

# The Effect of Suplatast Tosilate on TARC Production in Peripheral Blood Mononuclear Cells and TARC Plasma Levels

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## ABSTRACT

**Background:** Thymus and activation-regulated chemokine (TARC/CCL17) is a highly specific ligand for CCR 4. TARC may contribute to the recruitment, activation, and development of Th2 polarized cells that express CCR4. These characteristics have led investigators to hypothesize that TARC is involved in the development of Th2 responses. Suplatast tosilate ((±)-[2-[4-(3-ethoxy-2-hydroxy-propoxy) phenylcarbamoyl] ethyl] dimethylsulfonium p-toluenesulfonate) is an anti-allergic agent that selectively suppresses the synthesis of Th2 cytokines. We examined the effect of suplatast tosilate on TARC production and CCR-4 expression *in vitro*. Furthermore, we attempted to clarify whether TARC production was suppressed after clinical administration of suplatast tosilate.

**Methods:** Peripheral blood mononuclear cells (PBMCs) were isolated from patients with allergic rhinitis who tested positive to house dust. PBMCs were stimulated with mite antigen. TARC mRNA was detected by real time PCR. The amount of TARC was estimated using an ELISA kit. PBMCs expressing CCR-4 were sorted by flow cytometry. The plasma level of TARC was examined in patients with chronic allergic rhinitis before and after treatment with suplatast tosilate for 4 weeks.

**Results:** Suplatast tosilate significantly reduced TARC production by PBMCs. TARC mRNA was also suppressed in a concentration dependent manner. However, suplatast tosilate did not inhibit the expression of CCR-4 on PBMCs. The plasma level of TARC was significantly decreased in patients administered suplatast tosilate.

**Conclusions:** Suplatast tosilate suppressed TARC production by PBMCs and decreased the plasma level of TARC in patients with chronic allergic rhinitis.

## KEY WORDS

allergic rhinitis, chemokines, suplatast tosilate, TARC

## INTRODUCTION

Allergic rhinitis, as asthma and atopic dermatitis, is a manifestation of atopy characterized by the formation of IgE in response to environmental allergens. Eosinophils play an important role in the development of swelling and hyperreactivity of the nasal mucosa in response to non-specific stimulation.<sup>1-3</sup> Anti-

gen presentation to Th2 memory cells leads to the generation of interleukins (IL)-4, IL-13 and IL-5, which are involved in the stimulation of IgE production and the recruitment, activation and maintenance of eosinophils. They also stimulate the release of growth factors and chemokines from structural cells, such as epithelial cells, endothelial cells and fibroblasts. It has been reported that lymphocytic cell

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lines derived from Th1 cells selectively express CXCR3 and CCR5 *in vitro*, whereas lymphocytic cell lines derived from Th2 cells selectively express CCR3, CCR4 and CCR8.<sup>4-7</sup> Thymus and activation-regulated chemokine (TARC) was recently found to be a highly specific ligand for CCR4.<sup>8</sup> A number of cellular sources of TARC have been identified, including macrophages, dendritic cells, and natural killer cells. In addition, CD4 positive T cells, including naive T cells, Th2 cells and memory/effector T cells, were shown to produce TARC protein.<sup>9-11</sup> They may aid in the recruitment, activation, and development of Th2 polarized cells that express CCR4. These characteristics have led investigators to hypothesize that TARC is involved in the development of the Th2 response.<sup>12</sup> Therefore, the interaction between TARC and CCR4 is important in the pathogenesis of allergic inflammation. In a basic study, suplatast tosilate ((±)-[2-[4-(3-ethoxy-2-hydroxy-propoxy) phenylcarbamoyl] ethyl] dimethylsulfonium p-toluenesulfonate) inhibited an experimental asthma model of type I allergic reaction, passive cutaneous anaphylaxis and allergic rhinitis. Furthermore, Suplatast tosilate was confirmed to inhibit IgE antibody production in a class-specific manner without inhibiting IgE and IgG antibody production and to inhibit tissue infiltration by eosinophils, which is a cause of allergic inflammation. These actions are considered to be based on the inhibition of cytokine IL-4 and IL-5 produced by helper cells.<sup>13-20</sup> It also inhibits the allergen-induced increase of both eosinophils and CD4<sup>+</sup> T cells in airways and prevents allergen-induced goblet cell metaplasia.<sup>18,21</sup> These results strongly suggest that suplatast tosilate is a highly functional anti-allergic agent. In this study, we examined the effect of suplatast tosilate on TARC production from peripheral blood mononuclear cells (PBMCs) and CCR-4 expression *in vitro* and *in vivo*.

## METHODS

### EFFECTS OF SUPLATAST TOSILATE ON THE PRODUCTION OF TARC IN PBMCs

#### Subjects

This study enrolled 8 subjects with allergic rhinitis (6 men and 2 women, age 19–45 years, average age 25.2 ± 6.9 years) and 8 healthy controls (3 men and 5 women, age 18–41 years, average age 24.6 ± 7.7 years). All subjects underwent measurement of total serum IgE levels (IU/ml) and specific IgE antibody levels (UA/ml) to at least six inhalant allergens including house dust (HD; Greer Labs, Lenoir, NC, USA), mite (*Dermatophagoides pteronyssinus*), pollen of Japanese cedar (*Cryptomeria japonica*), common ragweed (*Ambrosia artemisiifolia*), cocksfoot (*Dactylis glomerata*), and mugwort (*Artemisia vulgaris*) by radioallergosorbent test (RAST). All patients with allergic rhinitis had a score of 2 or more on a RAST to *Dermatophagoides farinae* and a history of reasonably recurrent sneezing, watery rhinorrhea, and nasal ob-

struction. Neither patients nor controls had used any topical steroid or other medication for at least one month before the study. The control individuals were selected from among those who met the following conditions: absence of eosinophilia in nasal smears; RAST negative to the six allergens; IgE not higher than 300 U/ml; and absence of symptoms of nasal hyper-reactivity, such as sneezing and watery nasal discharge.

All patients were informed about the content of the study, and gave informed consent prior to participation. In addition this study was approved by the ethics committee of the Graduate School of Medicine, Chiba University.

#### Cells

PBMCs were purified by centrifugation at 88 *g* for 30 min at 20°C against a Ficoll-Hypaque density gradient. The cells at the interface (mononuclear cells) were collected and washed twice in RPMI 1640 supplemented with heat-inactivated 10% fetal calf serum (FCS). We selected only mononuclear cells with a viability of more than 95% as assessed by the trypan blue exclusion dye test. These mononuclear cells were suspended in RPMI 1640 containing 10% FCS and adjusted to a cell density of 2.5 × 10<sup>5</sup> cells/ml. They were incubated in the presence or absence of suplatast tosilate at various concentrations for 24 hours, followed by challenge with mite antigen (10 µg/ml) for up to 96 hours. As a control, PBMCs, isolated from healthy control subjects, were stimulated with or without mite antigen. The effect of dexamethasone on TARC production was also examined. The supernatants were separated by centrifugation at 200 *g* for 5 min at 4°C and stored at -80°C until use. Cells were harvested and immediately frozen in liquid nitrogen until use.

#### Real Time RT-PCR

Total RNA was extracted using an RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA concentration and purity were determined on a spectrophotometer (UV-1201, Shimadzu, Japan) by calculating the ratio of optical density at wavelengths of 260 and 280 nm. An Omniscript RT (Qiagen), a reverse transcriptase inhibitor (Lifetech, Rockville, MD, USA) and an Oligo dT primer (Lifetech) were used in the synthesis of complementary DNA.

PCR was performed using a Light Cycler Fast Start Hybridization Probes buffer (Roche Molecular Biochemicals)<sup>22,23</sup> by rapid cycling in a reaction volume of 20 µl with 0.5 mmol/L of each primer, 0.2 mmol/L of anchor probes, 0.4 mmol/L of detection probes and 50 ng of genomic DNA.

The Light Cycler Fast Start DNA Master Hybridization Probes buffer used was a 10-fold stock solution containing nucleotides, *Thermus aquaticus* DNA po-

**Table 1** Oligonucleotides used in this study.

b-actin	459bp	Sequence 5' - 3'
	Forward primer	CCAACCGCGAGCAGCTG
	Reverse primer	GGAAGGAAGGCTGGAAG
	Anchor probe	CCTCCCCATGCCATCCTGCGTC-FL
	Detection probe	LC-GGACCTGGCTGGCCGGGACCTG
TARC	241bp	
	Forward primer	TCTCTGCAGCACATCCACGC
	Reverse primer	GGCTTCAAGACCTCTCAAGG
	Anchor probe	GCTCCAGGGATGCCATCGTT-FL
	Detection probe	LC-TTGTAAGTGTGCAGGGCAGGG

lymerase and 10 mmol/L of Mg<sup>2+</sup>. The final Mg<sup>2+</sup> concentration in the reaction mixture was adjusted to 2 mmol/L for TARC and 3 mmol/L for  $\beta$ -actin. The samples were loaded into glass capillary cuvettes (Roche Molecular Biochemicals) and centrifuged to place the sample at the capillary tip before capping. After the initial denaturation at 95°C for 10 min, DNA was amplified through 40 cycles of denaturation (95°C for 15 sec), annealing (60°C for 15 sec) and extension (72°C for 10 sec). The temperature was programmed to decrease 20°C from denaturation to annealing, 20°C from annealing to extension, and 20°C from extension to denaturation. Fluorescence was measured at the end of the annealing period of each cycle to monitor amplification.

To detect TARC mRNA, four oligonucleotides were used in the assay; of them, two were the labeled anchor and detection probes and two served as amplification primers (Table 1).

All the hybridization probe PCR assays were performed in quadruplicate. The intraassay and interassay variation were 5.4% and 10.3%, respectively. The ratio of TARC mRNA to  $\beta$ -actin mRNA was determined.

#### ELISA for TARC

The amount of TARC was estimated using an ELISA kit (R & D Systems, Minneapolis, USA) and expressed in pg/mL; the lowest detection limit was 7 pg/mL. The ELISA used did not cross-react with other cytokines [IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-13, GM-CSF, IFN- $\gamma$  and TNF- $\alpha$ ] at levels of 50 ng/mL.

#### Flow Cytometric Analysis of CCR-4 Expression on PBMCs

In order to investigate the effect of suplatast tosilate on CCR-4 expression, we examined the expression of CCR-4 on PBMCs expressing CD4 or CD45RO before and after the clinical administration of suplatast tosilate. The cells were stained with PE-conjugated anti-CD4 monoclonal antibody (MoAb) (mouse IgG1, R & D Systems) or cyanine dye-conjugated anti-CD45 RO MoAb (mouse IgG1, R & D Systems) followed by

FITC-conjugated anti-CCR4 (KM-2160, mouse IgG1). This MoAb against CCR4 has been characterized in a previous report.<sup>12</sup> CCR-4 expression on CD4<sup>+</sup>CD45 RO<sup>+</sup>, CD4<sup>+</sup>CD45RO<sup>-</sup>, CD4<sup>-</sup>CD45RO<sup>+</sup>, or CD4<sup>-</sup>CD45 RO<sup>-</sup> PBMCs was examined.

The number of CCR-4<sup>+</sup> PBMCs expressing CD4<sup>+</sup>CD45RO<sup>+</sup> was counted. Labeled cells were analyzed using flow cytometry (FACScan, Becton-Dickinson, San Jose, CA, USA).

#### CHANGES IN TARC PLASMA LEVEL AFTER CLINICAL ADMINISTRATION OF SUPLATAST TOSILATE

The study was carried out on a group of 12 patients sensitized to house dust mites and with symptoms of perennial allergic rhinitis (7 men and 5 women, age 20–48 years, average age 28.6  $\pm$  6.1 years). All patients had a score of 2 or more on a RAST to *Derma-tophagoides farinae*. By using a patient diary, the severity of nasal symptoms, such as sneezing, rhinorrhea and nasal obstruction, was scored by patients each day during the study period in accordance with the Severity Classification of the Clinical Practice Guideline for Nasal Allergy and the Symptom Score (Table 2).<sup>24</sup> Symptom scores were determined by comparing the two different symptoms (Table 3). All patients had a score of 2 or more on this symptom score.

After a 1-week washout period, patients took oral suplatast tosilate (100 mg, three times per day) for 4 weeks. Before and after the administration, the plasma level of TARC was measured. The global improvement rating of "remarkably improved" was assigned when the symptom score decreased by 2 points or more. A rating of "moderately improved" was assigned when the symptom score decreased by 1 point. General laboratory tests (hematology, biochemical profile and urinalysis) were performed before and after treatment, in addition to monitoring subjective and objective adverse reactions. It has been reported that the adverse reactions of suplatast tosilate appeared in 5.0% (73/1460) of the patients.<sup>25</sup> Major adverse reactions included gastrointestinal symptoms such as stomach discomfort (0.5%) and

**Table 2** Evaluation criteria for determination of the symptom score

	Score (points)				
	4	3	2	1	0
Sneezing attacks *	$\geq 21$	20-11	10-6	5-1	0
Nasal discharge †	$\geq 21$	20-11	10-6	5-1	0
Nasal obstruction	Complete nasal obstruction all day	Nasal obstruction severe and breathing through the mouth performed for a considerable time	Nasal obstruction marked and breathing through the mouth performed several times a day	No breathing through the mouth, but nasal obstruction present	No nasal obstruction

\* Mean no. attacks per day

† Mean no. nose blows per day

**Table 3** Symptom score

	Sneezing attack or secretion score				
	4	3	2	1	0
Nasal obstruction score					
4	4	4	4	4	4
3	4	3	3	3	3
2	4	3	2	2	2
1	4	3	2	1	1
0	4	3	2	1	0

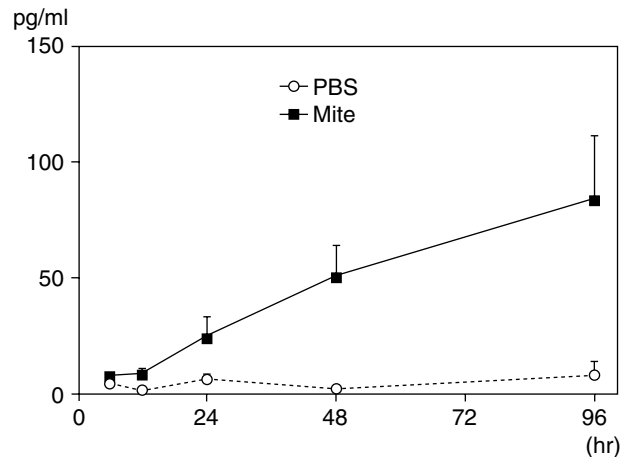
nausea (0.8%), hepatic function disorders such as increased ALT (GPT) (0.4%) and increased AST (GOT) (0.3%), sleepiness (0.6%), and rash (0.4%). All patients were informed about the content of the study, and gave informed consent prior to participation. In addition this study was approved by the ethics committee of the Graduate School of Medicine, Chiba University.

### Statistical Analysis

The results of the study were expressed as the mean values  $\pm$  SEM. Significance was determined using paired Wilcoxon tests. A *p* value of  $<0.05$  was considered to indicate a significant difference. The statistical software package SAS (SAS Institute Inc., Cary, NC, USA) was used for the statistical analysis.

### RESULTS

PBMCs were pre-incubated with suplatast tosilate ( $10^{-5}$  to  $10^{-8}$  M) or dexamethasone ( $10^{-7}$  M) for 24 hr and were then stimulated with mite antigen to investigate the effect of suplatast tosilate on the production of TARC from these stimulated PBMCs. As shown in Figure 1, mite antigen induced TARC production by PBMCs, isolated from patients with allergic rhinitis, in a time dependent manner. PBMCs from healthy control subjects did not produce significant amounts of TARC (data not shown). Dexamethasone ( $10^{-7}$  M) significantly inhibited the production of TARC (Fig. 2). Pre-incubation with suplatast tosilate also decreased TARC production in a concentration-dependent manner. At concentrations of  $1 \times 10^{-5}$  and  $1 \times 10^{-6}$  M, suplatast tosilate signifi-



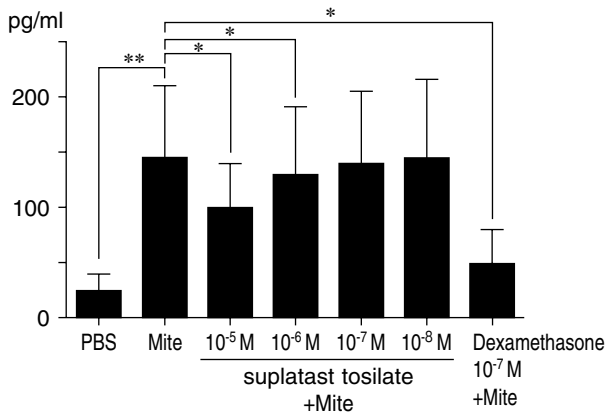
**Fig. 1** Time-course of TARC production by PBMCs after stimulation with mite antigen. Data are the mean + SEM of four experiments.

cantly reduced TARC production. Suplatast tosilate did not affect TARC production by unstimulated PBMCs (data not shown). The mRNA expression of TARC was suppressed by suplatast tosilate in a concentration-dependent manner (Fig. 3).

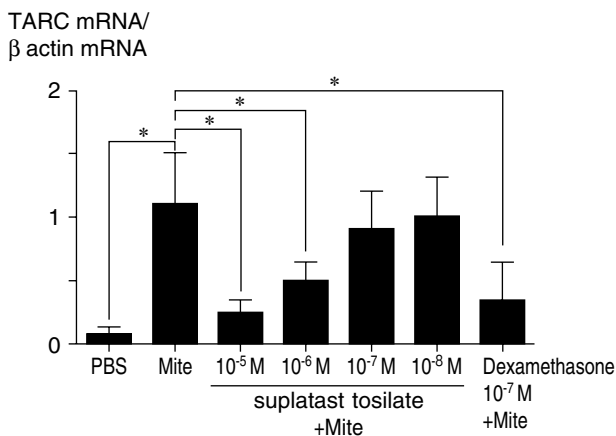
The effect of suplatast tosilate on the expression of CCR-4 on PBMCs was clarified by treating the cells with suplatast tosilate for 24 hr and examining the change in CCR-4 expression by flow cytometry.

CCR 4 is preferentially expressed on CD4<sup>+</sup>CD45RO<sup>+</sup> PBMCs in patients with nasal allergy. The mite antigen significantly upregulated CCR4 expression on PBMCs. However, suplatast tosilate did not exhibit a significant concentration-dependent inhibitory effect on the expression of CCR-4 on PBMCs at concentrations of  $1 \times 10^{-5}$ ,  $1 \times 10^{-6}$  and  $1 \times 10^{-7}$  M (Fig. 4).

Next, we attempted to clarify whether the production of TARC was suppressed after clinical administration of suplatast tosilate. Based on the records in an allergic symptom diary, the frequency of sneezing, frequency of nose blowing and nasal obstruction score were calculated for each patient in accordance

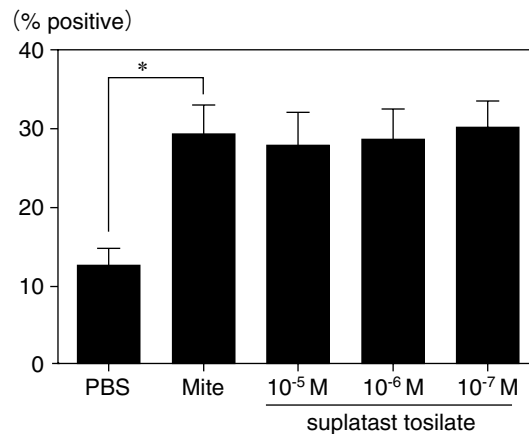


**Fig. 2** Effect of suplatast tosilate on TARC production by PBMCs. The supernatants were obtained 96 hr after stimulation with mite antigen. Suplatast tosilate significantly reduced TARC production at concentrations of  $1 \times 10^{-5}$  and  $1 \times 10^{-6}$  M. Data are the mean + SEM of eight experiments. \*  $p < 0.05$ , †  $p < 0.01$



**Fig. 3** Effect of suplatast tosilate on TARC mRNA expression in PBMCs. mRNA expression was normalized relative to that of  $\beta$ -actin mRNA. PBMCs were obtained 12 hr after specific antigen stimulation. Suplatast tosilate significantly reduced TARC mRNA expression at concentrations of  $1 \times 10^{-5}$  and  $1 \times 10^{-6}$  M. \*  $p < 0.05$

with the Severity Classification of the Clinical Practice Guideline for Nasal Allergy and the Symptom Score (Table 2).<sup>24</sup> After the start of the 4-week suplatast tosilate treatment, all clinical scores, particularly that of nasal obstruction, were significantly decreased (Fig. 5). More than two nasal obstruction scores were decreased in 6 out of 12 patients (Fig. 5c). According to a global improvement rating 3 patients were rated as “remarkably improved”, 5 as “moderately improved”, and 4 patients were rated as “unchanged”. No subjective/objective adverse reac-



**Fig. 4** Effect of suplatast tosilate on the expression of CCR-4 on PBMCs. Suplatast tosilate did not exhibit a significant inhibitory effect on the expression of CCR-4 on PBMCs. \*  $p < 0.05$ , compared with the values of the mite control.

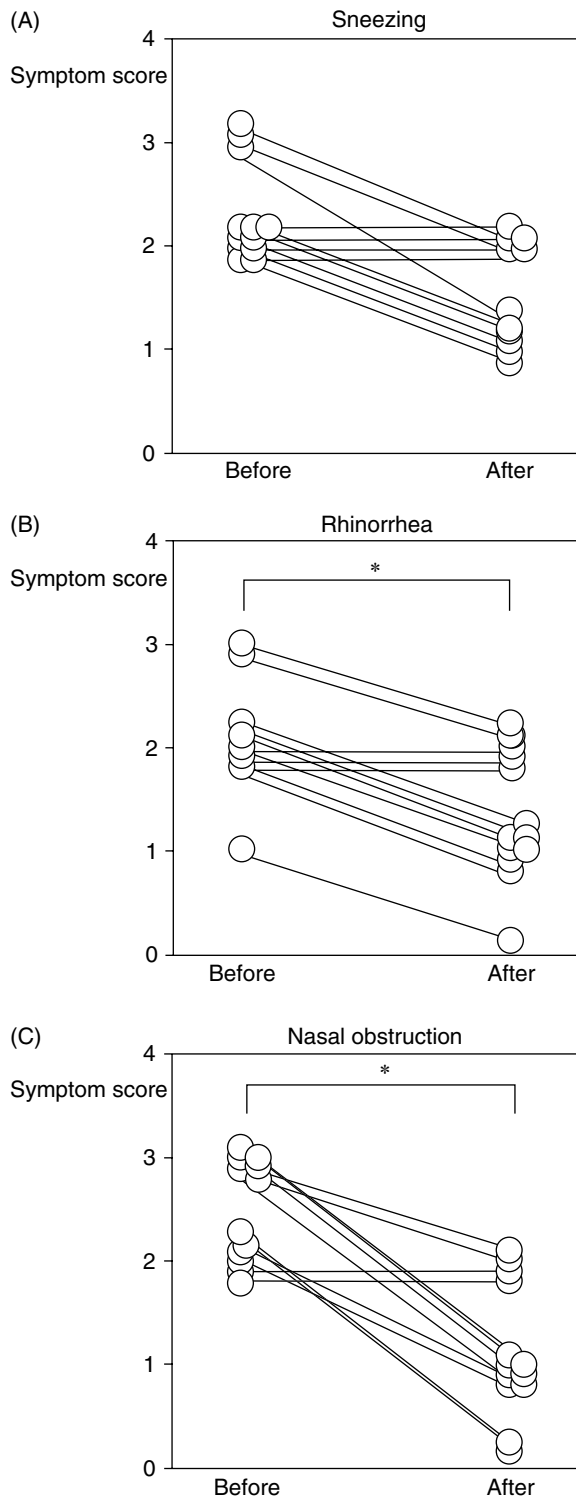
tions or abnormal changes in laboratory test results were observed in any of the 12 patients treated with suplatast tosilate for 4 weeks.

The plasma level of TARC was significantly decreased after the 4-week administration (Fig. 6). The correlation between plasma TARC concentration and these clinical parameters was tested. The percentage of decrease in plasma TARC concentration was significantly higher in the patients for whom suplatast tosilate proved more than ‘moderately improved’ (Fig. 7).

## DISCUSSION

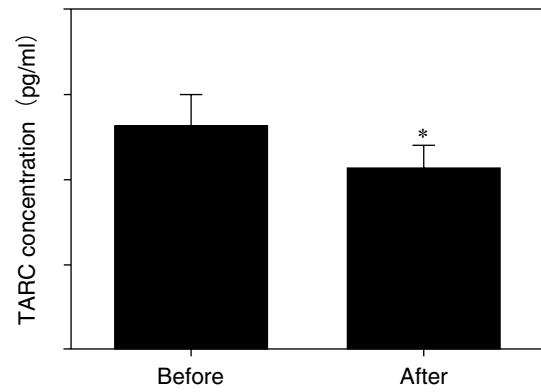
Suplatast tosilate is effective to control allergic diseases such as allergic rhinitis. Many authors have discussed the selective inhibitory effect of suplatast tosilate on IgE and Th2 cytokine production.<sup>13-20</sup> Allergic rhinitis is characterized by inflammatory cell infiltration, especially by eosinophils. It is probable that suplatast tosilate attenuates the PAF- and leukotriene-induced increase of edema and vascular permeability by reducing this eosinophilic infiltration of the nasal mucosa. This is possible because both IL-4 and IL-5 are implicated in the pathogenesis of eosinophilic inflammation<sup>26-28</sup> and because eosinophils are one of the major sources of PAF and leukotriene.<sup>29,30</sup>

In this study, we showed that suplatast tosilate inhibits TARC production by PBMCs stimulated with mite antigen and that the plasma TARC concentration decreased significantly in patients with chronic allergic rhinitis after 4-week administration of suplatast tosilate. These findings confirm the results of an earlier study showing that suplatast tosilate inhibited TARC production by antigen-specific human Th2 cells.<sup>31</sup>

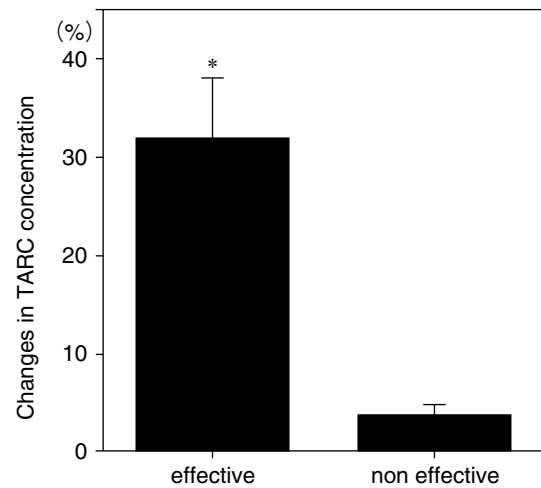


**Fig. 5** Effect of suptalast tosilate on nasal symptoms. Changes in symptom score of (A) Sneezing, (B) Rhinorrhea, (C) Nasal obstruction. \*  $p < 0.05$

The expression of TARC in the bronchial epithelium and sputum was significantly increased in astmat-



**Fig. 6** Effect of oral suptalast tosilate on the plasma level of TARC. The plasma level of TARC was significantly decreased after the 4-week administration. \*  $P < 0.05$



**Fig. 7** Comparison of changes in the plasma level of TARC between patients in whom suptalast tosilate was effective and those in whom it was not effective. \*  $P < 0.05$

ics.<sup>32,33</sup> Recently, we demonstrated that combined stimulation with IL-4 and TNF- $\alpha$ , as well as with IL-13 and TNF- $\alpha$ , synergistically induced TARC expression in epithelial cells. Furthermore, the amount of TARC induced by these cytokines was higher in epithelial cells obtained from patients with nasal allergy than in patients without allergy.<sup>27</sup> In addition, recent studies have demonstrated that in patients with allergic diseases CCR4 expression on peripheral blood CD4<sup>+</sup>T cells and serum TARC levels correlated with the severity of the disease.<sup>34,35</sup> Wakugawa *et al.* reported that in patients with atopic dermatitis (AD) CCR4 expression on peripheral blood CD4<sup>+</sup>T cells was significantly higher than in healthy subjects. Moreover, they observed that the symptoms improved with intensive topical corticosteroid therapy and that CCR4



expression on CD4<sup>+</sup>T cells gradually decreased as the symptoms improved.<sup>34</sup> Kakinuma *et al.* quantified serum TARC levels in patients with AD, and examined the correlation between TARC levels and disease activity.<sup>35</sup> The serum TARC level correlated with the severity of the disease and the number of eosinophils in peripheral blood. Both the serum TARC level and the number of eosinophils in peripheral blood decreased during treatment with topical corticosteroids and oral antihistaminics, and those changes were associated with an improvement of the skin conditions.

Suplatast tosilate inhibited TARC production at a concentration of  $1 \times 10^{-6}$  M, but showed no inhibitory effect at  $1 \times 10^{-7}$  M, the serum level attained following administration of the usual clinical dose. It appears that suplatast tosilate exhibits an inhibitory effect when it remains in contact with blood and tissues for a prolonged period. It may be necessary to increase its blood level to attain an appropriate inhibitory effect on TARC production induced by a specific antigen.

Next, we attempted to clarify whether the production of TARC was suppressed in patients with chronic allergic rhinitis after treatment with suplatast tosilate. As a result, the plasma TARC concentration decreased significantly after 4 weeks on suplatast tosilate. The percentage of decrease in plasma TARC concentration was significantly higher in the patients for whom suplatast tosilate was effective. In a previous study, we reported that compared with normal subjects, the plasma TARC concentration was significantly higher in patients with bronchial asthma, atopic dermatitis and allergic rhinitis.<sup>36</sup> Another study on the correlation between plasma TARC concentration and severity of clinical symptoms revealed that plasma TARC concentration was significantly higher in patients with severe allergic rhinitis than in those with mild or moderate allergic rhinitis (Terada *et al.* Unpublished data). The above findings suggest that TARC plays a crucial role in the pathogenesis and development of allergic rhinitis. Plasma TARC concentration is an important index of the severity of allergic rhinitis. Our present findings also suggest that suplatast tosilate suppresses the production of TARC and improves clinical symptoms of allergic rhinitis.

In conclusion, suplatast tosilate suppressed TARC production by PBMCs and decreased the plasma level of TARC after clinical administration. In addition, the percentage of decrease in plasma TARC concentration was significantly higher in patients who responded to treatment with suplatast tosilate. Suplatast tosilate was considered useful as a baseline drug for the treatment of allergic rhinitis.

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