

Effect of Suplatast Tosilate (IPD-1151T) on a Mouse Model of Asthma: Inhibition of Eosinophilic Inflammation and Bronchial Hyperresponsiveness

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Key Words

Asthma, bronchial · Eosinophil · Airway hyperresponsiveness · Cytokines · IgE

Abstract

Background: Suplatast tosilate (IPD) is a newly developed 'anti-allergic' drug. It seems to be a unique compound because of its ability to suppress IgE but not IgG or IgM production in vivo and cytokine production from type 2 helper T cells (Th2) in vitro. However, information on its in vivo effect on an animal model of asthma is limited. **Method:** BALB/c mice sensitized to ovalbumin (3 times, 2-week interval) were challenged with ovalbumin by inhalation (50 mg/ml for 20 min, once a day for 6 days). In this study, we explored the influence of IPD on eosinophil infiltration into the airways, bronchial hyperresponsiveness (BHR) to methacholine, specific IgE antibody production, and cytokines in bronchoalveolar lavage fluid (BALF) using this murine model. **Results:** Treatment with IPD significantly reduced the number of total cells and eosinophils in BALF (around –40%) and almost completely inhibited the development of antigen-induced BHR. Histological findings confirmed the reduction of submucosal cell infiltration in the lung, and disclosed the marked inhibition of bronchial epithelial cell

damage. Ovalbumin-specific IgE was slightly but significantly reduced. The levels of IL-4, IL-5 and IL-13 in BALF were significantly decreased in mice treated with the compound compared to those in untreated mice. **Conclusion:** These results suggest that IPD is capable of inhibiting the production of Th2 cytokines, which inhibit eosinophil infiltration into the murine airway, IgE synthesis, and development of BHR, in a murine model of asthma.

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Introduction

Eosinophilic airway inflammation is a distinguishing feature of bronchial asthma, and is considered to induce bronchial hyperresponsiveness (BHR) and remodelling of the airways [1–3]. To investigate the mechanisms of these characteristics, well-characterized animal models have been used. Among these models, the murine system has advantages for detailed analysis and greatly promoted our understanding of the pathophysiology of asthma. Accumulating evidence indicates that type 2 helper T cells (Th2) are essential and responsible for allergen-induced BHR and eosinophilic inflammation [3, 4]. For example, the adoptive transfer of Th2 cells to naive mice with trans-

bronchial exposure to antigen induces all features of atopic asthma, such as eosinophilic airway inflammation, antigen-specific IgE antibody production, and development of BHR [5, 6]. In this context, new therapeutic agents targeted to Th2 cells or Th2 cytokines, such as anti-CD4 monoclonal antibody, anti-interleukin-5 (IL-5) monoclonal antibody and cyclosporin, have been reported to have beneficial effects in the treatment of human asthma [7–9].

Suplatast tosilate (IPD) is a new so-called ‘anti-allergic’ drug. The compound was found by screening a series of dimethyl-sulfonium compounds in the search for IgE-suppressive agents. IPD can suppress IgE synthesis in both mice and humans without any influence on total IgG and IgM production [10, 11]. IPD did not suppress the production of IgE and IgG1 by splenic B cells stimulated with lipopolysaccharide and IL-4 [10]. Furthermore, IPD can inhibit both IL-4 and IL-5 production from Th2 cells in vitro [11]. Therefore, IgE-suppressive activity of IPD seems to be due to the inhibition of IL-4 production from T cells. Local eosinophilia induced by an adoptively transferred typical Th2 clone, D10.G4, into the abdominal cavity with antigen is markedly inhibited [12]. It can also inhibit the induction of mast cells from splenocytes in mice which may be dependent on Th2 cytokines [13].

All these facts indicate that IPD can inhibit the production of Th2 cytokines. Therefore, this compound would be effective at least in the murine model of asthma. However, there are a few studies concerning the effectiveness of IPD on animal models [14, 15]. Therefore, we performed this study to elucidate the effect of IPD on a mouse model of asthma, in terms of airway inflammation, production of specific antibodies, BHR to methacholine (MCh), and local production of cytokines.

Materials and Methods

Mouse Sensitization and Challenge

Sensitization and challenge were performed as described previously with some modifications [16]. Six- to eight-week-old female BALB/c mice (Charles River Laboratories, Shizuoka, Japan) were immunized intraperitoneally 3 times with 10 µg of ovalbumin (OVA) (grade V; Sigma Chemical Co., St. Louis, Mo., USA) plus 20 mg Al(OH)₃ in 0.2 ml saline at a 1-week interval (days 0, 7, and 14). A day after the third immunization, the sensitized mice were individually placed in a 50-ml plastic tube and exposed to aerosolized OVA (5%) dissolved in saline, delivered by a DeVilbiss 646 nebulizer (DeVilbiss Corp., Somerset, Pa., USA) driven by compressed air at 5 liters/min for 20 min, once a day for 6 consecutive days (days 15–20). For negative controls (saline control group), saline alone was used instead of OVA inhalation.

Administration of IPD

IPD was kindly provided by Taiho Pharmaceutical Co., (Tokyo, Japan). The compound was dissolved in sterile saline. In the treated group (IPD group), IPD (100 mg/kg/100 µl) was orally given to mice once a day for 21 days (days 0–20). The dose was determined by previous reports showing obvious inhibition of IgE synthesis [14, 15]. In the positive control group (OVA control group), saline alone was used instead of the compound.

Bronchoalveolar Lavage and Histologic Examination

Twenty-four hours after the final challenge (day 21), mice were sacrificed by cervical dislocation. Bronchoalveolar lavage fluid (BALF) was obtained by the slow injection of phosphate-buffered saline (PBS, 0.5 ml) into the trachea using a cannula 3 times (total 1.5 ml). This procedure always yielded >80% recovery of the infused fluid. The total number of cells in BALF was counted by a hemocytometer. A differential count was made by cytopspin preparations (150 g, 2 min, at room temperature). The cells were fixed and stained with May-Giemsa. Differential counts of at least 300 cells were done using standard morphological criteria to identify eosinophils. The absolute number of eosinophils in BALF was then calculated. The BALF was stored at –80°C until use. IL-4, IL-5, IL-13 and IFN-γ ELISA kits were purchased from Endogen (Boston, Mass., USA). The concentrations of these cytokines in BALF were assayed according to the procedures recommended by the manufacturer. The limit of detection was 5 pg/ml for IL-4, IL-5 and IL-13, and 15 pg/ml for IFN-γ.

Following BAL, lungs were perfused with PBS (10 ml) through the right ventricle. Then the lungs were removed and fixed with 4% paraformaldehyde. The resin (Jung Histo Resin plus, Reichert/Jung, Heidelberg, Germany)-embedded specimens were cut (vertically to the lower lobe bronchus) into 2-µm slices onto slides and stained with hematoxylin eosin or periodic acid-Schiff, according to a standard protocol.

The numbers of goblet cells (periodic acid-Schiff-positive cells) and total epithelial cells (total number of nuclei) were counted using an image analysis system. The stained slides were examined with a light microscope (AX80; Olympus, Tokyo, Japan), connected to a digital camera (HC-300; Fuji Film, Tokyo, Japan) and an image capture board (Photograb-300, Fuji Film). Images of the intrapulmonary airway epithelium (large airway in the specimen) were recorded from 5 consecutive high-power fields at ×400. Analysis was performed on a Power Macintosh G3 computer (Apple Computer, Cupertino, Calif., USA) using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>). Data are expressed as number of cells per millimeter subepithelial basement membrane.

OVA-Specific IgG2a and IgE Antibody

OVA-specific antibodies were measured by indirect ELISA. In brief, 96-well microtiter plates (ICN, Costa Mesa, Calif., USA) were coated with OVA at 4°C overnight. Then the plates were blocked with 1% BSA (Sigma) in PBS for 2 h at 37°C. Following washes, samples and control serum were incubated overnight at 4°C. Plates were incubated with horseradish peroxidase-conjugated rat anti-mouse IgE and IgG2a monoclonal antibodies (Pharmingen, San Diego, Calif., USA), diluted in 50% goat serum (Gibco, Gaithersburg, Md., USA)/PBS for 2 h at room temperature. After washing, *o*-phenylenediamine dihydrochloride substrate with hydrogen peroxide was added, and absorbance at 492–620 nm was measured by a

Table 1. Total cell count and cell differentials in BALF

Group	Total cell number × 10 ⁵ /ml	Cell differentials, %			Eosinophil number × 10 ⁵ /ml
		macrophages	eosinophils	lymphocytes	
Saline control	1.5 ± 0.5**	97.5 ± 0.7**	0.5 ± 0.4**	0.5 ± 0.3**	0.0 ± 0.0**
OVA control	14.8 ± 4.4	5.5 ± 1.6	91.3 ± 1.8	1.5 ± 0.7	13.5 ± 4.2
IPD	9.6 ± 2.9*	10.0 ± 3.4**	84.1 ± 3.3**	3.9 ± 1.0*	8.0 ± 2.5*

Data are shown as means ± SD (n = 6–7).

* p < 0.01, ** p < 0.001 versus OVA control group.

microplate reader. The antibody titers of the sample were calculated from the values of the control serum, in which OVA-specific IgE- and IgG2a titers of 100 U/ml for both were assigned.

Measurement of BHR

BHR was measured just before BAL by recording respiratory pressure curves by whole body plethysmography (Buxo, Troy, N.Y., USA) to aerosolized MCh, as described previously [17]. Before taking readings, the box was calibrated by rapid injection of 100 ml air into the main chamber. Pressure differences between the main chamber containing the mouse and a reference chamber were recorded. Readings were obtained at baseline and after exposure to aerosolized saline or MCh (3–50 mg/ml). Data were averaged for 3 min and expressed as the ‘enhanced pause’ (Penh):

$$\text{Penh} = [(\text{Te} - \text{Tr}) / \text{Tr}] \times (\text{PEP} / \text{PIP});$$

where Te is the expiratory time(s), Tr is the relaxation time (time of pressure decay to 36% of total box pressure during expiration); PEP is the peak expiratory pressure (ml/s), and PIP is the peak inspiratory pressure (ml/s). Results are expressed as percent increase of Penh following challenge with each concentration of MCh, where baseline Penh (after saline challenge) was expressed as 100%.

Statistics

Data are expressed as mean ± SD, or median (range). The differences of data from each group were calculated using Student’s t test following one-way ANOVA when applicable. The Mann-Whitney U test was applied for the comparison of cytokine concentrations. A value of p < 0.05 was considered as significant.

Results

The profile of cell counting and differentials of BALF is shown in table 1. The total cell number in BALF from the OVA control group was markedly higher than that in the negative saline control group (p < 0.001, respectively). Since more than 90% of the cells in BALF were eosinophils in the OVA control group, the absolute number of eosinophils was markedly increased. The lungs of the

Table 2. Number of goblet cells in the intrapulmonary bronchi

Group	Total epithelial cells	Goblet cells
Saline control	88.6 ± 6.9	0.0 ± 0.0**
OVA control	82.7 ± 6.5	26.0 ± 4.5
IPD	84.5 ± 7.6	15.2 ± 8.0*

Data are shown as means ± SD of cells per millimeter of basement membrane (n = 6–7).

* p < 0.05, ** p < 0.0001 versus OVA control group.

OVA control group had a marked thickened epithelium and goblet cell hyperplasia in the intrapulmonary bronchi, which were not found in the lungs of the saline control group (table 2). Submucosal cell infiltration with eosinophils and mononuclear cells was also observed in the OVA control but not in the saline control group. Representative figures are shown in figure 1a, b.

Treatment with IPD (100 mg/kg) resulted in a significant decrease in total cells and eosinophil numbers in BALF, compared with OVA control mice. The absolute number of eosinophils was significantly decreased (–40%), though there was a small but significant reduction in the percentage of eosinophils in BALF. Histological findings in the lungs of treated mice revealed an obvious reduction in thickness of epithelium and submucosal cell infiltration when compared with the OVA controls (fig. 1c). The number of goblet cells in the IPD group was significantly less than that in the OVA control group (table 2).

Serum OVA-specific IgE but not IgG2a antibody was detected in mice of the OVA control and the IPD group. Neither class of antibodies was detected in the saline control group. The OVA-specific IgE antibody production

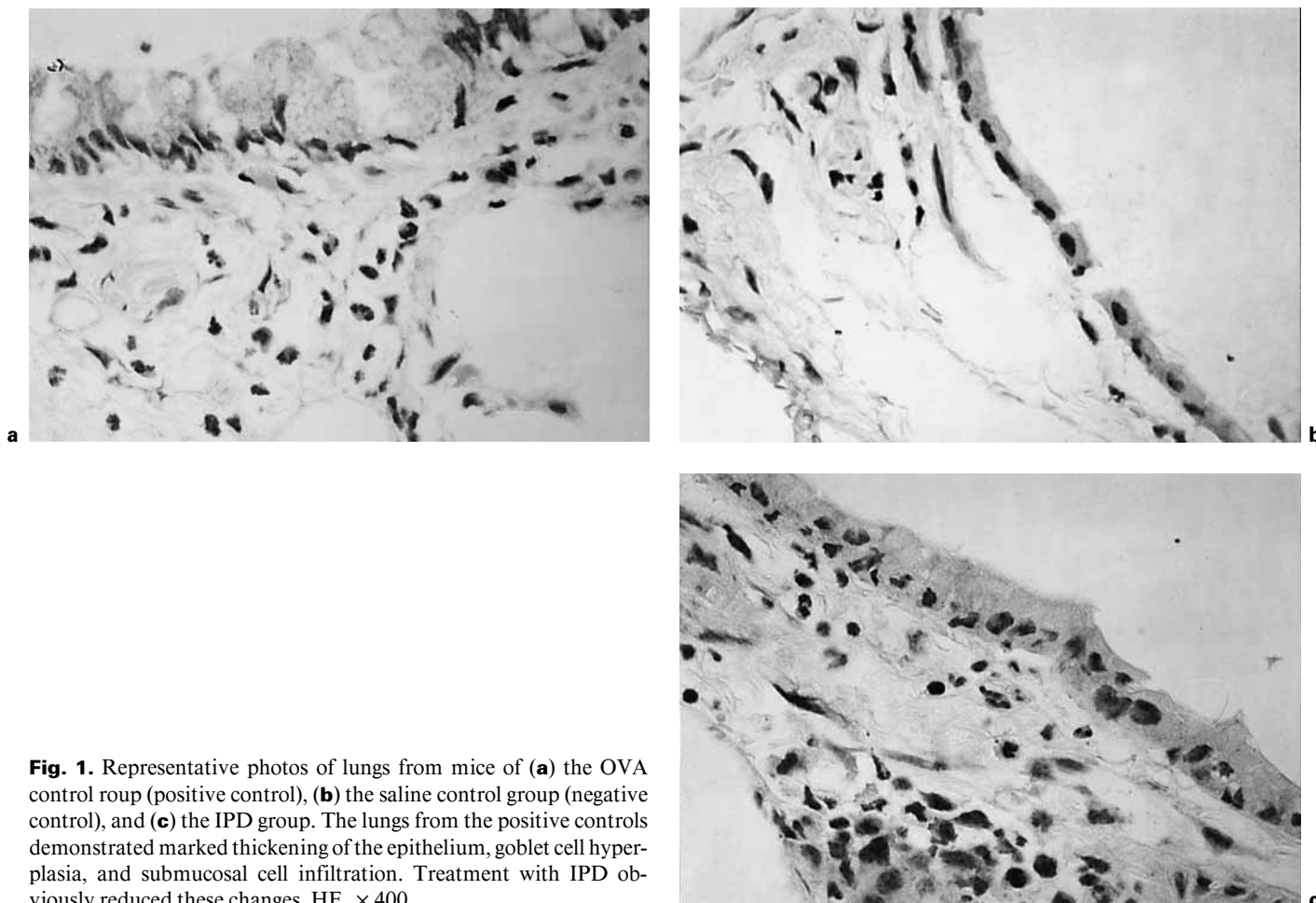


Fig. 1. Representative photos of lungs from mice of (a) the OVA control group (positive control), (b) the saline control group (negative control), and (c) the IPD group. The lungs from the positive controls demonstrated marked thickening of the epithelium, goblet cell hyperplasia, and submucosal cell infiltration. Treatment with IPD obviously reduced these changes. HE, $\times 400$.

was marginally but significantly decreased in mice treated with IPD compared with OVA control mice (fig. 2).

BHR to MCh was markedly increased in OVA control mice compared to saline control mice. The development of antigen-induced BHR was almost completely blocked by treatment with IPD (fig. 3).

We could detect IL-4, IL-5 and IL-13 but not IFN- γ in BALF from mice of both the OVA control and the IPD group (table 3). The levels of IL-4, IL-5 and IL-13 in mice of the IPD group were significantly lower than those in OVA control mice ($p < 0.01$, for each).

Discussion

The present study showed that IPD can significantly reduce airway inflammation, induce a small but significant decrease in specific IgE production, and inhibit

almost completely the development of antigen-induced BHR in a mouse model of asthma. Furthermore, the levels of IL-4, IL-5 and IL-13 in BALF, which have important roles in the pathophysiology of asthma, were decreased by treatment with IPD.

Our results demonstrated that BALF eosinophilia, after OVA challenge in sensitized mice, was significantly inhibited by treatment with IPD. The precise mechanism(s) by which this drug inhibits eosinophil recruitment is currently uncertain. IL-5 is the most important cytokine in eosinophilic inflammation [18–20]. Since IL-5 levels in BALF from treated mice were significantly inhibited, suppression of IL-5 production would be involved. In any way, eosinophil infiltration in mice treated with IPD was decreased to about half of that observed in OVA control mice. Eosinophils are considered to be key cells for epithelial damage via eosinophilic granules, such as major basic protein and eosinophil cationic protein [21, 22]. How-

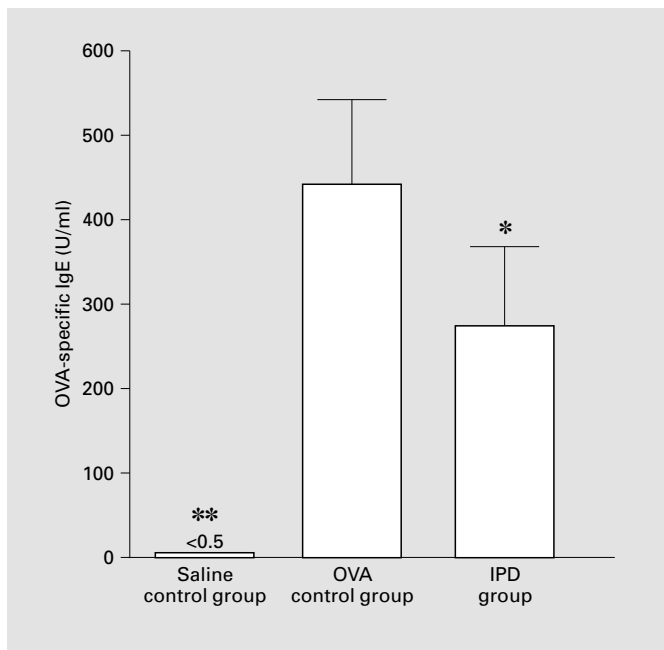


Fig. 2. Effects of IPD on OVA-specific IgE in serum. The titer of specific IgE was significantly decreased by treatment with IPD. Bars denote means $\pm$ SD for 6 mice in each group. * $p < 0.05$, ** $p < 0.01$, compared with the values of the OVA control group.

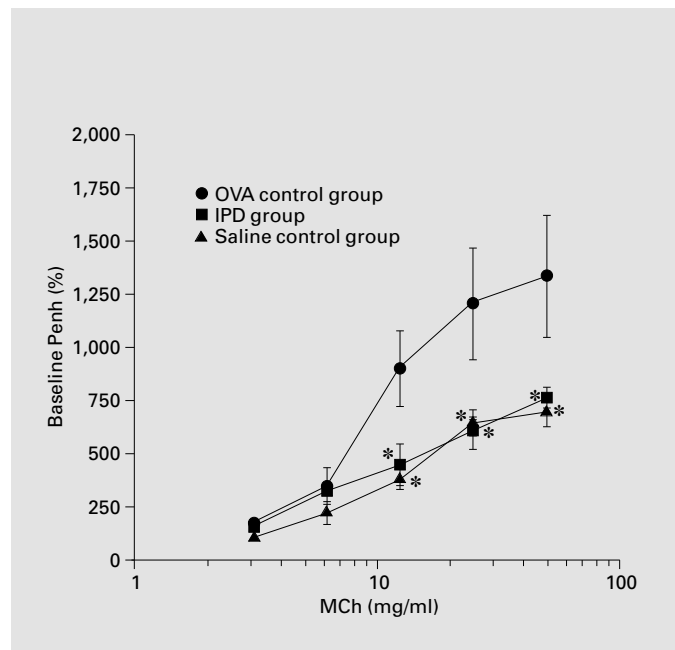


Fig. 3. Effect of IPD on the development of BHR to MCh. Data are expressed as percent increase of Penh (see Methods) following saline challenge for each concentration of MCh. Bars denote means $\pm$ SD for 6 mice in each group. * $p < 0.05$, compared with the values of the OVA control group.

ever, the development of antigen-induced BHR was almost completely inhibited by treatment with IPD, even in the presence of a significant number of eosinophils (fig. 3). According to the above facts, IPD may inhibit the degranulation of eosinophils, although this has not been proved.

There are two reports showing the inhibitory effect of IPD on BHR in animal models. Konno et al. [14] demonstrated that IPD could dose-dependently inhibit BHR to MCh in mice immunized and challenged with keyhole limpet hemocyanin. More than 30 mg/kg of IPD completely inhibited the development of BHR in that model. However, the degree or nature of airway inflammation was not evaluated in the report. Taniguchi et al. [15] also demonstrated that IPD could inhibit BHR to acetylcholine in a guinea pig model of asthma, in which the animal was sensitized twice and challenged once with aerosolized OVA. Although specific IgE was not detected in this model, eosinophils in BALF and in peribronchioles were significantly decreased to about half and one third, respectively, by IPD treatment of 100 mg/kg/day. Histological examination revealed that airway epithelial cells were preserved, which are markedly damaged in mice of the

OVA control group, by the treatment with IPD. This observation seemed to be connected to the diminished development of BHR.

The suppression of specific IgE antibody production observed in the present study, which is consistent with previous studies [10], may contribute to the inhibition of BHR. However, the reduction of specific IgE was not prominent, like the suppression of eosinophil infiltration. Therefore, such a mild decrease may not account for the almost complete inhibition of BHR. This notion could be supported by the fact that the presence of immunoglobulins including IgE is dispensable for the development of eosinophilic inflammation and BHR in this type of mouse model [23, 24]. Since IPD can inhibit the induction of mast cells in spleen cells of mice, the effect may be due to a decreased number of mast cells [13]. However, the role of mast cells in the development of eosinophilic inflammation and BHR is limited. It seems to be dependent on the strength of the reaction; i.e., an intense inflammatory response may develop regardless of the presence of mast cells, whereas a less severe inflammation may need mast cells for full development of the inflammation [25, 26].

Table 3. Cytokine concentration (pg/ml) in BALF

Group	IL-4	IL-5	IL-13	IFN- γ
Saline control	<5.0* (<5.0–5.0)	<5.0* (<5.0–5.2)	<5.0** (<5.0)	<15 (<15)
Ova control	9.5 (5.0–16)	8.8 (5.0–15)	111 (72–148)	<15 (<15)
IPD	<5.0* (<5.0–6.0)	<5.0* (<5.0–8.2)	19* (6.0–86)	<15 (<15)

Data are shown as median (range) (n = 6–7).

* p < 0.05, ** p < 0.01 versus OVA control group.

We observed that treatment with IPD decreased the concentration of IL-4, IL-5 and IL-13 in BALF, an information not reported previously. All these cytokines are considered to be important for the development of inflammation and BHR. In particular, it has recently been reported that IL-13 alone can induce eosinophilic inflammation, epithelial damage, and BHR [27, 28]. A relatively large amount of IL-13 was detected in BALF in comparison to the other cytokines (table 3). Suppression of this cytokine may contribute to the fact that IPD can inhibit BHR more efficiently than airway eosinophilia.

In any way, the combination of the effects mentioned above may contribute to marked inhibition of the devel-

opment of BHR, although further work will be needed before the mechanisms of action of this drug can be sorted out. A recent report showed that treatment with IPD resulted in diminished infiltration of eosinophils and improved BHR in patients with mild asthma [29]. It should be noted that IPD has beneficial therapeutic effects not only on bronchial asthma, but also on eosinophilic pneumonia, Kimura's disease and vitiligo [30–33]. The same mechanism demonstrated in our present study may possibly take place in humans.

In conclusion, our results disclosed that IPD can act as a possible anti-Th2 and anti-inflammatory drug in treating patients with bronchial asthma.

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