARC production is twice that for IL-4. The reasons for this discrepancy are not fully understood at present. The GATA-binding protein GATA-3 is reported to be essential for the production of Th2 cytokines, such as IL-4 (Chakir et al 2003). On the other hand, the production of CC chemokines, including TARC, requires GATA-1 activation (Richter et al 2003). From these reports, the difference in the susceptibility of GATA-1 and GATA-3 to epinastine HCl might be responsible for the phenomenon.

In conclusion, this study strongly suggests that the suppressive activity of epinastine HCl on TARC production from human peripheral blood $CD4^+$ T cells may underlie the therapeutic mode of action of epinastine HCl on allergic diseases, including allergic rhinitis.

References

Andrian, U. H., Mackay, C. R. (2000) T-cell function and migration. Two sides of the same coin. *N. Engl. J. Med.* **343**: 1020–1034

Asano, K., Kamakazu, K., Hisamitsu, T., Suzaki, H. (2001) Modulation of Th2 type cytokine production from human peripheral blood leukocytes by a macrolide antibiotic, roxithromycin, *in vitro*. *Int. Immunopharmacol.* **1**: 1913–1921

Asano, K., Kanai, K., Suzaki, H. (2004a) Suppressive activity of fexofenadine hydrochloride on the production of eosinophil chemo-attractants from human nasal fibroblasts *in vitro*. *Arzneimittelforschung/Drug Res.* **54**: 436–443

Asano, K., Inagaki, M., Kanai, K., Suzaki, H., Hisamitsu, T. (2004b) Influence of epinastine hydrochloride on the production of eosinophil chemo-attractants, RANTES and eotaxin, *in vitro* and *in vivo*. *Jpn. J. Pharmacol. Ther.* **32**: 561–567

Barnes, P. J., Jonsson, B., Klim, J. B. (1996) The costs of asthma. *Eur. Respir. J.* **9**: 636–642

Barnes, P. J., Pederson, S., Busse, W. W. (1998) Efficacy and safety of inhaled corticosteroids. New developments. *Am. J. Respir. Crit. Care Med.* **157**: S1–S35

Beltrani, V. S.(1999) The clinical spectrum of atopic dermatitis. *J. Allergy Clin. Immunol.* **104**: S87–S98

Bensch, G. W., Nelson, H. S., Borish, L. C. (2002) Evaluation of cytokines in nasal secretions after nasal antigen challenge: Lack of influence of antihistamines. *Ann. Allergy Asthma Immunol.* **88**: 457–462

Berin, M. C., Eckmann, L., Broide, D. H., Kagnoff, M. F. (2001) Regulated production of the T helper 2-type T-cell chemoattractant TARC by human bronchial epithelial cells *in vitro* and in human lung xenografts. *Am. J. Respir. Cell. Mol. Biol.* **24**: 382–389

Boushey, H. A., Fahy, J. V. (2000) Targeting cytokines in asthma therapy: round one. *Lancet* **356**: 2114–2116

Chakir, H., Wang, H., Lefebvre, D. E., Webb, J., Scott, F. E. (2003) T0bet/GATA-3 ratio as a measure of the Th1/Th2 cytokine profile in mixed cell populations: predominant role of GATA-3. *J. Immunol. Methods* **278**: 157–169

Chantry, D. (2000) Novel therapeutics for the treatment of asthma. *Expert. Opin. Emerging Drugs* **5**: 321–328

Chensue, S. W., Lukacs, N. W., Yang, T. Y., Shang, X., Frai, K. A., Kunkel, S. L., Kung, T., Wiekowski, M. T., Hedrick, J. A., Cook, D. N., Zingoni, A., Narula, S. K., Zlotnik, A., Barrat, F. J., O'Garra, A., Napolitano, M., Lira, S. A. (2001) Aberrant *in vivo* T helper type 2 cell response and impaired eosinophil recruitment in CC chemokine receptor 8 knockout mice. *J. Exp. Med.* **193**: 573–584

Coffman, R. L., Ohara, J., Bond, M. W., Carty, J., Zlotnik, A., Paul, W. E. (1986) B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells. *J. Immunol.* **136**: 4538–4541

Columbo, M., Horowitz, E. M., Patella, V., Kagey-Sobotka, A., Chilton, F. H., Lichtenstein, L. M. (1995) A comparative study of the effects of 1-acyl-2-acetyl-sn-glycero-3-phosphocholine and platelet activating factor on histamine and leukotriene C4 release from human leukocytes. *J. Allergy Clin. Immunol.* **95**: 565–573

Dabbagh, K., Takeyama, K., Lee, H. M., Ueki, I. F., Lausier, J. A., Nadel, J. A. (1999) IL-4 induces mucin gene expression and goblet cell metaplasia *in vitro* and *in vivo*. *J. Immunol.* **162**: 6233–6237

Doucet, C., Brouty-Boye, D., Pottin-Clemenceau, C., Jasmin, C., Canonica, G. W., Azzarone, B. (1998) IL-4 and IL-13 specifically increase adhesion molecule and inflammatory cytokine expression in human lung fibroblasts. *Int. Immunol.* **10**: 1421–1433

Fokkens, W. J., Godthelp, T., Holm, A. F., Blom, H., Klein-Jan, A. (1997) Allergic rhinitis and inflammation: the effect of nasal corticosteroid therapy. *Allergy* **52** (Suppl. 36): 29–32

Kakinuma, T., Nakamura, K., Wakugawa, M., Mitsui, H., Tada, Y., Saeki, H., Torii, H., Asahina, A., Onai, N., Matsushima, K., Tamaki, K. (2001) Thymus- and activation-regulated chemokine in atopic dermatitis: serum thymus- and activation-regulated chemokine level is closely related with disease activity. J. *Allergy Clin. Immunol.* **107**: 535–541

Kamei, C., Akagi, M., Mio, M., Kitazumi, K., Izushi, K., Masaki, S., Tasaka, K. (1992) Antiallergic effect of epinastine (WAL 801 CL) on immediate hypersensitivity reactions: (I). Elucidation of the mechanism for histamine release inhibition. *Immunopharmacol. Immunotoxicol.* **14**: 191–205

Kawasaki, S., Takizawa, H., Yoneyama, H., Nakayama, T., Fujisawa, R., Izumizaki, M., Imai, T., Yoshie, O., Homma, I., Yakamoto, K., Matsushima, K. (2001) Intervention of thymus-and activation-regulated chemokine attenuates the development of allergic airway inflammation and hyperresponsiveness in mice. *J. Immunol.* **166**: 2055–2062

Kobarg, J., Whitney, G. S., Palmer, D., Aruffo, A., Bowen, M. A. (1997) Analysis of the tyrosinephosphorylation and calcium fluxing of human CD6 isoforms with different cytoplasmatic domains. *Eur. J. Immunol.* **27**: 2971–2980

Lenschow, D. J., Walunas, T. L., Bluestone, J. A. (1996) CD28/B7 system of T cell costimulation. *Ann. Rev. Immunol.* **14**: 233–258

Milgrom, H., Fick, R. B., Su, J. Q. (1999) Treatment of allergic asthma with monoclonal anti-IgE antibody. RhuMAb-E25 study group. *N. Engl. J. Med.* **341**: 1966–1973

Mita, H., Shida, T. (1995) Comparison of anti-allergic activities of the histamine H1 receptor antagonists epinastine, ketotifen and oxatomide in human leukocytes. *Arzneimittelforschung/Drug Res* **45**: 36–40

Oda, N., Minoguchi, K., Tanaka, A., Yokoe, T., Minoguchi, H., Matsuo, H., Nakashima, M., Tasaki, T., Adachi, M. (2002) Suplatast tosilate inhibits thymus- and activation-regulated chemokine production by antigen-specific human Th2 cells. *Clin. Exp. Allergy* **32**: 1782–1786

O'Garra, A. (1998) Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* **8**: 275–283

Palanki, M. S. (2002) Inhibitors of AP-1 and NF-kappa B mediated transcriptional activation: therapeutic potential in autoimmune diseases and structural diversity. *Curr. Med. Chem.* **9**: 219–227

Pawankar, R., Okuda, M., Yssel, H., Okumura, K., Ra, C. (1997) Nasal mast cells in perennial allergic rhinitis exhibit increased expression of the Fc epsilon RI, CD40L, IL-4, and IL-13, and can induce IgE synthesis in B cells. *J. Clin. Invest.* **99**: 1492–1499

Raab, M., Cai, Y., Bunnell, S. C., Heyeck, S. D., Berg, L. J., Rudd, C. E. (1995) p56 Lck and p59 Fyn regulate CD28 binding to phosphatidylinositol 3-kinase, growth factor receptor-bound protein tyrosine kinase ITK: implications for T cell costimulation. *Proc. Natl Acad. Sci. USA* **91**: 8891–8895

Richter, M., Cantin, A. M., Beaulieu, C., Cloutier, A., Larivee, P. (2003) Zinc chelators inhibit eotaxin, RANTES, and MCP-1 production in stimulated human airway epithelium and fibroblasts. *Am. J. Physiol. Lung Cell Mol. Physiol.* **285**: L719–L729

Schwartz, R. H. (1992) Costimulation of T lymphocytes: the role of CD28, CTLA-4 and B7/BB1 in interleukin-2 production and immunotherapy. *Cell* **72**: 1065–1068

Sotsios, Y., Blair, P. J., Westwick, J., Ward, S. G. (2000) Disparate effects of phorbol esters, CD3 and the costimulatory receptors CD2 and CD28 on RANTES secretion by human T lymphocytes. *Immunology* **101**: 30–37

Terada, N., Nomura, T., Kim, W. J., Otsuka, Y., Takahashi, R., Kishi, H., Yamashita, T., Sugawara, N., Fukuda, S., Ikeda-Ito, T., Konno, A. (2001) Expression of C-C chemokine TARC in human nasal mucosa and its regulation by cytokines. *Clin. Exp. Allergy* **31**: 1923–1931

Ward, S. G., Westwick, J., Hall, N. D., Sansom, D. M. (1993) Ligation of CD28 receptor by B7 induces formation of D-3 phosphoinositides in T lymphocytes independently of T cell receptor/CD3 activation. *Eur. J. Immunol.* **23**: 2572–2577

Zhou, X. Y., Yashiro-Ohtani, Y., Nakahira, M., Park, W. R., Abe, R., Hamaoka, T., Naramura, M., Gu, H., Fujiwara, H. (2002) Molecular mechanisms underlying differential contribution of CD28 versus non-CD28 costimulatory molecules to IL-2 promoter activation. *J. Immunol.* **168**: 3847–3854