

Effect of Histamine H₁ Receptor Antagonists on TARC/CCL17 and MDC/CCL22 Production from CD14⁺ Cells Induced by Antigenic Stimulation in vitro

Naruo Shoji^a Kazuhito Asano^b Atsuko Furuta^a Kojiro Hirano^a Harumi Suzuki^a

^aDepartment of Otolaryngology, School of Medicine, Showa University, Tokyo, and ^bDivision of Physiology, School of Nursing and Rehabilitation Sciences, Showa University, Yokohama, Japan

Key Words

Antihistamine • CD14⁺ cells • Japanese cedar pollen • NF- κ B • Macrophage-derived chemokine • Thymus- and activation-regulated chemokine • Mitogen-activated protein kinases

Abstract

Background: Thymus- and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC) are accepted to be important molecules in the development and maintenance of allergic diseases. Although several types of histamine H₁ receptor antagonist (antihistamine) have been developed and used for the treatment of allergic diseases, the influence of antihistamines on TARC and MDC production is not well understood. **Objective:** The present study was undertaken to examine the influence of antihistamines on TARC and MDC production from CD14⁺ cells after antigenic stimulation in vitro. **Methods:** CD14⁺ cells prepared from patients with pollinosis to Japanese cedar pollen were stimulated with specific allergen extracted from Japanese cedar pollen (Cry j 1) in the presence of azelastine (AZE), ketotifen (KET), fexofenadine (FEX) and oxatomide (OXA) for 6 days. TARC and MDC levels in culture supernatants were examined by ELISA. We also examined the influence of FEX on TARC and MDC mRNA expression, phosphorylation of mitogen-activated protein kinases (MAPKs) and transcription factor activation in CD14⁺ cells after Cry j 1 stimulation. **Results:**

FEX at 250 ng/ml, which is almost equal to therapeutic blood levels, caused a significant inhibition of TARC and MDC production. However, AZE, OXA and KET required higher concentrations than their therapeutic blood levels to suppress production of these factors. FEX at 250 ng/ml also suppressed NF- κ B activation, phosphorylation of p38 MAPK and extracellular signal-regulated kinases 1 and 2 and expression of mRNA for TARC and MDC. **Conclusions:** These results suggest that antihistamines, especially FEX, suppress CC chemokine production from CD14⁺ cells through interference with antigen-mediated signaling and result in favorable modification of allergic disease states or conditions.

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Introduction

Histamine H₁ receptor antagonists, so-called antihistamines, are well known as first-choice drugs for the treatment of allergic disorders, including urticaria and allergic rhinitis. The clinical effects of these agents are generally believed to be mediated by their antagonistic action against histamine. On the other hand, there is much evidence that antihistamines, such as azelastine hydrochloride (AZE), fexofenadine hydrochloride (FEX), ketotifen fumarate salt (KET) and oxatomide (OXA), inhibit inflammatory cytokine production by T cells in vitro and in vivo [1–3]. It was also observed that second-

generation antihistamines, such as epinastine hydrochloride and FEX, suppressed production of the eosinophil chemoattractants RANTES and eotaxin by nasal fibroblasts in vitro [4, 5]. Furthermore, these two agents were reported to reduce nasal inflammation through the inhibition of RANTES and eotaxin production in response to antigenic stimulation when the agents were administered orally to patients with allergic rhinitis [4]. These reports strongly suggest that inhibition of the production of inflammatory mediators by these agents may be an additional beneficial therapeutic effect not directly related to their antihistamine activity in allergic patients.

Chemokines are chemoattractants that regulate the recruitment of various types of leukocytes from the blood to sites of inflammation [6]. They have been divided into four subfamilies, CXC, CC, CX3C and C, depending on the position of the first two N-terminal cysteine residues [6, 7]. Thymus- and activation-regulated chemokine (TARC; CCL17) is a member of the CC chemokine superfamily and is produced by macrophages, dendritic cells, endothelial cells and T helper 2 (Th2) T cells [8, 9]. Macrophage-derived chemokine (MDC; CCL22) is also a CC chemokine produced by antigen-presenting cells, especially dendritic cells and macrophages [10]. It was reported that these two chemokines may facilitate the recruitment, activation and development of Th2-polarized cells that express CC chemokine receptor 4 [8, 9]. Previous studies also showed that TARC and MDC levels are significantly increased in patients with allergic rhinitis and atopic allergy, and that the serum TARC and MDC levels are significantly correlated with the severity of allergic diseases [9, 11]. These reports indicate that TARC and MDC are potential targets in the management and treatment of allergic diseases. However, little is known about the effects of antihistamines on TARC and MDC production from immune cells of allergic patients. Therefore, in the present study we examined the influence of antihistamines on the production of TARC and MDC by CD14⁺ cells from patients with pollinosis after antigen-specific stimulation in vitro.

Materials and Methods

Reagents

FEX was kindly donated by Sanofi Aventis Pharmaceutical Co. Ltd. (Tokyo, Japan) as a preservative-free pure powder. KET, OXA and chlorpheniramine maleate (CHL) were purchased from Sigma-Aldrich Inc. (St. Louis, Mo., USA). AZE, a preservative-free pure powder, was also kindly donated by Eisai Co. Ltd. (Tokyo, Japan). The agents were dissolved in RPMI-1640 medium (Sigma-

Aldrich) supplemented with 10% heat-inactivated fetal calf serum (RPMI-FCS; Irvine Scientific, Santa Ana, Calif., USA) at appropriate concentrations for experiments, sterilized by passing through 0.2- μ m filters and used for experiments. Histamine was purchased from Sigma-Aldrich, dissolved in RPMI-FCS at appropriate concentrations, sterilized by passing through 0.2- μ m filters and used for experiments.

Antigen

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 Co. Ltd. (Okayama, Japan) as a preservative-free PBS solution. This contained 50 μ g/ml allergen and was diluted with RPMI-FCS at appropriate concentrations for experiments just before use. A purified protein derivative of tuberculin (PPD; Japan BCG Co. Ltd., Tokyo, Japan) was dissolved in RPMI-FCS at a concentration of 50 μ g/ml. These solutions were sterilized by passing through 0.2- μ m filters and used for experiments.

Preparation of CD14⁺ Cells

The subjects were 5 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 in accordance with the established criteria on the basis of patient history and rhinoscopic examination [12]. To confirm the diagnosis and demonstrate allergen-caused pollinosis, skin prick testing (mean wheal 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 Pharmaceutical Co., Ltd., Tokyo, Japan). The number of eosinophils in nasal secretions obtained after the provocation test was also examined using smears stained with Giemsa solution. The characteristics of the subjects investigated in this study are shown in table 1. Peripheral blood mononuclear cells were prepared from donors from whom informed consent (approved by the Ethics Committee of Showa University) was obtained, using Ficoll-Hypaque (Organon Technika, Durham, N.J., USA) density gradient centrifugation. The cells were suspended in RPMI-FCS at 5×10^6 cells/ml, transferred to plastic culture plates and allowed to adhere for 1 h at 37°C. The plates were then washed thoroughly 3 times with RPMI-FCS to remove nonadherent cells. The adherent cells were collected by scraping with rubber policeman, washed 3 times and resuspended in RPMI-FCS at a concentration of 1×10^5 cells/ml. The purity of CD14⁺ cells in cell suspensions was more than 93%, as determined using FITC-labeled CD14 monoclonal antibody (R&D Systems Inc., Minneapolis, Minn., USA) and FACS Calibur (Becton Dickinson Co. Ltd., Baltimore, Md., USA).

Cell Culture

To examine the influence of antihistamines on the ability of CD14⁺ cells to produce TARC and MDC in response to Cry j 1 stimulation, cells (1×10^5) were stimulated in triplicate with Cry j 1 in the presence of various concentrations of antihistamines in 24-well culture plates at 37°C. After 1–8 days, the culture supernatants (2.0 ml) were obtained and stored at –40°C until used. To prepare culture supernatants for stimulation with histamine and

PPD, 1.0 ml of cell suspension (1×10^5 cells) was introduced into each well of 24-well culture plates in triplicate that contained various concentrations of antihistamines. The cells were then stimulated with 10^{-5} M histamine [13] and 20 µg/ml PPD [14] in a final volume of 2.0 ml for 24 and 48 h, respectively. The culture supernatants were obtained and stored at -40°C until used. To examine the effect of the agents on the activity of the transcription factors NF-κB and GATA-1, as well as on chemokine mRNA expression, cells were cultured in a similar manner for 24 and 48 h and stored at -80°C until used. To examine the influence of antihistamines on the phosphorylation of mitogen-activated protein kinases (MAPKs) and proliferative activity, both of which are induced by Cry j 1 stimulation, 100 µl of cell suspension (1×10^4 cells) were cultured in flat-bottomed 96-well culture plates in a similar manner for 24 and 48 h, respectively. In all cases, treatment of cells with antihistamines was started 2 h before stimulation.

Assay for Chemokines

TARC and MDC levels in culture supernatants were examined in duplicate by commercially available ELISA test kits (R&D Systems) according to the manufacturer's recommendations. The minimum detectable level of TARC and MDC with these kits was 7.0 and 62.5 pg/ml, respectively.

Assay for Cell Proliferation

Cell proliferation induced by Cry j 1 stimulation was examined by a commercially available Cell Proliferation ELISA test kit (GE Healthcare Ltd., Buckinghamshire, UK) that contained sufficient reagents according to the manufacturer's recommended procedures. Briefly, cells (1×10^4 cells/well) stimulated with Cry j 1 for 48 h in 96-well flat-bottomed culture plates were labeled with 10 µM 5-bromo-2'-deoxyuridine (BrdU) for 2 h. After removing the BrdU solution, cells were blocked with blocking buffer for 30 min and then treated with peroxidase-labeled anti-BrdU monoclonal antibody for 90 min. After washing 3 times with washing buffer, 3,3',5,5'-tetramethylbenzidine (TMB) was added to each well and incubated for 30 min. After addition of 1 M sulphuric acid, the optical density at 450 nm was measured with an ELISA plate reader.

Assay for mRNA Expression

Poly A+ mRNA was separated from cultured cells with oligo(dT)-coated magnetic micro beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The first-strand cDNA was synthesized from 1.0 µg of Poly A+ mRNA using a Superscript cDNA synthesis kit (Invitrogen Corp., Carlsbad, Calif., USA) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was then carried out using a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, Calif., USA). The PCR mixture consisted of 2.0 µl of sample cDNA solution (100 ng/µl), 25.0 µl of SYBR Green Master Mix (Applied Biosystems), 0.3 µl of both sense and antisense primers and distilled water to give a final volume of 50.0 µl. The reaction was conducted as follows: 4 min at 94°C , followed by 40 cycles of 15 s at 95°C and 60 s at 60°C . Threshold cycle numbers were transformed using the comparative threshold cycle and relative value methods, as described by the manufacturer (Applied Biosystem), and were expressed relative to β-actin, which was used as a housekeeping gene. The nucleotide sequences of the primers were as follows: for TARC, 5'-CCACTGAAGATGCTGGCCCTG-3' (sense) and 5'-G-

Table 1. Characteristics of the subjects investigated in this study

	Controls	Patients
Number of subjects	5	5
Median age, years (range)	51 (26–55)	45 (23–58)
Sex	male	male
Disease severity	nonallergic	mild
Medication	none	none
Serum IgE, U/ml	39.5 ± 7.2	125.8 ± 19.5
IgE RAST score		
Cj	0	5.9 ± 0.6
Aa	0	0
Ap	0	0
Dg	0	0
Df	0	0
Af	0	0
Cd	0	0
Dd	0	0
Blood eosinophil count, %	3.3 ± 0.4	11.3 ± 1.4
Skin prick test	–	+++ ^a
Nasal provocation test		
Symptoms	–	+++ ^b
Smear cytology	<1% eosinophils	>25% eosinophils

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

^a Wheal reaction >13 mm and flare reaction >30 mm against *C. japonica* alone. ^b Positive for sneezing/itch, watery rhinorrhea and nasal blockage against *C. japonica* alone.

AGGCTTCAAGACCTCTCAAG-3' (antisense); for MDC, 5'-TGCCGTGATTACGTCCGTAC-3' (sense) and 5'-ATCGGCACAGATCTCCTTATCC-3' (antisense), and for β-actin, 5'-CGGAA-CCGCTCATTGCC-3' (sense) and 5'-ACCCACACTGTGCCC-ATCTA-3' (antisense).

Assay for Phosphorylation of MAPKs

Phosphorylation of p38 MAPK was analyzed by commercially available ELISA test kits (Active Motif Co. Ltd., Carlsbad, Calif., USA) that contained sufficient antibodies and reagents, in accordance with the manufacturer's recommendations. In brief, cells cultured in flat-bottomed 96-well plates in triplicate were fixed with 4% formaldehyde for 20 min. After removing the formaldehyde, cells were treated with antibody blocking buffer for 1 h at 25°C . After washing 3 times, 100 µl of primary antibody (phosphorylated p38 MAPK antibody) was added to each well, followed by incubation for a further 12 h at 4°C . Antirabbit HRP-conjugated IgG 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 TMB solution. Extracellular signal-regulated kinases 1 and 2 (ERK1/2) and c-Jun N-terminal kinase (JNK) were also examined with commercially available ELISA test kits (Active Motif) according to the manufacturer's recommendations.

Fig. 1. Influence of Japanese cedar pollen (Cry j 1) stimulation on the production of TARC and MDC from CD14⁺ cells in vitro. CD14⁺ cells from 5 patients with pollinosis to Cry j 1 were stimulated with various concentrations of allergen extracted from *C. japonica* for 6 days. TARC and MDC levels were examined by ELISA. The data are expressed as means $\pm$ SE.

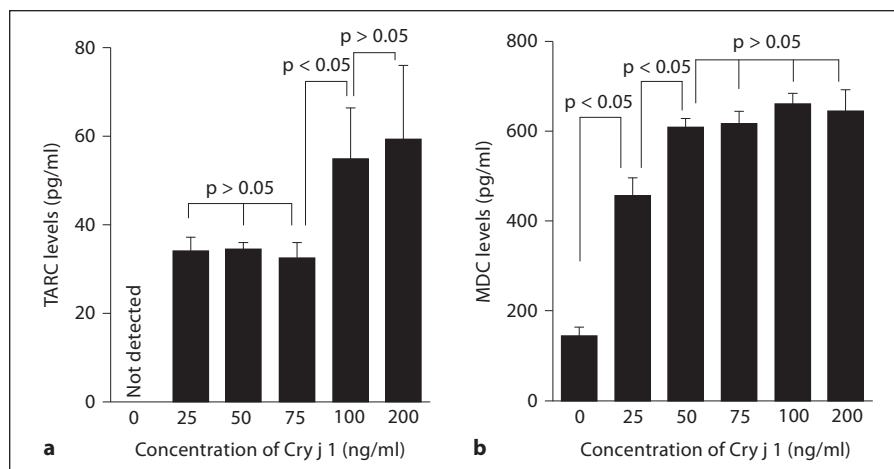
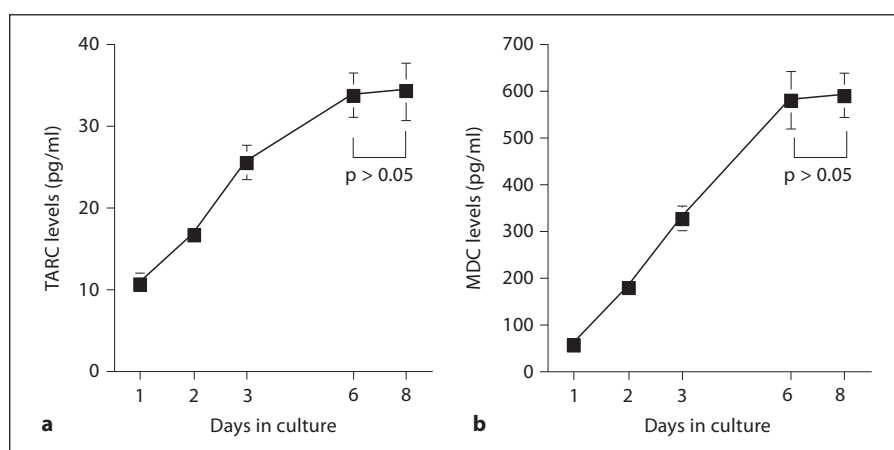


Fig. 2. Time course of TARC and MDC production from CD14⁺ cells after Cry j 1 stimulation in vitro. CD14⁺ cells from 5 patients with pollinosis to Cry j 1 were stimulated with 100 ng/ml allergen extracted from *C. japonica* for 1, 2, 3, 6 and 8 days. TARC (a) and MDC (b) levels in culture supernatants were examined by ELISA. The data are expressed as means $\pm$ SE.



Assay for Activation of Both NF- κ B and GATA-1

NF- κ B activity in cultured cells was analyzed by commercially available NF- κ B ELISA test kits (Active Motif) that contained sufficient reagents and monoclonal antibody against p65, in accordance with the manufacturer's recommended procedures. In brief, nuclear extract (5.0 μ g of protein) from CD14⁺ cells was introduced into each well of 96-well microplates precoated with oligonucleotide containing the NF- κ B consensus site (5'-GGGACTTTCC-3') in a volume of 20.0 μ l, followed by incubation for 1 h at 25°C. After washing 3 times, 100 μ l of monoclonal antibody against p65 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 μ l was then added and incubated for a further 1 h at 25°C. The absorbance at 450 nm was measured after the addition of TMB solution. GATA-1 activity was also measured with commercially available GATA-1 ELISA test kits (Active Motif) in a similar manner.

Statistical Analysis

All data are expressed as means $\pm$ SE. All results were analyzed using one-way analysis of variance. The level of statistical significance was set at $p < 0.05$.

Results

Influence of Cry j 1 Stimulation on TARC and MDC Production from CD14⁺ Cells

The first set of experiments was carried out to examine the influence of Cry j 1 stimulation on the production of both TARC and MDC from CD14⁺ cells in vitro. We initially optically examined the dose of Cry j 1 required to produce TARC and MDC. CD14⁺ cells were stimulated with various concentrations of Cry j 1 (0, 25, 50, 75, 100 and 200 ng/ml), and factor levels in culture supernatants were examined by ELISA on day 6. As shown in figure 1a, the ability of CD14⁺ cells to produce TARC was increased by Cry j 1 stimulation, and the maximum production was observed when the cells were stimulated with Cry j 1 at concentrations above 100 ng/ml. Cry j 1 stimulation also caused a significant increase in MDC levels in culture supernatants when the cells were stimulated with 25 ng/ml Cry j 1 (fig. 1b). The ability of CD14⁺

Fig. 3. Suppressive effect of FEX on TARC and MDC production from CD14⁺ cells in response to Cry j 1 stimulation in vitro. CD14⁺ cells from 5 patients with pollinosis to Cry j 1 were stimulated with 100 ng/ml allergen extracted from *C. japonica* in the presence of various concentrations of FEX for 6 days. TARC (a) and MDC (b) levels in culture supernatants were examined by ELISA. The data are expressed as means $\pm$ SE. Med. = Medium.

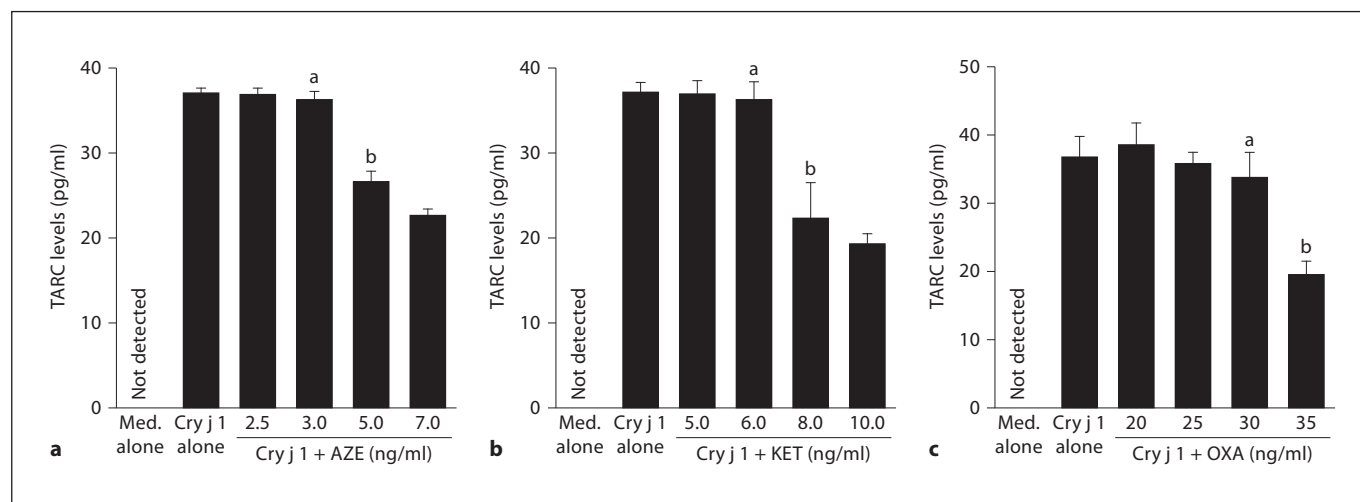
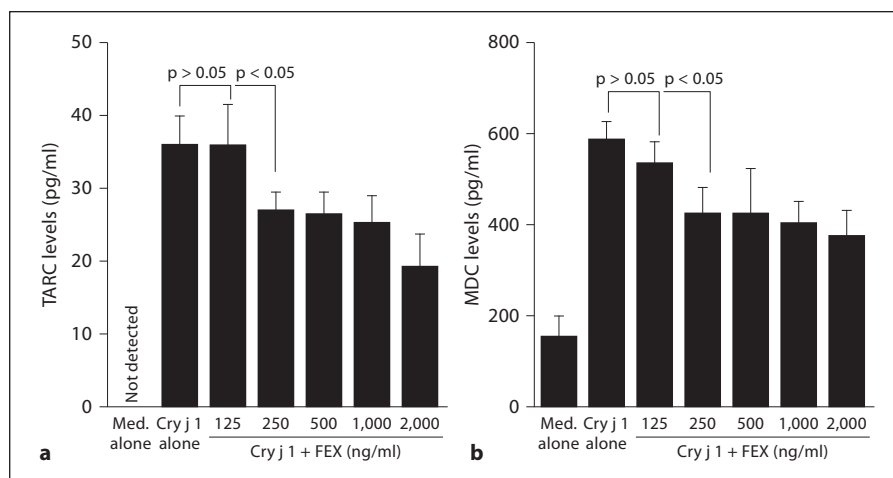


Fig. 4. Influence of antihistamines on the production of TARC from CD14⁺ cells in response to Cry j 1 stimulation in vitro. CD14⁺ cells from patients with pollinosis to Cry j 1 were stimulated with 100 ng/ml allergen extracted from *C. japonica* in the presence of various concentrations of the antihistamines AZE (a),

KET (b) and OXA (c) for 6 days. TARC levels in culture supernatants were examined by ELISA. The data are expressed as means $\pm$ SE. Med. = Medium. ^a $p > 0.05$ versus Cry j 1 alone; ^b $p < 0.05$ versus Cry j 1 alone.

cells to produce MDC upon Cry j 1 stimulation increased further, and much higher levels of MDC were detected in culture supernatants when the cells were stimulated with Cry j 1 at more than 50 ng/ml (fig. 1b). We then examined the time course of the production of both TARC and MDC. CD14⁺ cells were stimulated with 100 ng/ml Cry j 1, and culture supernatants were obtained 1, 2, 3, 6 and 8 days after culture. TARC and MDC levels in culture supernatants gradually increased and peaked on day 6 after culture (fig. 2a, b).

Influence of Antihistamines on TARC and MDC Production from CD14⁺ Cells

The second set of experiments was designed to examine the influence of antihistamines on TARC and MDC production from CD14⁺ cells in response to Cry j 1 stimulation. CD14⁺ cells were stimulated with 100 ng/ml Cry j 1 in the presence of antihistamines. TARC and MDC levels in 6-day-culture supernatants were examined by ELISA. We initially examined the influence of FEX on TARC and MDC production. As shown in figure 3a, FEX at more than 250 ng/ml significantly inhibited the abil-

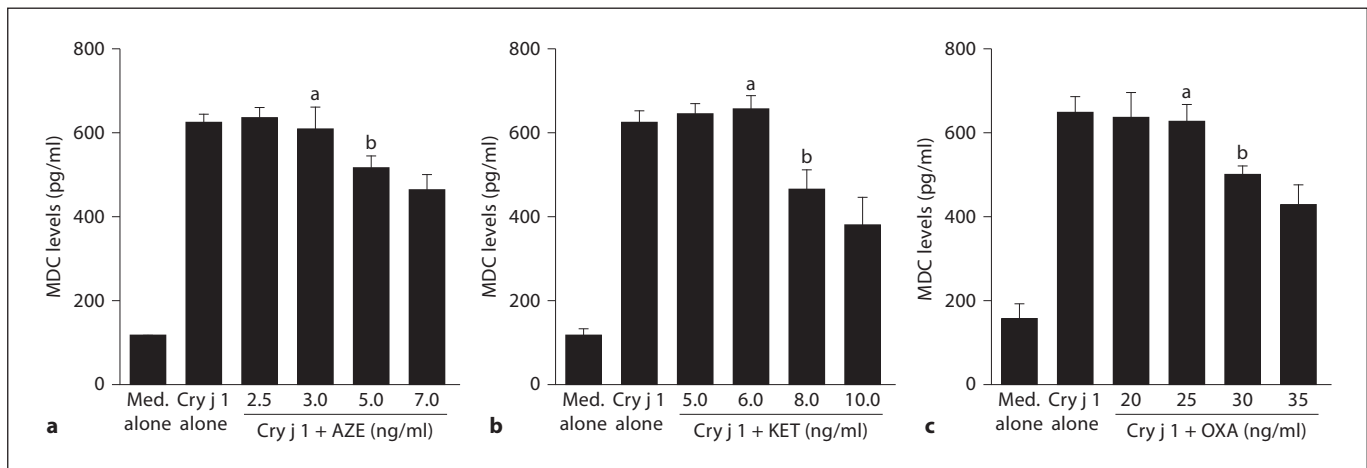
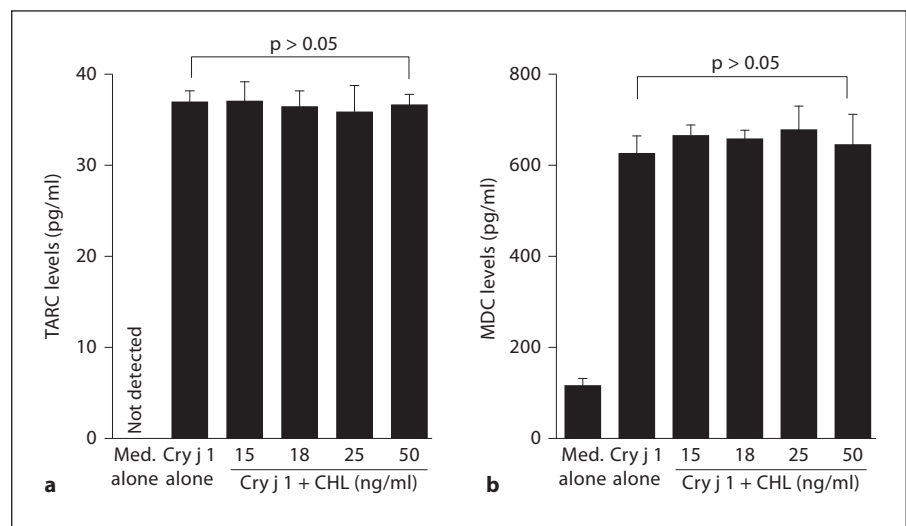


Fig. 5. Influence of antihistamines on the production of MDC from CD14⁺ cells in response to Cry j 1 stimulation in vitro. CD14⁺ cells from patients with pollinosis to Cry j 1 were stimulated with 100 ng/ml allergen extracted from *C. japonica* in the presence of various concentrations of the antihistamines AZE (a),

KET (b) and OXA (c) for 6 days. MDC levels in culture supernatants were examined by ELISA. The data are expressed as means $\pm$ SE. Med. = Medium. ^a $p > 0.05$ versus Cry j 1 alone; ^b $p < 0.05$ versus Cry j 1 alone.

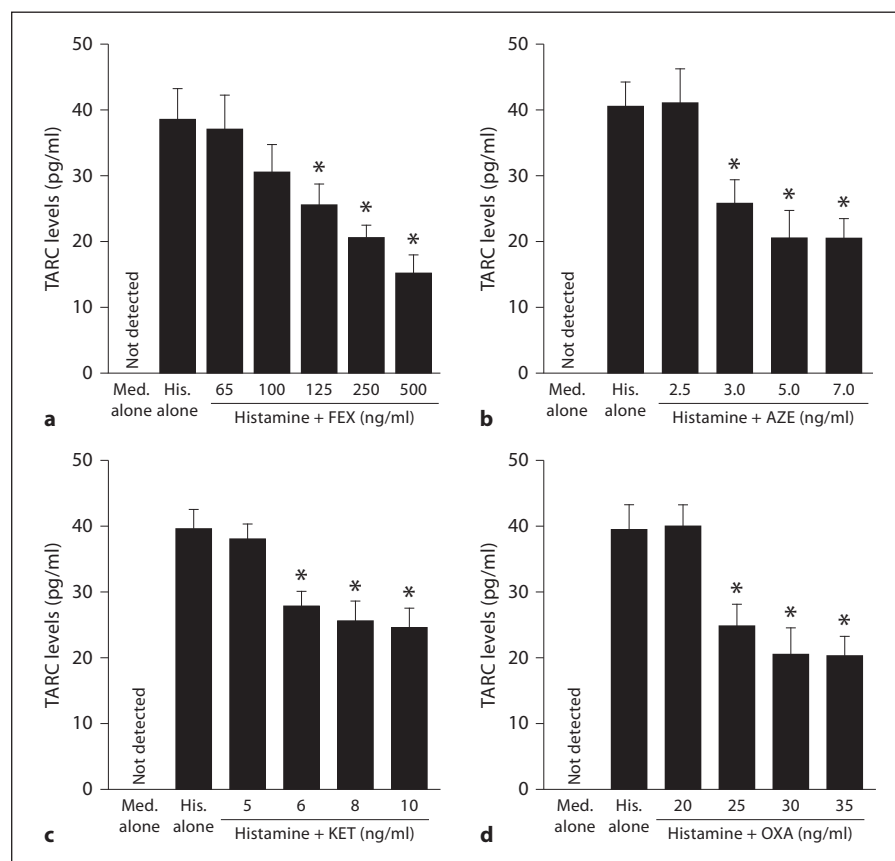
Fig. 6. Negative suppressive effect of CHL on TARC and MDC production from CD14⁺ cells in response to Cry j 1 stimulation in vitro. CD14⁺ cells from patients with pollinosis to Cry j 1 were stimulated with 100 ng/ml allergen extracted from *C. japonica* in the presence of various concentrations of CHL for 6 days. TARC (a) and MDC levels (b) in culture supernatants were examined by ELISA. The data are expressed as means $\pm$ SE. Med. = Medium.



ity of cells to produce TARC, which was increased by stimulation with Cry j 1. Figure 3b shows the suppressive activity of FEX on MDC production from CD14⁺ cells after Cry j 1 stimulation, which was firstly detected at 250 ng/ml. Since there are several types of antihistamines used for treatment of allergic disorders such as pollinosis and atopic allergy, we then examined the influence of AZE, KET, OXA and CHL on TARC and MDC production from CD14⁺ cells in response to Cry j 1 stimulation. AZE (fig. 4a), KET (fig. 4b) and OXA (fig. 4c) suppressed TARC production by CD14⁺ cells af-

ter Cry j 1 stimulation, and the minimum concentrations that caused significant suppression were 5.0, 8.0 and 35.0 ng/ml, respectively (fig. 4a–c). These agents also inhibited MDC production by CD14⁺ cells at similar concentrations to those observed in the case of TARC production (fig. 5a–c). In contrast, CHL did not inhibit TARC (fig. 6a) and MDC (fig. 6b) production by CD14⁺ cells in response to Cry j 1 stimulation, even when 50.0 ng/ml of the agent was added to cell cultures. Since it is well accepted that histamine is responsible for the development of clinical symptoms of allergic diseases, we examined

Fig. 7. Influence of antihistamines on the production of TARC from CD14⁺ cells in response to histamine stimulation in vitro. CD14⁺ cells from patients with pollinosis to Cry j 1 were stimulated with 10^{-5} M histamine (His.) in the presence of various concentrations of the antihistamines FEX (a), AZE (b), KET (c) and OXA (d) for 6 days. TARC levels in culture supernatants were examined by ELISA. The data are expressed as means $\pm$ SE. Med. = Medium. * $p < 0.05$ versus histamine alone.



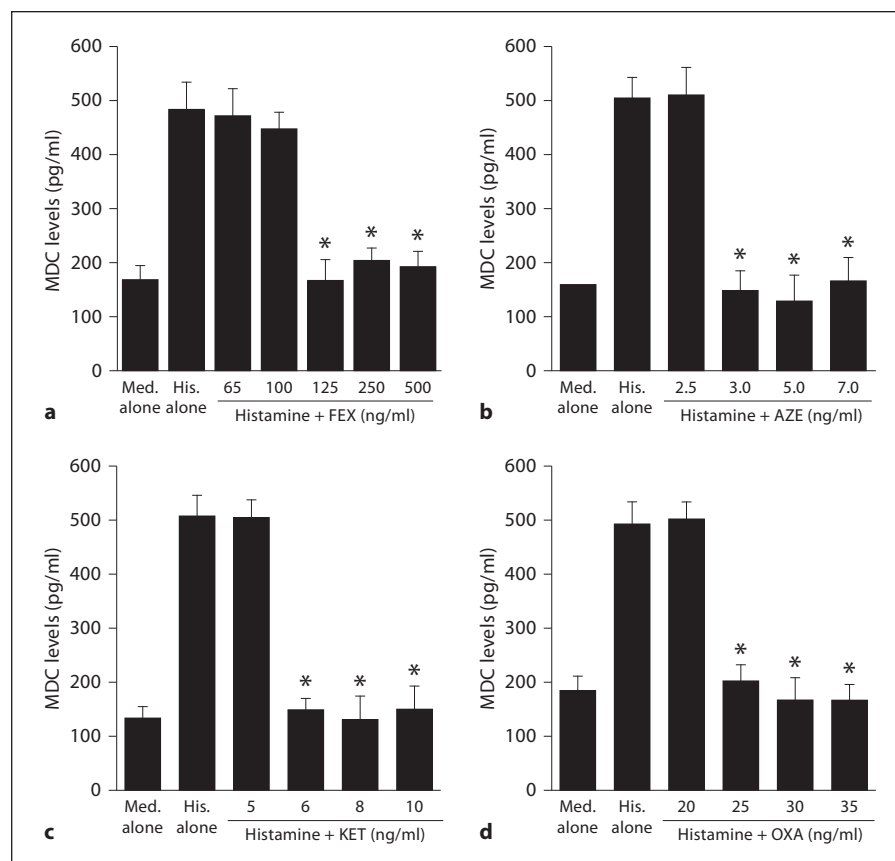
the influence of antihistamines on TARC and MDC production from CD14⁺ cells after histamine stimulation. CD14⁺ cells were stimulated with 10^{-5} M histamine in the presence of various doses of antihistamines. TARC and MDC levels in 24-hour-culture supernatants were examined by ELISA. Treatment of cells with antihistamines caused suppression of TARC production, which was increased with histamine stimulation, and the minimum concentration that caused significant suppression was 125 ng/ml for FEX, 3.0 ng/ml for AZE, 6.0 ng/ml for KET and 25 ng/ml for OXA (fig. 7a–d). These antihistamines also inhibited MDC production from CD14⁺ cells after histamine stimulation at similar concentrations to those observed in the case of TARC production (fig. 8a–d). We finally examined the influence of antihistamines on TARC and MDC production from CD14⁺ cells in response to antigenic, not allergen, stimulation. CD14⁺ cells obtained from nonallergic subjects were stimulated with 20.0 μ g/ml PPD in the presence of various concentrations of antihistamines. TARC and MDC levels in 24-hour-culture supernatants were examined by ELISA. As shown in figure 9a, stimulation of CD14⁺ cells with

PPD increased the ability of cells to produce TARC, and treatment of cells with FEX at 250 ng/ml caused significant inhibition of TARC production. The data in figure 9a also showed that FEX at 500 ng/ml completely suppressed TARC production; experimental culture supernatants contained undetectable levels of TARC (<7.0 pg/ml). Other antihistamines were also able to suppress TARC production from CD14⁺ cells after PPD stimulation. The minimum concentration which caused significant suppression was 3.0 ng/ml for AZE, 6.0 ng/ml for KET and 25 ng/ml for OXA (fig. 9b–d). Treatment of cells with the antihistamines FEX, AZE, KET and OXA also inhibited MDC production from CD14⁺ cells at similar concentrations to those observed in the case of TARC production, which was increased by PPD stimulation (fig. 10a–d).

Influence of Antihistamines on Cell Proliferation Induced by Cry j 1 Stimulation

The third set of experiments was carried out to examine the influence of antihistamines on Cry j 1-induced cell proliferation. CD14⁺ cells were stimulated with 100

Fig. 8. Influence of antihistamines on the production of MDC from CD14⁺ cells in response to histamine stimulation in vitro. CD14⁺ cells from patients with pollinosis to Cry j 1 were stimulated with 10^{-5} M histamine (His.) in the presence of various concentrations of the antihistamines FEX (a), AZE (b), KET (c) and OXA (d) for 6 days. MDC levels in culture supernatants were examined by ELISA. The data are expressed as means $\pm$ SE. Med. = Medium. * $p < 0.05$ versus histamine alone.



ng/ml Cry j 1 in the presence of various concentrations of antihistamines for 48 h. Cell proliferation was examined by ELISA. As shown in figure 11a, addition of FEX to cell cultures scarcely affected cell proliferation, and the optical density at 450 nm in experimental groups was similar (not significant; $p > 0.05$) to that observed in cells stimulated with Cry j 1 alone. The data in figure 11b–d also show the negative suppressive activity of the antihistamines AZE, KET and OXA on cell proliferation induced by Cry j 1 stimulation; the optical density at 450 nm in cells treated with these antihistamines at the maximum concentrations (5.0 ng/ml for AZE, 8.0 ng/ml for KET and 30.0 ng/ml for OXA) was nearly identical (not significant; $p > 0.05$) to that observed in the corresponding controls.

Influence of FEX on the Activation of Both NF- κ B and GATA-1

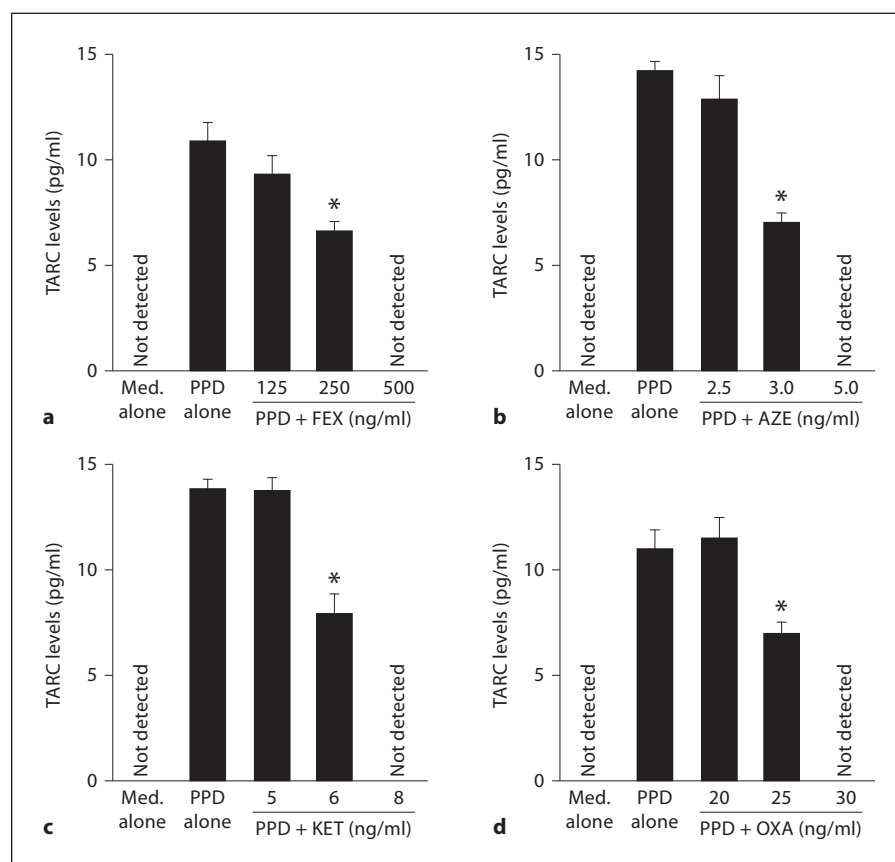
The fourth set of experiments was carried out to examine the influence of FEX on transcription factor activation after Cry j 1 stimulation. CD14⁺ cells were stimulated with 100 ng/ml Cry j 1 in the presence of various

concentrations of FEX for 24 h. The nuclear extracts were prepared, and NF- κ B and GATA-1 activities were measured by ELISA. The addition of FEX to cell cultures at more than 250 ng/ml caused significant suppression of NF- κ B p65 activation, which was increased by Cry j 1 stimulation; the optical density at 450 nm in the experimental groups was significantly lower than that in controls (fig. 12). However, FEX did not suppress GATA-1 activation induced by Cry j 1 stimulation, even when 500 ng/ml of the agent was added to cell cultures (fig. 12).

Influence of FEX on MAPKs

The fifth set of experiments was designed to examine the influence of FEX on the activation of MAPKs in CD14⁺ cells after Cry j 1 stimulation. CD14⁺ cells were stimulated with 100 ng/ml Cry j 1 in the presence of various concentrations of FEX for 24 h. The activation of the MAPKs p38 MAPK, ERK1/2 and JNK was examined by ELISA. As shown in figure 13, addition of FEX at more than 250 ng/ml significantly inhibited p38 MAPK and ERK1/2 activation, which was increased by Cry j 1 stimulation in CD14⁺ cells. On the other hand, Cry j 1 stimula-

Fig. 9. Influence of antihistamines on the production of TARC from CD14⁺ cells in response to PPD stimulation in vitro. CD14⁺ cells from nonallergic subjects were stimulated with 20 μ g/ml PPD in the presence of various concentrations of the antihistamines FEX (**a**), AZE (**b**), KET (**c**) and OXA (**d**) for 6 days. TARC levels in culture supernatants were examined by ELISA. The data are expressed as means $\pm$ SE. Med. = Medium. * $p < 0.05$ versus PPD alone.



tion did not change JNK phosphorylation, and FEX scarcely affected JNK phosphorylation (fig. 13).

Influence of FEX on Expression of mRNA for TARC and MDC

The final set of experiments was carried out to examine the influence of FEX on expression of mRNA for TARC and MDC in CD14⁺ cells after Cry j 1 stimulation. CD14⁺ cells were stimulated with 100 ng/ml Cry j 1 in the presence of various concentrations of FEX for 48 h. The level of mRNA expression was measured by real-time RT-PCR. The addition of FEX to cell cultures at more than 250 ng/ml caused significant suppression of TARC and MDC mRNA expression, which had been enhanced by Cry j 1 stimulation (fig. 14).

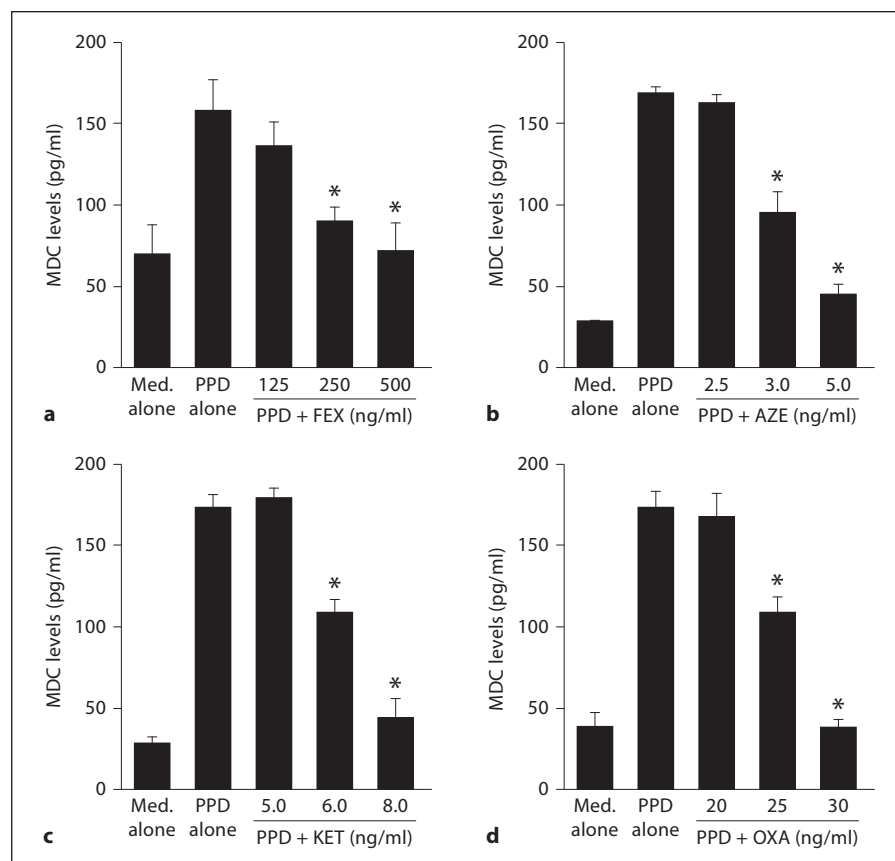
Discussion

Antihistamines such as FEX, AZE, KET and OXA are widely used for treating allergic diseases with remarkable success. The primary pharmacological target of these

agents is generally accepted to be the histamine H₁ receptor, with the goal of inhibiting chemical mediator release from mast cells and eosinophils. It has also been reported that these agents suppress the expression of costimulatory molecules on dendritic cells [15] and the secretion of inflammatory cytokines from T cells and dendritic cells [1, 2, 15, 16]. However, the influence of antihistamines on the production of chemokines, which attract Th2 T cells to allergic inflammatory sites, is not well defined [17].

Therefore, in the present study we examined the influence of antihistamines on TARC and MDC production from CD14⁺ cells in vitro. Our results clearly show that second-generation antihistamines, such as AZE, FEX, KET and OXA, but not a first-generation antihistamine, CHL, suppress the ability of CD14⁺ cells to produce TARC and MDC induced by stimulation with specific antigens and histamine without changes in cell proliferation by antigenic stimulation. We also demonstrated that FEX is the most effective agent in terms of the suppression of TARC and MDC production from CD14⁺ cells after Cry j 1 stimulation. This conclusion may be supported by the observation that FEX exerted a significant

Fig. 10. Influence of antihistamines on the production of MDC from CD14⁺ cells in response to PPD stimulation in vitro. CD14⁺ cells from nonallergic subjects were stimulated with 20 μ g/ml PPD in the presence of various concentrations of the antihistamines FEX (a), AZE (b), KET (c) and OXA (d) for 6 days. MDC levels in culture supernatants were examined by ELISA. The data are expressed as means $\pm$ SE. Med. = Medium. * $p < 0.05$ versus PPD alone.



suppressive effect at a concentration of 250 ng/ml, which is almost equal to therapeutic blood levels [18], but other agents, AZE, KET and OXA, required higher concentrations (approximately 1.5–2 times higher) than their therapeutic blood levels [19–21].

TARC and MDC are Th2 T cell chemokines that bind and attract CC chemokine receptor 4-positive Th2-type T cells [7–9]. It was reported that serum obtained from patients with atopic dermatitis contained much higher levels of both TARC and MDC than that from normal subjects [9, 11, 22] and that these chemokine levels were significantly correlated with the severity of these atopic diseases [9, 11, 22]. In the case of allergic rhinitis, nasal epithelial cells from patients with pollinosis released much higher levels of TARC than those from normal subjects after in vitro stimulation with inflammatory cytokines [23]. It has also been observed that monocyte-derived dendritic cells induced from patients with pollinosis could produce higher levels of MDC by stimulation with specific antigen through the enhancement of MDC mRNA expression [24]. Immunohistochemical analysis of nasal biopsy samples showed TARC immunoreactivity

in nasal epithelium [23]. These reports suggest that TARC and MDC may be strongly involved in the development and maintenance of allergic diseases, including atopic dermatitis and allergic rhinitis. Therefore, the present results showing the suppressive activity of the second-generation antihistamines, especially FEX, on TARC and MDC production may be interpreted as demonstrating that these antihistamines decrease the recruitment of Th2-type T cells to the site of allergic inflammation through the suppression of TARC and MDC production and reduce disease severity after antigenic stimulation in vivo. This may be supported by the observation that a monoclonal antibody against TARC could prevent airway hyperresponsiveness induced by antigenic stimulation in a murine asthma model [25]. Other observations showing the inhibitory action of a polyclonal antibody against mouse MDC on airway hyperresponsiveness and lung inflammation induced by provocation with ovalbumin when the antibody was administered to mice sensitized with ovalbumin also support our speculation [26].

CD14, a coreceptor along with the Toll-like receptor TLR-4 and MD-2 for the detection of lipopolysaccharide,

Fig. 11. Influence of antihistamines on cell proliferation induced by Cry j 1 stimulation in vitro. CD14+ cells from patients with pollinosis to Cry j 1 were stimulated with 100 ng/ml allergen extracted from *C. japonica* in the presence of various concentrations of the antihistamines FEX (a), AZE (b), KET (c) and OXA (d) for 48 h. Cell proliferation was examined by ELISA. The data are expressed as the mean optical density at 450 nm $\pm$ SE. Med. = Medium.

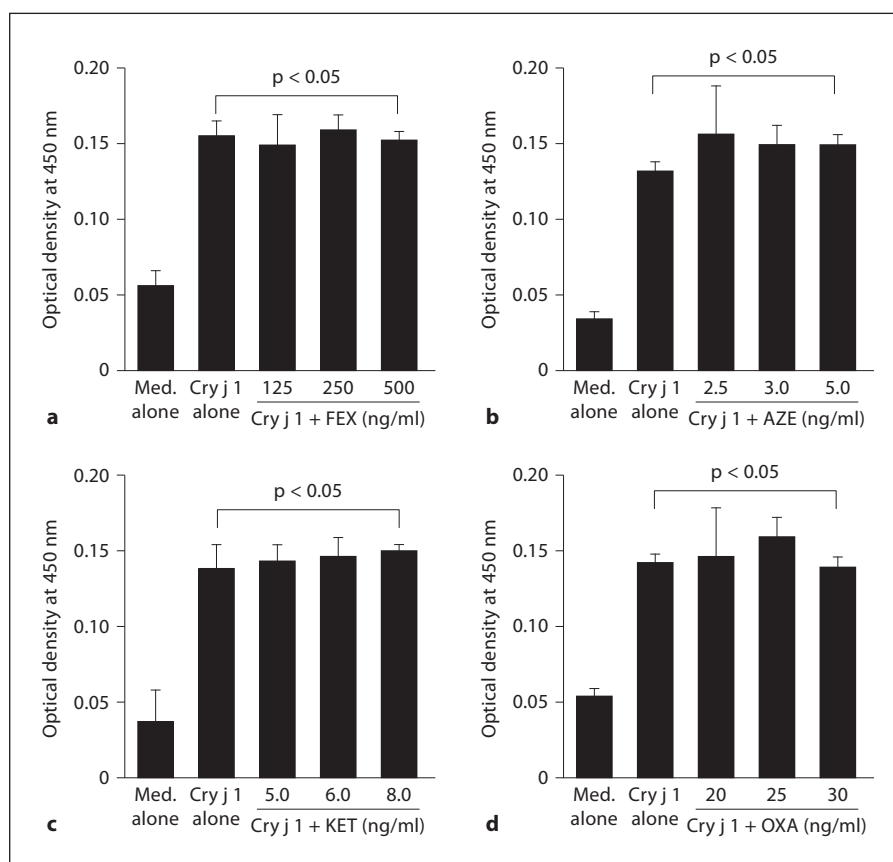
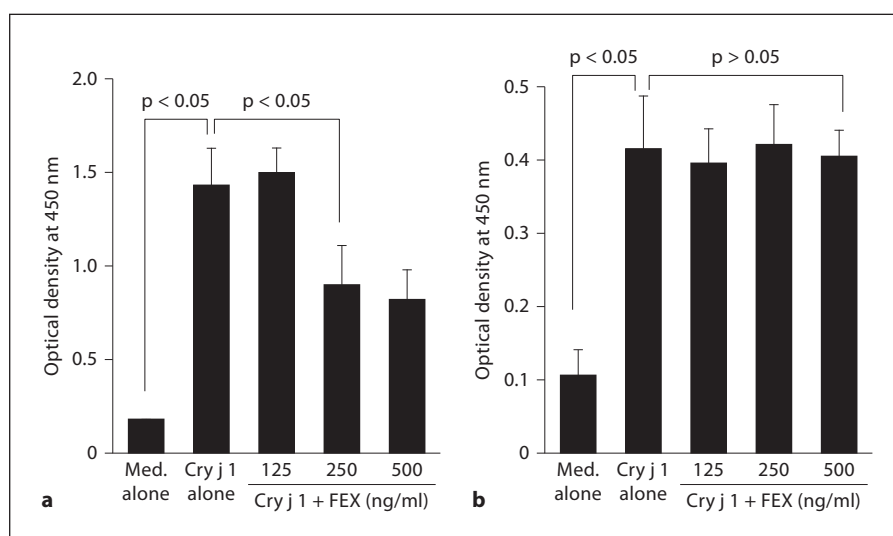


Fig. 12. Influence of FEX on transcription factor activation in CD14+ cells after Cry j 1 stimulation. CD14+ cells from patients with pollinosis to Cry j 1 were stimulated with 100 ng/ml allergen extracted from *C. japonica* in the presence of various concentrations of FEX for 24 h. NF- κ B p65 (a) and GATA-1 (b) activities were examined by ELISA. The data are expressed as the mean optical density at 450 nm $\pm$ SE. Med. = Medium.



is expressed mainly by monocytes/macrophages and by neutrophils, but at levels 10 times lower [27]. These receptors trigger inflammatory responses, activate innate immune responses and prime adaptive immunity to eradicate invading microbes [28]. Macrophages, especially

alveolar macrophages, from patients with allergic inflammation have a significantly greater capacity to stimulate IL-5 production from peripheral CD4+ T cells than those from healthy controls [29]. A lot of studies have shown that alveolar macrophages and peripheral blood

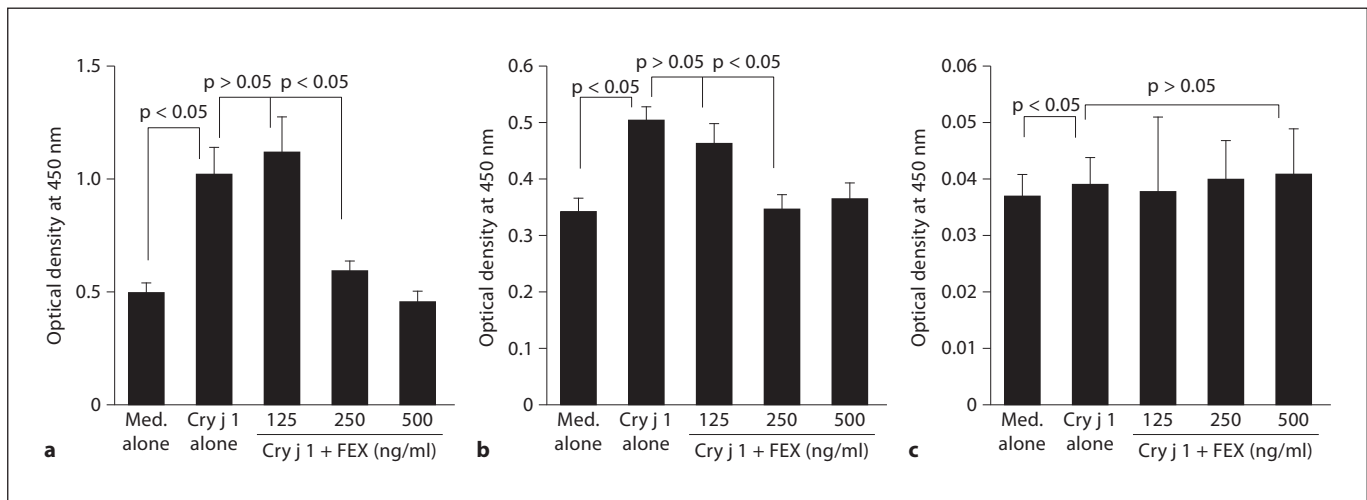
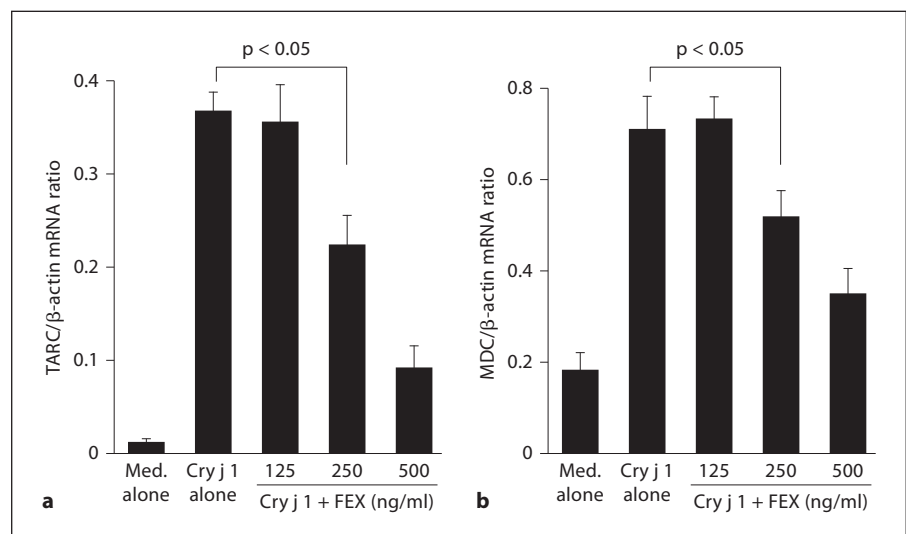


Fig. 13. Influence of FEX on the activation of MAPKs in CD14+ cells after Cry j 1 stimulation. CD14+ cells from patients with pollinosis to Cry j 1 were stimulated with 100 ng/ml allergen extracted from *C. japonica* in the presence of various concentra-

tions of FEX for 24 h. The activation of p38 MAPK (a), ERK1/2 (b) and JNK (c) in CD14+ cells was examined by ELISA. The data are expressed as the mean optical density at 450 nm $\pm$ SE. Med. = Medium.

Fig. 14. Suppressive activity of FEX on TARC and MDC mRNA expression in CD14+ cells after Cry j 1 stimulation. CD14+ cells from patients with pollinosis to Cry j 1 were stimulated with 100 ng/ml allergen extracted from *C. japonica* in the presence of various concentrations of FEX for 24 h. mRNA expression for TARC (a) and MDC (b) was examined by real-time RT-PCR and the results are expressed as means $\pm$ SE relative to β -actin expression. Med. = Medium.



monocytes have an altered phenotypic pattern with increased expression of CD14 after repeated allergen exposure and that these cellular changes correlated with the disease severity [30–32]. It has also been shown that antigenic challenge enhanced the ability of alveolar macrophages to produce increased amounts of superoxide anion as well as cytokines and chemokines, including MDC and TARC [33]. Furthermore, macrophages obtained from mice sensitized with ovalbumin and Al(OH)₃ could produce much higher levels of TARC and MDC in re-

sponse to in vitro stimulation with anti-CD40 antibodies [34]. In the case of atopic dermatitis, sensitization with extract of *Dermatophagoides farinae* in NC/Nga mice, an experimental model of atopic dermatitis, caused an increase in the accumulation of macrophages in atopic lesions, and the ability of these macrophages to produce TARC and MDC was significantly increased compared with those from nonsensitized mice [35]. These reports strongly indicate that CD14+ cells, especially macrophages, are implicated in the development and mainte-

nance of allergic diseases, such as asthma and atopic dermatitis. Moreover, these reports further support our speculation that the suppressive activity of antihistamines on TARC and MDC production from CD14+ cells by the stimulation of specific antigens and histamine consists, in part, of a therapeutic mode of action of the agents on allergic diseases.

The production of cytokines and chemokines by antigen-presenting cells such as CD14+ cells after antigenic stimulation is reported to require the activation of various signaling molecules, including ERK, JNK and p38 MAPK [17, 36, 37]. It has also been demonstrated that p38 MAPK contributes to the production of TARC and MDC in parallel with the activation of NF- κ B [38]. Therefore, we then examined the possible mechanisms by which antihistamines suppress TARC and MDC production from CD14+ cells in response to antigenic stimulation through the use of FEX, which is the most effective agent in terms of the inhibition of TARC and MDC production. The present results clearly showed that FEX at 250 ng/ml suppressed the phosphorylation of ERK and p38 MAPK, but not JNK, which were induced by Cry j 1 stimulation of CD14+ cells. This suggests that antihistamines, especially FEX, suppress the production of TARC and MDC by inhibiting NF- κ B activation through ERK and p38 MAPK pathways in CD14+ cells. There is evidence that GATA-1 is an important regulatory factor in the transcription of CC chemokines in human airway epithelial cells and fibroblasts, when these cells are stimulated with the inflammatory cytokine TNF- α [39]. However, the

present results clearly show that stimulation of CD14+ cells with a specific antigen, Cry j 1, did not increase GATA-1 activation, and FEX scarcely affected the activation, indicating that GATA-1 may be a minor transcription factor in the production of CC chemokine in CD14+ cells after antigenic stimulation.

The present results clearly show that antihistamines exert a suppressive effect on CC chemokine production by CD14+ cells in response to antigenic stimulation. However, the minimum concentration of the agent required for significant suppression of TARC and MDC production differs depending on the antihistamine used. The reasons for this discrepancy remain unclear; however, it is possible that the difference in the susceptibility of antihistamines to MAPKs (e.g. ERK and p38 MAPK) and NF- κ B might be responsible. Further experiments are required to clarify this point.

In conclusion, the present results strongly suggest that the attenuating effect of antihistamines, especially on TARC and MDC production by CD14+ cells after Cry j 1 stimulation, constitute at least part of the activity of these agents in treating allergic diseases, including atopic allergy and allergic rhinitis.

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