Regulatory Effects of Eotaxin, Eotaxin-2, and Eotaxin-3 on Eosinophil Degranulation and Superoxide Anion Generation[1]

A.P. BADEWA, C.E. HUDSON, AND A.S. HEIMAN[2]

Florida A & M University, College of Pharmacy and Pharmaceutical Sciences, Tallahassee, Florida 32307

Eosinophilic leukocytes have been implicated as primary effector cells in inflammatory and allergic diseases. When activated by cytokines, human eosinophils secrete and produce a variety of proinflammatory or tissue damaging substances. Although well known for their chemoattractant effects, little is known about the precise contribution of the eosinophil-selective chemokines, eotaxin, eotaxin-2, and eotaxin-3 to the effector functions of eosinophils. This forms the central focus of these investigations for which clone 15-HL-60 human eosinophilic cells were used as the *in vitro* model. Investigation results suggest that all three subtypes of eotaxin directly stimulate eosinophil superoxide anion generation that is inhibited by neutralizing eotaxin antibody or pretreatment of cells with the receptor antibody anti-CCR3. Pretreatment or co-treatment with each of the eotaxins augmented phorbol myristate-induced superoxide generation. Concentration-dependent degranulation of eosinophil peroxidase was noted for all three chemokines, and potentiation of calcium ionophore-induced degranulation was observed with eotaxin pretreatments. Results of interleukin-5 pretreatment studies suggest that the eotaxin chemokines may act cooperatively to enhance effector functions of eosinophils. Collectively, the present studies have advanced knowledge of the eotaxin family of chemokines to include eosinophil priming and modulation of eosinophil activation and secretion effector functions. [Exp Biol Med Vol. 227(8):645–651, 2002]

Key words: eotaxin; eotaxin-2; eotaxin-3; human eosinophils; superoxide anion; eosinophil peroxidase

Eosinophils (EOS) are considered the predominant effector cells in inflammatory and allergic diseases. The physiological/pathophysiological roles of EOS have been described as both tissue protective and tissue damaging. It is now well established that, following activation, human EOS secrete and produce a variety of proinflammatory or tissue-damaging substances, including cationic proteins, major basic protein, EOS peroxidase (EPO), and reactive oxygen species (1–3). Allergic/inflammatory airway diseases such as asthma are characterized by infiltration of EOS, the activation of which lead to long-term changes (4–6). Accumulation of EOS at inflammatory sites is likely to occur as a result of interaction and coordination of several events ranging from bone marrow stem cell EOS differentiation to generation and action of chemoattractants, EOS adhesion, EOS activation, and delayed apoptosis (7).

Among the agents that have been incriminated in EOS tissue infiltration are the recently discovered eotaxins. Of particular interest to these present investigations is the eotaxin family of chemokines. The identification of eotaxin (CCL11), eotaxin-2 (CCL24), and eotaxin 3 (CCL26) as primary EOS recruiting factors with a common receptor has led to extensive research into the EOS-selective functions of these chemokines. Positioning of the four cysteine residues places the eotaxins in the β-chemokine (CC) family of cytokines. All three eotaxins bind to the CCR3 G-protein coupled receptor, a member of the seven-transmembrane-spanning receptor family (8–11).

In human asthmatics, both CCL11 and CCL24 are present at significantly increased levels (7, 12, 13). In contrast, CCL26 levels are not elevated in asthmatics, but dramatically increase 24 hr after allergen challenge (13). In cutaneous responses of atopic subjects, it has recently been found that CCL11 may have a role in an early 6-hr EOS recruitment, whereas CCL24 appeared to be involved in the later 24-hr EOS infiltration (14). Together, these results suggest different mechanisms by which members of the eotaxin chemokines guide EOS allergic responses.

In vitro, effects of CCL11 on human EOS include che-

[1] Support for this research was provided in part by the National Institutes of Health (grants RR08111 and RR03020).

[2] To whom requests for reprints should be addressed at Florida A&M University, College of Pharmacy and Pharmaceutical Sciences, Tallahassee, FL 32307. E-mail: ann.heiman@famu.edu

Received October 15 , 2001.
Accepted April 11, 2002.

1535-3702/02/2278-0645$15.00

motaxis, actin polymerization, and induction of CD11b upregulation (6). Both CCL11 and CCL24 induce chemotaxis and transendothelial migration with approximately equal potency (15, 16). Rapid increases in intracellular calcium ion concentrations have been reported after treatment of human EOS with CCL11, CCL24, or CCL26 (9–11). Pretreatment with either an anti-CCR3 antibody or antagonists has been shown to abolish the eotaxin-induced responses (16–18).

Understanding processes that control selective EOS recruitment, activation, and secretion is a fundamentally important prelude to development of novel mechanism-based therapies for future treatments of allergic/inflammatory diseases. Thus, the present investigations were undertaken to test the hypothesis that the effector functions of the EOS-selective chemokines CCL11, CCL24, and CCL26 extend beyond recruitment and also directly act as EOS proinflammatory-priming/activating agents. To circumvent difficulties that arise when attempting to explore direct effects of cytokines on peripheral blood EOS, clone 15 HL-60 human eosinophilic cells were used.

Methods

EOS Culture and Cell Treatment. Clone 15 HL-60 EOS (clone 15 EOS; ATCC CRL-1964) were purchased from American Type Culture Collection (Rockville, MD) and were grown in suspension in RPMI 1640 (Cellgro; Mediatech, Inc., Herndon, VA) supplemented with 10% fetal calf serum (Atlanta Biologicals, Atlanta, GA), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% carbon dioxide at 35°C. Final differentiation was carried out by culturing cells at 5×10^5 cells/ml in the above medium containing 0.5 m*M* butyric acid for 7 days as previously described (2). Eosinophilic morphology was assessed in Wright-Giemsa-stained slide preparations and with the EOS-specific stain phloxine B and presence of EPO in cytoplasmic granules (2). Cells were maintained by biweekly centrifugation and dilution to a concentration of 3×10^5 cells/ml. Viability of cells harvested for experiments was assessed by trypan blue exclusion, and populations of cells with viability >95% were used.

For experiments, cells were centrifuged (100*g* for 5 min), washed two times in Hank's balanced salts solution (HBSS), and were then resuspended in HEPES-buffered HBSS containing 0.2% bovine serum albumin and aliquoted to 96-well plates. Chemokine stock solutions of CCL11, CCL24, or CCL26 (Atlanta Biologicals) were prepared at 10 μg/ml in the above buffer, aliquots were stored at −80°C, and they were then thawed and diluted immediately before use. Overnight treatments with cytokines were carried out as follows. Cells were centrifuged, washed one time in HBSS, and then resuspended in Dulbecco's modified Eagle's medium (DMEM) without phenol red (Cellgro; Mediatech, Inc.) and supplemented with 10% fetal bovine serum at 1×10^6 cells/ml in 6-well plates. Chemokines were added at the indicated concentrations. Following overnight incubation, cells were washed and suspended in HEPES-buffered HBSS and then stimulated as described above. For neutralization experiments, CCL11 stock solutions were preincubated with anti-eotaxin goat polyclonal IgG (at 1:250 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hr at 37°C, and were then used to stimulate clone 15 EOS (19). For neutralization of cell surface CCR3-mediated bioactivity, cells were preincubated with anti-human CCR-3 IgG (10 μg/ml; R&D Systems, Minneapolis, MN) for 30 min prior to chemokine stimulation.

Assessment of Superoxide Anion Generation. Superoxide anion generation was assessed in experiments conducted in microtiter plates with 1×10^5 cells/well in a total volume of 0.1 ml HEPES-buffered HBSS containing 0.2% bovine serum albumin and 0.5 mg ferricytochrome C with and without 0.65 m*M* superoxide dismutase. After treatment with recombinant human chemokines or phorbol myristate acetate (PMA) and incubation at 35°C in an atmosphere of 5% CO_2, absorbances were read at 550 nm to determine superoxide dismutase-inhibitable reduction of ferricytochrome C (20). Results were presented as nanomoles superoxide anion generated/10^6 EOS.

Assessment of EPO Degranulation. Because of the losses in recoverable product once EPO is secreted, release of this enzyme is expressed as the difference between the EPO content of the cell pellets before activation and the content of the pellets from activated cells using the following equation: net % secretion = $100\ ([EPO]_{f,\ control} - [EPO]_{f,\ experimental})/[EPO]_i$, where [EPO] represents the EPO concentration, subscript i is the initial or total EPO present in cells, subscript f, control is the final EPO levels in unstimulated cells, and subscript f, experimental is the final EPO levels in stimulated cells. After stimulation with chemokines and/or ionophore A23187, cells were centrifuged and supernatants were removed. EPO content in 0.1% Triton-X-100-solubilized pellets was quantified from the change in absorption at 490 nm in a microtiter-adapted enzymatic-colorimetric assay. Aliquots of sample (50 μl) were incubated with 50 μl of substrate solution containing 1.8 m*M* *o*-phenylenediamine and 0.4 m*M* H_2O_2 in 0.1 *M* phosphate buffer containing 0.1% Triton-X-100 (pH 8.0) for 30 min at 37°C followed by acidification with 4 *M* sulfuric acid (21, 22). Results are shown as the percentage of total EPO.

Data Handling and Analysis. Experiments were repeated on at least three to four separate occasions with the same outcome. Data shown are from a representative experiment and are expressed as mean ± SEM One-way analysis of variance (ANOVA) was applied to all experimental results. When indicated, ANOVA was followed either by the Dunnett's multiple comparisons post test or the Tukey-Kramer multiple comparisons post test to determine statistical significance ($P < 0.05$) between indicated groups.

Results

The Eotaxins CCL11, CCL24, and CCL26 Directly Stimulate Clone 15 EOS to Generate Superoxide Anion. Distinctions between "complete" activators and "incomplete" activators or "primers" have been proposed (23). In these investigations, complete activators are agents that singly induce superoxide anion production, whereas incomplete activators require a second agent prior to the cellular response. To investigate the direct effects of the chemokines as complete EOS-selective activating agents, clone 15 EOS were incubated with various concentrations of CCL11 or CCL24 (1–30 ng/ml), or CCL26 (3–100 ng/ml) for 2 hr. Results, depicted in Figure 1, indicate a concentration-dependent generation of superoxide anion reaching a maximum of 1.3 n$M/10^6$ cells with CCL11, 1.2 n$M/10^6$ cells with CCL24, and 1.1 n$M/10^6$ cells with CCL26. Although CCL11 or CCL24 generated statistically significant amounts of superoxide anion at 10–30 ng/ml, CCL26-induced increases were significant at 100 ng/ml. To demonstrate that chemokine stimulation was responsible for superoxide anion generation, clone 15 EOS were stimulated with CCL11 alone or CCL11 pretreated with anti-eotaxin goat polyclonal IgG. Results, shown in Table I, indicate very significant inhibition of superoxide generation by clone 15 EOS stimulated with antibody-treated eotaxin.

It has been reported that all three eotaxins bind to the CCR3 cell surface receptor (9–11). To determine whether CCL11, CCL24, or CCL26 were stimulating superoxide anion generation through the CCR3 receptor, clone 15 EOS were pretreated with human anti-CCR3 IgG at the reported half-maximal neutralization concentration of 10 μg/ml prior to chemokine stimulation. Results, depicted in Figure 2, indicate that all three eotaxins stimulate superoxide anion generation through the CCR3 receptor as indicated by significant inhibitions in the range of 50%–70% for CCL11 and CCL24 at 3–30 ng/ml and CCL26 at 30 ng/ml. Taken together, these results suggest that the chemokines CCL11, CCL24, and CCL26 can act through the CCR3 cell surface receptor as complete activators and can directly stimulate clone 15 EOS to generate modest amounts of superoxide anion.

Table I. Effect of Anti-Eotaxin Treatment on CCL11-Induced Superoxide Generation

CCL11 (ng/ml)	CCL11 alone	CCL11 + antieotaxin
0	0.686 ± 0.114	0.472 ± 0.257
1	1.54 ± 0.304	0.635 ± 0.198
3	2.22 ± 0.075	0.576 ± 0.260**
10	2.48 ± 0.345	0.422 ± 0.186**
30	2.54 ± 0.199	0.211 ± 0.043**

Note. Cells were washed, resuspended in HEPES-buffered HBSS, and were dispensed to 96-well plates (1×10^5 cells/well) that contained ferricytochrome C both in presence and absence of superoxide dismutase. CCL11 (1–30 ng/ml) or CCL11 preincubated with anti-eotaxin goat polyclonal IgG (1:250 dilution) was added and the cells were incubated for 2 hr. Absorbances were read at 550 nm to determine superoxide dismutase-inhibitable reduction of ferricytochrome C. Table values indicate nanomoles superoxide anion/10^6 cells from a representative experiment conducted in triplicate on three separate occasions. Asterisks indicate values that differed from cytokine alone treatments ($P < 0.01$).

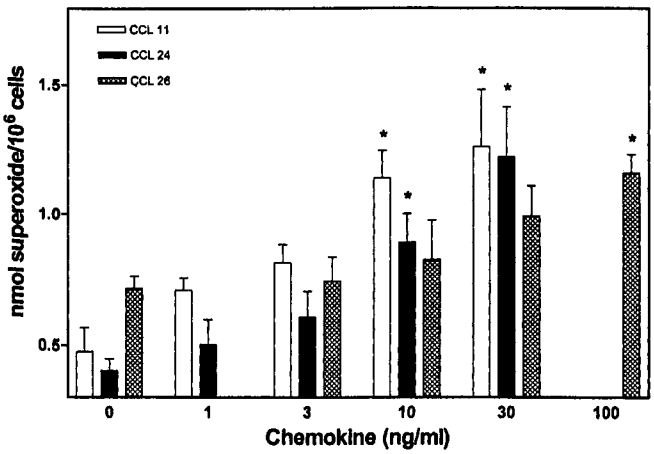

Figure 1. The eotaxins CCL11, CCL24, and CCL26 directly stimulate clone 15 EOS to generate superoxide anion. Cells were washed, resuspended in HBSS, and dispensed to 96-well plates (1×10^5 cells/well) that contained ferricytochrome C both in presence and absence of superoxide dismutase and indicated concentrations of CCL11, CCL24, or CCL26. Plates were incubated for 2 hr, and absorbances were read at 550 nm to determine superoxide dismutase-inhibitable reduction of ferricytochrome C. Data shown are the mean ± SEM of a representative experiment conducted in triplicate on three separate occasions. Asterisks indicate values that differed significantly from untreated controls ($P < 0.05$) assessed by ANOVA followed by Dunnett's multiple comparisons post test.

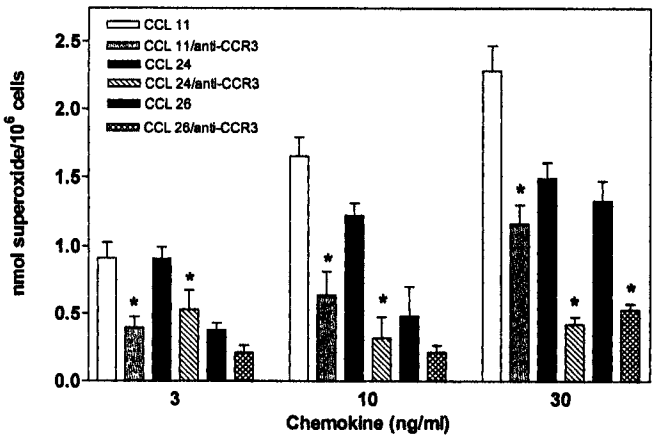

Figure 2. Treatment of clone 15 EOS with anti-CCR3 antibody inhibits CCL11, CCL24, and CCL26 stimulated superoxide anion generation. Following pretreatment of washed cells with 10 mg/ml human anti-CCR3 IgG for 30 min, experiments were carried out as described in Figure 1. Data shown are the mean ± SEM of a representative experiment conducted in triplicate on three separate occasions. Background generation of superoxide anion, which averaged 0.396 n$M/10^6$ cells, has been subtracted from the data shown. ANOVA was followed by the Tukey-Kramer multiple comparisons post test to determine significance between anti-CCR3 antibody pretreated versus no antibody pretreatment groups. Asterisks indicate values that differed significantly between groups at $P < 0.05$.

PMA-Stimulated Superoxide Anion Generation in Clone 15 EOS Is Potentiated by the Eotaxins. The priming effect of eotaxin (CCL11) on calcium ionophore-induced EOS superoxide anion generation has been reported (5). When PMA was used as the sole stimulating agent over a range of 3 pM–100 n*M*, clone 15 EOS routinely generated 2–20 n*M* superoxide/10^6 cells/3 hr (data not shown). In the present investigations, eotaxin priming effects were explored. As depicted in Figure 3, clone 15 EOS stimulated directly with PMA (1 n*M*) alone generated an average of 6.53 ± 0.325 n*M* superoxide anion/10^6 cells. EOS pretreated with CCL11 (1–30 ng/ml) or CCL24 (3–30 ng/ml) generated significant and concentration-dependent increases in superoxide anion. CCL26 treatment yielded a significant increase at 100 ng/ml. To demonstrate whether the eotaxin family of chemokines could augment superoxide anion production when present as co-stimuli, clone 15 EOS were concomitantly treated with the indicated concentrations of CCL11, CCL24, CCL26, and 1 n*M* PMA. Representative results, shown in Figure 4, indicate that co-stimulation with CCL11 significantly increased superoxide generation by 32%, and CCL24 by 45% when each was present at 30 ng/ml. In contrast, co-treatment with CCL26 increased superoxide anion release by 11%. Taken together, these results suggest that the eotaxins CCL11, CCL24, and CCL26 may influence clone 15 EOS effector functions as "priming" agents and may also augment effector functions when present as co-stimulants.

Effect of Interleukin (IL)-5 Pretreatment on Superoxide Anion Generation Induced by Eotaxins and PMA. It has recently been shown that CC chemokine receptors 1 and 3 are differentially regulated by IL-5 during maturation of clone 15 EOS (24). To explore the effect of IL-5 exposure on superoxide anion generation by CCL11, CCL24, or CCL26, EOS were treated for 72 hr without or with IL-5 (20 ng/ml), followed by stimulation with indicated concentrations of the chemokines then PMA. In the

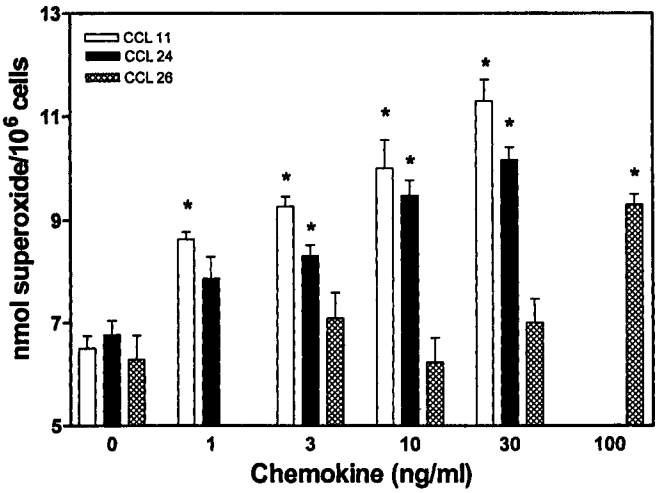

Figure 3. Pretreatment with CCL11, CCL24, or CCL26 increases clone 15 EOS superoxide anion generation in response to PMA. Experiments were carried out as described in Figure 1. Following a 3-hr incubation with the chemokines, cells were stimulated for an additional 2 hr with 1 n*M* PMA. Data shown are the mean ± SEM of a representative experiment conducted in triplicate on three separate occasions. Asterisks indicate values that differed significantly from cells treated with PMA alone as assessed by ANOVA ($P < 0.05$) followed by Dunnett's multiple comparisons post test.

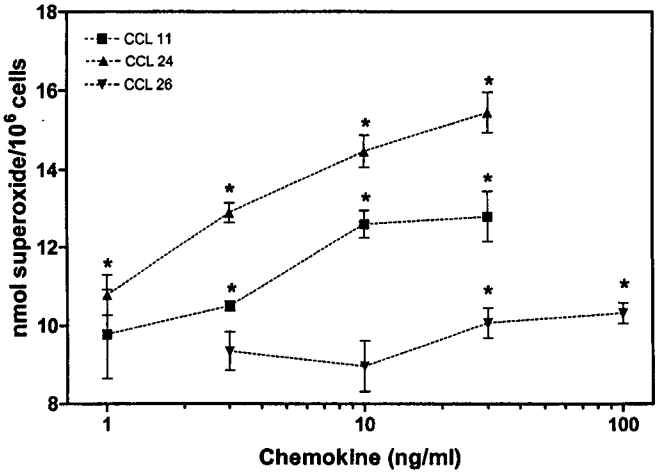

Figure 4. Co-stimulation of clone 15 EOS with the eotaxins and PMA increased superoxide anion generation. Experiments were carried out as described in Figure 1 with co-stimulation of cells with indicated concentrations of CCL11, CCL24, or CCL26 each with 1 n*M* PMA for 2 hr. In the absence of cytokines, cells generated 7.46 ± 0.324 n*M* superoxide anion/10^6 cells when stimulated with PMA alone. Data shown are the mean ± SEM of a representative experiment conducted in triplicate on three separate occasions. Asterisks indicate values that differed significantly from cells treated with PMA alone as assessed by ANOVA ($P < 0.05$) followed by Dunnett's multiple comparisons post test.

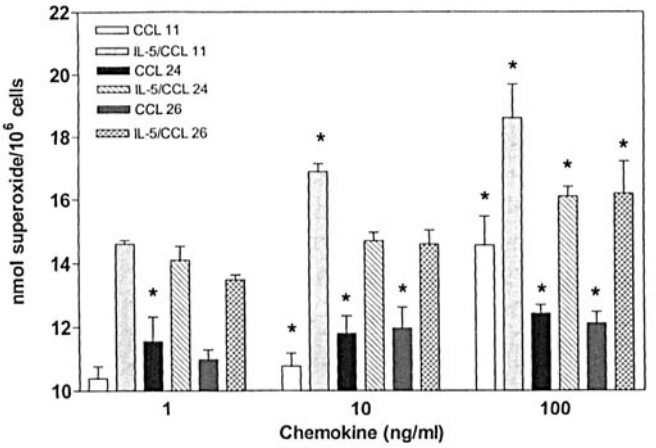

Figure 5. The chemokines CCL11, CCL24, or CCL26 potentiate PMA-stimulated superoxide anion generation by clone 15 EOS pretreated with IL-5. Cells were pretreated for 72 hr without or with 20 ng/ml IL-5, and were then washed, resuspended in HBSS, and dispensed to 96-well plates as described in Figure 1. Cells were stimulated with 1, 10, or 100 ng/ml CCL11, CCL24, or CCL26 for 3 hr followed by 1 n*M* PMA for an additional 2 hr. In the absence of chemokines and IL-5, cells generated 9.31 ± 0.241 n*M* superoxide anion/10^6 cells in response to PMA stimulation. Cells pretreated with IL-5 and stimulated with PMA generated 13.6 ± 0.492 n*M* superoxide anion/10^6 cells. Data shown are the mean ± SEM of a representative experiment conducted in triplicate on three separate occasions. ANOVA was followed by the Tukey-Kramer multiple comparisons post test to determine significance between IL-5 versus no IL-5 pretreatment groups. Asterisks indicate values that differed significantly between groups at $P < 0.05$.

absence of chemokines, IL-5 treatment increased PMA-induced superoxide generation from 9.3 to 13.6 n$M/10^6$ cells as described in Figure 5. At 10 and 100 ng/ml for CCL11, and 100 ng/ml for CCL24 and CCL26, superoxide anion generation was significantly augmented, suggesting that IL-5 and the eotaxin chemokines may act cooperatively to enhance effector functions of clone 15 EOS.

The Eotaxins CCL11, CCL24, and CCL26 Induce EPO Degranulation from EOS. It has recently been shown that chemokine family members induce EOS-derived neurotoxin degranulation through the CCR3 chemokine receptor (25). To investigate the direct effect of members of the eotaxin family on EOS degranulation, cells were incubated for 1 hr with 1–30 ng/ml CCL11 or CCL24 and 3–100 ng/ml CCL26 as the sole stimulating agents. The percentage of EPO released is depicted in Figure 6. Concentration-dependent degranulation was noted for all three chemokines with ranges of 10%–24% for CCL11, 9%–20% for CCL24 (1–30 ng/ml), and 8%–21% for CCL26 (3–100 ng/ml). When clone 15 EOS were pretreated with human anti-CCR3 IgG, CCL11-, CCL24-, or CCL26-stimulated degranulation of EPO was inhibited by 56%–80% (data not shown). Prolonged (18 hr) exposure of eosinophils to eotaxins on calcium ionophore-induced degranulation was then tested. EOS were pretreated with CCL11 or CCL24 (10 ng/ml) or CCL26 (100 ng/ml) for 18 hr and were then stimulated with 0.3–10 μg/ml calcium ionophore A23187. Results indicated significant potentiation of ionophore A23187-induced EPO degranulation by CCL11(1.51-fold increase), CCL24 (1.35-fold increase), and CCL26 (1.41-fold increase). These results suggest that the eotaxin family of chemokines may act through the CCR3 cell surface receptor as the sole degranulating agent or may augment the degranulating effector function of EOS in response to other secretagogs.

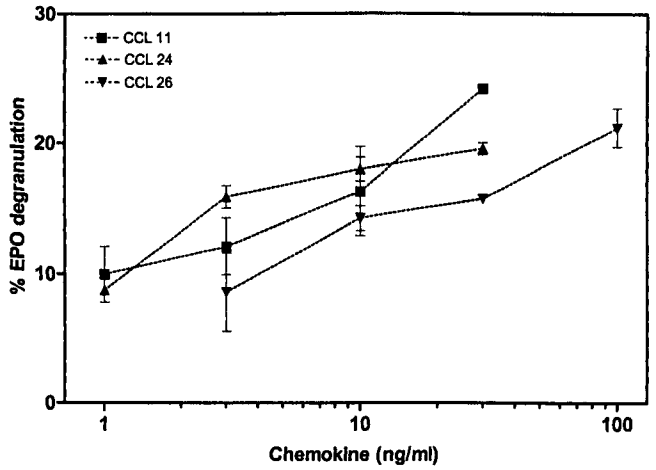

Figure 6. The eotaxins CCL11, CCL24, and CCL26 directly stimulate clone 15 EOS to degranulate EPO. Washed cells were dispensed to microcentrifuge tubes and were stimulated for 1 hr with the indicated concentrations of chemokines. Following incubation, cell were pelleted, washed, and residual EPO was assessed in Triton-X-lysed pellets. The percentage of EPO released was calculated by comparing absorbances of stimulated Triton-X-lysed pellets with those of untreated lysates. Data shown are the mean ± SEM of a representative experiment conducted in triplicate on three separate occasions.

Discussion

These investigations were conducted to explore the effects of the EOS-selective chemokines CCL11, CCL24, and CCL26 beyond their reported chemotactic functions. Clone 15 HL-60 human EOS were used as the *in vitro* model to circumvent the issue of employing *in vivo*-primed or -activated EOS that may be isolated from allergic and/or hypereosinophilic individuals and then studied *ex vivo*. It has recently been shown that the cells used in the present investigations express mRNA for the CCR3 receptor, express eotaxin binding sites, and exhibit calcium flux and chemotactic responses to the CCR3 ligand (24). CCL11-induced EOS responses, mediated through the CCR3 receptor, are blocked by anti-CCR3 (26). Thus, this cell line is a useful EOS *in vitro* model. However, results with this cell line may not be identical to results of experiments carried out with eosinophils from normal or atopic persons.

Results of the present investigations indicate a concentration-dependent superoxide anion generation of cells treated directly with CCL11, CCL24, or CCL26 as the sole stimulating agent (Fig. 1). After eotaxin neutralization with an anti-eotaxin antibody, superoxide anion generation was significantly reduced, suggesting the response was due to chemokine stimulation (Table I). Both eotaxin and eotaxin-2 have been shown to induce release of reactive oxygen species, as measured by lucigenin-dependent chemiluminescence in isolated, purified human EOS. It was suggested that in the same range of efficacy, both chemokines play important roles as chemoattractants and, indirectly, as tissue-damaging agents at sites of inflammation (27, 14). Results of the present investigations using the chemokines as the sole EOS stimulating agents corroborate these findings for superoxide anion generation and extend the findings to include the newly discovered chemokine CCL26. Results also suggest that the chemokine bioactivity is CCR3 mediated (Fig. 2).

CCL11, CCL24, or CCL26 pretreatment or co-stimulation significantly increased PMA-induced superoxide anion generation (Figs. 3 and 4). Potentiation of the response was most evident following treatments with either CCL11 or CCL24. As reported for chemotactic activity, CCL26 was less potent than the other eotaxins (18, 28). In similar investigations using both the modified anti-CD16 negative selection isolated peripheral blood EOS and the YY-1 eosinophilic cell line, eotaxin (CCL11) primed cells for calcium ionophore A23187 evoked reactive oxygen species production that was assessed by a lucigenin-dependent chemiluminescence reaction (5, 29). Results of the present investigation, which focus specifically on superoxide anion, extend the effector functions beyond CCL11 and include CCL24 and CCL26 as clone 15 EOS-priming/potentiating agents.

There is *ex vivo* and *in vitro* evidence to indicate that

IL-5 and CCL11 may cooperate to selectively and synergistically promote eosinophilia by transendothelial migration (30), and upregulate CCR3 receptors and CCR3-mediated chemotaxis and adhesion (31). Marked CCR3 upregulation has been shown in the IL-5/butyric acid-treated clone 15 HL-60 EOS used in these studies (24). IL-5 pretreatment followed by eotaxin and PMA stimulations resulted in significant increases of superoxide anion release (fig. 5). These results suggest that IL-5 and the chemokines CCL11, CCL24, and CCL26 may act cooperatively to enhance effector functions of EOS.

EOS granule proteins, which may be linked to chemokine stimulation, are toxic to respiratory epithelium, and concentrations of these proteins in the toxic range are present in respiratory secretions of asthmatics (1). In studies with magnetic bead-isolated EOS from peripheral blood of mildly atopic donors, increased Ca^{2+} influx and degranulation of EDN resulted from stimulation of the CCR3 receptor by either eotaxin or RANTES (25). Similar exocytosis of EDN and IL-4 were noted when normal human donor-derived EOS were stimulated with eotaxin (32, 33). Results of the present studies using the *in vitro* clone 15 HL-60 EOS model where CCL11, CCL24, and CCL26 could be used as the sole stimulating agents are in agreement with study results from EOS studied *ex vivo*. Each of the chemokines induced concentration-dependent degranulation of EPO (Fig. 6) and augmented degranulation induced by the calcium ionophore A23187. These present investigations extend the degranulating capabilities of chemokines to include EPO and the CCL24 and CCL26 members of the eotaxin family.

In conclusion, the present studies have advanced knowledge of the eotaxin family of chemokines and their possible roles in the pathogenesis of eosinophilic inflammation. Roles of the eotaxins CCL11, CCL24, and CCL26 extend beyond selective recruitment and include priming, activation, and secretion effector functions. Understanding processes that control selective recruitment, priming, and activation of EOS may be a fundamentally important prelude to development of novel mechanism-based therapies that focus on cytokine and/or chemokine downregulation.

1. Gleich GJ. Mechanisms of eosinophil-associated inflammation. J Allergy Clin Immunol **105:**651–663, 2000.
2. Heiman AS, Allen-Gipson D. Cytokines potentiate human eosinophil superoxide generation in the presence of N^{w}-nitro-L-arginine methyl ester. Int J Immunopharmacol **22:**171–181, 2000.
3. Giembycz MA, Lindsay MA. Pharmacology of the eosinophil. Pharmacol Rev **51:**213–340, 1999.
4. Gleich GJ, Adolphson CR. The eosinophil and bronchial asthma: evidence for a critical role in pathophysiology. In: Sanderson CJ, Ed. Interleukin-5: From Molecule to a Drug Target for Asthma. New York: Marcel Dekker, Vol. **125:**pp1037, 1998.
5. Honda K, Chihara J. Eosinophil activation by eotaxin: eotaxin primes the production of reactive oxygen species from eosinophils. Allergy **54:**1262–1269, 1999.
6. Tenscher K, Metzner B, Schopf E, Norgauer J, Czech W. Recombinant human eotaxin induces oxygen production, Ca^{++} mobilization, actin reorganization, and CD11b upregulation in human eosinophils via a pertussis toxin-sensitive heterotrimeric guanine nucleotide-binding protein. Blood **88:**3195–3199, 1996.
7. Nickel R, Beck LA, Schleimer R. Chemokines and allergic diseases. J Allergy Clin Immunol **104:**723–742, 1999.
8. Bjornsdottir US, Cypcar DM. Asthma: an inflammatory mediator soup. Allergy **54:**55–61, 1999.
9. White JR, Imburgia C, Dul E, Appelbaum E, O'Donnell K, O'Shannessey DJ, Brauner M, Fornwald J, Adamou J, Elshourbagy NA, Kaiser K, Foley JJ, Schmidt DJ, Johanson K, Macphee C, Moores K, McNulty D, Scott GF, Schleimer RP and Sarau HM. Cloning and functional characterization of a novel human CC chemokine that binds to the CCR3 receptor and activates human eosinophils. J Leukocyte Biol **62:**667–675, 1997.
10. Forssmann U, Uguccioni M, Loetscher P, Dahinden CA, Langen H, Thelen M, Baggiolini M. Eotaxin-2, a novel CC chemokine that is selective for the chemokine receptor CCR3 and acts like eotaxin in human eosinophil and basophil leukocytes. J Exp Med **185:** 2171–2176, 1997.
11. Kitaura M, Suzuki N, Imai T, Takagi S, Suzuki R, Nakajima T, Hirai K, Nomiyama H, Yoshie O. Molecular cloning of a novel human CC chemokine (eotaxin-3) that is a functional ligand of CC chemokine receptor-3. J Biol Chem **274:**27975–27980, 1999.
12. Wardlaw AJ. Molecular basis for selective eosinophil trafficking in asthma. J Allergy Clin Immunol **104:**917–926, 1999.
13. Berkman N, Ohnona S, Chung F, Breuer R. Eotaxin-3 but not eotaxin gene expression is upregulated in asthmatics 24 hours after allergen challenge. Am J Respir Cell Mol Biol **24:**682–687, 2001.
14. Ying S, Robinson DS, Meng Q, Barata LT, McEuen AR, Buckley MG, Walls AF, Askenase PW, Kay AB. C-C chemokines in allergen-induced late-phase cutaneous responses in atopic subjects. J Immunol **163:**3976–3984, 1999.
15. Elsner J, Petering H, Kluthe C, Kummig D, Smolarski R, Ponath P, Kapp A. Eotaxin-2 activates chemotaxis-related events and release of reactive oxygen species via pertussis toxin-sensitive G proteins in human eosinophils. Eur J Immunol **28:**2152–2158, 1998.
16. Shahabuddin S, Ponath P, Schleimer RP. Migration of eosinophils across endothelial cell monolayers: interactions among IL-5, endothelial-activating cytokines, and C-C chemokines. J Immunol **164:** 3847–3854, 2000.
17. White JR, Lee JM, Dede K, Imburgia CS, Jurewicz AJ, Chan G, Fornwald JA, Dhanak D, Christmann LT, Darcy MG, Widdowson KL, Foley JJ, Schmidt DB and Sarau HM. Identification of potent, selective non-peptide CC chemokine receptor-3 antagonist that inhibits eotaxin-, eotaxin-2-, and monocyte chemotactic peptide-4-induced eosinophil migration. J Biol Chem **275:**36626–36621, 2000.
18. Betrand CP, Ponath PD. CCR3 blockade as a new therapy for asthma. Expert Opin Invest Drugs **9:**43–52, 2000.
19. Ghaffar M, Hamid Q, Renzi PM, Allakhverdi Z, Molet S, Hogg JC, Shore SA, Luster AD, Lamkhioued B. Constitutive and cytokine-stimulated expression of eotaxin by human airway smooth muscle cells. Am J Respir Crit Care Med **159:**1933–1942, 1999.
20. Fabian I, Lass M, Kletter Y, Golde DW. Differentiation and functional activity of human eosinophilic cells from an eosinophil HL-60 subline: response to recombinant hematopoietic growth factors. Blood **80:**788–794, 1992.
21. Bach MK, Brashler JR, Sanders ME, Bienkowski MJ. Pitfalls in the quantitative estimation of the secretion of granule proteins by eosinophils. J Immunol Methods **142:**243–250, 1991.
22. Horie S, Gleich GJ, Kita H. Cytokines directly induce degranulation and superoxide production from human eosinophils. J Allergy Clin Immunol **98:**371–381, 1996.
23. Bischoff SC, Brunner T, DeWeck A, Dalhinden CA. Interleukin-5 modifies histamine release and leukotriene generation by human basophils in response to diverse agonists. J Exp Med **172:**1577–1582, 1990.
24. Tiffany HL, Alkhatib G, Combadiere C, Berger EA, Murphy PM. CC

chemokine receptors 1 and 3 are differentially regulated by IL-5 during maturation of eosinophilic HL-60 cells. J Immunol **160:**1385–1392, 1998.
25. Fujisawa T, Kato Y, Hagase H, Atsuta J, Terada A, Iguchi K, Kamiya H, Morita Y, Kitaura M, Kawasaki H, Yoshie O. and Hirai K. Chemokines induce eosinophil degranulation through CCR-3. J Allergy Clin Immunol **106:**507–513, 2000.
26. Heath H, Qin SX, Rao P, Wu JJ, Larosa G, Kassam N, Ponath PD, Mackay CR. Chemokine receptor usage by human eosinophils: the importance of CCR3 demonstrated using an antagonistic monoclonal antibody. J Clin Invest **99:**178–184, 1997.
27. Elsner J, Hochstetter R, Kimmig D, Kapp A. Human eotaxin represents a potent activator of the respiratory burst of human eosinophils. Eur J Immunol **26:**1919–1925, 1996.
28. Teran M. CCL chemokines and asthma. Immunol Today **21:**235–242, 2000.
29. Honda K, Yamada Y, Cui C, Saito N, Kayaba H, Lobayashi Y, Oyamada H, Kamada Y, Kuwasaki T, Tsurufuji S and Chihara J. Effect of eotaxin on the generation of reactive oxygen species from eosinophil cell line, YY-1. Int Arch Allergy Immunol **120**(Suppl 1):48–50, 1999.
30. Foster PS, Mould AW, Yang M, Mackenzie J, Mattes J, Hogan SP, Mahalingam S, Mckenzie AN, Rothenberg ME, Young IG, Matthae KI and Webb DC. Elemental signals regulating eosinophil accumulation in the lung. Immunol Rev **179:**173–181, 2001.
31. Zimmermann N, Daugherty BL, Stark JM, Rothenberg ME. Molecular analysis of CCR-3 events in eosinophilic cells. J Immunol **164:** 1055–1064, 2000.
32. El-Shazly A, Masuyama K, Nakano K, Eura M, Samejima Y, Ishakawa T. Human eotaxin induced eosinophil-derived neurotoxin release from normal human eosinophils. Int Arch Allergy Immunol **117**(Suppl 1):55–58, 1998.
33. Bandeira-Mela C, Sugiyama K, Woods LJ, Weller PF. Eotaxin elicits rapid vesicular transport-mediated release of preformed IL-4 from human eosinophils. J Immunol **166:**4813–4817, 2001.