

Short communication

Eotaxin in induced sputum of asthmatics: relationship with eosinophils and eosinophil cationic protein in sputum

Background: Eosinophilic inflammation is a crucial aspect of allergic diseases such as bronchial asthma. An eosinophil-active chemokine, eotaxin, may play a role in the pathogenesis of the tissue eosinophilia accompanying asthma.

Methods: Induced sputa were obtained from 53 patients with atopic asthma and six healthy subjects, and the concentration of eotaxin in the sputum was measured by ELISA. We investigated whether the sputum content of eotaxin is related to 1) asthma status or corticosteroid therapy, and 2) other sputum indices, including percentage of eosinophils and concentration of eosinophil cationic protein (ECP).

Results: The patients with stable or unstable asthma showed significantly higher concentrations of sputum eotaxin than the normal controls. The level of sputum eotaxin demonstrated a positive correlation with the percentage of eosinophils in stable asthmatics not receiving corticosteroid therapy, but not in stable patients treated with corticosteroids, or in unstable patients. Sputum eotaxin demonstrated a positive correlation with ECP in asthmatic patients who were either in a stable state or not receiving steroid therapy.

Conclusions: The elevated level of eotaxin detected in association with increased eosinophils and ECP in the sputum of asthmatics suggests that eotaxin is involved in the pathogenesis of eosinophilic airway inflammation. The relationship of eotaxin to airway eosinophilia may be modified by the stability status of asthma and corticosteroid therapy.

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Key words: bronchial asthma; ELISA; eosinophil cationic protein; eosinophils; eotaxin; induced sputum.

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Accepted for publication 30 November 1999

Massive accumulation of eosinophils in airways is a fundamental trait of bronchial asthma (1). Locally migrated eosinophils contribute to airway tissue damage through the release of diverse toxic mediators (1). Thus, it is important to analyze the processes involved in local eosinophilia to understand the pathogenesis of bronchial asthma. A novel CC chemokine, eotaxin, may play an integral role in tissue recruitment of eosinophils in the inflammation associated with allergic disorders (2). Increased expression of eotaxin has been demonstrated in the broncho-alveolar lavage (BAL) and in airway biopsies of mildly asthmatic patients (3, 4).

Recently, we developed an ELISA system sensitive enough to detect 3 pg/ml of human eotaxin (5–7). We used this ELISA to assay eotaxin in induced sputum from asthmatic patients, as well as normal subjects, in

order to clarify the involvement of this chemokine in the pathogenesis of asthma.

Material and methods

Subjects

Fifty-three patients with atopic asthma were randomly recruited from outpatients of the Division of Allergy and Respiratory Medicine, Doai Memorial Hospital, Tokyo, Japan (Table 1). The diagnosis of asthma and the classification of asthma severity were based on the reported criteria (8). According to their disease conditions, the asthmatic subjects were classified as of stable or unstable status. The stable group consisted of patients who fulfilled the following two criteria for at least 6 weeks before sputum induction:

Table 1. Subjects included in this study, and measured indices of their sputa

	Normal controls	Total asthmatics	Stable				Unstable			
			Steroid therapy				Steroid therapy			
			Subtotal	—	+	+	Subtotal	—	+	+
<i>n</i> (male/female)	6 (2/4)	53 (29/24)	23 (10/13)	15 (5/10)	8 (5/3)		30 (19/11)	19 (9/10)	11 (10/1)	
Age (years) (Range)	34.5 ± 8.3 (23–43)	53.0 ± 13.4 (19–74)	53.9 ± 14.5 (21–74)	49.1 ± 15.1 (21–73)	63.0 ± 7.8 (49–74)		52.3 ± 12.7 (19–72)	50.4 ± 13.5 (19–65)	55.5 ± 11.2 (30–72)	
Steroid (topical/systemic/both)	—	13/2/4	—	—	8/0/0		—	—	5/2/4	
Severity (mild/moderate/severe)	—	7/40/6	4/17/2	4/11/0	0/6/2		3/23/4	3/16/0	0/7/4	
FEV _{1,0} (l)	—	2.04 ± 0.69	2.04 ± 0.71	2.26 ± 0.69	1.64 ± 0.58		2.04 ± 0.69	2.09 ± 0.61	1.95 ± 0.83	
FEV _{1,0} (% predicted)	—	69.6 ± 12.2	71.7 ± 11.7	75.3 ± 8.7	64.8 ± 14.0		68.1 ± 12.6	71.4 ± 10.4	62.3 ± 14.3	
PC ₂₀ histamine (µg/ml)	—	627 ± 2789	1179 ± 5251	1687 ± 6029	547 ± 3165		382 ± 1415*	330 ± 1188**	498 ± 2053	
Eosinophils in blood (per µl)	—	(<i>n</i> = 50)	(<i>n</i> = 22)	(<i>n</i> = 15)	(<i>n</i> = 7)		(<i>n</i> = 28)	(<i>n</i> = 18)	(<i>n</i> = 10)	
		272 ± 173	259 ± 164	241 ± 152	299 ± 195		282 ± 181	300 ± 177	248 ± 194	
Serum total IgE (IU/ml)	—	(<i>n</i> = 51)	(<i>n</i> = 22)	(<i>n</i> = 15)	(<i>n</i> = 7)		(<i>n</i> = 29)	(<i>n</i> = 19)	(<i>n</i> = 10)	
		638 ± 1623	354 ± 719	437 ± 863	176 ± 131		854 ± 2051	964 ± 2504	645 ± 688	
Eotaxin in sputum (pg/ml)	14.3 ± 19.8	(<i>n</i> = 51)	(<i>n</i> = 22)	(<i>n</i> = 15)	(<i>n</i> = 7)		(<i>n</i> = 29)	(<i>n</i> = 19)	(<i>n</i> = 10)	
		353.6 ± 582.6	227.1 ± 255.6	289.0 ± 259.3	111.1 ± 217.5‡		450.6 ± 732.6	404.7 ± 521.5	529.9 ± 1027.7	
Eosinophils in sputum (%)	0.25 ± 0.47	8.71 ± 16.48	4.29 ± 5.75	2.41 ± 3.11	7.81 ± 7.93		12.10 ± 20.84	10.29 ± 18.28*	15.23 ± 25.32	
ECP in sputum (ng/ml)	5.0 ± 6.4	361.5 ± 700.1	268.2 ± 426.0	172.0 ± 177.8	448.5 ± 670.9		433.1 ± 853.9	386.0 ± 624.7	514.3 ± 1183.4	

Statistical significance assessed by Mann–Whitney U-test is shown as follows: * $P < 0.05$, ** $P < 0.01$, vs corresponding value of stable patients; ‡ $P < 0.05$ vs corresponding value of patients treated without corticosteroids. Numbers of patients assessed for PC₂₀, eosinophils in blood, or serum IgE are indicated in parentheses.

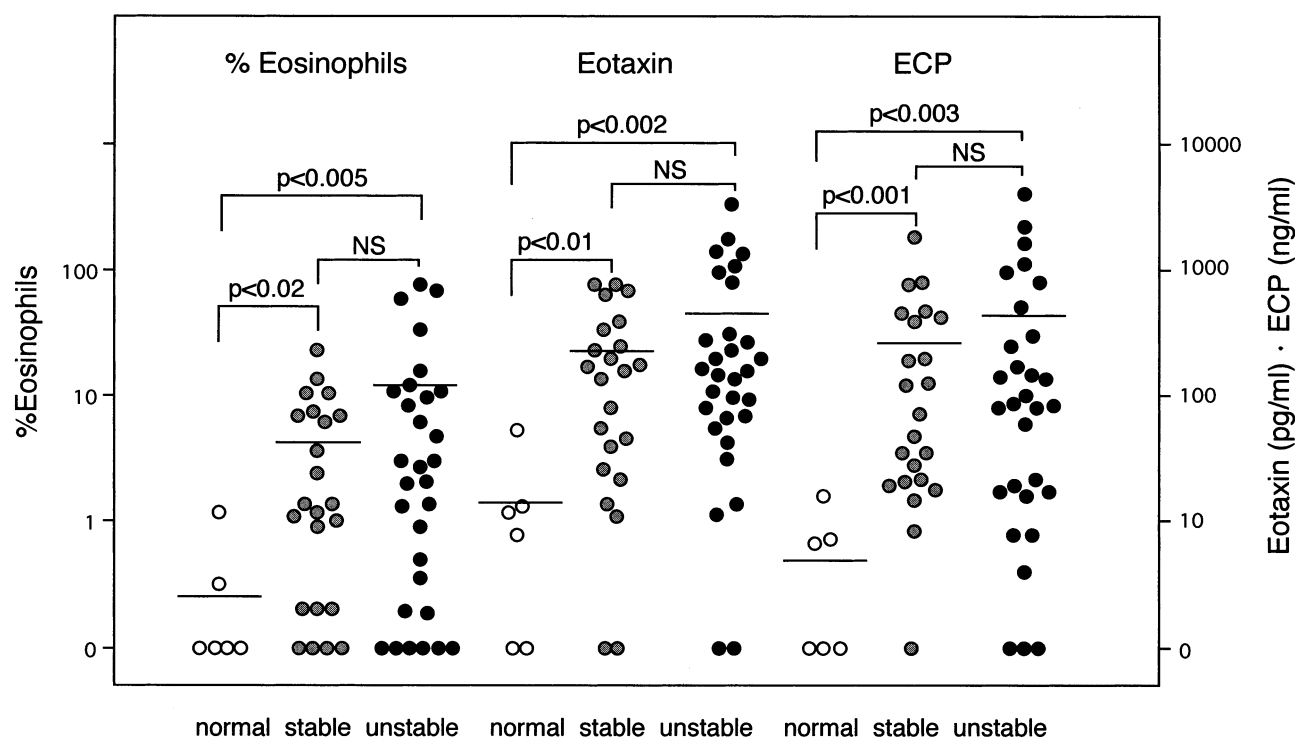


Figure 1. Percentage of eosinophils and concentrations of eotaxin and ECP in sputum of healthy, stable asthmatics and unstable asthmatics. Each symbol represents measured value from individual sputum sample obtained from healthy (open circle, $n=6$), stable asthmatic (shaded circle, $n=23$), or unstable asthmatic (closed circle, $n=30$) subjects. All three indices were significantly higher in sputum of stable and unstable asthmatics than in that of healthy subjects (Mann-Whitney U test; P values are indicated in figure). Horizontal bars show mean values. NS: not significant.

- 1) no asthma attacks or need for a change in medication
- 2) less than 15% daily variability in the peak expiratory flow rate (PEFR), use of a β_2 -agonist inhaler less than twice a week, and no drop in PEFR below 80% of the personal best level.

In addition, the patients were further classified into two categories based on their medication: treatment with or without corticosteroids. The corticosteroid (+) group included patients treated with topical and/or systemic corticosteroids, whereas the no corticosteroid (-) group consisted of asthmatics who had not been treated with corticosteroids for at least 2 months before sputum induction. The control subjects consisted of six healthy volunteers with no history of asthma or other allergic diseases. All of the asthmatic and control subjects were nonsmokers. Written informed consent was obtained before sputum induction.

Analysis of induced sputum

Induced sputa were obtained from the asthmatics and healthy volunteers after hypertonic saline inhalation, as described by Pin et al. (9). Plug selection of the induced expectorate was done to minimize salivary contamination (10). Half of the gently homogenized sputum was treated with 10 mM dithioerythritol (Boehringer Mannheim, Mannheim, Germany) and filtered. The

cells in the filtrate were cytocentrifuged onto glass slides and stained with May-Grünwald-Giemsa, and then the percentage of eosinophils was assessed. The rest of the sputum was processed for assay of soluble mediators. That is, the sample was mixed with an equal volume of D-PBS and centrifuged, and then the concentration of eotaxin in the supernatant was determined by a sandwich ELISA (5–7). Eosinophil cationic protein (ECP) was assayed with an RIA kit (Kabi Pharmacia Diagnostics AB, Uppsala, Sweden). The assays were performed at least in duplicate, and the mean value was calculated. The variation in the measured eotaxin levels was always less than 3% when samples were analyzed in duplicate on an ELISA plate.

Statistical analysis

Data are expressed as the mean $\pm$ SD. Statistical analysis was performed with commercial software (StatView v4.51.2, Abacus Concepts, Inc., Berkeley, CA, USA). A P value of <0.05 was considered to indicate significance.

Results

Demographics

Among the 53 patients with asthma, 23 were classified as stable. The stable and unstable asthmatics showed a

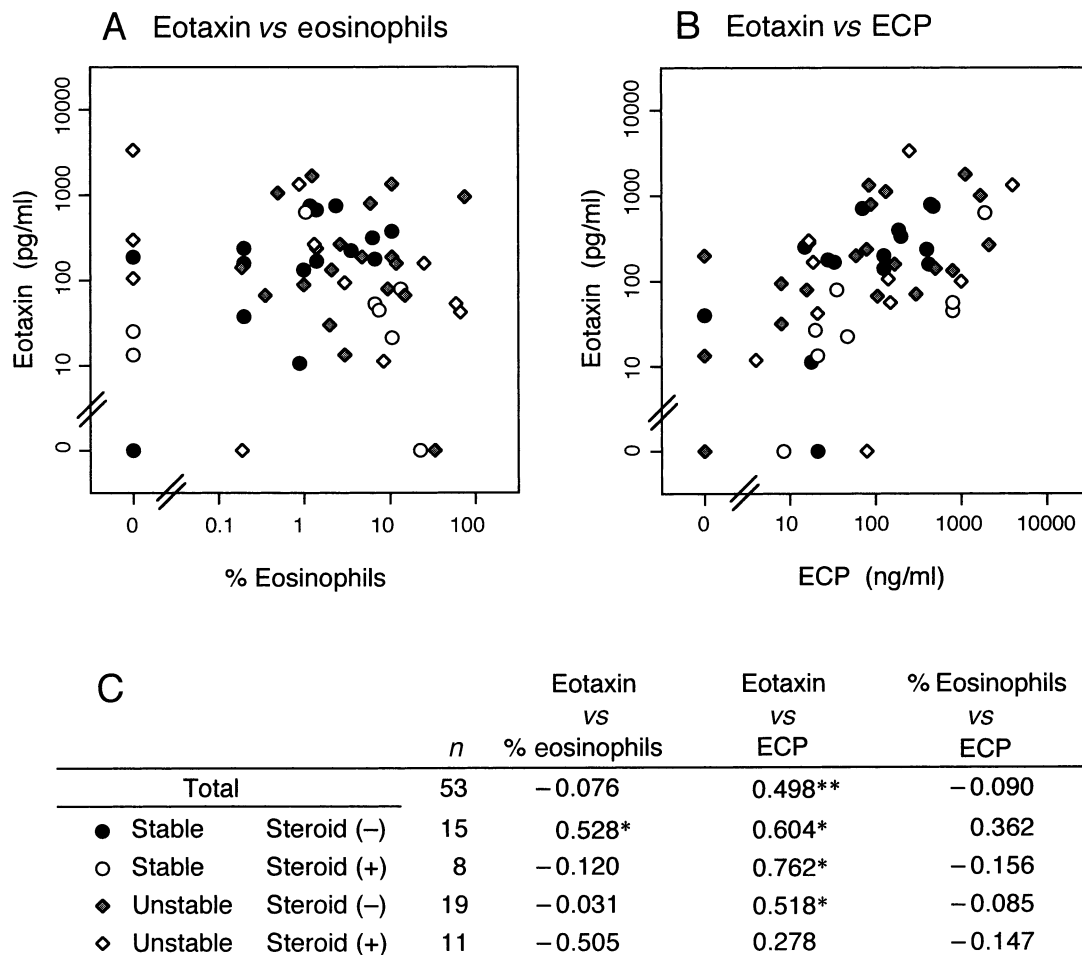


Figure 2. Relationships between percentage of eosinophils and eotaxin level (A), and ECP and eotaxin levels (B), and correlation coefficients (C) of percentage of eosinophils, and eotaxin and ECP levels in sputum of asthmatics. Each symbol (A, B) indicates measured value for induced sputum of asthmatic individuals according to their disease status and medication; stable asthmatics without (closed circles, $n=15$) or with (open circles, $n=8$) systemic and/or inhaled corticosteroids, or unstable asthmatics without (shaded diamonds, $n=19$) or with (open diamonds, $n=11$) corticosteroids. C) Correlation coefficient (r_s) and statistical significance were assessed by Spearman's rank correlation test; * $P<0.05$, ** $P<0.0005$.

statistically significant difference in the value of histamine PC₂₀ ($P<0.05$), but not in relation to the age, FEV_{1.0}, serum IgE, or blood eosinophil count (Table 1).

Percentage of eosinophils, and concentrations of eotaxin and ECP in sputa

The percentage of eosinophils in the sputa of both stable and unstable asthmatics was significantly increased compared with the normal controls (Table 1 and Fig. 1). The corticosteroid(-) stable asthmatics demonstrated a significantly lower percentage of eosinophils than the corticosteroid(-) unstable patients (Table 1). There was no apparent difference in the total cell density in the sputa among the normal subjects ($1.10 \pm 0.46 \times 10^6/\text{ml}$, $n=3$), stable asthmatics

($7.8 \pm 5.3 \times 10^6/\text{ml}$, $n=15$), and unstable asthmatics ($1.30 \pm 1.40 \times 10^6/\text{ml}$, $n=15$).

The sputa from the stable and unstable asthmatics contained a significantly higher concentration of eotaxin than that of the controls (Table 1 and Fig. 1). There was no obvious difference in the levels of sputum eotaxin between the stable and unstable asthmatics in total, but the unstable asthmatics included several patients with very high concentrations of eotaxin in the sputum (up to 3400 pg/ml). The corticosteroid-(+) stable asthmatics showed a significantly decreased level of sputum eotaxin compared with the corticosteroid(-) stable asthmatics (Table 1).

The sputum samples from the asthmatics contained significantly higher concentrations of ECP than that of the normal controls, consistent with previous reports (10, 11). No apparent difference was observed in the

concentration of ECP between the stable and unstable asthmatics.

Correlation between the percentage of eosinophils, the levels of eotaxin, and ECP in sputa of asthmatics

We found that the percentage of eosinophils correlated significantly with the sputum concentration of eotaxin only in the corticosteroid(-) stable asthmatics (correlation $r_s=0.528$, $P<0.05$) (Fig. 2). There was a positive correlation between the levels of eotaxin and ECP in the sputa from all 53 asthmatics ($r_s=0.498$, $P<0.0005$). However, in the corticosteroid-(+) unstable subgroup, there was no correlation between those two sputum indices.

The sputum level of eotaxin did not show any significant correlation with the patient's age, asthma severity (mild vs moderate), the level of serum IgE, or the airway hyperresponsiveness assessed by histamine challenge (PC₂₀) or FEV_{1.0} % (data not shown).

Discussion

Eotaxin induces a selective migration of allergic effector cells, i.e., eosinophils and basophils (12), but not of other leukocytes such as neutrophils and monocytes, because of the restricted expression of its high-affinity receptor, CC chemokine receptor 3 (CCR3). The selective action of this chemokine is unique, since other eosinophil-active chemokines, including RANTES (regulated upon activation, normal T expressed and secreted) and MCP-3, are not specific for CCR3 (13). In addition, the expression of eotaxin is upregulated by a Th2 cytokine, IL-4 (14–17), and downregulated by a Th1 cytokine, IFN- γ (17), suggesting that enhanced eotaxin production may be associated with Th2-polarized allergic inflammation, as in asthma.

We have demonstrated that the level of eotaxin protein is markedly increased in sputum samples of asthmatics compared with healthy subjects. It is important to elucidate which cell type in the airways is a major source of eotaxin. We have reported that eosinophils themselves possess eotaxin protein in their specific granules, but in only a small amount: approximately 20 pg/10⁶ cells (7). Quantitatively, the main cell source of eotaxin is thought to be mesenchymal cells such as fibroblasts (14–17) and airway epithelial cells (2–4). It can be reasonably surmised that the measured eotaxin levels in sputa directly reflect the concentration of this chemokine in airway mucous secretions. Because such high levels of eotaxin can reportedly induce eosinophil migration *in vitro*, eotaxin may be important *in vivo* in the pathogenesis of airway eosinophilia in asthmatics. In fact, we found a significant correlation between the percent eosinophils and the concentration of eotaxin in the sputa of corticosteroid(-) stable asthmatics. Moreover, the

level of eotaxin in the sputa of stable asthmatics demonstrated a significant positive correlation with the level of ECP, further supporting the involvement of eotaxin in the pathogenesis of local allergic inflammation in asthma.

In the stable asthmatics treated with corticosteroids, however, the decrease in the sputum eotaxin level was not accompanied with a parallel change in the sputum eosinophil count (compared to the corticosteroid(-) stable asthmatics in Table 1). Although the patients' background, such as the asthma severity and bronchial hyperresponsiveness, could affect the sputum indices, corticosteroids may also be a factor. We have observed that *in vitro* synthesis of eotaxin by fibroblasts is completely inhibited by corticosteroids at the protein and mRNA levels (T. Nakajima, et al., paper in preparation). Such a potential pharmacologic modulation of eotaxin production would make the relationship between sputum eosinophils and eotaxin ambiguous in patients treated with steroids.

In the unstable asthmatics, a positive correlation was not observed between the eotaxin level and eosinophil count in the sputum, possibly because of different time dynamics for the appearance of eotaxin and eosinophils in mucous secretions from airway tissues. In fact, patients experiencing an acute asthma attack often show a high level of eotaxin but nearly no eosinophils in the sputum (data from three such patients are included in Fig. 2). Another explanation may be that local eosinophil migration in unstable asthmatics is partly regulated by other eosinophil-active cytokines such as RANTES and MCP-4.

An increasing body of evidence has indicated that the sputum can serve as a useful sample for evaluating mediators involved in airway inflammation (10). It has been shown that indices such as the percentage of eosinophils and the level of ECP in induced sputa are reliable and reproducible markers of asthmatic airway inflammation (10). The levels of other chemokines have also been shown to be elevated in the sputa of asthmatics (18). We expect that our results presented herein and the findings of future studies (e.g., focusing on detailed analysis of eotaxin mRNA and protein in sputum and tissues) will collectively elucidate the specific role of this chemokine in the pathogenesis of asthma.

Acknowledgments

We thank Dr A. Morita (Shionogi & Co, Ltd) for providing the HRPO-conjugated Fab antibody preparation; Dr H. Saito and Ms H. Kusakabe for their advice and assistance regarding the method for examining sputum; Ms T. Imamura, Ms H. Ishiguro, Ms M. Imanishi, Ms S. Jibiki, and Ms Y. Asada for their excellent technical help; and Ms Y. Watanabe, Ms A. Kikutake, and Ms S. Takeyama for their expert secretarial assistance. This work was supported by a grant from the Manabe Medical Foundation; grants-in-aid from the Ministry of Health and Welfare of Japan; and grants-in-aid from the Ministry of Education, Science, Sports, and Culture of Japan.

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