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ORIGINAL ARTICLE

RANTES Production from Mononuclear Cells in Response to the Specific Allergen in Asthma Patients

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ABSTRACT

Background: Eosinophils are considered to be the major inflammatory cells in asthma. Since regulated on activation, normal T expressed and secreted (RANTES) is a potent chemoattractant for various important inflammatory cells such as eosinophils as well as memory T cells potentially recruiting these cells to an inflamed focus, RANTES has been considered to play a key role in various allergic disorders such as asthma.

Methods: To extend our understanding of the participation of eosinophils and T cells in relation to the production of RANTES in response to the specific allergen in asthma, we examined the production of RANTES from peripheral blood mononuclear cells cultured with specific allergen in atopic asthma patients by a sandwich enzyme-linked immunosorbent assay.

Results: It was revealed that mononuclear cells produced RANTES but not eotaxin in response to the specific allergen in asthma. RANTES production from mononuclear cells of asthma patients with eosinophilia was greater than that of asthma patients without eosinophilia. Moreover, in this study, no differences in RANTES production between CD4 negative cells and CD8 negative cells were observed.

Conclusions: Taken together, these findings may suggest that mononuclear cells play a crucial role in the pathogenesis, particular in eosinophil and T lymphocyte recruitment into the inflamed focus of asthma through RANTES production in response to the specific allergen.

KEY WORDS

asthma, chemokine, eosinophil, mononuclear cells, platelets, RANTES

INTRODUCTION

Much attention has recently been focused on the inflammatory reaction in diseases such as asthma. Eosinophils, together with T lymphocytes, are considered to be the major inflammatory cells in asthma,[1-6] since eosinophil specific granule proteins can damage bronchial mucosa cells resulting in bronchial hyperreactivity.[7] Mononuclear cells (MNC) and platelets also have been implicated as important cells in the inflammatory reactions, since they are an excellent source of a wide range of biologically active materials of relevance to the inflammatory process.[8-10]

Recently, the members of the chemokine family are 8−10 kD basic heparin-binding polypeptides which are related in primary structure, in particular being characterized by the presence of a four-cysteine motif.[11] Chemokines related to the beta subfamily, the so called C-C branch of platelet factor 4 (PF4)-related proteins[12] have been shown to stimulate human eosinophils or basophils, and are considered important mediators of inflammation.[11-13] Particularly, not only eotaxin but also RANTES has been considered to play an important role in various immune and allergic disorders,[14,15] since RANTES is a potent chemoattractant for various important inflammatory cells such as eosinophils[16] as well as memory

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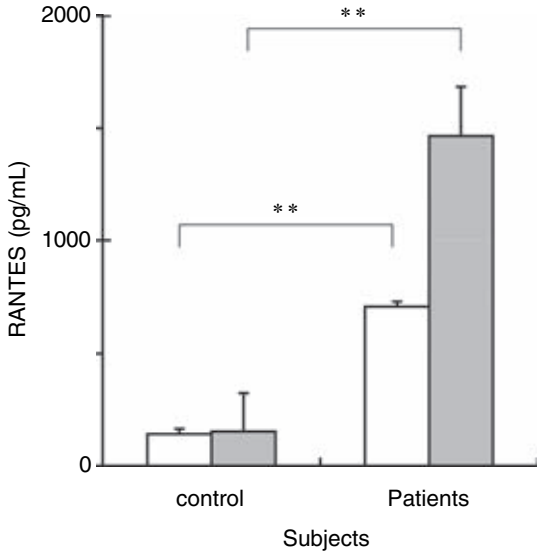

Fig. 1 Level of RANTES in the supernatant of PBMNC from patients (Patients) or controls (Control) cultured with (■) or without (□) specific (mite) allergen. PBMNC were suspended at the concentration of 2×10^6/mL in RPMI1640 with 10% heat-inactivated fetal calf serum and antibiotics. The cells were cultured in the presence or absence of mite-allergen at 37℃ in 5% CO_2 for 4 days (approximately for 84 hours) and then the supernatants were obtained. RANTES in the supernatant of PBMNC was measured by ELISA. ** $P < 0.01$, significant differences between with or without specific antigen.

T cells and monocytes,[17] potentially recruiting these cells from the circulation to an inflamed focus. Therefore, it is of great interest to study the source of RANTES production.

In this study, we examined the production of RANTES and eotaxin from peripheral blood mononuclear cells (PBMNC), cultured with specific allergen in patients with allergic asthma by a sandwich enzyme-linked immunosorbent assay (ELISA).

METHODS

SUBJECTS

Twelve normal control subjects (19–58 years old, average age of 39.2 years) and 18 patients with bronchial asthma during attack as defined by the American Thoracic Society[18] (16–61 years old, average age of 37.4 years) were involved in this study. All patients with asthma had mite-allergic asthma with an IgE RAST score for mite allergen of 3 or more with positive skin test.

PBMNC ISOLATION AND ISOLATION OF CD4 OR CD8 NEGATIVE PBMNC

PBMNC were collected from the heparinized peripheral venous blood of patients with mite-allergic asthma and that of normal control subjects, by Ficoll-Paque (Pharmacia, Uppsala, Sweden) centrifugation.

PBMNC isolated as described above were suspended in 2.0 mL of HBSS, anti-CD4 monoclonal antibody conjugated magnetic beads (Dynabeads, Dynal, Norway) were added at the proportion of 1 : 40 (MNC; anti-CD4 monoclonal antibody conjugated magnetic beads), and the mixture was incubated for 45 minutes at 4℃. These PBMNC that attached to the magnetic beads were subsequently reacted with a magnetic particle concentrator (MPC, Dynal, Norway) for 15 minutes, and then CD4 positive cells were removed to isolate the CD4 negative PBMNC. CD8 negative PBMNC were also isolated in the same manner, using anti-CD8 monoclonal antibody conjugated magnetic beads. Before being used for the experiments, the isolated CD4 and CD8 negative MNC were confirmed to contain <5% CD4 and CD8 positive MNC, respectively, by the indirect fluorescent antibody technique and a FACScan (Becton-Dickinson, Mountain View, CA, USA) as described elsewhere.[10, 19]

CELL CULTURE AND ELISA ASSAY FOR RANTES

Cells were suspended at the concentration of 2×10^6/mL in RPMI1640 (GIBCO, Grand Island, NY, USA) with 10% heat-inactivated fetal calf serum (FCS) (M. A. Biproducts, MD, USA) and antibiotics (100 μg/mL penicillin and 100 μg/mL streptomycin).[19] Mite allergen, which was the mite extract *Dermatophagoides farinae* (Torii Pharmaceutical Co, Tokyo, Japan), was added at a final concentration of 10 μg/mL as previously described.[19] The cells were cultured in the presence or absence of mite-allergen at 37℃ in 5% CO_2 for 4 days (approximately for 84 hours) and then the supernatants were obtained as described previously.[10-19]

RANTES in the supernatant of PBMNC was measured by ELISA as previously reported.[20] In brief, an ELISA kit (R & D systems, USA) with two antibodies to differentiate epitopes of RANTES was used for the assay. A 96-well plate was treated with anti-RANTES monoclonal antibody, and 100 μl of the standard solution or the test sample was added followed by 50 μl of horseradish peroxidase-labeled anti-RANTES antibody. Absorbance was determined at 450/540 nm with a Titertek Multiskan MCC/340 spectrophotometer (ICN, Labosystem Oy., Helsinki, Finland). A calibration curve was generated with the standard solution, and the RANTES concentration in each sample was determined from this curve (sensitivity 15.6 pg/ml or more). All determinations were performed in duplicate or triplicate.

The level of β-thromboglobulin (β-TG) in supernatants was assessed according to a method described in a previous study.[21]

Table 1 Time course of RANTES production from PBMNC

Subjects	Culture conditions[a]	RANTES in the supernatant (pg/mL)[b]			
		Culture Duration			
		3 hrs	24 hrs	48 hrs	84 hrs
Patients ($n = 4$)	without mite	537.00 ± 101.41	558.75 ± 96.53	565.73 ** ± 79.39	593.00 ** ± 112.33
	with mite	580.00 ± 88.48	677.50 ± 129.70	1079.75 ** ± 212.48	2014.50 ** ± 229.59
Controls ($n = 4$)	without mite	102.00 ± 9.15	115.20 ± 13.57	112.20 ± 10.75	100.13 ± 14.11
	with mite	132.38 ± 22.64	139.70 ± 27.48	120.98 ± 14.19	124.60 ± 12.06

[a] PBMNC were suspended at the concentration of 2×10^6/mL in RPMI1640 (GIBCO, Grand Island, NY) with 10% heat-inactivated fetal calf serum and antibiotics. The cells were cultured in the presence or absence of mite-allergen at 37℃ in 5% CO_2 for 3 hrs, 24 hrs, 48 hrs or 84 hrs and then the supernatant were obtained.

[b] RANTES in the supernatants of PBMNC was measured by ELISA.

** $P < 0.01$, significant differences between with or without mite antigen.

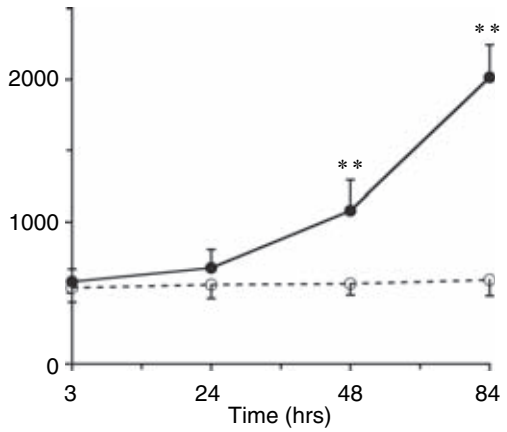

Fig. 2 The time course experiments of RANTES in the supernatant from PBMNC of the patients cultured in the presence (—) or absence (----) of mite-allergen. PBMNC were suspended at the concentration of 2×10^6/mL in RPMI1640 with 10% heat-inactivated fetal calf serum and antibiotics. The cells were cultured in the presence or absence of mite-allergen at 37℃ in 5% CO_2 for 3 hours, 24 hours, 48 hours, or 84 hours and then the supernatants were obtained. RANTES in the supernatant of PBMNC was measured by ELISA. ** $P < 0.01$, *vs* absence of mite-allergen.

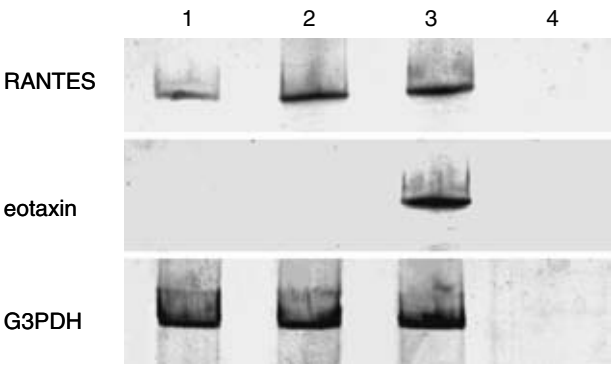

Fig. 3 RT-PCR analysis of RANTES mRNA expression in peripheral mononuclear cells. The amplified products were separated on 12.5% polyacrylamide gel, and the gel was subjected to silver staining. Lane 1: PBMNC from allergic patient cultured without mite-allergen; Lane 2: PBMNC from allergic patient cultured with mite-allergen; Lane 3: human bronchial epithelial cell line (NCI-H_{292}); Lane 4: negative control.

PRIMERS

The RANTES primers used were 5' primer, 5' CTGTCATCCTCATTGCTACT; and 3' primer, 5' ATCTCCAAAGAGTTGATGTAC (product size 244 bp). The eotaxin primers used were 5' primer, 5' GAAAGCTGTGATCTTCAAGAC; and 3' primer, 5' CATGCCCTTTGGACTGATAATGA (product size 224 bp). As a control, G3PDH primers were used (Clontech Co., USA).

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

Messenger RNA was isolated by the guanidinium thiocyanate method (QuickPrep Micro mRNA Purification kit, Pharmacia). A cDNA template for the polymerase chain reaction (PCR) was prepared by reverse transcription using mRNA and distilled water as negative control, Moloney murine leukemia virus (M-MuLV) reverse transcriptase, and random hexadeoxy-nucleotides (First-Strand cDNA product Synthesis kit; Pharmacia).

Six percent of each cDNA product was transferred

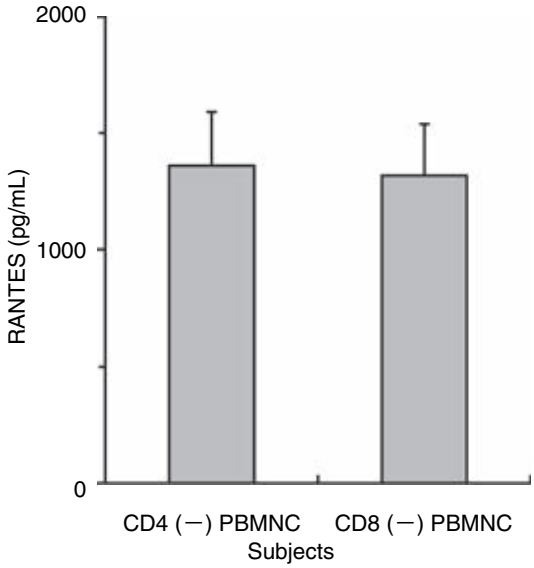

Fig. 4 A comparison of RANTES production of PBMNC between CD4 negative and CD8 negative PBMNC. CD4 negative or CD8 negative PBMNC were also isolated by magnetic isolation, using CD4 or CD8 conjugated magnetic beads. CD4 negative or CD8 negative PBMNC were suspended at the concentration of 2×10^6/mL in RPMI1640 with 10% heat-inactivated fetal calf serum and antibiotics. The cells were cultured in the presence of mite-allergen at 37℃ in 5% CO_2 for 4 days and then the supernatants were obtained. RANTES in the supernatants of PBMNC was measured by ELISA.

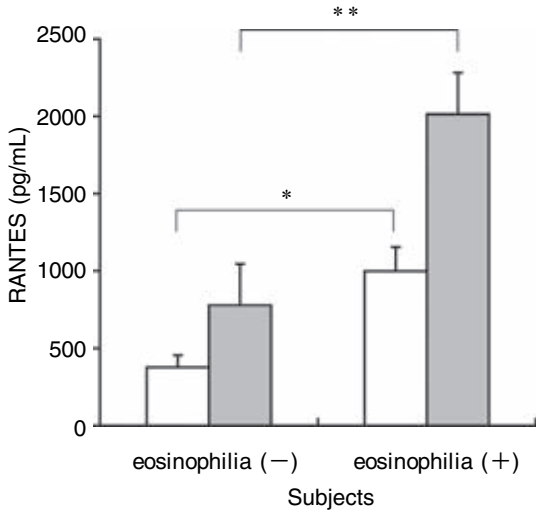

Fig. 5 Level of RANTES production from PBMNC of asthma patients with or without peripheral blood eosinophilia (more than 8%) cultured with (■) or without (□) mite-allergen. The cells were cultured in the presence or absence of mite-allergen at 37℃ in 5% CO_2 for 4 days and then the supernatants were obtained. RANTES in the supernatant of PBMNC was measured by ELISA. * $P<0.05$ and ** $P<0.01$, *vs* between with or without mite-antigen.

to individual tubes to which were added one of the 22 5'-oligonucleotide primers, 30 pmol, and a reaction mixture containing water, buffer, 10 mmol./l, deoxynucleotide triphosphates, and Ampli-TaqR DNA polymerase (Perkin-Elmer). The reaction was carried out with a Perkin-Elmer Gene-Amp PCR system 9600. The PCR was started with an initial 45-second denaturation step at 94℃, followed by 35 cycles of 94℃ denaturation for 10 seconds, annealing at 60℃ for 10 seconds, extension for 10 seconds at 72℃, with a final extension step of 10 minutes at 72℃. The amplified products were separated on 12.5% polyacrylamide gels (PhastGel, Parmacia) stained with the silver staining method (PhastGel DNA Silver Staining kit, Pharmacia).[22]

STATISTICAL ANALYSIS

Data were expressed as means ± SEM. Student's *t* test was used for comparison of means, and a *P* value of 0.05 or less was considered to indicate a significant difference.

RESULTS

LEVEL OF RANTES IN THE SUPERNATANT OF PBMNC CULTURED WITHOUT SPECIFIC ALLERGEN (MITE-ALLERGEN)

The RANTES level of the supernatants of PBMNC cultured without mite-allergen in patients with mite-allergic asthma was 709.54 ± 171.74 pg/mL (mean ± SEM), while that in the control was 141.83 ± 22.92 pg/mL. The RANTES level in the patients was significantly higher than that in the controls in the culture condition without mite allergen ($P<0.01$, Fig. 1), whereas eotaxin was not detectable in any supernatant (data not shown).

RANTES PRODUCTION FROM PBMNC IN RESPONSE TO SPECIFIC ALLERGEN

In the culture condition with mite-allergen, the RANTES level in the supernatant of PBMNC cultured with mite-allergen in the patients was 1464.61 ± 218.87 pg/mL, whereas that in the controls was 152.81 ± 22.18 pg/mL. The RANTES level in the patients was significantly higher than that in the culture without mite allergen in the same patients ($P<0.01$) and than in that the controls cultured with or without mite-allergen ($P<0.01$, Fig. 1), there being no significant differences between the controls cultured with mite allergen and without mite allergen (152.81 ± 22.18 *vs* 141.83 ± 22.92, $P>0.1$, Fig. 1).

Moreover, in the time course experiments, RANTES production from PBMNC in response to the spe-

cific allergen was significantly observed in the culture for 48 hours (Table 1, Fig. 2, $P < 0.01$).

In addition, gene (mRNA) expression for RANTES was observed in PBMNC cultured with mite-allergen, whereas eotaxin mRNA was not observed (Fig. 3).

To clarify the contamination of platelets, since platelets are an excellent source of RANTES,[15,17,21] β-TG was also examined. In the culture condition with mite-allergen, the β-TG level in the patients showed no significant difference with that in the control (118.6 ± 30.1 IU/mL *vs* 122.8 ± 32.6 IU/mL, $P > 0.1$).

COMPARISON OF RANTES PRODUCTION BETWEEN CD4 NEGATIVE AND CD8 NEGATIVE PBMNC

A comparative study on RANTES production between CD4 negative and CD8 negative PBMNC in response to mite allergen revealed no significant differences (CD4 negative: 1359.67 ± 230.76 *vs* CD8 negative: 1317.00 ± 221.84, $P > 0.1$, Fig. 4)

A COMPARATIVE STUDY ON RANTES PRODUCTION FROM ASTHMATIC PATIENTS WITH PERIPHERAL BLOOD EOSINOPHILIA AND WITHOUT EOSINOPHILIA

The level of RANTES production from PBMNC of patients with asthma with peripheral blood eosinophilia (more than 8%) cultured without mite-allergen were 998.3 ± 268.54 ($n = 10$) pg/mL, while that from those without eosinophilia was 377.01 ± 77.68 ($n = 8$) pg/mL. The levels in the patients with eosinophilia were significantly higher than those in the patients without eosinophilia ($P < 0.05$, Fig. 5) in the culture condition without specific allergen. Moreover, in response to the specific allergen, the level of RANTES production in the patients with eosinophilia was much more higher than that in the patients without eosinophilia (2014.20 ± 268.42 pg/mL ($n = 10$) *vs* 777.63 ± 156.86 pg/mL, $n = 8$, $P < 0.01$, Fig. 5).

DISCUSSION

Together with lymphocytes, eosinophils prominently infiltrate in the bronchial mucosa of patients with asthma, and are thought to be the cause of epithelial damage and characteristic airway hyperreactivity.[4,5,23,24] Several stimuli have been implicated in their recruitment and recent studies suggest that lymphocytes of the Th2 type, which migrate together with eosinophils into sites of late-phase reactions,[25] are an important source of chemoattractant cytokines and growth factors that prime eosinophils.[26,27]

RANTES is a member of the 8-kD cytokine family that has been shown to possess selective chemotactic activity for eosinophils.[17] Since RANTES attracts T lymphocytes of the memory type ($CD45RO^+$)[15] besides eosinophils, RANTES may be a common mechanism in various allergic or immunological reactions.[28-34] The allergen-induced late phase airway reaction in atopic subjects, in which both memory T cell infiltration and eosinophil accumulation are characteristic,[25,35] might represent a possible clinical model.

In this context, it is of great interest to clarify the source of RANTES production as well as eotaxin.[34] RANTES originally was identified as an apparently T cell-specific inducible gene, which was initially found to be expressed by cultured T cell lines that were antigen specific and growth factor dependent.[36] The present findings revealed that MNC could produce RANTES but not eotaxin in response to a specific allergen in asthma patients. Our present results of RANTES production from MNC of patients agree with a previous report of a murine model.[37] Eotaxin is also one of the most important chemokines. However, it may not be generated by MNC but by cytokines such as IL-4 produced from MNC.[38] We also examined the β-TG level to clarify the possible contamination by platelets, since they are an excellent source of RANTES.[15] No significant differences in β-TG levels were observed.

Moreover, in the present time course study, the RANTES production from MNC in response to the specific allergen was also revealed to apparently be observed after 48 hours. However, RANTES release from MNC without allergen in patients with asthma was elevated as compared with that of controls with or without mites, suggesting that the PBMNC of patients may have been stimulated *in vivo*. These findings suggest that RANTES plays a key role in the airway inflammation in the late asthmatic response in which eosinophilia is characterized.[25,35] Indeed, RANTES production from PBMNC of asthma patients with eosinophilia was greater than that of patients without eosinophilia.

Moreover, in this study, no significant differences in RANTES production between CD4 negative cells and CD8 negative cells were observed, in contrast with the role of CD4 positive cells in IL-5 production as we previously reported.[39]

While it was reported that RANTES was produced predominantly by $CD8^+$ as compared with $CD4^+$,[40] our present results suggest, as one of several possible explanations, that not only CD8 positive cells but also CD4 positive cells may be together involved in the production of RANTES or that PBMNC which are neither for CD4 nor for CD8 such as monocytes/macrophages may be involved. Indeed, expression of RANTES was initially considered to be T-cell specific.[36] However, subsequent studies have revealed that RANTES can be expressed by a broad range of cell types such as synovial fibroblasts,[41] monocytes/macrophages[42,43] and platelets.[17]

Taken together, these findings suggest that MNC play a crucial role in the pathogenesis, in particular in eosinophil and T lymphocyte recruitment into the inflamed focus of asthma through RANTES production

in response to specific allergen. The details of the mechanism of production of RANTES from MNC in the pathogenesis of bronchial asthma need to be further investigated.

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