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Upregulated response to chemokines in oxidative metabolism of eosinophils in asthma and allergic rhinitis

S. Sannohe*,#, T. Adachi*, K. Hamada*, K. Honda#, Y. Yamada*, N. Saito*, C-H. Cui*, H. Kayaba*, K. Ishikawa#, J. Chihara*

Upregulated response to chemokines in oxidative metabolism of eosinophils in asthma and allergic rhinitis. S. Sannohe, T. Adachi, K. Hamada, K. Honda, Y. Yamada, N. Saito, C-H. Cui, H. Kayaba, K. Ishikawa, J. Chihara. ©ERS Journals Ltd 2003.
ABSTRACT: Reactive oxygen species (ROS) from eosinophils are known to cause tissue damage in allergic inflammation. CC chemokines, especially eotaxin and regulated on activation, normal T-cell expressed and secreted (RANTES), are involved not only in chemotaxis but also in eosinophil activation, such as ROS production. It has been shown that eosinophils from allergic patients are not functionally equivalent to those from normal subjects. In the present study, the characteristics of chemokine-primed ROS production in eosinophils from allergic patients and normal controls were compared.

After pretreatment with chemokines, eosinophils were stimulated with calcium ionophore A23187. ROS production by eosinophils was measured using luminol-dependent chemiluminescence.

Both RANTES and eotaxin exhibited a priming effect on calcium ionophore-induced ROS production from eosinophils. Despite there being no difference in expression of CC chemokine receptor 3, the priming effect of RANTES and eotaxin was significantly enhanced in eosinophils from the patients. Interleukin-5 further enhanced the priming effect of chemokines in eosinophils from normal subjects, but not those from allergic subjects.

The present results suggest an upregulated response to chemokines in eosinophils from allergic patients, and that interleukin-5 can induce a similar phenotype to that found *in vivo* in allergic patients.
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Depts of *Clinical and Laboratory Medicine and #Oto-Rhino-Laryngology, Akita University School of Medicine, Akita, Japan.

Correspondence: J. Chihara, Dept of Clinical and Laboratory Medicine, Akita University School of Medicine, Hondo, Akita 010-8543, Japan.
Fax: 81 188362624
E-mail: chihara@hos.akita-u.ac.jp

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One characteristic feature of allergic disease is tissue inflammation, involving the activation of T-lymphocytes and eosinophils [1]. The severity of allergic disease is influenced by the degree of eosinophil activation. During the process of allergic inflammation, eosinophils migrate into tissues and release toxic granule proteins and reactive oxygen species (ROS), leading to tissue damage [2].

ROS production is elicited by several stimuli, such as immunoglobulins (Igs) and cytokines [3]. It has been previously reported that the signal from adhesion molecules plays a critical role in ROS production by eosinophils [4]. The CC chemokines, especially eotaxin and regulated on activation, normal T-cell expressed and secreted (RANTES), possess a selective chemotactic activity for eosinophils. Besides chemotaxis, these chemokines are involved in eosinophil activation. Indeed, it has been shown recently that chemokines prime ROS production by eosinophils [5, 6].

It has been shown that eosinophils from allergic patients are not equivalent in effector function to those from normal subjects [7–9]. However, the different response of eosinophils to chemokines has not been fully elucidated. Therefore, in the present paper, comparative studies were performed in allergic patients and normal subjects regarding the priming effects of chemokines on ROS production from eosinophils.

Materials and methods

Subjects

Venous blood was drawn from 12 healthy nonallergic adults (age 18–40 yrs, mean 27.3 yrs; four females) and from 15 patients with allergic diseases of the respiratory tract (23–32 yrs, mean 25.1 yrs; five females). Age and sex distribution were not significantly different between normal subject and patient groups. All subjects gave informed consent, and the study was carried out according to the principles of the Declaration of Helsinki. None of the subjects had received either any medication for $\geqslant$24 h or steroids for $\geqslant$2 weeks before blood collection. Normal subjects were defined on the basis of a lack of a clinical history of allergy or other similar diseases. All patients had allergic asthma and/or allergic rhinitis, IgE concentrations of >400 International Units (IU)·mL^{-1} and an IgE radioallergosorbent test result of higher than class 3 against at least one of the common airborne allergens, such as house dust mite, pollens or fungi. The numbers of patients with asthma and allergic rhinitis were seven and 11, respectively (three had both asthma and allergic rhinitis). Asthmatic patients participating in the present study met the American Thoracic Society's definition of asthma. All patients with allergic rhinitis showed symptoms at the

time of blood collection (nasal congestion, sneezing, rhinorrhoea, itchy eyes, *etc.*). The eosinophil counts in the peripheral blood of patients were significantly higher than those of normal subjects (683.3±374.0 *versus* 126.0±80.4 cells·mm^{-3}; $p<0.01$).

Eosinophil isolation

Eosinophils were isolated from heparinised venous blood using a modified CD16 negative selection method, as previously described [10]. In brief, cells obtained from the buffy coat were incubated with anti-CD16, anti-CD3, anti-CD20 and anti-CD14 monoclonal antibodies (mouse IgG; Nichirei, Tokyo, Japan), and subsequently reacted with antimouse IgG magnetic beads (Dynal, Oslo, Norway). CD16-, CD3-, CD20- and CD14-negative eosinophils were obtained using a magnetic cell-sorting system (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the eosinophils was >97%.

Luminol-dependent chemiluminescence

ROS production from eosinophils was examined by means of luminol-dependent chemiluminescence [5]. Previously, an apparent effect of eotaxin and RANTES on eosinophil oxidative metabolism was found after 15-min incubation [5, 6]. Thus purified eosinophils (1×10^6 cells·mL^{-1}) were suspended in Roswell Park Memorial Institute (RPMI) 1640 medium and incubated with 1–100 nM eotaxin (R & D Systems, Minneapolis, MN, USA) or RANTES (Sigma, St Louis, MO, USA) in 96-well flat-bottomed plates in the presence or absence of 1 ng·mL^{-1} interleukin (IL)-5 or granulocyte macrophage-colony stimulating factor (GM-CSF) (R & D Systems) for 15 min at 37°C. In some experiments, eosinophils were pretreated with an anti-IL-5 receptor alpha (IL-5Rα) antibody (mouse IgG1κ; Pharmingen, San Diego, CA, USA) or an isotype-matched control (Pharmingen), both at 0.2 μg·mL^{-1}, for 60 min at 4°C, or a CC chemokine receptor (CCR) 3 antagonist (Compound X; a gift from Banyu Pharmaceutical Co., Ltd., Tsukuba, Japan) for 30 min at 37°C. ROS production was evoked by adding 50 μL calcium ionophore A23187 (Sigma; final concentration 1×10^{-5} M) to 100 μL eosinophil suspension (5×10^4 cells) containing 0.25 mM luminol (Futaba Medical, Tokyo, Japan). Maximal and integral intensity chemiluminescence were determined for 60 min using an ARGUS-50/2D luminometer (Hamamatsu Photonics, Hamamatsu, Japan).

Flow cytometric analysis of eosinophil surface CC chemokine receptor 3

Purified eosinophils ($<1\times10^6$ cells) were incubated with a fluorescein isothiocyanate (FITC)-conjugated antihuman CCR3 monoclonal antibody (mouse IgG2; DAKO, Glostrup, Denmark; 0.5 μg·mL^{-1}) for 30 min at 37°C. An FITC-conjugated IgG2 isotype-matched control monoclonal antibody (Beckton-Dickinson, San Jose, CA, USA; 0.5 μg·mL^{-1}) was applied to assess the degree of nonspecificity. After washing the cells, the stained cells were analysed using a FACScan flow cytometer (Beckton-Dickinson).

Measurement of intracellular calcium concentration

Purified eosinophils from normal subjects were suspended in Hank′s balanced salt solution (HBSS) containing Ca^{2+} (0.14 g·mL^{-1} $CaCl_2$), Mg^{2+} (0.1 g·mL^{-1} $MgCl_2\cdot6H_2O$; 0.1 g·mL^{-1} $MgSO_4\cdot7H_2O$) and 2% foetal calf serum (Sigma) at a cell density of 2×10^6 cells·mL^{-1}. Fura-2-acetoxymethyl ester (DOJINDO, Kumamoto, Japan) was added at a final concentration of 2 μM. After incubation for 40 min, excess dye was removed by centrifugation for 5 min at 270×*g* at 4°C, and the cells were resuspended in HBSS containing 20 mM hydroxyethyl piperazine ethane sulphonic acid (HEPES) (pH 7.4) at a concentration of 2×10^6 cells·mL^{-1}. Calcium influx was measured using excitation at 340 and 380 nm in a fluorescence spectrometer (ARGUS; Hamamatsu Photonics).

Statistical analysis

Data were analysed using paired and unpaired t-tests, analysis of variance (ANOVA) or the Mann-Whitney U-test. A p-value of $\leqslant0.05$ was considered to indicate significance.

Results

Luminol-dependent chemiluminescence in eosinophils from normal and allergic subjects

ROS production by eosinophils was examined in terms of luminol-dependent chemiluminescence evoked by calcium

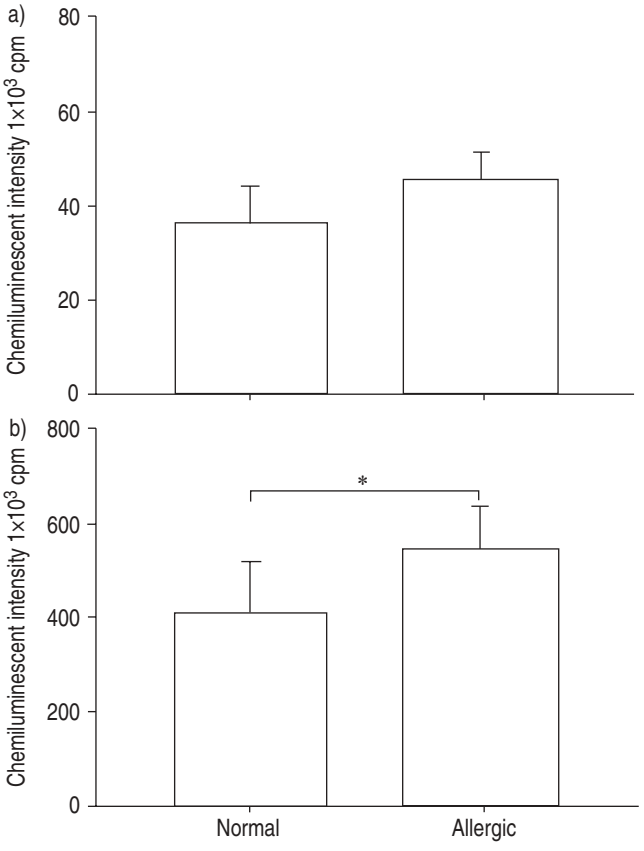

Fig. 1. – Reactive oxygen species production by eosinophils from normal (n=12) and allergic (n=15) subjects as determined by a) maximal and b) integral intensity luminol-dependent chemiluminescence for 60 min. Eosinophil stimulation was performed by adding 50 μL calcium ionophore A23187 (final concentration 1×10^{-5} M) to 100 μL eosinophil suspension (5×10^4 cells) containing 0.25 mM luminol. Data are presented as mean±SEM. cpm: counts per minute. *: $p<0.05$ *versus* normal subjects (unpaired t-test).

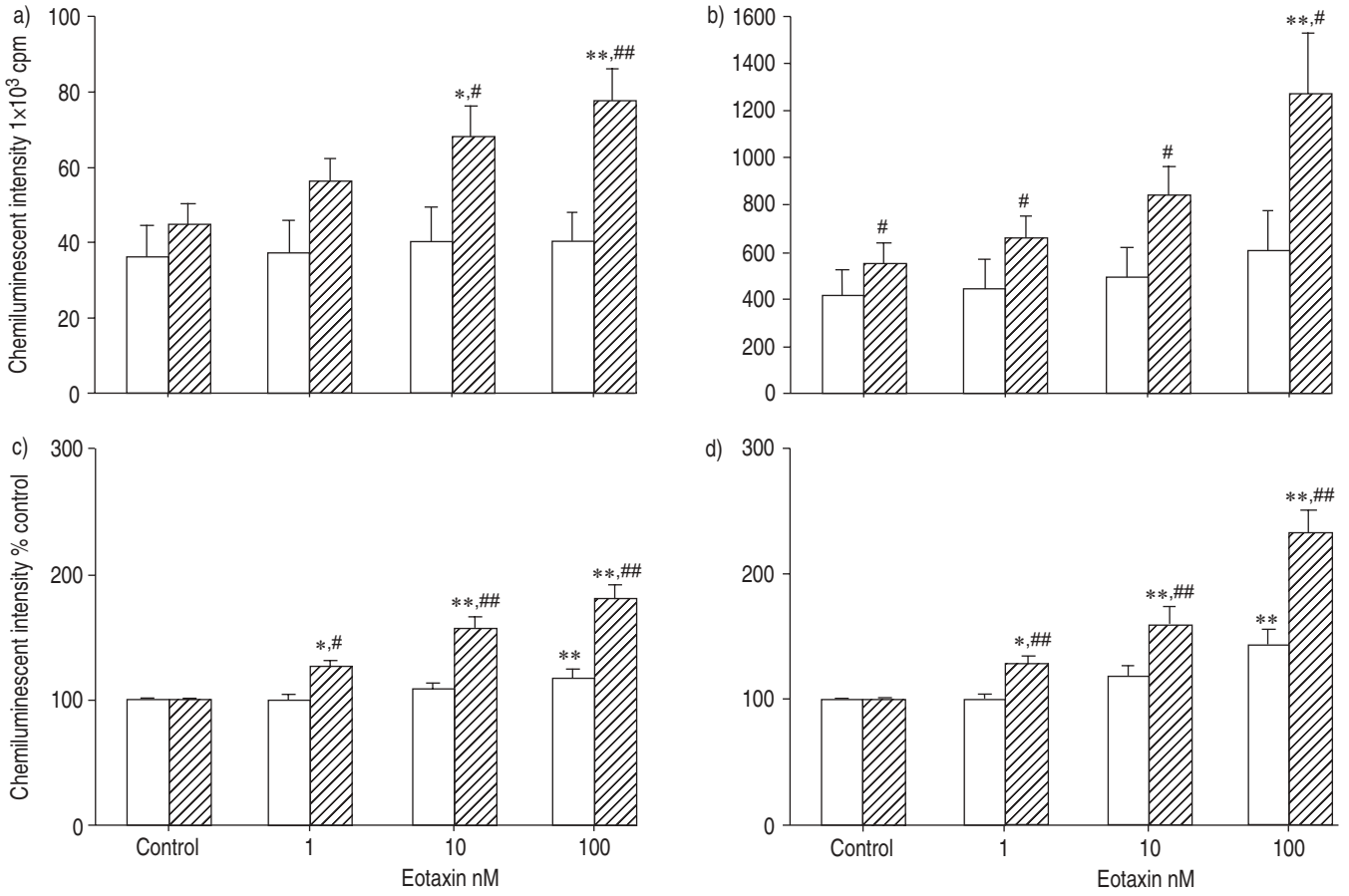

Fig. 2. – Reactive oxygen species (ROS) production by eosinophils treated with eotaxin (1–100 nM) from normal (□; n=12) and allergic (▨; n =15) subjects as determined by maximal (a, c) and integral (b, d) intensity luminol-dependent chemiluminescence for 60 min. Eosinophil stimulation was performed by adding 50 μL calcium ionophore A23187 (final concentration 1×10^{-5} M) to 100 μL eosinophil suspension (5×10^{4} cells) containing 0.25 mM luminol. Data are presented as mean±SEM. Preincubation of eosinophils with eotaxin enhances ROS production in allergic patients as well as normal subjects. The priming effect of eotaxin is more potent in eosinophils from allergic subjects than in those from normal subjects. cpm: counts per minute. *, **: $p<0.05$, $p<0.01$ *versus* control (analysis of variance); #, ##: $p<0.05$, $p<0.01$ *versus* normal subjects (unpaired t-test).

ionophore A23187, and compared between normal subjects and allergic patients. ROS production from eosinophils as measured *via* integral intensity was significantly greater in allergic patients than normal subjects (fig. 1) ($p<0.05$).

Effect of eotaxin and regulated on activation, normal T-cell expressed and secreted on reactive oxygen species production by eosinophils from normal and allergic subjects

The priming effect of eotaxin and RANTES on ROS production was compared in normal subjects and allergic patients. Figures 2a and b show the maximal and integral intensity. Preincubation of eosinophils with eotaxin clearly enhanced ROS production in allergic patients, but not in normal subjects. The difference between allergic and normal subjects in ROS production was much greater in the eotaxin-primed condition. In order to investigate the augmentative effect of eotaxin, results were also expressed in relation to those without chemokines (figs 2c and d). The augmentative effect of eotaxin was more potent in eosinophils from allergic patients than in those from normal subjects. A similar effect of RANTES was also observed (fig. 3).

Effect of CC chemokine receptor 3 antagonist on chemokine-primed reactive oxygen species production by eosinophils

In order to confirm the involvement of CCR3 in chemokine-primed ROS production, the effect of a CCR3 antagonist that inhibits the binding of eotaxin to human eosinophils [11] was investigated. The CCR3 antagonist completely inhibited eotaxin- and RANTES-primed ROS production (fig. 4).

CC chemokine receptor 3 expression on eosinophils from normal and allergic subjects

In order to investigate the different response of eosinophils from allergic patients, expression of CCR3, a common receptor for RANTES and eotaxin, was determined. The percentage of CCR3-positive cells and mean fluorescent intensity compared to controls were used as parameters of receptor expression. No significant differences in either were observed between eosinophils from allergic and normal subjects (fig. 5).

Effect of interleukin-5 on chemokine-primed reactive oxygen species production by eosinophils

IL-5 has been shown to enhance the effector function of eosinophils [12, 13]. IL-5 augments eosinophil responses to

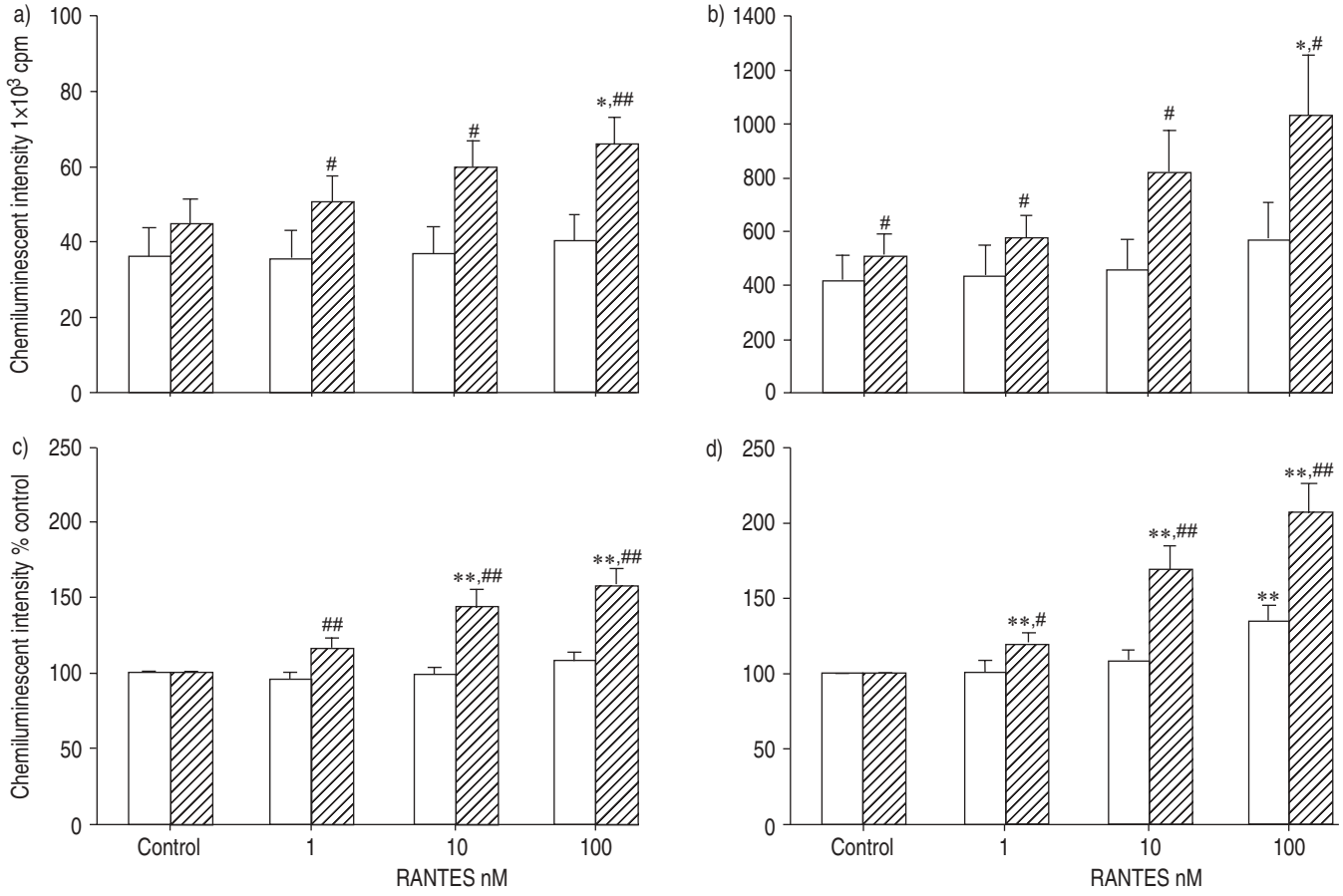

Fig. 3. – Reactive oxygen species (ROS) production by eosinophils treated with regulated on activation, normal T-cell expressed and secreted (RANTES; 1–100 nM) from normal (□; n=12) and allergic (▨; n=15) subjects as determined by maximal (a, c) and integral (b, d) intensity luminol-dependent chemiluminescence for 60 min. Eosinophil stimulation was performed by adding 50 μL calcium ionophore A23187 (final concentration 1×10^{-5} M) to 100 μL eosinophil suspension (5×10^{4} cells) containing 0.25 mM luminol. Data are presented as mean±SEM. Preincubation of eosinophils with RANTES enhances ROS production in allergic patients as well as normal subjects. The priming effect of RANTES is more potent in eosinophils from allergic subjects than in those from normal subjects. cpm: counts per minute. *, **: $p<0.05$, $p<0.01$ *versus* control (analysis of variance); #, ##: $p<0.05$, $p<0.01$ *versus* normal subjects (unpaired t-test).

platelet-activating factor, formyl-methionyl-leucyl-phenylalanine, platelet factor-4 and complement factor 5a [13, 14]. Therefore, the possible involvement of IL-5 in the different eosinophil responses to chemokines between normal subjects and patients was examined. In a preliminary study, a 1-ng·mL^{-1} dose of IL-5, as indicated in previous reports of serum concentrations in allergic patients [15], had no effect on ROS production by eosinophils in the absence of chemokines (fig. 6a). In normal subjects, the action of IL-5 further enhanced the priming effect of chemokines (figs 6b and c). Interestingly, this effect was not observed in eosinophils from allergic patients (fig. 6d).

In addition, GM-CSF (1 ng·mL^{-1}) did not affect the priming effect of eotaxin or RANTES (125.5±17.6 *versus* 119.7± 9.4% control integral chemiluminescent intensity, eotaxin alone *versus* eotaxin plus GM-CSF). Moreover, the CCR3 expression of eosinophils did not change after treatment with IL-5 (fig. 7).

In order to investigate whether blockade of IL-5Rα on allergic eosinophils is able to reverse this augmentative effect on chemokine priming, allergic eosinophils were preincubated with the anti-IL-5Rα antibody prior to eotaxin stimulation. Blockade of the IL-5 receptor did not affect the priming effect of eotaxin in allergic eosinophils (156.6±10.2 *versus* 159.6± 18.8% control integral chemiluminescent intensity, eotaxin alone *versus* eotaxin plus anti-IL-5Rα; n=4).

Effect of interleukin-5 on chemokine-induced calcium influx in eosinophils

In order to study whether IL-5 modulates the downstream signalling of CCR3 to enhance the response to eotaxin, the effect of IL-5 on chemokine-induced calcium influx was investigated. However, IL-5 did not affect the calcium influx induced by eotaxin (fig. 8).

Discussion

Several studies have reported that eosinophil function is highly dependent on the pathophysiological conditions of allergic disease [7, 16–19]. The present study shows upregulated oxidative metabolism in eosinophils obtained from allergic patients compared to those from normal subjects. A similar increase in ROS production by eosinophils was observed in allergic patients [16, 17]. It has also been demonstrated that eosinophils from subjects undergoing allergen challenge or patients with such symptoms exhibit enhanced ROS production [18, 19]. Taking the results of these studies together with the present observations, eosinophils from allergic patients may have already been activated in the peripheral blood stream before they infiltrate the tissues.

Moreover, in the present study, functional upregulation of

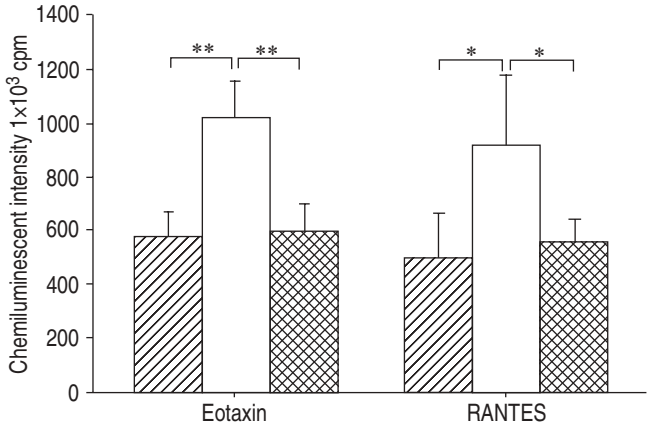

Fig. 4. – Effect of CC chemokine receptor (CCR)3 antagonist on chemokine-primed reactive oxygen species (ROS) production by eosinophils as determined by integral intensity luminol-dependent chemiluminescence for 60 min (▨: control; □: chemokine; ■: chemokine plus antagonist). Purified eosinophils (1×10^6 cells·mL^{-1}) obtained from allergic patients (n=5) were incubated with CCR3 antagonist (Compound X; 1×10^{-6} M) for 30 min. The eosinophils were then treated with eotaxin or regulated on activation, normal T-cell expressed and secreted (RANTES) (both 100 nM) for 15 min. Eosinophil stimulation was performed by adding 50 μL calcium ionophore A23187 (final concentration 1×10^{-5} M) to 100 μL eosinophil suspension (5×10^4 cells) containing 0.25 mM luminol. Data are presented as mean±SEM. CCR3 antagonist completely inhibited the priming effect of eotaxin and RANTES. cpm: counts per minute. *, **: $p<0.05$, $p<0.01$ (paired t-test).

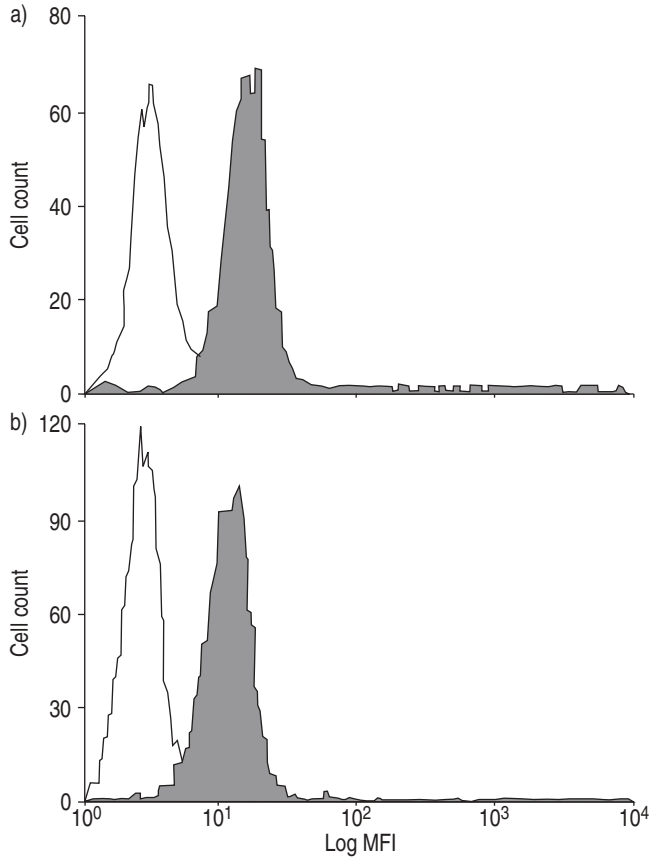

Fig. 5. – Fluorescence-activated cell-sorting analysis of CC chemokine receptor (CCR)3 expression on eosinophils treated with fluorescein isothiocyanate-conjugated monoclonal antibodies directed against CCR3 from a) normal subjects (n=7) and b) allergic patients (n=8) (□: immunoglobulin G2a (negative control); ■: CCR3). Representative histograms are shown. No significant difference in surface expression was observed between eosinophils from allergic and normal subjects (14.5±2.2 *versus* 13.9±1.8 difference in mean fluorescence intensity (MFI) from negative control; 82.2±6.7 *versus* 83.0±7.8% CCR3-positive cells).

the response to chemokines in ROS production by eosinophils obtained from allergic patients was observed. The priming effect of both RANTES and eotaxin on ROS production was significantly greater than that on eosinophils from normal subjects. Even at the suboptimal dose for eosinophils from normal subjects, eosinophils from allergic patients showed enhanced ROS production after treatment with chemokines. These results suggest that eosinophils from allergic patients are more sensitive and responsive to chemokines.

It has been reported that eotaxin and IL-5 cooperate to

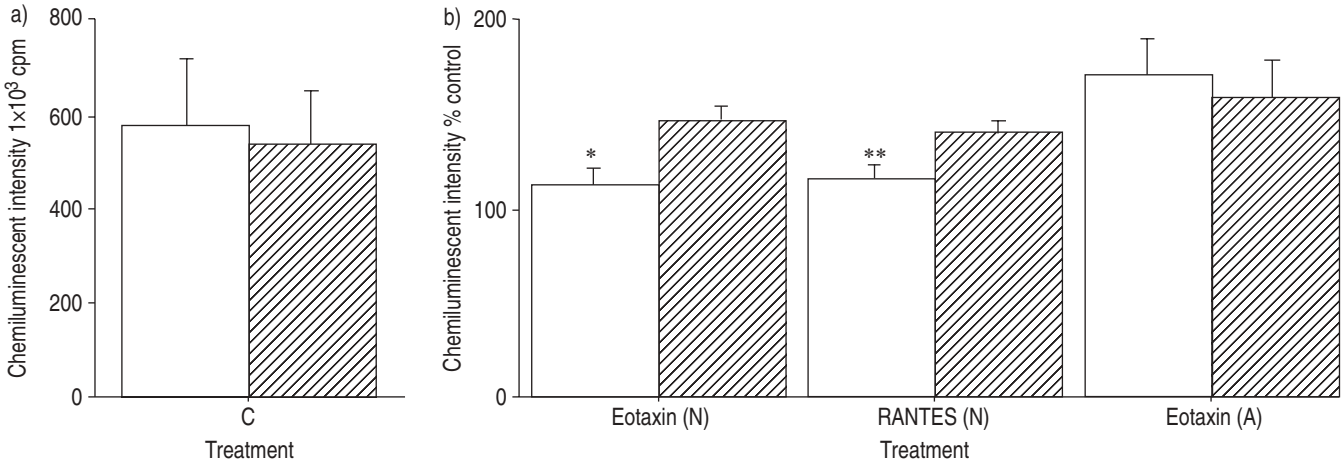

Fig. 6. – Effect of interleukin (IL)-5 on reactive oxygen species (ROS) production by a) control (C; normal subjects) and b) chemokine-primed (normal (N) or allergic (A) subjects) eosinophils as determined by integral-intensity luminol-dependent chemiluminescence for 60 min. Purified eosinophils (1×10^6 cells·mL^{-1}) obtained from normal (n=7) and allergic (n=6) subjects were preincubated with eotaxin or regulated on activation, normal T-cell expressed and secreted (RANTES) (both 10 nM) in the presence (▨) or absence (□) of IL-5 (1 ng·mL^{-1}). Eosinophil stimulation was performed by adding 50 μL calcium ionophore A23187 (final concentration 1×10^{-5} M) to 100 μL eosinophil suspension (5×10^4 cells) containing 0.25 mM luminol. Data are presented as mean±SEM. In a preliminary study, a 1-ng·mL^{-1} dose of IL-5 alone did not affect ROS production by eosinophils (a). In normal subjects, the action of IL-5 further enhances the priming effect of chemokines. This effect is not observed in eosinophils from allergic patients. cpm: counts per minute. *, **: $p<0.05$, $p<0.01$ *versus* chemokine plus IL-5 (paired t-test).

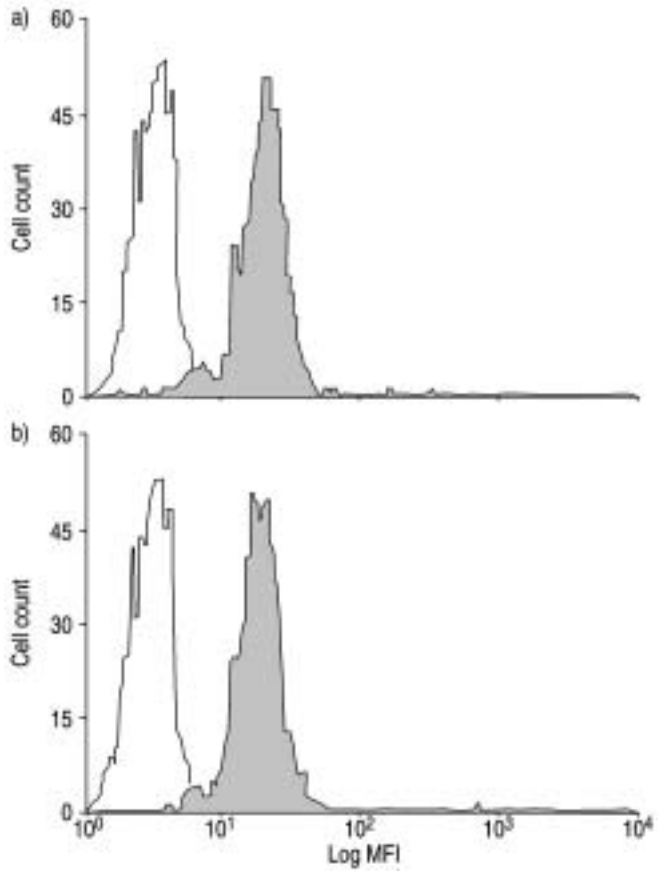

Fig. 7. – Fluorescence-activated cell-sorting analysis of CC chemokine receptor (CCR) 3 expression on eosinophils from normal subjects (n=5) treated with fluorescein isothiocyanate-conjugated monoclonal antibodies directed against CCR3 after incubation with a) phosphate-buffered saline (PBS) and b) interleukin (IL)-5 (1 ng·mL^{-1}) for 30 min (□: immunoglobulin G2a (negative control); ■: CCR3). Representative histograms are shown. Eosinophil CCR3 expression did not change after the 30-min treatment with IL-5 compared to PBS (14.8±2.5 *versus* 15.4±1.7 difference in mean fluorescence intensity (MFI) from negative control; 85.5±5.5 *versus* 89.7±3.5% CCR3-positive cells).

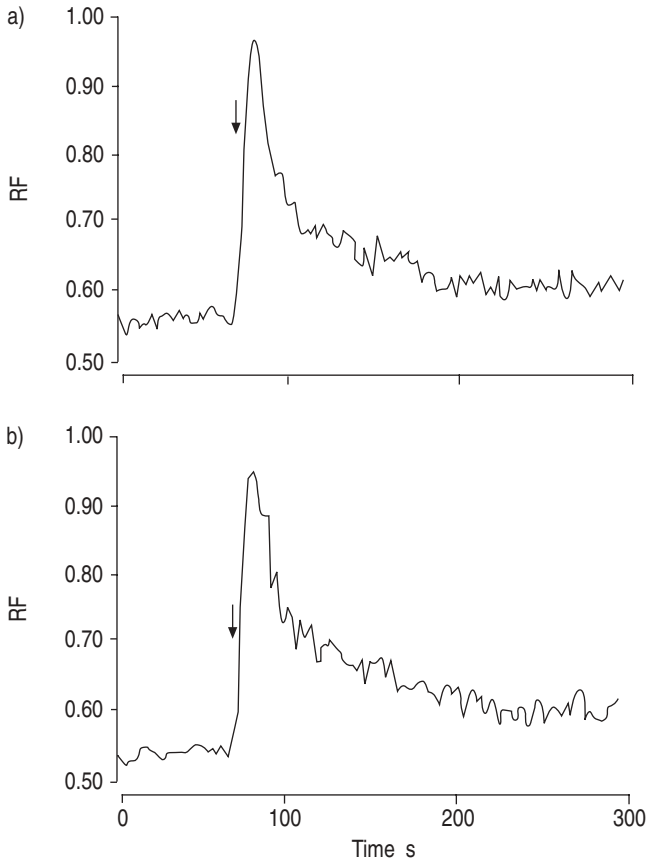

Fig. 8. – Effect of interleukin (IL)-5 on calcium influx into eosinophils induced by eotaxin. Eosinophils obtained from normal subjects were preincubated in the a) absence and b) presence of IL-5 (1 ng·mL^{-1}). The eosinophils were then stimulated with eotaxin at a final concentration of 100 nM (arrow) and calcium influx was measured as described in the Measurement of intracellular calcium concentration section. The data shown are representative of three independent analyses from different donors, each showing similar results. Pre-incubation with IL-5 did not affect calcium influx induced by eotaxin. RF: relative fluorescence.

regulate eosinophil trafficking during allergic inflammation [20, 21]. SCHWEIZER *et al.* [22] reported that chemokine-induced responses are very sensitive to priming by cytokines such as IL-5. Therefore, in order to extend understanding of these upregulated sensitivities and their responsiveness to chemokines, the possible involvement of cytokines, such as IL-5 and GM-CSF, in the priming effect of chemokines was examined. It was demonstrated that a low concentration (1 ng·mL^{-1}) of IL-5 enhanced chemokine-primed ROS production by eosinophils, suggesting that IL-5 may enhance the responsiveness to chemokines. Although a similar tendency has been observed in other eosinophil functions, such as degranulation and migration [14, 22, 23], this is the first report of a priming effect of IL-5 on chemokine-primed ROS production from eosinophils. Interestingly, no augmentative effect of GM-CSF was demonstrated despite the β subunit (βc) being common to both IL-5 and GM-CSF receptors. Although βc plays a major role in IL-5 signalling [24], recent evidence indicates that the specific IL-5 receptor IL-5Rα is also involved in signal transduction. GEIJSEN *et al.* [25] have cloned an IL-5Rα-associated molecule, syntenin, which is required for activation of the transcription factor Sox4. IL-5Rα also associates with a novel signalling molecule, IL-5 receptor-interacting protein, which activates Lyn and Hck in eosinophils [26]. Therefore, these IL-5Rα-specific molecules may be responsible for the distinct response to IL-5.

It has been reported that IL-5 is produced by eosinophils themselves, especially in allergic conditions [27]. One possibility is that allergic eosinophils can be primed by IL-5 produced by themselves. However, it was demonstrated that blockade of the IL-5 receptor on allergic eosinophils could not reverse the priming effect of chemokines. This result indicates that the upregulated response to chemokines observed in allergic eosinophils was not elicited by IL-5 produced after eosinophil isolation. Moreover, the augmentative effect of IL-5 was observed only in eosinophils from normal subjects and not in those from allergic patients. This distinct phenotype is in line with data demonstrating *in vivo* priming of adhesion-associated responses of peripheral blood eosinophils of patients with allergic diseases [13, 14]. Therefore, eosinophils from allergic patients may undergo IL-5 exposure in the blood stream, resulting in great enhancement of responsiveness to chemokines, as demonstrated in the present study.

The possibility of differences in expression of CCR3 as a means of explaining the different responses to chemokines was examined, but no significant difference was found in CCR3 expression between patients and normal subjects.

Furthermore, CCR3 expression of eosinophils did not change after treatment with IL-5. These observations suggest that functional upregulation of response through CCR3 in allergic patients does not depend on an increase in CCR3 expression. As regards signalling of eosinophils, it was examined whether IL-5 modulates the calcium mobilisation induced by chemokines. However, IL-5 did not affect the intracellular calcium influx induced by eotaxin. It has recently been reported that the baseline activity of phosphatidylinositol 3-kinase is elevated in allergic patients compared to normal subjects, together with involvement of IL-5 in phosphatidylinositol 3-kinase activation [28, 29]. Thus, it may be assumed that IL-5 modulates the downstream signalling of CCR3 to enhance the response to eotaxin. Beside the involvement of cytokines, such as IL-5, in the upregulated response of eosinophils from allergic patients to chemokines, it can be presumed that other mechanisms, such as CCR3 polymorphism [30] and change in affinity/avidity, are involved. Recently, CCR3 has become a target in the treatment of allergic diseases such as asthma, atopic dermatitis and allergic rhinitis. Indeed, an inhibitory effect of CCR3 antagonist on chemokine-mediated eosinophil function has been found (manuscript in preparation).

In conclusion, the present study has demonstrated an enhanced response to chemokines in the reactive oxygen species production of eosinophils from allergic patients, with the possible involvement of interleukin-5 in that enhancement, and without changes in CC chemokine receptor 3 expression. Further studies are required to elucidate the mechanisms of the different responses of CC chemokine receptor 3.

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