

Eotaxin-2 regulates newly produced and CD34⁺ airway eosinophils after allergen exposure

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Background: Eosinophils develop from CD34⁺ bone marrow progenitors, and evidence increasingly suggests that these progenitors are recruited to the airways in atopic asthmatics after allergen challenge. Moreover, it has been shown that the number of CD34⁺ progenitors in bronchial tissue correlates with asthma severity. To date, however, little is known about how these cells are recruited into the airways.

Objective: Our aim was to evaluate the role of 2 chemokines, eotaxin-1 and/or eotaxin-2, in the recruitment of newly produced and CD34⁺ eosinophils to the airways after allergen exposure.

Methods: BALB/c mice sensitized and exposed to ovalbumin were pretreated intraperitoneally or intranasally with a neutralizing anti-eotaxin-1 and/or anti-eotaxin-2 antibody. A thymidine analogue, 5-bromo-2'-deoxyuridine (BrdU), was used to mark newly produced cells. Bronchoalveolar lavage (BAL), blood, and bone marrow were collected 24 hours after the final exposure.

Results: Anti-eotaxin-1 and/or anti-eotaxin-2 given intranasally (ie, locally to airways) significantly decreased BAL eosinophils. This decrease was paralleled with a decrease in both BrdU⁺ and CD34⁺ eosinophils. In contrast, systemically administered (ie, intraperitoneally) anti-eotaxin-1 and/or anti-eotaxin-2 resulted in a significant decrease in BAL eosinophils only when the combined treatment was of a sufficiently high dosage to produce measurable concentrations in the airways.

Furthermore, neither of the treated groups showed any significant decrease in blood or bone marrow eosinophils.

Conclusion: Both eotaxin-1 and eotaxin-2 are involved locally within the airways in the regulation of the recruitment of newly produced and CD34⁺ eosinophils after allergen exposure. (J Allergy Clin Immunol 2004;113:1109-16.)

Key words: Eotaxin-1, eotaxin-2, eosinophils, BrdU, CD34⁺ progenitors, airway, allergy

Abbreviations used

BAL: Bronchoalveolar lavage
BALF: Bronchoalveolar lavage fluid
BM: Bone marrow
BrdU: 5-Bromo-2'-deoxyuridine
CCR3: Chemokine receptor 3
UK: United Kingdom

Allergic airway inflammation is dominated by eosinophils,¹⁻³ which originate from CD34⁺ hematopoietic bone marrow (BM) progenitors via the influence of IL-5.⁴⁻⁶ Although hematopoietic BM events may trigger the increased production and release of mature eosinophils from the BM in response to allergen, there is increasing evidence that CD34⁺ progenitors also migrate to the airways, resulting in localized eosinophil differentiation.⁷ Furthermore, by using a thymidine analogue (5-bromo-2'-deoxyuridine [BrdU]) to label proliferating inflammatory cells undergoing mitosis within the BM, we have previously shown increases in both BrdU⁺ cells and BrdU⁺ eosinophils in bronchoalveolar lavage fluid (BALF) 24 hours after allergen exposure. These increases most likely reflect the traffic to the airways of newly divided myeloid cells in response to allergen exposure.⁵

Studies of allergic airway inflammation in guinea pig and mouse models suggest that eotaxin-1 plays a key role in the traffic of eosinophils into allergic inflammation sites.^{8,9} Furthermore, the airway concentration of eotaxin-1 is increased in patients with asthma.¹⁰ Recently, a novel, relatively eosinophil-specific chemokine, eotaxin-2, has been identified. This chemokine is structurally different from eotaxin-1, with only ~30% homology to eotaxin-1, and the gene is located in a different chromosome position. However, eotaxin-2 acts on the same receptor as eotaxin-1, chemokine receptor 3 (CCR3), and eotaxin-2 is also expressed in tissues in which allergic inflammation is present.^{11,12}

Currently, it is not known which chemotactic factors orchestrate the recruitment of CD34⁺ cells into the airways during an allergen response. However, it has recently been shown that CCR3 is upregulated in CD34⁺ cells within the BM in asthmatics after allergen challenge.¹³

The aim of the current study was therefore to determine whether eotaxin-2 is involved in the recruitment of newly produced and CD34⁺ eosinophils to the airways after allergen exposure, and whether any observed effect is regulated systemically or locally within the airways. We

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thus hypothesized that the eotaxin/CCR3 pathway is involved in the recruitment of specifically CD34⁺ eosinophils and new eosinophils (BrdU⁺) locally in airways after allergen exposure. To test this, we used a mouse model of allergic airway inflammation. Animals were pretreated with neutralizing antibodies (anti-eotaxin-1 and/or anti-eotaxin-2), administered either by the airway (intranasally) or a systemic route (intraperitoneally), because it is known that intranasal doses of antibodies will lead to high local tissue concentration and low systemic concentration, whereas the opposite is true for intraperitoneal injection.⁵ BrdU was used to label newly produced inflammatory cells. To elucidate further whether eotaxin-1 and/or eotaxin-2 had any effect on the presence of progenitor cells in different compartments, immunocytochemical staining of CD34⁺ eosinophils in BM, blood, and bronchoalveolar lavage (BAL) was performed.

METHODS

Animals

This study was approved by the Animal Ethics Committee in Gothenburg, Sweden. Male BALB/c mice 5 to 6 weeks old were purchased from Mollegaard-Bommice A/S (Ry, Denmark). The mice were kept under animal housing conditions and provided with food and water ad libitum.

Sensitization and allergen exposure

All mice were immunized twice, at an interval of 5 days by intraperitoneal injections of 0.5 mL alum-precipitated antigen containing 8 µg ovalbumin (Sigma-Aldrich, St Louis, Mo) bound to 4 mg Al(OH)₃ (Sigma-Aldrich) in PBS. Eight days after the second sensitization, the mice were briefly anesthetized with Isoflurane (Baxter, Deerfield, Ill) and received an intranasal administration of 100 µg ovalbumin in 25 µL PBS on 5 consecutive days. Twenty-four hours after the last ovalbumin exposure, the mice were killed, and blood, BM, and BALF cells were collected.

In vivo protocol for anti-eotaxin-1 and/or anti-eotaxin-2 treatment

The effects of anti-eotaxin-1 and/or anti-eotaxin-2 in allergic airway eosinophilia were investigated by pretreatment of the animals with the eotaxin-neutralizing mAb, anti-eotaxin-1 (clone 42285.111; R&D Systems Europe, Abingdon, United Kingdom) and anti-eotaxin-2 (clone 106521; R&D Systems) or a combination of both. Four series of experiments were performed. (1) Mice were treated intranasally with either anti-eotaxin-1 or anti-eotaxin-2 in 3 different doses (5–50 µg per animal in 25 µL PBS) or isotype control (20 µg per animal in 25 µL PBS, rat IgG_{2a}, clone 54447; R&D Systems) on 5 consecutive days, 1 hour before exposure. (2) The animals were treated intranasally with both anti-eotaxin-1 and anti-eotaxin-2 (5 + 5 µg to 50 + 50 µg per animal in 25 µL PBS) or isotype control antibody (40 µg per animal in 25 µL PBS) on 5 consecutive days, 1 hour before exposure. (3) Mice were treated with 30 to 300 µg anti-eotaxin-1, anti-eotaxin-2, or isotype control (100 µg) by intraperitoneal injection 1 hour before exposure on the first day of exposure. (4) The animals were treated intraperitoneally with both anti-eotaxin-1 (30–300 µg per animal) and anti-eotaxin-2 (30–300 µg per animal) or isotype control (200 µg per animal) on the first day of exposure.

In vivo protocol for evaluation of newly produced inflammatory cells

5-Bromo-2'-deoxyuridine is a thymidine analogue that is incorporated into the DNA during the S-phase of the cell cycle by replacing thymidine.¹⁴ The animals were given BrdU (Roche Diagnostics Scandinavia AB, Bromma, Sweden) to pulse-label newly produced eosinophils and thereby evaluate the contribution of enhanced eosinophilopoiesis to allergen-induced airway eosinophilia. The BrdU was given at a dose of 1 mg in 250 µL PBS by intraperitoneal injection on 2 occasions, 8 hours apart on day 1 and day 3 of ovalbumin exposure.

Cell collection and sample processing

Samples were collected 24 hours after the final ovalbumin exposure. The animals were deeply anesthetized with a mixture of xylazine (130 mg/kg, Rompun; Bayer, Gothenburg, Sweden) and ketamine (670 mg/kg, Ketalar; Pfizer, Täby, Sweden). Animals were killed by puncture of the right ventricle, also used for collecting blood samples. Animals were then tracheostomized, and BAL was performed by instilling 0.25 mL PBS through the tracheal cannula, followed by gentle aspiration and repeated with a second volume of 0.2 mL PBS. After this, 1 femur was cut at the epiphysis, and the BM cells were flushed with 2 mL PBS.

Blood

Two hundred µL of blood was mixed with 800 µL 2 mmol/L EDTA in PBS, and red blood cells were lysed in 0.1% potassium bicarbonate and 0.83% ammonium chloride (lysing solution) for 15 minutes at 4°C. After washing, white blood cells were resuspended in 0.03% BSA in PBS (all reagents from Sigma-Aldrich). Serum was obtained from the remaining volume of blood after centrifugation at 950g for 10 minutes at 4°C, which was used for measurement of rat IgG_{2a}.

BALF and BM

Bronchoalveolar lavage fluid and BM samples were centrifuged at 100g for 10 minutes at 4°C. The cells were resuspended in 0.03% BSA in PBS. BALF supernatant was used for measurement of rat IgG_{2a}.

The total number of cells in blood, BALF, and BM was counted with a standard hemocytometer. Cytospins of blood, BALF, and BM samples were prepared and stained with May-Grünwald-Giemsa stain for differential cell counts. Cell differentiation was determined by counting 300 to 400 cells with a light microscope (Zeiss Axioplan 2; Carl Zeiss, Jena, Germany). Serum and BALF supernatant samples were stored at –80°C until measurement of rat IgG_{2a}.

Immunocytochemical detection of BrdU⁺ cells

Intranuclear incorporated BrdU was detected in BAL, BM, and blood cytopins by using a monoclonal BrdU antibody (clone BU1/75, no. MAS 250p; Harlan-Sera Lab, Loughborough, United Kingdom). Bound antibodies were visualized by DAB substrate-chromogen system (no. K3466; DAKO, Glostrup, Denmark). The slides were stained for eosinophils by using 2% eosin, followed by counterstaining with Mayer hematoxylin (DAKO). All slides were evaluated with the light microscope (Zeiss Axioplan 2) in random fields of view. Cells with any nuclear brown staining in combination with pink staining in the cytoplasm were counted as BrdU⁺ eosinophils (Fig 1, A). Staining was also included on BrdU[–] slides. No false-positive staining was detected.

Immunocytochemical detection of CD34⁺ eosinophils

Cytospin preparations from BM, blood, and BAL were stained for CD34⁺ eosinophils by using a monoclonal biotinylated rat antimouse

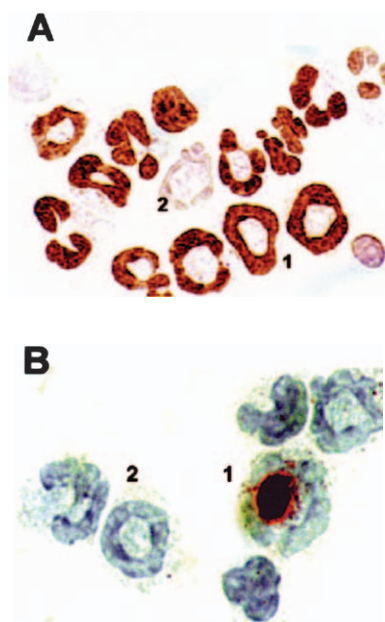


FIG 1. Photomicrograph (original magnification, $\times 1000$) of **(A)** BAL cells stained positively for BrdU in ovalbumin-sensitized/exposed Balb/c mouse. Cells with nuclear brown staining together with pink staining in the cytoplasm were counted as BrdU⁺ eosinophils. 1, BrdU⁺ eosinophil; 2, BrdU⁻ eosinophil. **B**, BAL eosinophil stained positively for CD34 in ovalbumin-sensitized/exposed Balb/c mice. CD34 staining is red; Luxol staining is green. Cells with both red and green staining in cytoplasm were counted as CD34⁺ eosinophils. 1, CD34⁺ eosinophil; 2, CD34⁻ eosinophil.

CD34 antibody (clone RAM34; BD Biosciences, Erembodegem, Belgium). Bound antibodies were visualized with a Vector Red Alkaline Phosphatase Substrate kit (Vector Laboratories, Burlingame, Calif). The slides were stained with Luxol Fast Blue and counterstained with Mayer hematoxylin (DAKO). Five hundred cells were evaluated in random fields of view. Cells with both red cytoplasm (CD34⁺) and green Luxol staining (eosinophil) were counted as CD34⁺ eosinophils (Fig 1, B).

Quantification of rat IgG_{2a} in mice treated with anti-eotaxin-1 and/or anti-eotaxin-2

Levels of rat IgG_{2a} in serum and BALF supernatants were measured in both systemically and locally treated animals by the ELISA method. Mouse antirat IgG_{2a} mAb (clone B46-7; BD Biosciences) was used for capture in combination with biotin-conjugated mouse antirat IgG_{2a} mAb (clone RG7/1.30; BD Biosciences). A serial dilution of antirat IgG_{2a} (clone 54447; R&D Systems) was used as a standard. The detection limit for rat IgG_{2a} was 3.9 ng/mL.

Statistical analysis

All data are expressed as means $\pm$ SEMs. Statistical analysis was carried out by using a nonparametric analysis of variance (Kruskal-Wallis test) to determine the variance among more than 2 groups. If significant variance was found, an unpaired 2-group test (Mann-Whitney *U* test) was used to determine significant differences between individual groups. *P* < .05 was considered statistically significant. The Spearman rank correlation test was used to determine whether there was any correlation between the amount of rat IgG_{2a} measured in BALF or serum and BrdU⁺ or CD34⁺ eosinophilic number in the treated groups of animals.

TABLE I. Antibody concentrations (rat IgG_{2a}) in BAL and serum*

	BALF	Serum (IgG _{2a} ng/mL)
Intraperitoneal treatment		
IgG _{2a} (100 μ g $\times$ 1)	129 (21)	8397 (328)
Anti-eotaxin-1 (30 μ g $\times$ 1)	0	626 (426)
Anti-eotaxin-1 (100 μ g $\times$ 1)	135 (21)	7904 (1221)
Anti-eotaxin-1 (300 μ g $\times$ 1)	155 (34)	10650 (5221)
Anti-eotaxin-2 (30 μ g $\times$ 1)	0	0
Anti-eotaxin-2 (100 μ g $\times$ 1)	10 (4)	1647 (890)
Anti-eotaxin-2 (300 μ g $\times$ 1)	66 (6)	4462 (293)
IgG _{2a} (200 μ g $\times$ 1)	268 (21)	14869 (2301)
Anti-eotaxin-1+2 (30 + 30 μ g $\times$ 1)	18 (7)	596 (95)
Anti-eotaxin-1+2 (100 + 100 μ g $\times$ 1)	156 (28)	9888 (1783)
Anti-eotaxin-1+2 (300 + 300 μ g $\times$ 1)	405 (46)	16744 (6306)
Intranasal treatment		
IgG _{2a} (20 μ g $\times$ 5)	455 (101)	119 (12)
Anti-eotaxin-1 (5 μ g $\times$ 5)	104 (49)	23 (3)
Anti-eotaxin-1 (20 μ g $\times$ 5)	428 (98)	142 (12)
Anti-eotaxin-1 (50 μ g $\times$ 5)	1083 (314)	281 (36)
Anti-eotaxin-2 (5 μ g $\times$ 5)	42 (24)	0
Anti-eotaxin-2 (20 μ g $\times$ 5)	245 (105)	21 (7)
Anti-eotaxin-2 (50 μ g $\times$ 5)	2023 (644)	121 (30)
IgG _{2a} (40 μ g $\times$ 5)	1585 (536)	173 (11)
Anti-eotaxin-1 + 2 (5 + 5 μ g $\times$ 5)	222 (57)	7 (3)
Anti-eotaxin-1 + 2 (20 + 20 μ g $\times$ 5)	792 (123)	77 (7)
Anti-eotaxin-1 + 2 (50 + 50 μ g $\times$ 5)	1778 (225)	194 (16)

*The concentration of rat IgG_{2a} (ng/mL) in BALF and serum from mice treated intraperitoneally or intranasally with different doses of anti-eotaxin-1 and/or anti-eotaxin-2 or isotype control antibody (IgG_{2a}). Data are shown as means (SEMs).

RESULTS

Concentration of neutralizing antibody in BALF and serum

The intraperitoneal administration of antibody resulted in a high concentration of antibodies in serum and low concentrations in BALF, whereas intranasal administration resulted in a low concentration in serum but high concentrations in BALF (Table I).

Effects of local airway anti-eotaxin-1 treatment

Anti-eotaxin-1 administered intranasally significantly decreased BAL eosinophils compared with isotype controls (54 ± 15 vs $172 \pm 18 \times 10^4$ /mL; *P* < .01). The inhibiting effect of intranasally administered anti-eotaxin-1 on BAL eosinophils was paralleled with a decrease in BrdU⁺ BAL eosinophils when compared with isotype controls (28 ± 8 vs $138 \pm 18 \times 10^4$ /mL; *P* < .01; Fig 2, A). Furthermore, intranasally administered anti-eotaxin-1 significantly decreased the number of CD34⁺ eosinophils in BAL compared with isotype controls (16 ± 6 vs $93 \pm 10 \times 10^4$ /mL; *P* < .05; Fig 2, B). However, intranasal anti-eotaxin-1 treatment had no effect on allergen-induced BM or blood eosinophil numbers (see Table E1 in the Journal's Online Repository at www.mosby.com/jaci).

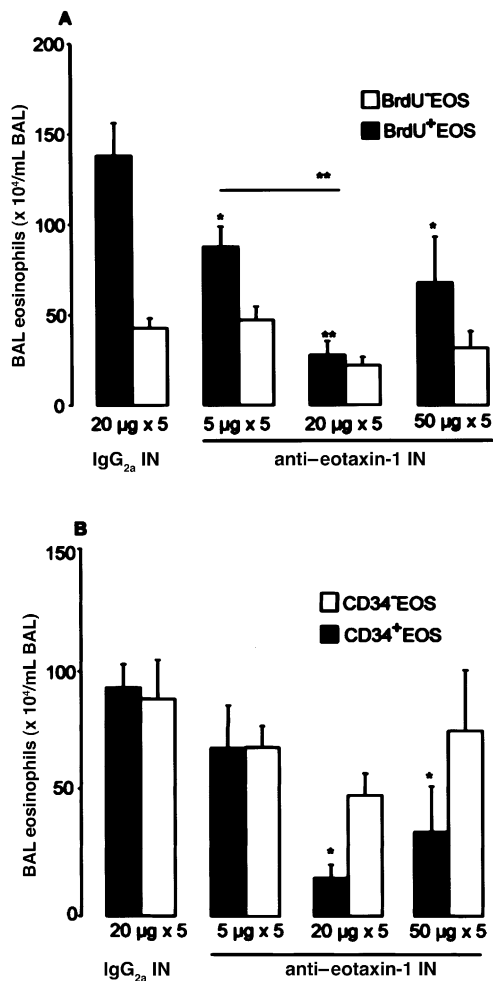


FIG 2. Effect of in vivo treatment with the neutralizing anti-eotaxin-1 mAb versus its isotype control (rat IgG_{2a}) in BAL on (A) BrdU⁺ eosinophils (EOS; dark column) and (B) CD34⁺ eosinophils (dark column) in ovalbumin-sensitized/exposed Balb/c mice. The antibody was administered intranasally (IN; 5–50 µg on 5 consecutive days) 1 hour before exposure. Data are shown as means ($\pm$ SEMs); $n = 4$ to 8. * $P < .05$ and ** $P < .01$ compared with the rat IgG_{2a}-treated group.

Effects of local airway anti-eotaxin-2 treatment

Anti-eotaxin-2 administered intranasally significantly decreased BAL eosinophils compared with isotype controls (84 ± 18 vs $172 \pm 18 \times 10^4/\text{mL}$; $P < .05$). The inhibiting effect of intranasally administered anti-eotaxin-2 on BAL eosinophils corresponded with a decrease in BrdU⁺ eosinophils compared with isotype controls (60 ± 15 vs $138 \pm 18 \times 10^4/\text{mL}$; $P < .05$; Fig 3, A). Moreover, an inverse relationship was found between the BrdU⁺ eosinophils and the amount of rat IgG_{2a} measured in BALF and serum in the groups treated with anti-eotaxin-2 (see Table E2 in the Online Repository at www.mosby.com/jaci). Furthermore, locally administered anti-eotaxin-2 significantly decreased the number of CD34⁺ eosinophils compared with isotype controls

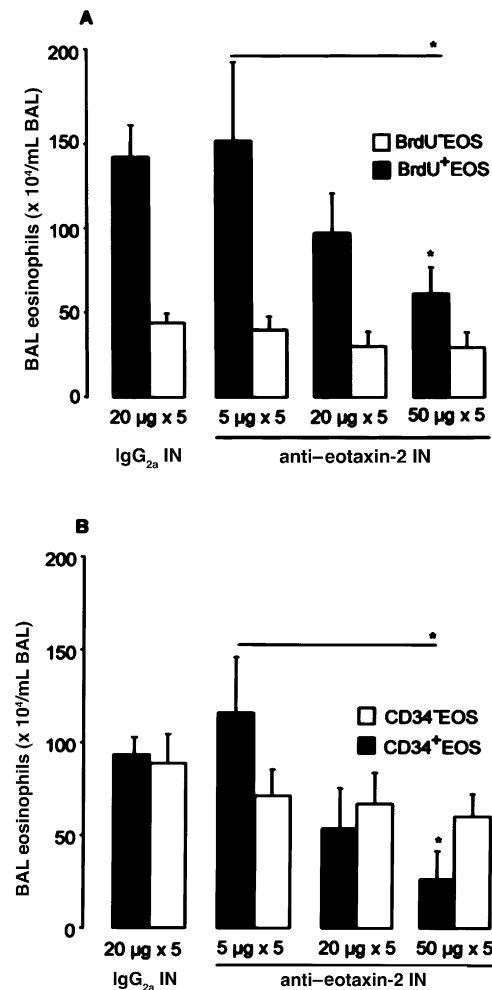


FIG 3. Effect of in vivo treatment with the neutralizing anti-eotaxin-2 mAb versus its isotype control (rat IgG_{2a}), in BAL on (A) BrdU⁺ eosinophils (EOS; dark column) and (B) CD34⁺ eosinophils (dark column) in ovalbumin-sensitized/exposed Balb/c mice. The antibody was administered intranasally (IN; 5–50 µg on 5 consecutive days) 1 hour before exposure. Data are shown as means ($\pm$ SEMs); $n = 5$ to 8. * $P < .05$ compared with the rat IgG_{2a}-treated group.

(26 ± 15 vs $93 \pm 10 \times 10^4/\text{mL}$; $P < .05$; Fig 3, B). Here, an inverse relationship was also found between the CD34⁺ eosinophils and the amount of rat IgG_{2a} measured in BALF and serum in the groups treated with anti-eotaxin-2 (Table E2). However, intranasal anti-eotaxin-2 treatment had no effect on allergen-induced BM or blood eosinophil numbers (Table E1).

Effects of combined anti-eotaxin-1 and anti-eotaxin-2 local airway treatment

The combination of anti-eotaxin-1 and anti-eotaxin-2 administered intranasally significantly decreased BAL eosinophils. In contrast, the combined treatment showed a decrease in BAL eosinophils compared with isotype controls (103 ± 24 vs $234 \pm 31 \times 10^4/\text{mL}$; $P < .05$),

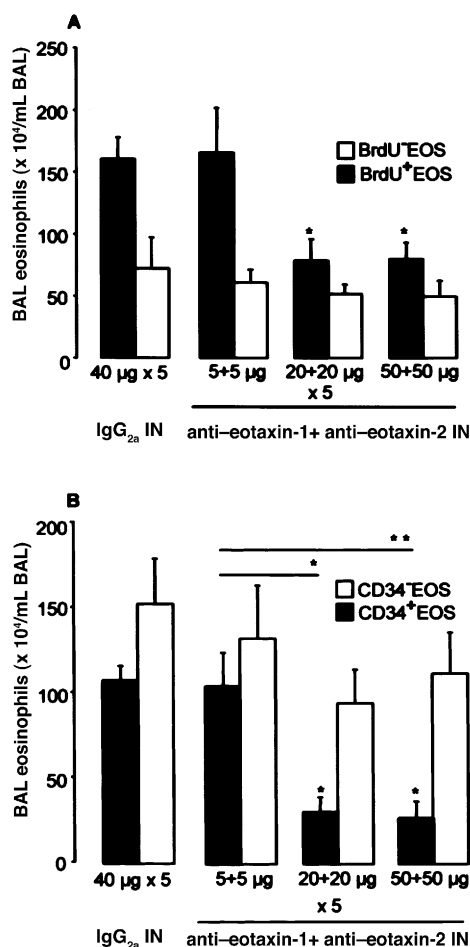


FIG 4. Effect of *in vivo* treatment with neutralizing anti-eotaxin-1 and anti-eotaxin-2 mAbs versus its isotype control (rat IgG_{2a}), in BAL on (A) BrdU⁺ eosinophils (EOS; dark column) and (B) CD34⁺ eosinophils (dark column) in ovalbumin-sensitized/exposed Balb/c mice. The antibody was administered intranasally (IN; 5-50 µg on 5 consecutive days) 1 hour before exposure. Data are shown as means (± SEMs); n = 5 to 8. **P* < .05 compared with the rat IgG_{2a}-treated group.

although numerically there was no difference compared with either of the individual treatments. The decrease of BAL eosinophils as a result of intranasally administered anti-eotaxin-1 and anti-eotaxin-2 paralleled a decrease in BrdU⁺ eosinophils in this compartment (80 ± 14 vs $161 \pm 17 \times 10^4/\text{mL}$; *P* < .05; Fig 4, A). Moreover, there was an inverse correlation between the BrdU⁺ eosinophils and the amount of rat IgG_{2a} measured in BALF in the groups treated with both anti-eotaxin-1 and anti-eotaxin-2 (Table E2). Furthermore, intranasally administered anti-eotaxin-1 and anti-eotaxin-2 significantly decreased the CD34⁺ eosinophils in BAL (27 ± 10 vs $108 \pm 10 \times 10^4/\text{mL}$; *P* < .05; Fig 4, B). In this case, there was also an inverse relationship between the amount of rat IgG_{2a} measured in BALF and CD34⁺ eosinophils in BAL in the groups treated with both anti-eotaxin-1 and anti-eotaxin-2 (Table E2). However, the combination of intranasally administered anti-eotaxin-1 and anti-eotaxin-2 had no

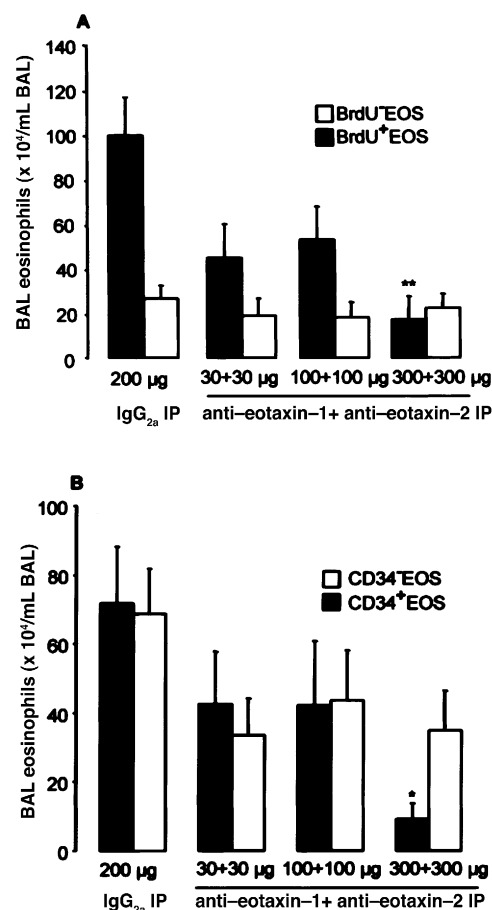


FIG 5. Effect of *in vivo* treatment with neutralizing anti-eotaxin-1 and anti-eotaxin-2 mAbs versus its isotype control (rat IgG_{2a}), in BAL on (A) BrdU⁺ eosinophils (EOS; dark column) and (B) CD34⁺ eosinophils (dark column) in ovalbumin-sensitized/exposed Balb/c mice. The antibody was administered intraperitoneally (IP; 30-300 µg) 1 hour before exposure on the first day of exposure. Data are shown as means (± SEMs); n = 4 to 8. **P* < .05 and ***P* < .01 compared with the rat IgG_{2a}-treated group.

effect on allergen-induced BM or blood eosinophil numbers (Table E1).

Effects of systemic anti-eotaxin-1 or anti-eotaxin-2 treatment

Systemic administration of anti-eotaxin-1 or anti-eotaxin-2 had no statistical effect on allergen-induced BAL, BM, or blood eosinophils when compared with isotype control groups (data not shown). Moreover, there were no differences in BrdU⁺ eosinophils or CD34⁺ eosinophils between any of the treatment groups.

Effects of combined systemic anti-eotaxin-1 and anti-eotaxin-2 treatment

The high dose (300 µg + 300 µg) but not the lower doses (30 µg + 30 µg or 100 µg + 100 µg) of the combination of anti-eotaxin-1 and anti-eotaxin-2 administered intraperitoneally significantly decreased BAL

eosinophils compared with isotype controls (36 ± 11 vs $126 \pm 18 \times 10^4/\text{mL}$; $P < .05$). The decrease in BAL eosinophils by intraperitoneally administered anti-eotaxin-1 and anti-eotaxin-2 corresponded with a decrease in BrdU⁺ eosinophils in this compartment (18 ± 11 vs $99 \pm 17 \times 10^4/\text{mL}$; $P < .05$; Fig 5, A). Furthermore, the anti-eotaxin-1 and anti-eotaxin-2 combination in high doses intraperitoneally significantly decreased the CD34⁺ eosinophils in BAL (9 ± 5 vs $72 \pm 16 \times 10^4/\text{mL}$; $P < .05$; Fig 5, B). However, there was no correlation between the amount of rat IgG_{2a} measured in BALF or serum in relation to BAL eosinophils (data not shown). The combination of intraperitoneally administered anti-eotaxin-1 and anti-eotaxin-2 had no statistically significant effect on allergen-induced BM or blood eosinophilia regardless of the dose given (Table E1).

DISCUSSION

This study provides evidence that eotaxin-2 is intricately involved in parallel with eotaxin-1 in the eosinophil traffic into the airways after allergen exposure. It is important to note that neutralization of either or both of these chemokines is effective only when treatment results in sufficient concentrations of neutralizing antibodies in BALF. No significant effects were observed in BM or blood after either local airway or systemic administration of the antibodies. In general, the effects of both anti-eotaxin-1 and anti-eotaxin-2 on eosinophilia are caused only by the reduction of newly produced eosinophils (BrdU⁺) and CD34⁺ eosinophils.

It is known that eotaxin-1 knockout mice have a reduced number of eosinophils in the airways after allergen exposure.¹⁵ It has also been shown that a polyclonal anti-eotaxin-1 antibody will lessen the degree of eosinophilic inflammation, but this reduction is only partial.¹⁶ Our study, for the first time using a mAb administered locally to the airways, confirms the role of eotaxin-1, because an approximately 50% reduction in BAL eosinophilia was observed. Interestingly, the effects of anti-eotaxin-1 were almost exclusively confined to the number of new eosinophils (BrdU⁺) and CD34⁺ eosinophils. However, no effects were observed with anti-eotaxin-1 on blood or BM eosinophils, which strongly suggests that this chemokine is exclusively involved locally at the site of inflammation. In fact, eotaxin-1 expression is seen in airways but to a much lesser extent in BM after airway allergen exposure.¹⁷⁻¹⁹ The expression of CCR3 is upregulated during maturation of CD34 cells to eosinophils, to be maximized in blood eosinophils that presumably are being conditioned for traffic into inflamed tissue.²⁰

Eotaxin-2 is a newly discovered chemokine and is structurally different from eotaxin-1.^{11,12} It was previously known that eotaxin-2 can act as a chemokine for eosinophils in skin.²¹ However, it has recently been shown that eotaxin-2 cooperates with IL-5 within the lung to induce airway eosinophilia and hyperreactivity in mice.²²

The current study shows for the first time that neutralizing eotaxin-2 by treatment with a mAb administered directly into the airways significantly reduces allergen-induced airway eosinophilia. In addition, the effects of anti-eotaxin-2, as with anti-eotaxin-1, were mainly evident on the number of new eosinophils (BrdU⁺), as well as CD34⁺ eosinophils. As with anti-eotaxin-1, no effects were observed with anti-eotaxin-2 on blood or BM eosinophils, which suggests that this chemokine is also involved, primarily within the airways. It is argued that eotaxin-2 is mainly expressed in airway epithelium, which further indicates the role of peripheral tissue, as opposed to BM.²³

Surprisingly, combined local treatment (ie, intranasally) with anti-eotaxin-1 and anti-eotaxin-2 had inhibitory effects similar to those of individually administered mAbs on BAL eosinophils, BrdU⁺ eosinophils, and CD34⁺ eosinophils, irrespective of dose. This finding tends to disprove any synergistic or additive effect of eotaxin-1 and eotaxin-2 in allergic airway inflammation. Both of these chemokines also act on CCR3; therefore, it seems that other chemokines may be involved in the CCR3 response. It has been shown that other chemokines, such as RANTES and monocyte chemoattractant proteins 3 and 4, act via this receptor.²³ Thus, the partial effect observed in our study suggests that other chemokines are involved in the traffic of eosinophils in parallel with eotaxin-1 and eotaxin-2. Interestingly, blocking CCR3 with high doses of a mAb has shown a more pronounced effect than blocking any one of the possible chemokines,²⁴ suggesting that this receptor is crucial in allergic inflammation. Furthermore, CCR3 knockout mice also had a greatly reduced eosinophilic inflammatory response to allergen, but surprisingly, other cells, such as mast cells and basophils, increased in number.²⁵ It is therefore not yet clear whether targeting CCR3 in the treatment of allergic inflammation or asthma is feasible or clinically helpful.

The combined systemic treatment with anti-eotaxin-1 and anti-eotaxin-2 showed a significant decrease in BAL eosinophils compared with isotype controls, but it must be emphasized that this was observed only with the highest dose of mAbs ($300 \mu\text{g} + 300 \mu\text{g}$), which resulted in measurable concentrations of antibody in the BALF. The observed decrease was equal to the reduction of newly produced eosinophils (BrdU⁺) as well as CD34⁺ eosinophils in this compartment. Although trends in blood or BM eosinophil changes were observed, these were not statistically significant (Table E1). Therefore, some role of eotaxin-1 and/or eotaxin-2 in the periphery cannot be excluded.

Compartmentalization of the mAb treatment to the lungs and airways was achieved by administration in the form of intranasal instillation. This resulted in much higher concentrations in BALF compared with intraperitoneal administration (Table I), whereas the latter resulted in higher concentrations in serum. Thus, when evaluating effects of intraperitoneally versus intranasally administered anti-eotaxin-1 and/or anti-eotaxin-2, BAL eosinophilia at antibody doses showing a similar concentration

of rat IgG_{2a} in serum and BALF can be compared. This approach of measuring the antibody concentration in different compartments may be crucial in understanding the site of the effect of this type of treatment. For example, we have previously shown that anti-IL-5 has to have systemic effects to produce a significant reduction of eosinophils in airways after airway allergen exposure.⁵ In some experiments, a higher concentration of control antibodies was found in BALF compared with the specific mAbs, despite administration of an identical dose in the same way at the same time point. It can be speculated that this discrepancy in measured concentration is caused by the binding of specific antibodies to locally released ligands, thus influencing the assay.

A clear finding from our current study is that treatment with either anti-eotaxin-1 or anti-eotaxin-2 will need to reach the airways in sufficiently high concentrations to reduce eosinophils. We can conclude that the concentration of the neutralizing antibody (rat IgG₂) in BALF must be at least 400 ng/mL in the treated animals to result in a significant decrease of BAL eosinophilia. This concentration was achieved by local administration (ie, intranasally) of both anti-eotaxin-1 and anti-eotaxin-2 individually and in combination as well as by high doses of the combined systemic (ie, intraperitoneal) treatment (300 µg + 300 µg; Table I).

The significant, although nonlinear, relationship between the concentration of antibodies in BALF and eosinophils, BrdU⁺ eosinophils, and CD34⁺ eosinophils suggests that the effect of the antibody is concentration-dependent (Table E2). This is clear regarding local treatment with both anti-eotaxin-2 and the combination of anti-eotaxin-1 and anti-eotaxin-2. When these parameters were correlated with the groups given anti-eotaxin-1 locally, the correlation was nonsignificant, probably a result of the fact that a maximal effective dose was already evident at the medium antibody dose. However, we were unable to show any correlation between the antibody measured in BALF or serum and eosinophils in the groups treated systemically (ie, intraperitoneally) with anti-eotaxin-1, anti-eotaxin-2, or a combination of the two.

5-Bromo-2'-deoxyuridine is used as a marker of newly produced eosinophils. The treatment with anti-eotaxin-1 or anti-eotaxin-2 or both significantly reduced these cells. CD34⁺ eosinophils are also reduced in number by the anti-eotaxin-1 and anti-eotaxin-2 treatments. Nevertheless, these cells are likely to be newly produced and relatively less mature because they have not fully shed this surface protein, which is a surface marker for BM stem cells. A recent study by Sehmi et al¹³ shows that CCR3 is upregulated on CD34⁺ cells within the BM in asthmatic patients after allergen challenge. These data together with our results give us reason to speculate that newly produced eosinophils are more responsive to eotaxins, possibly because of the expression of more CCR3. Moreover, it has previously been shown that the expression of CCR3 mRNA is colocalized to mature and immature eosinophils within the BM in patients with atopic asthma.²⁶

An increase in CD34⁺ cells has also been reported in the blood of atopic asthmatic patients, as well as in nasal polyp tissue.²⁷⁻²⁹ The finding of CD34⁺ cells in nasal polyps could suggest that progenitors may be recruited into the airways, where they may differentiate into more mature cells within the site of allergic inflammation (ie, *in situ* hematopoiesis).^{29,30} It is known that CD34⁺ cells in human cord blood express CCR3, which would allow these cells to traffic in a milieu where eotaxin-1 or eotaxin-2 is being released. Thus, our study argues that both eotaxin-1 and eotaxin-2 may be involved in the CD34⁺ cell trafficking.

In conclusion, our study suggests a regulatory role for eotaxin-2 that parallels eotaxin-1 in allergen-induced airway eosinophilia. This effect is mainly localized within the airways, because significant airway concentrations of neutralizing antibodies must be achieved to produce a reduction of eosinophils. Both anti-eotaxin-1 and anti-eotaxin-2 reduce the number of newly produced eosinophils and CD34⁺ eosinophils, which argues for an effect in quite young cells. Targeting eotaxin-1 or eotaxin-2, or perhaps CCR3, may be effective in reducing allergen-induced airway eosinophilia.

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