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Original article

Inhibitory effects of ketotifen on eotaxin-dependent activation of eosinophils: consequences for allergic eye diseases

Background: The aim of this study was to investigate the effects of ketotifen on different parameters of human eosinophil functions, namely chemotaxis, oxidative metabolism and mediator release, induced after 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 potent chemoattractants: formyl-methionine-leucine-phenylalanine (fMLP), interleukin (IL)-5 and eotaxin. Oxidative metabolism was determined by a luminol-dependent chemiluminescence assay after activation with eotaxin or secretory immunoglobulin A (sIgA). The release of eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN) was measured by radioimmunoassay after activation with sIgA.
Results: At pharmacologically active concentrations and in a dose-dependent manner, ketotifen significantly inhibited the chemotaxis of eosinophils to fMLP, IL-5 and eotaxin. The production of reactive oxygen species induced by eotaxin and sIgA was decreased by ketotifen, showing a more pronounced effect when cells were activated by eotaxin. Activation by sIgA resulted in ECP and EDN release, which was partially inhibited by ketotifen.
Conclusions: Through inhibition of chemotaxis, ketotifen might limit the number of eosinophils at the inflammation site during allergic reaction. Furthermore, inhibition by ketotifen of main inflammatory mediators release suggests a potential role of the drug in limiting the pathological potential of eosinophils.

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Key words: chemiluminescence; chemotaxis; conjunctivitis; degranulation; eosinophils; eotaxin; interleukin-5; ketotifen.

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Eosinophils are major effector cells in various allergic diseases and asthma. The eye, in particular, is a common site of allergic inflammation. In allergic conjunctivitis (AC), a first immediate reaction initiated by mast cells take place, followed by a late phase reaction characterized by a massive influx of eosinophils, as well as neutrophils (1). In normal conjunctiva, eosinophils are not normally found in the epithelium, but their numbers are increased in atopic keratoconjunctivitis (AKC) and vernal keratoconjunctivitis (VKC), both in the conjunctival epithelium, subepithelium and tears (2). Eosinophils contain toxic cationic proteins such as eosinophil peroxidase (EPO), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and major basic protein (MBP), which, when secreted, can lead to serious corneal epithelium damage. Deposition of MBP has been observed in AC (3), which was shown to be responsible for the inhibition of corneal epithelial wound healing (4).

Abbreviations: AKC, atopic keratoconjunctivitis; VKC, vernal keratoconjunctivitis; ROS, reactive oxygen species; sIgA, secretory IgA; LT, leukotrienes; AC, allergic conjunctivitis; LDH, lactate dehydrogenase.

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 (5). Among these, formyl-methionine-leucine-phenylalanine (fMLP), interleukin (IL)-5 and eotaxin have been shown to exert potent chemotactic activity on eosinophils *in vitro* (6). Increased levels of IL-5 have been found in tears of VKC patients (7), suggesting its potential role in eosinophil recruitment. During this migration process to inflammatory sites, eosinophils become activated and may cause tissue damage by releasing not only eosinophil-specific granule proteins, but also reactive oxygen species (ROS). Secretory immunoglobulin A (sIgA) is the predominant isotype produced by lymphocytes at all secretory effector sites, and the most abundant immunoglobulin class in external secretions, in particular in lacrimal glands and tears (8). Previous studies have shown that one of the more potent stimulus of eosinophil activation is immobilized or cross-linked sIgA, able to induce the release of eosinophil cationic proteins and cytokines (9, 10). The role of eotaxin as major eosinophil activator

has also been described, in inducing the release of ROS and calcium mobilization (11).

Drugs employed in the treatment of AC are generally classified as anti-inflammatory compounds, vasoconstrictors, antihistamines and mast cell stabilizers. Ketotifen fumarate shares properties with the two latter groups of compounds (12). Clinical studies have shown ketotifen to be effective in the treatment of asthma (13, 14) and AC (15, 16). It exerts anti-anaphylactic and antihistamine activities, mainly through inhibition of the release of chemical mediators such as histamine and leukotrienes (LT) from sensitized mast cells (17, 18). It also inhibits platelet activating factor (PAF)-induced acute bronchoconstrictor response, airway hyperresponsiveness and accumulation of eosinophils in the airways in guinea-pigs (19), as well as allergen-induced degranulation of eosinophils in allergic patients (20).

Altogether, these observations suggest a pathophysiologic role of eosinophils in allergic eye inflammation. Therefore, drugs modulating various aspects of eosinophil function could play a primary role in the treatment of allergic eye diseases. We investigated the possibility that ketotifen could act directly on eosinophil chemotaxis induced by potent chemoattractants, namely fMLP, IL-5 and eotaxin, and also on eosinophil activation induced by eotaxin and sIgA, as measured by ROS, ECP and EDN release.

Materials and methods

Reagents

Ketotifen fumarate was provided by Novartis Ophthalmics AG (Basel, Switzerland). Anti-human CD16- and CD3-conjugated magnetic beads and the magnetic cell separation (MACS) system were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Percoll was obtained from Pharmacia (Uppsala, Sweden). RPMI 1640 medium, glutamine, penicillin, streptomycin and foetal calf serum (FCS) were from Gibco BRL Life Technologies (Paisley, UK). Human sIgA, fMLP and luminol were purchased from Sigma (St Louis, MO). Anti-human IgA was from Immunotech (Coulter Corp., Miami, FL). Recombinant human eotaxin and IL-5 were obtained from Peprotech (Rocky Hill, NJ). Annexin-V fluoresceine isothiocyanate (FITC) was purchased from Pharmingen (San Diego, CA).

Patients

A total of 18 different patients with hypereosinophilia associated with skin diseases, allergy, hypereosinophilic syndromes (HES) and hematologic disorders, and four normal donors was selected for this study, after informed consent.

Eosinophil and neutrophil purification

Eosinophils and neutrophils were isolated by immunomagnetic separation technique using the MACS system, as previously described (10, 21). 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 (negative selection) and neutrophils were recovered from the column after detachment of the cells out of the magnetic field (positive selection). After isolation, cell preparations were cytocentrifuged and cytospins were stained with May–Grünwald–Giemsa (RAL 555; Bordeaux Technopolis, Martillac, France). The purity of eosinophil and neutrophil preparations was normally above 95%.

Chemotaxis assay

The measurement of leukocyte chemotaxis was assessed by a modification of the Boyden micropore filter technique, as previously described (6). Briefly, the assay is carried out in a 48-well microchemotaxis assembly (Neuroprobe; Cabin John, MD). Cell suspensions are placed with or without ketotifen (at different concentrations) in the upper chamber separated from the leukoattractant solution by a 5-μm (eosinophils) or 3-μm (neutrophils) pore size polycarbonate membrane filter (Nucleopore Co., Pleasanton CA). The stimulus compartment received either control buffer for spontaneous migration measurement, or the attractant reagent at optimal concentrations determined by preliminary dose-dependent experiments. Migration is carried out at 37°C in humidified air with 5% CO_2 for 2 h (eosinophils) or 30 min (neutrophils). The filters are then fixed and stained with May–Grünwald–Giemsa. The numbers of cells that had migrated were enumerated microscopically in four random high-power fields in quadruplicated wells.

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

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

Chemiluminescence assay

Reactive oxygen species from eosinophils and neutrophils were examined with luminol-dependent chemiluminescence. For eotaxin activation, purified eosinophils (5×10^5) were preincubated with or without ketotifen in the presence of IL-5 for 1 h at 37°C, before addition of eotaxin (100 ng/ml) and luminol. For sIgA activation, cells were incubated with 15 μg/ml sIgA with or without ketotifen. After 1 h at 37°C, anti-IgA (20 μg/ml) and luminol (8.3 μg/ml) were added to the cells. Neutrophils were incubated with or without ketotifen for 1 h at 37°C, before addition of fMLP (10^{-6} M) and luminol.

Chemiluminescence was immediately measured with a luminometer (Victor^{2TM} Wallac; Perkin–Elmer, Shelton, CT). Kinetic was performed at 37°C over a 40-min time period and for each timepoint, chemiluminescence was counted for 5 s. Results were expressed in counts per second (CPS).

The percentage of inhibition obtained with ketotifen was calculated by comparison with the positive control (ROS production of activated cells in the absence of ketotifen), according to the formula:

$$\%\ \text{Inhibition} = 100 - \frac{\text{ROS with ketotifen} - \text{spontaneous ROS}}{\text{ROS without ketotifen} - \text{spontaneous ROS}} \times 100$$

Eosinophil mediator release

Highly purified eosinophils (2×10^6/ml) were incubated with sIgA and anti-IgA monoclonal antibody (mAb) at a respective final concentration of 15 and 20 μg/ml in RPMI medium supplemented with 10% heat inactivated FCS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. After 18 h at 37°C, the cells were centrifuged, the supernatants were collected and stored at −20°C until the measurements of granule proteins could be performed. The determination of ECP and EDN was carried out by radioimmunoassay (Pharmacia, Uppsala, Sweden) and results were expressed in ng/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.

Eosinophil viability

Lactate dehydrogenase assay. Eosinophil cytolysis was followed by the measurement of one cytoplasmic marker, lactate dehydrogenase (LDH), in the supernatants of eosinophils incubated for 90 min or 18 h with medium alone, ketotifen at the concentration used for chemotaxis or with Triton X-100 as positive control. The LDH was evaluated by a colorimetric assay (Boehringer Mannheim, Mannheim, Germany).

Apoptotic analysis. Annexin-V binding was chosen as a marker of cell apoptosis (22). Eosinophils were incubated with medium alone or ketotifen in the conditions used for chemotaxis (90 min) or mediator release (18 h). Cells were harvested and stained with FITC-labeled Annexin-V for 15 min. After washing, samples were analyzed by flow cytometry on a FACSCalibur™ using the CellQuest™ software (Becton Dickinson, Mountain View, CA). Ten thousand events were usually acquired per sample.

Statistical analysis

Statistical significance was determined using the Wilcoxon signed rank test for paired group and P values <0.05 were considered as significant.

Results

Inhibitory effect of ketotifen on human eosinophil chemotaxis

Highly purified eosinophils have been studied in chemotaxis experiments, either with control buffer (negative control), fMLP (10^{-6} M), IL-5 (10 ng/ml) or eotaxin (1 ng/ml). These optimal concentrations of each chemotactic agent have been defined after preliminary dose-dependent studies. The inhibitory effects of ketotifen have been evaluated for each donor and for the three chemotactic agents, in function of the doses of the drug ranging from 10^{-8} to 10^{-4} M. These concentrations of ketotifen are relevant with the concentrations reached *in vivo*, as an ophtalmic solution of 0.025% corresponds to ca. 8×10^{-4} M concentration. As shown in Fig. 1, a significant and dose-dependent inhibition by ketotifen of eosinophil chemotactic response to the three potent chemotactic factors was obtained, reaching more than 85% inhibition at the highest concentration (10^{-4} M). Fifty percent inhibition was usually obtained between 10^{-7} and 10^{-6} M. This effect of ketotifen was specific to eosinophils as no inhibition of the chemotactic response of neutrophils to fMLP was observed (Fig. 1). Interestingly, eosinophil chemotaxis to eotaxin appeared to be more susceptible to ketotifen effect, as a 37.5% inhibition was already obtained with ketotifen at 10^{-8} M (as compared with 20.3 and 16% for chemotaxis to fMLP and IL-5, respectively).

In order to be able to compare our *in vitro* results with the *in vivo* situation, where ketotifen is applied in the eyes before eosinophil infiltration, chemotaxis was performed without direct contact between the drug and the cells (ketotifen in the lower chamber). As shown in Fig. 2, ketotifen was still able to inhibit eosinophil migration, although higher concentrations were needed to reach the same effect. At the highest drug concentration (10^{-4} M), maximal inhibition was obtained in both chemotaxis protocols. Eosinophil chemotaxis to eotaxin was again more susceptible to ketotifen effect than chemotaxis to IL-5. No statistical differences in eosinophil migration to eotaxin were obtained, whether the drug was in direct contact with the cells or not. In the case of chemotaxis to IL-5, 50% inhibition was only reached at 10^{-5} M, as compared with 10^{-7} M when eosinophils were incubated with the drug. Statistical differences between the two protocols were obtained for all concentrations, except at the maximal concentration (10^{-4} M).

Effect of ketotifen on eosinophil oxidative metabolism

The effects of ketotifen have been evaluated on the oxidative metabolism of eosinophils induced either by eotaxin or by 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 ketotifen would have an effect on the total ROS production or on specific time-points (delayed production). Total ROS production was inhibited by ketotifen and, therefore, results were considered at the maximal ROS production: 3 and 10 min for eotaxin and sIgA activation, respectively (data not shown).

The results presented in Fig. 3 indicate that ketotifen was able to inhibit in a dose-dependent manner the production of ROS from human eosinophils activated by eotaxin, reaching 85% at the highest concentration of the drug (10^{-4} M). Activation with sIgA, which also resulted in ROS production, was only partially inhibited by ketotifen (Fig. 4). Inhibition was maximal at 10^{-5} M ketotifen, reaching 40%. As for chemotaxis, eotaxin activation was more susceptible to ketotifen effect. The same inhibition (39%) was obtained at 10^{-7} and 10^{-5} M ketotifen for eotaxin and sIgA activation, respectively. This effect of ketotifen on eosinophils was specific as no inhibition of ROS production was

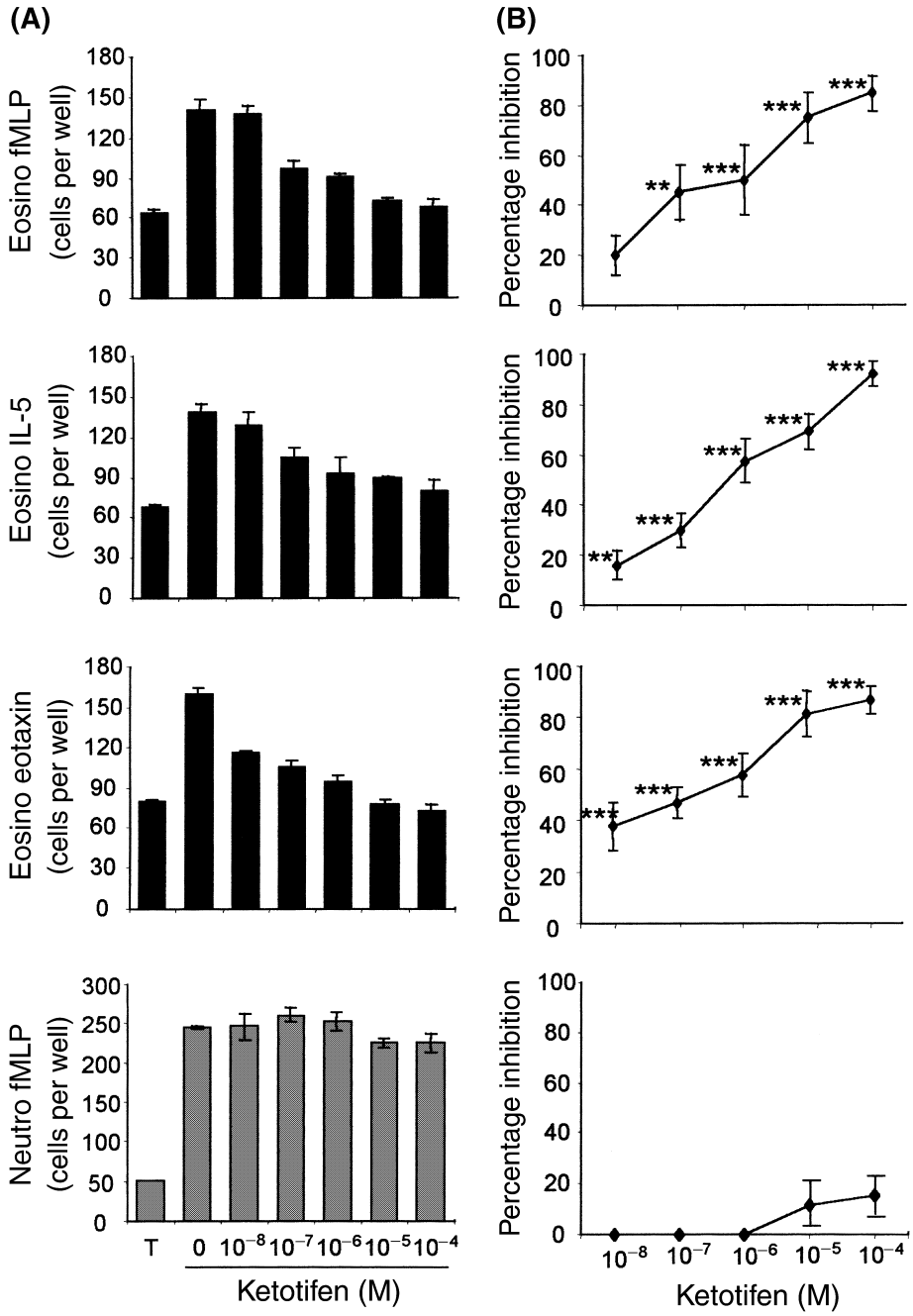

Figure 1. Inhibitory effect of ketotifen on the chemotaxis of human eosinophils to fMLP, IL-5, and eotaxin. (A) Results from one representative experiment are expressed as absolute values of the number of cells that have migrated. (B) Data are expressed as percent inhibition of the chemotactic response in the presence of the drug by comparison with the positive control (chemoattractant alone). Results are presented as mean ± SEM (eosinophil $n = 8$; neutrophil $n = 3$). Statistical differences are analyzed by Wilcoxon signed rank test. $^{**}P < 0.01$ and $^{***}P < 0.001$.

obtained following activation of neutrophils by fMLP (data not shown).

Effect of ketotifen on eosinophil mediator release

The effects of ketotifen were investigated on the release of ECP and EDN by eosinophils after activation with sIgA. Results presented in Fig. 5A showed that ketotifen at 10^{-6} M induced a decrease of ECP release (mean inhibition = 53%). However, this inhibitory effect was dependant on the dose of the drug, giving a biphasic curve; at low concentration, an increase of the inhibition level, followed by a decreased inhibitory effect at high dose (Fig. 5B). Such a dual effect of ketotifen has been already

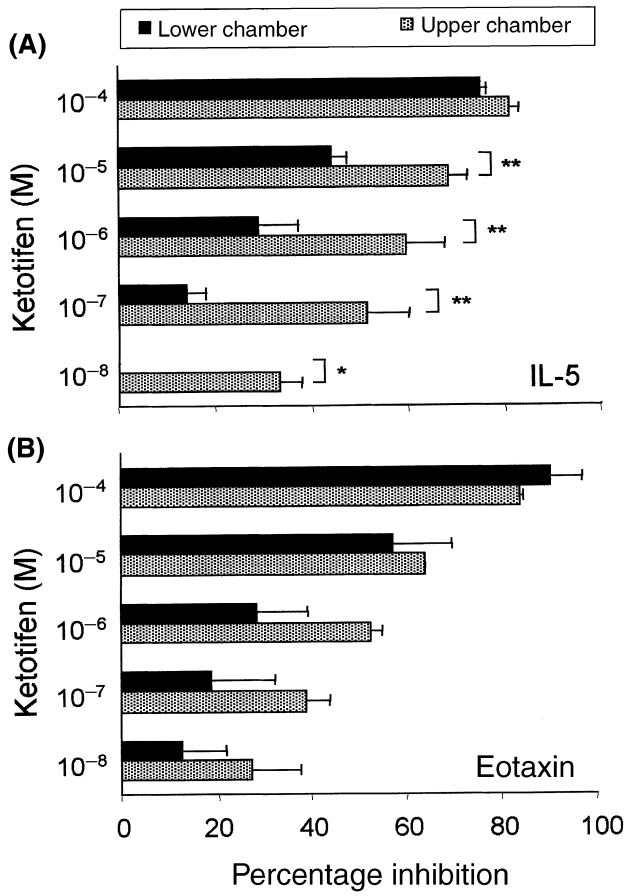

Figure 2. Effect of ketotifen on the chemotactic response of human eosinophils with or without direct contact with the cells. Chemotaxis to IL-5 (A) or eotaxin (B) was performed by incubating the drug either with the cells (upper chamber) or without the cells (lower chamber). Results are expressed as percent inhibition of the chemotactic response (mean ± SEM, $n = 2$), as in Fig. 1. Statistical differences are analyzed by Wilcoxon signed rank test. $*P < 0.05$ and $**P < 0.01$.

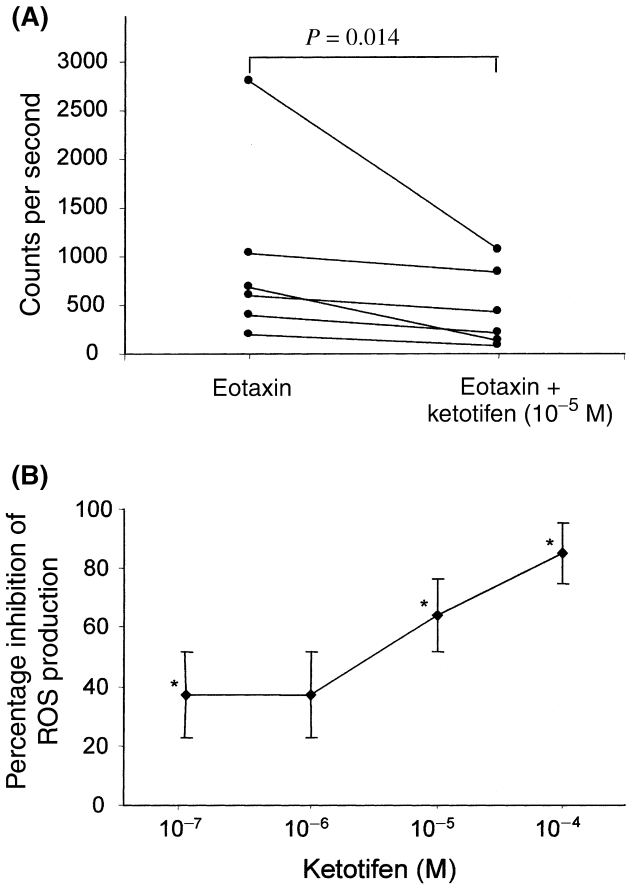

Figure 3. Effect of ketotifen on the eosinophil oxidative metabolism induced by eotaxin. The production of ROS was measured in the supernatants by a luminol-dependent chemiluminescence assay. (A) Results are expressed as absolute values of chemiluminescence (counts per second). (B) Data are expressed as percent inhibition of the chemiluminescence response in the presence of the drug by comparison with the positive control (stimulus alone). Results are presented as mean ± SEM ($n = 6$). Statistical differences are analyzed by Wilcoxon signed rank test. $*P < 0.05$.

demonstrated in the case of the release of histamine by activated rat and human conjunctival mast cells (23, 24), however at higher concentrations than those encountered in the 0.025% ophthalmic formulation (Ch. Schoch, personal communication). The EDN release was induced after IgA activation and was also inhibited by ketotifen (mean inhibition = 46% at 10^{-6} M) (Fig. 6A). However, no clear dose-dependent inhibition of EDN release was observed (range 35–46%) (Fig. 6B). These results suggest either that eosinophil degranulation is not a major target for ketotifen effect or that activation by sIgA is less sensitive to ketotifen (as demonstrated for chemotaxis and ROS production).

Eosinophil viability

In order to exclude the possibility that the inhibitory effect observed was beacuse of a toxic effect of ketotifen on eosinophils, we evaluated the viability of the cells after culture with ketotifen. Two parameters were analysed: the binding of Annexin-V as a measure of apoptosis and the LDH release as a marker of cytolysis. Cells incubated for 90 min with ketotifen did not show any sign of apoptosis (Table 1). During overnight culture, eosinophil apoptosis was induced, as 29% of the cells were Annexin-V positive. However, cell cytolysis was minimal, as only 10% LDH release was measured, indicating that the release of ECP and EDN were not the result of cell degradation. No change of eosinophil viability was detected in the presence of ketotifen, indicating that the drug was not toxic at the cell level.

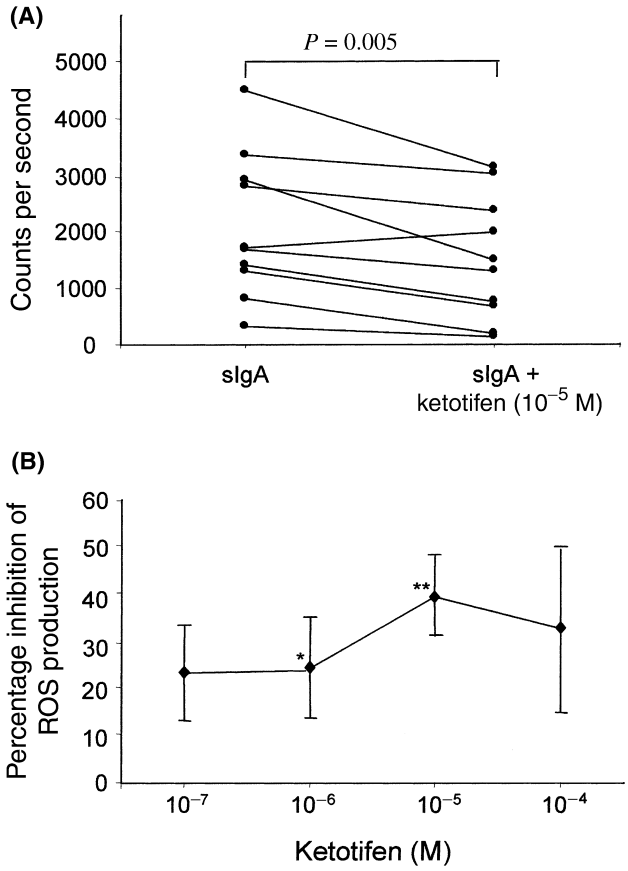

Figure 4. Effect of ketotifen on the eosinophil oxidative metabolism induced by sIgA. The production of ROS was measured in the supernatants by a luminol-dependent chemiluminescence assay. (A) Results are expressed as absolute values of chemiluminescence (counts per second). (B) Data are expressed as percent inhibition of the chemiluminescence response in the presence of the drug by comparison with the positive control (stimulus alone). Results are presented as mean ± SEM ($n = 5$–10). Statistical differences are analyzed by Wilcoxon signed rank test. *$P < 0.05$ and **$P < 0.01$.

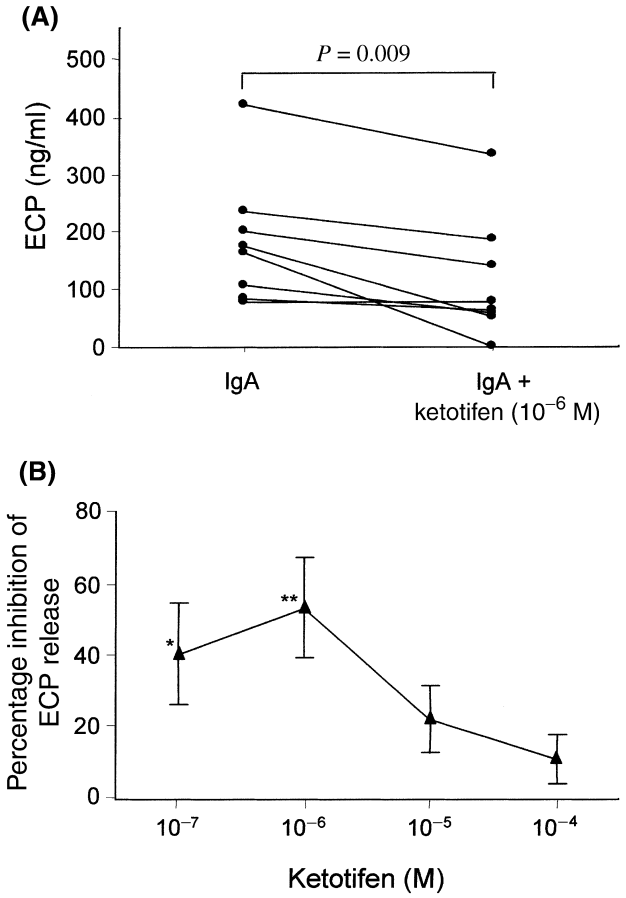

Figure 5. Effect of ketotifen on sIgA-induced ECP release by eosinophil. ECP was measured in the supernatants by RIA. (A) Results are expressed as absolute values of release, from which the spontaneous values have been subtracted for each patient. (B) Data are expressed as percent inhibition of ECP release in the presence of the drug by comparison with the positive control (stimulus alone). Results are presented as mean ± SEM ($n = 5$–8). Statistical differences are analyzed by Wilcoxon signed rank test. *$P < 0.05$ and **$P < 0.01$.

Discussion

The presence of eosinophils or of their mediators often correlates with pathologic manifestations (25–28). Inhibiting the inflammatory influx of eosinophils and/or the release of toxic mediators would allow significant treatment of allergic inflammation. Our previous *in vitro* studies have shown the usefulness of anti-allergic drugs by demonstrating that molecules, such as cetirizine or lodoxamide, could have a direct effect on eosinophil functions, allowing better understanding of the drug mechanism (6, 29). In this study, we investigated the effect of ketotifen, a drug with anti-anaphylactic and antihistaminic properties already used in the treatment of asthma and AC, on eosinophil chemoattraction and on the release of inflammatory mediators, such as ROS, ECP, and EDN by activated eosinophils. Our results clearly show that ketotifen is a potent inhibitor of eosinophil chemotactic response to fMLP, IL-5 and eotaxin and this effect is specific for eosinophils as no inhibition of the chemotactic response of neutrophils to fMLP is evidenced. Such an effect on eosinophil migration has been suggested by Nabe et al (30). They demonstrated that ketotifen at 10 μM significantly inhibited PAF-induced eosinophil chemotaxis (in a range of 28–50% inhibition). However, in our experiments, the effect of ketotifen was much more pronounced, as statistically significant inhibitions were already obtained at lower concentrations. In addition, our studies have been extended to eotaxin, a potent chemokine involved in eosinophil tissue migration. Indeed, we could show that eotaxin-induced eosinophil migration was highly susceptible to ketotifen effect. Eosinophils are recruited to sites of inflammation by locally released chemotactic agents. The presence of high concentrations of eotaxin in tears of

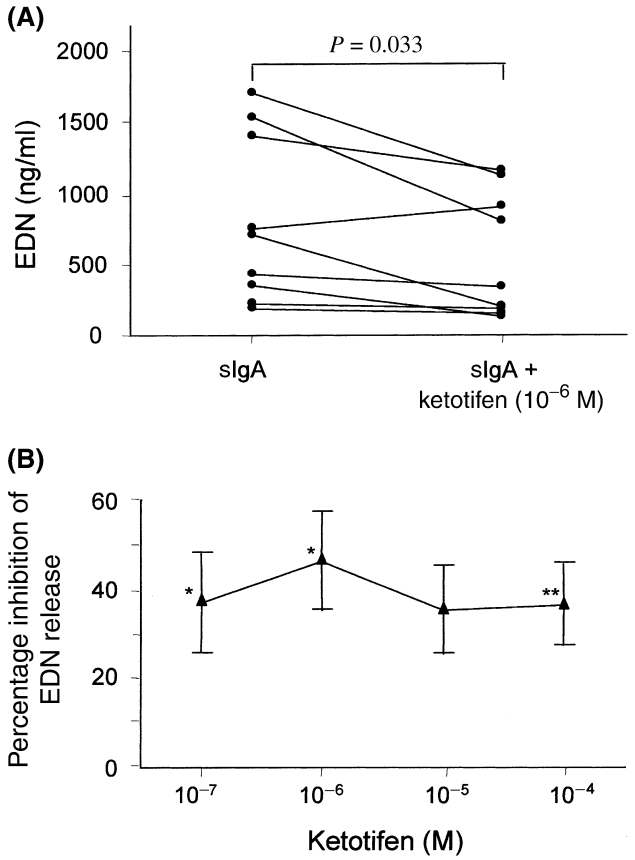

Figure 6. Effect of ketotifen on sIgA-induced EDN release by eosinophil. EDN was measured in the supernatants by RIA. (A) Results are expressed as absolute values of release, from which the spontaneous values have been subtracted for each patient. (B) Data are expressed as percent inhibition of EDN release in the presence of the drug by comparison with the positive control (stimulus alone). Results are presented as mean ± SEM ($n = 8$–9). Statistical differences are analyzed by Wilcoxon signed rank test. $^{*}P < 0.05$ and $^{**}P < 0.01$.

patients with AKC and severe corneal damage has been reported, that correlated with the number of infiltrated eosinophils (31). Another group has detected higher tear IL-5 levels in patients with diseases associated with proliferative lesions (VKC and AKC) than those in AC and normal controls (32). These observations suggest that eotaxin and IL-5 in tears may play an important role in eosinophil recruitment during allergic ocular diseases. The present chemotaxis results, together with these *in vivo* observations, indicate that ketotifen could effectively impair the inflammatory influx of eosinophils directed by IL-5 and more particularly by eotaxin. *In vivo* study on patients with atopic asthma receiving ketotifen has shown a reduced infiltration of activated eosinophils and T cells in the bronchial mucosa (33), pointing out the efficacy of ketotifen in the treatment of asthma.

Besides toxic cationic proteins, ROS released by activated eosinophils are believed to be responsible for tissue injury at the inflammation sites. Activation by eotaxin of the respiratory burst of eosinophils has been demonstrated, resulting in the release of ROS (11). Previous studies have also shown that sIgA, the main Ab isotype secreted in the mucosa, activates eosinophils by inducing the release of various mediators (EPO, ECP, EDN, and ROS) (9, 34) and cytokines (IL-4, IL-5, and IL-10) (35–37). In the present work, we show that ketotifen is able to inhibit ROS production by eotaxin and sIgA-activated eosinophils. More interesting is the observation that ROS production induced by eotaxin is almost totally inhibited, whereas ROS production induced by sIgA activation is only partially inhibited by ketotifen. As for chemotaxis, no inhibition of ROS production was observed when neutrophils were stimulated by fMLP in the presence of ketotifen, suggesting a specific effect of this drug on eosinophils. Such an effect on the release of ROS by eosinophils activated with either PAF, IL-5 or eotaxin has also been shown for some anti-asthmatic drugs (38, 39). However, in his work, Ezeamuzie et al. failed to show any significant effect of ketotifen on eosinophils activated with IL-5 or PAF (38). These results indicate that the eotaxin activation pathway seems extremely sensitive to ketotifen effect. Furthermore ketotifen may protect tissue damage at inflamed sites, at least partially by inhibiting the release of ROS from eosinophils.

The deleterious potential of eosinophils in tissues is also the result of the release of cytolytic granule contents. Among the factors involved in this process, IgA, IgE and IgG immune complexes have been shown to be involved in eosinophil degranulation (9). From our results, ketotifen inhibited partially and significantly the release of ECP and EDN induced by sIgA activation. Interestingly, a previous study on eosinophils from a patient with milk allergy had already reported the inhibitory effect of ketotifen on eosinophil degranulation, although the results were only based on electron microscopic analysis (20). On the contrary, a more recent study on eosinophils isolated from healthy or mildly atopic donors failed to demonstrate any effect of ketotifen on EPO release induced by fMLP or C5a (38). One explanation to this discrepancy on the effect of ketotifen could be that the activation mechanism inducing eosinophil degranulation does not show the same sensitivity to the drug, although we have demonstrated that chemotaxis to fMLP is inhibited. The effects of ketotifen on mast cell activation, and specially histamine release, have been extensively studied. Comparison of the results among the different *in vitro* studies clearly showed an heterogeneity of ketotifen action depending on the species, the tissues and the activators used (18, 23, 24, 40–42). Although some studies showed low potency of ketotifen, most of them indicated that ketotifen inhibited mast cell degranulation and mediator release ($IC50 = 10^{-5}$–10^{-4} M). They represent higher concentrations, than those observed on eosinophils, giving IC_{50} values of 10^{-7}–10^{-6} M for chemotaxis, 5×10^{-6} M for eotaxin-induced ROS production and 8×10^{-7} M for IgA-induced ECP release.

Table 1. Absence of effect of ketotifen on eosinophils apoptosis or lysis

	90 min		18 h	
Ketotifen (M)	% Annexin-V*	% LDH†	% Annexin-V*	% LDH†
0	1.5 ± 0.1	3.8 ± 3.0	29.1 ± 4.6	9.9 ± 5.0
10^{-4}	1.2 ± 0.3	3.8 ± 2.6	24.4 ± 3.8	10.5 ± 3.1
10^{-5}	1.6 ± 0.3	4.8 ± 3.5	20.9 ± 3.7	9.4 ± 3.0
10^{-6}	1.2 ± 0.3	4.1 ± 2.1	23.3 ± 3.4	9.9 ± 2.5
10^{-7}	1.0 ± 0.1	3.8 ± 2.8	24.3 ± 1.9	10.7 ± 3.5
10^{-8}	1.5 ± 0.3	4.0 ± 3.0	19.5 ± 0.4	10.2 ± 4.2

Results are expressed as mean ± SEM of three different experiments.
* Percent of Annexin-V positive cells as a measure of apoptosis.
† Percent cell lysis as determined by LDH release.

A biphasic effect of ketotifen on histamine release by mast cells has been previously described by some groups (23, 24). Such an effect has been also obtained for ECP release by eosinophils in our experiments. The actions of ketotifen on the release of other eosinophil mediators, and particularly of (LT) have also been demonstrated. The generation of LTC_4, D_4 and E_4 after cell activation with anti-IgG or A23187 was in all cases inhibited by ketotifen, although this effect reached only 25% inhibition (30, 43). These observations suggest that the effect of ketotifen might not only be limited to eosinophil function, such as chemotaxis and degranulation, but also to different aspects of eosinophil activation. This finding is particularly important, as it has been shown that the expression of cell surface antigens, such as intercellular adhesion molecule 1 (ICAM-1), CD4, IL-2R and human leukocyte antigen (HLA-DR) are increased and vary in different ocular allergic disorders, indicating a greater eosinophil activation state (44). Moreover, bearing in mind the capacity of eosinophils to produce rapidly various cytokines in a local concentration that may modulate the immune response (45), it would be of interest to investigate whether ketotifen could also inhibit cytokine production by eosinophils. At the present time, only one study has reported an inhibitory effect of ketotifen on cytokine production. In this work, the Df-induced IL-5 production by peripheral blood mononuclear cells (PBMC) of *Dermatophagoides farinae* extract (Df)-sensitive donors was inhibited by 100 μM ketotifen, indicating that the anti-allergic effect of the drug might be broader than first evaluated (46).

Our results clearly show a significant inhibitory effect of ketotifen on eosinophil activated with eotaxin. This observation is particularly important, because it is well-recognized that eotaxin is one of the most potent activator of eosinophils and it does so through binding to its specific receptor CCR3 (11). Whether ketotifen can or cannot modulate CCR3 expression at the cell membrane of eosinophils has not been reported. However, Saito et al. had investigated the effect of the anti-allergic drug emedastine on chemokine-elicited eosinophil migration (47). In their work, they showed that emedastine, like ketotifen, inhibited eosinophil chemotaxis to eotaxin. Furthermore, this effect was not the result of an altered CCR3 expression, but rather of decreased activities of protein and /or tyrosine kinases. The importance of the eotaxin-CCR3 activation pathway in allergic eye diseases has been recently demonstrated by Fukagawa et al. (48). The authors first showed that eosinophils in tears from allergic patients express CCR3 on their surfaces. They went on to show that culture supernatants from corneal keratocytes and tears from allergic patients with corneal ulcer induced eosinophil migration. Finally, these eosinophil chemotactic responses were almost totally inhibited by anti-CCR3 Ab, indicating that eotaxin, produced by keratocytes and present in tear samples may play a preponderant role in eosinophil activation in ocular allergy. Further supporting this idea is the detection of increased eotaxin expression in the conjunctiva of patients with VKC (49). Therefore, it seems of interest that ketotifen, which preferentially inhibits eosinophil activation by eotaxin, may be effective in the treatment of patients with allergic eye diseases.

Ophthalmic solution of ketotifen (0.025% in US and Europe, and 0.05% in other countries) has already proven its efficacy in numbers of clinical studies, in terms of relief of ocular symptoms (redness, itching, sneezing, lacrimation) (15, 16). However, none of these clinical studies have reported on the effect of ketotifen on ECP levels in nasal or lacrymal fluids, although it is well-recognized that ECP is increased in tears during AC and that ECP is correlated with signs, symptoms, and number of eosinophils in tears. Interestingly, by looking at the effect of ketotifen treatment during the pollen season, Kato et al. have reported a decrease in blood eosinophil count, as well as a decrease in serum ECP or MBP, suggesting a possible direct effect of ketotifen on peripheral blood eosinophil activation and degranulation and maybe on tissue eosinophils (50, 51). In another study, Leonardi et al. have described that the level of ECP in tears of patients with VKC was decreased after treatment with lodoxamide, another mast cell stabilizer and anti-allergic drug (52). These *in vivo* observations were then further supported by our *in vitro* results demonstrating a direct inhibitory effect of lodoxamide on EPO, ECP and EDN release from eosinophil after sIgA activation, as well as on the chemotactic reponses to fMLP and IL-5 (6). Therefore, drugs modulating several aspects of eosinophil function are likely to play a primary role in the treatment of allergic eye diseases.

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