

Ebastine inhibits T cell migration, production of Th2-type cytokines and proinflammatory cytokines

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Summary

Background Cytokine imbalance and cellular migration to inflammatory sites are critical components of allergic diseases. Redirecting cytokine imbalance and inhibiting cell migration therefore represent important therapeutic strategies for the treatment of these disorders.

Objectives To study the *in vitro* effect of ebastine, a novel non-sedating H1 receptor antagonist, on cytokine secretion and migration of activated T cells, as well as production of pro-inflammatory cytokines by macrophages.

Methods Peripheral T cells obtained from healthy volunteers were cultured in wells coated with the combination of anti-CD3 monoclonal antibody (mAb) and anti-CD26 mAb, anti-CD3 mAb and anti-CD28 mAb, or anti-CD3 mAb with PMA, in the presence or absence of ebastine. T cell proliferation and the production of cytokines were measured by [³H]thymidine incorporation assay and ELISA, respectively. In addition, transendothelial migration of T cells and production of pro-inflammatory cytokines by macrophages were examined.

Results Ebastine inhibited T cell proliferation and the production of IL-4, IL-5, IL-6, and TNF- α by T cells under each co-stimulatory condition tested, whereas it exhibited no effect on the production of IL-2 or IFN- γ . In addition, T cell migration and the production of such pro-inflammatory cytokines as TNF- α and IL-6 by macrophages were inhibited by ebastine.

Conclusions These results indicate that ebastine has a specific inhibitory effect on Th2-type cytokine production. Moreover, ebastine inhibited T cell migration and pro-inflammatory cytokine production by T cells and macrophages, suggesting that ebastine might be useful for the treatment of T cell-mediated allergic inflammatory disorders, including asthma, atopic dermatitis, and Th2-type autoimmune diseases.

Keywords CD3, CD26, CD28, ebastine, IL-4, IL-5, IL-6, T cell co-stimulation, T cell migration, TNF- α

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Introduction

Ebastine (4-diphenylmethoxy-1 [3-(4-terbutyl-benzoyl)-propyl] piperidine) is a selective and potent H1 receptor antagonist with no anticholinergic or sedative effect, and it is widely used for seasonal allergic rhinitis and chronic urticaria [1, 2]. Although it has a similar chemical structure as terfenadine, another H1 receptor antagonist whose side-effect is Q-T elongation and arrhythmia, ebastine displays few cardiac side-effects [3–5]. Its clinical effect is believed to be mediated by its antagonistic action against histamine receptors [6]. On

the other hand, recent studies have suggested that terfenadine may possess additional mechanisms of action besides its ability to block histamine receptor [7, 8]. It is therefore conceivable that the effectiveness of ebastine in the treatment of allergic diseases may be due to other biological mechanisms.

In a hypersensitivity allergic reaction, various inflammatory cells contribute to the initial triggering and subsequent maintenance of the reaction through cell–cell interactions or the production of soluble biological factors. Helper T (Th) cells are specifically regarded as the effector cells and play important roles in regulating the hypersensitivity reaction. Based on the profile of cytokine production, two subsets of CD4⁺ T cells have been identified [9, 10]. The first is the T helper 1-type cell (Th1), which releases IL-2 and IFN- γ [9, 10]. The second is the T helper 2-type cell (Th2), which

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releases IL-4 and IL-5 [9, 10]. In some allergic diseases, Th2 cells are reported to accumulate predominantly at the inflammation site, and these cells trigger the hypersensitive reaction [11–13]. Thus, specific inhibition of Th2 cell functions may lead to the successful treatment of allergic diseases or hypersensitivity.

In the initial stages of a hypersensitivity reaction, a certain allergen is engaged by the TCR, which is followed by the release of allergic cytokines. However, this process alone is insufficient for the induction of all necessary events accompanying T cell activation. Accumulating evidence indicates the importance of the so-called co-stimulatory signals that occur through the engagement of additional T cell surface molecules, which are independent of the CD3/TCR complex [14]. These co-stimulatory signals are indispensable for the full activation of T cells, which is characterized by T cell proliferation and cytokine production. Therefore, the triggering of co-stimulatory signals plays an important role in the generation of a hypersensitivity immune reaction. Such co-stimulatory signals may be provided by a number of accessory molecules, including CD28/CTLA-4 [15–18]. Furthermore, we have identified CD26 as a novel co-stimulatory molecule. It is preferentially expressed on CD4⁺ memory T cells [19–21], and is speculated to be involved in the functions of effector T cells migrating to the focus of inflammation in immune-mediated diseases [22, 23].

To further determine the mechanism of action of ebastine that is involved in its inhibition of an allergic reaction, we evaluated its effect on T cell proliferation and cytokine production through distinct co-stimulatory signaling pathways. Moreover, we examined the effect of ebastine on T cell migration and pro-inflammatory cytokine production by macrophages. The results revealed that ebastine inhibited T cell proliferation in a dose-dependent manner under each co-stimulatory condition. Of great interest and potential significance is the fact that ebastine clearly inhibited the production of IL-4 and IL-5 as well as IL-6 and TNF- α by T cells, whereas it did not affect Th1-associated IL-2 and IFN- γ production. Furthermore, ebastine inhibited T cell migration and the production of such pro-inflammatory cytokines as IL-6 and TNF- α by macrophages. Our findings indicate that ebastine specifically inhibits Th2-type cytokine production by human peripheral T lymphocytes, and T cell migration as well as pro-inflammatory cytokine production by macrophages. Our data thus suggest that ebastine may be useful for the treatment of T cell-mediated allergic diseases, including asthma, atopic dermatitis as well as Th2-type autoimmune disorders.

Methods

Cells and reagents

Human peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteer donors by Ficoll-Hypaque (Pharmacia Biotech Inc., Piscataway, NJ, USA) density gradient centrifugation [19]. Unfractionated mononuclear cells were separated into an E rosette-positive (E⁺) population and were used as resting T cells. Monocytes were

depleted by adherence to plastic plates for 24 h at 37 °C followed by incubation with 5 mM L-leucine methyl ester HCl (Sigma Chemical Co., St Louis, MO, USA) for 1 h. The purity of T cells in these preparations was always over 95%, which was determined by flow cytometry for CD3 antigen (data not shown). The monoclonal antibody (mAb) OKT3 was obtained via American Tissue Culture Collection (ATCC, Rockville, MD, USA) and CD26 (1F7) has been previously described [19]. Anti-CD28 mAb, 4B10, was developed in our laboratory as previously described [16]. Ebastine was generously supplied by Meiji Seika Ltd. (Tokyo, Japan). Ketotifen was purchased from Sigma. Ebastine and ketotifen were dissolved in DMSO and further diluted in the culture media consisting of RPMI-1640 and 10% human AB serum.

T cell proliferation assays

The proliferative response of T cells was evaluated by [³H]thymidine incorporation assay. Ninety-six-well flat-bottomed plates (Costar, Cambridge, MA, USA) were coated in triplicate with 100 μ L of phosphate-buffer saline (PBS) containing 0.05 μ g/mL of OKT3 with or without 1 μ g/mL of 1F7, or 2 μ g/mL of 4B10 mAb, and incubated overnight at 4 °C, as described previously [20]. Prior to use, these plates were washed once with 200 μ L of PBS. Highly purified T cells were resuspended at 1×10^5 in 200 μ L of RPMI medium containing 10% human AB serum, along with four different concentrations of ebastine (0, 1, 5, and 10 μ M). To assess phorbol myristate acetate (PMA) stimulation, the cell suspension supplemented with 5 ng/mL of PMA was applied into OKT3-coated wells. Cells were incubated at 37 °C in 5% CO₂ humidified atmosphere for 3 days. Cells were pulsed with 1 μ Ci/well of [³H]thymidine (ICN Radiochemicals, Irvine, CA, USA) 8 h prior to harvest onto a glass-fibre filter (Wallac, Turku, Finland), and the incorporated radioactivity was quantitated by a liquid scintillation counter (Wallac).

Cytokine production assays

Cytokine production by T cells and macrophages was measured by ELISA. Antibody-coated plates and purified T cells were prepared in the same manner as described above, with the exception of OKT3 concentration being 0.5 μ g/mL. For the measurement of cytokines produced by T cells and macrophages, we chose 24 h after stimulation for T cells, and 8 h for macrophages, since that time point was optimal for each cell type. Cytokine production by T cells was assayed in triplicate in 96-well, flat-bottomed plates as described above for the T cell proliferation assay. After 24 h of incubation, culture supernatants were subjected to ELISA (IL-2 and IL-4: Biosource International, Camarillo, CA, USA; IFN- γ and IL-5: R&D Systems, Minneapolis, MN, USA) to measure the levels of IL-2, IFN- γ , IL-4, and IL-5 as well as TNF- α and IL-6. For TNF- α and IL-6 production by macrophages, macrophages were enriched from E⁺ cells by adherence to plastic plates. Macrophages (1×10^6 /mL) were suspended in 10% FCS-RPMI-1640 and stimulated with 1 μ g/mL of LPS (Sigma). After 8-h culture, culture supernatants were

harvested and subjected to ELISA (TNF- α and IL-6: R&D Systems, Minneapolis, MN, USA).

Assessment of cell viability

The trypan blue dye exclusion test was used to assess cell viability. In all experiments, the viability was found to be over 95% at each point measured (data not shown).

Transendothelial migration assay

Transendothelial migration activity was assessed using a kind of Boyden-chamber assay as described previously with some modifications [24]. The human endothelial cell line ECV304 obtained from ATCC was pre-cultured to make a monolayer sheet on Transwell cell culture inserts with 3.0 μ m pore size (Corning Costar, Cambridge, MA, USA) for 48 h. Ebastine was first dissolved in DMSO and further diluted in the assay medium consisting of RPMI-1640 and 0.6% bovine serum albumin, and then added to culture plates in a final volume of 600 μ L (the lower chamber) just before migration assay. PHA-activated T cells (1×10^6 cells/well) were added to each insert in a volume of 200 μ L simultaneously with the same concentration of ebastine as in the corresponding culture wells (the upper chamber). The spontaneous migration (chemokinesis) assay was performed at 37 °C for 8 h with or without ebastine, then harvested and counted by flow cytometry (FACS Calibur, Nippon Becton-Dickinson, Tokyo, Japan) for 1 min.

Immunofluorescence analysis

PHA-activated T cells were incubated with or without the indicated doses of ebastine at 37 °C for 8 h in 5% CO₂/95% air and then washed. All the cells were incubated with mAb against various cell surface molecules (1 μ g/ml) on ice for 30 min. The cells were further incubated with fluorescein isothiocyanate labelled goat anti-mouse IgG (Sigma) after being washed three times with PBS containing 2% newborn calf serum and 0.02% sodium azide (Sigma), and then subjected to flow cytometry. We used isotype-matched control antibodies for the respective antibodies used in this study. The results are after subtraction of these control values. The mAbs used for this experiment were anti-CD3 (OKT3), anti-CD11a (BD PharMingen, NJ, USA), anti-CD25 (BD PharMingen), anti-CD26 (1F7), anti-CD29 (4B4), anti-CD44 (BD PharMingen), and anti-CD47 (BD PharMingen).

Statistical analysis

Statistical analysis was performed by the two-tailed Student's *t*-test for all the assays (³H]thymidine incorporation assay, ELISA, transendothelial migration assay, and flow cytometry). The comparison was made between drug-free control (DMSO alone) and each dose of reagents (ebastine or ketotifen). In the case with comparison of more than two related samples such as dose-response analysis, we first performed the variance analysis test between the different concentration points. When it was significant, we further proceeded to the Student's *t*-test on two-by-two data.

Results

Effect of ebastine on T cell proliferation through different co-stimulatory pathways

To determine the effect of ebastine on T cell proliferation, we used three different co-stimulatory pathways. The first one includes the phorbol ester PMA; the second involves stimulation through one of the representative T cell co-stimulatory molecules, CD28; and the third is mediated by CD26, which is preferentially expressed on CD4⁺ memory T cells. All three stimuli were combined with submitogenic doses of anti-CD3 mAb. As a control, we used ketotifen, which is a representative antihistamine drug. Purified T cells were stimulated with immobilized anti-CD3 mAb alone, anti-CD3 mAb and anti-CD26 mAb, anti-CD3 mAb and anti-CD28 mAb, or anti-CD3 mAb with PMA in the presence of various concentrations of ketotifen or ebastine. As shown in Fig. 1, the stimulus through CD3 alone resulted in the induction of low levels of T cell proliferation. Marked T cell proliferation was observed when stimulation through CD3 was combined with an additional second signal, such as anti-CD26 mAb, anti-CD28 mAb, or PMA. Under these conditions, ebastine clearly inhibited T cell proliferation in a dose-dependent manner. Ebastine at 1, 5, and 10 μ M provided significant inhibition of T cell proliferation in each of the co-stimulatory conditions (Fig. 1a), whereas ketotifen did not inhibit T cell proliferation at any tested concentration under the same experimental conditions (Fig. 1b). It should be noted that this inhibitory effect was not due to non-specific cytotoxicity by ebastine, since over 95% T cells were viable at the time of assay. We did observe a tendency for T cell proliferation induced by anti-CD26 to be slightly resistant to the inhibitory effect of ebastine.

Effect of ebastine on Th1-type cytokine production through different costimulatory pathways

To examine the potential inhibitory effect of ebastine on cytokine production following stimulation with the agents described above, we first measured the levels of IL-2 and IFN- γ in the culture supernatants. In cytokine production assays, peripheral T cells did not secrete any detectable cytokines when stimulated with CD3 alone, therefore the results with CD3 alone were omitted in the figures. As shown in Fig. 2a, ebastine, even at 10 μ M, did not inhibit IL-2 production under all costimulatory conditions. In addition to IL-2, ebastine did not display any apparent effect on the production of IFN- γ (Fig. 2b) at any tested concentration (1–10 μ M). Similarly, ketotifen did not inhibit both IL-2 and IFN- γ production (Fig. 2c and d) at any tested concentration (1–10 μ M). These findings therefore indicated that ebastine and ketotifen did not inhibit the Th-1-type cytokine production in our experimental conditions.

Effect of ebastine on Th2-type cytokine production through different co-stimulatory pathways

Since Th-2-type CD4⁺ T cells play an important role in allergic disorders, we examined the effect of ebastine on the Th2-type cytokine production of IL-4 and IL-5. As shown in

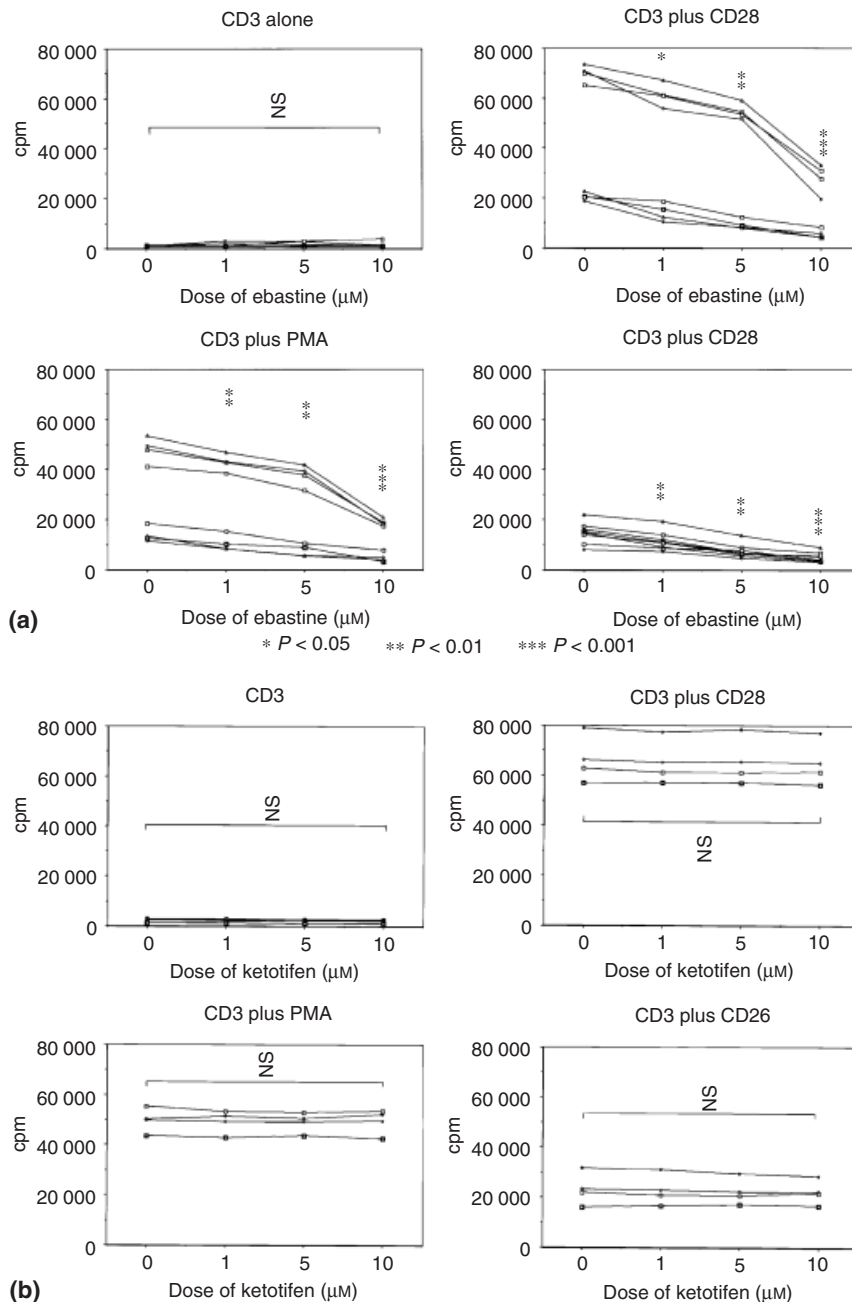


Fig. 1. Effect of ebastine and ketotifen on the proliferative response of peripheral T cells stimulated with CD3, CD3 plus CD28, CD3 plus PMA, and CD3 plus CD26. (a) Effect of ebastine on the proliferative response of peripheral T cells. The cpm value in the case of T cells without any stimuli was near the background level (data not shown). Mean cpm values from triplicate samples were plotted from eight different donors. Variance analysis tests were statistically significant ($P < 0.05$). The P -values calculated by the two-tailed Student's t -test were indicated in each figure between 0 and 1 μM , 0 and 5 μM , and 0 and 10 μM of ebastine, respectively (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). NS means not significant judged by a variance analysis test. (b) Effect of ketotifen on the proliferative response of peripheral T cells. The experiments were carried out in the same manner as in (a), with the exception that mean values were from four different donors.

Fig. 3a, ebastine inhibited IL-4 production under each co-stimulatory condition in a dose-dependent manner. In particular, at 10 μM , treatment with ebastine led to an almost complete inhibition of IL-4 production from T lymphocytes. In contrast, ketotifen did not inhibit IL-4 production at the same tested concentrations (Fig. 3c). We next examined the effect of ebastine on IL-5 production. As shown in Fig. 3b, ebastine inhibited IL-5 production under each co-stimulatory condition in a dose-dependent manner, whereas treatment with similar concentrations of ketotifen did not inhibit IL-5 production (Fig. 3d). It should be noted that compared to the effect of ebastine on IL-4 production, IL-5 production by T cells was more susceptible to ebastine, since over 50% of

IL-5 production was inhibited at 5 μM . Similar results were reproducibly obtained in five different donors.

Effect of ebastine on pro-inflammatory cytokine production through different co-stimulatory pathways

We next examined the effect of ebastine on the production of such pro-inflammatory cytokines as IL-6 and TNF- α . As shown in Fig. 4, production of the pro-inflammatory cytokines IL-6 (a) and TNF- α (b) was significantly inhibited by ebastine in a dose-dependent manner under each co-stimulatory condition. It should be noted that ketotifen did not inhibit IL-6 and TNF- α production at the same

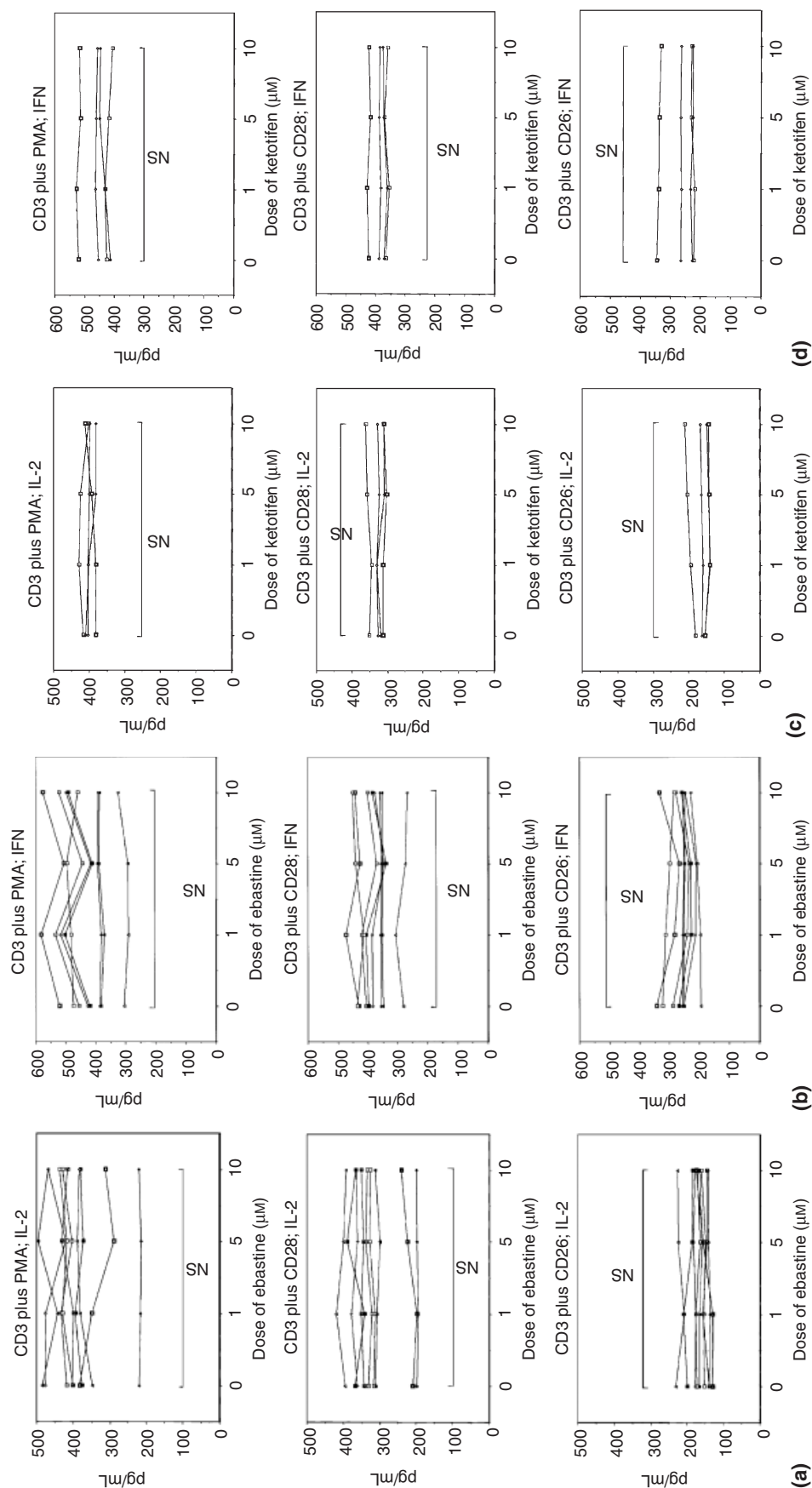


Fig. 2. Effect of ebastine and ketotifen on the production of IL-2 and IFN- γ by peripheral T cells stimulated with CD3 plus PMA, CD3 plus CD28, CD3 plus PMA, and CD3 plus CD26. Effect of ebastine on the production of IL-2 (a) IFN- γ (b) by peripheral T cells. T cells were stimulated for 24 h, and the culture supernatants were assayed for the concentration of IL-2 and IFN- γ by ELISA. Mean values from duplicate samples were plotted from nine different donors (IL-2) or eight different donors (IFN- γ). The cytokine levels in the case of T cells stimulated with CD3 alone were always the background level (data not shown). Effect of ketotifen on the production of IL-2 (c) and IFN- γ (d) by the peripheral T cells. The experiments were carried out in the same manner as in (a) and (b), with the exception that mean values were from four different donors. NS means not significant judged by a variance analysis test.

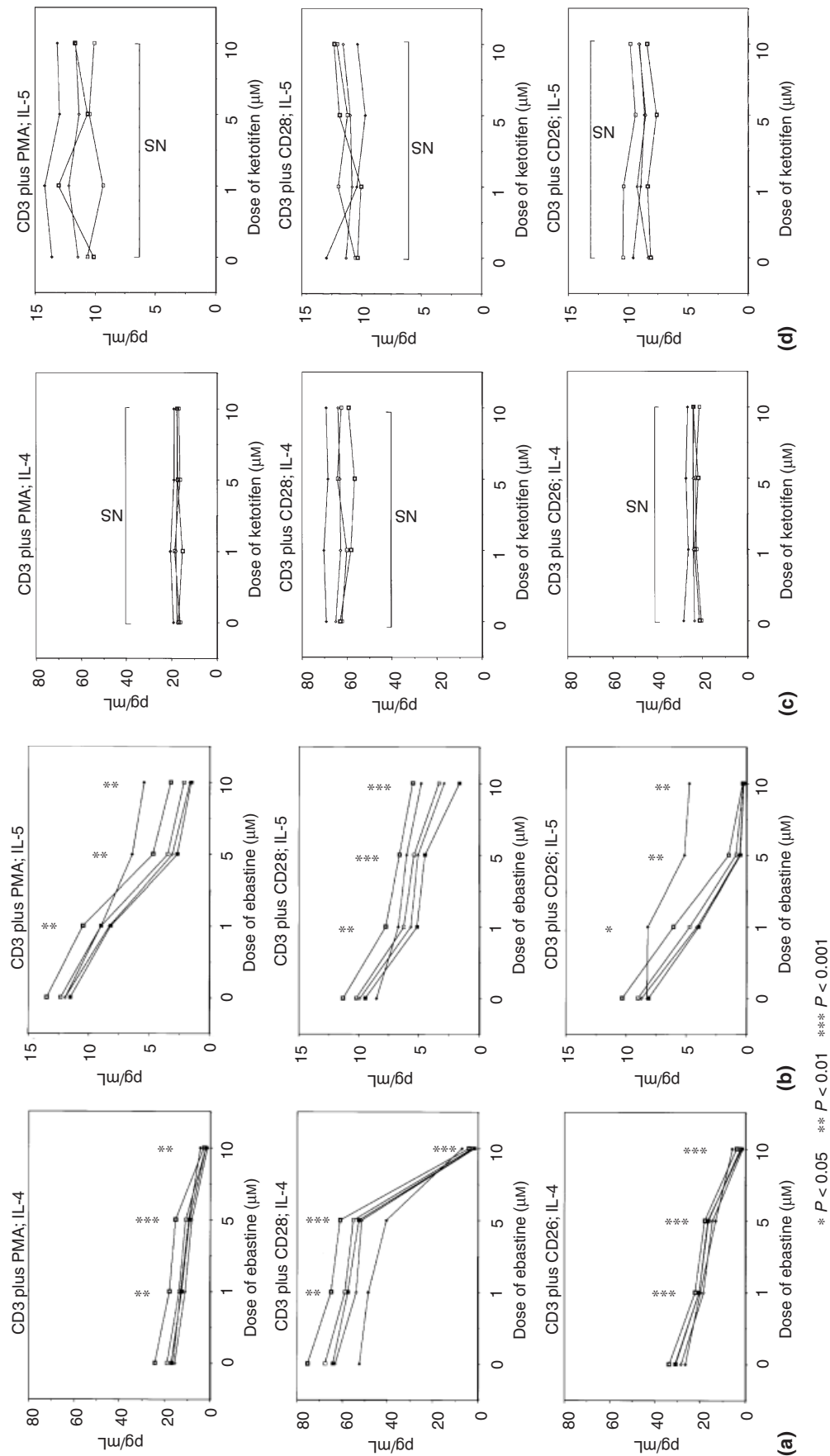


Fig. 3. Effect of ebastine and ketotifen on the production of IL-4 and IL-5 by peripheral T cells stimulated with CD3 plus PMA, and CD3 plus CD28. Effect of ebastine on the production of IL-4 (a) and IL-5 (b) by peripheral T cells. T cells were stimulated for 24 h, and the culture supernatants were assayed for the concentration of IL-4 and IL-5 by ELISA. Mean values from duplicate samples were plotted from five different donors. The cytokine levels in the case of T cells stimulated with CD3 alone were always the background level (data not shown). Variance analysis tests were statistically significant ($P < 0.05$). The P -values calculated by the two-tailed Student's t -test were indicated in each figure between 0 and 1 µM, 0 and 5 µM, 0 and 10 µM of ebastine, respectively (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Effect of ketotifen on the production of IL-4 (c) IL-5 and (d) by peripheral T cells. The experiments were carried out in the same manner as in (a) and (b), with the exception that mean values were from four different donors. NS means not significant judged by a variance analysis test.

concentrations (data not shown). Similar results were reproducibly obtained in four different donors.

Effect of ebastine on transendothelial migration of activated T cells

To gain insights into the effect of ebastine on T cell extravasation *in vivo*, we examined its effect on transendothelial migration of PHA-stimulated T cells. Since resting T cells hardly migrate through the endothelial cell monolayer, we used activated T lymphocytes for the migration assay, with T cell activation being accomplished with PHA treatment (10 µg/mL). These pre-activated T cells spontaneously migrate from the upper chamber to the lower chamber through the endothelial monolayer, which is therefore regarded as chemokinesis. As shown in Fig. 5, T cell migration was significantly inhibited in a dose-dependent manner when ebastine was present during the endothelial migration assay. It should be noted that at 5 µM, the inhibition of T cell migration achieved a plateau level. In contrast, when we pre-treated ECV304 with various concentrations of ebastine for 48 h, and then washed and exposed to PHA-stimulated T cells, ebastine did not affect the migration of T cells through ECV304 even at the highest concentration tested (10 µM) (data not shown). Ketotifen also did not inhibit T cell migration (data not shown).

Effect of ebastine on the expression of T cell surface adhesion molecules

To further explore the mechanism involved in ebastine-mediated inhibition of T cell migration, we utilized flow cytometry to evaluate cell surface expression of such adhesion molecules as CD11a, CD29, CD44 and CD47, and activation antigens such as CD25 and CD26 on PHA-activated T cells following ebastine treatment. As shown in Table 1, among all the adhesion molecules and activation antigens tested, the expression of CD26, CD29, and CD47 on T cells was significantly affected after an 8-h treatment with ebastine. At a dose of 5 µM ebastine, the expression levels of CD26, CD29, and CD47 were reduced by approximately 14, 21, and 31%, respectively. On the other hand, the expression levels of CD3,

CD11a, CD25, and CD44 were virtually unaffected. It should be noted that ebastine did not affect ICAM-1 expression on the endothelial cell line ECV304 (data not shown). Since CD26, CD29, and CD47 appear to be involved in cellular migration, our data may explain at least in part the inhibitory effect of ebastine on T cell migration.

Effect of ebastine on pro-inflammatory cytokine production by macrophages

Since macrophages play a primary role in the host defence against infections and in the local modulation of immune and inflammatory responses [25], we also examined the effect of ebastine on the production of the pro-inflammatory cytokines IL-6 and TNF-α by macrophages. For this purpose, macrophages isolated from PBMC were stimulated by LPS (1 µg/mL) and the culture supernatants were assayed for IL-6 and TNF-α production. The preliminary time course experiment showed that 8 h after stimulation was optimal for LPS-stimulated pro-inflammatory cytokine production by macrophages; we chose the different time point from the case with T cells, which was 24 h after stimulation (data not shown). As shown in Fig. 6, at 1 µM of ebastine, there was greater than 50% inhibition of cytokine production, while at 10 µM of ebastine, macrophage production of these cytokines was almost totally inhibited. It should be noted that ketotifen treatment did not inhibit IL-6 and TNF-α production by macrophages at the same concentrations (data not shown).

Discussion

In the present study, we demonstrated that ebastine clearly inhibited production of the Th2-type cytokines IL-4 and IL-5 as well as the pro-inflammatory cytokines IL-6 and TNF-α by T cells, with virtually no effect on the production of the Th1-type cytokines IL-2 and IFN-γ. In addition, ebastine inhibited T cell migration and the production by macrophages of the pro-inflammatory cytokines IL-6 and TNF-α. Moreover, ebastine-mediated inhibition of T cell migration appeared to result partly from the reduced expression of CD26, CD29, and CD47 on the T cell surface. On the other hand, treatment with another H1 receptor antagonist, ketotifen, did not have similar effects.

Ebastine is a novel, non-sedating H1 histamine receptor antagonist, which is clinically used for the treatment of allergic rhinitis, urticaria, and other allergic reactions [1–3, 26]. The interaction of histamine with histamine H1 receptors induces a series of biological effects, such as contraction of bronchial smooth muscle and increased vascular permeability. Furthermore, there is stimulation of peripheral nerve endings to cause pruritus and pain, with stimulation of the vagus nerve endings resulting in reflex bronchoconstriction and cough [27]. The effectiveness of ebastine has been mainly attributed to its inhibition of histamine release from basophils and mast cells [27, 28]. Moreover, it is reported that ebastine blocks the release of anti-IgE-induced prostaglandin D2 and leukotriene C4/D4 from human nasal polyp cells *in vitro* after allergen challenge [29]. Therefore, ebastine appears to exert additional biological effects besides its blockade of the

Table 1. Effect of ebastine on the expression levels of cell surface molecules on peripheral T cells

Dose of ebastine	0 µM	1 µM	5 µM
(–)	0.3	0.3	0.3
CD3	10.6 ± 0.26	10.6 ± 0.2: NS	10.9 ± 0.26: NS
CD11a	53.1 ± 0.44	53.8 ± 0.44: NS	53.9 ± 0.4: NS
CD25	18.2 ± 0.56	18.3 ± 0.35: NS	16.5 ± 0.26: **
CD26	102.0 ± 0.69	99.1 ± 0.36: **	87.8 ± 0.36: ***
CD29	28.0 ± 0.56	25.0 ± 0.26: *	22.1 ± 0.36: ***
CD44	48.7 ± 0.62	47.6 ± 0.30: NS	47.9 ± 0.36: NS
CD47	14.5 ± 0.35	11.4 ± 0.17: **	10.0 ± 0.26: ***

PHA-activated T cells were incubated with or without the indicated doses of ebastine at 37°C for 8 h, and analysed for the expression of CD3, CD11a, CD25, CD26, CD29, CD44, and CD47 by flow cytometry. The data shown are representative of three independently performed experiments. The *P*-values calculated by the two-tailed Student's *t*-test were indicated in each figure between 0 and 1 µM, 0 and 5 µM, and 0 and 10 µM of ebastine, respectively (**P* < 0.05; ***P* < 0.01; ****P* < 0.001; NS: not significant).

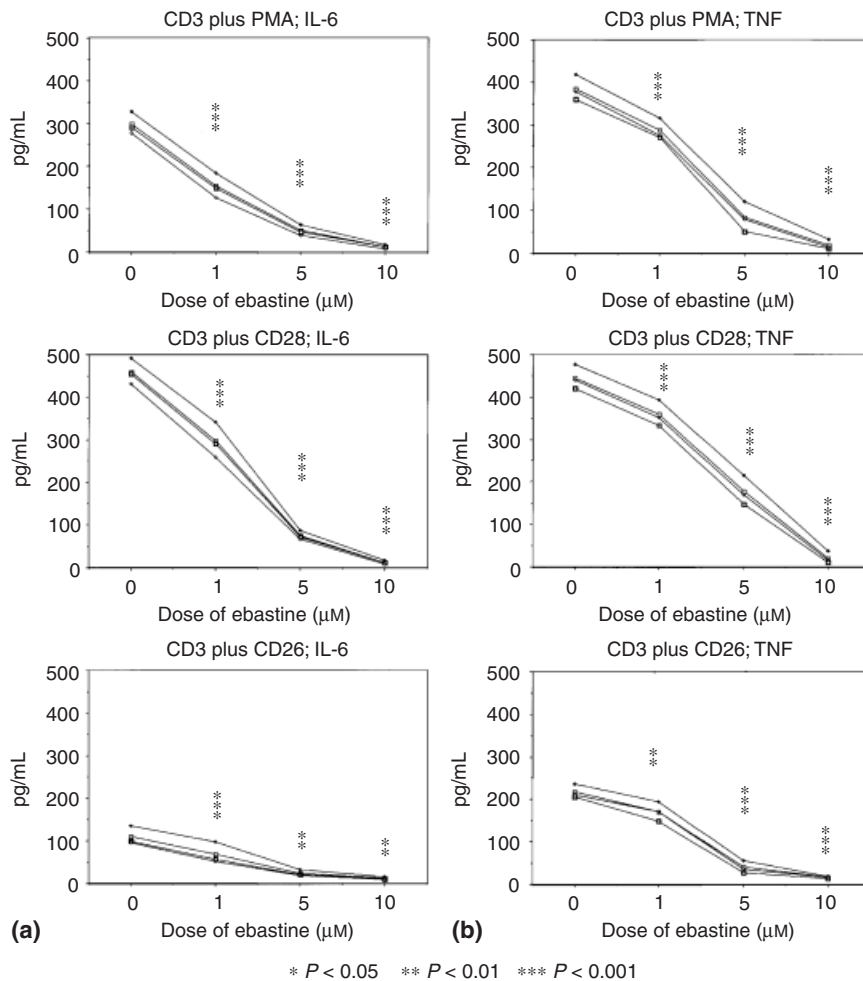


Fig. 4. Effect of ebastine on the production of IL-6 and TNF- α by peripheral T cells stimulated with CD3 plus CD28, CD3 plus PMA, and CD3 plus CD26. Effect of ebastine on the production of IL-6 (a) and TNF- α (b) by peripheral T cells. T cells were stimulated for 24 h, and the culture supernatants were assayed for the concentration of IL-6 and TNF- α by ELISA. Mean values from duplicate samples were plotted from four different donors. The cytokine levels in the case of T cells stimulated with CD3 alone were always the background level (data not shown). Variance analysis tests were statistically significant ($P < 0.05$). The P -values calculated by the two-tailed Student's t -test were indicated in each figure between 0 and 1 μ M, 0 and 5 μ M, and 0 and 10 μ M of ebastine, respectively (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

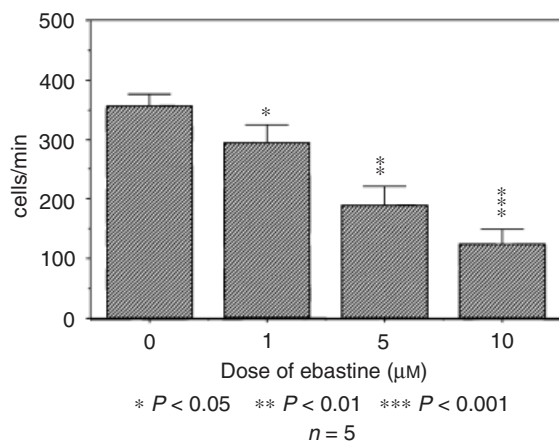


Fig. 5. Inhibitory effect of ebastine on transendothelial migration (chemokinesis) of PHA-activated T cells. ECV304 cells were grown on Transwell cell culture inserts for 48 h. After washing of the monolayers with the assay medium (0.6% BSA RPMI-1640), PHA-activated T cells were layered onto them with or without three different concentrations of ebastine. T cells that spontaneously migrated to the lower chambers without any exogenous chemokines were counted in each experiment by flow cytometry. Bars show the mean values and SD of five different experiments. Variance analysis tests were statistically significant ($P < 0.05$). The P -values calculated by the two-tailed Student's t -test were indicated in each figure between 0 and 1 μ M, 0 and 5 μ M, and 0 and 10 μ M of ebastine, respectively (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

histamine H1 receptor. Recently, Jutel et al. [30] have demonstrated that the histamine H1 receptor is preferentially expressed on Th1 cells, and that mice lacking the H1 receptor displayed suppression of IFN- γ and dominant secretion of Th2 cytokines (IL-4 and IL-13), which seems to be in contrast to our results obtained in the human system. One explanation is that it might be due to the difference in species. Indeed, we sometimes encounter difficulty in separating Th1-type and Th2-type responses clearly in the human system. The second is that ebastine might inhibit the production of Th2-type and inflammatory cytokines through a mechanism other than inhibition of the H1 receptor. It is particularly interesting when ineffectiveness of another H1 receptor antagonist, ketotifen, is taken into account. Further detailed analysis is necessary on the mechanism and site of action by which ebastine inhibits proliferative response and cytokine production.

In a hypersensitivity response, helper CD4⁺ T cells play a key role in triggering the allergic inflammatory response. Namely, cytokine production by Th2-type cells is indispensable to initiating and maintaining the hypersensitive inflammatory response [9, 10]. Trafficking of activated T cells into inflammatory sites is a tightly controlled process directed by multiple molecules, in particular adhesion

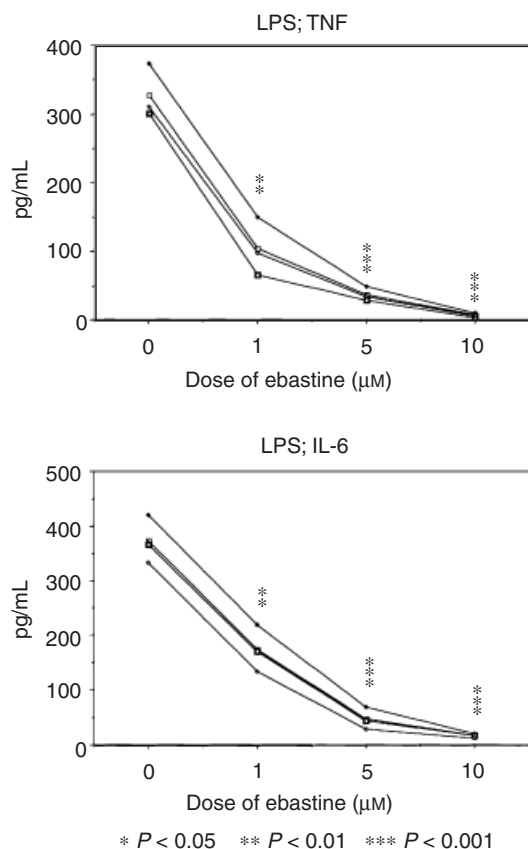


Fig. 6. Effect of ebastine on the production of IL-6 and TNF- α by macrophages stimulated with LPS. Macrophages were stimulated by LPS (1 μ g/mL) for 8 h, and the culture supernatants were assayed for the concentration of IL-6 and TNF- α by ELISA. Mean values from duplicate samples were plotted from four different donors. Variance analysis tests were statistically significant ($P < 0.05$). The P -values calculated by the two-tailed Student's t -test were indicated in each figure between 0 and 1 μ M, 0 and 5 μ M, and 0 and 10 μ M of ebastine, respectively (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

molecules and chemokines [31]. Three pairs of adhesion molecule and counter receptor families are implicated in the interaction between lymphocytes and their target cells in the endothelium and tissues: (1) integrins, which target molecules of the immunoglobulin superfamily (IgSF) or ECM components; (2) selectins, which interact with sialylated carbohydrate determinants O-linked to mucin-like molecules; and (3) CD44 with binding affinity for matrix and cell surface components.

These are implicated in the transendothelial migration of lymphocytes, which comprises multiple steps: (1) the capture of lymphocytes by the luminal surface of the endothelium; (2) cell rolling and cell arrest in the vasculature; (3) binding of lymphocytes to the high endothelial venule of the lymph node (firm adhesion); (4) a process of transendothelial migration; and (5) subsequent cell lodgement in organ parenchyma.

Selective recruitment of subsets of CD4⁺ effector T cells into sites of inflammation may contribute to the development of different pathological conditions. Activated Th2-type of CD4⁺ T cells producing IL-4, IL-5, and IL-13 has been identified at the sites of allergic inflammation and in bronchoalveolar lavage (BAL) and bronchial biopsies of

both atopic and non-atopic asthmatic patients, and is currently placed at the centre of mechanistic schemes for the presumed pathophysiology of asthma [32, 33]. Therefore, Th2-type of CD4⁺ T cells and their cytokine production are considered as important therapeutic targets. Our study clearly showed that ebastine inhibited T cell proliferation and the production of such Th2-type cytokines as IL-4 and IL-5 by human T cells through various co-stimulatory signals in a dose-dependent manner. On the other hand, ebastine exhibited no apparent effect on the production of such Th1-type cytokines as IL-2 and IFN- γ . In this regard, we previously showed that terfenadine, a histamine H1 receptor antagonist with a chemical structure that is similar to ebastine, inhibited the production of the Th2-type cytokines IL-4 and IL-5 but not the Th1-type cytokines IL-2 and IFN- γ by T cells induced by the same stimuli [34]. Although terfenadine has been associated with Q-T elongation and potentially fatal ventricular tachyarrhythmias on rare occasions [35], ebastine has not been associated with serious adverse cardiovascular effect in clinical trials, and available data indicated that it did not adversely affect the Q-T interval even at doses up to five times the therapeutic dose [3–5]. Terfenadine and other H1 receptor antagonists are reported to inhibit eosinophil and neutrophil chemotaxis and to reduce the expression of ICAM molecule on epithelial cell lines [36], whereas their ability to inhibit T cell migration has yet to be clarified. In this present study, ebastine clearly inhibited T cell migration in a dose-dependent manner. To define the mechanism of the inhibitory effect of ebastine on transendothelial T cell migration, we determined the level of surface expression of various adhesion molecules on T cells. Ebastine treatment led to down-regulation of CD26, CD29, and CD47 without affecting the expression of CD3, CD11a, and CD44 on T cells, or ICAM-1 molecule on endothelial cells.

Accumulating evidence has suggested that CD26 and CD29 have important roles in the interaction of T cells and endothelial cells as well as cellular migration [37, 38]. More recently, it has been reported that CD47 is an integrin-associated protein with a role in cellular migration [39]. The statistical analysis showed that ebastine significantly but partially down-regulated the expression of those molecules (Table 1). However, this partial down-regulation alone could not explain the total inhibitory effect of ebastine on the migratory behaviour of activated T cells. Further analysis would be important for exploring the inhibitory mechanism by ebastine, especially on the function (activation state) of adhesion molecules and cytoskeletal reorganization.

Ebastine also inhibited production of the pro-inflammatory cytokines IL-6 and TNF- α by T cells and human macrophages stimulated by LPS. In this regard, it is reported that terfenadine inhibited IL-4 and IL-13 release from human basophils [40]. Moreover, ebastine inhibited the release of TNF- α , IL-8, and granulocyte macrophage-colony stimulating factor (GM-CSF) from human nasal polyp cells [29]. The macrophage represents the predominant cellular component in the airways and one of the most abundant cells in the lung parenchyma of both healthy individuals and asthmatic patients [25]. The macrophage synthesizes and releases a large spectrum of pro-inflammatory mediators, enzymes,

cytokines, and chemokines [41]. The major cytokines produced by lung macrophages are TNF- α and IL-6 [42, 43]. Current studies suggest that TNF- α , which is produced in considerable quantities in asthmatic airways, may potentially be involved in the development of bronchial hyper-responsiveness by directly altering the contractile properties of the airway smooth muscle (ASM) [43]. IL-6 is a multi-functional cytokine released in inflammatory diseases. This cytokine has been implicated in human mast cell development and maturation and in the regulation of IgE synthesis [44]. Increased levels of IL-6 have been detected in the blood and BAL after bronchial challenge of patients with asthma, and bronchial biopsies of these patients reveal an increased expression of IL-6 [45]. Therefore, inhibition of TNF- α and IL-6 production by T cells and macrophages by ebastine may also have important therapeutic implications for asthma.

Our present study showed that ebastine exerted a novel functional effect on T cells as well as macrophages in addition to the antihistamine effect. Since ebastine inhibited Th2 cytokine secretion by T cells, T lymphocyte migration and production of the pro-inflammatory cytokines IL-6 and TNF- α by T cells and macrophages, our data strongly suggest that ebastine may be an effective therapy for the treatment of T cell-mediated allergic disorders, including asthma, atopic dermatitis, and Th2-type autoimmune disorders such as lupus nephritis. Moreover, anti-TNF- α therapy has been recently reported to be effective for the treatment of patients with rheumatoid arthritis and Crohn's disease [46, 47]. Since T cells and macrophages at the inflammatory sites of such diseases and pro-inflammatory cytokines such as TNF- α and IL-6 play a key role in triggering and maintaining the pathophysiology of these disorders, ebastine may be effective in treating these conditions.

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