

CD28 and secretory immunoglobulin A-dependent activation of eosinophils: inhibition of mediator release by the anti-allergic drug, suplatast tosilate

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Summary

Background Eosinophils are major effector cells in allergic diseases. After their recruitment to sites of inflammation, they contribute to the pathophysiology of the disease by releasing granule proteins and cytokines. Suplatast tosilate (IPD-1151T), a new anti-allergic agent, has shown beneficial effect in the treatment of asthma, associated with reduced bronchoalveolar lavage eosinophil infiltration and eosinophilic cationic protein (ECP) release in serum and sputum.

Objective We investigated whether suplatast tosilate could exert direct effects on human eosinophil activation.

Methods Eosinophils from hypereosinophilic patients or normal donors were purified by Percoll gradient and the magnetic cell separation system. Chemotaxis was studied using the Boyden chamber technique using three chemoattractants, formyl-methionine-leucine-phenylalanine (fMLP), IL-5 and eotaxin. Oxidative metabolism was determined by a luminol-dependent chemiluminescence assay after activation with eotaxin or secretory IgA (sIgA). The release of ECP and eosinophil derived neurotoxin (EDN) was measured by radioimmunoassay and cytokine production was determined by ELISA following activation with sIgA or anti-CD28.

Results The chemotactic response to fMLP, IL-5 and eotaxin was significantly inhibited by IPD-1151T. Suplatast tosilate was partially inhibiting the release of reactive oxygen species (ROS) induced by eotaxin and sIgA. Activation by sIgA and CD28 ligation resulted in the release of ECP and EDN, which was inhibited by IPD-1151T. Upon activation by anti-CD28, only IL-13 production was inhibited by IPD-1151T, whereas release of IL-2 and IFN- γ was not affected. IL-10 release induced by sIgA was also inhibited by IPD-1151T. Additionally, the pro-inflammatory cytokine IL-6, which was secreted following anti-CD28 and sIgA stimulation, was strongly inhibited by IPD-1151T.

Conclusion Through inhibition of chemotaxis, IPD-1151T might limit the number of eosinophils at the inflammation site. Furthermore, it could reduce the pathological potential of eosinophils by inhibiting the release of ROS and cationic proteins, main inflammatory mediators produced by eosinophils. Moreover, the inhibition of immunoregulatory cytokines released by eosinophils could locally modify the immune response.

Keywords activation, allergy, CD28, eosinophils, IPD-1151T, sIgA

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Introduction

Allergic disorders, such as asthma, atopic dermatitis (AD) and rhinoconjunctivitis are accompanied by inflammatory responses including infiltration of eosinophils. In asthmatics, the level of eosinophilic inflammation in the airway correlates with the severity of asthma. The migration of eosinophils from the blood to the tissues is controlled by membrane adhesion molecules and by soluble factors including various cytokines and chemokines, as well as small molecular weight substances [1]. Among those, formyl-methionine-leucine-phenylalanine (fMLP), IL-5 and eotaxin have been shown

to exert potent chemotactic activity on eosinophils *in vitro* [2]. Eosinophils contain a variety of pro-inflammatory mediators, including cytoplasmic granule proteins and lipid mediators, as well as a large number of immunoregulatory cytokines and chemokines. At the site of inflammation, eosinophils become activated and release cytotoxic products, such as cationic proteins (eosinophil peroxidase, major basic protein, eosinophil cationic protein (ECP), eosinophil derived neurotoxin (EDN)) and reactive oxygen species (ROS), all of which contribute to tissue damage. Eosinophils may be activated by a large number of molecules (cytokines, chemokines, and molecules of the immunoglobulin superfamily). One of the more potent stimulus of eosinophil activation is immobilized or cross-linked secretory IgA (sIgA), able to induce the release of ECPs, cytokines and ROS [3–5]. The costimulatory molecule CD28, which is expressed by eosinophils, is also a

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potent activator of eosinophils in inducing the release of type-1 cytokines (IL-2 and IFN- γ) [4], as well as the type-2 cytokine IL-13 [6]. The role of eotaxin as the major eosinophil activator has also been described, in inducing the release of reactive oxygen species and calcium mobilization [5, 7].

Suplatast tosilate (IPD-1151T) is a new dimethylsulphonium agent used therapeutically in Japan for the treatment of allergic diseases, such as bronchial asthma, allergic rhinitis (AR) and AD [8–10]. This anti-allergic agent was reported to suppress specifically IgE antibody production [11]. It has also been shown to selectively inhibit the production of IL-4 and IL-5 in human and mouse T helper (Th) 2 clones [12, 13]. Inhibition by IPD-1151T of chemical mediator release from mast cells [14] and murine mast cell differentiation [15] have been demonstrated. In the mouse model of asthma, eosinophil infiltration and airway hyper-responsiveness are impaired by IPD-1151T treatment [16, 17]. In humans, such an effect has also been obtained in patients with asthma-receiving suplatast tosilate, allowing doses reduction of inhaled corticosteroids [8, 18]. However, no information about direct effects of IPD-1151T on eosinophil function and activation has been reported. Therefore, we investigated the potential role of IPD-1151T on eosinophil migration induced by three potent chemoattractants (fMLP, IL-5 and eotaxin), and on eosinophil activation, as determined by ROS production, cationic protein release and cytokine secretion induced by activators, such as eotaxin, sIgA and CD28 ligation.

Materials and methods

Patients

A total of 25 different patients with hypereosinophilia associated with allergy (11), skin diseases (11) and drug hypersensitivity (three), and 12 normal donors was selected for this study, after informed consent.

Cell purification

Eosinophils were isolated from the venous blood of patients with various eosinophilia-associated diseases, by the method previously described using immunomagnetic beads and the magnetic cell separation system (MACS) [6]. Briefly, after density centrifugation on Percoll of diluted whole blood, the granulocyte pellet was incubated with anti-CD16-immunomagnetic beads. Eosinophils were eluted by passage of the cells through the field of a permanent magnet and the purity of eosinophil preparations was normally above 95%, with lymphocytes and few neutrophils as contaminating cells. Neutrophils were isolated from the blood of healthy donors after Percoll density centrifugation and erythrocyte lysis and preparation with 99% neutrophils were routinely obtained.

Reagents

Suplatast tosilate (IPD-1151T) was provided by Taiho Pharmaceutical Co. (Tokyo, Japan). Anti-human CD16-magnetic beads and the MACS were purchased from Miltenyi Biotec (Bergish Gladbach, Germany). Percoll was obtained from Pharmacia (Uppsala, Sweden). RPMI 1640 medium, gluta-

mine, penicillin, streptomycin and fetal calf serum (FCS) were from Gibco BRL Life Technologies (Paisley, UK). Eotaxin and IL-5 were purchased from Peprotech (Rocky Hill, NJ, USA). Human sIgA, fMLP, luminol and anti-mouse IgG F(ab')₂ were obtained from Sigma (St Louis, MO, USA) and anti-human IgA mAb from Immunotech (Coulter Corp., Miami, FL, USA). Anti-CD28 and isotype control mouse monoclonal antibodies were from Diaclone (Besançon, France).

Chemotaxis assay

The measurement of leucocyte chemotaxis was assessed by a modification of the Boyden micropore filter technique, as previously described [5]. Briefly, the assay is carried out in a 48-well microchemotaxis assembly (Neuroprobe, Cabin John, MD, USA). Cell suspensions of eosinophils or neutrophils were placed, with or without IPD-1151T, in the upper chamber separated from the leukoattractant solution by a 5 μ m (eosinophils) or 3 μ m (neutrophils) pore size polycarbonate membrane filter (Nucleopore Co., Pleasanton, CA, USA). The stimulus compartment received either control buffer or the attractant reagent. Migration was carried out at 37 °C in humidified air with 5% CO₂ for 2 h (eosinophils) or 60 min (neutrophils). The filters were then fixed and stained with May-Grünwald Giemsa (RAL, Paris, France). The numbers of eosinophils that had migrated were enumerated microscopically in four random high-power fields in quadruplicated wells.

The percentage of inhibition obtained with IPD was calculated by comparison to the positive control (migration of untreated cells in response to the respective chemoattractant reagent), according to the formula:

$$\% \text{ inhibition} = 100 - \frac{\text{migration with IPD} - \text{spontaneous migration}}{\text{positive control migration} - \text{spontaneous migration}} \times 100$$

Chemiluminescence assay

The production of ROS from eosinophils was examined with luminol-dependent chemiluminescence [5]. Purified eosinophils (5×10^5) were incubated with 15 μ g/mL sIgA with or without IPD. After 1 h at 37 °C, anti-IgA (20 μ g/mL) and luminol (8.3 μ g/mL) were added to the cells.

For eotaxin activation, cells were pre-incubated with or without IPD in the presence of IL-5 for 1 h at 37 °C, before addition of eotaxin (100 ng/mL) and luminol. Chemiluminescence was immediately measured with a luminometer (Victor²™ Wallac, Perkin Elmer, Shelton, CT, USA). Kinetic was performed at 37 °C during 40 min and for each time-point, chemiluminescence was counted for 5 s.

The percentage of inhibition was calculated by comparison to the positive control (ROS production of activated cells in the absence of IPD), according to the above formula.

Cell culture

Purified eosinophils (2×10^6 /mL) were cultured in RPMI 1640 medium supplemented with 10% heat inactivated FCS, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 μ g/mL streptomycin. Cells were activated by incubation with IgA with or without IPD (1 h) followed by anti-IgA mAb at a respective final concentration of 15 and 20 μ g/mL. Activation

by CD28 ligation was performed as previously described [4]. Wells were first coated with 40 µg/mL anti-mouse IgG F(ab')₂ for 2 h at 37 °C, followed by 10 µg/mL anti-CD28 or isotype-matched control mAb for 2 h at 37 °C. After washing, cells were added into the wells with or without IPD-1151T. After 18 h at 37 °C, the cells were centrifuged, the supernatants were collected and stored at -20 °C until the measurement of granule proteins and cytokines could be performed.

Eosinophil mediator measurements

The determination of ECP and EDN was carried out by radioimmunoassay (Pharmacia) and results were expressed in nanograms per mL on the basis of a standard curve obtained with purified ECP and EDN. The percentage inhibition was calculated using the above formula, in which the spontaneous release values, in the absence of stimulus, have been subtracted.

Cytokine measurements

Cytokines were assayed in eosinophil supernatants using specific ELISA kits (Diaclone). The lower detection limit was 1.5 pg/mL for IL-13, 5 pg/mL for IL-10, IL-6 and IFN-γ and 10 pg/mL for IL-2.

Statistical analysis of data

Statistical significance was determined using Wilcoxon's signed rank test, in order to compare results in the absence and in the presence of different concentrations of IPD-1151T. The general effect of the drug was considered by using Mann-Whitney *U*-test. *P*-values <0.05 were considered to be significant.

Results

Inhibitory effect of IPD on human eosinophil migration

The eosinophil chemotactic response to fMLP, IL-5 or eotaxin has been studied and the inhibitory effect of IPD has been evaluated for the three chemotactic agents, in function of the doses of the drug ranging from 10⁻⁸ to 10⁻⁴ M. As shown in Fig. 1, a significant and dose-dependent inhibition by IPD-1151T of eosinophil chemotactic response to the three potent chemotactic factors was obtained, reaching more than 80% inhibition at the highest concentration (10⁻⁴ M). This effect of IPD-1151T was specific to eosinophils as no inhibition of the chemotactic response of neutrophils to fMLP was observed (Fig. 1). Whereas, at high concentration, maximal inhibition was obtained with the three chemoattractants, the inhibitory effect of IPD seemed to depend on the chemotactic pathway, especially for low drug concentration. Indeed, eosinophil chemotaxis to eotaxin appeared to be more susceptible to IPD effect, as 47% inhibition was already obtained with IPD at 10⁻⁸ M, as compared to 38% for chemotaxis to IL-5 and only 12% for chemotaxis to fMLP (at 10⁻⁷ M). This observation was confirmed by the determination of the IC₅₀ values (drug concentration leading to 50% inhibition): 5 × 10⁻⁶ M, 6.5 × 10⁻⁷ M and 6.5 × 10⁻⁸ M for fMLP, IL-5 and eotaxin, respectively.

Effect of IPD on eosinophil oxidative metabolism

The effects of IPD have been evaluated on the oxidative metabolism of eosinophils induced either by eotaxin or sIgA immune complexes. Kinetic experiments were performed with various concentrations of the drug (ranging from 10⁻⁷ to 10⁻⁴ M) in order to evaluate whether IPD would have an effect on the total ROS production or on specific time points (delayed production). Total ROS production was inhibited by IPD and, therefore, results were considered at the maximal ROS production: 16 and 6 min for eotaxin and sIgA activation respectively (Figs 2a and c).

The production of ROS from human eosinophils activated by eotaxin was inhibited in a dose-dependent manner by IPD (Figs 2a and b). However, this inhibition was only partial, reaching 34% at the highest concentration of the drug (10⁻⁴ M). Activation by sIgA, which induced an even higher release of ROS as compared to eotaxin, was also partially inhibited by IPD, but with a constant level of inhibition (28%) (Figs 2c and d).

Inhibition by IPD-1151T of eosinophil mediator release

The effects of IPD were then investigated on the release of ECP and EDN by eosinophils after activation with sIgA. Results presented in Fig. 3a showed that IPD at 10⁻⁵ M induced a significant decrease of ECP release (mean inhibition = 76%). This inhibition was dose-dependent, reaching a maximum of 85% inhibition at 10⁻⁴ M (Fig. 3b). Similarly, the release of EDN was also reduced by IPD with a maximal inhibition of 60% (Figs 3c and d).

Furthermore, ligation of CD28, which has been shown to induce the secretion of immunoregulatory cytokines [4, 6], also induced eosinophil ECP and EDN release, in the same extent as sIgA activation (Figs 4a and c). This CD28-induced degranulation was inhibited by IPD in a dose-dependent manner and reached a maximum of 86% inhibition and 70% inhibition for ECP and EDN release, respectively (Figs 4b and d). Interestingly, the release of ECP induced by both stimulation appeared to be more sensitive to IPD-1151T than EDN release, as a difference of two log drug concentrations were needed to obtain the same inhibition level. This observation suggests that different mechanisms are implicated in eosinophil degranulation.

Inhibition of immunoregulatory cytokine release

We have previously shown that ligation of CD28 was inducing the release of IFN-γ, IL-2 and IL-13, whereas activation by sIgA immune complexes induced the release of IL-10 by eosinophils [4, 6]. Therefore, we investigated whether IPD could inhibit the release of type-1 and type-2 cytokines after CD28 or sIgA activation. As shown in Fig. 5, IPD had no effect on IL-2 and IFN-γ secretion induced by anti-CD28 activation, although some non-significant decreased IFN-γ production was detected at the highest concentration of the drug (10⁻⁴ M). Contrastingly, the production of IL-13 was inhibited significantly and in a dose-dependent manner, with a maximal inhibition of 60% (IC₅₀ = 1.5 × 10⁻⁵ M) (Fig. 5). Activation by anti-CD28 also released IL-6, which was inhibited by IPD-1151T, reaching 47% maximal inhibition (Fig. 5).

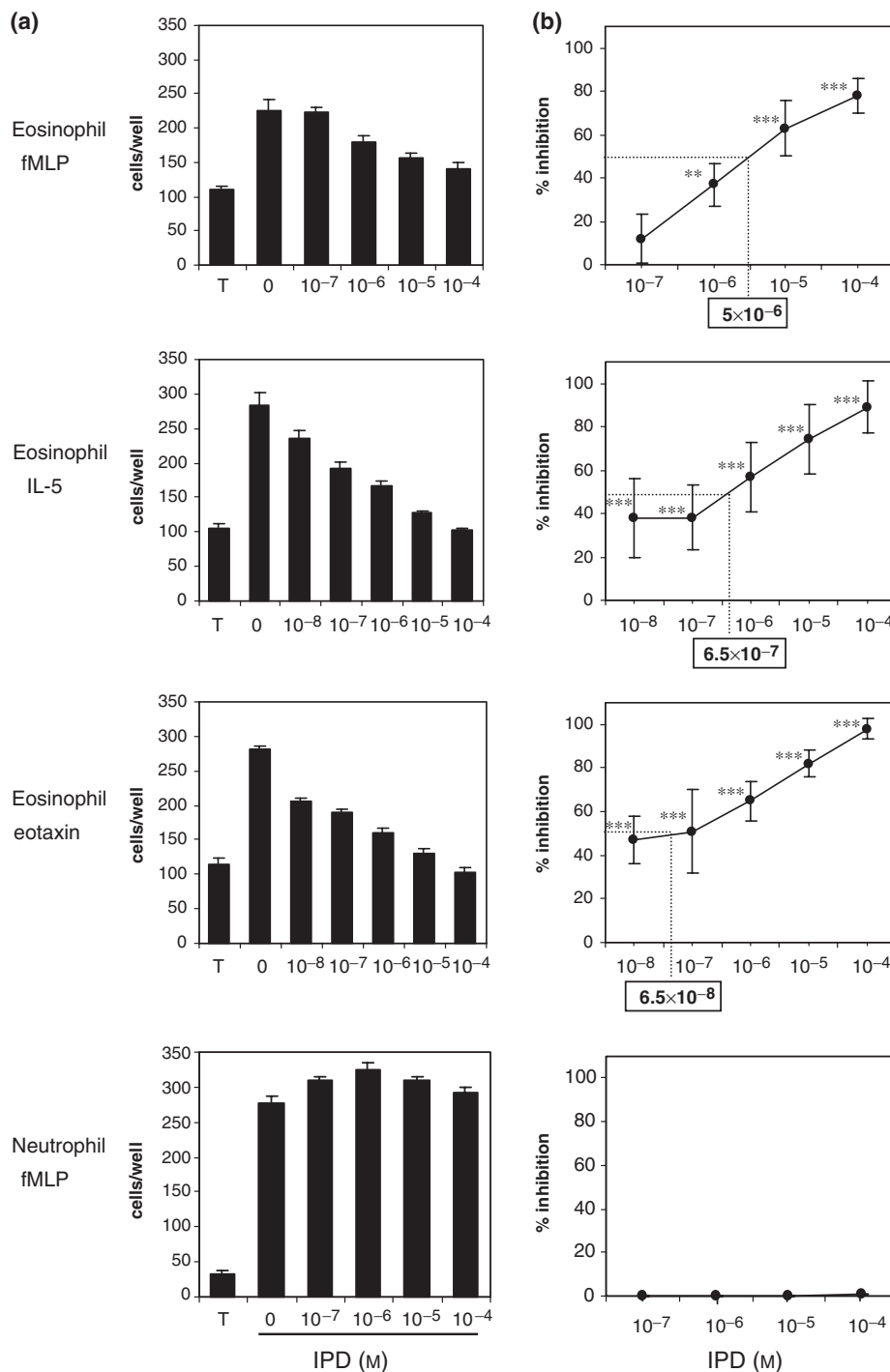


Fig. 1. Inhibitory effect of IPD-1151T on the chemotaxis of human eosinophils to formal-methionine-leucine-phenylalanine (fMLP), IL-5 and eotaxin. Human eosinophils were incubated in a Boyden chamber with increasing concentrations of IPD-1151T in the upper compartment and the stimulus (RPMI medium or chemoattractants) was placed in the lower compartment. Migration was carried out for 90 min (eosinophils) or 60 min (neutrophils). (a) Results from one representative experiment are expressed as absolute values of the number of cells that have migrated. (b) Data are expressed as percent inhibition of the chemotactic response in the presence of the drug by comparison to the positive control (chemoattractant alone). Results are presented as mean $\pm$ SEM (eosinophil $n = 4 - 6$; neutrophil $n = 3$). The IC₅₀ values are indicated in the box. Statistical differences are analysed by Wilcoxon-signed rank test. ** $P < 0.01$ and *** $P < 0.001$.

Eosinophil activated by sIgA immune complexes released IL-10, which was inhibited by IPD: 68% maximal inhibition and IC₅₀ = 10^{-5} M (Fig. 6). In addition, 60% inhibition of IL-6 release was already reached at 10^{-7} M (87% maximal inhibition), when IL-6 was produced after sIgA activation (Fig. 6).

Discussion

The presence of eosinophils or of their mediators often correlates with pathological manifestations [19–21]. Inhibiting the inflammatory influx of eosinophils and/or the release of toxic mediators would allow significant reduction of allergic

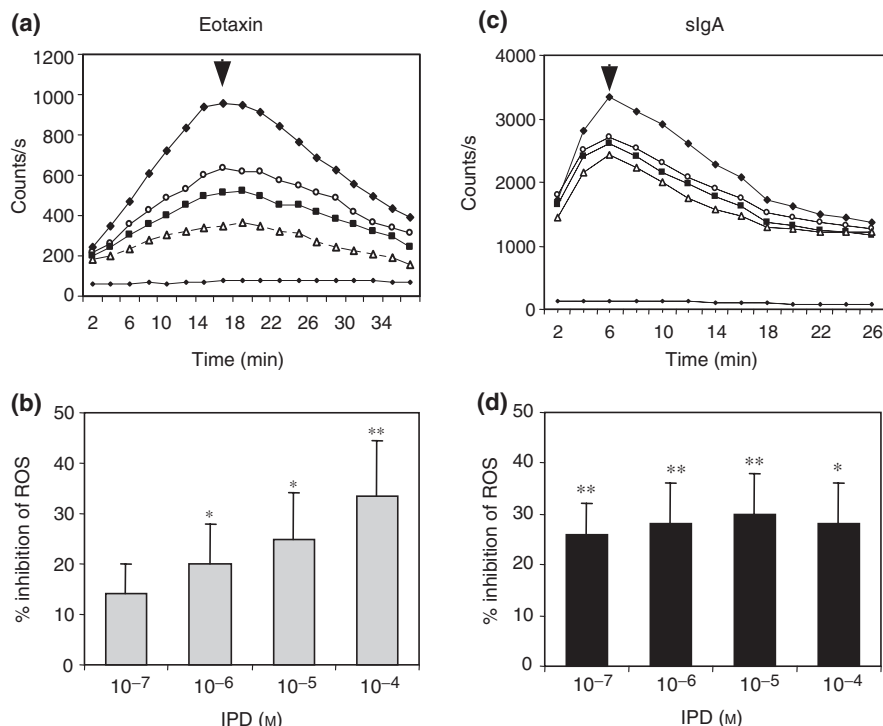


Fig. 2. Effect of IPD on the eosinophil oxidative metabolism induced by eotaxin (a, b) and secretory IgA (c, d). The production of reactive oxygen species (ROS) was measured in the supernatants by a luminol-dependent chemiluminescence assay. (a, c) Results from one representative experiment are expressed as absolute values of chemiluminescence (counts/s). $\blacklozenge$, 0; $\circ$, 10^{-6} M; $\blacksquare$, 10^{-5} M; $\triangle$, 10^{-4} M IPD-1151T and $\bullet$, phosphate-buffered saline. Arrows indicate the time point for maximal ROS production. (b, d) Data are expressed as percent inhibition, at the maximal ROS production, of the chemiluminescence response in the presence of the drug by comparison to the positive control (stimulus alone). Results are presented as mean $\pm$ SEM ($n = 9$). Statistical differences are analysed by Wilcoxon signed rank test. * $P < 0.05$ and ** $P < 0.01$.

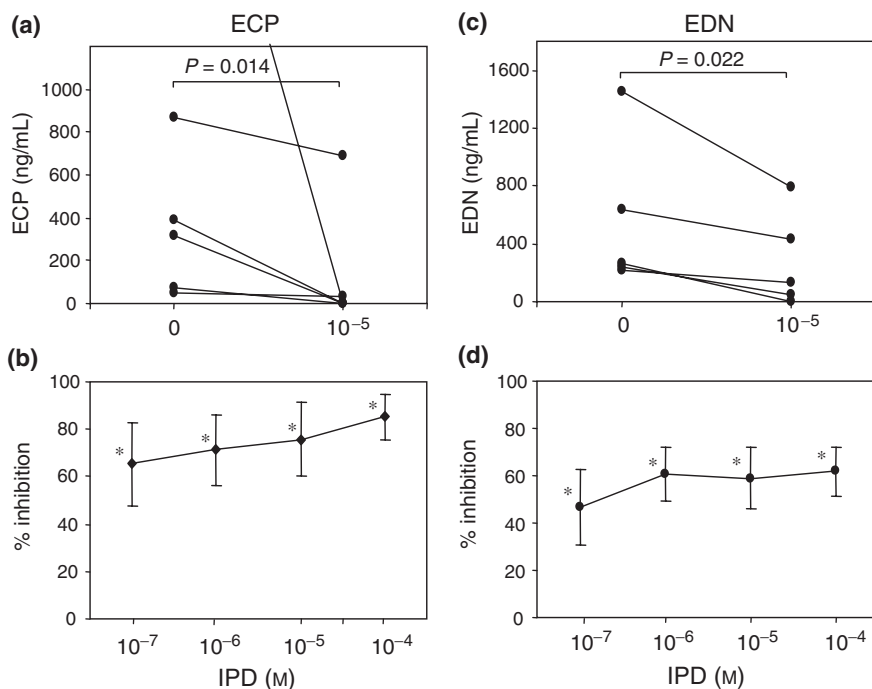


Fig. 3. Effect of IPD on secretory IgA-induced eosinophil cationic protein (ECP) (a, b) and eosinophil derived neuroxin (EDN) (c, d) release by eosinophils. (a, c) Results are expressed as absolute values of release, from which the spontaneous values have been subtracted for each patient. (b, d) Data are expressed as percent inhibition of release in the presence of the drug by comparison to the positive control (stimulus alone). ECP and EDN were measured in the supernatants by radioimmunoassay. Results are presented as mean $\pm$ SEM ($n = 5-6$). Statistical differences are analysed by Wilcoxon-signed rank test. * $P < 0.05$.

inflammation. In the present study, we investigated the effects of suplatast tosilate (IPD-1151T), an anti-allergic drug, used for the treatment of allergic diseases in Japan, on eosinophil chemotaxis and on various parameters of eosinophil activation, such as ROS production, cationic protein and cytokine release. The migration of eosinophils induced by three chemoattractants, namely fMLP, IL-5 and eotaxin, was strongly and specifically inhibited by IPD-1151T. Furthermore, a gradient of sensitivity to IPD was observed as the

IC₅₀ values were decreasing from fMLP to IL-5 and to eotaxin. Until now, only one study has reported on a direct inhibitory effect of IPD-1151T on eosinophil migration to platelet activating factor and the inhibition levels obtained were comparable to our results for chemotaxis to fMLP (IC₅₀ $\sim 10^{-5}$ M) [22]. These results indicate that, although IPD-1151T shows inhibitory property to eosinophil migration, sensitivity to IPD-1151T seems to depend on the chemoattractant pathway and, particularly, eosinophil

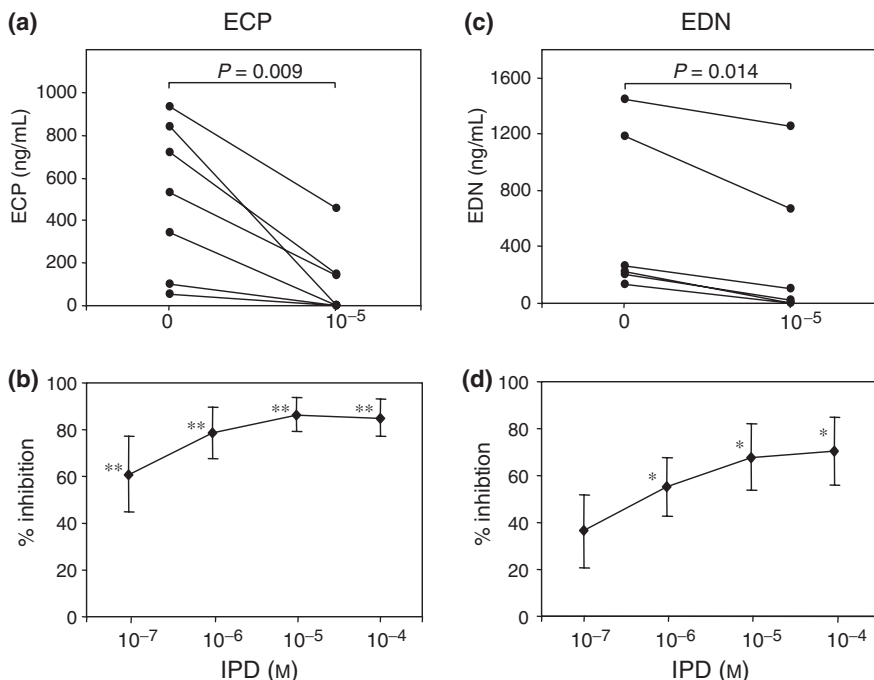


Fig. 4. Effect of IPD on anti-CD28-induced eosinophil cationic protein (ECP) (a, b) and eosinophil derived neuroxin (EDN) (c, d) release by eosinophils. (a, c) Results are expressed as absolute values of release, from which the spontaneous values have been subtracted for each patient. (b, d) Data are expressed as percent inhibition of release in the presence of the drug by comparison to the positive control (stimulus alone). ECP and EDN were measured in the supernatants by RIA. Results are presented as mean $\pm$ SEM ($n = 6-7$). Statistical differences are analysed by Wilcoxon-signed rank test. * $P < 0.05$ and ** $P < 0.01$.

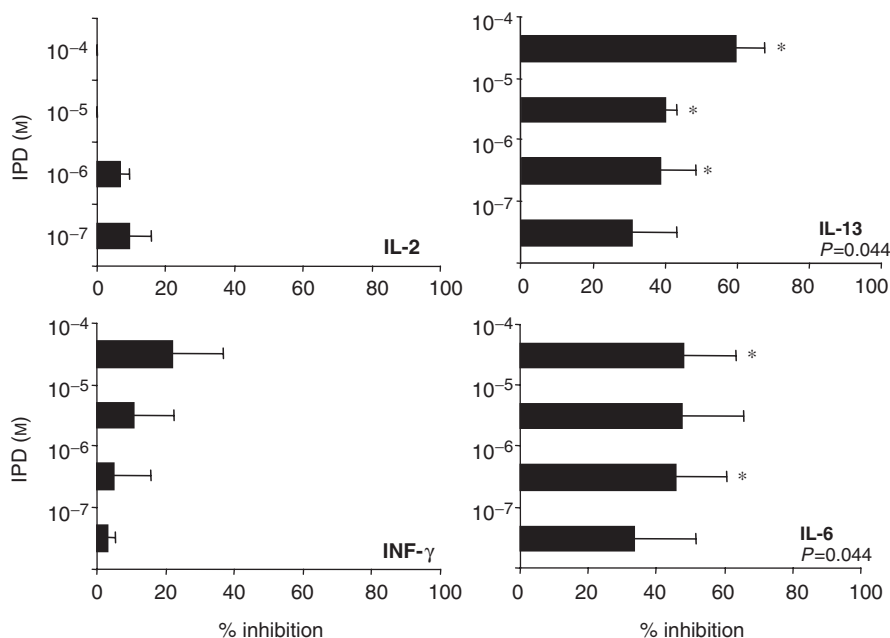


Fig. 5. Effect of IPD on the release of cytokines upon CD28 ligation. Eosinophils were incubated in anti-CD28- or isotype control mAb-coated plates for 18 h. Cytokines were measured in the supernatants by specific ELISA. The magnitude of cytokine production were [32–696 pg/mL] for IL-2, [20–160 pg/mL] for IFN- γ , [39–73 pg/mL] for IL-13 and [1–8.2 ng/mL] for IL-6. Data are expressed as percent inhibition of release in the presence of the drug by comparison to the positive control (stimulus alone). Results are presented as mean $\pm$ SEM ($n = 4-5$). Statistical differences between groups are analysed by Wilcoxon-signed rank test. * $P < 0.05$. Significance of the global effect of IPD-1151T as compared to the positive control is determined by Mann-Whitney U -test, for which the P -value is indicated.

migration towards eotaxin appears to be highly susceptible to IPD-1151T effect. IL-5 has been identified as a major regulator of eosinophil function (differentiation, migration, activation and survival). However, the failure of anti-IL-5 treatments to completely abolished tissue eosinophilia has led to the conclusion that IL-5 was only minimally implicated in eosinophil migration. Indeed, several studies have shown that eotaxin was the key factor involved in tissue recruitment of eosinophils [23–27]. Therefore, by inhibiting eosinophil migration, IPD-1151T could effectively impair the inflammatory influx of eosinophils directed by IL-5 and more particularly by eotaxin.

Oxidative stress occurs in many allergic and immunologic disorders. Number of studies have shown an increased production of ROS in asthma, AR and AD, which contribute, in part, to tissue injury at sites of inflammation [28]. Respiratory burst of eosinophils activated by eotaxin has been demonstrated, leading in ROS production [5, 7]. sIgA, the main antibody isotype secreted by the mucosa, also activates eosinophils, resulting in the release of various mediators, such as cationic proteins [3], ROS [5] and cytokines [4]. From our results, IPD-1151T was able to partially inhibit ROS production from eosinophils activated by eotaxin and sIgA. The absence of a dose-dependent effect

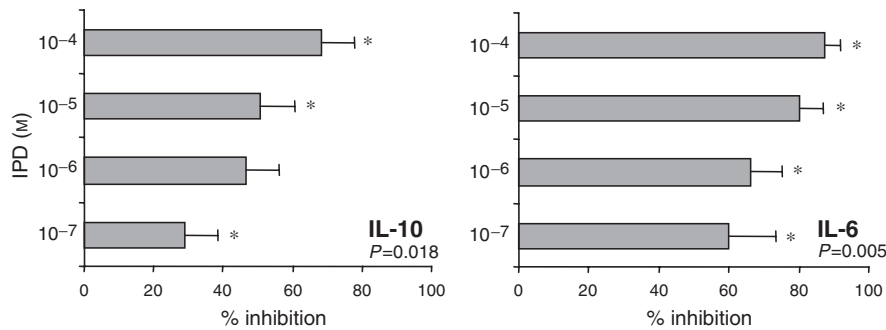


Fig. 6. Effect of IPD on cytokine released following secretory IgA activation. Eosinophils were activated by incubation with secretory IgA with or without IPD-1151T, followed by anti-IgA mAb for 18 h. Cytokines were measured in the supernatants by specific ELISA. The magnitude of cytokine production were [26–52 pg/mL] for IL-10 and [1–25.9 ng/mL] for IL-6. Data are expressed as percent inhibition of release in the presence of the drug by comparison to the positive control (stimulus alone). Results are presented as mean $\pm$ SEM ($n = 5$). Statistical differences between groups are analysed by Wilcoxon-signed rank test. * $P < 0.05$. Significance of the global effect of IPD-1151T as compared to the positive control is determined by Mann–Whitney U -test, for which the P -value is indicated.

of IPD-1151T on sIgA activation, as compared to eotaxin activation, is either due to a higher sensitivity of this activation pathway to the drug and therefore the maximum inhibition level ($\sim 30\%$) is already obtained at low concentration, or to the fact that ROS production induced by sIgA is too strong (3.5-fold higher than for eotaxin) to be efficiently inhibited by this drug. These results bring great interest as, Sanders et al. have shown that, in allergic subjects and during antigen challenge, the major source of ROS in bronchoalveolar lavage (BAL) fluid was eosinophils and not mononuclear cells [29]. Therefore, by its capacity to partially impair oxidative metabolism of activated eosinophils, IPD-1151T may protect tissue damage at the sites of inflammation.

Besides ROS, toxic cationic proteins are also responsible for tissue injury at inflamed sites. Among the factors involved in this process, sIgA immune complexes have been shown to be involved in eosinophil degranulation [3]. In previous studies, we demonstrated that the co-stimulatory molecule CD28 was expressed on eosinophils, and that CD28 activation resulted in cytokine release by these cells [4, 6]. We now showed that CD28 ligation could induce the release of cytolytic granule contents (ECP and EDN) from eosinophils, comparatively to sIgA activation. Interestingly, eosinophil degranulation induced by anti-CD28 or sIgA activation was highly sensitive to IPD-1151T effect, indicating that this drug could very efficiently impair the damage of the respiratory epithelium. Until now, two studies have mentioned an effect of IPD-1151T on mediator release, one in mast cells and the other in purified basophils, where IPD inhibited antigen-induced histamine release [14, 30].

Eosinophils have the capacity to produce rapidly various cytokines, which may, in the near proximity, modulate the immune response [31]. We have previously shown that anti-CD28 activation resulted in the release of type-1 cytokines (IL-2, IFN- γ) and the type-2 cytokine IL-13, whereas activation by sIgA immune complexes led to IL-10 release [4, 6]. Following anti-CD28 activation of eosinophils, release of IL-13 was inhibited by IPD-1151T, whereas the production of IL-2 and IFN- γ was unaffected. Furthermore, IPD-1151T also inhibited IL-10 release induced by sIgA stimulation. These results are in agreement with number of studies, both in human and mouse, which have demonstrated a specific inhibitory effect of suplatast tosylate on type-2 cytokines (IL-4

and IL-5) production by Th2 clones, whereas type-1 cytokines (IL-2 and IFN- γ) production by Th1 clones was not affected [12, 13, 32]. Moreover, Shichijo et al. [30] have shown that IL-13 production by human basophils stimulated by mite antigen is also inhibited by IPD-1151T. In addition, we demonstrated here that eosinophils produced the pro-inflammatory cytokine IL-6 in response to anti-CD28 and sIgA activation, which was inhibited by IPD-1151T. Interestingly, the sensitivity to the drug was totally dependent on the activation pathway, as IL-6 produced by sIgA activation was more strongly inhibited than IL-6 produced after anti-CD28 activation. An inhibitory effect of IPD-1151T on the production of IL-6 by phytohaemagglutinin-stimulated peripheral blood mononuclear cell of normal donors has also been reported [11]. The fact that suplatast tosylate has the ability to suppress the production of immunoregulatory cytokines such as IL-13, IL-10 and IL-6 by activated eosinophils might be of importance. Effectively, cytokines-derived eosinophils could locally influence the function of other cells, such as Th cells and endothelial cells, resulting in some immunomodulatory reactions, which could be impaired by IPD-1151T.

Signalling through IL5R (β c) or CCR3, the G protein-coupled seven transmembrane domain receptor for eotaxin, leads to extracellular-regulated kinase (ERK)/mitogen-activated protein (MAP) kinase activation pathway in many cell types. In eosinophils, number of studies have demonstrated the role of MAP kinases, including the ERK 1 and 2 and the p38 MAP kinase, in eosinophil functions. Indeed, by the use of specific inhibitors of these kinases, eosinophil chemotaxis, degranulation and cytokine/chemokine release in response to IL-5 or eotaxin were inhibited, indicating the major role play by the MAP kinase cascade in various eosinophil function [33–35]. More recently, the implication, in eosinophil chemotaxis and ROS production, of the G protein Rho and its substrate ROCK, as well as the myosin light-chain kinase (MLCK), has been reported [36, 37]. Altogether, eosinophil chemotaxis and activation seem to be regulated by multiple signalling pathways that involve at least ROCK, ERK and p38 MAP kinase. Therefore, it is tempting to speculate that IPD-1151T could act by blocking the activity of such kinases. Supporting this hypothesis is the only work of Saito et al. [38] whose results showed that the anti-allergic drug emedastine was inhibiting CC-chemokine-elicited eosinophil migration

by decreasing the activities of tyrosine kinase and/or protein kinases A and C [38].

Suplatast tosilate has proved his efficacy in *in vivo* study, both in asthmatic patients and in animal models of asthma [10, 17, 39]. Most of them have reported a decreased infiltration of eosinophils in BAL and submucosa, as well as an improved bronchial hyper-responsiveness. Moreover, studies in asthmatic patients have demonstrated significant decreased concentrations of serum ECP and ECP level in sputum after suplatast tosilate administration [18, 40, 41]. However, all these *in vivo* studies could not argue on a direct or indirect effect of IPD-1151T on eosinophils. From our results, it becomes clear that IPD-1151T can act directly on eosinophils, by first inhibiting their chemotactic response towards various chemoattractants. Secondly, at sites of inflammation, IPD-1151T significantly decreases eosinophil mediator release implicated in tissue damage. Thirdly, the production of cytokines by eosinophils, susceptible to locally modulate the immune response, is also impaired by IPD-1151T. Altogether, by its direct inhibitory effect on eosinophils, suplatast tosilate exerts important anti-inflammatory property in allergic diseases.

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