A Novel Anti-Allergic Drug, Betotastine Besilate, Suppresses Interleukin-5 Production by Human Peripheral Blood Mononuclear Cells

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The effect of a novel anti-allergic drug, betotastine besilate (betotastine) on interleukin (IL)-5 production by human peripheral blood mononuclear cells (PBMC) was investigated. PBMC of Dermatophagoides farinae extract (Df)-sensitive donors produced IL-5 and showed a proliferative response upon stimulation with relevant antigen (10 µg/ml). Df-induced IL-5 production by PBMC was significantly inhibited by betotastine at 10 and 100 µM. Betotastine also suppressed proliferation of PBMC with less potency. The effect of betotastine on IL-5 production was enhanced and significant even at 0.1 µM when the drug was added 120 min before antigen stimulation. Ketotifen and cetirizine also inhibited IL-5 production, but the effects of these drugs were significant only at 100 µM. These findings indicate that the suppression of IL-5 production may be involved in the anti-allergic effect of betotastine.

Key words allergy; atopy; betotastine besilate; eosinophil; IL-5; T cell

Chronic eosinophilic inflammation of peripheral tissues has been recently recognized in the pathophysiology of allergic diseases such as atopic dermatitis, chronic rhinitis and bronchial asthma. 1,2) Accumulating evidence suggests that activated T cells and T cell-derived interleukin (IL)-5 play crucial roles in eosinophilic inflammation. 3–5) IL-5 enhances the proliferation, terminal differentiation and survival of eosinophils in vitro. 6,7) In allergic patients, serum IL-5 concentration was elevated 8) and activated T cells expressing IL-5 mRNA were detected in the inflammatory site. 9) Altogether, IL-5 seems to be the key cytokine involved in the allergic diseases associated with eosinophilic inflammation.

We have developed a novel anti-allergic drug, betotastine besilate (betotastine; (+)-(S)-4-{[4-(chlorophenyl)[2-pyridyl]methoxy]piperidino}butylic acid monobenzenesulfonate). It has been reported that betotastine inhibited histamine- and antigen-induced bronchoconstriction in guinea pigs. 9) It also suppressed histamine release from rat peritoneal mast cells in vitro. 10) Additionally, betotastine inhibited antigen-induced eosinophilic inflammation in mice 11) and guinea pigs (Ueno M. et al., submitted for publication). It has been demonstrated by several experimental models that the local recruitment of eosinophils is dependent on IL-5 produced by helper T cells, 4,12,13) suggesting that betotastine suppresses IL-5 production by T cells.

In this study, we report the effect of betotastine on IL-5 production by human peripheral blood mononuclear cells (PBMC). The effect was further characterized in comparison with other anti-allergic drugs, ketotifen and cetirizine.

MATERIALS AND METHODS

Subjects Six adult male volunteers (age: 30–45 years) sensitive to Dermatophagoides farinae extract (Df) were subjects of this study. None was receiving corticosteroids or anti-allergic drugs. Informed written consent was obtained from each individual.

Reagents Betotastine, ketotifen and cetirizine were obtained from Ube Industries, Ltd. (Tokyo). Df was purchased from Torii Pharmaceutical Co. (Tokyo). Purified rat anti-mouse/human IL-5 monoclonal antibody and biotinylated rat anti-human IL-5 monoclonal antibody were from Pharmingen (San Diego, CA). Other drugs used were Cell Titer 96™ Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI) and AIM-V medium (Gibco BRL, Gaithersburg, MD).

Cell Cultures Heparinized venous blood was taken between 9 and 10 a.m. PBMC were prepared by Ficoll-Paque density gradient centrifugation as described previously. 14) Cells were washed and suspended in AIM-V medium (2×10^6/ml), then cultured with or without Df (10 µg/ml) for 2–10 d. Each test compound was added at the start of cultures. In some experiments, betotastine was added 120 min before antigen stimulation. For cytokine assays, supernatants were harvested, and then frozen at −70°C until used.

Quantitation of IL-5 in Culture Supernatants IL-5 concentration in the culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA) as described, 14) using purified rat anti-mouse/human IL-5 monoclonal antibody and biotinylated rat anti-human IL-5 monoclonal antibody as the capture and the detecting antibody, respectively. The range of detection of the assay system was from 0.02 to 10 ng/ml.

Cell Proliferation Assay After PBMC (2×10^5/well) were cultured for 6 d with Df and test compound in 96 well flat-bottom culture plates, proliferation was assayed by the bioreduction of tetrazolium salt into formazan as described by Roehm et al. 15) with Cell Titer 96™ Aqueous Non-Radioactive Cell Proliferation Assay kit according to the manufacturer's manual. Briefly, 20 µl of tetrazolium assay solution was added to 100 µl of each well culture. After incubation for 4 h at 37°C, the absorbance of each well at 490 nm was measured. Result was expressed as stimulation index, which was calculated as the ratio of values in stimulated cultures to those in control cultures.

Statistics All data are presented as mean±S.E.M. Statistical analysis was performed by paired t-test, and p values less than 0.05 were considered to be statistically significant.
RESULTS

**Effect of Betotastine on IL-5 Production by PBMC of Df-Sensitive Donors** The first experiment was carried out to examine the production of IL-5 by PBMC of Df-sensitive donors upon specific antigen challenge in vitro. PBMC were stimulated with Df and the resulting supernatants were assayed for IL-5. No detectable amount of IL-5 was produced spontaneously by PBMC (data not shown). A significant increase in IL-5 production by PBMC stimulated with Df was detected after 3 days of incubation and production reached a maximum on day 6 (3.2 ± 1.0 ng/ml, Df; 10 μg/ml, n = 6). The optimal concentration of antigen in most subjects was 10 μg/ml. Other inflammatory cytokines, IL-2 and IL-4 were not detected in the culture supernatants even with antigen stimulation (data not shown).

Subsequently, we examined the effect of betotastine on antigen-induced IL-5 production by PBMC. No concentration of the test drugs used in this study affected the viability of PBMC (data not shown). Betotastine suppressed antigen-induced IL-5 production in a concentration-dependent manner and its effect was significant at 10 and 100 μM (Fig. 1). Ketotifen and cetirizine also inhibited IL-5 production by PBMC in a concentration-dependent manner, though the effective doses of these drugs were higher than that of betotastine (Fig. 1).

We have confirmed that the effect of betotastine in some *in vitro* studies was enhanced when the target cells were pretreated with this drug for several hours prior to stimulation (unpublished data). Therefore, after PBMC was preincubated with betotastine for 120 min, the effect of the drug on IL-5 production was examined. The effect of betotastine was enhanced and significant even at 0.1 μM (Fig. 1).

**Effect of Betotastine on Proliferative Response of PBMC of Df-Sensitive Donors** We also examined another antigen-specific response of PBMC, proliferation, in order to confirm whether betotastine suppressed IL-5 production as a result of growth inhibition. An antigen-specific proliferative response of PBMC was detected on day 2 and reached a maximum on day 6. Stimulation index was the highest at an antigen concentration of 10 μg/ml (2.3 ± 1.1, p < 0.05, compared with non-stimulated control by paired t-test). Betotastine significantly inhibited antigen-specific proliferation of PBMC at 100 μM (Table 1). Ketotifen and cetirizine also suppressed the proliferation at 100 μM.

DISCUSSION

Our study clearly demonstrated that the novel anti-allergic drug, betotastine, suppressed antigen-stimulated IL-5 production by human PBMC; significant suppression was obtained at 10 and 100 μM. The effect of betotastine on IL-5 production was enhanced and significant even at 0.1 μM when PBMC was preincubated with this drug before antigen stimulation. It has been reported in clinical study that the serum concentration of betotastine reached a maximum of 0.28 μM after a single oral administration at 10 mg.16 Betotastine therefore has a potential to inhibit IL-5 production *in vivo*. A time course study of the drug preincubation time remains to be further examined.

Chronic allergic diseases have recently been believed to be a consequence of eosinophilic inflammation of peripheral tissues.1,2) Accumulating evidence suggests that IL-5, which promotes terminal differentiation of the committed eosinophil precursor6 and prolongs the survival of eosinophils,7 is a key factor in inducing eosinophilic inflammation. It has been demonstrated in several experimental allergic models that administration of anti-IL-5 antibody inhibited local recruitment of eosinophils.4,15) In addition, we recently demonstrated that the existence of IL-5-producing T cells is sufficient for the development of peripheral eosinophilic inflammation.13 Therefore, the inhibitory effect of betotastine on late-phase eosinophil accumulation in antigen-challenged mice11 and guinea pigs (Ueno M. et al., submitted for publication) can be explained at least in part by the suppression of IL-5 synthesis by T cells.

Betotastine also suppressed antigen-specific proliferative response of PBMC, though the effect of this drug on proliferation was less potent than that on IL-5 production. Because we previously demonstrated that IL-5 produced by PBMC is derived exclusively from CD4+ T cells,17 we assume that betotastine suppressed IL-5 production by CD4+ T cells. Other cell types such as B cells, monocytes and basophils are also included in PBMC. For analysis of the target cell and the mechanism of betotastine in suppressing IL-5 production, examination of purified human T cell clones is an effective

![Fig. 1. Effects of Betotastine, Ketotifen and Cetirizine on IL-5 Production by Human PBMC](image)

Table 1. Effects of Betotastine, Ketotifen and Cetirizine on Antigen-Induced Proliferative Response in Human Peripheral Blood Mononuclear Cells

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Betotastine</th>
<th>Ketotifen</th>
<th>Cetirizine</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>2.9 ± 6.3</td>
<td>-71 ± 24</td>
<td>-3.9 ± 13</td>
</tr>
<tr>
<td>100</td>
<td>30 ± 6.2**</td>
<td>61 ± 20</td>
<td>93 ± 12**</td>
</tr>
</tbody>
</table>

PBMC (2×10^6/ml) was incubated with Df (10 μg/ml) and betotastine (open circles), ketotifen (open squares) or cetirizine (open triangles) for 6 days. The proliferation of PBMC was measured by non-radioactive cell proliferation assay system. Percent inhibition of proliferation compared with the control culture is shown (n = 3 - 6). * p < 0.05, ** p < 0.01; compared with Df-stimulated control (paired t-test).
means and is currently underway.

Ketotifen and cetirizine also inhibited IL-5 production and proliferation of PBMC. Both drugs have been reported to suppress local eosinophilic inflammation in clinical and animal studies. These findings also suggest that betotastine inhibits eosinophilic inflammation via the suppression of IL-5 production in vivo.

However, it has been reported that other mechanisms such as IgE-mast cell pathway are also involved in the development of eosinophilic inflammation in human allergic diseases. Therefore, the effect of betotastine on the eosinophilic inflammation should be explored further in clinical study.

In conclusion, betotastine which suppresses IL-5 production by human PBMC has potential as a treatment of allergic diseases because of its inhibition of eosinophilic inflammation.

REFERENCES