

今年度の研究成果

II 呼気凝縮液分析による気道過敏性検査の代用

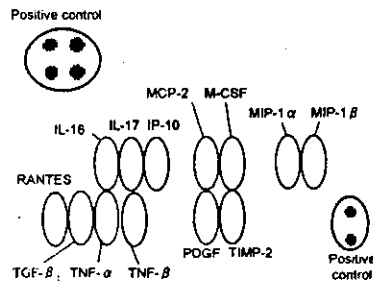
気道過敏性検査との関連性

1. 呼気凝縮液中のcysLTs濃度と気道過敏性の間には有意な相関は認めなかったが、プロモチロシンやクロチロシンとは弱いながら相関を認めた。
2. 呼気凝縮液内分子の発現様式(関与細胞)と気道過敏性の関連性が示唆された。

研究計画

1. 気道過敏性に対する生理学的アプローチの再考
2. 呼気凝縮液分析による気道過敏性検査の代用
方法論の確立:安全性・妥当性・他の検査との比較
気道過敏性検査との関連性
分析物質の絞込み

呼気凝縮液中のサイトカイン・ケモカイン発現（一ノ瀬）

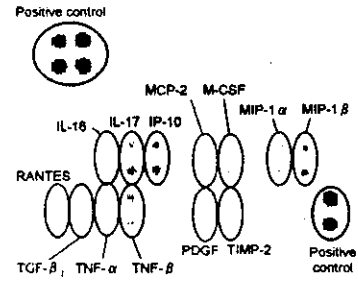


Intensity: 0.0 - 1.0

Case: 36y, Male

Smoking: Non-smoker

VC	4.86 L (116.5%)
FVC	4.77 L (114.4%)
FEV ₁	3.98 L (83.4%)
%FEV ₁	102.1%
PC ₂₀₀	25mg/ml



ICC: 0.000 - 0.01000

Case: 61y, Female

Therapy: Steroid naive

VC	2.16 L (92.0%)
FVC	2.28 L (97.3%)
FEV ₁	1.54 L (78.3%)
%FEV ₁	67.5%
PEF Variability	310/350 (12.5%)
PC ₂₀₀	0.78mg/ml

Cytokine Arrayにて呼気凝縮液中に検出された物質（一ノ瀬）

Interleukines	IL-16, IL-17
CC Chemokines	MIP-1 α , MIP-1 β RANTES, MCP-2
CXC Chemokine	IP-10
CSF	M-CSF
Growth factor	TGF- β ₁ , PDGF
Others	TNF- α , TNF- β TIMP-2

Cytokine Arrayにて呼気凝縮液中に検出された物質 (一ノ瀬)

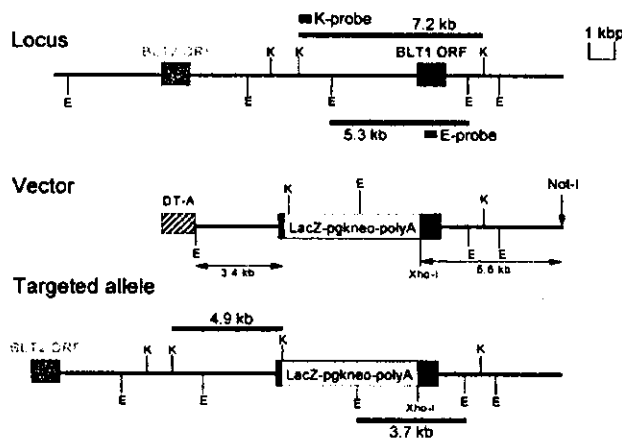
喘息重症化に伴い、発現が増強していた物質

IL-17, IP-10, TNF- β

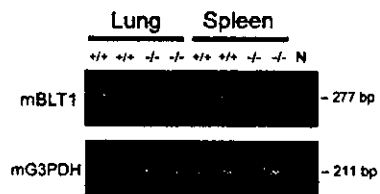
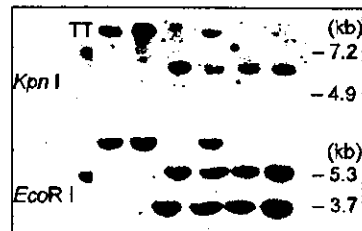
喘息重症度で発現に大きな変化を認めなかった物質

MIP-1 α , MIP-1 β , M-CSF, TIMP-2

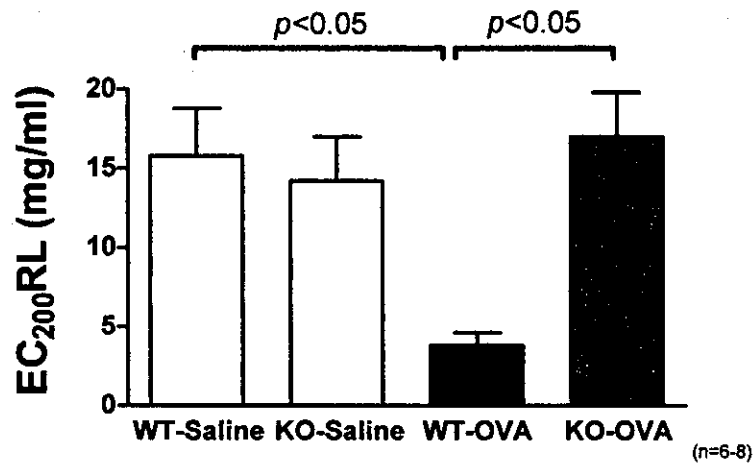
Gene Targeting of BLT1 (長瀬)



Southern blot



LTB受容体欠損によるアレルギー反応後の気道過敏性亢進の消失
(長瀬)



今年度の研究成果

II 呼気凝縮液分析による気道過敏性検査の代用

分析物質の絞込み

1. Human Cytokine Arrayを用いた喘息患者呼気凝縮液検討で、サイトカイン(M-CSF、TNF- α 、VEGF、IGF-1)、CCケモカイン(RANTES、TARC)、CXCケモカイン(SDF-1)を検出可能であった。
2. 動物モデルのデータ(LTB etc)を加味し検討を加える。

成果に対する評価

1. ピークフローの週内変動がより簡便な気道過敏性の生理学的指標として代用できる可能性を示した。
2. 他の検査法との比較による呼気凝縮液の妥当性を示すとともに、種々の炎症関連物質の測定により、いくつかの物質については臨床応用の可能性が示唆された。

→今年度の目的は概ね達成でき、臨床応用への端緒となった意義は大きいと考えられる。

今後の課題

1. 生化学的指標の絞込み。
2. 簡便性・迅速性の向上による臨床応用化。

V. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
H Sugiura, M Ichinose, et al	Quantitative assessment of protein-bound tyrosine nitration in airway secretions from patients with inflammatory airway disease	Free Radical Research	38	49-57	2004
M Ichinose, PJ Barnes :	Cytokine-directed therapy in asthma. Current Drug Targets	Inflammation & Allergy	3	263-269	2004
K Matsunaga, M Ichinose, et al :	Effect of a leukotriene receptor antagonist on the prevention of recurrent asthma attacks after an emergency room visit	Allergology International	53	341-347	2004
Y Fukuchi, M Ichinose, et al :	COPD in Japan : the Nippon COPD Epidemiology Study	Respirology	9	458-465	2004
Ishii S, Nagase T, Shindou H, Takizawa H, Ouchi Y, Shimizu T	Platelet-activating factor receptor develops airway hyperresponsiveness independently of airway inflammation in a murine asthma model	J Immunol	172	7095-7102	2004
Takai D, Nagase T, Shimizu T	New therapeutic key for cystic fibrosis : a role for lipoxins	Nature Immunol (News and Views) (論文解説)	5	357-358	2004
Jo T, Nagata T, Iida H, Imuta H, Iwasawa K, Ma J, Hara K, Omata M, Nagai R, Takizawa H, Nagase T, Nakajima T	Voltage-gated sodium channel expressed in cultured human smooth muscle cells : Involvement of SCN9A	FEBS Lett	567	339-343	2004
一ノ瀬正和	気道過敏性検査の臨床の現状	呼吸	23	9-17	2004
一ノ瀬正和	気道過敏性の意義とそれに代わる指標	診断と治療	92	1325-1332	2004
松永和人, 一ノ瀬正和, 他	急性喘息治療後の再発作に対するロイコトリエン受容体拮抗薬の有用性に関する検討	呼吸	23	S37-S39	2004
池田剛司, 一ノ瀬正和, 他	外来気管支喘息患者に対する自覚症状アンケート調査およびプラシランカストによる長期自覚症状改善作用の検討	呼吸	23	726-733	2004

南方良章, 一ノ瀬正和	気道平滑筋とその機能	呼吸と循環	52	169-175	2004
山縣俊之, 一ノ瀬正和	－喘息の長期管理における薬物療法－ 長時間作用型 β_2 刺激薬に関する臨床エ ビデンス	現代医療	36	500-505	2004
松永和人, 一ノ瀬正和	IgE と気道過敏性	喘息	17	58-62	2004
一ノ瀬正和	医学と医療の最前線。気道過敏性におけ る最近の知見	日本内科学会 雑誌	93	162-167	2004
松永和人, 一ノ瀬正和	気管支喘息関連遺伝子	医学のあゆみ	210	867-870	2004
山縣俊之, 一ノ瀬正和	吸入ステロイド薬の使い方 3) ベクロメ タゾン (とくにキュパール®)	呼吸器科	6	119-124	2004
平野綱彦, 一ノ瀬正和, 他:	アレルギー診療 Update. アレルギー治 療薬の上手な使い方. 気管支喘息 成人吸 入ステロイド薬 (解説/特集)	Medicine	41	378-380	2004
平野綱彦, 一ノ瀬正和, 他:	Annual Review 呼吸器 2005 4. 呼気凝縮液による病態解明	Annual Review 呼吸器 2005		85-91	2005

VI. 研究成果の刊行物

Quantitative Assessment of Protein-bound Tyrosine Nitration in Airway Secretions from Patients with Inflammatory Airway Disease

HISATOSHI SUGIURA^{a,*}, MASAKAZU ICHINOSE^b, MASAFUMI TOMAKI^a, HIROMASA OGAWA^a, AKIRA KOARAI^a, TOMOMI KITAMURO^a, YUICHI KOMAKI^a, TAKEFUMI AKITA^a, HIROHITO NISHINO^c, SHINICHIRO OKAMOTO^d, TAKAAKI AKAIKE^d and TOSHIO HATTORI^a

^aDivision of Respiratory and Infectious Diseases, Tohoku University Graduate School of Medicine, Sendai 980-8574, Japan; ^bThird Department of Internal Medicine, Wakayama Medical University, Wakayama 641-0012, Japan; ^cEicom Corporation, Kyoto 612-8474, Japan; ^dDepartment of Microbiology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto 860-8556, Japan

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Because reactive nitrogen species (RNS) have potent inflammatory activity, they may be involved in the inflammatory process in pulmonary diseases. We recently reported increased numbers of 3-nitrotyrosine immunopositive cells, which are evidences of RNS production, in the sputum of patients with chronic obstructive pulmonary disease (COPD) and patients with asthma compared with healthy subjects. In the present study, we attempted to quantify this protein nitration in the airways by means of high-performance liquid chromatography (HPLC) used together with an electrochemical detection system that we developed. Sputum samples were obtained from 15 stable COPD patients, 9 asthmatic patients and 7 healthy subjects by using hypertonic saline inhalation. The values for the molar ratio of protein-bound 3-nitrotyrosine/tyrosine in patients with asthma ($4.31 \pm 1.13 \times 10^{-6}$, $p < 0.05$) and patients with COPD ($3.04 \pm 0.36 \times 10^{-6}$, $p < 0.01$) were significantly higher than those in healthy subjects ($1.37 \pm 0.19 \times 10^{-6}$). The levels of protein-bound 3-nitrotyrosine in the airways were not significantly different in asthmatic patients and COPD patients. A significant negative correlation was found between values for protein-bound 3-nitrotyrosine/tyrosine and % FEV₁ values in patients with COPD ($r = -0.53$, $p < 0.05$) but not in patients with asthma. These results suggest that our HPLC-electrochemical method is useful for quantifying RNS production in human airways. More importantly, they show that increased RNS production in the airways seems to contribute in a critical way to the pathogenesis of COPD, and that the effects of RNS in airways may differ in asthma and COPD.

Keywords: COPD; Asthma; Reactive nitrogen species; 3-Nitrotyrosine

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a serious medical problem worldwide.^[1] Although the pathogenesis has not yet been fully elucidated, airway and parenchymal inflammation seems to play a key role.^[2] Reactive nitrogen species (RNS) are formed from nitric oxide (NO) and superoxide anion^[3] or via the H₂O₂/peroxidase-dependent nitrite oxidation pathway.^[4] These RNS cause airway inflammation^[5] by means of activation of matrix metalloproteinase (MMP),^[6] inactivation of α_1 -antitrypsin,^[7] and enhancement of the production of the potent neutrophil chemoattractant interleukin-8 (IL-8)^[8] and the proinflammatory cytokine tumor necrosis factor- α (TNF- α).^[9] Therefore, RNS may be involved in the pathophysiology of the inflammatory process in COPD.

Production of RNS was reported to be upregulated in the airways of asthmatic patients.^[10] More recently, we reported that RNS production by inflammatory cells in the sputum was increased in patients with COPD as well as those with asthma compared with that of healthy subjects, as assessed by immunochemical staining for 3-nitrotyrosine.^[11] However, because this method was only semi-quantitative, a procedure for the accurate quantification of the amount of 3-nitrotyrosine, which is a footprint of RNS production, is still required. RNS cause

*Corresponding author. Tel.: +81-22-717-8220. Fax: +81-22-717-8221. E-mail: sugiura@intl.med.tohoku.ac.jp

the nitration of tyrosine residues of proteins^[5] and, as a result, may change the function of the proteins.^[7,12] Therefore, quantification of protein-bound 3-nitrotyrosine is important. In the present study, we developed a new technique that utilizes high-performance liquid chromatography (HPLC) with electrochemical detection to quantify 3-nitrotyrosine formed in the airways of asthmatic and COPD patients. By means of this technique, we found that protein-bound 3-nitrotyrosine levels in the airways of COPD patients as well as asthmatic patients were increased compared with those of healthy subjects. Furthermore, protein-bound 3-nitrotyrosine levels were negatively correlated with the % predicted FEV₁ values for COPD patients but not for asthmatic patients.

MATERIALS AND METHODS

Subjects

Fifteen stable COPD patients, 9 asthmatic patients and 7 healthy subjects took part in the present study. Table I gives the characteristics of the study subjects. All subjects in all groups had smoked, but all had stopped smoking at least 1 year before the study. All healthy subjects were not atopic and had normal lung function. They did not have clinical manifestations of bronchial asthma such as recurrent episode of wheezing. All COPD patients were diagnosed with the disease according to the criteria of the American Thoracic Society (ATS).^[13] The lungs of all COPD patients showed low-attenuation areas in computed tomographic studies. Ten of the COPD patients were diagnosed as having pulmonary emphysema by pulmonary function tests, including measurement of lung volume (total lung capacity exceeded 120% of predicted values) and carbon monoxide diffusing capacity of the lungs (DL_{CO}) (<60% of predicted values). The remaining five COPD patients were diagnosed as having chronic bronchitis on the basis of clinical manifestations of a chronic, productive cough for 3 months/year at

least two successive years. Asthmatic patients had their disease diagnosed on the basis of recurrent episodes of wheezing, airway hyperresponsiveness (provocative methacholine testing causing a 2-fold increase in respiratory resistance, <10 mg/ml) and airway eosinophilia as assessed by sputum examination. All asthmatic patients had a positive diagnosis of immediate-type hypersensitivity made via allergen skin prick tests and a radioallergosorbent assay, and an improvement in FEV₁ of more than 12% from the predicted values or an absolute value of 200 ml after inhalation of 400 µg of salbutamol. No subject had a respiratory tract infection during the month preceding the test. Patients were excluded from the study if they had had systemic glucocorticoid therapy during the 2 months before the study or inhaled glucocorticoid treatment during the month before the study. The study was conducted with the approval of the Tohoku University Committee on Clinical Investigation, and informed written consent was obtained from all patients.

Pulmonary Function Testing

Pulmonary function was assessed with a dry rolling seal spirometer (CHESTAC 11; Chest Co., Tokyo, Japan) according to ATS criteria.^[14] The normal predicted value of FEV₁ derived by Knudson *et al.* was used in the present study.^[15] The thoracic gas volume was measured by using a constant-volume, pressure-compensated, whole body plethysmograph (2800J Autobox; Gould Electronics, Dayton, OH, USA) with the subject panting at a frequency of 2 Hz and a peak-to-peak flow of 2–3 l/s.^[16] DL_{CO} was assessed with a total pulmonary function analyzer (FUDAC-70; Fukuda Electronics Co., Tokyo, Japan) according to ATS recommendations for the standard technique.^[17]

Measurement of Exhaled NO

A rapid response chemiluminescent analyzer (280NOA; Sievers Instruments Inc., Boulder, CO,

TABLE I Characteristics of the study subjects

	Healthy subjects	Asthmatic patient	COPD patients
Age (years)	65.7 ± 3.9	40.1 ± 4.5	70.8 ± 1.7
Sex (M:F)	6:1	6:3	13:2
Pack-years	27.6 ± 3.6	20.0 ± 4.2	29.7 ± 2.7
FVC (l)	4.25 ± 0.16	4.04 ± 0.41	2.89 ± 0.17 ^{*,#}
FEV ₁ (l)	3.68 ± 0.19	3.12 ± 0.41	1.44 ± 0.11 ^{*,##}
FEV ₁ /FVC	86.8 ± 3.2	74.1 ± 4.5	56.8 ± 3.6 ^{*,##}
% FEV ₁	122 ± 4.4	93.9 ± 7.7	64.7 ± 6.3 ^{*,#}
% TLC	ND	ND	121 ± 2.3
% DL _{CO}	ND	ND	40.3 ± 2.8
eNO (ppb)	9.0 ± 2	51.0 ± 13*	11.0 ± 2 [#]

Data are given as mean values ± SEM. COPD, chronic obstructive pulmonary disease; eNO, exhaled NO; FVC, forced vital capacity; FEV₁, forced expiratory volume in 1 s; TLC, total lung capacity; DL_{CO}, carbon monoxide diffusing capacity of the lungs; ND, not done. **p* < 0.05, ***p* < 0.01 compared with the values for the healthy subjects; #*p* < 0.05, ##*p* < 0.01 compared with the values for asthmatic patients.

USA) was used for analysis of exhaled NO (eNO). We measured the eNO as previously described.^[11,18] Exhalations were repeated until we obtained three values of NO that varied by less than 10%.

Sputum Induction and Processing

Sputum was induced and processed according to the method described in previous studies.^[19,20] All subjects inhaled 400 μg of salbutamol, both to assess lung function and to avoid hypertonic saline-induced bronchoconstriction. Fifteen minutes after salbutamol inhalation, subjects inhaled 4% hypertonic saline by means of an ultrasonic nebulizer (MU-32; Sharp Co. Ltd., Osaka, Japan). The nebulizer generated particles with a mean mass median diameter of 5.4 μm at an output of 2.2 ml/min. We eliminated saliva contamination by using visual inspection and inverted microscope examination. Hypertonic saline inhalation was performed for 15–30 min until the sputum volume was approximately 1 ml. Sputum samples were immediately treated with Sputasol (Oxoid Ltd., Basingstoke, Hampshire, UK) at 4 times the volume to dissociate the sulfide bonds in the mucus. The mixtures were vortexed for 15 s and gently aspirated in and out of a Pasteur pipette to ensure mixing. Samples were rocked for 15 min, and 10 mM phosphate-buffered saline (PBS; pH 7.4) was then added to stop the effect of Sputasol. After samples were centrifuged at 790g for 10 min, supernatants were obtained. All supernatant samples were stored at -80°C until measurement of 3-nitrotyrosine.

Measurement of Protein-bound 3-Nitrotyrosine

The protein-bound 3-nitrotyrosine level in the sputum was measured by using the protein hydrolysate prepared according to the method reported in previous studies.^[21,22] Briefly, a supernatant sample was first centrifuged at 9000g for 20 min. The supernatant sample was centrifuged again at 9000g for 40 min to concentrate it (by 100-fold); proteins of more than 10 kDa were obtained by use of an Ultrafree-MC centrifugal filter (Millipore Corp., Bedford, MA, USA). The protein concentration of the condensed supernatant was determined by the Lowry method,^[23] and this concentration was adjusted to 5–20 mg/ml. The sputum protein thus recovered was enzymatically hydrolyzed to liberate tyrosine and 3-nitrotyrosine residues. Samples were mixed with a freshly prepared solution of *Streptomyces griseus* Pronase (Calbiochem, Darmstadt, Germany), which had been extensively dialyzed against PBS (pH 7.4) before use, to yield 5 mg/ml sputum protein and 1 mg/ml Pronase in a volume of 1 ml. The Pronase-treated samples were incubated at 50°C for 18 h. The hydrolysate was centrifuged at 9000g with filtration for 30 min with an Ultrafree-MC centrifugal filter (10-kDa cutoff), and then the filtrates were analyzed for tyrosine and 3-nitrotyrosine by HPLC under the conditions described below.

HPLC-electrochemical Analysis

The configuration of the HPLC-electrochemical system used in this study is shown in Fig. 1A. The sample (injection volume, 10 μl) was subjected to

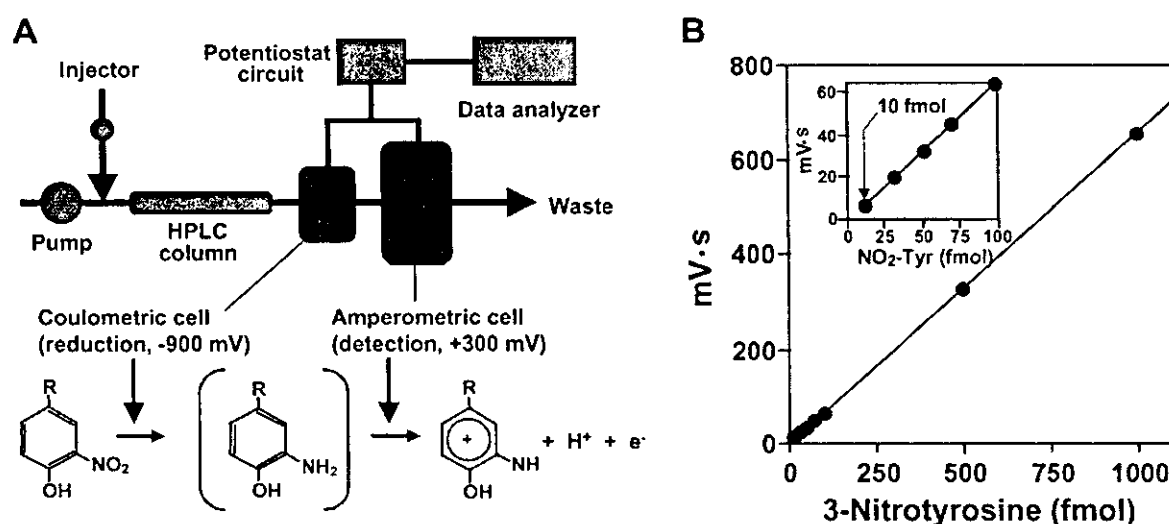


FIGURE 1 Diagram of the HPLC-electrochemical system used in this study (A), and dose-dependent electrochemical responses as a function of authentic 3-nitrotyrosine concentration (B). 3-Nitrotyrosine was electrochemically reduced at the coulometric cell to form 3-aminotyrosine, which was then detected by an oxidation (amperometric) cell, as shown in the diagram in (A). The inset in (B) shows the detection limit of our electrochemical method, which exceeded 10^{-10} M (1 fmol/10 μl).

a reverse phase column (C18: 3 × 150 mm; Eicom, Kyoto, Japan) and eluted under isocratic conditions with 0.1 M sodium phosphate buffer (pH 5.0) containing 5% methanol, at a flow rate of 0.5 ml/min. The eluate was continuously applied to the analytical electrochemical system that consisted of two electrochemical cells. The upstream electrochemical (coulometric) cell was made of porous carbon and was used for reduction of 3-nitrotyrosine at -900 mV. The downstream cell was made of amperometric glassy carbon to detect the reduced form of 3-nitrotyrosine (i.e. 3-aminotyrosine) at an oxidation potential of +300 mV. 3-Nitrotyrosine was quantified by the response at the oxidation (amperometric) cell on the basis of a standard curve of electrochemical responses as a function of authentic 3-nitrotyrosine (Sigma Chemical Co., St. Louis, MO, USA) concentration. Assignment of the peak and quantification of protein-bound 3-nitrotyrosine from each group were determined in a blinded manner to avoid identification of the group. The specificity of the detection of the peak for 3-nitrotyrosine by this system was confirmed according to the following three criteria. (1) Comparison of the retention time of the peak with that of authentic 3-nitrotyrosine, which is 13.98 min under these HPLC conditions. (2) Disappearance of the peak after treatment of the sample with 10 mM sodium hydrosulfite (Na₂S₂O₄) in PBS (pH 7.4) for 30 min at 37°C. (3) Nullification of the peak with use of the reduction potential of -600 mV.

The amount of 3-nitrotyrosine in each sample was corrected by the amount of tyrosine in the same sample, which was determined in a separate process by using a 12-channel HPLC-electrochemical array analysis (CoulArray 5600; ESA, Inc., Chelmsford, MA, USA), as described earlier.^[22,24] Briefly, for determination of the tyrosine content, the hydrolysate (10 µl) was injected into a reverse phase column (4.6 × 150 mm; TSK gel ODS-80Ts, Tosoh, Tokyo, Japan) and eluted to separate tyrosine under isocratic conditions (50 mM sodium acetate buffer, pH 4.7, plus 5% methanol; flow rate, 0.8 ml/min). The tyrosine peak from the HPLC column was detected with the CoulArray instrument equipped with 12 detector cells operating at oxidative potentials that increased by 50 mV between +200 and +750 mV. Tyrosine eluted under these conditions at a 5.35-min retention time, showed a typical profile of electrochemical responses with a peak response at +600 mV. The peak amplitude for various amounts of authentic tyrosine was linear over at least two orders of magnitude (0.1–10 µM; $r = 0.999$). The amount of tyrosine in a sample was determined from the peak response obtained after injection of appropriately diluted hydrolysate, as compared with a standard curve for the electrochemical responses of authentic tyrosine.

In some experiments, spike-recovery analyses were performed with samples obtained from each group (healthy subjects, asthmatic patients and COPD patients). Specifically, authentic 3-nitrotyrosine or tyrosine was added to the hydrolysate of the sputum protein at the same concentration of 3-nitrotyrosine or tyrosine in each hydrolysate sample, as determined by the HPLC-electrochemical analysis as just described, and the mixture was subjected to analysis for 3-nitrotyrosine. The spike recovery was compared with that of the same amount of 3-nitrotyrosine or tyrosine without the sputum hydrolysate and with that of the hydrolysate alone. This spike-recovery analysis showed that the percentage of recovery of tyrosine and 3-nitrotyrosine was more than 90%, and no appreciable increase in 3-nitrotyrosine was observed. We thus confirmed that tyrosine derivatives were recovered quantitatively and that no artifactual tyrosine nitration occurred.

Statistical Analysis

Data were expressed as means ± SEM. Nonparametric analysis of variance (Kruskal–Wallis test) was used to assess variance among the three groups. If significant variance was found, an unpaired two-group test was performed to determine significant differences between individual groups. Data were tested by means of Pearson's correlation analysis to assess the correlation between the 3-nitrotyrosine level and lung function. Probability values of less than 0.05 were considered significant.

RESULTS

Sensitivity of the HPLC-electrochemical Analysis

We first examined the sensitivity of our HPLC-electrochemical analysis system and the dose-dependent profile of the electrochemical response for authentic 3-nitrotyrosine obtained by this system. A linear electrochemical response was observed with a wide range of authentic 3-nitrotyrosine concentrations (10 fmol–1 nmol in absolute amounts) in the present system (Fig. 1B). The detection limit of our electrochemical method exceeded 10^{-10} M (>1 fmol), which is superior to other methods previously reported, including HPLC-electrochemical array detection.^[21,22,25]

3-Nitrotyrosine Assay

We then analyzed the formation of free 3-nitrotyrosine in supernatants of sputum samples by using filtrates obtained with an ultrafiltration tube (cutoff size, 10 kD). However, the level of 3-nitrotyrosine in such a low-molecular-weight fraction was below

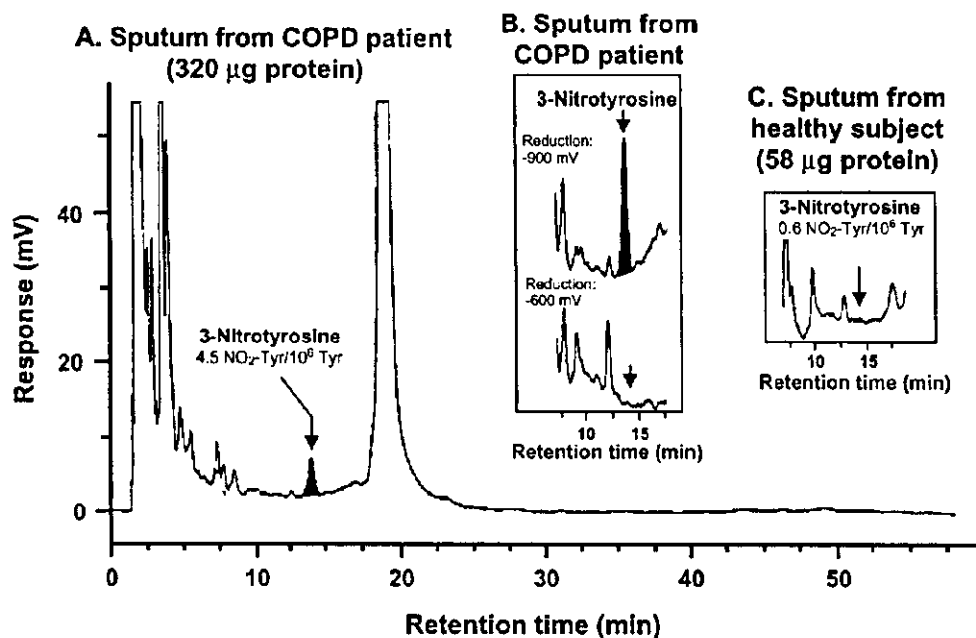


FIGURE 2 Elution profiles of hydrolysates of sputum proteins in samples obtained from a COPD patient (A,B) and from a healthy subject (C) obtained by using HPLC-electrochemical detection. (B) shows the electrochemical response of the same COPD sample as in (A). The relative amount of 3-nitrotyrosine ($\text{NO}_2\text{-Tyr}/\text{Tyr}$) identified by each analysis is also shown.

the detection limit. Thus, in the present study, we mainly focused on protein-bound 3-nitrotyrosine as a biomarker for biological nitration occurring during airway inflammation.

Furthermore, the variation in 3-nitrotyrosine measurement was tested by using different protein preparations obtained from the sputum. Specifically, sputum samples from six different individuals including healthy subjects and COPD and asthmatic patients were used for preparation of protein hydrolysates via Pronase digestion. Then, these six hydrolysates were subjected to HPLC-electrochemical analysis for quantification of 3-nitrotyrosine. The coefficient of variation obtained for values of triplicate determinations for each sample was 5–10%. This result indicates that determination of protein-bound 3-nitrotyrosine by the present analysis was highly reproducible regardless of the disease condition of the subjects tested.

Figure 2 shows an actual trace for 3-nitrotyrosine detected by the HPLC-electrochemical method. The amount of 3-nitrotyrosine was higher in the sputum of COPD patients (Fig. 2A,B) than in that of healthy subjects (Fig. 2C). The specificity of the peak response for 3-nitrotyrosine was verified by nullification of the peak with a change to a reduction potential of -600 mV , as illustrated in the lower panel of Fig. 2B. Similarly, the peak response disappeared after treatment of the hydrolysate of the sputum protein applied to the HPLC column with sodium hydrosulfite (data not shown).

Figure 3A,B shows the content of tyrosine and 3-nitrotyrosine, respectively, for each group.

The 3-nitrotyrosine values corrected for protein content for both asthmatic patients ($0.64 \pm 0.21\text{ nmol/mg protein}$, $p < 0.05$) and COPD patients ($0.57 \pm 0.07\text{ nmol/mg protein}$, $p < 0.05$) were significantly higher than those for healthy subjects ($0.13 \pm 0.01\text{ nmol/mg protein}$) (Fig. 3B). This result is consistent with the data illustrated in Fig. 3C showing the molar ratio of 3-nitrotyrosine to tyrosine in the induced sputum from healthy subjects and asthmatic and COPD patients. Specifically, the values of the ratio 3-nitrotyrosine/tyrosine were significantly higher for asthmatic patients ($4.31 \pm 1.13 \times 10^{-6}$, $p < 0.05$) and COPD patients ($3.04 \pm 0.36 \times 10^{-6}$, $p < 0.01$) than values for healthy subjects ($1.37 \pm 0.19 \times 10^{-6}$). However, there was no significant difference in tyrosine content corrected for the amount of protein in the sputum between the two groups of patients and the healthy subjects, as illustrated in Fig. 3A. Also, the levels of protein-bound 3-nitrotyrosine in the airways were not significantly different in asthmatic patients and COPD patients.

There was a significant negative correlation between the sputum 3-nitrotyrosine/tyrosine values and the % predicted FEV_1 values in COPD patients ($r = -0.53$, $p < 0.05$) (Fig. 4), but no significant relationship between these measures in asthmatic patients.

Levels of eNO

We also monitored the level of eNO for each subject (Table I). The eNO value for asthmatic patients

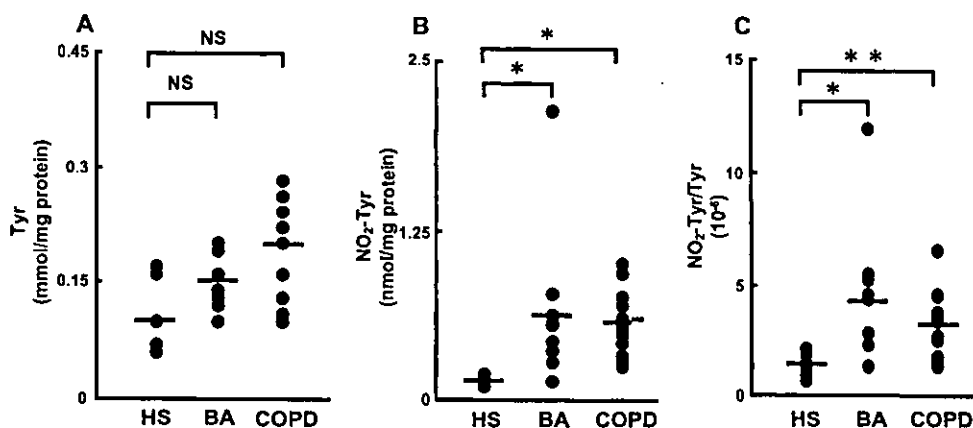


FIGURE 3 Levels of tyrosine (Tyr) (A), 3-nitrotyrosine (NO₂-Tyr) (B) and NO₂-Tyr/Tyr (C) formed in protein of the sputum from healthy subjects (HS), asthmatic patients (BA) and COPD patients (COPD) * $p < 0.05$, ** $p < 0.01$ compared with values for healthy subjects. NS, not significant.

(51 ± 13 ppb, $p < 0.01$) was significantly higher than that for healthy subjects (9.0 ± 2 ppb). The eNO value for COPD patients (11 ± 2 ppb, $p < 0.05$) was significantly lower than that for asthmatic patients. No significant difference in the eNO value between in the healthy and COPD groups was found. We had noted that a similar trend of only slight increase in eNO in asthmatic patients compared with COPD patients in our earlier study.^[11] Therefore, tyrosine nitration in the airway seems to be a more useful biomarker for RNS production than is eNO, particularly for COPD patients.

DISCUSSION

Until now, reproducible quantification of 3-nitrotyrosine in human airway secretion samples had not been possible. Our present work has confirmed that our HPLC-electrochemical analysis system can be clinically applied to measurement of protein-bound 3-nitrotyrosine in human airway secretions.

We quantified protein-bound 3-nitrotyrosine in airway secretions (sputum induced with hypertonic saline) from healthy subjects, asthmatic patients, and COPD patients. 3-Nitrotyrosine formation was increased in secretions in airways affected by asthma and COPD, which indicates that RNS production was elevated in these diseases. Furthermore, we showed that protein-bound 3-nitrotyrosine levels in the sputum from COPD patients had a significantly negative correlation with % FEV₁ values, which suggests that this increased RNS production may contribute to the pathogenesis of COPD.

3-Nitrotyrosine is thought to be a useful biomarker for RNS generated during airway inflammation, because a number of reports have shown that upregulation of the inducible isoform of NO synthase, to produce excessive amounts of NO, often accompanies the formation of 3-nitrotyrosine at the site of inflammation.^[5,26,27] Two pathways are thought to be involved in RNS-dependent tyrosine nitration that occurs in biological systems. One is peroxynitrite-mediated tyrosine nitration,^[3] and

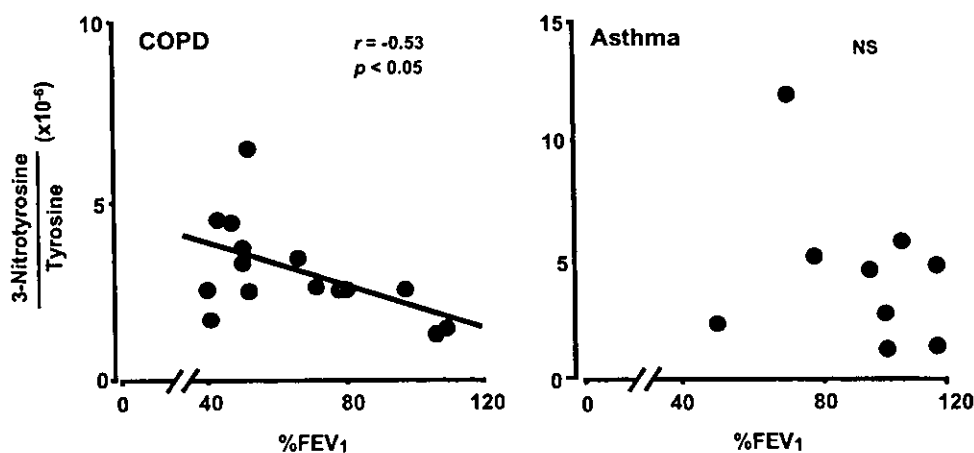


FIGURE 4 Correlation between protein-bound 3-nitrotyrosine/tyrosine and % predicted FEV₁ values in COPD and asthmatic patients. The straight line and p value correspond to the fitted regression equation of Pearson's correlation analysis. NS, not significant.

the other is nitration mediated by peroxidase-dependent nitrite oxidation.^[4] In a separate experiment, we counted the number of neutrophils and eosinophils, which are the major contributors to peroxidase-mediated tyrosine nitration, in the same sputum sample that was used for 3-nitrotyrosine quantification. The neutrophil and eosinophil counts were not significantly correlated with 3-nitrotyrosine content (data not shown). It is difficult, therefore, to establish which pathway served as the major mechanism for nitration of protein-bound tyrosine observed in the present study.

In our earlier work, we assessed nitrate stress in the airways of asthmatic and COPD patients with an immunocytochemical method to localize 3-nitrotyrosine. This method was a semi-quantitative one for assessing RNS production. 3-Nitrotyrosine levels can be measured quantitatively by using an enzyme-linked immunosorbent assay (ELISA) or semi-quantitatively by means of immunochemical staining. Such immunological techniques, however, may suffer from certain methodological shortcomings.^[28,29] Because the ELISA measures nitration products of both free tyrosine and protein-bound tyrosine, the actual nitration of protein by RNS cannot be determined. Immunochemical staining shows only the cellular localization of 3-nitrotyrosine; it does not provide a quantitative 3-nitrotyrosine level. Thus, a more accurate and convenient approach for analysis of 3-nitrotyrosine would be beneficial so as to quantify the biological effects of RNS.

In this study, we were able to measure protein-bound 3-nitrotyrosine quantitatively by using a newly developed HPLC-electrochemical method that provides high sensitivity and the required specificity for 3-nitrotyrosine. With this technique, we measured the 3-nitrotyrosine/tyrosine ratio in sputum protein at an order lower than $\mu\text{mol/mol}$. To our knowledge, this report is the first one to provide the exact amount of 3-nitrotyrosine in human airway secretions. Recently, Kenyon *et al.* measured the level of 3-nitrotyrosine in mouse pulmonary tissue sections.^[30] They showed that 3-nitrotyrosine levels increased about 3-fold after ozone exposure.^[30] The values for the nitration products of protein-bound tyrosine in their study were about $\mu\text{mol/mol}$, which are compatible with our data for human sputum.

The precise reason for the negative correlation of the values of % FEV₁ with those of 3-nitrotyrosine content in COPD patients, shown in Fig. 4A, is still unclear. However, because the decrease in % FEV₁ indicates airway constriction and obstruction, such a negative correlation suggests that RNS, which are produced in excess in COPD as assessed by elevated levels of 3-nitrotyrosine, may exacerbate airway inflammation and oxidative stress, which would

result in obstruction of the airways. Oxidative stress is known to be involved in the pathogenesis of COPD.^[5,26,31,32] Among the molecules that induce oxidative stress, RNS seem to be important because of their potent chemical reactivities.^[5] RNS not only cause tissue injury directly^[3-5] but also enhance the production of proinflammatory cytokines and chemokines such as TNF- α ,^[9] IL-6^[9] and IL-8.^[8] Increased production of these mediators was reported in COPD airways.^[2,32] Furthermore, activation of MMP^[6] and inactivation of antiprotease by RNS lead to an imbalance in protease/antiprotease,^[7] the destruction of lung parenchyma and a decrease in lung elastic recoil.^[2] Also, 3-nitrotyrosine itself causes microtubule dysfunction in an alveolar type II epithelial cell line.^[12] Therefore, RNS may contribute to pathological changes of airway inflammation in COPD via induction of both oxidative stress and nitrate stress.

In asthmatic patients, in contrast, no significant correlation was observed between the level of protein-bound 3-nitrotyrosine and the degree of airway obstruction as assessed by % FEV₁, a finding that also agrees with our previous report.^[11] This lack of significant correlation in asthma is quite intriguing, and its mechanism remains to be clarified. A beneficial effect of NO in asthma is suggested because of the amelioration of bronchial constriction and oxidative stress by NO.^[33,34] Although nitrate stress is induced by RNS, NO itself or its *S*-nitrosated derivatives such as *S*-nitrosoglutathione may conversely improve airway obstruction. Thus, the lack of an apparent correlation between % FEV₁ and 3-nitrotyrosine content in asthma may reflect the overall effect of NO.

In our current study, the levels of nitration products of protein-bound tyrosine in the airways were not significantly different in asthmatic patients and COPD patients. This result is in contrast to our previous study that showed significantly higher 3-nitrotyrosine-immunopositive cell counts in the sputum from COPD patients than in sputum from asthmatic patients.^[11] There are two possible explanations for this discrepancy. First, because in our previous study we counted 3-nitrotyrosine-immunopositive cells, only the localization of 3-nitrotyrosine was determined, not the quantitative amount of 3-nitrotyrosine. Second, the formation of 3-nitrotyrosine by inflammatory cells, which are the main cells recovered in the sputum, may be more extensive in COPD than in asthma. However, tyrosine nitration in other cells, such as epithelial cells^[10] or endothelial cells,^[35] may be enhanced in asthma compared with COPD. Thus, these data may provide a plausible explanation of our finding of equal total amounts of nitration products of protein-bound tyrosine in the sputum of COPD patients and asthmatic patients.

Protein-bound tyrosine was used as an internal standard in the present work, as in previous studies.^[22,30] Tyrosine may be oxidized and lost because of oxidative stress in asthma and COPD, which could affect the values for 3-nitrotyrosine corrected for tyrosine content. However, when the tyrosine content was corrected for the protein content in each sputum preparation, there was no apparent difference between the disease groups and healthy subjects, as shown in Fig. 3A. More important, the values of 3-nitrotyrosine content (expressed as amounts per mg protein), as for the values of the molar ratio of 3-nitrotyrosine/tyrosine (Fig. 3C), were significantly higher in asthmatic patients ($p < 0.05$) and COPD patients ($p < 0.05$) than in healthy subjects (Fig. 3B). Therefore, the oxidation of tyrosine, if any did occur, did not have a significant effect on the estimation of protein-bound 3-nitrotyrosine corrected for tyrosine content in the sputum.

In conclusion, by use of HPLC-electrochemical analysis we successfully quantified protein nitration in human airways as indicated by the level of protein-bound 3-nitrotyrosine. Protein nitration was greater in airways of asthmatic and COPD patients than in airways of healthy subjects. Moreover, we showed that airway tyrosine nitration in COPD patients correlated well with the degree of airway obstruction, which suggests that RNS may have a key role in the pathogenesis of COPD. Accurate measurement of protein nitration in human airways now warrants further study, to clarify the molecular pathogenesis of inflammatory airway diseases such as COPD and asthma as well as to develop novel approaches to their treatment.

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References

- [1] Murray, C.J. and Lopez, A.D. (1996) "Evidence-based health policy—lessons from the Global Burden of Disease Study", *Science* **274**, 740–743.
- [2] Barnes, P.J. (2000) "Mechanisms in COPD: differences from asthma", *Chest* **117**, 10S–14S.
- [3] Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A. and Freeman, B.A. (1990) "Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide", *Proc. Natl Acad. Sci. USA* **87**, 1620–1624.
- [4] Eiserich, J.P., Hristova, M., Cross, C.E., Jones, A.D., Freeman, B.A., Halliwell, B. and van der Vliet, A. (1998) "Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils", *Nature* **391**, 393–397.
- [5] van der Vliet, A., Eiserich, J.P., Shigenaga, M.K. and Cross, C.E. (1999) "Reactive nitrogen species and tyrosine nitration in the respiratory tract", *Am. J. Respir. Crit. Care Med.* **160**, 1–9.
- [6] Okamoto, T., Akaike, T., Nagano, T., Miyajima, S., Suga, M., Ando, M., Ichimori, K. and Maeda, H. (1997) "Activation of human neutrophil procollagenase by nitrogen dioxide and peroxynitrite: a novel mechanism for procollagenase activation involving nitric oxide", *Arch. Biochem. Biophys.* **342**, 261–274.
- [7] Whiteman, M., Szabo, C. and Halliwell, B. (1999) "Modulation of peroxynitrite- and hypochlorous acid-induced inactivation of α_1 -antitrypsin by mercaptoethylguanidine", *Br. J. Pharmacol.* **126**, 1646–1652.
- [8] Filep, J.G., Beauchamp, M., Baron, C. and Paquette, Y. (1998) "Peroxynitrite mediates IL-8 gene expression and production in lipopolysaccharide-stimulated human whole blood", *J. Immunol.* **161**, 5656–5662.
- [9] Matata, B.M. and Galinanes, M. (2002) "Peroxynitrite is an essential component of production mechanism in human monocytes through modulation of nuclear factor- κ B DNA binding activity", *J. Biol. Chem.* **277**, 2330–2335.
- [10] Saleh, D., Ernst, P., Lim, S., Barnes, P.J. and Giaid, A. (1998) "Increased formation of the potent oxidant peroxynitrite in the airways of asthmatic patients is associated with induction of nitric oxide synthase: effect of inhaled glucocorticoid", *FASEB J.* **12**, 929–937.
- [11] Ichinose, M., Sugiura, H., Yamagata, S., Koarai, A. and Shirato, K. (2000) "Increase in reactive nitrogen species production in chronic obstructive pulmonary disease airways", *Am. J. Respir. Crit. Care Med.* **162**, 701–706.
- [12] Eiserich, J.P., Estevez, A.G., Bamberg, T.V., Ye, Y.Z., Chumley, P.H., Beckman, J.S. and Freeman, B.A. (1999) "Microtubule dysfunction by posttranslational nitrotyrosination of α -tubulin: a nitric oxide-dependent mechanism of cellular injury", *Proc. Natl Acad. Sci. USA* **96**, 6365–6370.
- [13] American Thoracic Society (1995) "Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease", *Am. J. Respir. Crit. Care Med.* **152**, S77–S120.
- [14] American Thoracic Society (1995) "Standardization of spirometry", *Am. J. Respir. Crit. Care Med.* **152**, 1107–1136.
- [15] Knudson, R.J., Slatin, R.C., Lebowitz, M.D. and Burrows, B. (1976) "The maximal expiratory flow-volume curve. Normal standards, variability, and effects of age", *Am. Rev. Respir. Dis.* **113**, 587–600.
- [16] Ichinose, M., Nakajima, N., Takahashi, T., Yamauchi, H., Inoue, H. and Takishima, T. (1992) "Protection against bradykinin-induced bronchoconstriction in asthmatic patients by neurokinin receptor antagonist", *Lancet* **340**, 1248–1251.
- [17] American Thoracic Society (1987) "Single breath carbon monoxide diffusing capacity (transfer factor)", *Am. Rev. Respir. Dis.* **136**, 1299–1307.
- [18] Ichinose, M., Takahashi, T., Sugiura, H., Endoh, N., Miura, M., Mashito, Y. and Shirato, K. (2000) "Baseline airway hyperresponsiveness and its reversible component: role of airway inflammation and airway calibre", *Eur. Respir. J.* **15**, 248–253.
- [19] Pizzichini, E., Pizzichini, M.M.M., Efthimiadis, A., Evans, S., Morris, M.M., Squillace, D., Gleich, G.J., Dolovich, J. and Hargreave, F.E. (1996) "Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid-phase measurements", *Am. J. Respir. Crit. Care Med.* **154**, 308–317.
- [20] Tomaki, M., Ichinose, M., Miura, M., Hirayama, Y., Yamauchi, H., Nakajima, N. and Shirato, K. (1995) "Elevated substance P content in induced sputum from patients with asthma and chronic bronchitis", *Am. J. Respir. Crit. Care Med.* **151**, 613–617.
- [21] Hensley, K., Maitt, M.L., Yu, Z., Sang, H., Markesbery, W.R. and Floyd, R.A. (1998) "Electrochemical analysis of protein 3-nitrotyrosine and dityrosine in the Alzheimer brain indicates region-specific accumulation", *J. Neurosci.* **18**, 8126–8132.

- [22] Crow, J.P. (1999) "Measurement and significance of free and protein-bound 3-nitrotyrosine, 3-chlorotyrosine, and free 3-nitro-4-hydroxyphenylacetic acid in biologic samples: a high-performance liquid chromatography method using electrochemical detection", *Methods Enzymol.* **301**, 151–160.
- [23] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) "Protein measurement with the Folin phenol reagent", *J. Biol. Chem.* **193**, 265–275.
- [24] Hensley, K., Maitt, M.L., Pye, Q.N., Stewart, C.A., Wack, M., Tabatabaie, T. and Floyd, R.A. (1997) "Quantitation of protein-bound 3-nitrotyrosine and 3,4-dihydroxyphenylalanine by high-performance liquid chromatography with electrochemical array detection", *Anal. Biochem.* **251**, 187–195.
- [25] Moore, K.P. and Mani, A.R. (2002) "Measurement of protein nitration and S-nitrosothiol formation in biology and medicine", *Methods Enzymol.* **359**, 256–268.
- [26] John, E.R., Bast, A. and Lankhorst, I. (1997) "Oxidative stress in chronic obstructive pulmonary disease", *Am. J. Respir. Crit. Care Med.* **156**, S341–S357.
- [27] Akaike, T., Okamoto, S., Sawa, T., Yoshitake, J., Tamura, F., Ichimori, K., Miyazaki, K., Sasamoto, K. and Maeda, H. (2003) "8-Nitroguanosine formation in viral pneumonia and its implication for pathogenesis", *Proc. Natl Acad. Sci. USA* **100**, 685–690.
- [28] Tsikas, D., Schwedhelm, E. and Frolich, J.C. (2002) "Methodological considerations on the detection of 3-nitrotyrosine in the cardiovascular system", *Circ. Res.* **90**, E70.
- [29] Tarpey, M.M. and Fridovich, I. (2001) "Methods of detection of vascular reactive species: nitric oxide, superoxide, hydrogen peroxide, and peroxynitrite", *Circ. Res.* **89**, 224–236.
- [30] Kenyon, N.J., van der Vliet, A., Schock, B.C., Okamoto, T., McGrew, G.M. and Last, J.A. (2002) "Susceptibility to ozone-induced acute lung injury in iNOS-deficient mice", *Am. J. Physiol.* **282**, L540–L545.
- [31] Hageman, G.J., Larik, I., Pennings, H.J., Haenen, G.R., Wouters, E.F. and Bast, A. (2003) "Systemic poly (ADP-ribose) polymerase-1 activation, chronic inflammation, and oxidative stress in COPD patients", *Free Radic. Biol. Med.* **35**, 140–148.
- [32] Barnes, P.J. (2003) "New concepts in chronic obstructive pulmonary disease", *Annu. Rev. Med.* **54**, 113–129.
- [33] Tanaka, H., Jing, L., Takahashi, S. and Ito, Y. (1996) "The possible role of nitric oxide in relaxations and excitatory neuroeffector transmission in the cat airway", *J. Physiol.* **499**, 785–791.
- [34] Gaston, B., Sears, S., Woods, J., Hunt, J., Ponaman, M., McMahon, T. and Stamler, J.S. (1998) "Bronchodilator S-nitrosothiol deficiency in asthmatic respiratory failure", *Lancet* **351**, 1317–1319.
- [35] Sugiura, H., Ichinose, M., Oyake, T., Mashito, Y., Ohuchi, Y., Endoh, N., Miura, M., Yamagata, S., Koarai, A., Akaike, T., Maeda, H. and Shirato, K. (1999) "Role of peroxynitrite in airway microvascular hyperpermeability during late allergic phase in guinea pigs", *Am. J. Respir. Crit. Care Med.* **160**, 663–671.

Cytokine-Directed Therapy in Asthma

Masakazu Ichinose*[§] and Peter J. Barnes[‡]

[§]Third Department of Internal Medicine, Wakayama Medical University, Wakayama, Japan

[‡]National Heart & Lung Institute, Imperial College, London, UK

Abstract: Increasing evidence of the pathological roles of multiple cytokines in orchestrating and perpetuating inflammation in asthma has prompted the evaluation of novel anti-cytokine therapies. Anti-IL-5 antibody markedly reduces peripheral blood and airway eosinophils, but does not appear to be effective in symptomatic asthma. Inhibition of IL-4, despite promising early results in asthma has been discontinued and blocking IL-13 might be more effective. Inhibitory cytokines, such as IL-10, interferons and IL-12 are less promising, as systemic delivery produces side effects. Inhibition of TNF- α may be useful in severe asthma. Agents that target IL-13 are still early in the development process. Many chemokines are involved in the inflammatory response of asthma and several small molecule inhibitors of chemokine receptors are in development. CCR3 antagonists, which block eosinophil chemotaxis, are in clinical development for asthma therapy. Because so many cytokines are involved in asthma, drugs that inhibit the synthesis of multiple cytokines may prove to be more useful; several such classes of drug are now in clinical development and any risk of side effects with these non-specific inhibitors may be reduced by the inhaled route.

Keywords: Interleukin-4, interleukin-5, interleukin-10, interleukin-12, interleukin-13, chemokines.

INTRODUCTION

Cytokines are important for maintaining physiological condition, however, these proteins also play a critical role in the orchestration of chronic inflammation in all diseases, including asthma. Multiple cytokines and chemokines have been implicated in the pathophysiology of asthma [1, 2]. There is now an intensive search for more specific therapies in asthma. Inhibitors of cytokines and chemokines figure prominently in these novel therapeutic approaches [3-5] (Table I).

There are several possible approaches to inhibiting specific cytokines (Fig. 1). These range from drugs that inhibit cytokine synthesis (glucocorticoids, cyclosporin A, tacrolimus, rapamycin, mycophenolate, Th2 selective inhibitors), humanized blocking antibodies to cytokines or their receptors, soluble receptors to mop up secreted cytokines, small molecule receptor antagonists or drugs that block the signal transduction pathways activated by cytokine receptors. On the other hand, there are cytokines that themselves suppress the allergic inflammatory process and these may have therapeutic potential in asthma [6, 7]. This review focuses on the cytokine-inhibiting strategies recently adopted as asthma therapy.

I. INHIBITION OF TH2 CYTOKINES

T helper 2 (Th2) lymphocytes play a key role in orchestrating the eosinophilic inflammatory response in asthma, suggesting that blocking the release or effects of these cytokines may have therapeutic potential. This has been strongly supported by studies in experimental animals, including mice with deletion of the specific Th2 cytokine genes.

1. Anti-IL-5

IL-5 plays an essential role in orchestrating the eosinophilic inflammation of asthma [8]. In IL-5 gene knock-out mice the eosinophilic response to allergen and the subsequent airway hyperresponsiveness (AHR) are markedly suppressed, and yet animals have a normal survival, validating the strategy to inhibit IL-5. This has also been achieved using blocking antibodies to IL-5. Blocking antibodies to IL-5 inhibit eosinophilic inflammation and AHR in animal models of asthma, including primates [9]. This blocking effect may last for up to 3 months after a single intravenous injection of antibody in primates, making treatment of chronic asthma with such a therapy a feasible proposition. Humanized monoclonal antibodies to IL-5 have been developed and a single intravenous infusion of one of these antibodies (mepolizumab) markedly reduces blood eosinophils for several weeks and prevents eosinophil recruitment in to the airways after allergen challenge in patients with mild asthma [10] (Fig. 2). However this treatment has no significant effect on the early or late response to allergen challenge or on baseline AHR, suggesting that eosinophils may not be of critical importance for these responses in humans (Fig. 3). A clinical study in patients with moderate to severe asthma who had not been controlled on inhaled corticosteroids therapy

confirmed a profound reduction in circulating eosinophils, but no significant improvement in either asthma symptoms or lung function [11]. In both of these studies it would be expected that high doses of corticosteroids would improve these functional parameters. These surprising results question the critical role of eosinophils in asthma and indicate that other strategies aimed at inhibiting eosinophilic inflammation might not be effective. More recently a biopsy study has demonstrated that anti-IL-5 antibody, while profoundly reducing eosinophils in the circulation (by over 95%), is less effective at reducing eosinophils in bronchial biopsies (by ~50%), which may explain why this treatment is not clinically effective [12]. Nevertheless, this suggests that blocking IL-5 is not likely to be a useful approach to asthma therapy.

Table I. Cytokine Modulators for Asthma

Anti-cytokines	Inhibitory cytokines
Anti-IL-5 Anti-IL-4 Anti-IL-13 Anti-IL-9 Anti-IL-25 Anti-IL-1 Anti-TNF- α	IL-1 receptor antagonist IL-10 IL-12 Interferons (IFN- α , IFN- γ) IL-18 IL-23
Chemokine inhibitors	Cytokine synthesis inhibitors
CCR3 antagonists CCR2 antagonists CCR4 antagonists CCR8 antagonists	Corticosteroids Immunomodulators Phosphodiesterase-4 inhibitors NF- κ B inhibitors (IKK2 inhibitors) p38 MAP kinase inhibitors

Abbreviations: IL = interleukin, NF- κ B = nuclear factor- κ B, IKK = I kappa B kinase, MAP = mitogen-activated protein

Somewhat similar findings have previously been reported in some studies in mice where anti-IL-5 antibodies reduced eosinophilic responses to allergen, but not AHR, whereas AHR was reduced by anti-CD4 antibody which depletes helper T cells [13] and suggests that T cell derived cytokines other than IL-5 must be playing a more important role in AHR.

Non-peptidic IL-5 receptor antagonists would be an alternative strategy and there is a search for such compounds using molecular modeling of the IL-5 receptor α -chain and through large-scale throughput screening. One such molecule YM-90709 appears to be a relatively selective inhibitor of IL-5-receptors [14]. However, the lack of clinical benefit of anti-IL-5 antibodies has made this a less attractive approach. It is possible that eosinophils are associated with more chronic aspects of asthma, such as airway remodeling, and in mice a blocking anti-IL-5 antibody prevents the increased collagen deposition in airways associated with repeated allergen exposure [15]. Eosinophils may be an important source of transforming growth factor- β in asthmatic airways, resulting in structural changes [16].

2. Anti-IL-4

IL-4 is critical for the synthesis of IgE by B-lymphocytes and is also involved in eosinophil recruitment to the airways [17]. A unique function

*Address correspondence to this author at the Third Department of Internal Medicine, Wakayama Medical University, Kimiidera 811-1 Wakayama 641-8508, Japan; Tel: +81 73 441 0619; Fax: +81 73 447 2201; E-mail: masakazu@wakayama-med.ac.jp

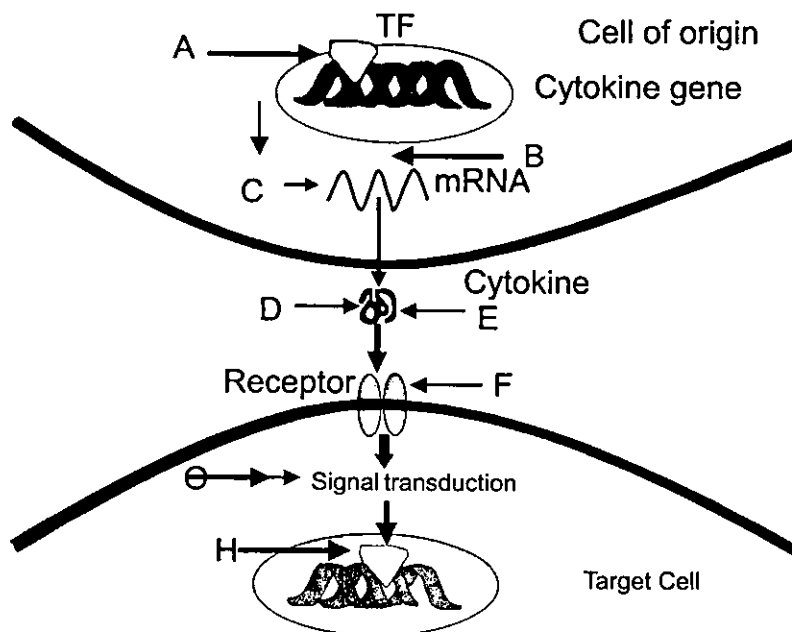


Fig. (1). Strategies for inhibiting cytokines include inhibition of cytokine synthesis, inhibition of secreted cytokines using blocking antibodies or soluble receptors, and blocking of cytokine receptors and their signal transduction pathways. Horizontal arrows indicate inhibition strategies: A and H: transcription factor (TF) inhibitor; B: synthesis inhibitor; C: antisense oligonucleotide; D: monoclonal antibody; E: soluble receptor; F: receptor antagonist; G: kinase inhibitor. mRNA: messenger ribonucleic acid.

of IL-4 is to promote differentiation of Th2 cells and therefore acts at a proximal and critical site in the allergic response, making IL-4 an attractive target for inhibition (Fig. 4).

IL-4 blocking antibodies inhibit allergen-induced AHR, goblet cell metaplasia and pulmonary eosinophilia in a murine model [18]. Inhibition

of IL-4 may therefore be effective in inhibiting allergic diseases, and soluble humanized IL-4 receptors (sIL-4r) have been tested in clinical trials. A single nebulized dose of sIL-4r prevents the fall in lung function induced by withdrawal of inhaled corticosteroids in patients with moderately severe asthma [19]. Subsequent studies have demonstrated that

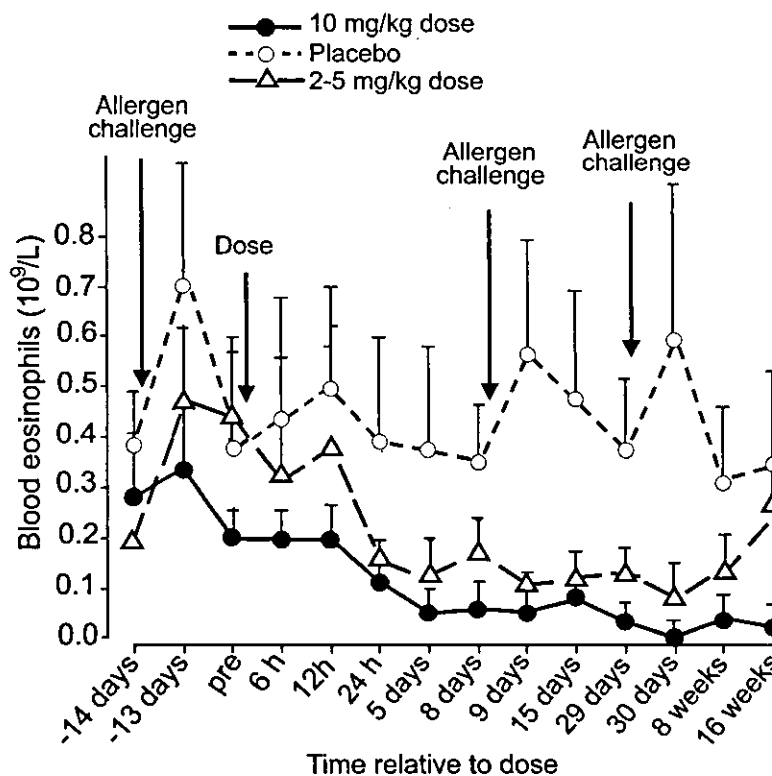


Fig. (2). The effect of a humanized monoclonal antibody against interleukin-5 (mepolizumab) on circulating eosinophils in patients with mild asthma, demonstrating a profound and very prolonged inhibitory effect. Reproduced from reference [10].