cytokines, MMP-9, and fibrosis in lung. Am. J. Physiol., Lung Cell Mol. Physiol. 279, L895–L902.

van der Pouw Kraan, T.C., Boeije, L.C., de Groot, E.R., Stapel, S.O., Snijders, A., Kapsenberg, M.L., van der Zee, J.S., Aarden, L.A., 1997. Reduced production of IL-12 and IL-12-dependent IFN-gamma release in patients with allergic asthma. J. Immunol. 158, 5560–5565.

Vernooy, J.H., Kucukaycan, M., Jacobs, J.A., Chavannes, N.H., Buurman, W. A., Dentener, M.A., Wouters, E.F., 2002. Local and systemic inflammation in patients with chronic obstructive pulmonary disease: soluble tumor necrosis factor receptors are increased in sputum. Am. J. Respir. Crit. Care Med. 166, 1218–1224.

Walter, D.M., McIntire, J.J., Berry, G., McKenzie, A.N., Donaldson, D.D., DeKruyff, R.H., Umetsu, D.T., 2001. Critical role for IL-13 in the development of allergen-induced airway hyperreactivity. J. Immunol. 167, 4668–4675.

Ward, S.G., Finan, P., 2003. Isoform-specific phosphoinositide 3-kinase inhibitors as therapeutic agents. Curr. Opin. Pharmacol. 3, 426–434.

Ward, S., Sotsios, Y., Dowden, J., Bruce, I., Finan, P., 2003. Therapeutic potential of phosphoinositide 3-kinase inhibitors. Chem. Biol. 10, 207–213.

White, J.R., Lee, J.M., Young, P.R., Hertzberg, R.P., Jurewicz, A.J., Chaikin, M. A., Widdowson, K., Foley, J.J., Martin, L.D., Griswold, D.E., Sarau, H.M., 1998. Identification of a potent, selective non-peptide CXCR2 antagonist that inhibits interleukin-8-induced neutrophil migration. J. Biol. Chem. 273, 10095–10098.

White, J.R., Lee, J.M., Dede, K., Imburgia, C.S., Jurewicz, A.J., Chan, G., Fornwald, J.A., Dhanak, D., Christmann, L.T., Darcy, M.G., Widdowson, K. L., Foley, J.J., Schmidt, D.B., Sarau, H.M., 2000. Identification of potent, selective non-peptide CC chemokine receptor-3 antagonist that inhibits eotaxin-, eotaxin-2-, and monocyte chemotactic protein-4-induced eosinophil migration. J. Biol. Chem. 275, 36626–36631.

Widdowson, K.L., Elliott, J.D., Veber, D.F., Nie, H., Rutledge, M.C., McCleland, B.W., Xiang, J.N., Jurewicz, A.J., Hertzberg, R.P., Foley, J.J., Griswold, D.E., Martin, L., Lee, J.M., White, J.R., Sarau, H.M., 2004. Evaluation of potent and selective small-molecule antagonists for the CXCR2 chemokine receptor. J. Med. Chem. 47, 1319–1321.

Wills-Karp, M., Chiaramonte, M., 2003. Interleukin-13 in asthma. Curr. Opin. Pulm. Med. 9, 21–27.

Wills-Karp, M., Luyimbazi, J., Xu, X., Schofield, B., Neben, T.Y., Karp, C.L., Donaldson, D.D., 1998. Interleukin-13: central mediator of allergic asthma. Science 282, 2258–2261.

Wymann, M.P., Bjorklof, K., Calvez, R., Finan, P., Thomast, M., Trifilieff, A., Barbier, M., Altruda, F., Hirsch, E., Laffargue, M., 2003. Phosphoinositide 3-kinase gamma: a key modulator in inflammation and allergy. Biochem. Soc. Trans. 31, 275–280.

Yang, G., Volk, A., Petley, T., Emmell, E., Giles-Komar, J., Shang, X., Li, J., Das, A.M., Shealy, D., Griswold, D.E., Li, L., 2004. Anti-IL-13 monoclonal antibody inhibits airway hyperresponsiveness, inflammation and airway remodeling. Cytokine 28, 224–232.

Yang, G., Li, L., Volk, A., Emmell, E., Petley, T., Giles-Komar, J., Rafferty, P., Lakshminarayanan, M., Griswold, D.E., Bugelski, P.J., Das, A.M., 2005. Therapeutic dosing with anti-interleukin-13 monoclonal antibody inhibits asthma progression in mice. J. Pharmacol. Exp. Ther. 313, 8–15.

Ying, S., Robinson, D.S., Meng, Q., Rottman, J., Kennedy, R., Ringler, D.J., Mackay, C.R., Daugherty, B.L., Springer, M.S., Durham, S.R., Williams, T. J., Kay, A.B., 1997. Enhanced expression of eotaxin and CCR3 mRNA and protein in atopic asthma. Association with airway hyperresponsiveness and predominant co-localization of eotaxin mRNA to bronchial epithelial and endothelial cells. Eur. J. Immunol. 27, 3507–3516.

Ying, S., Meng, Q., Zeibecoglou, K., Robinson, D.S., Macfarlane, A., Humbert, M., Kay, A.B., 1999. Eosinophil chemotactic chemokines (eotaxin, eotaxin-2, RANTES, monocyte chemoattractant protein-3 (MCP-3), and MCP-4), and C-C chemokine receptor 3 expression in bronchial biopsies from atopic and nonatopic (Intrinsic) asthmatics. J. Immunol. 163, 6321–6329.

Zhou, Y., McLane, M., Levitt, R.C., 2001. Th2 cytokines and asthma. Interleukin-9 as a therapeutic target for asthma. Respir. Res. 2, 80–84.

Zuany-Amorim, C., Haile, S., Leduc, D., Dumarey, C., Huerre, M., Vargaftig, B. B., Pretolani, M., 1995. Interleukin-10 inhibits antigen-induced cellular recruitment into the airways of sensitized mice. J. Clin. Invest. 95, 2644–2651.

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

Kazuto Matsunaga, MD, PhD, Satoru Yanagisawa, MD, Tomohiro Ichikawa, MD, Kazuhito Ueshima, MD, Keiichirou Akamatsu, MD, Tsunahiko Hirano, MD, PhD, Masanori Nakanishi, MD, PhD, Toshiyuki Yamagata, MD, PhD, Yoshiaki Minakata, MD, PhD, and Masakazu Ichinose, MD, PhD *Wakayama City, Japan*

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-naive 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 1α and 1β 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, FEV_1 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 2006;118:84-90.)

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

From the Third Department of Internal Medicine, Wakayama Medical University, School of Medicine.

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.
Received for publication January 20, 2006; revised March 17, 2006; accepted for publication April 7, 2006.
Available online May 28, 2006.

Reprint requests: Masakazu Ichinose, MD, PhD, Third Department of Internal Medicine, Wakayama Medical University, School of Medicine, 811-1 Kimiidera, Wakayama City, Wakayama, Japan 641-0012. E-mail: masakazu@wakayama-med.ac.jp.

0091-6749/$32.00

doi:10.1016/j.jaci.2006.04.020

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

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 ± 6.6	37.1 ± 12.6
FVC (L)	3.38 ± 0.82	3.19 ± 0.58
FEV_1 (L)	3.10 ± 0.70	2.47 ± 0.47
FEV_1% (%)	92.2 ± 3.1	77.5 ± 5.2
%FEV_1 (%)	103.9 ± 9.0	81.3 ± 8.9

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

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 breath 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 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

FEV_1 and forced vital capacity were measured with a Vitalograph Pneumotrac 6800 (Vitarograph 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 loudspeaker 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 ± 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-1α	1000	CXC-chemokine	IL-8	1
	IL-1β	100		Mig	1
	IL-2	25		IP-10	10
	IL-3	100	CC-Chemokine	I-309	1000
	IL-4	1		MIP-1α	20
	IL-6	1		MIP-1β	10
	IL-6sR	20		MIP-1δ	100
	IL-7	100		RANTES	2,000
	IL-10	10		MCP-1	3
	IL-11	10,000		MCP-2	100
	IL-12 p40	1000		Eotaxin-1	1
	IL-12 p70	10		Eotaxin-2	1
	IL-13	100	Colony-stimulating factor	G-CSF	2,000
	IL-15	100		GM-CSF	100
	IL-16	1		M-CSF	1
	IL-17	10	Growth factor	TGF-β	200
TNF	TNF-α	50		PDGF	1000
	TNF-β	1000	Others	TIMP-2	1
	sTNF RI	100		ICAM-1	50,000
	sTNF RI	10		IFN-γ	100

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 ± 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) 1α, and MIP-1β 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 FEV_1 ($r = -0.72$, $P < .01$ [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-1α, and MIP-1β 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

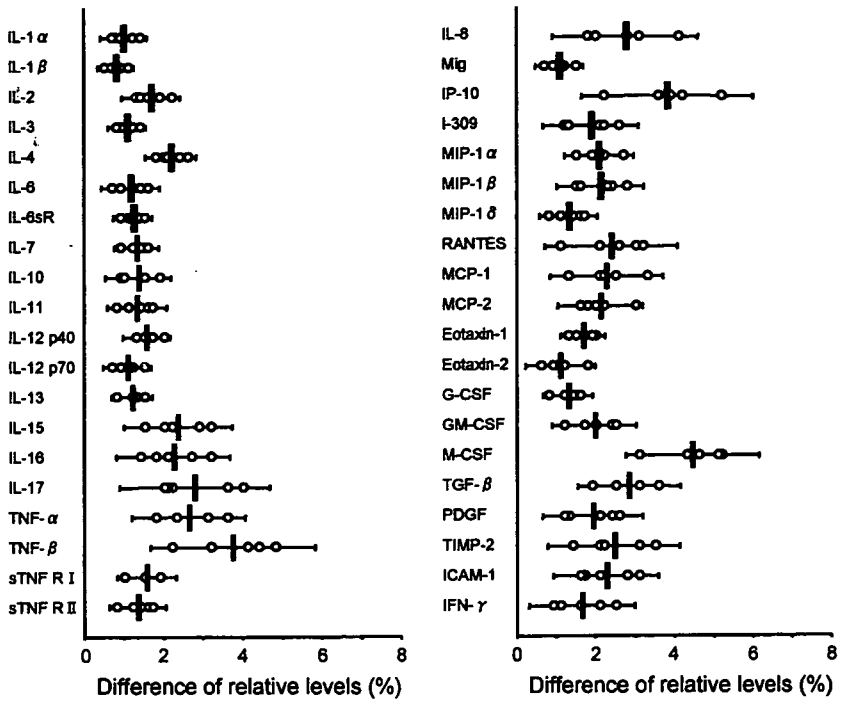

FIG 1. Within-subject reproducibility of relative cytokine levels. Data are presented as *bold vertical bars* and *whisker plots* showing the mean difference and the limit of agreement (±2 SDs of the differences). Differences in the individual relative levels between the first and second EBC samples (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 metalloprotease 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-1α	4.0 ± 2.1	5.2 ± 1.3	1.30	IL-8	5.4 ± 2.1	8.3 ± 1.9*	1.52
IL-1β	4.6 ± 0.9	4.2 ± 2.0	0.92	Mig	4.2 ± 1.4	4.1 ± 1.5	0.97
IL-2	4.9 ± 1.7	4.1 ± 2.0	0.83	IP-10	8.4 ± 1.3	22.7 ± 6.4*	2.72
IL-3	5.7 ± 1.4	5.0 ± 2.0	0.88	I-309	3.5 ± 1.5	3.5 ± 2.2	1.00
IL-4	5.2 ± 1.7	8.2 ± 1.6*	1.56	MIP-1α	6.3 ± 1.3	9.2 ± 2.0*	1.47
IL-6	5.2 ± 1.2	4.7 ± 1.7	0.91	MIP-1β	6.5 ± 1.5	10.2 ± 3.7*	1.58
IL-6sR	5.1 ± 1.3	4.6 ± 1.8	0.91	MIP-1δ	3.7 ± 1.3	5.4 ± 2.9	1.45
IL-7	2.6 ± 0.8	3.2 ± 1.5	1.24	RANTES	6.2 ± 1.5	10.4 ± 2.5*	1.69
IL-10	5.4 ± 1.8	5.7 ± 1.6	1.04	MCP-1	6.5 ± 2.1	7.9 ± 2.2	1.20
IL-11	5.6 ± 1.8	5.2 ± 1.8	0.93	MCP-2	4.1 ± 1.7	4.3 ± 1.5	1.04
IL-12 p40	4.8 ± 1.4	4.2 ± 1.8	0.88	Eotaxin-1	4.6 ± 2.2	5.0 ± 2.3	1.09
IL-12 p70	2.8 ± 1.4	3.4 ± 2.1	1.24	Eotaxin-2	3.9 ± 1.7	4.3 ± 1.3	1.11
IL-13	4.0 ± 1.0	5.5 ± 2.3	1.37	G-CSF	3.6 ± 1.7	3.1 ± 1.5	0.88
IL-15	7.3 ± 2.8	7.4 ± 3.4	1.01	GM-CSF	3.8 ± 1.0	3.4 ± 1.6	0.92
IL-16	6.2 ± 1.8	6.5 ± 4.3	1.04	M-CSF	9.7 ± 3.4	9.4 ± 4.7	0.97
IL-17	8.6 ± 1.5	12.6 ± 4.1*	1.46	TGF-β	6.6 ± 1.2	11.6 ± 3.4*	1.69
TNF-α	7.0 ± 1.0	12.4 ± 3.8*	1.76	PDGF	6.8 ± 1.6	7.6 ± 1.8	1.12
TNF-β	27.7 ± 7.4	27.6 ± 8.3	1.00	TIMP-2	9.5 ± 2.9	9.0 ± 3.0	0.94
sTNF RI	4.8 ± 1.8	5.4 ± 1.4	1.13	ICAM-1	3.4 ± 0.8	3.4 ± 2.1	1.00
sTNF RII	5.1 ± 1.6	4.6 ± 1.5	0.90	IFN-γ	5.4 ± 2.2	5.5 ± 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 metalloprotease 2; *sTNF-R*, soluble TNF receptor; *ICAM-1*, intracellular adhesion molecule 1.
*$P < .01$ compared with control subjects.

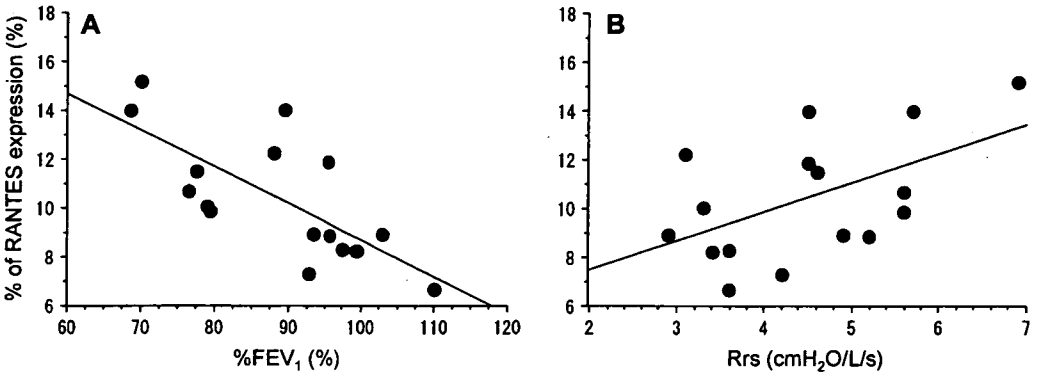

FIG 2. Relationship between the relative expression level of RANTES and parameters of airflow limitation: **A**, FEV_1 (percentage of predicted value; $r = -0.72$, $P < 0.01$); **B**, initial Rrs ($r = 0.53$, $P < .05$). The *lines* correspond to the fitted regression equation.

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-1α	$r = 0.09$, $P = .75$	$r = 0.22$, $P = .41$	$r = 0.03$, $P = .92$	$r = -0.01$, $P = .96$
MIP-1β	$r = -0.16$, $P = .57$	$r = 0.29$, $P = .41$	$r = -0.21$, $P = .45$	$r = 0.16$, $P = .56$

PD_{200}, 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 chemiluminescene-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 FEV_1 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 FEV_1 after allergen challenge are

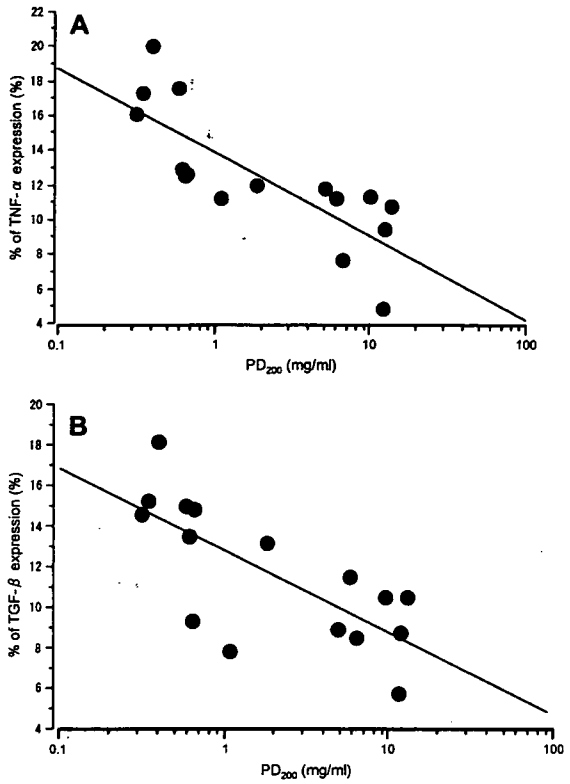

FIG 3. Relationship between airway responsiveness to methacholine (cumulative provocative dose of methacholine causing a 100% increase in Rrs [PD_{200}]) and the relative expression levels of TNF-α ($r = -0.80$, $P < .01$; **A**) and TGF-β ($r = -0.73$, $P < 0.01$; **B**). The *lines* correspond to the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

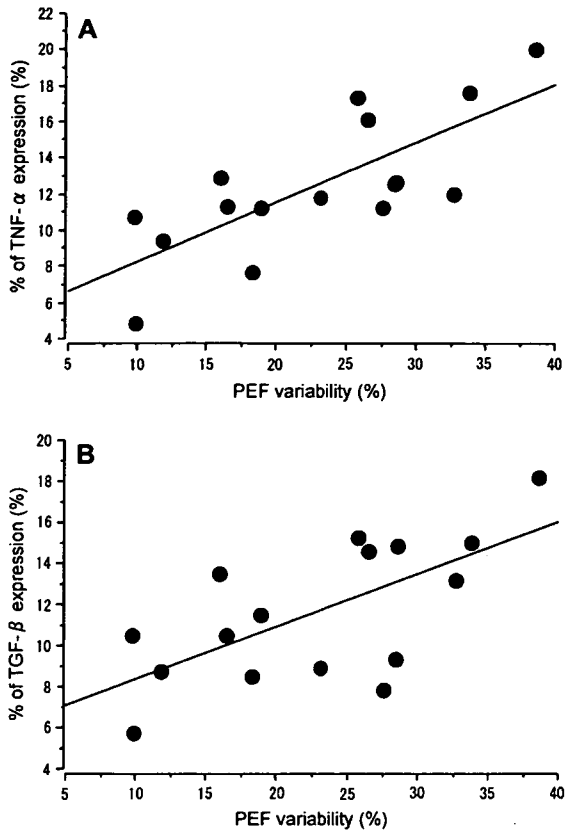

FIG 4. Relationship between PEF variability for the week and the relative expression levels of TNF-α ($r = 0.75$, $P < .01$; **A**) and TGF-β ($r = 0.66$, $P < 0.01$; **B**). The *lines* correspond to the fitted regression equation.

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.

We thank Mr Brent Bell for reading this manuscript.

REFERENCES

1. National Heart, Lung, and Blood Institute/World Health Organization. Global initiative for asthma, global strategy for asthma management and prevention. Bethesda: National Institutes of Health; 2002.
2. Horvath I, Hunt J, Barnes PJ. Exhaled breath condensate: methodological recommendations and unresolved questions. Eur Respir J 2005;26:523-48.
3. Montuschi P, Barnes PJ. Exhaled leukotrienes and prostaglandins in asthma. J Allergy Clin Immunol 2002;109:615-20.
4. Shahid SK, Kharitonov SA, Wilson NM, Bush A, Barnes PJ. Increased Interleukin-4 and decreased Interferon-γ in exhaled breath condensate of children with asthma. Am J Respir Crit Care Med 2002;165:1290-3.
5. Mutlu GM, Garey KW, Robbins RA, Danziger LH, Rubinstein I. Collection and analysis of exhaled breath condensate in humans. Am J Respir Crit Care Med 2001;164:731-7.
6. Huang R. Detection of multiple proteins in an antibody based protein microarray system. J Immunol Methods 2001;255:1-13.
7. Ying L, Huang R, Cao X, Wang SM, Shi Q, Huang RP. Detection of multiple cytokines by protein arrays from cell lysate and tissue lysate. Clin Chem Lab Med 2003;41:139-45.
8. Huang RP, Huang R, Fan Y, Lin Y. Simultaneous detection of multiple cytokines from conditioned media and patient's sera by an antibody-based protein array system. Anal Biochem 2001;294:55-62.
9. Turtinen LW, Prall DN, Bremer LA, Nauss RE, Hartsel SC. Antibody array generated profile of cytokine release from THP-1 leukemic monocytes exposed to different amphotericin B formulations. Antimicrob Chemother 2004;48:396-403.
10. American Thoracic Society. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. Am Rev Respir Dis 1987;136:225-44.
11. Montuschi P, Collins JV, Ciabattoni G, Lazzeri N, Corradi M, Kharitonov SA, et al. Exhaled 8-isoprostane as an in vivo biomarker of lung oxidative stress in patients with COPD and healthy smokers. Am J Respir Crit Care Med 2000;162:1175-7.
12. National Heart, Lung, and Blood Institute. Statement on technical standards for peak flow meters. National Asthma Education Program, expert panel report. Bethesda: National Heart, Lung, and Blood Institute; 1990.
13. Enright PL, Burchette RJ, Peters JA, Lebowitz MD, McDonnell WF, Abbey DE. Peak flow lability; association with asthma and spirometry in an older cohort. Chest 1997;112:895-901.
14. American Thoracic Society. Standardization of spirometry—1994 update. Am J Respir Crit Care Med 1995;152:1107-36.
15. Gimeno F, Berg WC, Slutier HJ, Tammeling GJ. Spirometry-induced bronchial obstruction. Am Rev Respir Dis 1972;105:68-74.
16. Takishima T, Hida W, Sasaki H, Suzuki S, Sasaki T. Direct writing recorder of the dose-response curves of the airway to methacholine. Chest 1981;80:600-6.
17. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1986;1:307-10.
18. Bousquet J, Chanez P, Lacoste JY. Eosinophilic inflammation in asthma. N Engl J Med 1990;323:1033-9.
19. Smith DL, Deshanzo RD. Bronchial lavage in asthma. An update and perspective. Am Rev Respir Dis 1993;148:523-32.
20. Pin I, Gibson PG, Kolendowicz R. Use of induced sputum cell counts to investigate airway inflammation in asthma. Thorax 1992;47:25-9.
21. Chung KF, Barnes PJ. Cytokines in asthma. Thorax 1999;54:825-57.
22. Medoff BD, Sauty A, Tager AM, Maclean JA, Smith RN, Mathew A, et al. IFN-{gamma}-inducible protein 10 (CXCL10) contributes to airway hyperreactivity and airway inflammation in a mouse model of asthma. J Immunol 2002;168:5278-86.
23. Brightling CE, Ammit AJ, Kaur D, Black JL, Wardlaw AJ, et al. The CXCL10/CXCR3 axis mediates human lung mast cell migration to asthmatic airway smooth muscle. Am J Respir Crit Care Med 2005;171:1103-8.
24. Wiggs BR, Bosken C, Pare PD, James A, Hogg JC. A model of airway narrowing in asthma and in chronic obstructive pulmonary disease. Am Rev Respir Dis 1992;145:1251-8.
25. Gauvreau GM, Watson RM, O'Byrne PM. Kinetics of allergen-induced airway eosinophilic cytokine production and airway inflammation. Am J Respir Crit Care Med 1999;160:640-7.
26. Ichinose M, Takahashi H, Sugiura N, Endoh M, Miura Y, Mashito Y, et al. Baseline airway hyperresponsiveness and its reversible component: role of airway inflammation and airway caliber. Eur Respir J 2000;15:248-53.
27. Thomas PJ, Heywood G. Effects of inhaled tumor necrosis factor alpha in subjects with mild asthma. Thorax 2002;57:774-8.
28. Howarth PH, Babu KS, Arshad HS, Lau L, Buckely M, McConnell W, et al. Tumor necrosis factor (TNFα) as a novel therapeutic target in symptomatic corticosteroid dependent asthma. Thorax 2005;60:1012-8.
29. Vignola AM, Chanez P, Chiappara G. Transforming growth factor-beta expression in mucosal biopsies in asthma and chronic bronchitis. Am J Respir Crit Care Med 1997;160:1001-8.
30. Redington A, Madden J, Frew A. Transforming growth factor beta1 in asthma: measurement in bronchoalveolar lavage fluid. Am J Respir Crit Care Med 1997;156:642-7.
31. Chetta A, Foresi A, Del Donno M, Consigli GF, Bertorelli G, Pesci A, et al. Bronchial responsiveness to distilled water and methacholine and its relationship to inflammation and remodeling of the airways in asthma. Am J Respir Crit Care Med 1996;153:910-7.
32. Chu HW, Halliday JL, Martin RJ, Leung DY, Szefler SJ. Collagen deposition in large airways may not differentiate severe asthma from milder forms of the disease. Am J Respir Crit Care Med 1998;158:1936-44.

Allergology International. 2006;55:347-351

CASE REPORT

Two Cases of Asthma in Handicapped Elderly Persons in Which Assisted Inhalation Therapy Was Effective

Kazuto Matsunaga[1], Satoru Yanagisawa[1], Tomohiro Ichikawa[1], Kazuhito Ueshima[1], Keiichirou Akamatsu[1], Tsunahiko Hirano[1], Masanori Nakanishi[1], Toshiyuki Yamagata[1], Yoshiaki Minakata[1] and Masakazu Ichinose[1]

ABSTRACT

Background: Chronic airway inflammation is a basic pathology of bronchial asthma and it is important to control the inflammation by anti-inflammatory therapy mainly with steroids. However, in asthma in the elderly, there are cases where physicians hesitate to introduce the inhaled corticosteroid (ICS) therapy based on the diagnosis that the use of inhalants is difficult due to the existence of a functional lesion accompanying asthma.

Methods & Results: In cases where self-administrated inhalation therapy is difficult to execute due to the accompaniment of a functional lesion and in cases where sufficient curative effects of steroids are not produced in self-inhalation, administration of assisted inhalation resulted in improvement of clinical symptoms and pulmonary function and was proven effective.

Conclusions: Assisted inhalation therapy is expected to be useful in general and also in terms of expanding the application of ICS in the asthma in the elderly.

KEY WORDS

assistance, bronchial asthma, elderly persons, handicapped persons, inhaled corticosteroid

INTRODUCTION

A basic pathology in bronchial asthma is chronic airway inflammation, in which various cells such as eosinophils, lymphocytes, mast cells, and airway epithelial cells and various cytokines, chemokines, and inflammatory mediators produced from them are involved.[1] In addition, chronic airway inflammation is also involved in the enhancement of airway hyperresponsiveness, which is another pathology of bronchial asthma.[2,3] Therefore the basics of the therapy against bronchial asthma is to control inflammation caused by these wide range of cells and the drug of first alternative at this point is inhaled corticosteroids (ICS). ICS improves enhancement of airway hyperresponsiveness as well as controls airway inflammation by their powerful anti-inflammation action.[3] ICS are positioned as the drug of first choice for asthma controllers in the 2002 GINA Guidelines as well.[4]

Devices for inhalants are developed assuming that the circumstances where patients use inhalants by themselves. In actual clinical conditions, however, there are cases where physicians hesitate to introduce ICS therapy because of the diagnosis that the use of inhalants is difficult due to the accompaniment of a functional lesion, and asthma cases where sufficient curative effects of steroids are not produced in self- inhalation mostly among the elderly. Even in such cases, however, we experience cases where asthma control is improved with execution of accurately manipulated ICS therapy assisted by a caregiver for a part of the inhalation procedure (Fig. 1). Such being the case, this paper presents handicapped elderly asthma cases where clinical symptoms and pulmonary functions were improved by providing assistance for inhalation to discuss problems in inhalation therapy against asthma in the elderly. Written informed consent was obtained from

[1]Third Department of Internal Medicine, Wakayama Medical University, School of Medicine, Wakayama, Japan.
Correspondence: Masakazu Ichinose, M.D., Ph.D., Third Department of Internal Medicine, Wakayama Medical University, School of Medicine, 811-1 Kimiidera, Wakayama City, Wakayama 641-0012, Japan.
Email: masakazu@wakayama-med.ac.jp
Received 8 September 2005. Accepted for publication 20 December 2005.

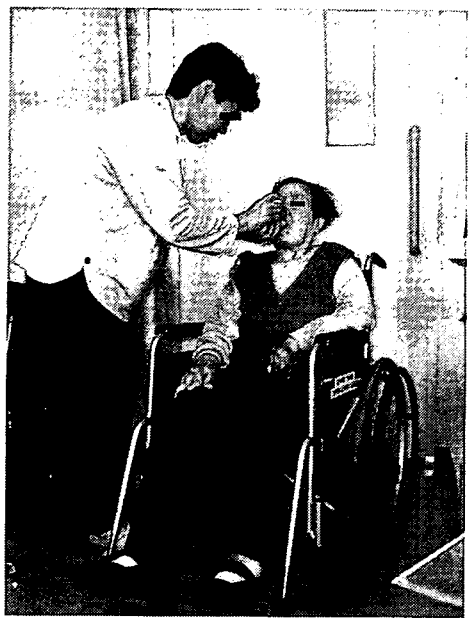

Fig. 1 Actual Assisted Inhalation Therapy. An asthma case where self-inhalation is impossible due to finger deformation associated with rheumatoid arthritis. A caregiver will spray the inhalant in a timely manner for the patient's inspiration and make sure of the inhalation condition.

both patients.

CLINICAL SUMMARY

CASE 1

Case: 81-year-old, woman

Chief complaints: Wheezing, coughing, sputum

Past history: She suffered from infantile asthma until nine years of age and has been suffering from rheumatoid arthritis since the age of 50.

Personal history: She has no smoking history, and occasionally drinks alcohol.

History of the present illness: The patient has been aware of coughing, sputum, and wheezing developing mostly during the night and continuing to the early morning for approximately four years and was treated with cough suppressants after being diagnosed as suffering from chronic bronchitis. After being admitted to our hospital for work-up of low back pain and rehabilitation, the patient was referred to our department for work-up of coughing.

Laboratory findings: FVC (forced vital capacity) 1.49 (L), $FEV_{1.0}$ (forced expiratory volume in one second) 1.05 (L), $FEV_{1.0}$% 70.5 (%), %$FEV_{1.0}$ 62.5 (%), %PEF (Peak expiratory flow) 50.2 (%)

Clinical course: With bronchial asthma suspected from the clinical history, administration of inhalational β_2 stimulants and monitoring of PEF under assistance started. We used the predicted PEF values in the Japanese report by Tsukioka *et al.*[5] After administration of inhalational β_2 stimulants, %PEF value reversed by approximately 20% and symptoms improved with no other cardiopulmonary diseases accompanied, the patient was diagnosed as suffering from bronchial asthma. Thus, self-administrated ICS therapy with 400 μg / day of hydrofluoroalkane-beclometasone (HFA-BDP) was started. With no improvement recognized both in the subjective symptoms and in the %PEF, which remained on the order of 59.1%, after eight weeks passed since the introduction of the ICS therapy, inhalation guidance was provided again, where it was found that inhalation was conducted with an inaccurate technique. Because it was considered difficult for the patient to manipulate inhaled steroids on her own due to a swan-neck deformity resulting from rheumatoid arthritis recognized in the inter-phalangeal joints, we introduced administration of assistance for inhalation with the cooperation of the patient's family. After administration of assistance for eight weeks, the clinical symptoms disappeared almost completely and the %PEF relative to the predictive values improved from approximately 59% to 90% (Fig. 2).

PATHOLOGICAL FINDINGS

CASE 2

Case: 82-year-old, woman

Chief complaints: Wheezing, dyspnea, insomnia

Past history: She suffered from asthma since the age of 70 and has been suffering from vascular dementia since the age of 76.

Personal history: She has no smoking history, and occasionally drinks alcohol.

History of the present illness: The patient became aware of paroxysmal dyspnea since the 70 years of age. Diagnosed as suffering from asthma by a general physician, the patient was receiving treatment with a theophylline drug. From approximately six months prior to admission, the frequency of wheezing and paroxysmal dyspnea increased and the patient became aware of insomnia due to nighttime asthma symptoms on several occasions a week. The patient was additionally administered with a patch-type β_2 stimulant but no sufficient improvement was noted in the asthma symptoms. The patient was referred to our hospital for the purpose of controlling the asthma.

Laboratory findings: FVC 1.48 (L), $FEV_{1.0}$ 1.12 (L), $FEV_{1.0}$% 75.7 (%), %$FEV_{1.0}$ 59.1 (%), %PEF 40.2 (%)

Clinical course: After administration of inhalational β_2 stimulants, $FEV_{1.0}$ improved by approximately 280 ml with no simultaneous onset of other cardiopulmonary diseases recognized in blood tests, thoracic radiographs, and electrocardiography. From the above findings, the asthma was diagnosed as being under control. With no problem in the patient's short term memory notwithstanding the accompaniment of vascular dementia, self-administrated ICS therapy

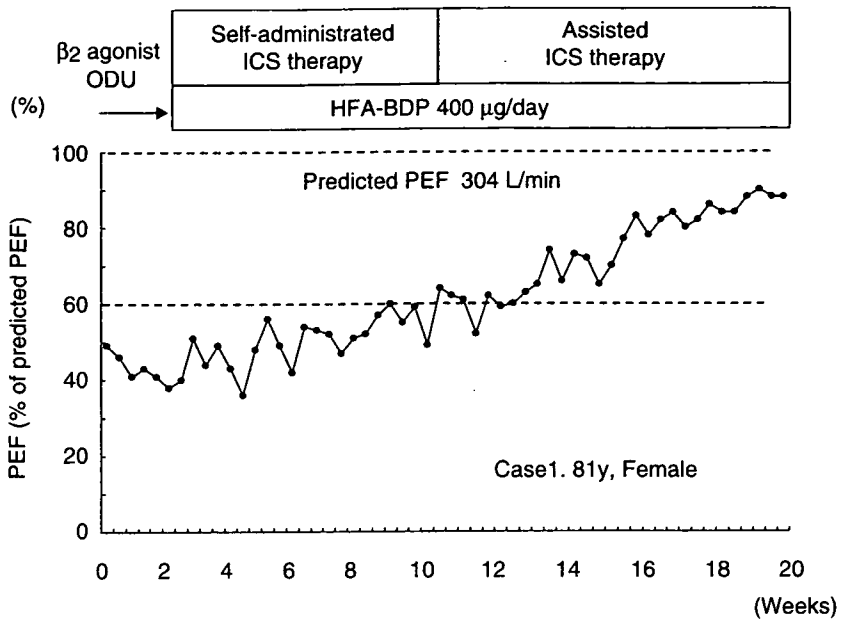

Fig. 2 Clinical Course of Case 1. PEF: peak expiratory flow; ODU: on demand use; ICS: inhaled corticosteroid; HFA-BDP: hydrofluoroalkane-beclometasone

was diagnosed as possible in inhalation guidance by a physician and ICS therapy with 400 μg/day of fluticasone propionate (FP) was introduced. In order to monitor the pulmonary function, the PEF values were measured by the physician three times from 9:00 to 11:00 in the morning and the highest value indicated in the measurement was regarded as the PEF value at that point. No improvements were recognized both in the subjective symptoms and in the %PEF, which remained on the order of 39.1%, after eight weeks passed since the introduction of the self-administrated ICS therapy. Inhalation guidance was provided again, where it was found that inhalation was conducted not only with an inaccurate technique but also in poor compliance with the prescription. To cope with the situation, assisted ICS therapy with a FP introduced under the cooperation of her family was administered with the objective of continuing inhalation with an accurate technique. After eight weeks of assisted administration of FP, insomnia associated with the asthma symptoms developing during the night time as well as the wheezing and dyspnea disappeared. The %PEF relative to the predictive value also improved from approximately 42% to 76% (Fig. 3).

DISCUSSION

In a case where self inhalation therapy is difficult to perform due to motor dysfunction associated with rheumatoid arthritis, and in a case where a problem was recognized in the inhalation technique and in the compliance to the prescription due to intellectual disability associated with vascular dementia, execution of assisted ICS therapy resulted in improvement in the clinical symptoms and the pulmonary function parameters.

It has been proven in many clinical studies that periodical use of ICS for persistent asthma results in improvement in pulmonary function, improvement in airway hyperresponsiveness, improvement in asthma symptoms, improvement in frequency and severity of attacks, and improvement in QOL.[6-9] In order to produce sufficient curative effects of ICS therapy, inhalation by an accurate technique is imperative.[4] It is often experienced, however, that complications accompanying asthma inhibit the inhalation technique in cases of asthma mostly in the elderly. While intellectual disability is a complication causing lack of understanding about the inhalation technique, motor dysfunction is a complication resulting in impairment of the inhalation technique. An inaccurate inhalation technique will not bring about improvement in asthma control because it will not produce sufficient curative effects of inhaled steroids, which may also result in lowering of compliance to an inhalation prescription. Assisted inhalation therapy is "an inhalation therapy performed with the assistance of caregivers for patients with diseases for which the therapy is indicated, in cases that the patients have difficulty in inhalation by themselves due to some functional disorders". Introduction of assisted ICS therapy is expected to be useful for improvement of asthma control in handicapped persons, in cases where self inhalation therapy is difficult to execute due to accompani-

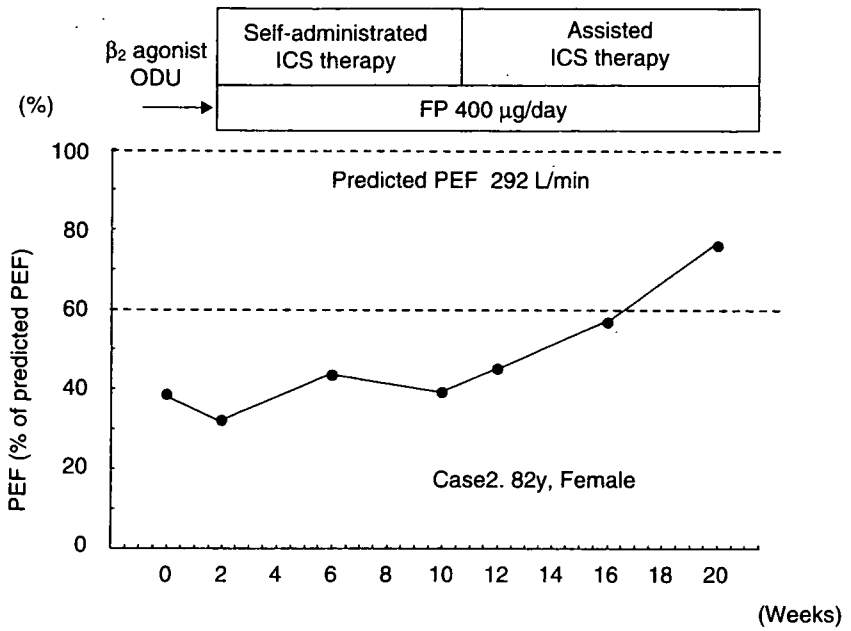

Fig. 3 Clinical Course of Case 2. PEF: peak expiratory flow; ODU: on demand use; ICS: inhaled corticosteroid; FP: fluticasone propionate

ment of a functional lesion or in cases where sufficient curative effects of steroids are not produced in self- inhalation, as presented this time. In the future, more handicapped asthma cases, for which the assisted ICS therapy is applicable, need to be accumulated to prove the clinical utility of assistance for inhalation.

The asthma mortality in the Japanese population, which continued to be on the order of approximately 6,000 people per annum in the early 1990s, has improved to less than 4,000 people per annum in the recent years.[10] The prevalence of anti-inflammatory therapy mostly with ICS is considered to have made a substantial contribution to the decrease in the asthma mortality.[11] On the contrary, according to a study on the change of asthma mortalities by generation over the years, however, the asthma mortality in the elderly aged 80 or higher has been increasing.[12] Chronic airway inflammation is a basic pathology of asthma in the elderly too and therefore it is important to control the inflammation by a therapy centering on ICS. However, it has been pointed out that the usage of ICS is extremely low in asthma in the elderly.[13,14] Assisted inhalation therapy is expected to be useful also in terms of expanding the application of ICS in the asthma in the elderly.

REFERENCES

1. Ichinose M. Inflamatory mechanisms in bronchial asthma and COPD. *Tohoku J. Exp. Med.* 2003;**200**:1-6.
2. Haley KJ, Drazen JM. Inflammation and airway function in asthma: what you see is not necessarily what you get. *Am. J. Respir. Crit. Care Med.* 1998;**157**:1-3.
3. Ichinose M, Takahashi T, Sugiura H *et al.* Baseline airway hyperresponsiveness and its reversible component: role of airway inflammation and airway calibre. *Eur. Respir. J.* 2000;**15**:248-253.
4. National Heart, Lung, and Blood Institute/World Health Organization. *Global Initiative for Asthma, Global Strategy for Asthma Management and Prevention.* Bethesda: NIH Publication, 2002.
5. Peak Expiratory Flow in Normal, Healthy Japanese Subjects (revised). In: Tsukioka K (ed). *Peak Expiratory Flow in Japanese Subjects.* Tokyo: Kyowa Kikaku Publication, 2002.
6. Haahtela T, Jarvinen M, Kava T, Kiviranta K, Koskinen S, Lehtonen K. Comparison of a beta 2-agonist, terbutaline, with an inhaled corticosteroid, budesonide, in newly detected asthma. *N. Engl. J. Med.* 1991;**325**:388-392.
7. Barnes PJ, Pedersen S, Busse WW. Efficacy and safety of inhaled corticosteroids. New developments. *Am. J. Respir. Crit. Care Med.* 1998;**157**:S1-53.
8. Suissa S, Ernst P, Benayoun S, Baltzan M, Cai B. Low-dose inhaled corticosteroids and the prevention of death from asthma. *N. Engl. J. Med.* 2000;**343**:332-336.
9. Jeffery PK, Godfrey RW, Adelroth E, Nelson F, Rogers A, Johansson SA. Effects of treatment on airway inflammation and thickening of basement membrane reticular collagen in asthma. A quantitative light and electron microscopic study. *Am. Rev. Respir. Dis.* 1992;**145**:890-899.
10. Ministry of Health, Labor and Welfare, Immunology and Allergy Research Group. *Asthma Prevention and Management Guideline 2003, Japan (Updated from: JGL1998).* Tokyo: Kyowa Kikaku Publication, 2003.
11. Suissa S, Ernst P. Use of anti-inflammatory therapy and asthma mortality in Japan. *Eur. Respir. J.* 2003;**21**:101-104.
12. Nakazawa T, Kawakami Y, Sudo M *et al.* Asthma death

among adults in Japan 1995-1997. *Arerugi* 2000;**49**:505-511.
13. Enright PL, McClelland RL, Newman AB, Gottlieb DJ, Lebowitz MD. Underdiagnosis and undertreatment of asthma in the elderly. *Chest* 1999;**116**:603-613.
14. Sin DD, Tu JV. Underuse of inhaled steroid therapy in elderly patients with asthma. *Chest* 2001;**119**:720-725.

CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Inhibition of reactive nitrogen species production in COPD airways: comparison of inhaled corticosteroid and oral theophylline

T Hirano, T Yamagata, M Gohda, Y Yamagata, T Ichikawa, S Yanagisawa, K Ueshima, K Akamatsu, M Nakanishi, K Matsunaga, Y Minakata, M Ichinose

Thorax 2006;**61**:761–766. doi: 10.1136/thx.200X.058156

See end of article for authors' affiliations

Correspondence to: Professor M Ichinose, Third Department of Internal Medicine, Wakayama Medical University School of Medicine, 811-1 Kimiidera, Wakayama 641-8509, Japan; masakazu@wakayama-med.ac.jp

Received 26 December 2005
Accepted 1 May 2006
Published Online First 31 May 2006

Background: Reactive nitrogen species (RNS) are thought to be one of the important factors in the pathogenesis of chronic obstructive pulmonary disease (COPD). A study was undertaken to examine the effects of theophylline and fluticasone propionate (FP) on RNS production in subjects with COPD.
Methods: Sixteen COPD subjects participated in the study. Theophylline (400 mg/day orally) or FP (400 μg/day inhalation) were administered for 4 weeks in a randomised crossover manner with a washout period of 4 weeks. Induced sputum was collected at the beginning and end of each treatment period. 3-nitrotyrosine (3-NT), which is a footprint of RNS, was quantified by high performance liquid chromatography with an electrochemical detection method as well as by immunohistochemical staining.
Results: Theophylline significantly reduced the level of 3-NT in the sputum supernatant as well as the number of 3-NT positive cells (both $p<0.01$). FP also reduced 3-NT formation, but the effect was smaller than that of theophylline. Theophylline also significantly reduced the neutrophil cell counts in the sputum ($p<0.01$), while FP treatment had no effect on the number of inflammatory cells in the sputum, except eosinophils.
Conclusions: Theophylline reduces nitrative stress and neutrophil infiltration in COPD airways to a larger extent than inhaled corticosteroid.

Airway inflammation is the pathophysiological feature of chronic obstructive pulmonary disease (COPD). Although a number of cells and mediators are involved in the pathophysiology of COPD, neutrophilic airway inflammation[1–4] and oxidative stress[5 6] in the lung are thought to play an important part in its development.

Reactive oxygen/nitrogen species (ROS/RNS) have a potent pro-inflammatory action[7] causing airway inflammation,[8] and therefore are thought to be one of the important factors in the pathogenesis of COPD, which results in airway epithelial injury, neutrophil migration and protease/antiprotease imbalance.[9] 3-Nitrotyrosine (3-NT) is a footprint of RNS. We have previously shown that the number of 3-NT positive cells and the level of 3-NT are increased in COPD airways, and these increases are correlated with the airflow limitation of COPD.[10 11] These data strongly suggest that ROS/RNS could have a key role in the pathogenesis of COPD, and that a reduction in ROS/RNS would lead to an anti-inflammatory effect.

More recently we have shown that inhaled corticosteroid can cause a small but significant reduction in RNS production in COPD airways.[12] On the other hand, a recent paper has reported that theophylline reduces the number of neutrophils via a reduction of interleukin (IL)-8 in COPD airways.[13] However, it is still unclear whether theophylline can suppress nitrative stress in COPD airways.

This study was undertaken to assess the anti-inflammatory effects of oral theophylline and inhaled corticosteroid in COPD using a crossover design. Neutrophilic airway inflammation and production of RNS were quantified by measuring 3-NT immunoreactivity in induced sputum. In addition, in order to evaluate the production of RNS in COPD airways in more detail, the levels of 3-NT were measured using high performance liquid chromatography (HPLC) with electrochemical detection (HPLC/ECD) analysis.

METHODS

Subjects

Sixteen patients with COPD regularly visiting Wakayama Medical University Hospital were recruited after giving informed consent. All patients satisfied the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria.[14] None of the patients had either asthma or atopy. The study was approved by the local ethics committee. All patients had a smoking history of at least 10 pack-years. The basal lung function of the patients is shown in table 1. All patients were stable and had had no exacerbation including viral or bacterial infection for at least 3 months before the study. None of the patients had been treated with inhaled or oral corticosteroid for at least 4 weeks before the study.

Study design

The study was a randomised crossover design to compare the effects of theophylline 200 mg twice daily with fluticasone propionate (FP) 200 μg twice daily. Each treatment was administered for 4 weeks with a 4 week washout period between treatments. All patients were assessed at the start and end of the treatment period (fig 1). Nitrotyrosine immunoreactivity, differential cell counts, and protein bound 3-NT levels in induced sputum were measured at that time.

Lung function testing

Lung function was evaluated using a dry rolling seal spirometer (System 7; Minato Medical Science, Osaka, Japan). Before and after treatment with theophylline or FP,

Abbreviations: COPD, chronic obstructive pulmonary disease; FEV_1, forced expiratory volume in 1 second; FP, fluticasone propionate; FVC, forced vital capacity; HDAC, histone deacetylase; HPLC/ECD, high performance liquid chromatography with electrochemical detection; IL, interleukin; 3-NT, 3-nitrotyrosine; RNS, reactive nitrogen species

Table 1 Characteristics of study subjects

Age (years)	71 (2)
Sex (F/M)	1/15
Smoking (current/ex)	4/12
Pack-years	68 (8)
FVC (l)	3.17 (0.19)
FEV_1 (l)	1.58 (.16)
FEV_1/FVC (%)	46.8 (3.9)
FEV_1 (% predicted)	53.8 (6.6)

Values are mean (SD).
FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 second.

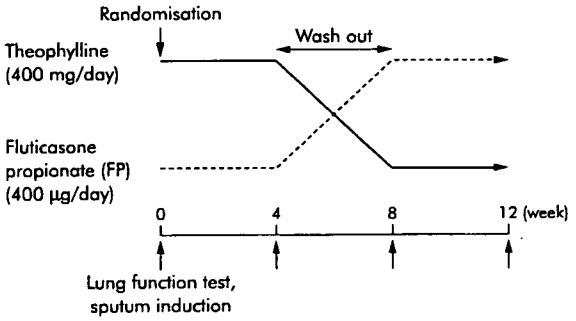

Figure 1 Study design.

forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC) were measured. Each measurement was performed 15 minutes after inhalation of 400 μg salbutamol via a metered dose inhaler.

Sputum induction and processing

Sputum was induced and processed as described in previous studies.[10 15] Briefly, after 15 minutes pretreatment with 400 μg salbutamol, all patients inhaled 4% hypertonic saline using an ultrasonic nebuliser (UN-701; AICA Co Ltd, Tokyo, Japan). Contamination of saliva was eliminated by visual inspection and examination with an inverted microscope. Hypertonic saline inhalation was performed for 15–30 minutes until the sputum volume was approximately 1 ml. The sputum sample was immediately treated with dithiothreitol (4 mg/g sputum) to dissociate the sulfide bonds of the mucus. Cell viability was determined by the trypan blue exclusion method. The total and absolute number of cells per ml processed sputum was calculated using a haemocytometer. 100 μl of the cell suspension adjusted to 1.0×10^6/ml were centrifuged in a Cytospin 4 cytocentrifuge (ThermoShandon, ThermoBioAnalysis, Tokyo, Japan) at 30*g* for 5 minutes. The preparation was stained with Hansel's stain (Torii Pharmaceutical, Tokyo, Japan) to assess the cell differential counts and stored at −80°C until immunocytochemical analysis.

Immunocytostaining

Samples were immunostained with antisera against 3-NT as described in previous studies.[10] Briefly, the preparation was fixed in 4% paraformaldehyde fixative solution for 30 minutes. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide in phosphate buffered saline (PBS) for 15 minutes at room temperature. After washing in PBS, the preparations were incubated with anti-nitrotyrosine rabbit polyclonal IgG (1:100 dilution; Upstate Biotechnology, Lake Placid, NY, USA) for 12 hours at 4°C. Non-specific binding to the antibody was prevented by preincubation with 4% skimmed milk in PBS containing 0.3% Triton-X for 30 minutes. The immunoreactions were visualised by the indirect immunoperoxidase method using Envision polymer reagent which is goat anti-rabbit IgG conjugated with peroxidase labelled dextran (Dako Japan Ltd, Kyoto, Japan) for 1 hour at room temperature. Diaminobenzidine reaction was performed, followed by counterstaining with Hansel's stain. The numbers of immunopositive cells were counted by two blinded investigators and the mean of the two values was registered. Cell types were distinguished by cell size, cell form, nuclear segmentation, and nuclear-cytoplasmic ratio.

Quantification of serum IL-8

The levels of serum IL-8 were measured using a commercially available ELISA kit (DuoSet ELISA Development Systems, R&D Systems, Minneapolis, MN, USA) according to the instructions provided by the manufacturer. The minimum detectable concentration of IL-8 was 31.2 pg/ml. A standard curve was obtained with serial dilution of the supplied recombinant human IL-8 by linear regression. The concentration of IL-8 in each sample was obtained by interpolation of its absorbance from a standard curve, and the mean value of the duplicate samples was then taken as the representative value.

Quantification of 3-nitrotyrosine

The levels of 3-NT in the cell-free supernatant were measured by HPLC/ECD as described previously.[11] Briefly, the cell debris was removed by additional centrifugation of the sputum at 3000*g* for 15 minutes at 4°C and, to condense the samples, 400 μl of supernatant were centrifuged using an Ultrafree-MC centrifugal filter (Millipore Corp, Bedford, MA, USA) at 9000*g* for 30 minutes at 4°C. This filter can collect protein of over 10 kDa. After centrifugation, the protein concentration of the sample was determined by the Lowry method.[16] After recovering the sputum protein, it was hydrolysed at 50°C for 18 hours with a freshly prepared solution of *Streptomyces griseus* Pronase (Calbiochem, Darmstadt, Germany) to liberate tyrosine and 3-NT residues. The hydrolysate was centrifuged at 9000*g* with filtration for 30 minutes with an Ultrafree-MC centrifugal filter and the filtrates were then analysed by HPLC/ECD.

50 μl of the sample were injected into a reverse phase column (C18: 3×150 mm; Eicom, Kyoto, Japan) at a flow rate of 0.5 ml/min. Eluents consisting of 5% methanol and 5 mg/l EDTA-2Na in 100 mM sodium phosphate buffer (pH 5.0) were continuously applied to the analytical electrochemical cells. The upstream electrochemical cell (coulometric cell) was used at −900 mV of applied potential for the reduction of 3-NT. The downstream cell (amperometric cell) was used at an oxidation potential of +300 mV for the detection of the reduced form of 3-NT. 3-NT was detected at a 13.5 minute retention time by the response at the oxidation cell on the basis of a standard curve of electrochemical responses as a function of the authentic 3-NT (Sigma Chemical Co, St Louis, MO, USA) concentration. We checked whether this peak was 3-NT as follows:[11] (1) there was no difference in the retention time of the peak between the standard 3-NT and the sputum samples under these HPLC conditions; and (2) when the reduction potential was changed from −900 mV to −600 mV, only the peak at 13.5 minutes disappeared.

The effect of treatment with dithiothreitol on the 3-NT level was determined. Levels of 3-NT with and without treatment with dithiothreitol showed quite good correlation ($r = 0.998$, $p<0.0001$), so it was considered that the processing of induced sputum with dithiothreitol had no influence on the measurement of 3-NT.

Table 2 Differential cell counts in induced sputum

	Theophylline		Fluticasone propionate	
	Before	After	Before	After
Total cells	2.53 (1.79–3.26)	1.63 (1.01–2.24)†	2.53 (1.86–3.19)	1.65 (0.70–2.60)
Neutrophils	1.89 (1.35–2.42)	1.15 (0.80–1.49)†	1.87 (1.33–2.42)	1.16 (0.46–1.87)
(%)	74.7	70.6	73.9	70.3
Macrophages	0.46 (0.18–0.74)	0.42 (0.10–0.74)	0.48 (0.24–0.72)	0.42 (0.08–0.76)
(%)	18.2	25.8	19.0	25.5
Eosinophils	0.06 (0.03–0.07)	0.03 (0.01–0.04)†	0.05 (0.04–0.07)	0.02 (0.01–0.04)†
(%)	2.4	1.8	2.0	1.2

Values are median (interquartile range) $\times 10^6$/ml.
†$p<0.01$ *v* pretreatment values.

The amount of tyrosine in the same sample was also determined in a separate process using HPLC analysis. Briefly, 1 µl of each sample was injected into a reverse phase column (Wakopak C30.5: 4.6 mm×300 mm, Wako Pure Chemical, Osaka, Japan) at a flow rate of 0.8 ml/min maintaining the temperature at 37°C. The eluents consisted of 5% methanol in 50 mM sodium acetate buffer (pH 4.7). Tyrosine was detected at a retention time of 8.47 minutes with the electrochemical response set at +600 mV. The amount of tyrosine in a sputum sample was determined based on the peak area compared with the standard curve of tyrosine (Wako Pure Chemical). The level of 3-NT was shown as a ratio to the total tyrosine concentration.

As shown in our previous report, the spike recovery analysis indicated that the percentage of recovery of 3-NT and tyrosine was more than 90%.[11] In addition, the coefficient of variation of 3-NT measurement in sputum samples previously performed in triplicate was 5–10%, indicating that the determination of 3-NT by this technique is highly reproducible.[11]

Statistical analysis

All data were expressed as median (interquartile range). Comparison of outcomes between the theophylline and FP groups was performed using repeated measures ANOVA. Wilcoxon's signed rank sum test was used to compare the effect of treatment on the total and differential cell counts and pulmonary function. Pearson's correlation analysis was used to assess the correlations between changes in the RNS marker and those in the differential cell counts. A value of $p<0.05$ was considered to be significant.

RESULTS

The mean (SD) plasma theophylline level during theophylline administration was 6.32 (0.9) mg/l, which is lower than the clinically recommended concentration as a bronchodilator (10–20 mg/l). Because of this low concentration of theophylline, neither FP nor theophylline had a significant effect on FVC and FEV_1 after 4 weeks of administration (FVC: before theophylline 3.34 (2.64–4.03) l; after theophylline 3.49 (2.81–4.16) l; before FP 3.41 (2.86–3.96) l; after FP 3.48 (2.89–4.07) l; FEV_1: before theophylline 1.51 (1.11–1.91) l; after theophylline 1.60 (1.09–2.11) l; before FP 1.48 (0.91–2.05) l; after FP 1.59 (1.06–2.11) l).

Theophylline administration significantly reduced the total number of inflammatory cells in the sputum from 2.53 (1.79–3.26)$\times 10^6$/ml to 1.63 (1.01–2.24)$\times 10^6$/ml ($p<0.01$, table 2, fig 2A). Consistent with this, the number of neutrophils in the sputum also decreased significantly from 1.89 (1.35–2.42)$\times 10^6$/ml to 1.15 (0.80–1.49)$\times 10^6$/ml ($p<0.01$, table 2,

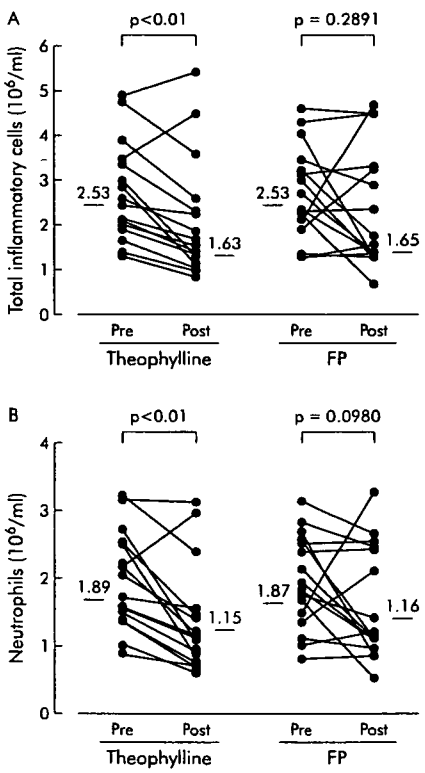

Figure 2 Effect of theophylline and fluticasone propionate (FP) on inflammatory cells. Theophylline significantly reduced the total number of inflammatory cells (A) and neutrophils (B), whereas FP had no apparent effect. Bars indicate median values.

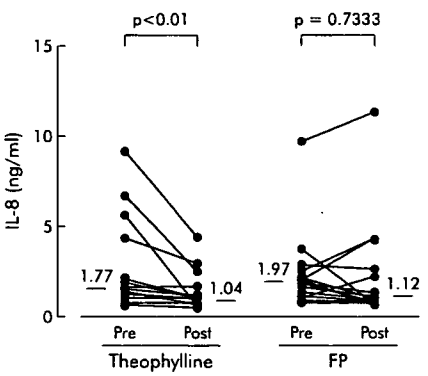

Figure 3 Effect of theophylline and fluticasone propionate (FP) on the concentration of interleukin (IL)-8. The IL-8 concentration in the sputum supernatant was significantly decreased by theophylline while FP had no effect. Bars indicate median values.

Table 3 Positive cell counts of 3-nitrotyrosine (3-NT) in induced sputum

	Theophylline		Fluticasone propionate	
	Before	After	Before	After
Total cells	1.24 (0.75–1.72)	0.73 (0.28–1.18)†	1.35 (0.86–1.83)	0.83 (0.25–1.41)*
Neutrophils	0.97 (0.52–1.42)	0.53 (0.22–0.83)†	0.89 (0.47–1.32)	0.52 (0.12–0.93)
(%)	78.2	72.6	65.9	44.6
Macrophages	0.31 (0.07–0.55)	0.18 (0.02–0.34)	0.40 (0.19–0.61)	0.42 (0.08–0.76)
(%)	23.8	24.7	29.6	50.6

Values are median (interquartile range) $\times 10^6$/ml.
†$p<0.01$; *$p<0.05$ *v* values before treatment.

fig 2B), while FP treatment did not affect the numbers of any inflammatory cells in the sputum with the exception of eosinophils (table 2, fig 2A, B). Neither theophylline nor FP had any effect on the number of macrophages in the sputum. The number of eosinophils was quite small, but significant decreases were seen with both theophylline and FP (table 2).

To determine the mechanism of the decrease in neutrophils, we next measured the concentration of IL-8 in the sputum supernatant which is one of the well known chemoattractants of neutrophils. As shown in fig 3, theophylline significantly reduced the level of IL-8 in the sputum supernatant (from 1.77 (0.03–3.50) ng/ml before treatment to 1.04 (0.39–1.70) ng/ml after treatment ($p<0.01$), while FP did not. There was no apparent correlation between the serum levels of IL-8 and the number of neutrophils.

We then compared the effects of theophylline and FP on nitrative stress in the airway inflammation of COPD. As shown in table 3 and fig 4A–C, after 4 weeks of treatment with theophylline the total number of 3-NT positive cells in the induced sputum was decreased from 1.24 (0.75–1.72)$\times 10^6$/ml to 0.73 (0.28–1.18)$\times 10^6$/ml. Theophylline also decreased the number of immunopositive neutrophils for 3-NT from 0.97 (0.52–1.42)$\times 10^6$/ml to 0.53 (0.22–0.83)$\times 10^6$/ml ($p<0.01$, table 3, fig 4D). In contrast, although FP also decreased the total number of 3-NT positive cells (from 1.35 (0.86–1.83)$\times 10^6$/ml to 0.83 (0.25–1.41)$\times 10^6$/ml, $p<0.05$), the effect was milder than that of theophylline and there was no apparent effect on the number of 3-NT positive neutrophils (0.89 (0.47–1.32)$\times 10^6$/ml before treatment, 0.52 (0.12–0.93)$\times 10^6$/ml after treatment; table 3, fig 4C, D).

We next measured the levels of 3-NT in sputum. There was a possibility that current smoking may affect the levels of 3-NT, but no significant difference in 3-NT levels was seen between current smokers and ex-smokers, at least in the

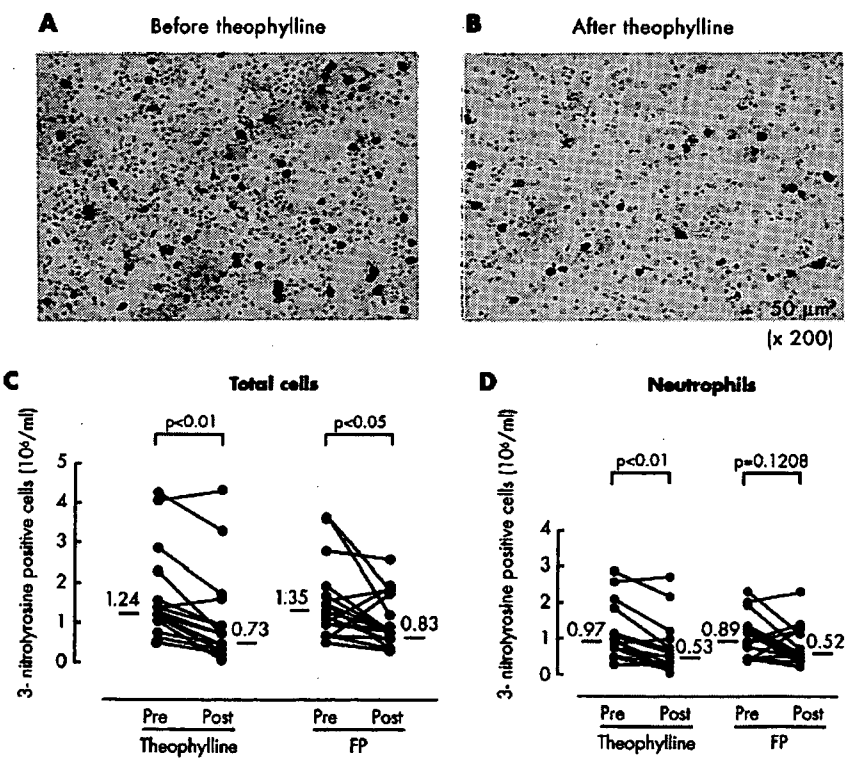

Figure 4 Effect of theophylline and fluticasone propionate (FP) on immunocytochemical staining against 3-nitrotyrosine (3-NT). Immunopositive inflammatory cells for 3-NT in the induced sputum were reduced after treatment with theophylline (B) compared with before treatment (A). Theophylline significantly reduced the total immunoreactivity of 3-NT in inflammatory cells (C) and neutrophils (D). FP also reduced the total immunoreactivity of 3-NT in inflammatory cells, but not in neutrophils. Bars indicate median values.

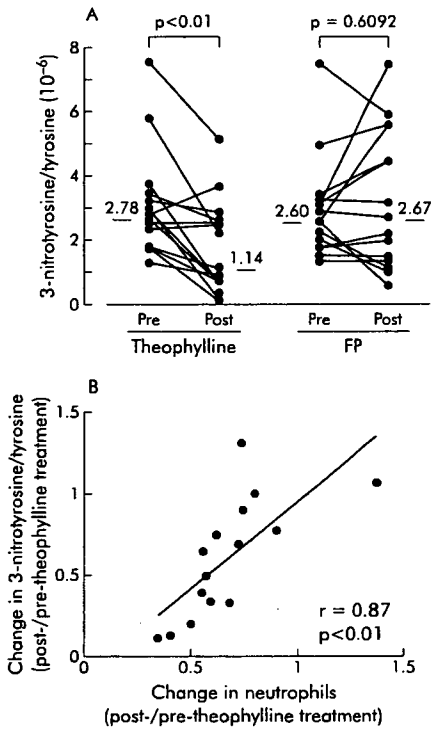

Figure 5 Effect of theophylline and fluticasone propionate (FP) on 3-nitrotyrosine (3-NT) levels (A) Theophylline significantly reduced the level of 3-NT in induced sputum as measured by HPLC/ECD, while FP had no effect. (B) There was a significant correlation between the decrease in neutrophils and the decrease in the 3-NT level after theophylline treatment. Bars indicate median values.

present study (current smokers 2.14×10^{-6}, ex-smokers 3.43×10^{-6} 3-NT/tyrosine). Consistent with the results of immunocytochmeical staining, the level of 3-NT in the sputum supernatant was significantly decreased after 4 weeks of treatment with theophylline ($p<0.01$, fig 5A), while no apparent effect was seen after treatment with FP. As shown in fig 5B, there was a significant positive correlation between the decrease in the number of neutrophils in the induced sputum and the reduction in 3-NT levels in the sputum supernatant in those treated with theophylline ($r=0.87$, $p<0.01$). There was no significant correlation between the serum IL-8 levels and the levels of 3-NT in sputum.

DISCUSSION

We have shown for the first time that treatment with low dose theophylline significantly reduces RNS production and neutrophil infiltration to a greater extent than inhaled corticosteroid in COPD airways.

We have previously reported the effect of inhaled corticosteroid on the suppression of nitrative stress in COPD airways. In that study, administration of inhaled corticosteroid for 4 weeks significantly reduced total 3-NT immunoreactivity of inflammatory cells, macrophages and neutrophils in the induced sputum.[12] Inhaled corticosteroid also reduced the inducible nitric oxide synthase (iNOS) immunoreactivity in those cells. The formation of nitrotyrosine depends on the oxidation of nitric oxide (NO), which reacts with superoxide anion to produce the more potent RNS, peroxynitrite.[17] Peroxynitrite causes tyrosine nitration. Because iNOS is one of the main sources of NO production, it is suggested that the mechanism of nitrative stress inhibition by inhaled corticosteroid, as in our present study, could be due mainly to the reduction of iNOS.

Low dose theophylline reduced nitrative stress in COPD airways to a larger extent than inhaled corticosteroid. Theophylline also inhibited neutrophilic inflammation in COPD airways. In addition, as shown in fig 5, there was a significant positive correlation between the reduction in 3-NT levels and the decrease in the number of neutrophils after theophylline administration. An alternative pathway for the formation of 3-NT is via the neutrophil myeloperoxidase (MPO) effect on NO.[17 18] Nitrite produced by the reaction of NO with oxygen is oxidised by MPO which results in the formation of reactive nitrogen intermediates. These products are also involved in tyrosine nitration.[17] It is therefore possible that the theophylline induced inhibition of nitrative stress seen in the present study was due to the inhibition of neutrophil infiltration.

We also observed a significant reduction in IL-8 production after theophylline administration, which is a possible mechanism for the inhibition of neutrophilic inflammation by theophylline. This is compatible with the findings of a previous study.[13] The precise mechanism of IL-8 reduction by theophylline is unclear. However, it has recently been shown that theophylline can inhibit the release of IL-8 from respiratory epithelial cells in vitro.[19] The direct effect of theophylline on respiratory epithelial cells might therefore be one possible mechanism.

A new anti-inflammatory mechanism by theophylline in the treatment of COPD has recently been proposed by Barnes and co-workers.[20–22] The activity of histone deacetylases (HDACs), which mediate inflammatory gene repression, is reduced in patients with COPD.[23] Although the precise mechanism of this inactivation of HDACs is not yet clear, oxidative/nitrative stress might be involved via the nitration of tyrosine residues in the active centre of HDACs by peroxynitrite or other RNS.[24 25] Theophylline has been reported to restore the decreased HDAC activity in patients with COPD.[20] In this study we have shown, for the first time, the reduction in tyrosine nitration by theophylline using electrochemical as well as immunohistochemical techniques. Our results support the hypothesis of Barnes and colleagues. It is considered that combined evaluation of the HDAC activity and nitrative stress by HPLC/ECD could clarify the precise mechanism of action of theophylline.

Recent investigations have shown that RNS has an important role in the pathogenesis of COPD, causing cell injury,[26] activation of metalloproteinases,[27] inactivation of α_1-antiproteinase,[28] and enhanced IL-8 production.[29] Neutrophilic airway inflammation is another important feature of COPD,[1–3] and neutrophils are an important source of RNS.[10] It is considered that both neutrophilic inflammation and oxidative/nitrative stress could have critical roles in the development of COPD. The findings of our study suggest that theophylline might be a useful therapeutic tool for COPD treatment by inhibiting both neutrophilic airway inflammation and nitrative stress.

In conclusion, treatment with theophylline reduces nitrative stress as well as neutrophilic inflammation in COPD. Because there is a significant positive correlation between the decrease in the number of neutrophils and the reduction in 3-NT levels, the reduction in nitrative stress is considered to be due mainly to the inhibition of neutrophilic inflammation. Since the suppression of nitrative stress seems to be effective in inhibiting the inflammatory process and subsequent obstructive changes in COPD airways, theophylline may slow the progression of airway obstruction in COPD. Further large, long term, placebo controlled studies with a range of concentrations of theophylline and different severities of COPD are needed to confirm this hypothesis.

ACKNOWLEDGEMENTS

The authors thank Koji Fukui for his technical help with the study and Brent Bell for reading the manuscript.

Authors' affiliations

T Hirano, T Yamagata, M Gohda, Y Yamagata, T Ichikawa, S Yanagisawa, K Ueshima, K Akamatsu, M Nakanishi, K Matsunaga, Y Minakata, M Ichinose, Third Department of Internal Medicine, Wakayama Medical University School of Medicine, Wakayama, Japan

Competing interests: none declared

REFERENCES

1 **Keatings VM**, Collins PD, Scott DM, *et al.* Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am J Respir Crit Care Med* 1996;**153**:530–4.
2 **Peleman RA**, Rytila PH, Kips JC, *et al.* The cellular composition of induced sputum in chronic obstructive pulmonary disease. *Eur Respir J* 1999;**13**:839–43.
3 **Stockley RA**. Neutrophils and the pathogenesis of COPD. *Chest* 2002;**121**:151–5S.
4 **Barnes PJ**. Mediators of chronic obstructive pulmonary disease. *Pharmacol Rev* 2004;**56**:515–48.
5 **Rahman I**, Morrison D, Donaldson K, *et al.* Systemic oxidative stress in asthma, COPD, and smokers. *Am J Respir Crit Care Med* 1996;**154**:1055–60.
6 **MacNee W**. Pathogenesis of chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2005;**2**:258–66.
7 **Nathan C**. Nitric oxide as a secretory product of mammalian cells. *Faseb J* 1992;**6**:3051–64.
8 **Barnes PJ**. Reactive oxygen species and airway inflammation. *Free Radic Biol Med* 1990;**9**:235–43.
9 **MacNee W**. Pulmonary and systemic oxidant/antioxidant imbalance in chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2005;**2**:50–60.
10 **Ichinose M**, Sugiura H, Yamagata S, *et al.* Increase in reactive nitrogen species production in chronic obstructive pulmonary disease airways. *Am J Respir Crit Care Med* 2000;**162**:701–6.
11 **Sugiura H**, Ichinose M, Tomaki M, *et al.* Quantitative assessment of protein-bound tyrosine nitration in airway secretions from patients with inflammatory airway disease. *Free Radic Res* 2004;**38**:49–57.
12 **Sugiura H**, Ichinose M, Yamagata S, *et al.* Correlation between change in pulmonary function and suppression of reactive nitrogen species production following steroid treatment in COPD. *Thorax* 2003;**58**:299–305.
13 **Culpitt SV**, de Matos C, Russell RE, *et al.* Effect of theophylline on induced sputum inflammatory indices and neutrophil chemotaxis in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2002;**165**:1371–6.
14 **Global Initiative for Chronic Obstructive Lung Disease (GOLD)**. *Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease.* Bethesda, MD: National Institutes of Health, National Heart Lung and Blood Institute, 2005.
15 **Pin I**, Gibson PG, Kolendowicz R, *et al.* Use of induced sputum cell counts to investigate airway inflammation in asthma. *Thorax* 1992;**47**:25–9.
16 **Lowry OH**, Rosebrough NJ, Farr AL, *et al.* Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;**193**:265–75.
17 **van der Vliet A**, Eiserich JP, Shigenaga MK, *et al.* Reactive nitrogen species and tyrosine nitration in the respiratory tract: epiphenomena or a pathobiologic mechanism of disease? *Am J Respir Crit Care Med* 1999;**160**:1–9.
18 **Eiserich JP**, Hristova M, Cross CE, *et al.* Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* 1998;**391**:393–7.
19 **Koyama S**, Sato E, Masubuchi T, *et al.* Procaterol inhibits IL-1beta- and TNF-alpha-mediated epithelial cell eosinophil chemotactic activity. *Eur Respir J* 1999;**14**:767–75.
20 **Ito K**, Lim S, Caramori G, *et al.* A molecular mechanism of action of theophylline: induction of histone deacetylase activity to decrease inflammatory gene expression. *Proc Natl Acad Sci USA* 2002;**99**:8921–6.
21 **Cosio BG**, Tsaprouni L, Ito K, *et al.* Theophylline restores histone deacetylase activity and steroid responses in COPD macrophages. *J Exp Med* 2004;**200**:689–95.
22 **Barnes PJ**. Theophylline in chronic obstructive pulmonary disease: new horizons. *Proc Am Thorac Soc* 2005;**2**:334–9.
23 **Ito K**, Ito M, Elliott WM, *et al.* Decreased histone deacetylase activity in chronic obstructive pulmonary disease. *N Engl J Med* 2005;**352**:1967–76.
24 **Ito K**, Hanazawa T, Tomita K, *et al.* Oxidative stress reduces histone deacetylase 2 activity and enhances IL-8 gene expression: role of tyrosine nitration. *Biochem Biophys Res Commun* 2004;**315**:240–5.
25 **Barnes PJ**, Adcock IM, Ito K. Histone acetylation and deacetylation: importance in inflammatory lung diseases. *Eur Respir J* 2005;**25**:552–63.
26 **Beckman JS**, Beckman TW, Chen J, *et al.* Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 1990;**87**:1620–4.
27 **Okamoto T**, Akaike T, Nagano T, *et al.* Activation of human neutrophil procollagenase by nitrogen dioxide and peroxynitrite: a novel mechanism for procollagenase activation involving nitric oxide. *Arch Biochem Biophys* 1997;**342**:261–74.
28 **Whiteman M**, Szabo C, Halliwell B. Modulation of peroxynitrite- and hypochlorous acid-induced inactivation of alpha1-antiproteinase by mercaptoethylguanidine. *Br J Pharmacol* 1999;**126**:1646–52.
29 **Filep JG**, Beauchamp M, Baron C, *et al.* Peroxynitrite mediates IL-8 gene expression and production in lipopolysaccharide-stimulated human whole blood. *J Immunol* 1998;**161**:5656–62.

IMPORTANCE OF ASSISTANCE BY CAREGIVERS FOR INHALED CORTICOSTEROID THERAPY IN ELDERLY PATIENTS WITH ASTHMA

To the Editor: Complications accompany asthma in elderly patients more often than in other age groups, and some complications could interfere with the procedure of inhalation therapy.[1–3] Even in such cases, regular and accurately calibrated inhaled corticosteroid (ICS) therapy can be made possible with the assistance of a caregiver.[3] Although assisted inhalation therapy is currently in practice, no clinical studies have evaluated its effects. This objective of this study was to investigate the usefulness of assistance by caregivers during the inhalation procedure in elderly patients with asthma whose pulmonary functions were not improved by self-administered ICS therapy.

Patients aged 65 and older with severe asthma and less than 60% of percentage peak expiratory flow (PEF) received self-administered ICS therapy (equivalent to 800 μg beclomethasone dipropionate (BDP)/d) for 8 weeks. Of the patients who received the above therapy, 10 cases with PEF improvement of less than 15% were enrolled. While investigating the causes of the poor responsiveness to self-administered ICS therapy, we conducted a survey on the caregivers' preferences concerning the devices used for assistance and their reasons by giving them three types of ICS devices for training: one metered-dose inhaler (MDI; hydrofluoroalkane–BDP) and two dry powder inhalers (DPIs: fluticasone propionate and budesonide). After that, assisted ICS therapy (equivalent to 800 μg BDP/d) was administered for 8 weeks using the devices selected by the caregivers to prospectively evaluate the effects of assisted inhalation on PEF and forced expiratory volume in 1 second (FEV_1). Statistical analysis was performed using the multiple comparison Tukey tests.

The subjects consisted of five men and five women with an average age of 81.6. All the patients had severe asthma with a mean percentage FEV_1 of 58.0% and a mean percentage PEF of 43.2%. Poor responsiveness to self-administered ICS therapy was attributed to problems with the inhalation technique in seven cases and compliance in nine cases. The presence of one or more complications was observed in all patients. These complications consisted of rheumatoid arthritis, diabetic neuropathy, or cerebral infarction causing motor dysfunction in seven cases; Alzheimer's disease or cerebral infarction causing intellectual dysfunction in five cases; and chronic obstructive pulmonary disease (COPD) in four cases.

PEF was increased more than 15% in 70% of the study subjects with assisted ICS therapy. Significantly greater improvement was observed in PEF and FEV_1 at the end of assisted ICS therapy than with self-administered ICS therapy (Figure 1; $P < .001$ in both cases). With regard to the three cases in which the assisted ICS therapy did not bring about improvement in pulmonary function, one was attributed to failure in compliance because of the complication of stomatitis, and the other two were attributed to airflow limitation with less reversibility associated with COPD or the progress of asthmatic airway remodeling. Nine caregivers selected MDI inhalers for assisted inhalation. The reasons for selecting the MDI inhaler were that MDI inhalers were easy to use, because they enable one-step inhalation and that MDI inhalers could be used without anxiety, because the spraying and inhaling conditions can be observed visually.

It is often the case that functional disorders accompanying asthma in elderly patients interfere with their inhalation technique, and this has led to hesitation on the part of physicians to prescribe inhalation therapy for such patients. One study reported that the more complications an elderly