

CC-Chemokine Receptor 3: A Possible Target in Treatment of Allergy-Related Corneal Ulcer

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PURPOSE. To determine the suppressive effects of antibodies (Abs) against CC-chemokine receptor (CCR)-1 and CCR-3 on eosinophil chemotaxis induced by culture supernatant from corneal keratocytes and by tears from severely allergic patients with corneal ulcer.

METHODS. Primary cultures of human corneal keratocytes were incubated with interleukin (IL)-4 (33.3 ng/mL) and tumor necrosis factor (TNF)- α (33.3 ng/mL) for 48 hours. In tear samples collected from five severely allergic patients and three nonallergic control subjects, eosinophils were immunostained for CCR. Next, eosinophils purified from peripheral blood were preincubated with or without anti-CCR-1 and anti-CCR-3 Abs before a Boyden chamber assay was conducted. Recombinant human (rh) eotaxin, rh-regulated on activation normal T-cell expressed and secreted (rh-RANTES), culture supernatant from human corneal keratocytes, and tear samples were used as chemoattractants.

RESULTS. Eosinophils in tears from allergic patients expressed CCR-1 and -3 on their surfaces. Anti-CCR-1 and -3 Abs each inhibited eosinophil chemotaxis induced by rh-RANTES. Anti-CCR-3 Ab (but not anti-CCR-1 Ab) also inhibited eosinophil chemotaxis induced by rh-eotaxin. Anti-CCR-1 and -3 Abs, respectively, inhibited up to 75.2% and 94.6% of eosinophil chemotaxis induced by culture supernatant, as well as 27.8% and 74.5% of chemotaxis induced by tear samples.

CONCLUSIONS. Anti-CCR-1 and -3 Abs inhibited eosinophil chemotaxis induced by culture supernatant from corneal keratocytes and tear samples from severely allergic patients. Anti-CCR-3 Ab was more effective than anti-CCR-1 Ab. Inhibition of CCR-3 on eosinophils may be a treatment for corneal ulcer in patients with ocular allergy. (*Invest Ophthalmol Vis Sci.* 2002; 43:58–62)

Corneal ulcer is among the most severe and treatment-resistant complications associated with ocular allergic diseases, such as atopic keratoconjunctivitis (AKC)^{1,2} and vernal keratoconjunctivitis (VKC).^{3,4} Eosinophils (EOSs) and eosinophil cationic protein (ECP) have been found in conjunctival tissue and in tears of patients with AKC and VKC.^{4,5} EOS major basic protein (MBP), detected in corneal plaques in VKC⁶ has

been shown to inhibit epithelial migration.⁷ EOSs therefore appear to participate in corneal damage in ocular allergic diseases.

CC chemokines, such as eotaxin^{8,9} and the protein regulated on activation normal T-cell expressed and secreted (RANTES),¹⁰ are important in recruiting EOS into tissue affected by allergy. RANTES has been found in tears of patients with allergic conjunctivitis¹¹ and is produced by conjunctival¹² and corneal cells.¹³ We have reported that eotaxin is present in tears of allergic patients with severe corneal damage, correlating with the number of EOSs in tears.¹⁴ We also have found that interleukin (IL)-4 induces eotaxin production in human corneal keratocytes.¹⁵

EOSs are attracted when chemokines interact with CC chemokine receptors (CCR)-1 and -3 on their surfaces.¹⁶ Although RANTES activates EOS through both CCR-1 and -3, eotaxin is a specific ligand for CCR-3.^{17,18} We hypothesized that blocking CCR-1 or -3 would suppress EOS recruitment and could represent a new approach in the treatment of corneal damage in ocular allergic diseases. We therefore investigated suppressive effects of anti-CCR-1 and -3 antibodies (Abs) on EOS chemotaxis in vitro induced by culture supernatant from corneal keratocytes incubated with IL-4 and also by tears from patients with severe ocular allergic disease.

MATERIALS AND METHODS

Tear Collection

All the experiments in this study followed the tenets of the Declaration of Helsinki. After informed consent was obtained, 100- μ L tear samples were collected from five patients with AKC involving corneal ulcer (five eyes) and from three nonallergic normal control subjects (three eyes; Table 1). To obtain unstimulated basal tears, the tear samples were collected with microcapillary tubes at the lateral canthus of the eyelid, in supine patients with heads tilted to the side. No anesthetic was used. Tear samples were centrifuged immediately at 4°C to remove cells and transferred to new tubes. Tear samples were stored at -70°C until further examination.

Immunocytochemistry

Cells from a tear sample (tear 4) were resuspended and centrifuged by cytospin techniques onto three glass slides for each sample. Cells on these three slides were stained with anti-CCR-1 Ab (1 μ g/mL), anti-CCR-3 Ab (1 μ g/mL), or control murine IgG₁ (1 μ g/mL) by the following method. Cells were fixed with 4% paraformaldehyde for 30 minutes at 4°C and treated with 3% H₂O₂ in methanol for 10 minutes at room temperature (RT). After blocking with Tris-buffered saline (TBS) at pH 7.6 containing 10% normal rabbit serum for 10 minutes at RT, the cells were allowed to react for 1 hour at RT with monoclonal IgG₁ murine anti-human CCR-1 Ab (R&D, Minneapolis, MN), dissolved at 1 μ g/mL in phosphate-buffered saline (PBS) supplemented with 10% fetal calf serum (FCS); anti-human CCR-3 murine monoclonal IgG₁ Ab (clone 444-11; Sato et al.¹⁸), dissolved at 1 μ g/mL in PBS supplemented with 10% FCS; or control mouse IgG₁ (R&D), dissolved at 1 μ g/mL in PBS supplemented with 10% FCS. After they were washed three times with PBS, cells were incubated with biotinylated rabbit anti-mouse Ig (Hist-

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TABLE 1. Patients Involved in the Study

Category and Subject No.	Age (y)	Sex (M/F)	Allergic Disease	Eye Condition	Eye Drops	Systemic Drugs	EOS Chemotaxis (EOS/HPF)	% Inhibition by Anti-CCR1 Ab (%)	% Inhibition by Anti-CCR3 Ab (%)
Nonallergic control subjects									
1	39	M	None	w.n.l.	None	None	4.2	ND	ND
2	18	F	None	w.n.l.	None	None	2.7	ND	ND
3	15	M	None	w.n.l.	None	None	3.7	ND	ND
Patients with AKC									
4	10	M	AKC, AD, AS	Plaque	CR, Dex, CsA	None	43.0	6.7	57.5
5	10	M	AKC, AD, AS	Ulcer	CR, Dex, CsA	None	20.0	18.0	67.9
6	15	M	AKC, AD, AS	Ulcer	CR, Dex, CsA	None	17.0	39.5	81.5
7	14	F	AKC, AD	Ulcer	CR, Dex, CsA	None	23.3	47.1	90.8
8	13	M	AKC, AD	Ulcer	CR, Dex, CsA	None	3.3	ND	ND

w.n.l., within normal limit; ND, not done; AD, atopic dermatitis; AS, asthma; CR, sodium cromoglycate; Dex, dexamethasone; CsA, cyclosporine.

ofine SAB-PO kit; Nichirei, Tokyo, Japan) for 10 minutes at RT. Cells were then treated with peroxidase-conjugated streptavidin (Histofine SAB-PO kit, Nichirei) for 5 minutes. Enzyme activity was developed using substrate solution (Histofine DAB substrate kit; Nichirei) for 5 minutes at RT in darkness. Cells were then washed and counterstained with hematoxylin.

Cell Culture

Human corneas were obtained from the American Eye Bank Association. Human corneal keratocytes were established in culture, as previously described by Cubitt et al.¹⁹ Cells were cultured in collagen-coated 35-mm culture dishes (Iwaki Co., Tokyo, Japan) and were studied at the second passage. Purity of each cell type was assessed by cell morphology and differential activity to anti-cytokeratin Abs. Corneal keratocytes were stained by for anti-vimentin Abs (Roche Molecular Biochemicals, Indianapolis, IN), but not anti-keratin AE1/AE3 (Progen Biotechnik GMBH, Heidelberg, Germany).

At the second passage, cells were removed from culture dishes by diluting cultures 1:10 with 0.05% trypsin-0.53 mM EDTA (Gibco BRL, Grand Island, NY) in PBS and incubating for 5 minutes. Keratocytes were resuspended in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) containing 15% FCS, and 100 μ L of the cell suspension was preincubated overnight in a 96-well culture plate. After a wash in PBS, the culture medium was changed to DMEM without FCS for 24 hours. Cells were then incubated with rhIL-4 (33.3 ng/mL) and TNF- α (33.3 ng/mL; all from R&D) for 48 hours. Samples were collected by pipetting, and the remaining cells were removed by centrifugation at 800g for 5 minutes. Supernatants were stored at -70°C until further study.

Immunoreactive eotaxin concentrations in supernatants was measured using a double-ligand immunoassay using two different mouse monoclonal Abs (clone 164-4 and clone 174-4) against human eotaxin. The procedure has been established and described elsewhere.²⁰ For chemotactic assay, culture supernatants were diluted with culture medium to result in an eotaxin concentration of 50 ng/mL.

EOS Purification

Human granulocytes were isolated from EDTA-anticoagulated venous blood from an atopic volunteer (41-year-old male volunteer with asthma), by using a gradient centrifugation (1.090 g/mL, Percoll; Pharmacia Upjohn, Uppsala, Sweden;) at RT. Procedures after centrifugation were performed at 4°C . Red blood cells were removed by hypotonic lysis. CD16-positive cells were removed using an immunomagnetic bead technique (MACS). EOS purity (according to examination of stained cytospin preparations; Diff-Quick, Kokusai-shiyaku, Kobe, Japan) was $99\% \pm 1\%$, and viability based on trypan blue dye exclusion test results was $99\% \pm 1\%$ ($n = 10$).

Chemotaxis Experiments

Chemotaxis experiments were performed using a modified Boyden chamber technique. Briefly, 28 μ L of medium (RPMI 1640) alone or medium containing various concentrations of stimulant was placed in triplicate in the lower chamber. For the experiments with cell culture supernatants, cell culture medium including IL-4 plus TNF- α was used as a control. A polycarbonate membrane with a 5- μ m pore size (Nucleopore, Pleasanton, CA) separated the upper and lower chambers. EOSs (5×10^4) resuspended in RPMI 1640 (50 μ L) were placed in each well of the upper chamber, on the upper surface of the membrane. For inhibition studies using anti-CCR-1 and -3 Abs, EOSs were preincubated with these Abs for 30 minutes at RT. The chamber then was incubated for 30 minutes at 37°C in a mixture of 5% CO_2 and air and then disassembled. The membrane was removed and washed in PBS to remove nonmigrating EOSs from the upper surface, scraped, and stained with Diff-Quik. EOSs were counted in five random high-power fields (HPFs) by light microscopy, and chemotactic activity was expressed as the mean number of EOS per HPF.

Statistical Analysis

Statistical analysis was performed by using analysis of variance (ANOVA) with a post hoc analysis (Fisher protected least significant difference [PLSD]). $P < 0.05$ was considered to indicate significance. Analysis was performed on computer (Statview 4 software; Abacus Concepts, Berkeley, CA).

RESULTS

CCR-1 and -3 Expression on EOSs in Tear Samples

Immunocytochemistry showed CCR-1 and -3 expression on EOSs in a tear sample from a patient with allergic corneal ulcer (tear 4; Fig. 1). Conjunctival epithelial cells did not express these receptors.

Suppressive Effect of Anti-CCR-1 and -3 Abs on EOS Chemotaxis Induced by Eotaxin and RANTES

rh-Eotaxin (0–100 ng/mL) and rh-RANTES (0–100 ng/mL) each increased EOS chemotaxis in a dose-dependent manner, from 4.33 ± 1.51 at the baseline to as much as 123.3 ± 23.2 EOS/HPF and from 4.33 ± 1.51 to 118.0 ± 27.3 EOS/HPF, respectively (data not shown). EOS chemotaxis induced by eotaxin (50 ng/mL) was suppressed by 0.1 to 10 μ g/mL anti-CCR-3 Ab (84.4%–98.5% suppression), but not by anti-CCR-1 Ab (Fig. 2a). EOS chemotaxis induced by RANTES (50 ng/mL)

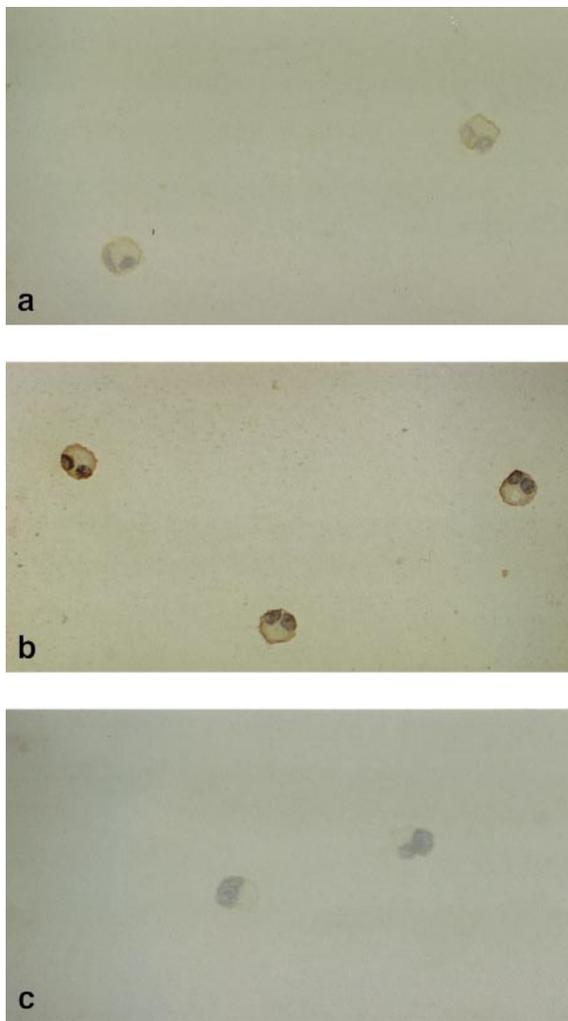


FIGURE 1. Photomicrograph of CCR-1 and -3 on EOSs in tears from a patient with AKC involving severe corneal damage. The cells in the AKC tear samples (tear 4) were incubated with anti-CCR-1, anti-CCR-3, or control IgG₁ Abs. Photomicrographs demonstrate immunoreactive CCR-1 (a) and -3 (b). (c) Photomicrograph demonstrates the absence of staining with control Ab (mouse IgG₁). Magnification, $\times 132$.

was suppressed by 0.1 $\mu\text{g/mL}$ anti-CCR-1 or -3 Ab (63.5% and 80.8% suppression, respectively; Fig. 2b). The percentage of inhibition was calculated after subtraction of EOS chemotaxis by culture medium only. RhIL-8 (50 ng/mL) induced EOS chemotaxis (25.5 ± 2.08 EOS/HPF), which was not inhibited by anti-CCR-1 Ab (1 $\mu\text{g/mL}$) or anti-CCR-3 Ab (1 $\mu\text{g/mL}$; data not shown).

Suppressive Effect of Anti-CCR-1 and -3 Abs on EOS Chemotaxis Induced by Culture Supernatant from Corneal Keratocytes

Culture supernatant from corneal keratocytes diluted to contain 50 ng/mL eotaxin induced EOS chemotaxis (58.5 ± 14.11 EOS/HPF) compared with control medium including IL-4 and TNF- α (24.25 \pm 2.50 EOS/HPF). EOS chemotaxis induced by culture supernatant was suppressed by anti-CCR-1 Ab (0.1–10 $\mu\text{g/mL}$) and also by anti-CCR-3 Ab (0.1–10 $\mu\text{g/mL}$) in a dose-dependent manner (from 35.0% to 75.2% and from 25.4% to 94.6% suppression, respectively). The combination of anti-CCR-1 Ab (0.1 $\mu\text{g/mL}$) and anti-CCR-3 Ab (0.1 $\mu\text{g/mL}$) suppressed 68.1% of EOS chemotaxis induced by the culture supernatant of corneal keratocytes. Anti-CCR-1 Ab (1 $\mu\text{g/mL}$)

plus anti-CCR-3 Ab (1 $\mu\text{g/mL}$) nearly eliminated EOS chemotaxis (91.2%; Fig. 3). The percentage of inhibition was calculated after subtraction of EOS chemotaxis by culture medium containing IL-4 and TNF- α (24.3 EOS/HPF).

Suppressive Effect of Anti-CCR-1 and -3 Abs on EOS Chemotaxis Induced by Tear Samples

Four of five tear samples from patients with AKC induced EOS chemotaxis (17.0–43.0 EOS/HPF), whereas tear samples from nonallergic volunteers did not (Table 1). Anti-CCR-1 Ab (1 $\mu\text{g/mL}$) suppressed the EOS chemotaxis induced by two of the four chemotactically active samples ($72.2\% \pm 18.7\%$ net chemotaxis, $P = 0.018$; Fig. 4, Table 1). Anti-CCR-3 Ab (1 $\mu\text{g/mL}$) suppressed EOS chemotaxis induced by all four active tear samples ($25.5\% \pm 14.5\%$ net chemotaxis, $P < 0.0001$; Fig. 4, Table 1). The suppressive effect of anti-CCR-3 Ab was significantly greater than that of anti-CCR-1 Ab ($P = 0.0009$). Net percentages of chemotaxis and inhibition were calculated after subtraction of EOS chemotaxis by nonallergic control tears (3.5 EOS/HPF).

DISCUSSION

In this study, anti-CCR-1 and -3 Abs inhibited EOS chemotaxis induced by culture supernatant from corneal keratocytes or by tear samples from severely allergic patients. Anti-CCR-3 Ab was more effective than anti-CCR-1 Ab. Inhibition of CCR-3 on the EOS surface may represent a treatment strategy for corneal ulcer in patients with ocular allergy.

We showed that rh-RANTES and rh-eotaxin induced EOS chemotaxis in a dose-dependent manner. Whereas RANTES activated EOS through both CCR-1 and -3, eotaxin was specific for CCR-3. In this study, EOS chemotaxis induced by rh-RANTES was suppressed by anti-CCR-1 or -3 Ab, whereas EOS chemotaxis induced by rh-eotaxin was suppressed only by anti-CCR-3 Ab. Moreover, EOS chemotaxis induced by rhIL-8 was not inhibited by these Abs. These results verify that the Abs and experimental design used in this study were appropriate.

We showed the expression of CCR-1 and -3 on the surface of EOSs in tear samples from a patient with AKC. The expression of CCR-3 on the surface of EOSs is constitutive, whereas CCR-1 is inducible. EOSs migrating into tears are thought to be activated.

IL-4²¹ and TNF- α ²² both have been identified in tears from allergic patients. We have reported that IL-4 and TNF- α induce eotaxin production in human corneal keratocytes.¹⁵ RANTES has been reported to be produced in keratocytes by TNF- α .¹³ In this study, culture supernatant from human corneal keratocytes incubated with IL-4 and TNF- α induced EOS chemotaxis. Our in vitro method appears to be a good model for examining EOS recruitment to the cornea in T-helper (Th)-2-dominant situations.

EOS recruitment induced by culture supernatant from human corneal keratocytes was suppressed by anti-CCR-1 Ab and was more effectively suppressed by anti-CCR-3 Ab. Although we did not determine the expression of CCRs on the surface of EOSs used in the chemotactic study, the EOSs were obtained from a volunteer with asthma, and therefore may have been activated. The suppressive effect of either anti-CCR Ab alone was greater than that expected from the results of simultaneous suppression using both anti-CCR Abs. From these results, CCR-1 and -3 are likely to interact with each other in EOS chemotaxis. Although the suppressive effect by the combina-

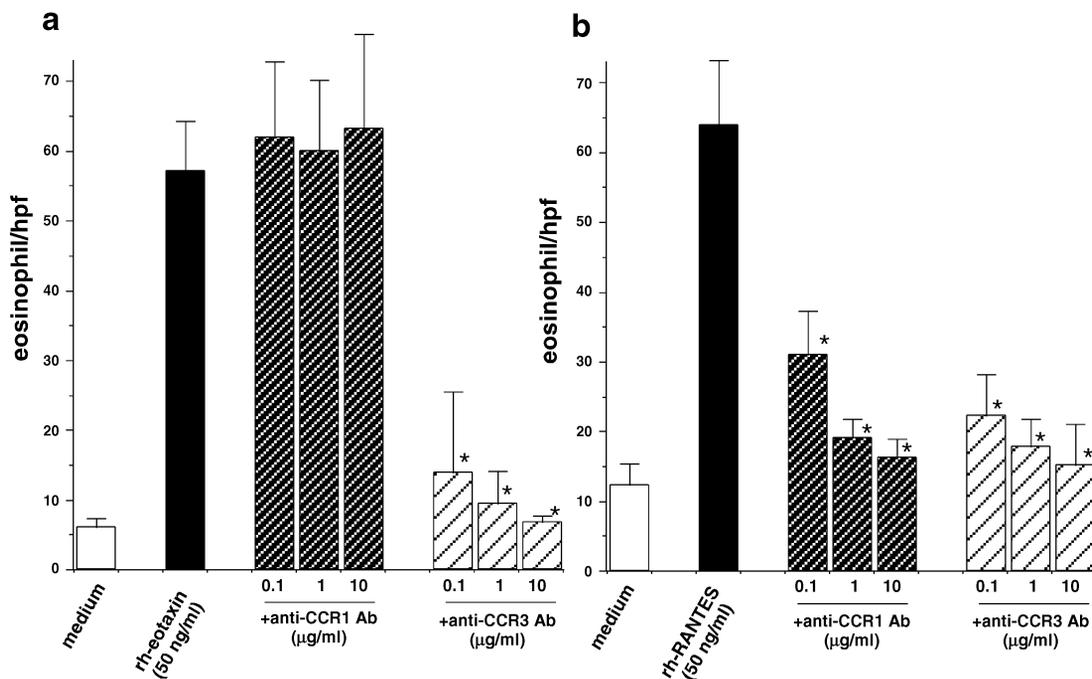


FIGURE 2. The suppressive effect of anti-CCR-1 and -3 Abs on EOS chemotaxis induced by rh-eotaxin and rh-RANTES. In vitro EOS chemotaxis assays were performed on (a) rh-eotaxin (50 ng/mL) and (b) rh-RANTES (50 ng/mL). The medium alone (RPMI 1640) was used in negative control experiments. EOSs were preincubated with or without anti-CCR-1 and/or -3 Abs (0.1–10 µg/mL) for 30 minutes. EOS migration, assessed by a modified Boyden chamber technique, is expressed as the mean number of EOSs per high-power field (hpf). Results are expressed as the mean ± SD of three separate experiments. *Significant suppression in migration compared with positive migration induced by rh-eotaxin or rh-RANTES ($P < 0.05$).

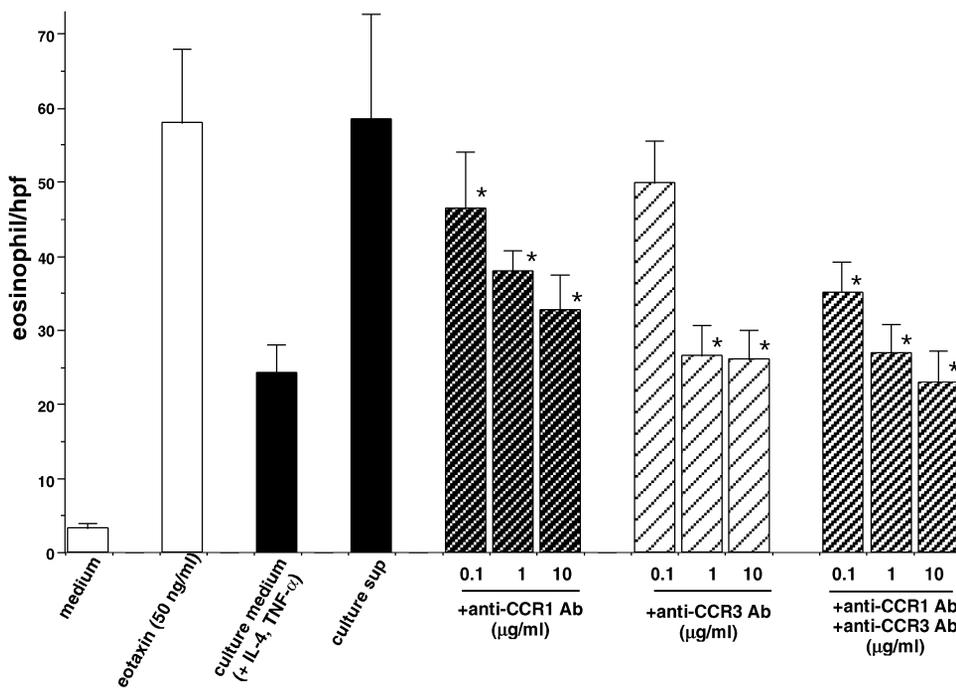
tion of anti-CCR-1 Ab and -3 Abs seemed to be greater than that by each anti-CCR Ab, the combined effect was not significant.

Four of five tear samples from patients with AKC induced EOS chemotaxis. Although the difficulties of tear sampling without stimulation precluded obtaining sufficient tear sample volume to determine chemokine concentrations, tear samples that induced EOS chemotaxis showed activity comparable to 10 to 50 ng/mL of rh-eotaxin. To our knowledge, this is the first

report to examine EOS chemotaxis in response to tear samples. EOS chemotaxis induced by tears from allergic subjects may explain EOS recruitment to affected ocular surfaces.

The tear-induced chemotaxis of EOSs was significantly, but not completely, inhibited by anti-CCR-3 mAb in this study, indicating there may be some other factors that induce EOS migration through the receptors other than CCR-1 or -3, such as IL-8, substance-P, or platelet-activating factor (PAF). How-

FIGURE 3. The suppressive effect of anti-CCR-1 and -3 Abs on EOS chemotaxis induced by culture supernatant of human corneal keratocytes. In vitro EOS chemotaxis assays were performed on diluted culture supernatants from human corneal keratocytes incubated with IL-4 (33.3 ng/mL) plus TNF-α (33.3 ng/mL) for 48 hours. The culture supernatants were diluted until eotaxin concentration was 50 ng/mL. The medium alone (RPMI 1640) and medium with IL-4 and TNF-α were used as the negative controls. Recombinant human eotaxin (50 ng/mL) was used as the positive control. EOSs were preincubated with or without anti-CCR-1 and/or -3 Abs (0.1–10 µg/mL) for 30 minutes, EOS migration, assessed by a modified Boyden chamber technique, is expressed as the mean number of EOS per high-power field (hpf). Results are expressed as the mean ± SD for three separate experiments. *Significant suppression in migration compared with culture medium with IL-4 and TNF-α; $P < 0.05$.



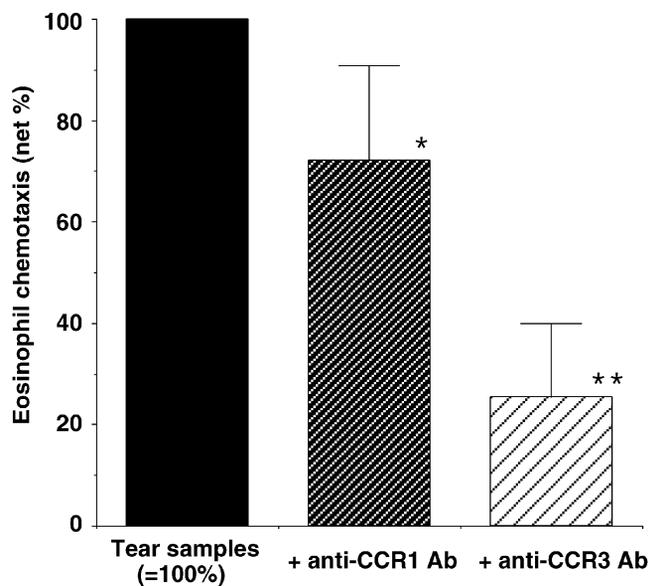


FIGURE 4. The suppressive effect of anti-CCR-1 and -3 Abs on EOS chemotaxis induced by tears of patients with severe ocular allergic corneal damages. In vitro EOS chemotaxis assays were performed on tears of patients with severe ocular allergic corneal damage. Tears of nonallergic normal control subjects were used as a negative control. EOSs were preincubated with or without anti-CCR-1 and -3 Abs (1 μ g/mL) for 30 minutes. EOS migration, assessed by a modified Boyden chamber technique, is expressed as the net percentage of EOS chemotaxis. Results are expressed as the mean \pm SD ($n = 4$). Percentage of net chemotaxis was calculated after subtraction of EOS chemotaxis from nonallergic control tears (3.5 EOS/HPF). *Significant suppression of migration compared with positive migration by tear samples from patients; $P = 0.0182$); **Significant suppression of migration compared with positive migration by tear samples from patients ($P < 0.0001$) and also by anti-CCR-1 Ab ($P = 0.0009$).

ever, anti-CCR-3 Ab suppressed EOS chemotaxis induced by all four chemotactically active tear samples in this study, indicating that CCR-3 may be a new target for treatment of corneal tissue damage in severe ocular allergic disease.

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