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# A Novel Antiallergic Drug Epinastine Inhibits IL-8 Release from Human Eosinophils

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**Eosinophils are believed to be one of the important sources of cytokines at the site of allergic inflammation. A novel antiallergic agent epinastine showed a dose- and time-dependent suppressive effect on IL-8, one of the chemokines for eosinophils, released from eosinophils isolated from atopic diseases. The time-dependent accumulation of IL-8 inhibited by cycloheximide and the evaluation of the IL-8 mRNA levels by reverse transcriptase-polymerase chain reaction suggested that its action occurred in the posttranscripitional processes. It was suggested that epinastine might prevent the autocrine cycle for recruitment of human eosinophils by inhibiting IL-8 release from these cells.** 

Bronchial asthma is characterized by profound bronchoconstriction induced by a variety of chemical mediators released from mast cells and basophils. Antiallergic drugs are generally capable of inhibiting mediator release from these cells; and therefore are useful for therapy of bronchial asthma. However, recent studies strongly suggest that persistent airway inflammation with dense eosinophil infiltration is important in the pathogenesis of asthma. Activated eosinophils cause airway mucosal injury which is closely linked to airway hyperresponsiveness (1). These cells are now also considered to be potent sources of chemokines which play a critical role in the recruitment of inflammatory cells. Among the chemotactic factors for eosinophils, IL-8 (2) and RANTES (3) (regulated upon activation in normal T cell expressed and secreted) have attracted attention, since they were elevated in bronchoalveolar lavage fluids from patients with asthma (4).

Epinastine (3-amino-9,13b-dihydro-1H-dibenz[c,f]-imidazo [1,5-a]azeptine hydrochloride, WAL810CL; CAS80012-43-7) is a new antiallergic drug(5,6) that has been reported to inhibit antigen-induced histamine release from lung pieces of actively sensitized guinea pigs (5). Moreover, chronic treatment with epinastine decreased airway hyperresponsiveness in rats (7). However, it remains unclear whether epinastine has any effect on the activation of eosinophils, one of the most important effector cells in asthma. The purpose of the present study was to clarify the effect of epinastine on the release of IL-8 and RANTES from human eosinophils.

## MATERIALS AND METHODS

*Reagents.* Calcium ionophore A23187 and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Percoll solution was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Hanks' balanced salt solution (HBSS) without calcium and magnesium, phosphate buffered saline (PBS), heat inactivated fetal calf serum (FCS) and RPMI1640 medium were purchased from GIBCO (Grand Island, NY, USA). Dextran70 was obtained from Green Cross (Osaka, Japan). Magnetic cell sorting (MACS) and anti-CD16-bound micromagnetic beads were purchased from Miltenyi Biotec Inc. (Sunnyvale, CA, USA). Human recombinant granulocyte-macrophage colony stimulating factor (GM-CSF), IL-5 and platelet-activating factor (PAF) were purchased from Intergen, NY, USA, Cosmo Bio, Tokyo, Japan and Bachem, CA, USA, respectively. Specific ELISA kits were from SCETI, Ltd., Tokyo, Japan for IL-8 and from R&D System, Inc., MN for RANTES. Epinastine was generously provided from Nippon Boehringer Ingelheim Co. (Tokyo, Japan) and dissolved in distilled water as stock solutions.

*Purification of blood eosinophils.* Eosinophils were isolated by modification of the previous report (8). Briefly, eosinophils were

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Abbreviations used: RANTES, regulated upon activation in normal T cells, expressed and secreted; EDTA, ethylenediaminetetraacetic acid; HBSS, Hanks' balanced salt solution; PBS, phosphate buffered saline; FCS, fetal calf serum; MACS, magnetic cell sorting; GM-CSF, granulocyte-macrophage colony stimulating factor; PAF, platelet-activating factor; RT-PCR, reverse transcriptase-polymerase chain reaction.



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separated from peripheral blood of consenting patients who had allergic disease such as bronchial asthma and atopic dermatitis. These patients received neither systemic nor inhaled corticosteroid treatment. After sedimentation with Dextran70 for 90 minutes, granulocyte rich plasma was collected and centrifuged at 400g for 10 minutes at 20°C. After washing procedure, the cells suspended in Percoll solution at the density of 1.088 g/ml were centrifuged at 400g for 30 minute at 4°C. The cell pellet was collected and washed followed by hypotonic lysis to exclude residual erythrocytes. After washing, the pellet of highly purified granulocytes was then incubated with anti-CD16-bound micromagnetic beads for 30 minute at 4°C. Magnetic labeled neutrophils were then depleted by passing the steel wire column (MACS type B) in the field of a permanent magnet. The number of isolated eosinophils was counted by a hemocytometer after staining with Randorph's stain and the cell differentials were studied with cytocentrifuged preparations stained with Diff Quik stain (Kobe, Japan). Eosinophils were shown to be 97.4 ± 1.2% pure, with a few contaminating neutrophils (mean 1.2%) or mononuclear cells (mean 0.8%).

*Culture of eosinophils.* Purified eosinophils ($2.5 \times 10^5$/ml) were cultured in RPMI1640 supplemented with 10% FCS using 48-well flat-bottom plates (Coster, Cambridge, USA). To determine whether epinastine had any effect on the IL-8 release from human blood eosinophils, the cells were incubated in duplicates with epinastine at different concentrations (0, 1, 5 and 25$\mu$g/ml) for 1, 3, 6, 16 and 24 hours in a humidified atmosphere at 37°C and 5%$CO_2$. In additional series of experiments, we performed 15 minutes' preincubation of eosinophils with calcium ionophore, PAF, GM-CSF or IL-5 for eosinophil activation before the treatment with epinatine. Supernatants were harvested by centrifugation and stored at −70°C until assay. Cell lysates were obtained from eosinophils by the treatment with 0.5% NP-40 (Iwai, Tokyo, Japan).

*Measurement of IL-8 and RANTES by ELISA.* The amounts of IL-8 and RANTES in the supernatants were measured in duplicates by ELISA kits according to the instructions of the manufacturer. The lower detection limit was 3 pg/ml. Inter- and intra- assay variations were less than 10%. The each kit was specific for IL-8 and RANTES, and did not show crossreactivity with other cytokines.

*IL-8 mRNA detection by reverse transcriptase-polymerase chain reaction (RT-PCR).* To determine the effect of epinastine on IL-8 mRNA levels in eosinophils, a quantitative assay utilizing reverse transcription and polymerase chain reaction previously reported(9) was peformed. Briefly, total RNA was isolated by RNesy (Qiagen, Inc., Chatsworth, CA) and was reverse transcribed by Avian Myeloblastosis Virus reverse transcriptase (Takara, Tokyo, Japan). Then the PCR was done with the IL-8 primers with the sequences of (1) 5′ primer: 5′ ATGACTTCCAAGCTGGCCGTGCT 3′ (2) 3′ primer: 5′ TCTCAGCCCTCTTCAAAAACTTCTC 3′, and with the $\beta$-Actin primers: (1) 5′ primer: 5′ ATGGATGATGATATCGCCGCG 3′ (2) 3′ Primer: 5′ CTAGAAGCATTTGCGGTGGGACGATGGAGGGGCC 3′ (Clonetech Laboratories, INC. Palo Alto, CA). The predicted sizes of the amplified IL-8 and $\beta$ actin DNA products were 289 and 1126 bp, respectively.

*Statistical analysis.* Data were shown as mean ± SEM. The results were analyzed by non-parametric equivalents of analysis of variance for paired and unpaired data.

## RESULTS

Eosinophils from atopic patients spontaneously released IL-8 into media after 24 hours (186.6±68.5 pg/ml, n=9) as previously reported (10), and stimulation with calcium ionophore significantly increased IL-8 release by these cells in a dose-dependent fashion (1 $\mu$M: 659.2±233.6 pg/ml, n=9, $p < 0.05$). However, pathophysiologically relevant stimulants such as PAF ($10^{-8}$ to $10^{-7}$ M), GM-CSF (0.2 to 20ng/ml) and IL-5 (0.2 to 20ng/ml) did not show any significant effect of IL-8 release (data not shown). Time-course experiments showed that there was an accumulation of IL-8 released by cultured eosinophils in 0 to 24 hrs (Fig. 1). Cycloheximide treatment (10$\mu$g/ml) diminished IL-8 release, suggesting a de novo protein synthesis. Studies by RT-PCR showed a constitutive expression of IL-8 mRNA in human eosinophils which was increased with calcium ionophore as described(8,10) (data not shown). Human isolated eosinophils also released immunoreactive RANTES as assessed by specific ELISA (15.75 ± 7.19 pg/ml, n=3).

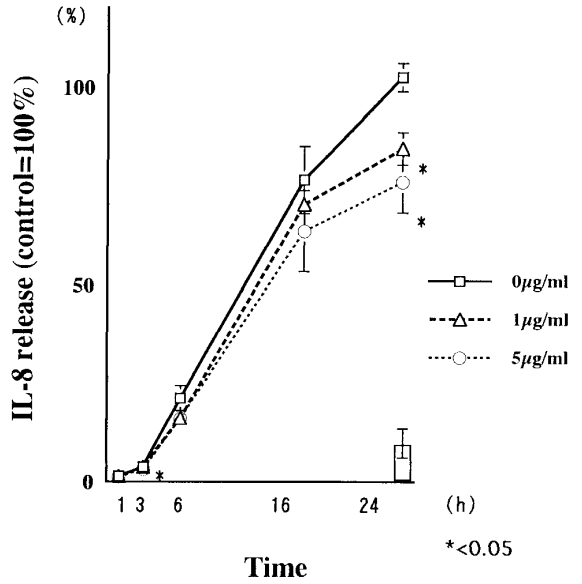


**FIG. 1.** Time course of IL-8 release from human eosinophils and effect of epinatine. Eosinophils ($2.5\times10^5$/ml) were incubated with epinastine (0, 1, 5, 25 $\mu$g/ml) in a humidified atmosphere at 37°C and 5% $CO_2$. Each point represents the mean of four experiments. The results are expressed as percent of IL-8 amount when the values for control group (24 hrs without epinastine) are calculated as 100%. The open column indicates the amount of IL-8 released in 24hrs by eosinophils with the treatment of cycloheximide (10 $\mu$g/ml).

Epinastine showed a dose-dependent suppressive effect on constitutive IL-8 release from eosinophils of atopic patients at the concentrations of 1, 5 and 25 $\mu$g/ml (the mean %inhibition at 5$\mu$g/ml, 29.5%). Epinastine further showed suppressive effect on IL-8 release by stimulated eosinophils with calcium ionophore at 25 $\mu$g/ml (mean %inhibition, 40.4%), but not at 1 and 5$\mu$g/ml (Fig. 2a). Epinastine significantly inhibited IL-8 release at 5$\mu$g/ml in 3hrs, and at 1 and 5$\mu$g/ml in 24hrs (Fig. 1). Eosinophil viability did not significantly change with epinastine treatment as assessed by typan blue dye exclusion technique (data not shown). We ob-

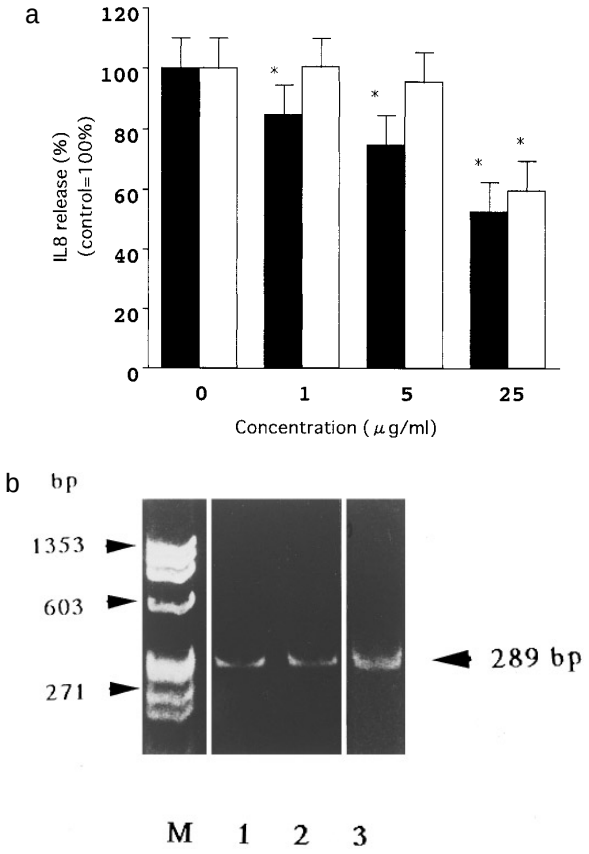


**FIG. 2.** (a) Effect of epinastine on IL-8 release from human eosinophils with or without calcium ionophore (1 $\mu$M). Closed columns indicated a dose-dependent inhibitory effect of epinastine on IL-8 release from unstimulated eosinophils (*$p<0.001$). Epinastine also inhibited IL-8 release from eosinophils stimulated with calcium ionophore (open columns). (b) Effect of epinastine on IL-8 mRNA levels evaluated by RT-PCR technique. Total RNA was extracted and equivalent amount of RNA was reverse transcribed. The PCR cycle was determined by preliminary experiments showing linear relationship between PCR cycles and intensity of signals on ethidium bromide stained agarose gels. For semi-quantitative evaluation of IL-8 and $\beta$-actin, 35 and 30 cycles were chosen respectively. Intensity of IL-8 signals did not statistically change with epinastine treatment (relative ratio of IL-8/$\beta$-actin transcripts showed no significant difference, $p>0.05$, paired t test). This was also the case with stimulation of calcium ionophore (not shown). M, molecular marker; lane 1, eosinophils with no treatment; lane 2, eosinophils treated with epinastine (5 $\mu$g/ml), lane 3, neutrophils as positive control.

tained cell lysates from eosinophils and evaluated changes in intracellular IL-8 levels. Epinastine (1 to 25$\mu$g/ml) did not affect the intracellular concentration of IL-8 (at 25$\mu$g/ml, 99.9 $\pm$ 28.1% when control values calculated as 100%, $p>0.05$). Finally, IL-8 mRNA as evaluated by RT-PCR technique showed no significant change by epinastine when normalized by $\beta$-actin transcripts (Fig. 2b). Epinastine did not show any significant effect on release of RANTES (1, 5, 25 $\mu$g/ml) (epinastine 5$\mu$g/ml, 93.1 $\pm$ 11.8% when control values calculated as 100%, $p>0.05$, $n=3$).

## DISCUSSION

In the present study, epinastine inhibited IL-8 release from human eosinophils in a dose- and time-dependent fashion. This effect was not due to cytotoxicity and found at comparable concentrations to previous reports in mast cells(5,6). Cycloheximide study as well as time-dependent accumulation suggested that IL-8 production required protein synthesis. Epinastine showed no effect on the intracellular concentration of IL-8. RT-PCR studies with no apparent effect on IL-8 mRNA levels suggested epinastine acted on the post-transcriptional processes. Human eosinophils also released low levels of RANTES, but epinastine had no significant effect on the release of this chemokine. It might be due to the low levels of synthesis of this chemokine in eosinophils, or it was possible that epinastine selectively affected IL-8 release.

Epinastine is considered to be a potent mast cell stabilizer (5). This agent inhibited antigen induced mediator release from mast cells *in vitro*(5,6). Furthermore, *in vivo* experiments suggested that epinastine reduced airway hyperresponsiveness by inhibition of late asthmatic response in rats (7). Since eosinophils are believed to play a crucial role in late responses(11,12), it is important to evaluate its potency on the function and recruitment of eosinophils. IL-8 (2,13,14) is a potent eosinophil chemoattractant accumulated in the sites of allergic inflammation(4). Interestingly, eosinophils themselves express and secrete IL-8 (8)(10). Therefore, it is likely that activated eosinophils produce IL-8 and play important roles of migration and activation of themselves by an autocrine mechanism.

In conclusion, epinastine inhibited release of IL-8, but not RANTES, from human eosinophils, and thereby might improve allergic inflammation by reducing the local recruitment of these cells. Our present findings highlit a new mode of action of one of the anti-allergic drugs epinastine for its effectiveness on allergic diseases.

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