

Priming effect of RANTES on eosinophil oxidative metabolism

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Background RANTES has been shown to possess chemotactic activity for eosinophils, which have also been considered to play a role in allergic inflammation through reactive oxygen species. Thus, in this study, we examined the effect of RANTES on radical oxygen products from eosinophils.

Methods Purified eosinophils by CD16-negative selection or an eosinophilic cell line (EoL-1) were incubated with or without RANTES (2.5×10^{-6} M). To the mixture of eosinophils and luminol, calcium ionophore (A23187) or opsonized zymosan (OZ) was added, and radical oxygen products were determined by luminol-dependent chemiluminescence for 600 s.

Results Eosinophil-mediated radical oxygen products of untreated eosinophils produced with A23187 gave a peak value of 14.09 ± 2.40 (mean $\pm$ SE, $n = 12$) relative light units (RLU) and an integrated value of 3232.20 ± 513.09 RLU. However, with treatment with RANTES, a peak value of 18.66 ± 2.40 RLU and an integrated value of 5301.05 ± 561.02 RLU were obtained. Eosinophil oxidative metabolism-induced A23187 or OZ was apparently augmented by the preincubation with RANTES. In addition, the radical oxygen products of EoL-1 showed similar results.

Conclusions Thus, we concluded that RANTES may play an important role in the pathogenesis of allergic inflammation through its involvement in eosinophil activation, as evidenced by oxygen products, as well as in selective eosinophil infiltration as selective eosinophil chemoattractant.

Key words: bronchial asthma; chemiluminescence; chemokines; eosinophils; platelet factor 4 (PF4); radical oxygen products; RANTES.

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Recently, attention has focused on the allergic inflammatory reaction in asthma, especially in the late asthmatic response and intractable asthma. Many types of cells, such as eosinophils, macrophages, lymphocytes, and platelets, participate in the inflammatory reactions in the respiratory tract. Eosinophils are considered the major inflammatory cells in bronchial asthma (1), since eosinophil-specific granule proteins can damage bronchial mucosa cells (2). Platelets also have been implicated as mediators of inflammatory reactions, since they are an excellent source of a wide range of

biologically active materials of relevance to the inflammatory process (3, 4).

Chemokines related to the beta subfamily, the so-called platelet factor 4 (PF4) superfamily (5), have recently been shown to stimulate human eosinophils or basophils, and are considered important mediators of inflammation (5). RANTES, which is released not only from early activated T cells but also from thrombin-stimulated platelets, is a member of the 8-kDa cytokine family that has been shown to possess chemotactic activity for eosinophils (6).

However, in inflammation, reactive oxygen species (ROS) generated by eosinophils have been considered capable of causing airway tissue injury at the inflamed site. Thus, in this study, we examined the effect of RANTES on oxygen metabolism by eosinophils in terms of luminol-dependent chemiluminescence.

Abbreviations. CR3: complement receptor type 3; HBSS: Hanks' balanced salt solution; IL: interleukin; mAb: monoclonal antibody; OZ: opsonized zymosan; PBS: phosphate-buffered saline; PF4: platelet factor 4; RANTES: regulated on activation, normal T expressed and secreted; RLU: relative light unit; ROS: reactive oxygen species.

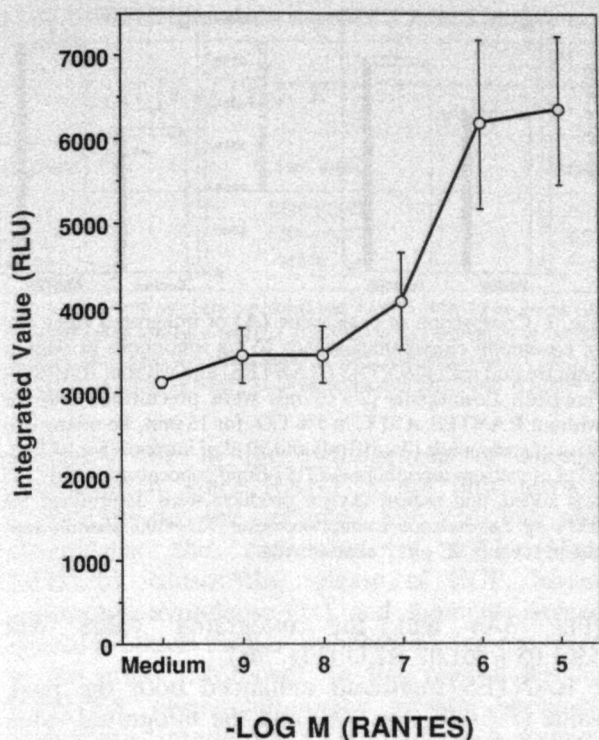


Fig. 1. Dose-response relationship for priming effect of RANTES. Purified eosinophils were incubated at 37°C in 5% CO₂ at 2×10^6 /ml in RPMI 1640 with various concentrations of RANTES for 15 min. To mixture of 50 μ l of eosinophils (2×10^6 /ml) and 50 μ l of luminol (5×10^{-4} M), 50 μ l of calcium ionophore A23187 (final concentration: 10^{-5} M) was added, and radical oxygen products were determined for 600 s and integrated value was determined.

Material and methods

Eosinophil isolation

Eosinophils were isolated from the heparinized venous blood of bronchial asthmatic patients by the modified CD16-negative depletion method (7), as previously described (8). In brief, after addition of 1/4 volume of 6% high-molecular-weight dextran solution (Wako Chemicals, Osaka, Japan) to the heparinized peripheral venous blood, the mixture was allowed to stand for 40 min at room temperature, and the buffy coat was obtained. Cells in the buffy coat were washed twice with Hanks' balanced salt solution (HBSS), and then the cells were reacted with anti-CD16 monoclonal antibody (mAb) (MG38: Nichirei, Tokyo, Japan), anti-CD3 mAb (Nu-pan T: Nichirei), anti-CD20 mAb (Nu-B2: Nichirei), and anti-CD14 mAb (CLB-Mon/1: Nichirei) for 45 min at 4°C. After the reaction, the cells were again washed twice with HBSS. Cells were reacted with anti-mouse IgG magnetic beads (Dynabeads, Dynal, Norway) (cell:bead ratio=1:20) at 4°C for 60 min. Then, positive cells for CD16, CD3, CD20, or CD14 were

removed by a magnetic particle concentrator (MPC, Dynal, Norway), and cells negative for CD16, CD3, CD20, or CD14 were harvested as eosinophils.

The availability of a human eosinophilic cell line, named EoL-1, established from a patient with eosinophilic leukemia (9), has facilitated studies on eosinophil activation (10). Thus, EoL-1 cells, as well as human eosinophils isolated as described above, were used in this study.

Preparation of opsonized zymosan (OZ)

An amount of 5 mg of zymosan (Sigma Chemical Co., St Louis, MO, USA) was suspended with 1 ml of PBS (10 mM phosphate-buffered saline, pH 7.2) and autoclaved for 10 min. The suspension was then incubated with 1 ml of human AB serum.

These zymosan preparations were washed four times and suspended in PBS at 20 mg/ml and then stored at -20°C until use.

Luminol-dependent chemiluminescence

Radical oxidative products in terms of luminol-dependent chemiluminescence were examined. Purified eosinophils and an eosinophilic cell line (EoL-1) were incubated at 37°C in 5% CO₂ at 2×10^6 /ml in RPMI 1640 with the RANTES (2.5×10^{-6} M) (Sigma Chemical Co.) dose indicated in previous reports (6, 11) for 15 min, because our preliminary results revealed that the effect of RANTES on eosinophil oxidative metabolism was apparent after 15-min incubation (data not shown). To a mixture of 50 μ l of eosinophils (2×10^6 /ml) and 50 μ l of luminol (5×10^{-4} M), 50 μ l of calcium (Ca) ionophore A23187 (final concentration: 10^{-5} M) or 50 μ l of OZ (20 mg/ml) was added, and radical oxygen products were determined for 600 s with TD-4000 (Laboscience Co., Tokyo, Japan). While measurements were made, the cell suspensions were maintained at 37°C. The cells were kept suspended in the buffer by frequent gentle agitation. A peak value and an integrated value were determined. All determinations were performed in duplicate or triplicate. Cell viability was maintained (95% exclusion of trypan blue dye) throughout the experiments.

Statistical analysis

Data were expressed as mean $\pm$ SE. Student's *t*-test was used for comparison of means. A *P* value of 0.05 or less was considered significant.

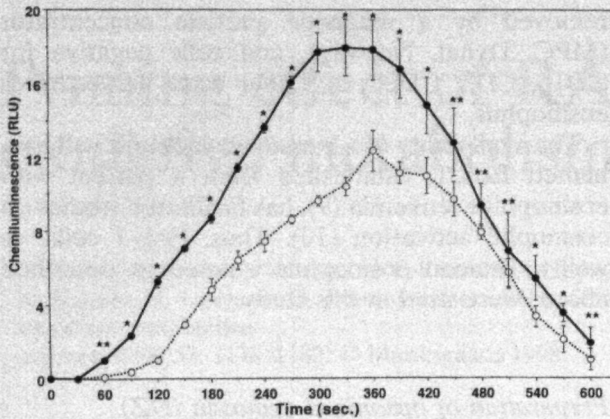


Fig. 2. Results represent mean $\pm$ SE of three experiments (out of 12) of chemiluminescence response to calcium ionophore (A23187) in eosinophils treated with RANTES (—●—) and without RANTES (---○---). Eosinophils (2×10^6 /ml) were precultured with or without RANTES at 37°C in 5% CO_2 for 15 min. * $P < 0.01$; ** $P < 0.05$.

Results

The dose-response study on the priming effect of RANTES revealed that optimal priming of RANTES was observed at 10^{-6} M (Fig. 1). Thereafter, eosinophils were preincubated with RANTES at a concentration of 2.5×10^{-6} M. Eosinophil oxidative metabolism was apparently augmented by preincubation with RANTES (Fig. 2). Eosinophil-mediated radical oxygen products determined by luminol-dependent chemiluminescence of untreated eosinophils induced with A23187 gave a peak value of 14.09 ± 2.40 (mean $\pm$ SE, $n=12$) relative light units (RLU) and an integrated value of 3232.20 ± 513.09 RLU. On the other hand, when they were treated with RANTES, the peak value was 18.66 ± 2.40 RLU

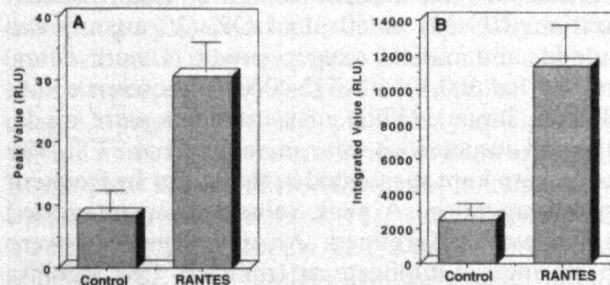


Fig. 4. Comparison of peak value (A) or integrated value (B) of eosinophil chemiluminescence to opsonized zymosan (OZ) in eosinophils treated with RANTES (RANTES) and without RANTES (Control). Eosinophils (2×10^6 /ml) were precultured with or without RANTES at 37°C in 5% CO_2 for 15 min. To mixture of 50 μl of eosinophils (2×10^6 /ml) and 50 μl of luminol (5×10^{-4} M), 50 μl of OZ (20 mg/ml) was added, and radical oxygen products were determined for 600 s by Laboscience Lumiphotometer TD-4000. Results represent mean $\pm$ SE of five experiments.

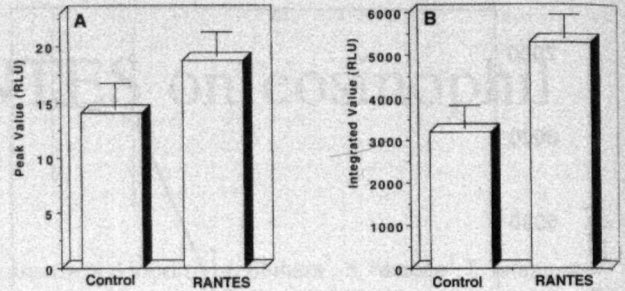


Fig. 3. Comparison of peak value (A) or integrated value (B) of eosinophil chemiluminescence to Ca ionophore in eosinophils treated with RANTES (RANTES) and without RANTES (control). Eosinophils (2×10^6 /ml) were precultured with or without RANTES at 37°C in 5% CO_2 for 15 min. To mixture of 50 μl of eosinophils (2×10^6 /ml) and 50 μl of luminol (5×10^{-4} M), 50 μl of calcium ionophore A23187 (final concentration: 10^{-5} M) was added, and radical oxygen products were determined for 600 s by Laboscience Lumiphotometer TD-4000. Results represent mean $\pm$ SE of 12 experiments.

(Fig. 3A) and the integrated value was 5301.05 ± 561.02 RLU (Fig. 3B).

RANTES treatment enhanced both the peak value ($P < 0.01$, Fig. 3A) and the integrated value ($P < 0.01$) (Fig. 3B) compared to those of untreated cells (Fig. 3). In the radical oxygen products from eosinophils evoked by OZ, radical oxygen products from eosinophils treated with RANTES were augmented as compared to those from untreated eosinophils at both peak value (30.61 ± 4.80 RLU vs 8.30 ± 3.39 RLU, $P < 0.01$, Fig. 4A) and integrated value (11375.00 ± 2133.19 RLU vs 2530.50 ± 708.84 RLU, $P < 0.01$, Fig. 4B). In addition, the radical oxygen products of EoL-1 showed similar results (Table 1) in response to A23187 or OZ.

Discussion

RANTES augmented eosinophil-induced radical oxygen products, which are considered one of the most important inflammatory mediators. It has been considered that ROS are closely related to the pathogenesis of a wide variety of allergic inflammatory diseases. Stimulated eosinophils produce a marked respiratory burst (12, 13). It was found that purified human eosinophils consumed twice as much oxygen as neutrophils from the same individuals (14), suggesting the importance of ROS from eosinophils in inflamed sites. Moreover, peripheral blood eosinophils from patients with allergic rhinitis produced more luminol-dependent chemiluminescence than normal, and the response was greatest under the worst conditions (15). Thus, ROS generated by eosinophils might be capable of causing airway tissue injury at inflamed sites in bronchial asthma. Therefore, RANTES might play

RANTES augments eosinophil oxidative metabolism

Table 1. Chemiluminescence response to ionophore or opsonized zymozan of EoL-1 treated with or without RANTES

Conditions ^a	Chemiluminescence of EoL-1 ^b			
	A23187		OZ	
	Peak value	Integrated value	Peak value	Integrated value
Control	0.616 ± 0.287	77.17 ± 5.06	0.622 ± 0.033	70.38 ± 4.04
RANTES	0.833 ± 0.372	96.33 ± 5.94	0.904 ± 0.040	110.86 ± 10.31
P value	P < 0.02	P < 0.01	P < 0.01	P < 0.01

^a EoL-1 cells (2×10^6 /ml) were precultured with RANTES (RANTES) or without RANTES (control) at 37°C in 5% CO₂ for 15 min.

^b To mixture of 50 µl of EoL-1 cells (2×10^6 /ml) and 50 µl of luminol (5×10^{-4} M), 50 µl of calcium (Ca) ionophore A23187 (final concentration: 10^{-5} M) or opsonized zymozan (OZ: 20 mg/ml) was added, and radical oxygen products were determined for 600 s by Laboscience Lumiphotometer TD-4000.

Results are expressed as mean ± SE of six independent experiments in A23187 and four experiments in OZ.

a major role in the pathogenesis of allergic inflammation, since RANTES not only attracts eosinophils to the inflamed site (6) but also upregulates the expression of CR3 (Mac-1:CD11b), causes the release of ECP, makes eosinophils hypodense (11), and augments oxygen radical products from eosinophils, as demonstrated in this study. Moreover, in this regard, besides RANTES, eosinophilopoietic growth cytokines, particularly interleukin-5 (IL-5), have a priming effect on eosinophil function (16). Therefore, it is a matter of great interest to compare the priming effect of RANTES with that of IL-5.

Moreover, these results showing that RANTES, which might be released from activated platelets (6), enhances the capability of radical oxygen products from eosinophils could be considered evidence of the participation of platelets in allergic inflammation (3, 4). In the context of platelet involvement in allergic inflammation, we also have observed that platelet factor 4 (PF4) might be involved in eosinophil radical oxygen products as well as in eosinophil chemotaxis (17), eosinophil Fc receptor expression (17, 18), hypodense eosinophils (19), and adhesion molecule expression such as CR3 (CD11b/CD18) (20, 21). Thus, we conclude that platelets may play an important role in the pathogenesis of allergic inflammation through the involvement in selective eosinophil infiltration and eosinophil activation by releasing RANTES as well as PF4. Indeed, we demonstrated that the elevation of the plasma level of RANTES during asthma attacks is correlated with the elevation of β -thromboglobulin (22).

Because of the effects of C-C chemokines on eosinophils, their ability to attract and activate eosinophils (5, 6, 23), and their potential role in eosinophil recruitment (5, 6, 11), these molecules have emerged as the most potent stimulators of eosinophil accumulation and activation in allergic diseases. Many of the previously described eosinophil chemotactic factors may actually correspond to

C-C chemokines. Therefore, besides RANTES, it is important to determine whether these chemokines also have a similar priming effect on ROS generation of eosinophils.

Since ROS represent released products of oxidative metabolism that may be toxic to tissue, it is indeed possible that this augmented activity can contribute to respiratory tract inflammation in the pathogenesis of asthma. Further studies are needed to clarify this possibility. Nevertheless, the present data provide additional evidence that eosinophil inflammatory function may be augmented by RANTES in allergic diseases.

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