

Suppressive Activity of Fexofenadine Hydrochloride on Thymus- and Activation-Regulated Chemokine Production from Human Peripheral Blood Leukocytes in Response to Antigenic Stimulation in vitro

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Key Words

H₁-receptor antagonist · Fexofenadine · Thymus- and activation-regulated chemokine · Human peripheral blood leukocyte · Pollinosis · Japanese cedar pollen

Abstract

Background: Thymus- and activation-regulated chemokine (TARC) is accepted as being an important molecule in the development and maintenance of allergic diseases. However, there is little information about the influence of antiallergic agents on TARC production after allergen challenge. The aim of this study is to examine the influence of fexofenadine hydrochloride (FEX), an H₁-receptor antagonist, on TARC production from human peripheral blood leukocytes (PBL) using an in vitro cell culture technique. **Methods:** PBL prepared from donors with pollinosis were cultured with either Japanese cedar pollen allergen, Cry j 1, or interleukin (IL)-4 in the presence of various doses of FEX for 6 days. Levels of TARC and the T cell cytokines IL-4 and interferon (IFN)- γ in culture supernatants were examined by ELISA. **Results:** FEX did not affect PBL proliferation induced by Cry j 1 stimulation, even when 500 ng/ml of the agent, twice the therapeutic blood levels, was added to cell cultures as assessed by measuring ³H-thymidine incorporation into DNA. On the other hand, FEX at 250 ng/ml (but not 125 ng/

ml), similar to therapeutic blood levels, significantly inhibited the ability of PBL to produce IL-4 (but not IFN- γ), which was enhanced by Cry j 1 stimulation. FEX at concentrations of more than 250 ng/ml also exerted a suppressive effect on TARC production from PBL in response to Cry j 1 and IL-4 stimulation in vitro. **Conclusion:** This inhibitory action of FEX may be partially responsible for the attenuating effect of the agent on allergic diseases.

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Introduction

Pollinosis is well recognized as an allergy characterized by sneezing, itchy and watery eyes, a runny nose and a burning sensation in the palate and throat. These clinical conditions are known to result, at least in part, from lipid mediators and other toxic proteins from both eosinophils and mast cells (including basophils) [1, 2]. Enhancement of these cell functions, such as endothelial adhesion and degranulation, is mediated by several types of cytokines and C-C chemokines, which are mainly produced from T helper (Th)2-type T cells [3, 4].

Fexofenadine hydrochloride (FEX), a newly developed antiallergic agent, is the carboxylic acid metabolite of terfenadine and shows a similar level of H₁-receptor-block-

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ing activity to that of terfenadine, but does not have the rare cardiovascular effects associated with terfenadine [5–7]. Although the precise mechanisms of the action of FEX are not fully understood, FEX is reported to prevent allergen-induced alterations in pulmonary inflammation and airway function, such as hyperresponsiveness, in sensitized mice [8]. Furthermore, FEX reduces nasal inflammation by modulating the release of inflammatory mediators, such as RANTES and interleukin (IL)-8, from human nasal epithelial cells in vitro [9], suggesting that the inhibitory action of FEX on inflammatory mediator production may underlie the therapeutic mode of action, which could explain the beneficial effect of FEX on allergic diseases.

Thymus- and activation-regulated chemokine (TARC) is a member of the C-C chemokine family. TARC is likely to be produced by dendritic cells, endothelial cells and Th2 cells and may facilitate the recruitment, activation and development of Th2-polarized cells [10, 11]. Plasma TARC levels in patients with either allergic rhinitis or atopic dermatitis are significantly higher than those of healthy subjects [12, 13]. These reports suggest that inhibition of TARC production in response to antigenic stimulation is important in the management and treatment of allergic diseases, including pollinosis and atopic allergy, among others. In the present study, therefore, we examined the influence of FEX on the production of TARC and T cell cytokines by peripheral blood leukocytes (PBL) from patients with pollinosis after antigen-specific stimulation in vitro.

Subjects and Methods

Reagents

FEX was kindly donated by Aventis Pharmaceutical Co. Ltd. (Tokyo, Japan) as a preservative-free pure powder. The agent was dissolved in dimethyl sulfoxide (Sigma Chemical Co., Ltd., St. Louis, Mo., USA) at 5.0 mg/ml and stored at –20 °C until used. All dilutions used in this study were prepared from this stock solution by dilution with RPMI-1640 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Irvine Scientific, Santa Ana, Calif., USA), 100 U/ml penicillin and 100 µg/ml streptomycin (RPMI-FCS) just before use. ³H-thymidine (³H-TdR) was purchased from PerkinElmer Life Sciences, Inc. (Boston, Mass., USA; specific activity 6.7 Ci/mmol). For in vitro use, this was diluted with phosphate-buffered saline (PBS) at 1.0 µCi/10 µl. The reagents used for reverse transcriptase-polymerase chain reaction (RT-PCR) were purchased from Invitrogen Corp. (Carlsbad, Calif., USA) and Takara Co., Ltd. (Tokyo, Japan), respectively. In a preliminary experiment, we confirmed the negative suppressive effects of 0.1% dimethyl sulfoxide on PBL functions such as proliferation and IL-4 production after antigenic stimulation in vitro.

Allergen

Japanese cedar pollen allergen (Cry j 1), purified from crude antigen by anti-Cry j 1 monoclonal antibody immobilized column chromatography, was purchased from Hayashibara Biochemical Laboratories Inc. (Okayama, Japan) as a preservative-free PBS solution. This contained 50 µg/ml allergen, and was diluted with RPMI-FCS at appropriate concentrations for experiments just before use.

Preparation of PBL

The subjects were 6 patients with Japanese cedar (*Cryptomeria japonica*) pollen-sensitized rhinitis and 5 nonallergic volunteers who served as healthy controls. Patients and control subjects were recruited from the Otolaryngology Outpatient Clinic of the Showa University Hospital. Pollinosis was diagnosed by otorhinolaryngologists according to the established criteria based on history and rhinoscopic examination [14]. To confirm diagnosis and exactly detect allergen-caused pollinosis, skin prick testing (wheal mean diameter at least 4 mm greater than negative control) and a nasal provocation test were performed with commercial crude extracts used in vitro and in vivo (Torii Pharmaceuticals, Tokyo, Japan). The number of eosinophils in nasal secretions obtained after the provocation test was also examined using the smears stained with Giemsa's solution. Healthy control donors had no history of allergic symptoms and exhibited negative skin prick tests and nasal provocation tests. The characteristics of subjects investigated in this study are shown in table 1. PBL were prepared from donors after obtaining written informed consent (approved by the Ethics Committee of Showa University), using Ficoll-Hypaque (Organon Technika, Durham, N.J., USA) density gradient centrifugation [15]. The cells were suspended in RPMI-FCS at 1×10^6 cells/ml.

Cell Culture

To examine the influence of FEX on the blastic activity of PBL, 100 µl of cell suspension (1×10^5 cells) was introduced into each well of 96-well flat-bottomed culture plates in triplicate. The cells were then stimulated with Cry j 1 in the presence of various concentrations of FEX in a final volume of 200 µl. After 4–7 days, the cells were pulsed with 1.0 µCi of ³H-TdR for the final 8 h of the culture, and ³H-TdR incorporation into DNA was measured as previously described [15]. In the case of examining the effect of FEX on the ability of PBL to produce TARC, IL-4 and interferon (IFN)-γ in response to Cry j 1, PBL were cultured in duplicate with Cry j 1, in the presence of various concentrations of FEX, for 6 days in a final volume of 2.0 ml. PBL was also stimulated with 10.0 ng/ml IL-4 (R&D Systems, Minneapolis, Minn., USA) [16] in the presence of various concentrations of FEX in a similar manner. Culture supernatants were collected after pelleting cells by centrifugation at 1,500 g for 10 min at 4 °C and stored at –40 °C until used. The pelleted cells were also stored at –80 °C for the preparation of mRNA.

Assay for Cytokines and Chemokine

IL-4, IFN-γ and TARC levels in culture supernatants were examined in duplicate by commercially available ELISA test kits (R&D Systems) for IL-4, IFN-γ and TARC according to the manufacturer's recommended procedure. The sensitivity of these kits for IL-4, IFN-γ and TARC was 2.0, 1.0 and 7.0 pg/ml, respectively.

Assay for TARC mRNA Expression

Poly A⁺ mRNA was separated from cultured cells with oligo(dT)-coated magnetic micro beads (Miltenyi Biotec, Bergisch Gladbach,

Table 1. Characteristics of subjects investigated in this study

	Controls	Patients
Number of subjects	5	6
Age, years (median, range)	40 (25–50)	44 (32–48)
Sex	male	male
Disease severity	nonallergic	mild
Medication	none	none
Serum IgE, U/ml	48.9 ± 9.6	127.9 ± 20.4
IgE RAST score		
Cj	0	5.8 ± 0.7
Aa	0	0
Ap	0	0
Dg	0	0
Df	0	0
Af	0	0
Cd	0	0
Dd	0	0
Blood eosinophil count, %	3.8 ± 0.5	10.5 ± 1.2
Skin prick test	–	+++ ¹
Nasal provocation test		
Symptoms	–	+++ ²
Smear cytology	<1 % eosinophils	>20 % eosinophils

Cj = *Cryptomeria japonica*; Aa = *Ambrosia artemisiaefolia*; Ap = *Artemisia princeps*; Dg = *Dactylis glomerata*; Df = *Dermatophagoides farinae*; Af = *Aspergillus fumigatus*; Cd = cat dander; Dd = dog dander.

¹ Wheal reaction >13 mm and flare reaction >30 mm against *Cryptomeria japonica* alone.

² Positive for sneezing/itch, watery rhinorrhea and nasal blockage against *Cryptomeria japonica* alone.

Germany). The first-strand cDNA was synthesized from 1 µg of Poly A⁺ mRNA using a Superscript cDNA synthesis kit (Invitrogen Corp., Carlsbad, Calif., USA) according to the manufacturer's instructions. Amplification of cDNA was performed with a Takara PCR Amplification kit (Takara Shuzo, Co., Ltd.) using specific primers for TARC, IL-4 and β-actin in a final volume of 30 µl. The nucleotide sequences of the primers were as follows: for TARC, 5'-CCACTGA-AGATGCTGGCCCTG-3' and 5'-GAGGCTTCAAGACCTCTCA-AG-3'; for β-actin, 5'-CGGAACCGCTCATTGCC-3' and 5'-ACC-CACACTGTGCCCATCTA-3' [16]; for IL-4, 5'-ATGGGTCTCAC-CTCCCACTGCT-3' and 5'-CGAACACTTTGAATATTTCTCTCTCAT-3' [17]. The PCR process was performed with a DNA Thermal Cycler (Takara Shuzo), and the durations and temperatures were as follows: for TARC and β-actin transcripts, 5 min at 95°C, and then 60 s at 94°C, 60 s at 58°C and 120 s at 72°C for 30 cycles [16]; for IL-4, 120 s at 94°C, followed by 30 cycles of 60 s at 94°C, 60 s at 60°C and 60 s at 72°C for 30 cycles [17]. After cycling, there was a DNA extension period of 10 min at 72°C [16, 17]. Each PCR product (10 µl) was run on 3% agarose gels, visualized by a UV illuminator after SYBR Green staining and photographed. Intensity of mRNA levels was corrected by β-actin transcripts calculated by a densitometer.

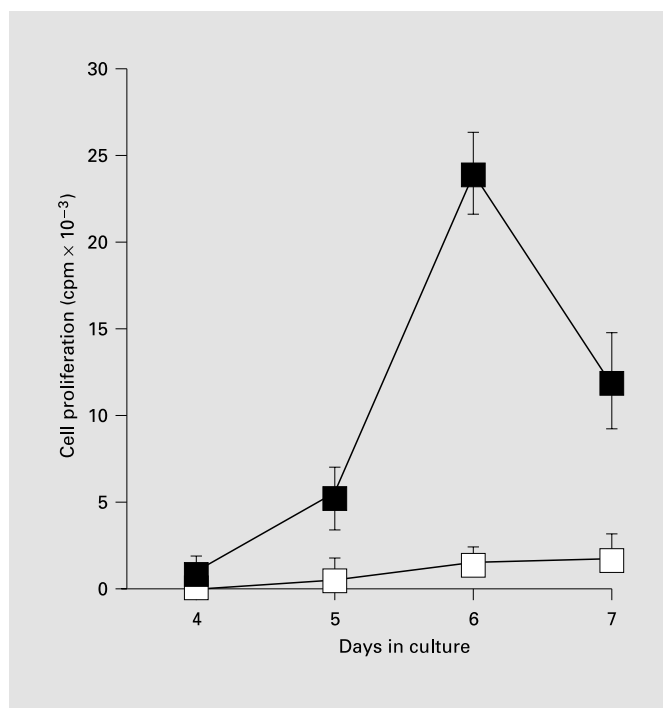


Fig. 1. Time course of proliferative response of PBL induced by Cry j 1 stimulation in vitro. PBL from patients with Japanese cedar pollinosis (1.0×10^6 cells/ml) were stimulated with 100 ng/ml Japanese cedar pollen allergen (Cry j 1) in a final volume of 200 µl. Cell proliferation was assessed by adding 1.0 µCi of ³H-TdR for the final 8 h of culture. The data are expressed as the mean ± SE of the different subjects. Open squares = Healthy controls (n = 5); closed squares = patients with pollinosis (n = 6).

Statistical Analysis

Comparisons were calculated by one-way ANOVA with Fisher's PLSD test. Values are expressed as the mean ± SE, and the level of statistically significant difference between groups was defined as $p < 0.05$.

Results

Influence of FEX on Proliferative Response of PBL Induced by Cry j 1 Stimulation in vitro

The first set of experiments was undertaken to examine the influence of FEX on the proliferative response of PBL after Cry j 1 stimulation in vitro. To do this, we firstly examined the influence of Cry j 1 on the proliferative response of PBL. PBL from patients and healthy subjects were cultured with 100 ng/ml Cry j 1 in a final volume of 200 µl. Cell proliferation was assessed by measuring ³H-TdR incorporation into DNA. As shown in figure 1, PBL

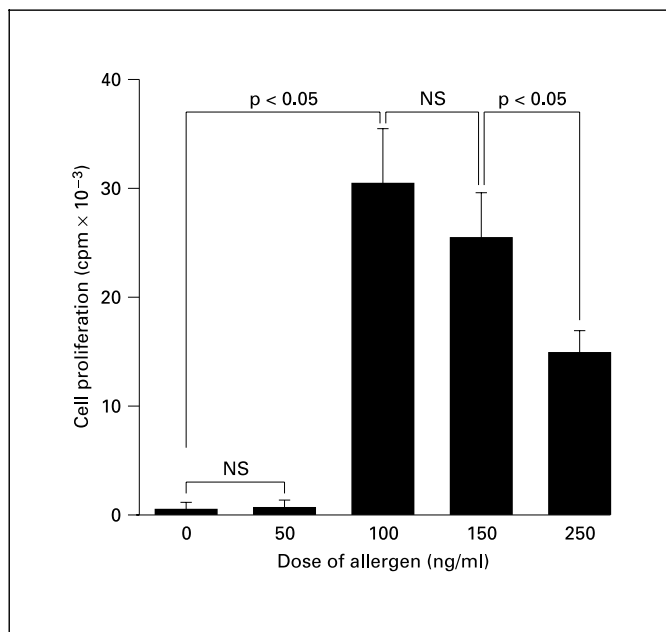


Fig. 2. Dose-response profile of PBL proliferation induced by Cry j 1 stimulation in vitro. PBL from patients with Japanese cedar pollinosis (1.0×10^6 cells/ml) were stimulated with various concentrations of Japanese cedar pollen allergen (Cry j 1) in a final volume of 200 μ l. Cell proliferation was assessed by adding 1.0 μ Ci of ^{3} H-TdR for the final 8 h of culture. The data are expressed as the mean $\pm$ SE of 6 different subjects. NS = Not significant.

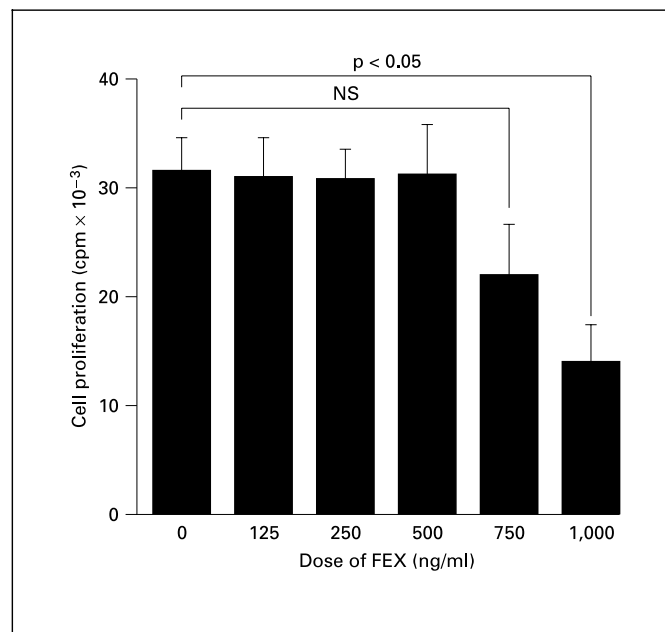
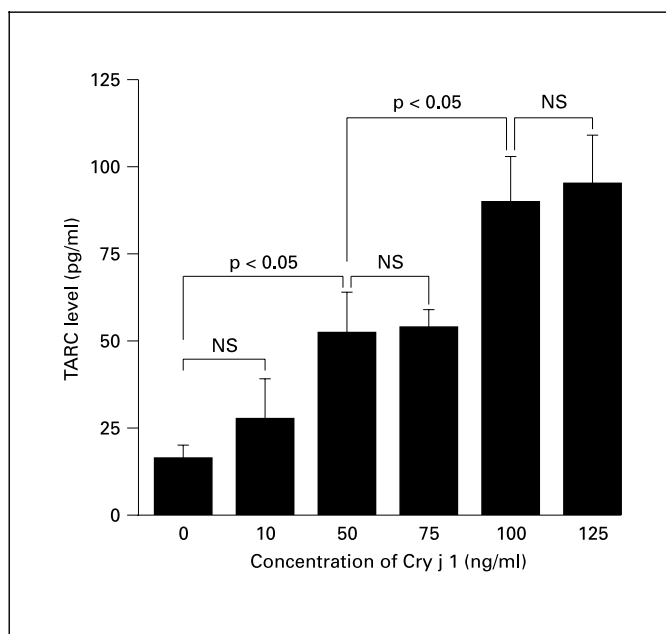


Fig. 3. Influence of FEX on Cry j 1-specific proliferation of PBL in vitro. PBL from patients with Japanese cedar pollinosis (1.0×10^6 cells/ml) were stimulated with 100 ng/ml Japanese cedar pollen allergen (Cry j 1) in the presence of various concentrations of FEX in a final volume of 200 μ l. Cell proliferation was assessed by adding 1.0 μ Ci of ^{3} H-TdR for the final 8 h of culture. The data are expressed as the mean $\pm$ SE of 6 different subjects. NS = Not significant.

from healthy subjects did not show proliferative responses to Cry j 1 stimulation. However, PBL from patients were highly responsive to in vitro stimulation with Cry j 1, and the maximum response was observed on day 6 in culture (fig. 1). The optimal concentration of Cry j 1 to induce proliferative responses of PBL was examined in the second set of experiments. PBL from patients were cultured with various concentrations of Cry j 1 for 6 days, and ^{3} H-TdR incorporation into DNA was measured. PBL did not show proliferative responses when the cells were stimulated with 50 ng/ml Cry j 1 (fig. 2). However, the maximum proliferation of PBL was induced when the cells were stimulated with 100 ng/ml Cry j 1, and 150 ng/ml or more was inhibitory (fig. 2). The influence of FEX on the proliferative responses of PBL after Cry j 1 stimulation was examined in the third set of experiments. FEX could not suppress the proliferative responses of PBL in response to Cry j 1 stimulation even when 500 ng/ml of the agent, twice the therapeutic blood levels [18], was added to cell cultures (fig. 3).

Suppressive Activity of FEX on TARC and Cytokine Production from PBL after Cry j 1 Stimulation

The second set of experiments was undertaken to examine the influence of FEX on TARC and cytokine production from PBL in response to Cry j 1 stimulation. The optimum concentration of Cry j 1 to produce the mediators from PBL was firstly assessed by measuring the TARC level in culture supernatants. The cells from patients were stimulated with various concentrations of Cry j 1 in the absence of FEX. After 6 days, culture supernatants were collected, and the TARC level was examined by ELISA. As shown in figure 4, Cry j 1 stimulation of PBL at a concentration of 10.0 ng/ml scarcely affected TARC production from PBL. However, Cry j 1 at more than 50.0 ng/ml caused a significant increase in TARC level as compared with the nonstimulated control (fig. 4). The maximum production of TARC from PBL was induced when the cells were stimulated with Cry j 1 at 100 and 125 ng/ml (fig. 4). We then examined the influence of FEX on TARC production. Addition of FEX to cell cultures significantly suppressed the ability of PBL to pro-



duce TARC in response to Cry j 1 stimulation (fig. 5a) in a dose-dependent manner. The minimum concentration that caused significant suppression was 250 ng/ml. The data in figure 5b also show the clear inhibition of TARC production by FEX when the cells were stimulated with IL-4 in the presence of more than 250 ng/ml of the agent. We then examined the influence of FEX on T cell cytokine production induced by Cry j 1 stimulation. As shown

Fig. 4. Influence of Cry j 1 on TARC production from PBL in vitro. PBL from patients with Japanese cedar pollinosis (1.0×10^6 cells/ml) were stimulated with various concentrations of Japanese cedar pollen allergen (Cry j 1) in a final volume of 2.0 ml for 6 days. TARC levels in supernatants were measured by ELISA. The data are expressed as the mean $\pm$ SE of 6 different subjects. NS = Not significant.

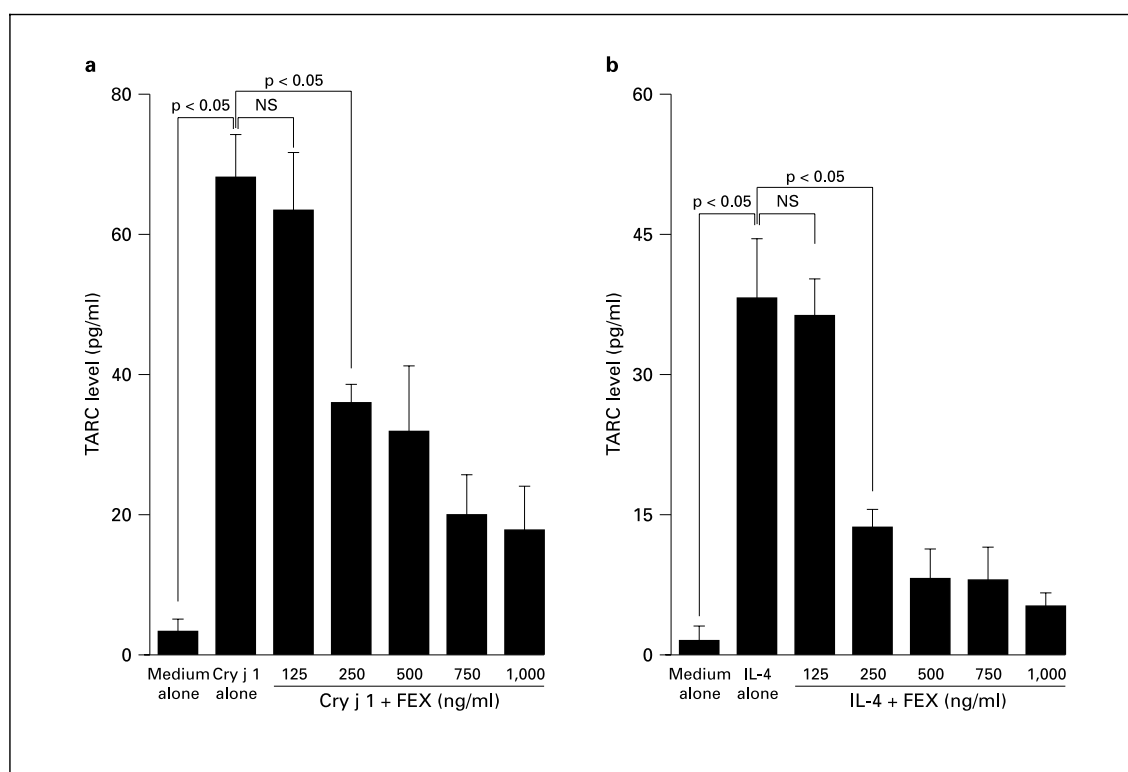


Fig. 5. Suppressive activity of FEX on TARC production from PBL stimulated with either Cry j 1 (a) or IL-4 (b) in vitro. PBL from patients with Japanese cedar pollinosis (1.0×10^6 cells/ml) were stimulated with either 100 ng/ml Japanese cedar pollen allergen (Cry j 1) or 10 ng/ml IL-4 in the presence of various concentrations of FEX in a final volume of 2.0 ml for 6 days. TARC levels in supernatants were measured by ELISA. The data are expressed as the mean $\pm$ SE of 6 different subjects. NS = Not significant.

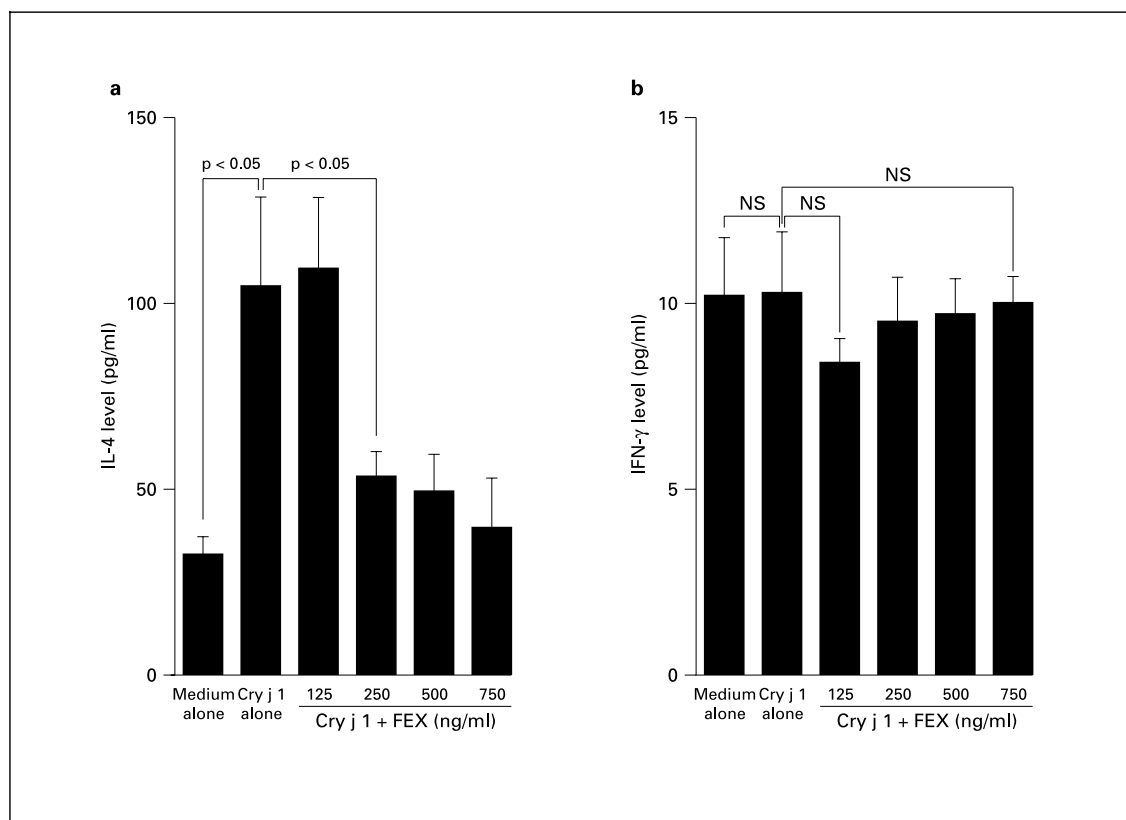


Fig. 6. Suppressive activity of FEX on IL-4 production from PBL stimulated with Cry j 1 in vitro. PBL from patients with Japanese cedar pollinosis (1.0×10^6 cells/ml) were stimulated with 100 ng/ml Japanese cedar pollen allergen (Cry j 1) in the presence of various concentrations of FEX in a final volume of 2.0 ml for 6 days. IL-4 (**a**) and IFN- γ (**b**) levels in supernatants were measured by ELISA. The data are expressed as the mean $\pm$ SE of 6 different subjects. NS = Not significant.

in figure 6, the ability of PBL to produce IL-4 was increased by Cry j 1 stimulation, and this ability was suppressed by the addition of FEX to cell cultures at more than 250 ng/ml (fig. 6a). On the other hand, Cry j 1 stimulation of PBL did not cause an increase in IFN- γ level in culture supernatants (fig. 6b). The data in figure 6b also show the negative suppressive effect of FEX on IFN- γ production from PBL.

Influence of FEX on mRNA Expression in PBL after Cry j 1 Stimulation

The final set of experiments was designed to examine the possible mechanisms by which 250 ng/ml FEX suppressed TARC and IL-4 production from PBL after Cry j 1 stimulation. PBL was stimulated with Cry j 1 in the presence of various concentrations of FEX for 6 days, and mRNA expression was examined using RT-PCR. The photographs in figure 7 clearly show that addition of FEX

at more than 250 ng/ml could inhibit TARC and IL-4 mRNA expression, which was enhanced by Cry j 1 stimulation. These were confirmed by graphs showing the ratio of targets to β -actin obtained from 6 different subjects.

Discussion

FEX, the active carboxylic acid metabolite of terfenadine, has been approved for the relief of symptoms of seasonal allergic rhinitis and atopic dermatitis [5–7, 9]. Although its effect was mainly described from the perspective of a histamine receptor antagonist that affected mast cells [5–7], the precise therapeutic mechanisms of FEX are not well understood.

The present results clearly showed that FEX could not inhibit the proliferative responses of sensitized PBL induced by Cr j 1 stimulation in vitro even when 500 ng/ml

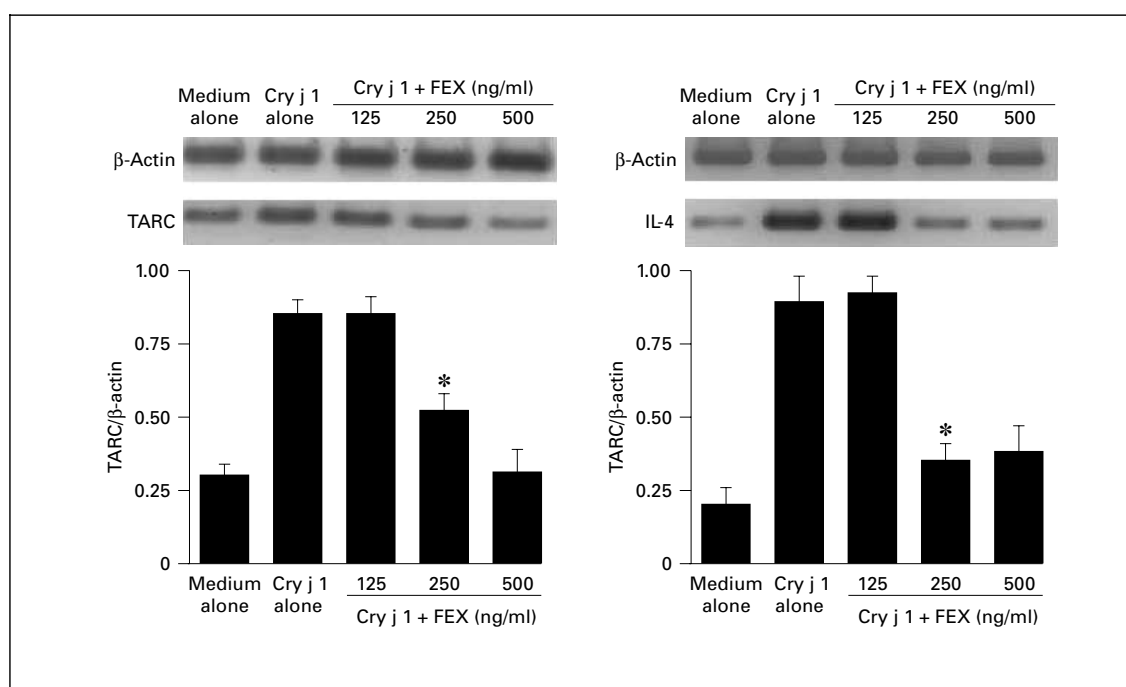


Fig. 7. Inhibitory effects of FEX on TARC and IL-4 mRNA expression in vitro. PBL (1.0×10^6 cells/ml) were stimulated with 100 ng/ml Japanese cedar pollen allergen (Cry j 1) in the presence of various concentrations of FEX in vitro. After 6 days, cells were collected and assayed for mRNA expression by RT-PCR. The PCR products were electrophoresed through 3% agarose gel and stained with SYBR Green. One representative of six is shown. Densitometric analysis of the results of RT-PCR is shown with the intensity of the TARC mRNA. * $p < 0.001$ versus Cry j 1 alone.

of the agent, twice the therapeutic blood levels [18], was added to cell cultures. In addition, we observed that FEX could suppress TARC, a Th2 chemokine, and IL-4, a Th2 cytokine, from sensitized PBL after Cry j 1 stimulation, whereas it virtually did not affect secretion of IFN- γ , a Th1 cytokine. Furthermore, the present data clearly show that the suppressive activity of FEX on TARC and IL-4 secretion was dose dependent and firstly observed at 250 ng/ml, which is a concentration apparently achievable in the blood of patients receiving the agent at conventional therapeutic doses [18].

Recent reports have shown several chemokines to be key molecules involved in the recruitment of inflammatory cells to the sites of allergic diseases [10, 19, 20]. TARC and macrophage-derived chemokine are C-C chemokines that selectively attract Th2-type T cells [19, 20]. Nasal epithelial cells obtained from patients with pollinosis can release much higher levels of TARC than those from normal subjects after IL-4 stimulation in vitro [13]. Immunohistochemistry applied to nasal biopsies also showed TARC immunoreactivity in nasal airway epithelium [13],

indicating that TARC may play important roles in the development and maintenance of allergic diseases. In light of these reports, the present results showing the suppressive activity of FEX on TARC production from PBL after allergen stimulation may be interpreted as demonstrating that FEX may decrease the recruitment of Th2 cells into the airway, including the nasal cavity, through inhibition of TARC production, and reduce the airway inflammatory responses by antigenic stimulation.

Allergic immune responses can be classified into two different phases, an early- or an immediate-phase allergic response and a late inflammatory phase of reactivity. The immediate responses are caused by biochemical mediators such as histamine and leukotrienes, and are responsible for the majority of acute symptoms in allergic rhinitis, asthma and urticaria. In conjunction with these immediate responses, many patients also show late inflammatory reactions, which occur during a typical allergen season and upon exposure to an allergen via nasal provocation [6]. Th2-type T cells are well known to orchestrate the late responses through the secretion of several types of cyto-

kines [6]. IL-4 has been reported to modulate functions of T and B cells, as well as those of mast cells and macrophages, and to promote selective adhesion of inflammatory cells to vascular endothelium [21–23]. On the other hand, the Th1 cytokine IFN- γ exerts selective inhibitory effects on Th2-type T cell function, including proliferation and cytokine production [24]. IFN- γ is also reported to oppose the effects of IL-4 on IgE production from B cells [25]. In light of these reports, the present results showing the suppressive effects of FEX on IL-4 (but not IFN- γ) production strongly suggest that oral administration of FEX to patients with pollinosis may regulate Th1/Th2 polarization, including Th1 and Th2 cytokine balance, and result in favorable modification of the clinical status of this disease.

Studies of the mechanisms of TARC production have shown that the Th2 cytokine IL-4 induces TARC production by macrophages and respiratory epithelial cells [26]. It is also reported that the ability of PBL to produce TARC is enhanced by IL-4 stimulation but is not affected by IFN- γ [16, 26]. In the present study, we found that FEX at more than 250 ng/ml could suppress TARC production from PBL induced by IL-4 stimulation, suggesting that FEX modulates the IL-4-dependent signal pathway responsible for expression of TARC mRNA, and results in inhibition of TARC production.

Th1 T cell activation, including cytokine production (e.g. IFN- γ), is reported to be regulated by a transcription factor, T-box, expressed in T cells (T-bet) [27]. On the

other hand, the GATA-binding protein GATA-3 is reported to be essential for Th2 T cell activation and production of Th2 cytokines, such as IL-4 [27]. In addition to these two transcriptional factors, the importance of several types of signal transducer and activator of transcription (STAT) in Th1 and Th2 T cell activation has also been recognized; STAT1 and STAT4 are essential for IFN- γ production and STAT6 for IL-4 and chemokine production [28–30]. These reports suggest that FEX modulates the function (or activation) of the transcription factors GATA-3 and STAT6 and results in inhibition of both mRNA expression and protein production. This suggestion may be supported by the experimental evidence showing that histamine H₁-receptor antagonists used for clinical treatment of diseases suppress the activation of transcription factors and downregulate inflammatory mediator mRNA expression [31, 32].

In conclusion, our findings indicate that FEX selectively inhibits production of both IL-4 and TARC, which are important factors in the development of allergic responses, from sensitized PBL in vitro. Therefore, FEX may be a useful agent for the treatment of allergic diseases

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