

Airway cytokine expression measured by means of protein array in exhaled breath condensate: Correlation with physiologic properties in asthmatic patients

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Background: Simultaneous monitoring of airway inflammation and physiology might be useful for asthma management.

Objective: We examined the upregulated molecules in asthmatic airways. Furthermore, we investigated the relationship between these molecules and the airway physiologic properties of asthma.

Methods: Ten nonsmoking healthy subjects and 16 steroid-naïve asthmatic patients were enrolled. Exhaled breath condensate (EBC) sampling, spirometry, and methacholine inhalation challenge were performed on one occasion in this cross-sectional study. Peak expiratory flow was also measured for 4 weeks. Airway cytokine–chemokine–growth factor production was analyzed with a protein array.

Results: The expressions of IL-4, IL-8, IL-17, TNF- α , RANTES, IFN- γ -inducible protein 10, TGF- β , and macrophage inflammatory protein 1 α and 1 β were significantly upregulated in asthmatic airways compared with those of nonsmoking healthy subjects. Among the upregulated molecules, RANTES expression was significantly correlated with the parameters that represent airway caliber, FEV₁ and respiratory resistance values. In addition, the levels of both TNF- α and TGF- β were significantly correlated with the methacholine threshold and peak expiratory flow variability for the week.

Conclusion: Inflammatory molecule analysis with EBC appeared to be useful for monitoring the asthmatic airway condition.

Clinical implications: Measurements of cytokine levels in EBC might be a promising approach to assess the efficacy of pharmacologic interventions and to investigate the pathophysiology of asthma. (*J Allergy Clin Immunol* ■■■■;■■■;■■■-■■■.)

Key words: Airway hyperresponsiveness, airway lability, airflow limitation, bronchial asthma, exhaled breath condensate, protein array, RANTES, TGF- β , TNF- α

Asthma is a chronic inflammatory disorder of the airways.¹ The inflammation causes airway physiologic changes, such as airway obstruction and airway hyperresponsiveness (AHR). Therefore establishing a simple monitoring system of airway inflammation would be useful for asthma management. In addition, examination of the relationship between the physiologic properties and molecules upregulated during inflammation would also be important.

Exhaled breath condensate (EBC), which is formed by breathing through a cooling system, contains both volatile compounds and nonvolatile compounds.²⁻⁵ Analyses of EBC could provide useful information for possible clinical applications. Because this method is noninvasive, repeated measurements can be made, which could be useful for monitoring the airway inflammation.²

Several inflammatory molecules, such as eicosanoids and cytokines, have been identified in the EBC,^{3,4} which is likely to reflect the composition of the airway-lining fluid.⁵ In the present study the cytokine expression in EBC obtained from asthmatic airways was simultaneously analyzed by using a chemiluminescence-based membrane protein array.⁶⁻⁹ Furthermore, we examined the relationship between these molecules and the physiologic properties of asthma, such as airway obstruction and AHR.

METHODS

Study subjects

Ten nonsmoking healthy subjects and 16 nonsmoking, steroid-naïve asthmatic patients took part in the study after providing informed consent. The study was approved by the local ethics committee. All patients satisfied the American Thoracic Society criteria for asthma.¹⁰ The clinical characteristics of these subjects are shown in Table I. All asthmatic patients were stable and had been without regular asthma treatment, including steroid therapy, before

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Abbreviations used

AHR: Airway hyperresponsiveness
 EBC: Exhaled breath condensate
 IP-10: IFN- γ -inducible protein 10
 MIP: Macrophage inflammatory protein
 PEF: Peak expiratory flow
 Rrs: Respiratory resistance

the study, but rescue use of short-acting inhaled β_2 -agonists as needed for symptom relief was permitted.

Study design

The study was cross-sectional. Subjects attended the outpatient clinic at the Wakayama Medical University hospital on one occasion for clinic examination, spirometry, EBC collection, and methacholine inhalation challenge. Peak expiratory flow (PEF) monitoring had been performed for at least 4 weeks before this attendance.

EBC collection

The EBC was collected by using a condenser, which permitted noninvasive collection of condensed exhaled air and froze it to -20°C (Ecoscreen; Jaeger, Hoechberg, Germany).¹¹ The subjects breathed through a mouthpiece and a 2-way nonrebreathing valve, which also served as a saliva trap. Subjects were asked to breathe at a normal frequency and tidal volume while wearing a nose clip for 15 minutes. The collected EBC was melted and transferred to 1-mL Eppendorf tubes and immediately stored at -70°C . The mean volume collected was 1.6 mL (range, 1.2–2.0 mL).

Cytokine measurements were performed within 4 weeks after the collection of the EBC samples.

Cytokine measurements

Human Inflammation Antibody III (Ray Biotech Inc, Norcross, Ga), consisting of 40 different cytokine and chemokine antibodies spotted in duplicate onto a membrane, was used.^{6–9} Briefly, the membranes were blocked with 10% BSA in Tris-buffered saline, and then 1.0 mL of EBC obtained from either healthy subjects or asthmatic subjects was added and incubated at room temperature for 2 hours. The membranes were washed, and 1.0 mL of primary biotin-conjugated antibody was added and incubated at room temperature for 2 hours. After a thorough wash, the membranes were incubated with 2.0 mL of horseradish peroxidase-conjugated streptavidin at room temperature for 1 hour. The intensity of signals was detected directly from the membranes by using a chemiluminescence imaging system (Luminocapture AE6955; Atto Co, Tokyo, Japan). Exposure times ranged from 30 seconds to 2 minutes. Chemiluminescence was quantified with Atto imaging and analysis software. Horseradish peroxidase-conjugated antibody served as a positive control at 6 spots and was also used to identify the membrane orientation. For each spot, the net intensity gray level was determined by subtracting the background gray levels from the total raw intensity gray levels. The relative intensity levels in the cytokine amount were normalized with reference to the amount present on the positive control in each membrane on the basis of the average of the cytokine spot intensity levels divided by the average of the positive control spot intensity levels and indicated as a percentage. A list of examined cytokines and their sensitivities is shown in Table II.

Reproducibility for the profiles of cytokine expression was assessed in 5 asthmatic patients in a randomized design in which a

TABLE I. Baseline characteristics of the study subjects

	Control subjects	Asthmatic subjects
Number	10 (F/M = 7/3)	16 (F/M = 12/4)
Age (y)	34.4 $\pm$ 6.6	37.1 $\pm$ 12.6
FVC (L)	3.38 $\pm$ 0.82	3.19 $\pm$ 0.58
FEV ₁ (L)	3.10 $\pm$ 0.70	2.47 $\pm$ 0.47
FEV ₁ % (%)	92.2 $\pm$ 3.1	77.5 $\pm$ 5.2
%FEV ₁ (%)	103.9 $\pm$ 9.0	81.3 $\pm$ 8.9

F, Female; M, male; FVC, forced vital capacity.

second EBC sample was collected while the patient was clinically stable within 7 days of obtaining the first sample.

PEF measurements

PEF was measured twice a day with an Assess peak flowmeter (Respironics HealthScan Co, Cedar Grove, NJ) for at least 4 weeks, according to the standard procedure.¹² The average of the 2 largest values of daily PEF variability from the recent week was determined to represent the PEF variability for the week.¹³

Pulmonary function

FEV₁ and forced vital capacity were measured with a Vitalograph Pneumotrac 6800 (Vitalograph Co, Ennis, Ireland), according to the standard procedure.¹⁴

Methacholine inhalation challenge

Thus far, the bronchial provocation test for estimating the hyperresponsiveness of the airways has been generally examined by means of spirometric measurement. However, forced expiration itself might introduce bronchoconstriction.¹⁵ To avoid a forced expiratory maneuver during provocation testing, airway responsiveness to inhaled methacholine was measured with a device (Astograph Jupiter21; Chest Co, Tokyo, Japan) that displays respiratory resistance (Rrs) measured by means of the forced oscillation method during tidal breathing with continuous inhalation of the aerosolized drug.¹⁶ Briefly, it consists of an aerosol delivery system, a loud-speaker box system that generates a constant-amplitude sine wave pressure at 3 Hz, and a system for measuring Rrs automatically from the mouth flow and mouth pressure. Aerosols were generated by using a Bird nebulizer (Bird Co, Palm Springs, Calif), each containing 4 mL of solution driven with a constant airflow of 6 L/min by an air compressor to elicit an output of approximately 0.15 mL/min. The output was determined by measuring the change in weight of the nebulizer chamber. Methacholine (Sigma Co, St Louis, Mo) was prepared in 0.9% saline in 2-fold increasing concentrations ranging from 0.049 to 25 mg/mL. After it was confirmed that a 1-minute inhalation of saline did not change the baseline Rrs, each concentration of methacholine solution was inhaled for 1 minute until Rrs reached approximately twice the baseline value or until the maximum concentration was administered. The index of the airway responsiveness was defined as the cumulative provocative dose of methacholine causing a 100% increase in Rrs.

Statistical analysis

Comparisons between 2 groups were performed by using the Kruskal-Wallis test, followed by the pairwise Mann-Whitney *U* test. Pearson correlation coefficients were calculated to determine the correlation between the relative cytokine levels and pulmonary physiologic parameters. All data were expressed as means $\pm$ SD, and significance was defined as a *P* value of less than .05.

TABLE II. List of 40 examined molecules

	Cytokine	Sensitivity (pg/mL)		Cytokine	Sensitivity (pg/mL)
IL	IL-1 α	1000	CXC-chemokine	IL-8	1
	IL-1 β	100		Mig	1
	IL-2	25		IP-10	10
	IL-3	100	CC-Chemokine	I-309	1000
	IL-4	1		MIP-1 α	20
	IL-6	1		MIP-1 β	10
	IL-6sR	20		MIP-1 δ	100
	IL-7	100		RANTES	2,000
	IL-10	10		MCP-1	3
	IL-11	10,000		MCP-2	100
	IL-12 p40	1000	Colony-stimulating factor	Eotaxin-1	1
	IL-12 p70	10		Eotaxin-2	1
	IL-13	100		G-CSF	2,000
	IL-15	100		GM-CSF	100
	IL-16	1	Growth factor	M-CSF	1
	IL-17	10		TGF- β	200
	IL-17	10		PDGF	1000
TNF	TNF- α	50	Others	TIMP-2	1
	TNF- β	1000		ICAM-1	50,000
	sTNF RI	100		IFN- γ	100
	sTNF RI	10			

Mig, Monokine induced by IFN- γ ; *IL-6sR*, IL-6 soluble receptor; *MCP*, Monocyte chemoattractant protein; *G-CSF*, granulocyte colony-stimulating factor; *M-CSF*, macrophage colony-stimulating factor; *PDGF*, platelet-derived growth factor; *TIMP-2*, tissue inhibitor of metalloprotease 2; *sTNF-R*, soluble TNF receptor; *ICAM-1*, intracellular adhesion molecule 1.

RESULTS

Reproducibility of measurements

Differences in the individual relative levels between the first and second EBC samples and the limits of agreement of each cytokine are shown in Fig 1 ($n = 5$). Within-subject reproducibility of the relative cytokine levels was expressed as the limit of agreement (mean difference $\pm$ 2 SDs of the differences).¹⁷

Cytokine expression in asthmatic airways

Selective upregulation of several molecules in EBC from both groups was detectable on the microarray membranes. The results of comparison analysis of the relative cytokine levels in 2 groups are summarized in Table III. The array analyses indicated that IL-4, IL-8, IL-17, TNF- α , RANTES, IFN- γ -inducible protein 10 (IP-10), TGF- β , macrophage inflammatory protein (MIP) 1 α , and MIP-1 β were the molecules with significantly upregulated expression in asthmatic airways compared with those of healthy subjects ($P < .01$).

Relationship between cytokine expression and pulmonary physiologic parameters

Among the upregulated molecules, correlations between the molecules and the physiologic properties of asthma, such as airway obstruction, airway lability, and AHR, were found (Table IV). The relative level of RANTES was significantly correlated with the percentage of FEV₁ ($r = -0.72$, $P < .01$ [Fig 2, A]) and Rrs values ($r = 0.53$, $P < .05$ [Fig 2, B]). In addition, the levels of both TNF- α and TGF- β were significantly correlated with the methacholine threshold ($r = -0.80$,

$P < .01$ [Fig 3, A] and $r = -0.73$, $P < .01$ [Fig 3, B], respectively) and PEF variability for the week ($r = 0.75$, $P < 0.01$ [Fig 4, A] and $r = 0.66$, $P < .01$ [Fig 4, B], respectively).

DISCUSSION

In the present study the array analyses indicated that IL-4, IL-8, IL-17, TNF- α , RANTES, IP-10, TGF- β , MIP-1 α , and MIP-1 β were the molecules significantly upregulated in asthmatic airways compared with those of healthy subjects. Furthermore, we have shown that among the increased molecules the relative level of RANTES was significantly correlated with the parameters of airflow limitation. Both the TNF- α and TGF- β values were significantly correlated with the degree of airway responsiveness and airway lability.

A basic pathologic feature of asthma is airway inflammation, in which various inflammatory cells and inflammatory molecules produced from them are involved.¹⁸ Both invasive (eg, bronchoalveolar lavage fluid) and semi-invasive (eg, induced sputum) methods have been used to quantify airway inflammatory molecules in many studies.^{19,20} However, these relatively invasive approaches are unsuitable to monitor airway inflammation repeatedly.

By contrast, collection of EBC samples is easy to perform, and because it is noninvasive, it can be done repeatedly. In the present study increased levels of several cytokines–chemokines–growth factors in EBC obtained from asthmatic subjects were demonstrated. The upregulation of these inflammatory molecules in asthmatic

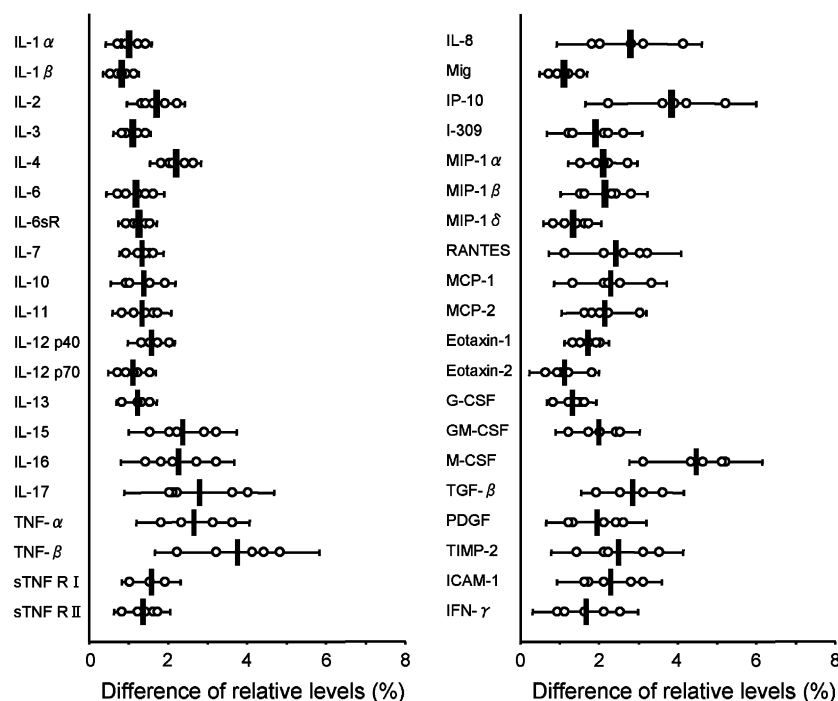


FIG 1. Within-subject reproducibility of relative cytokine levels. Data are presented as **bold vertical bars** and **whisker plots** showing the mean difference and the limit of agreement (± 2 SDs of the differences). Differences in the individual relative levels between the first and second EBC samples ($n = 5$) are superimposed. *IL-6sR*, IL-6 soluble receptor; *sTNF-R*, soluble TNF receptor; *Mig*, monokine induced by IFN- γ ; *MCP*, monocyte chemoattractant protein; *G-CSF*, granulocyte colony-stimulating factor; *M-CSF*, macrophage colony-stimulating factor; *PDGF*, platelet-derived growth factor; *TIMP-2*, tissue inhibitor of metalloproteinase 2; *ICAM-1*, intercellular adhesion molecule 1.

TABLE III. Relative cytokine levels in EBC

Cytokine	Control subjects (%) ^(a)	Asthmatic subjects (%) ^(b)	Fold increase ^(b/a)	Cytokine	Control subjects (%) ^(a)	Asthmatic subjects (%) ^(b)	Fold increase ^(b/a)
IL-1 α	4.0 $\pm$ 2.1	5.2 $\pm$ 1.3	1.30	IL-8	5.4 $\pm$ 2.1	8.3 $\pm$ 1.9*	1.52
IL-1 β	4.6 $\pm$ 0.9	4.2 $\pm$ 2.0	0.92	Mig	4.2 $\pm$ 1.4	4.1 $\pm$ 1.5	0.97
IL-2	4.9 $\pm$ 1.7	4.1 $\pm$ 2.0	0.83	IP-10	8.4 $\pm$ 1.3	22.7 $\pm$ 6.4*	2.72
IL-3	5.7 $\pm$ 1.4	5.0 $\pm$ 2.0	0.88	I-309	3.5 $\pm$ 1.5	3.5 $\pm$ 2.2	1.00
IL-4	5.2 $\pm$ 1.7	8.2 $\pm$ 1.6*	1.56	MIP-1 α	6.3 $\pm$ 1.3	9.2 $\pm$ 2.0*	1.47
IL-6	5.2 $\pm$ 1.2	4.7 $\pm$ 1.7	0.91	MIP-1 β	6.5 $\pm$ 1.5	10.2 $\pm$ 3.7*	1.58
IL-6sR	5.1 $\pm$ 1.3	4.6 $\pm$ 1.8	0.91	MIP-1 δ	3.7 $\pm$ 1.3	5.4 $\pm$ 2.9	1.45
IL-7	2.6 $\pm$ 0.8	3.2 $\pm$ 1.5	1.24	RANTES	6.2 $\pm$ 1.5	10.4 $\pm$ 2.5*	1.69
IL-10	5.4 $\pm$ 1.8	5.7 $\pm$ 1.6	1.04	MCP-1	6.5 $\pm$ 2.1	7.9 $\pm$ 2.2	1.20
IL-11	5.6 $\pm$ 1.8	5.2 $\pm$ 1.8	0.93	MCP-2	4.1 $\pm$ 1.7	4.3 $\pm$ 1.5	1.04
IL-12 p40	4.8 $\pm$ 1.4	4.2 $\pm$ 1.8	0.88	Eotaxin-1	4.6 $\pm$ 2.2	5.0 $\pm$ 2.3	1.09
IL-12 p70	2.8 $\pm$ 1.4	3.4 $\pm$ 2.1	1.24	Eotaxin-2	3.9 $\pm$ 1.7	4.3 $\pm$ 1.3	1.11
IL-13	4.0 $\pm$ 1.0	5.5 $\pm$ 2.3	1.37	G-CSF	3.6 $\pm$ 1.7	3.1 $\pm$ 1.5	0.88
IL-15	7.3 $\pm$ 2.8	7.4 $\pm$ 3.4	1.01	GM-CSF	3.8 $\pm$ 1.0	3.4 $\pm$ 1.6	0.92
IL-16	6.2 $\pm$ 1.8	6.5 $\pm$ 4.3	1.04	M-CSF	9.7 $\pm$ 3.4	9.4 $\pm$ 4.7	0.97
IL-17	8.6 $\pm$ 1.5	12.6 $\pm$ 4.1*	1.46	TGF- β	6.6 $\pm$ 1.2	11.6 $\pm$ 3.4*	1.69
TNF- α	7.0 $\pm$ 1.0	12.4 $\pm$ 3.8*	1.76	PDGF	6.8 $\pm$ 1.6	7.6 $\pm$ 1.8	1.12
TNF- β	27.7 $\pm$ 7.4	27.6 $\pm$ 8.3	1.00	TIMP-2	9.5 $\pm$ 2.9	9.0 $\pm$ 3.0	0.94
sTNF RI	4.8 $\pm$ 1.8	5.4 $\pm$ 1.4	1.13	ICAM-1	3.4 $\pm$ 0.8	3.4 $\pm$ 2.1	1.00
sTNF RII	5.1 $\pm$ 1.6	4.6 $\pm$ 1.5	0.90	IFN- γ	5.4 $\pm$ 2.2	5.5 $\pm$ 2.2	1.00

Relative cytokine levels to positive control in EBC obtained from either healthy subjects (a) or asthmatic subjects (b).

Mig, Monokine induced by IFN- γ ; *IL-6sR*, IL-6 soluble receptor; *MCP*, Monocyte chemoattractant protein; *G-CSF*, granulocyte colony-stimulating factor; *M-CSF*, macrophage colony-stimulating factor; *PDGF*, platelet-derived growth factor; *TIMP-2*, tissue inhibitor of metalloproteinase 2; *sTNF-R*, soluble TNF receptor; *ICAM-1*, intracellular adhesion molecule 1.

* $P < .01$ compared with control subjects.

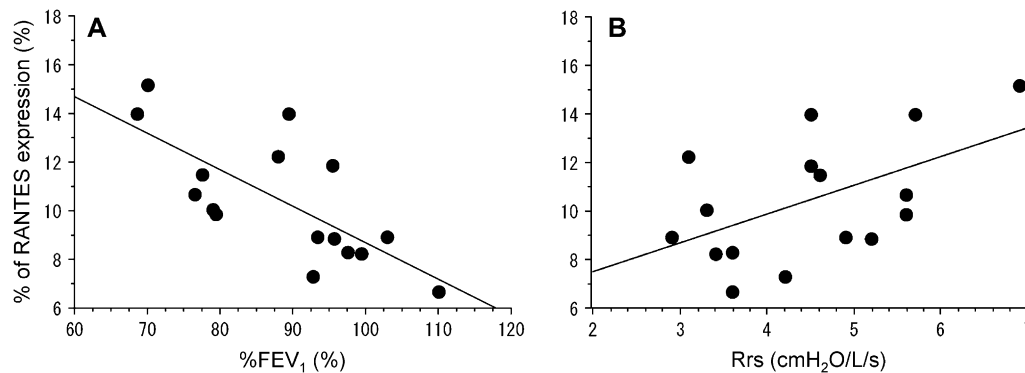


FIG 2. Relationship between the relative expression level of RANTES and parameters of airflow limitation: **A**, FEV₁ (percentage of predicted value; $r = -0.72$, $P < 0.01$); **B**, initial Rrs ($r = 0.53$, $P < .05$). The lines correspond to the fitted regression equation.

TABLE IV. Correlation between the molecules and airway physiologic parameters

Cytokine	% FEV ₁	Rrs	PD ₂₀₀	PEF variability
IL-4	$r = -0.20$, $P = .46$	$r = 0.12$, $P = .66$	$r = -0.22$, $P = .42$	$r = 0.35$, $P = .19$
IL-8	$r = 0.39$, $P = .14$	$r = 0.15$, $P = .58$	$r = 0.11$, $P = .68$	$r = -0.05$, $P = .86$
IL-17	$r = -0.40$, $P = .13$	$r = 0.49$, $P = .06$	$r = -0.16$, $P = .57$	$r = 0.15$, $P = .57$
TNF- α	$r = -0.35$, $P = .19$	$r = 0.30$, $P = .26$	$r = -0.80$, $P < .01$	$r = 0.75$, $P < .01$
RANTES	$r = -0.72$, $P < .01$	$r = 0.53$, $P < .05$	$r = 0.01$, $P = .96$	$r = -0.08$, $P = .79$
IP-10	$r = -0.24$, $P = 0.37$	$r = 0.45$, $P = .08$	$r = -0.28$, $P = .30$	$r = 0.31$, $P = .25$
TGF- β	$r = -0.38$, $P = .15$	$r = 0.36$, $P = .17$	$r = -0.73$, $P < .01$	$r = 0.66$, $P < .01$
MIP-1 α	$r = 0.09$, $P = .75$	$r = 0.22$, $P = .41$	$r = 0.03$, $P = .92$	$r = -0.01$, $P = .96$
MIP-1 β	$r = -0.16$, $P = .57$	$r = 0.29$, $P = .41$	$r = -0.21$, $P = .45$	$r = 0.16$, $P = .56$

PD₂₀₀, Cumulative provocative dose of methacholine causing a 100% increase in Rrs.

airways is in agreement with the findings of previous studies with bronchoalveolar lavage fluid,²¹ supporting the hypothesis that a nonvolatile molecule in the airway-lining fluid can be transported in the form of aerosols in exhaled breath.⁵

The chemiluminescence-based cytokine array, a type of proteomics approach, is a simple and rapid method of analysis of multiple proteins. It has been confirmed that the amount of increase in protein expression agrees with the added protein amount in this method.⁶⁻⁸ Furthermore, it has been shown that the relative levels obtained by using this method correlated well with the actual levels obtained by means of quantitative assays.^{8,9} Additionally, in the present study the reproducibility of EBC analysis by means of protein array was expressed as the limits of agreement. Thus analysis of EBC by means of protein array would be a simple and useful monitoring system of airway inflammatory molecules.

Among the upregulated molecules, there was a striking difference between the IP-10 levels in EBC obtained from asthmatic patients and that from healthy subjects. IP-10 is regarded as a marker of T_H1 activity because its expression is induced by IFN- γ . However, in a mouse model of asthma, IP-10 expression increased after allergen challenge, and IP-10-transgenic mice experience a T_H2 inflammatory response and AHR.²² A recent study indicated that IP-10 plays a key role in the migration of mast

cells into the airway smooth muscle bundles in asthma.²³ In addition, several upregulated molecules, such as IL-4, IL-17, and IL-8, are regarded as principal molecules in the pathophysiology of asthma.²¹ The present analytic system might be helpful to assess the potential role of these inflammatory molecules in asthma.

In the present study the RANTES level in EBC was significantly correlated with FEV₁ and Rrs, which are the indices of airflow limitation. The airflow limitation of asthma is multifactorial. The major cause is the contraction of smooth muscle provoked by mediators released from various inflammatory cells. This bronchoconstriction is exaggerated by thickening of the airway wall caused by mucosal edema, cellular infiltration, mucus plugging, and airway remodeling.²⁴ All of these features are related to the airway inflammation. RANTES, a member of the CC chemokines, is a powerful chemoattractant of eosinophils, T lymphocytes, and basophils.²¹ It also activates these immune cells and induces the exocytosis of bronchoconstrictive mediators, such as histamine and cysteinyl leukotrienes from basophils and eosinophilic cationic protein from eosinophils.²¹ Therefore RANTES might be involved in inflammatory cell recruitment and the induction of bronchoconstrictive mediators from cells, resulting in airflow limitation. A previous report has shown that RANTES-positive sputum eosinophils and the percentage of FEV₁ after allergen challenge are

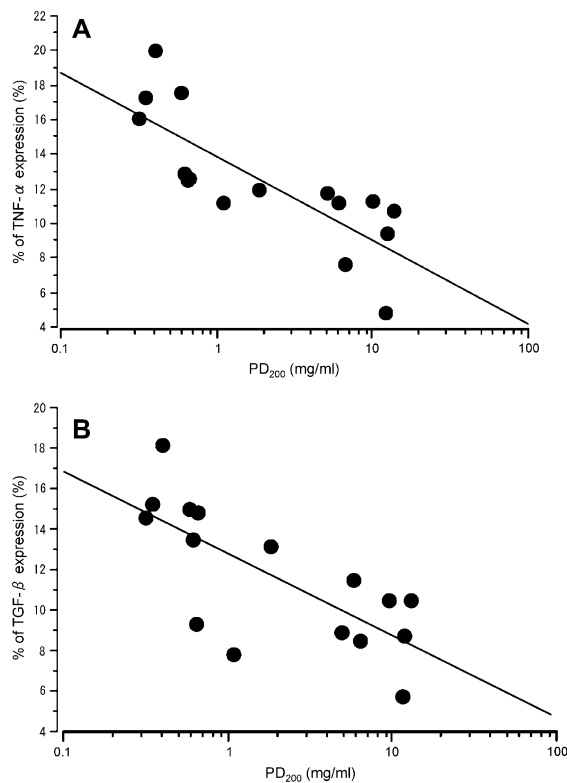


FIG 3. Relationship between airway responsiveness to methacholine (cumulative provocative dose of methacholine causing a 100% increase in Rrs [PD_{200}]) and the relative expression levels of TNF- α ($r = -0.80$, $P < .01$; **A**) and TGF- β ($r = -0.73$, $P < 0.01$; **B**). The lines correspond to the fitted regression equation.

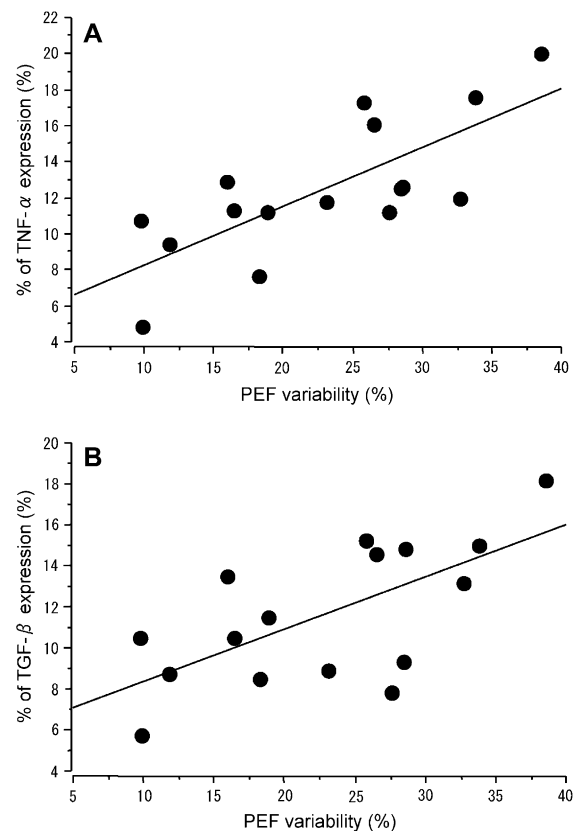


FIG 4. Relationship between PEF variability for the week and the relative expression levels of TNF- α ($r = 0.75$, $P < .01$; **A**) and TGF- β ($r = 0.66$, $P < 0.01$; **B**). The lines correspond to the fitted regression equation.

significantly correlated in asthmatic patients, which is compatible with our result.²⁵

AHR is another important physiologic property of asthma.¹ Several mechanisms, such as airway inflammation, increased neural reflexes, airway geometric factors, and genetic factors, have been proposed to explain the AHR.^{1,26} Among these mechanisms, airway inflammation has been reported to be a key factor, and it seems to cause AHR through 2 mechanisms.²⁶ One mechanism is active inflammation through the release of chemical mediators from immune cells, and another is modification of the airway resident cells through chronic inflammation, resulting in airway remodeling.

In the present study the degree of airway responsiveness correlated with both the TNF- α and TGF- β values in EBC. TNF- α is a proinflammatory cytokine produced by many cells and plays an important role in amplifying asthmatic inflammation.²¹ TNF- α acts on epithelial cells to release a variety of molecules, including GM-CSF and RANTES, which then amplify the inflammatory response and lead to the influx of inflammatory cells.²¹ In a previous study the inhalation of TNF- α increased AHR in human subjects.²⁷ Additionally, the possible effectiveness of TNF blockade with soluble TNF receptors for AHR in patients with severe asthma has been

demonstrated.²⁸ The present result is in agreement with these reports.

TGF- β appears to play an integral role in promoting the structural changes of airway remodeling.^{29,30} In asthma increased TGF- β mRNA expression in bronchial tissue is seen, and its level of expression correlates with the depth of subepithelial fibrosis.²⁹ In addition, the degree of thickening of the subepithelial layer is significantly correlated with the degree of airway responsiveness.³¹ In contrast, it has been shown that there are no identifiable differences in collagen deposition or TGF- β -expressing cells in the large airways of patients with mild asthma when compared with those of patients with severe asthma.³² Although the relationship between airway collagen deposition and physiologic parameters remains controversial, TGF- β might be involved in the mechanism of AHR through its promotion of airway remodeling.

The clinical consequences of AHR are an exaggerated variation in the airway caliber known as airway lability. Although the precise mechanism of airway lability in asthma is still unclear, it has been reported that the variability of PEF correlates better than any other indices with the degree of AHR.¹ In fact, in the present study both the TNF- α and TGF- β values were correlated with not only AHR but also the degree of PEF variability.

In conclusion, inflammatory molecule analysis with EBC appeared to be useful for monitoring the asthmatic airway condition and might be a promising approach to assess the efficacy of pharmacologic intervention and to investigate the pathophysiology of asthma.

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