

A Specific CCR3 Chemokine Receptor Antagonist Inhibits Both Early and Late Phase Allergic Inflammation in the Conjunctiva

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Abstract

Allergic inflammation manifests as one of a number of diseases, including asthma, dermatitis, food allergy, vernal keratoconjunctivitis, and systemic anaphylaxis. Together these diseases affect nearly 25% of the Western world and are a leading health-care problem. The diseases are often biphasic, with an early phase driven primarily by mast cell degranulation and a late phase characterized by leukocyte recruitment. While chemokines are well known to be critical for leukocyte recruitment, their importance in early-phase reactions is poorly defined. We show here that administration of a single oral dose of a high affinity and highly selective CCR3 antagonist ablates both the early and late phase reactions in a mouse model of allergic conjunctivitis. A direct analysis of mast cells in the conjunctiva demonstrates that antagonism of the CCR3 receptor stabilizes the mast cell *in vivo*, thereby leading to the impaired early phase reaction. The late phase reaction is also strongly inhibited as characterized by both reduced eosinophilia and neutrophilia. These results constitute the first direct evidence that antagonism of CCR3 has clear potential for the treatment of allergic diseases.

Key Words

Chemokine receptor
Antagonist
Mast cell stabilization

Chemokines are well known to play an essential role in the recruitment of inflammatory cells in late-phase allergic inflammation (1). Recent studies of CCR3 have suggested that the receptor may be important for both CCL11-mediated eosinophil recruitment and

mast cell-driven airway hyperresponsiveness (2). The effects on eosinophil recruitment were expected, as CCL11 and CCR3 were discovered via a biological screen for eosinophil chemoattractants (3,4). However, the data on mast cell responses were remarkable, as it

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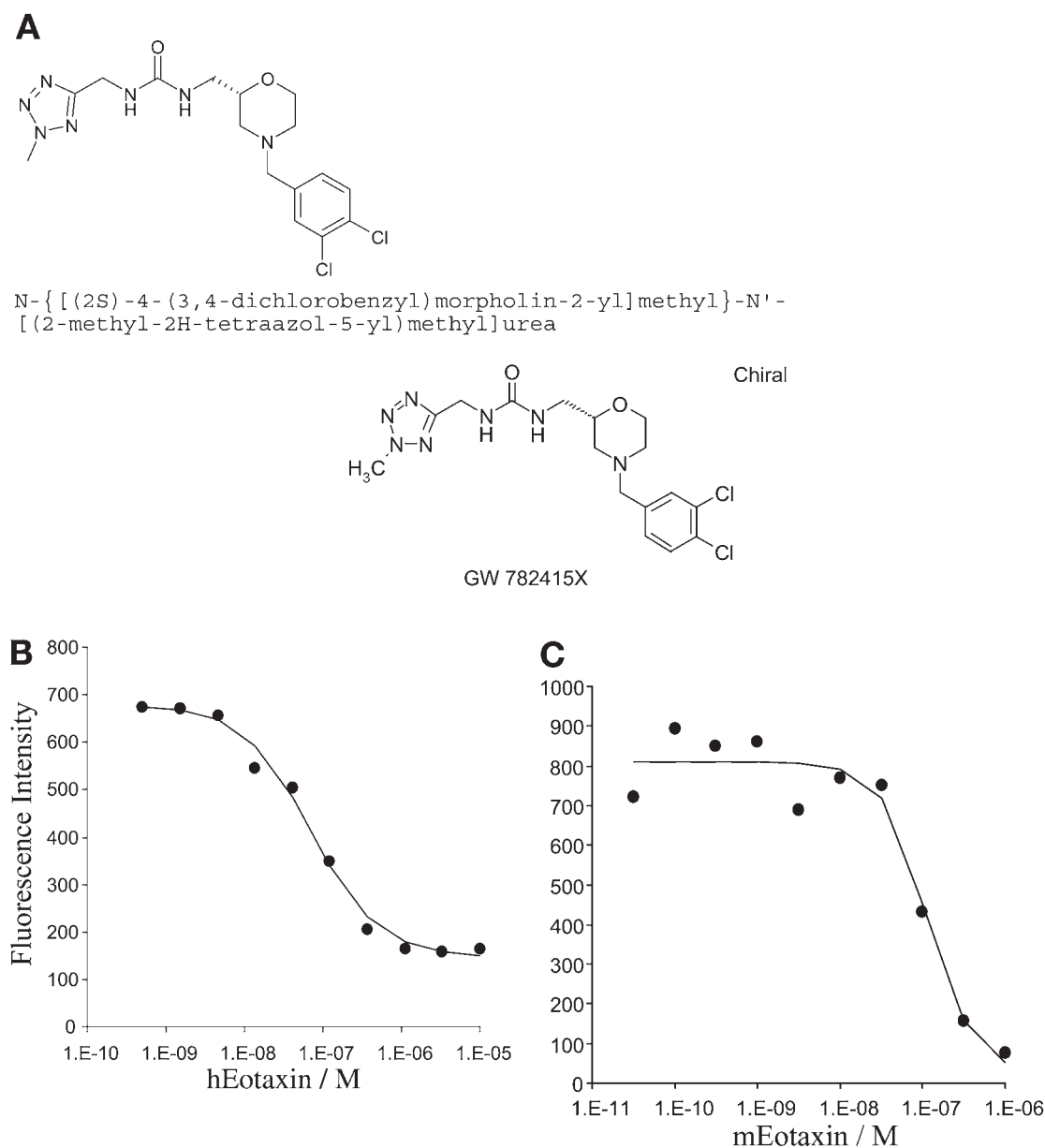


Fig. 1. (A) Structure of the CCR3-antagonist (GW782415X). *N*-{[(2*S*)-4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}-*N'*-[(2-methyl-2*H*-tetrazol-5-yl)methyl]urea. (B) Effect of the CCR3-antagonist on human eosinophil chemotaxis toward 3 nM eotaxin. Data are representative of 11 experiments in different donors. (C) Effect of the CCR3-antagonist on murine eosinophil chemotaxis towards 10 nM eotaxin. Data are representative of five similar experiments.

Detailed methods for Figure 1: Leukocyte suspensions enriched in murine eosinophils were obtained from Balb/c mice. Pooled bone marrow cells from normal Balb/c mice were cultured at an initial density of 1×10^7 cells/6 cm Petri dish in Iscove's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 U/mL penicillin, 0.1 mg/mL streptomycin, and 20 ng/mL recombinant human IL-5. The non-adherent cells were transferred to fresh medium on d 3 and harvested for chemotaxis assays on d 6 or 7. The proportion of eosinophils in the cultures was determined from cytopins stained with Leishmann's stain. Under these conditions the final cultures contained approx 50% eosinophils. For murine chemotaxis assays, the cultures were pooled, centrifuged at 400g for 5 min, and resuspended at 10^6 /mL in RPMI 1640 medium containing 10% heat-inactivated FBS. Cells were incubated with antagonists or vehicle (1% DMSO) for 30 min at 37°C before aliquots (50 μ L) were transferred to the upper surface of a 96-well 5- μ m-pore chemotaxis plate with murine eotaxin in the lower chamber. After incubation for 45 min at 37°C, the cell suspension was wiped from the upper surface of the plate. Cells that had migrated into the lower chamber were incubated with calcein-AM (15 μ M) for 30 min at 37°C. The cells were recovered by centrifugation (900g for 5 min), washed with 100 μ L PBS, and centrifuged again at 900g for 5 min. The fluorescence intensity of the cell pellet was determined on a Cytofluor 2300 fluorimetric plate reader exciting at 485 nm and reading emitted light at 530 nm. For human eosinophil chemotaxis assays, blood from hypereosinophilic human donors was taken into one-seventh of the volume of acid-citrate-dextrose anticoagulant, mixed with an equal volume of HEAS-steril, and incubated at room temperature for 40 min to allow the red cells to settle. The upper (leukocyte-rich) layer was collected and centrifuged at 400g for 5 min. The granulocytes were isolated by Percoll density gradient centrifugation and resuspended at 1×10^9 /mL PBS containing 1% BSA (PBS BSA) at 4°C and an equal volume of anti-CD16 magnetic microbeads was added. Cells were incubated at 4°C for 30 min, resuspended in 5 mL of PBS BSA (4°C) and applied to a Supermacs magnetic separation column that had previously been prepared by washing with 150 mL of PBS and 75 mL of 4°C PBS BSA. The eosinophils were eluted with PBS BSA (4°C), centrifuged (400g for 5 min), and resuspended in 10 mL of phenol red-free RPMI 1640 medium containing 10% heat-inactivated FBS (assay buffer). Calcein-AM (15 μ M) was added and the cells incubated at 37°C for 30 min. The cell suspension was centrifuged at 400g for 5 min and resuspended at 2.2×10^6 /mL in 37°C assay buffer. Aliquots of cell suspension were incubated with antagonist or vehicle (1% DMSO) for 30 min at 37°C and transferred to the upper surface of a 96-well 5- μ m-pore chemotaxis plate with human eotaxin in the lower chamber. The plates were incubated at 37°C for 45 min after which the cell suspension was gently wiped from the upper surface of the plate. Calcein fluorescence in the lower chamber was quantified using a Cytofluor 2300 fluorimetric plate reader exciting at 485 nm and reading emitted light at 530 nm.

revealed an additional role for the CCL11/CCR3 system. Analysis of mast cells in CCR3-deficient mice has indicated that CCR3 signaling may be important for the development of the mast cell, with mast cells from CCR3-deficient mice failing to migrate normally in certain mucosal tissues (2). However, as the data were obtained using CCR3-deficient mice, it is therefore difficult to ascertain at what developmental point(s) CCR3 signaling might regulate mast cell function. To conditionally interfere with CCR3 function in vivo, and to extend studies to another mucosal tissue, we have generated a high-affinity and highly selective small molecule antagonist of CCR3, and evaluated its ability to inhibit early- and late-

phase inflammation in a mouse model of allergic conjunctivitis. The selectivity of the antagonist for CCR3 over CCR1 exceeds by 80-fold that for CCR1 as measured on human receptors. The data show that CCR3 antagonism delivered as a brief pulse prior to allergen challenge is sufficient to inhibit both early- and late-phase inflammation in a sensitized mouse.

We have developed a potent and specific CCR3 antagonist, GW782415X (*N*-[[(2*S*)-4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl]-*N'*-[(2-methyl-2*H*-tetrazol-5-yl)methyl]urea) via a high-throughput chemical screen. The chemical structure of the antagonist is shown in Fig. 1A. Figures 1B,C illustrate the ability of the antagonist to antagonize CCR3-mediated

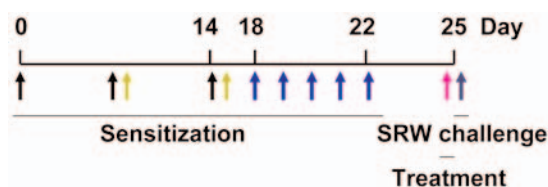


Fig. 2. Protocol of Immunization and GW782415X treatment. Mice were sensitized by intraperitoneal injection of 100 μ g SRW with alum or alum only on d 0, 7, and 14 (black $\uparrow$), and by eye drops of 100 μ g SRW with alum or alum only on d 8 and 15 (light grey $\uparrow$) followed by eye drops of 400 μ g SRW once a day for 5 d between d 18 and d 22 (dark grey $\uparrow$). Challenge of 400 μ g SRW (final $\uparrow$) was topically administered 30 min after the treatment by GW782415X (penultimate $\uparrow$) orally on d 25, and followed by the evaluation of the early-phase reaction clinically and histologically as well as the late phase reaction by histologically. *Detailed methods:* Female 8-wk-old A/J mice were obtained from Harlan UK (Oxon, UK). Mice were sensitized by intraperitoneal injection (ip) of 100 μ g short ragweed pollen (SRW; Greer, Lenoir, NC) with 1 mg aluminum hydroxide (alum; Sigma, St. Louis, MO) or 1 mg alum only on d 0, 7, and 14, and by topical treatment (eye drops) of 100 μ g SRW with 25 μ g alum or 25 μ g alum only on d 8 and 15 followed by eye drops of 400 μ g SRW once a day for 5 d between d 18 and d 22. The experimental challenge of 400 μ g SRW in phosphate-buffered saline (PBS) was administered topically on d 25 (Fig. 1). Mice were treated with 8 μ g per 1 g mouse body weight GW782415X in 1% NaCl with 1% methylcellulose was administered orally 30 min before the challenge (Fig. 1). All data are the mean $\pm$ SE of results. Data were analyzed using ANOVA, and $p < 0.05$ was considered significant.

eosinophil chemotaxis. GW782415X inhibited chemotaxis of human eosinophils (B) toward 3 nM eotaxin with a pK_i of 8.08 ± 0.11 (SEM, $n = 11$) and of murine eosinophils (C) toward 10 nM eotaxin with a similar affinity ($pK_i = 7.85 \pm 0.23$, $n = 5$).

We tested the ability of the CCR3-antagonist to inhibit early- and late-phase allergic inflammation in the conjunctiva using a well-established murine model of the disease (5–7). Mice were sensitized with ragweed allergen as previously described, so as to gen-

Table 1. Characteristics of Mice Groups

Group	Sensitization	Challenge	Treatment
1	–	–	PBS
2	–	+	PBS
3	+	–	PBS
4	+	+	PBS
5	+	+	Vehicle
6	+	+	GW782415X

erate a potent and specific hypersensitivity reaction in the conjunctiva. Figure 2A illustrates the sensitization scheme used in this experiment. Thirty minutes prior to final allergen challenge, mice were treated with a single oral administration of the CCR3 antagonist or vehicle control. The mice were then challenged in the conjunctiva, and evaluated in a double-blind manner for clinical symptoms of early-phase inflammation. Conjunctival redness, lid edema and redness, tearing and discharge, and behavioral changes were monitored in all of the mice via both direct examination of the challenged eyes and computer-assisted analysis of digital photos and movies. The total clinical response (as well as each symptom measured) was markedly inhibited by treatment with the CCR3 antagonist (Fig. 3A). Each symptom was significantly inhibited (Figs. 3A,B). Figure 3C shows a representative photo of eyes from mice either treated with the CCR3-antagonist or vehicle alone.

As the early-phase reaction is often driven by mast cell degranulation, we next examined mast cell integrity *in situ* in naive, sensitized, vehicle-treated and CCR3 antagonist-treated mice (Fig. 4). Because the degree of mast cell involvement in models of allergic inflammation can vary depending on the route of sensitization, we first verified that the early-phase response in this model was mast cell-dependent, using mast cell-deficient W/W^v mice (8,9). The analysis of these and control mice

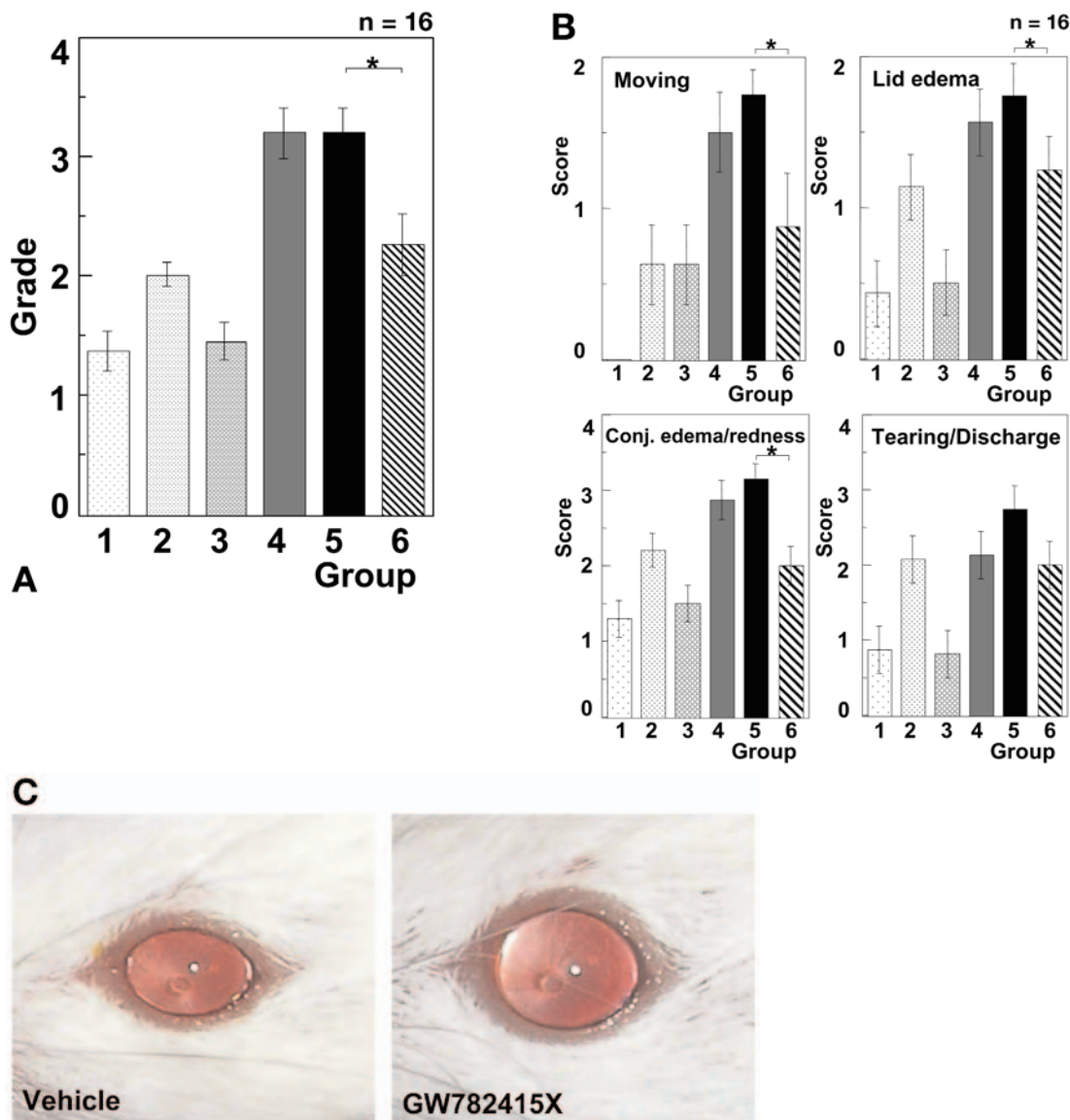


Fig. 3. Effect of CCR3-antagonist on clinical symptoms of SRW allergic conjunctivitis. SRW sensitized/nonsensitized mice were challenged by SRW/mock topically with/without treatment by GW782415X orally. Clinical symptoms were evaluated 20 min after the challenge. Group designations are as described in Table 1. **(A)** Treatment by GW782415X orally shows less allergic conjunctivitis significantly than that by vehicle. **(B)** All clinical symptoms, such as moving, lid edema, conjunctival edema/redness, and tearing/discharge, show significantly lower score by treatment of GW782415X. $n=16/\text{group}$, $*p<0.05$. **(C)** Representative picture shows smaller size of the eye by treatment of GW782415X than by that of vehicle, which suggests that GW782415X treatment prevents progression of lid edema induced by SRW challenge. *Detailed methods:* Mice were observed in a double-blind study for clinical symptoms of immediate hypersensitivity response 20 min after the topical challenge of SRW. Both the behaviors of mice in the cage (moving) and specific clinical symptoms in the eye, such as tearing/discharge, conjunctival edema/redness, and lid edema were scored using video photography, digital photography, and microscopy. Eventually, the severity of the disease was evaluated by the sum of these scores. After the challenge, each clinical symptom was scored on scale of 0–2 (0, absent; 1, mild; 2, severe). Then the severity of the disease was evaluated and graded by the sum of each score (symptom) on scale of 0–4 (0 none, 0 point; 1 minimal, 1–3 points; 2 mild, # of severe symptoms is less than 2 or more than 4 points; 3 moderate, # of severe symptoms is 3 or 4; 4 severe, # of severe symptoms is more than 5). Lethargy in the mice correlated with severe inflammation (grade 4).

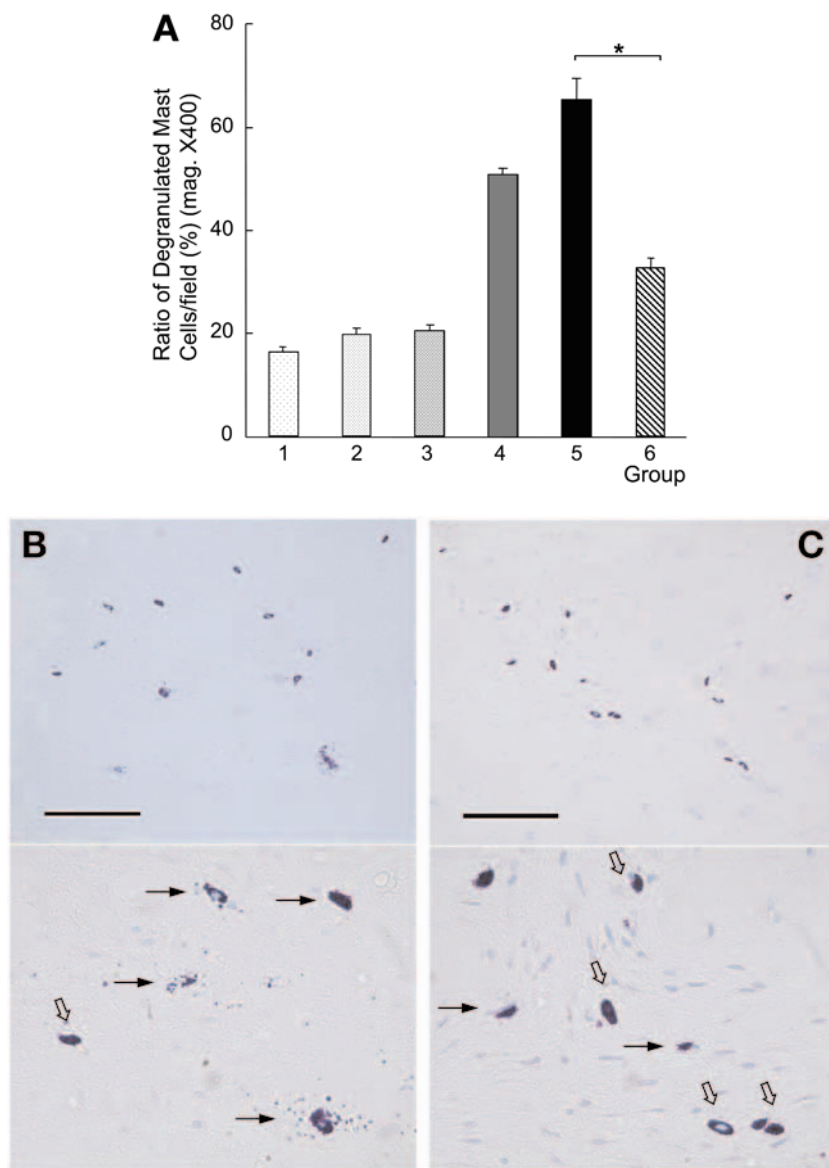


Fig. 4. Effect of CCR3-antagonist on early-phase reactions of SRW allergic conjunctivitis. Ratio of degranulated mast cells of early-phase reactions were evaluated for histology 2 h after challenge. **(A)** Bar graphs showing the mean number of ratio of mast cell degranulation. Mast cells present in the conjunctiva were counted under a X200 field ($n = 8$ eyes or more per group). Compared to the vehicle group, there is significantly lower ratio of degranulated mast cells from GW782415X treatment group ($*p < 0.001$). Histological findings of the conjunctiva: **(B)** Upper: low magnification, bottom: high magnification, note the mast cell degranulation in the conjunctiva from vehicle mice. **(C)** Upper: low magnification, bottom: high magnification, nondegranulated mast cells is predominant in mice treated with GW782415X. **(B and C)** Giemsa; scale = 100 μ m. Arrows indicate degranulated mast cells and open arrows indicate nondegranulated mast cells. *Detailed methods:* To evaluate the cellular infiltrates in the conjunctiva, whole eye tissue were excised 2 and 24 h after the challenge, fixed in 4% paraformaldehyde, embedded in JB-4 (Polysciences, Inc., Warrington, PA), and serially sectioned to 3 μ m thickness. The sagittal sections were stained with Giemsa for mast cells and hematoxylin and eosin for the identification of eosinophils and neutrophils. Eight (for mast cells) and six (for eosinophils and neutrophils) sections from each eye tissue were examined and counted by a masked observer under a microscope, using a $\times 200$ and $\times 400$ high-powered field, respectively.

clearly showed that early-phase inflammation in this model is mast cell-dependent (Miyazaki and Ono, submitted for publication). Inspection of the mast cell degranulation index in vehicle- and CCR3 antagonist-treated mice shows clearly that a single oral dosing of CCR3 antagonist significantly ablates allergen-driven mast cell degranulation in the conjunctiva (Fig. 4). This decrease in mast cell degranulation was clearly consistent with the mast cell dependence of this model and the decreased clinical symptoms (Fig. 3). We note, because the CCR3 antagonist was given as a single dose prior to allergen challenges, that the effect of the antagonist in these experiments is unlikely to be operative at the level of mast cell development (as appears to be the case in CCR3-deficient mice).

Because the CCL11/CCR3 system is clearly important in eosinophil recruitment, we also examined the effect of the CCR3 antagonist on eosinophil and neutrophil recruitment in the late phase reaction. Eosinophilia and neutrophilia are hallmarks of chronic inflammation in cases of severe seasonal allergic conjunctivitis and vernal keratoconjunctivitis (10). Conjunctival eosinophilia was evaluated by counting eosinophils attracted to the forniceal region of the conjunctiva. Conjunctival challenge of SRW-sensitized mice resulted in a massive eosinophil recruitment as previously observed. The single oral dose of CCR3 antagonist markedly inhibited this eosinophilia (Figs. 5A,B). This is consistent with the important role CCR3 plays in eosinophil recruitment as shown previously in studies using antibody blockade or CCR3-deficient mice (11,12).

Because neutrophilia is a hallmark of the late-phase reaction in severe seasonal allergic conjunctivitis, we also evaluated whether CCR3 antagonist had any effect on neutrophil attraction. As shown in Figs. 5A,B, conjunctival challenge of SRW-sensitized mice showed significant neutrophilia in the con-

junctival fornix. This was markedly reduced in the mice receiving the single oral dose of CCR3-antagonist (Figs. 5A,B). This was surprising because we have previously documented a CCL3/likely CCR1 dependence of neutrophil recruitment in the conjunctiva. As the antagonist has exquisite specificity for CCR3 (80-fold over CCR1), this might suggest that neutrophil recruitment to the conjunctiva may also be dependent on CCR3. Alternatively, neutrophil recruitment *in vivo* may be facilitated by the CCR3-dependent eosinophilia.

In conclusion, these results suggest that (1) signaling from the CCR3 receptor is essential to prime conjunctival mast cells for Fc ϵ RI-mediated activation, (2) that a single CCR antagonist can inhibit both CCR3- and CCR1-mediated leukocyte recruitment, and (3) that antagonism of CCR3 has clear potential for the treatment of allergic diseases. There are several reasons that we believe that CCR3 signaling provides an obligatory "priming" signal to conjunctival mast cells (and perhaps other mast cells). First, the brief pulse of CCR3 antagonist administered to the presensitized mice makes it unlikely that the mice have suffered a block in mast cell development or in allergen sensitization (as could occur in CCR3-deficient mice). Second, we do not detect changes in the number or location of conjunctival mast cells in CCR3-antagonist treated mice. Finally, *in vitro* analyses of Fc ϵ RI-mediated conjunctival mast cell degranulation in CCL11-deficient mice suggest that an impaired degranulation of these cells can be rapidly rescued via CCL11-priming (Miyazaki, Nakamura, and Ono, manuscript in preparation). This is not to say that a lack of a CCR3 signal may not block mast cell development (as suggested by the CCR3-deficient mice), but that there are likely critical downstream signals from CCR3 that are obscured by the early block in mast

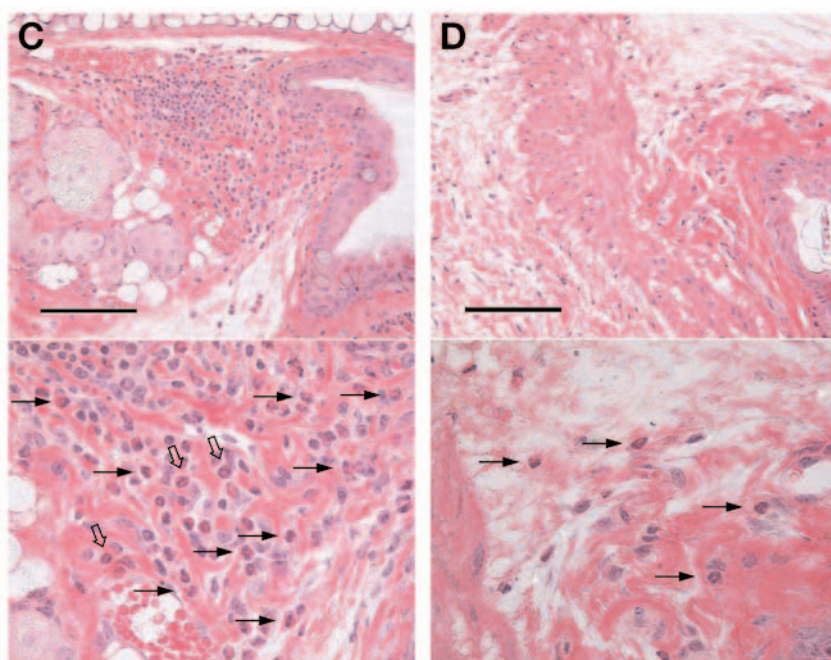
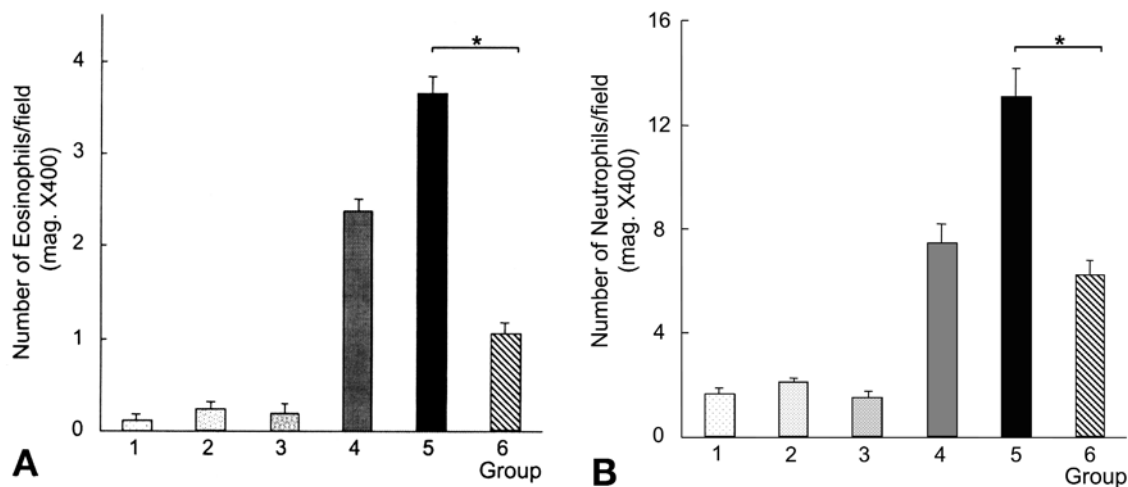


Fig. 5. Effect of GW782415X treatment on late-phase reactions of SRW allergic conjunctivitis. Inflammatory cellular infiltrations of late-phase reactions were evaluated for histology 24 h after challenge. Bar graphs showing the mean number of eosinophils (**A**) and neutrophils (**B**). Inflammatory cells present in the fornix conjunctiva were counted under a $\times 400$ field ($n = 8$ eyes or more per group). Compared to the vehicle group, there are significantly loss eosinophil or neutrophil recruitment into the conjunctiva of GW782415X treatment group ($*p < 0.001$). (**C**) Upper: low magnification, inflammatory cell infiltration is seen in the conjunctiva from vehicle mice. Bottom: high magnification, substantial neutrophils and eosinophils infiltrations are found. (**D**) Upper: low magnification, reduction of inflammatory cells is observed in the mice treated with GW782415X. Bottom: high magnification, a few neutrophils are remaining in the conjunctival tissue. (**C** and **D**) Hematoxylin and eosin; scale = 100 μm . Arrows indicate neutrophils and open arrows indicate eosinophils.

cell development observed in the CCR3-deficient mice.

Why are both eosinophilia and neutrophilia inhibited by CCR3-antagonist treatment? One possibility is that the CCR3 antagonist used may also antagonize CCR1 or CCR5 *in vivo*. While the exquisite specificity of the reagent has been tested *in vitro*, perhaps this may be a different scenario than that which occurs *in vivo*. Alternatively, the data might imply that neutrophil recruitment to the conjunctiva is actually CCR3-dependent, or that neutrophil recruitment is facilitated by the coordinate or early recruitment of eosinophils. Further experiments are required to distinguish between these possibilities.

Apart from the fundamental questions raised by this study, perhaps most important, the data obtained in these experiments indicate that antagonism of the CCR3 chemokine receptor is potentially of therapeutic value for allergic diseases. The inhibition of both early- and late-phase responses indicate that this single target can have broad protective effects in both mild and severe/chronic allergy. Future experiments will likely survey the range of allergic diseases where CCR3 antagonist will be of therapeutic value, the duration of protection afforded by a single dose of antagonist, and its comparative safety/efficacy (relative to other treatments) in human disease.

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