Importance of environmental fungi and IgE non-mediated mechanism in atopic eough

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アトピー咳嗽におけるIgE非依存性機序と環境真菌の重要性

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序

我々は、1989年以来、慢性乾性咳嗽の原因疾患として「アトピー咳嗽(atopic cough」を提唱し、通常の気管支生検に加え気管生検を実施することにより、本疾患の基本病態が、Eosinophilic tracheobronchitis with cough hypersensitivity であることを解明し、その咳嗽にヒスタミンH1-拮抗薬およびステロイド薬が有効であることを報告した(Clin Exp Allergy 2000、Allergology International 2000)。また、アトピー咳嗽は、喘息に移行しないという長期予後においても、咳喘息とは異なる新しい疾患概念であることを確認した(Thorax 2003)。

上記の研究を進める中で、慢性咳嗽の診断と治療に関する臨床研究も推進した。その結果、日本における慢性咳嗽の主要疾患が、咳喘息、アトピー咳嗽、副鼻腔気管支症候群であり、欧米とは全く異なることを明らかにし、日本咳嗽研究会での討論を通して、2003年に「慢性咳嗽に関する診断と治療の指針」を発刊した。さらに、2005年には日本呼吸器学会から「咳嗽に関するガイドライン」の発刊に成功した。これらの業績は、我が国における慢性咳嗽診療に方向性とエビデンスを与えるものとなった。

一方、アトピー咳嗽患者の中には、Trichosporon asahii、Pichia guilliermondii、Streptomyces albusなどの環境真菌が原因抗原と考えられた難治性の症例が存在することを経験した(順に Int Arch Allergy Immunol 1998、日呼吸会誌1999 Allergology International 2000)。特に好熱真菌である Humicola fuscoatra (HF)が環境に出現すると、喀痰中の好酸球が増加するアトピー咳嗽の1例を経験した(J Asthma 2002)。また、喀痰、咽頭および勤務する職場から Humicola fuscoatra (HF)が繰り返し培養された難治性アトピー咳嗽の1例を経験した。アトピー咳嗽患者64名を対象とした臨床的検討の結果、アトピー咳嗽の治療抵抗性(難治度)は、

著者寄贈

HFの環境および咽頭の培養陽性と関連し(順にp=0.046, p=0.0008)、多変量解析の結果、咽頭培養陽性と最も強く関連した。

以上の成績から、アトピー咳嗽や咳喘息の治療抵抗性（難治性）と環境真菌の関係が示唆されたため、本研究を計画した。

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咽頭に生着した Basidiomycetes（BM）は、AC の難治化因子の一つであるが、アレルギー性気道疾患全体においても、環境落下真菌が重要である可能性がある。慢性乾性咳嗽患者における咽頭真菌培養の陽性頻度と、その季節性に関する検討を行った。真菌培養陽性患者は、アトピー咳嗽では 73 名中 14 名(19.2%)(Candida 8 名、BM6 名)、咳喘息では 27 名中 5 名(18.5%)(Candida 2 名、BM 2 名、Penicillium 1 名)、咳優位型気管支喘息では 22 名中 6 名(Candida 4 名、BM 1 名、Penicillium 1 名、Aspergillus flavis 1 名）であり、慢性乾性咳嗽患者全体の咽頭真菌培養陽性率は 18.4%であった。BM の陽性頻度（6.4%）は Candida に次いで 2 番目に多かった。

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平成13年4月1日から10月31日の間に当院に通院した、慢性乾性咳嗽患者90名(アトピー咳嗽64名、咳喘息26名)と気管支喘息85名に対して、喀痰および 咽頭拭い液の真菌培養、環境落下真菌培養、HFの抗原抽出液を用いた皮内テストを実施した。対照として 、健常人19名に対しても同抗原液を用いた皮内テストを実施した。

1)アレルギー性気道疾患(アトピー咳嗽; AC、咳喘息; CVA、気管支喘息; BA)において、環境真菌培養の陽性率は 88.9～100%であり各疾患群に差はなく、また Humicola fuscoatra (HF)の検出率(31.1～44.4%)も、各疾患群に有意差はみられなかった。

2)喀痰真菌培養陽性率は 57.0～72.7%と 各疾患群に差はなく、またHFの検出率(16.7～27.3%)も、各疾患群に差はみられなかった。

3)咽頭の真菌培養では、 HFの出現頻度はAC患者がBA患者より有意に高かった(p=0.027)が、CVA患者とは有意差を認めなかった。

4)HFに対する即時型皮内反応の陽性率は、健常者(5.3%)に比較して、AC(18.8%), CVA (26.9%) および BA (22.4%) の各アレルギー性気道疾患群の方が高かった。

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慢性咳嗽患者171名の中、39名の喀痰からBMが培養同定され、その中21名が本研究に参加した。10名は2週間のイトラコナゾール（50 mg/day)投与を受け、残りの11名は2週間のプラセボ（n=10)投与を受けた。イトラコナゾール投与群は咳スコアの有意な改善がみられ、プラセボ群と有意差(P<0.001)を認めた。

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呼気中NOは、好酸球性気道炎症のバイオマーカーとして注目されている。実際に喘息や咳喘息では上昇していることが報告されている。アトピー咳嗽患者9名、咳喘息患者11名、喘息患者10名を対象として呼気NO濃度を測定し

た。呼気NO濃度は、アトピー咳嗽患者では咳喘息患者および喘息患者より有意に低値を示した。この結果は、アトピー咳嗽の好酸球性気道炎症が中枢側に限局していることに一致する。

VII The effect of pressure stress to airway on cough reflex sensitivity in guinea pigs

モルモットの咳受容体感受性に対する圧ストレスの影響　　P79-101

仮説「気道壁への咳嗽ストレスは咳反射回路の自己増幅を招来する」を立証するために、モルモットを用いて実験した。麻酔・人工呼吸下のモルモットに咳嗽ストレスに類似すr急峻な陰圧負荷を加え、覚醒後に経時的にカプサイシン咳感受性測定と気管支肺胞洗浄（BAL）を実施した。カプサイシン咳感受性とBAL好中球は圧ストレス6時間後に増加したが、24時間以内に正常化した。Hydroxyurea処置は、圧ストレス6時間後のカプサイシン咳感受性亢進とBAL好中球増加の両者を抑制した。すなわち、圧ストレス一咳感受性亢進の自己増幅悪循環回路の存在が示唆された。

I. 慢性乾性咳嗽患者における咽頭真菌培養の陽性頻度とその季節性に関する臨床的検討

Fungal culture from pharyngeal swab and seasonal influence in patients with chronic non-productive cough

SUMMARY

It has been suggested that Basidiomycetes is an important fungal aeroallergen. We have reported that Basidiomycetes may be important for allergic airway disorders such as atopic cough, cough variant asthma and bronchial asthma. In this study, 141 patients with chronic nonproductive cough underwent pharyngeal swab culture for fungi, and 18.4% of all patients was positive for Candida (10.6%) and Basidiomycetes (6.4%). Positive result of the cultured Basidiomycetes was relatively frequent in August and September. It is suggested that Basidiomycetes is important as a fungus colonizing in the pharynx, which is causative and/or exacerbating for allergic airway disorders presenting with chronic non-productive cough.

抄録

咽頭に生着した Basidiomycetes (BM) は、AC の難治化因子の一つであるが、アレルギー性気道疾患全体においても、環境落下真菌が重要である可能性がある。今回我々は、2002 年 4 月 1 日から 1 年間に、慢性咳嗽を主訴に当院呼吸器科を受診した 156 名のうち、とくに乾性咳嗽を主訴とした患者 141 名を対象として、慢性乾性咳嗽患者における咽頭真菌培養の陽性頻度と、その季節性に関する検討を行った。真菌培養陽性患者は、アトピー咳嗽では 73 名中 14 名 (19.2%) (Candida 8 名、BM6 名)、咳喘息では 27 名中 5 名 (18.5%) (Candida 2 名、BM 2 名、Penicillium 1 名)、咳優位型気管支喘息では 22 名中 6 名 (Candida 4 名、BM 1 名、Penicillium

1名、Aspergillus flavis 1名）であり、慢性乾性咳嗽患者全体の咽頭真菌培養陽性率は18.4%であった。BMの陽性頻度（6.4%）はCandidaに次いで2番目に多く、とくに8月と9月に多かった。咽頭に生着したBMが慢性咳嗽を呈するアレルギー性気道疾患の原因または増悪因子である可能性を追求する必要がある。

はじめに

これまでに、種々の環境真菌が原因抗原と考えられた難治性のアトピー咳嗽(atopic cough 以下 AC[1-7]や、咳喘息(cough variant asthma 以下、CVA[8]の症例を報告してきた。とくに、Basidiomycetes(BM)[8]が原因抗原であった症例では、BM の環境への出現に伴い喀痰中の好酸球が増加した[7]、また生活環境にBMが存在するとBMに対する即時型皮内反応の陽性率が高くなる[9]ことより、環境真菌であるBMと気道アレルギー疾患が密接に関係している可能性が示唆された。

日常診療における慢性咳嗽の診断と治療には、日本咳嗽研究会が中心になって執筆された「慢性咳嗽の診かた」(藤村政樹編)[10]が極めて有用であり、 咽頭に生着した環境真菌が、原因あるいは増悪因子として働くような難治性のアトピー咳嗽患者の治療においては、環境真菌を念頭において診療にあたることの重要性についてもく言及されている。これまでに我々は、アンフォテリシンBシロップによる含嗽や、少量のイトラコナゾールの内服が有効であった難治性咳嗽症例を経験した[11,12]。

2005 年に、咳嗽に関するガイドラインが日本呼吸器学会から提出されるが、今回我々は、環境真菌の除菌なしでは治療しえない症例が実際にどのくらい存在するのかを明確にするために、その前段階として、慢性乾性咳嗽患者における咽頭真菌培養の陽性頻度とその季節性に関して臨床的検討を行った。

【方法】

2002年4月1日から1年間に、慢性咳嗽を主訴に当院呼吸器科を受診した156名のうち、とくに乾性咳嗽を主訴とした患者141名を対象とした。慢性乾性咳嗽の原因疾患は、咳嗽研究会が定める慢性咳嗽の診断基準（D:厳しい基準、P:あまい基準）に基づいて診断し、咽頭真菌培養を実施した。

呼吸機能検査は、スパイロメーター（チェスタック）を用い、咳受容体感受性は、カプサイシンを用いた咳閾値(C5)を測定した[14]。

気道過敏性は、非特異的な気管支平滑筋の反応性を評価する指標として、PC20を測定した。また、気管支拡張薬に対する気道の可逆性として、Salbutamol 300μgの吸入前後に肺機能検査を実施し、FEV1.0の増加率を測定した。なお、咳閾値と気道可逆性の測定は初診時に、気道過敏性の測定は再診時に実施した。

咽頭真菌培養は初診時に実施し、咽頭後壁を滅菌綿棒で拭い、サブロー培地にまんべんなく塗布し、7ー10日後に同定した。

【結果】

1) 慢性咳嗽患者の内訳(Table1)

慢性咳嗽患者156名から、副鼻腔気管支症候群10名、心因性咳嗽4名(D:1名、P:3名)、GERD(P:1名）を除いた141名の内訳は、アトピー咳嗽73名(D:17名、P:56名)(51.8%)、咳喘息27名(D:17名、P:10名)(19.2%)、咳優位型気管支喘息22名(15.6%)、不明19名(13.5%)であった。

アトピー咳嗽患者は、男性32名、女性41名であり、年齢は43.1±13.7歳だった。一般肺機能検査では、1秒量（FEV1）(%predicted)は104.1±15.5%、1秒率（FEV1/FVC）は82.2±6.9%、メサコリン気道過敏性（PC20）の幾何平均は17.7(幾何標準誤差（GSEM）1.06) mg/ml、咳閾値の幾何平均は14.3(GSEM, 1.15) μM、気管支拡張薬に対する可逆性は2.7±2.7%であった。咳喘息患者は、男性18名、女性9名であり、年齢は40.3±14.4歳だった。FEV1(%predicted)は95.6

±21.0%、FEV1/FVC は 82.9±7.9%、PC20 は 3.39(GSEM, 1.32) mg/ml、咳閾値は 33.8 (GSEM, 1.31) μM、気管支拡張薬に対する可逆性は 4.8±3.9%であった。咳優位型喘息患者は、男性 11 名、女性 11 名であり、年齢は 56.9±14.8 歳だった。FEV1(%predicted) は 84.8±19.4%、FEV1/FVC は 68.3±11.2%、PC20 は 0.31(GSEM, 1.49) mg/ml、咳閾値は 60.4(GSEM, 1.81) μM、気管支拡張薬に対する可逆性は、9.0±9.0%であった。

2) 月別慢性乾性咳嗽患者 141 名の分布と、咽頭真菌培養陽性患者 26 名の分布。

慢性乾性咳嗽患者は一年を通して当院を受診したが、とくに 5 月、6 月に多かった(Figure 1)。また、咽頭真菌培養陽性患者は 26 名(18.4%) (Candida 15 名、Basidiomycetes 9 名、その他 3 名) であり、 BM が陽性の患者は、4、5、7、8、9 月に見られ、とくに 8 月と 9 月に多かった(Figure 2)。

3) 疾患別咽頭真菌培養分離菌の内訳

真菌培養陽性患者は、アトピー咳嗽では 73 名中 14 名(19.2%)(Candida 8 名、BM 6 名)、 咳喘息では 27 名中 5 名(18.5%)(Candida 2 名、BM 2 名、Penicillium 1 名)、咳優位型気管支喘息では 22 名中 6 名 (27.3%) (Candida 4 名、BM 1 名、Penicillium 1 名、Aspergillus flavis 1 名) であった(Figure 3)。

【考察】我々は、1989 年以来、慢性乾性咳嗽の原因疾患として「アトピー咳嗽 (atopic cough; AC) 」を提唱し[1]、通常の気管支生検と気管支肺胞洗浄に加えて気管生検を実施することにより、 AC の基本病態が、Eosinophilic tracheobronchitis with cough hypersensitivity であることを解明し、その咳嗽にヒスタミン H1-拮抗薬およびステロイド薬が有効であることを報告した[2]。また、長期予後においても、AC は喘息に移行せず、咳喘息[15]とは異なる新しい疾患概念であることを確認した[3]。

アトピー咳嗽(AC)の標準的な治療法は、我々よってほぼ確立されている[2,3]が、一部には治療に難渋する症例もあり、過敏性肺臓炎や好酸球性肺炎[16,17]など

と同様に、原因抗原の追究[13]が治療の糸口を見いだす手掛かりになる。

とくに、環境真菌が原因、あるいは増悪因子であった難治性のアトピー咳嗽[4-7]や咳喘息の症例[8]では、抗真菌薬の少量内服や含嗽が有効である症例が存在するため、環境真菌が、どの程度気道アレルギー疾患に関与しているかについて検討することは重要である。

今回の検討では、慢性乾性咳嗽患者の咽頭から培養同定された真菌において、Basidiomycetes が Candida に次いで２番目に出現頻度の高い真菌であることが判明した。Basidiomycetes は、担子菌門に属する無性性真菌である。ラッカーゼ陰性で、かすがい連結（clampconnection）を持たない。人体に病原性を有する担子菌門の真菌は、Cryptococcus neoformans や Trichosporon のような Basidiomycetous yeast などに限られ、担子菌門に属する他の真菌でも、亀井ら[18]が Schizophyllum commune（スエヒロタケ）によるアレルギー性気管支肺真菌症を報告しているにすぎない。

しかしながら、Basidiomycetes（BM）の空中浮遊真菌（環境真菌）としての重要性は古くから認識されていて、1952年 の Gregory と Hirst らの報告[15]に遡る。Manuel ら[19]もアトピー型喘息患者の BM に対する即時型皮内反応陽性率が42-68%であったと報告している。また、 著者らも、職場と喀痰から繰り返し BM が検出されたアトピー咳嗽患者の職場職員 19名に実施した BM の即時型皮内反応の陽性率（３１.6%）が、健常人(3.4%)より有意に高率であることを示し[9]、無症状であっても 環境に BM が存在すれば、BM の即時型皮内反応の陽性率が高くなり、ひいては、アレルギー性気道疾患を惹起する可能性を報告した。

著者らは、難治性咳嗽症例の治療経験を通して、Basidiomycetes(BM)の重要性を報告してきたが、今回の検討では、慢性乾性咳嗽患者の咽頭真菌における BM の出現頻度からも、広く気道アレルギー疾患の病像を修飾している可能性が示唆された。また、環境真菌調査の実施に際しては、最も滞在時間が長いと考えられる寝室、職場や自家用車の空調も含めた居住環境についての詳しい問診を基

礎として、住居、職場など広く環境真菌培養を実施するとともに、季節性についても留意することが有用と考えられた。

【結論】

慢性乾性咳嗽患者における咽頭真菌培養陽性率は 18.4%であった。BM の陽性頻度（6.4%）は Candida に次いで 2 番目に多く、とくに 8 月と 9 月に多かった。抗真菌薬による含嗽が、咳嗽治療に役立つ症例が存在する可能性がある。

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Table 1 Characteristics of subjects (mean ±SD)

	Gender Male/female	Age (years)	FEV1 (% pred.)	FEV1/FVC (%)	PC20 (mg/ml)	C5 (mM.)	Bronchial Reversibility (%.)
Atopic cough	32/41	43.1 ±13.7	104 ±16	82 ±7	17.7 (1.06)	14.3 (1.15)	2.7 ±2.7
Cough variant asthma	19/9	40.3 ± 14.4	96 ± 21	83 ± 8	3.39 (1.32)	33.8 (1.31)	4.8 ± 3.9
Bronchial asthma	11/11	56.9 ±14.8	85 ±19	68 ±11	0.31 (1.49)	60.4 (1.81)	9.0 ±9.0
Others	11/11	42.9 ±15.7	105 ±16	80 ±7	5.00 (4.00)	23.1 (1.47)	2.9 ±2.9

( ): geometric standard error of the mean

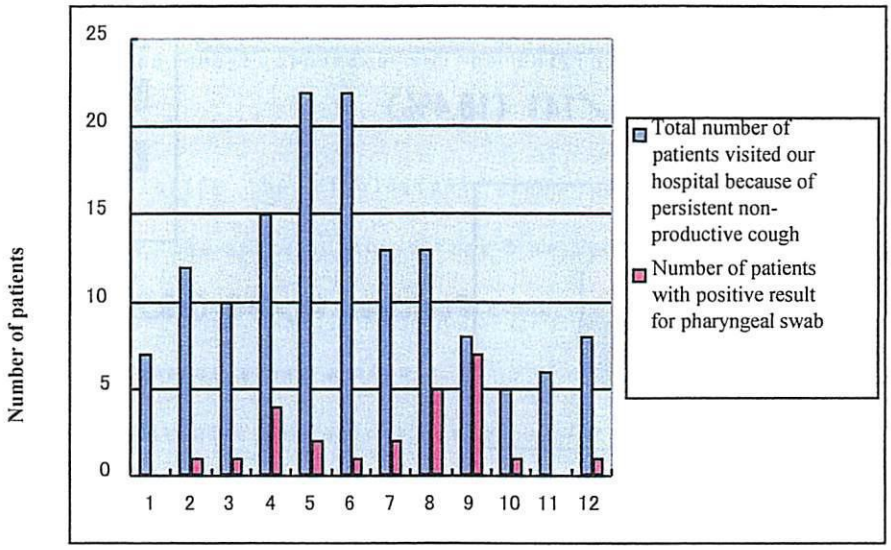

Figure 1 月別慢性乾性咳嗽患者141名の分布と、咽頭真菌培養陽性患者26名の分布

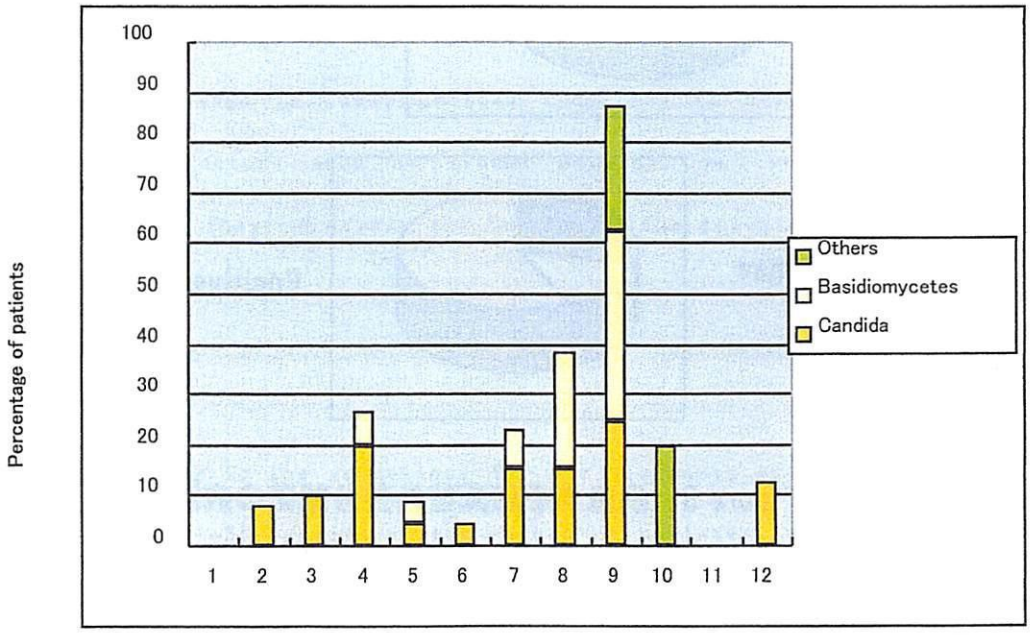

Figure 2 咽頭真菌培養陽性患者は26名(18.4%)(Candida 15名Basidiomycetes 9名、その他3名)の月別分布

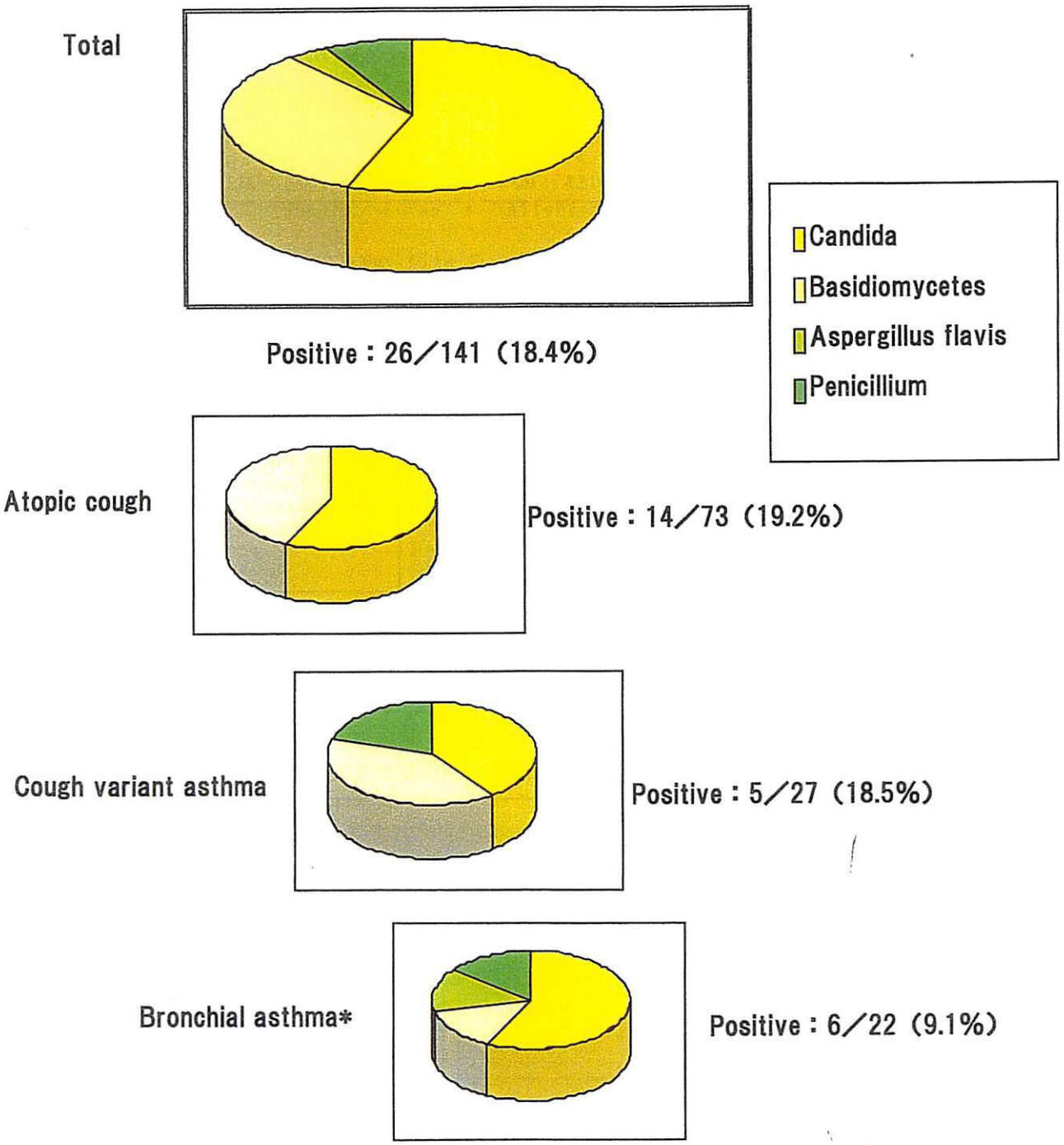

Figure 3　疾患別咽頭真菌培養分離菌の内訳

II The importance of *Bjerkandera adusta* as a potential etiological agent of fungus-associated cough syndrome (FACS)

Abstract

[Background] We have reported the importance of basidiomycetous fungi (BM) in allergic airway disorders, which present as chronic nonproductive cough. The recognition of a new clinical concept-FACS-an acronym for fungus-associated cough syndrome may be helpful for the diagnosis and management of intractable chronic cough.

[Methods] To clarify the etiological class of basidiomycetous fungi relating to FACS, the sputa cultured from 8 patients randomly selected out of 21 patients of FACS were identified and described in detail by using 28S rDNA (D1/D2) sequencing and analysis.

[Results] The molecular biological analyses of the basidiomycetous fungi cultured from the sputa of 8 subjects of FACS revealed all of them to be *Bjerkandera adusta* (Yakeirotake in Japanese) (100% homology to DDBJ/EMBL/GenBank DNA database accession number AB096738).

[Conclusions] Although the major species of basidiomycetous fungi relating to FACS may change in their profile according to change in natural environmental factors such as global warning, we contend that the basidiomycetous fungi-*B. adusta*-may be one of the potential etiological agents of FACS.

Introduction

Despite adhering to the guidelines for the diagnosis and management of chronic cough [1-3], an increasing number of patients continue to seek

medical help for their chronic intractable cough. Based on our clinical experience of the 21 patients with chronic cough whose sputum yield basidiomycetous fungi (BM) [4], we have recently proposed a new clinical concept-FACS-an acronym for fungus-associated cough syndrome [5]. It exhibits the following clinical features: (1) chronic cough, (2) environmental fungi, particularly BM fungi, in the sputum, and (3) clinical response to antifungal drugs. The recognition of FACS may be helpful for the diagnosis and management of intractable chronic cough. To confirm this new clinical concept, at first, the etiological class of basidiomycetous fungi cultured from the sputa of 8 patients with FACS were identified and described in detail by using 28S rDNA (D1/D2) sequencing and analysis [6-8].

Materials and methods

Patients

From April 2005 to May 2006, 171 patients visited our hospital for the diagnosis and treatment of chronic cough, which was defined as isolated persistent cough lasting for 8 weeks or more. BM was detected in the sputa of 39 of the 171 patients (22.8%). Of these 39 patients, diagnosis of FACS was made in 21 patients who were not included in currently prevalent diagnoses. Eight patients were randomly selected out of the 21 patients with FACS, and enrolled to this study.

The cause of chronic cough in each patient was diagnosed based on a questionnaire, blood examination findings, chest and sinus X-rays, induced-sputum examination, pulmonary function tests [9], test for cough sensitivity to capsaicin [10], bronchial reversibility in response to bronchodilators, bronchial responsiveness to methacholine [11], and the

efficacy of individual-specific treatments. The examinations were performed in accordance with the diagnostic criteria for each cause, as recommended by the Japanese Cough Research Society [1], Japanese Respiratory Society [2], and the American College of Chest Physicians (ACCP) evidence-based practice guidelines [3].

Strain isolations and morphological observations

Sputum samples taken from the 8 patients with FACS were inoculated on Sabouraud' s dextrose agar (SDA). These cultures were incubated at 22-27℃ for 2 weeks. The colonial characteristics of the strains were observed by culture on SDA plates at 22-27℃ for 14 d. The morphological features of the strains were observed by using the slide culture method (22-27℃ for 2 weeks) and lactophenol cotton blue staining.

DNA preparation from fungal cells

All fungal strains were grown on SDA at 27℃ for 5 d. The rapid preparation of DNA from the strains was performed according to the method described previously [6]. A small amount of mycelium grown on SDA was placed in a lysis buffer (200 mM Tris-HCl, pH8.0, 0.5% w/v SDS, 250 mM NaCl, 25 mM EDTA) and crushed with a conical grinder. It was then incubated at 100℃ for 15 min and mixed with 150 µl of 3.0 M sodium acetate, kept at -20 ℃ for 10 min, and then centrifuged at 10,000 __g for 5 min. The supernatant was extracted once with phenol-chloroform-isoamyl alcohol (25:24:1, v/v) and subsequently extracted once with chloroform. DNA was precipitated with an equal volume of isopropanol at -20℃ for 10 min, washed with 0.5 ml of 99% ethanol, dried, and suspended in 50 µl of ultrapure water (Milli-Q Synthesis A10 ; Millipore). Of the resulant solution, 1 µl was used as the template for the polymerase chain reaction (PCR).

Oligonucleotide

The oligonucleotide primers for 28S-rDNA partial sequence (D1/D2 region)-specific PCR [7], NL-1 (5 GCATATCAATAAGCGGAGGAAAAG) and NL-4 (5 GGTCCGTGTTTCAAGACGG), were obtained from Amersham Pharmacia Biotech Co., Ltd. (Tokyo).

PCR

Each PCR mixture contained 10μl of 10__reaction buffer; 100 μM each of dATP, dCTP, dGTP, and dTTP; and 2.5 U of Taq polymerase (all from Amersham Pharmacia Biotech), as well as 30 pmol of each primer and the DNA template solution. Ultrapure water was added to each of these mixture in order to increase the volume to 100 μl. Each mixture was heated to 94℃ for 5 min, and PCR was performed under the following conditions: 94℃, 1 min; 60℃, 15 sec; and 72℃, 15 sec; 25 cycles. Thermal cycles were terminated by polymerization at 72℃ for 10 min. The products were detected as a single band of 0.5 kbp by agarose gel electrophoresis and UV irradiation.

28S rDNA (D1/D2) sequencing and analysis

Both the strands of the PCR products were directly sequenced using a DNA Sequencing Kit (Applied Biosystems) with primers and an automatic sequencer (Genetic Analyzer 310; Applied Biosystems) according to the manufacturer' s instructions. The DDBJ/EMBL/GenBank nucleotide sequence databases were searched for matches of the confirmed sequences using BLAST programs [8].

Results

Clinical findings

The study subjects comprised 5 males and 3 females with FACS; their mean age was 50.4 ± 17.1 (SD) years (Table 1). Chest and sinus X-rays were normal

for all the patients. Chronic airflow limitation, which is defined as the ratio of forced expiratory volume in 1 s (FEV1) to forced vital capacity (FVC) (FEV1/FVC) < 0.7, was not investigated in all the patients. Further, FEV1 of <80% of the predicted value was not observed in any of the 8 patients.

Cough reflex sensitivity [10] was increased in 3 patients (Patient 1, 2, and 4.) Bronchial responsiveness to methacholine [11] was exaggerated in only 1 patient (patient 7).

The bronchodilator therapy was effective in only 3 patients (patients 3, 7, and 8) and not in the other 5 patients. Histamine H1 antagonists and inhaled corticosteroids produced a partial response in 4 patients (patients 1, 2, 4, and 8). Patients 5 and 6 were resistant to these standard therapies; however, it was remarkable that antifungal treatment with low-dose of itraconazole 50 mg/d for 2 weeks was efficacious in all of the 8 patients with FACS.

Identification of the molds

All strains demonstrated the same characteristics; i.e., the hyphae appeared white along with the presence of septa; however, neither conidia nor clumps were observed. Therefore, morphological identification was not applicable to the isolates. In order to identify the strains; 28S rDNA (D1/D2) sequence-based molecular biological identification was performed. The base sequences of 28S rDNA (D1/D2) are species-specific and are widely used for species identification of fungi that are difficult to identify on the basis of their morphological features. In the 28S rDNA (D1/D2) sequence analysis, all the strains were shown to be identical to *Bjerkandera adusta* (Yakeirotake in Japanese) (100% homology to DDBJ/EMBL/GenBank DNA database

accession number AB096738). From the molecular biological analyses above, the strains were confirmed to be as *B. adusta*.

Discussion

Only a limited number of reports have documented infectious diseases caused by BM fungi [4]. *Schizophyllum commune* and the *Coprinus* species have been shown to cause endocarditis [12], sinusitis [13], and allergic bronchopulmonary mycosis [14]. Following our previous report of a typical case of chronic cough with sputum eosinophilia caused by an allergic reaction to basidiomycetous fungi (BM) (it was termed *Humicola fuscoatra* at that time) [15], we have focused on the possible role of BM as a fungal aeroallergen [16,17] in chronic cough such as atopic cough [18-20], cough variant asthma [21], and cough-predominant asthma. Furthermore, based on our clinical experiment during several years of intractable chronic cough [22-27] who could not be assigned to any currently prevalent diagnoses, we noticed that there exists a new clinical entity of chronic cough related with BM.

Recently, we have proposed a new clinical concept-FACS-an acronym for fungus-associated cough syndrome [5]. It exhibits the following clinical features: (1) chronic cough, (2) environmental fungi, particularly BM fungi, in the sputum, and (3) clinical response to antifungal drugs. Though the recognition of FACS may be helpful for the diagnosis and management of intractable chronic cough, the association between exposure to environmental fungi and precipitation of chronic cough has not been recognized clearly.

Since the basidiomycetous spores are generally small (3-7 ・m in diameter),

it is easy to deposit within the airway [28].

It is well-known that the Basidiomycetes class, the most morphologically complex of the fungi, consists of approximately 20,000 to 25,000 species, we aimed to identify and describe the etiological class of Basidiomycetous fungi relating to FACS by using 28S rDNA (D1/D2) sequencing and analysis [6-8].

In this study, the detailed examination of the basidiomycetous fungi cultured from the sputa of 8 patients with FACS revealed all of them to be *Bjerkandera adusta* [29].

Lignin occurs as one of the major components of woody and herbaceous plants and is the most abundant renewable aromatic polymer an earth. It has been reported that multiple lignin peroxidase isozymes are secreted by B. adusta [30]. Therefore, this mold is well known in the field of agricultural science.

Gloria et al [31] studied a total of 44 clinical isolates of Basidiomycetous fungi, namely, *S. commune* (n=5), the *Coprinus species* (n=8), *B. adusta* (n=14), and sterile, uncharacterized Basidiomycetes (n=17). In their report, the white-rot Basidiomycetes-*B. adusta*-were detected from bronchial washes (n=8), sputum (n=3), skin, pleural fluid, and lung tissue (n=1 for each); however, the clinical significance of each isolate was not clearly mentioned.

Global warming in recent years results from the growing level of greenhouse gases such as carbon dioxide may result in an upward tendency in the temperature of seawater and frequent arrival of typhoon, thus contributing to thickly growing of a lot of kinds of mushrooms on the fallen woods in the hills or bush.

The family Basidiomycetes (mushrooms, puffballs, rusts, smuts, and bracket fungi) deserves special attention because of the high atmospheric concentration of basidiospores in the near future.

Concerning these natural environmental factors [31], although the major species of basidiomycetous fungi relating to FACS may change in their profile or increase in the amount, at least based on our result of this study, we contend that the basidiomycetous fungi－*B. adusta*－may be one of the potential etiological agents of FACS.

Now it is becoming clear that we may not be able to neglect the influence of global warming in the treatment and management of the allergic airway disorder, and future worldwide studies on FACS are required as same as the efforts to lower emissions of greenhouse gases.

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Table 1 Characteristics of subjects

	Age	Gender	FEV1 (L)	FEV1/FVC (%)	Bronchial reversibility (%)	RT-Meth (mg/ml)	Cough threshold (μM)
1	20	M	5.24	88.8	3.2	>20	1.95
2	46	M	4.08	83.3	2	>20	0.49
3	58	F	2.41	72.6	7.8	5	3.9
4	66	F	2.38	77.1	0	>20	0.98
5	40	M	2.79	74.6	2.2	>20	312
6	68	M	2.33	76.9	0	>20	312
7	39	M	3.61	86.4	0	1.25	312
8	66	F	1.99	73.7	8.6	5	1.95

III.慢性乾性咳嗽患者におけるHumicola fuscoatraの重要性に関する臨床的検討

【背景】

1）我々は、1992年以来、慢性乾性咳嗽の原因疾患として「アトピー咳嗽(atopic cough; AC)」を提唱し、通常の気管支生検に加え気管生検を実施することにより、ACの基本病態が、Eosinophilic tracheobronchitis with cough hypersensitivity であることを解明し、その咳嗽にヒスタミンH1-拮抗薬およびステロイド薬が有効であることを報告した（Clin Exp Allergy 2000、Allergology International 2000)。また、ACは、喘息に移行しないという長期予後においても、咳喘息とは異なる新しい疾患概念であることを確認した（Thorax 2003)。

2）AC患者の中には，Trichosporon asahii、Pichia guilliermondii、Streptomyces albusなどの環境真菌が原因抗原と考えられた、難治性のAC症例が存在し（順に Int Arch Allergy Immunol 1998、日呼吸会誌1999 Allergology International 2000)する。特に好熱真菌である Humicola fuscoatra (HF)が環境に出現すると、喀痰中の好酸球が増加するACの1例を経験し報告した（J Asthma 2002)。

3）喀痰、咽頭および勤務する職場から Humicola fuscoatra (HF)が繰り返し培養された難治性ACの1例を経験した。その職場職員19名に実施したHFの即時型皮内反応の陽性率(31.6%)は、健常者19名の陽性率(5.3%)より有意に高く(P=0.036)、HFの皮内反応陽性は、環境にHFが存在する可能性を示唆した。

4）咳嗽研究会が定める『慢性咳嗽の診断基準』に基づいて診断した、アトピー咳嗽患者64名を対象とした臨床的検討の結果、アトピー咳嗽の治療抵抗性（難治度）は、HFの環境および咽頭の培養陽性と関連し(順にp=0.046, p=0.0008)、多変量解析の結果、咽頭培養陽性と最も強く関連した。

【目的】

アトピー咳嗽の難治化因子として重要と考えられる Humicola fuscoatra (HF)のアレルギー性気道疾患における臨床的意義を明らかにする。

【方法】

平成13年4月1日から10月31日の間に当院に通院した、慢性乾性咳嗽患者90名（アトピー咳嗽64名、咳喘息26名）と気管支喘息85名に対して、喀痰および 咽頭拭い液の真菌培養、環境落下真菌培養、 HFの抗原抽出液を用いた皮内テストを実施した（Table 1）。対照として 、健常人19名に対しても同抗原液を用いた皮内テストを実施した。 各疾患の診断は、咳嗽研究会が定める『慢性咳嗽の診断基準』に準じた。

【結果】

1）アレルギー性気道疾患（アトピー咳嗽; AC、咳喘息; CVA、気管支喘息; BA）において、環境真菌培養の陽性率は 88.9～100%であり各疾患群に差はなく、また Humicola fuscoatra (HF)の検出率(31.1～44.4%)も、各疾患群に有意差はみられなかった（Figure 1A）。

2）喀痰真菌培養陽性率は 57.0～72.7%と 各疾患群に差はなく、またHFの検出率(16.7～27.3%)も、各疾患群に差はみられなかった（Figure 1B）。

3）咽頭の真菌培養では、 HFの出現頻度はAC患者がBA患者より有意に高かった(p=0.027)が、CVA患者とは有意差を認めなかった（Figure 1C）。

4）HFに対する即時型皮内反応の陽性率は、健常者(5.3%)に比較して、AC(18.8%), CVA（26.9%）および BA（22.4%）の各アレルギー性気道疾患群の方が高かった（Figure 2）。

5）HFの即時型皮内反応の陽性・陰性は、AC患者では、同真菌の咽頭および環境の培養陽性・陰性と関連したが（順にp=0.0007, p=0.005)、喀痰培養陽性・陰性とは関連しなかった。また、CVA患者やBA患者においては、このような関連はみられなかった（Table 2）。

【結論】

1）好熱真菌であるHumicola fuscoatra（HF）は居住環境に広く存在した。

2）HFは、アレルギー性気道疾患の真菌アレルゲンとして重要である可能性が示唆された。

3）アトピー咳嗽患者におけるHFの咽頭への生着は、他のアレルギー性気道疾患患者におけるよりも高頻度である可能性が示された。

Table 1 Characteristics of subjects (mean ±SD)

	Gender Male/female	Age (years)	FEV1 (% pred.)	FEV1/FVC (%)	PC20 (mg/ml)	C5 (mM.)	Bronchial Reversibility (%.)
Atopic cough	29/35	44.5 ±13.7	107 ±13	84 ±6	15.3 (1.07)	4.7 (1.16)	2.4 ±3.9
Cough variant asthma	11/15	42.4 ± 16.8	95 ± 15	79 ± 8	1.96 (1.37)	8.1 (1.31)	5.8 ± 4.3
Bronchial asthma	47/38	52.4 ±16.8	87 ±19	70 ±12	0.72 (1.29)	60.4 (1.37)	7.0 ±7.1
Others	9/10	34.5 ±7.3					

( ): geometric standard error of the mean

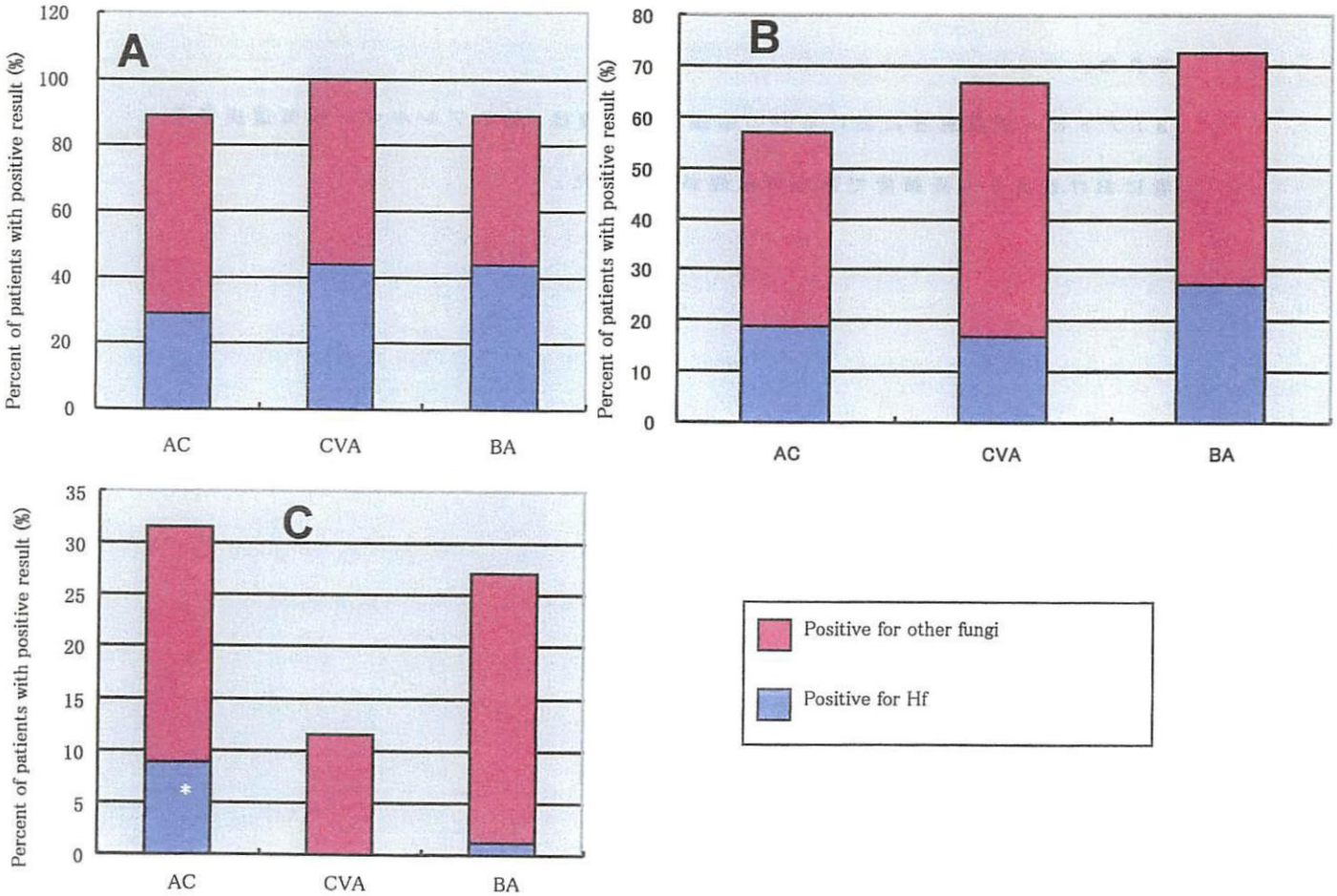

Figure 1 Detection of fungi from bedroom (A), sputum (B) and pharyngeal swab (C) in patient with atopic cough, cough variant asthma and bronchial asthma

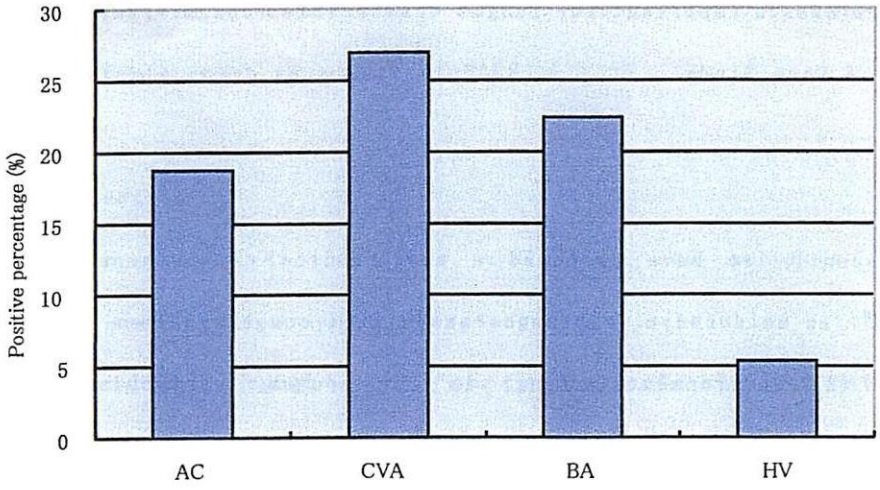

Figure 2 Immediate type skin reaction for Humicola fuscoatra

Table 2 Correlation of immediate type skin reaction to fungal culture from sputum (A), pharyngeal swab (B) or environmental survey (C) for Humicola fuscoatra in patients with atopic cough

		A			B			C		
		Fungal culture from sputum			Fungal culture from pharyngeal swab			Environmental survey		
		Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total
Skin reaction	Positive	4 (36.4%)	7 (63.6%)	11 (26.2%)	4 (33.3%)	8 (66.7%)	12 (21.1%)	8 (61.5%)	5 (38.5%)	13 (28.9%)
	Negative	4 (12.9%)	27 (87.1%)	31 (73.8%)	1 (2.2%)	44 (97.8%)	45 (78.9%)	6 (18.8%)	26 (81.2%)	32 (71.1%)
	Total	8 (19.0%)	34 (81.0%)	42 (100%)	5 (8.8%)	52 (91.2%)	57 (100%)	14 (31.1%)	31 (68.9%)	45 (100%)
				NS			p = 0.0007			p = 0.005

IV Is *Bjerkandera adusta* important for fungus— associated cough syndrome as an allergen? A Case Study

Abstract

[Background] Recently we have proposed a new clinical comprehensive syndrome "FACS" as an acronym for fungus-associated cough syndrome (1) chronic cough, (2) environmental fungi in the sputum, particularly basidiomycetous (BM) fungi, and (3) clinical response to antifungal drugs. But the association between exposure to environmental fungi and appearance of the cough attacks has not been clearly understood. From the results of further examination with using the molecular biological analyses of the BM fungi, we assumed that *Bjerkandera adusta* (*B. adusta*) is one of the important potential etiological agents of FACS.

[Methods] We selected 8 patients with chronic cough in which sputum examination was revealed to yield *B. adusta* beforehand, and we conducted allergological tests such as immediate-type skin test, serological test, bronchoprovocation test, and lymphocyte stimulation test(LST) by using the antigenic solution of *B. adusta*. Efficacy of each therapy and clinical manifestation of 8 patients were examined.

[Results] All patients were diagnosed as FACS. Though 3 patients who had neither a positive reaction to the bronchoprovocation test nor LST showed excellent clinical response to anti-fungal drugs, 5 patients who showed a positive reaction to the bronchoprovocation test and/or LST seemed to be more intractable with long duration for complete remission, recurrence of cough and/or onset of typical asthma.

[Conclusions] It is concluded that allergic fungal cough (AFC), which is

characterized by sensitization with *B. adusta*, may be included in chronic intractable cough as a part of FACS.

Introduction

Despite extensive diagnostic evaluation and numerous treatment guidelines [1-4], a number of patients remain troubled by chronic and uncontrollable cough. From our series of clinical studies on fungus-associated allergic cough [5-8], we have focused on the possible role of BM fungi as a fungal aeroallergen [9, 10], and recently from the result of the controlled study we have concluded that low dose itraconazole has been an effective antitussive in patients with chronic cough in which sputum examination yielded BM fungi. Thus we have proposed a new clinical comprehensive syndrome "FACS" as an acronym for fungus-associated cough syndrome [11] (1) chronic cough, (2) environmental fungi in the sputum, particularly basidiomycetous (BM) fungi, and (3) clinical response to antifungal drugs.

To examine in what kind of mechanism the BM fungi is related to cough, at first, the identification of BM fungi obtained from the sputum of 4 patients with fungus-associated chronic cough we have previously reported [12-13] was made in detail by using 28S rDNA (D1/D2) sequencing and analysis. Because the molecular biological analyses of the BM fungi obtained from the 4 patients revealed all of them to be *Bjerkandera adusta* (Yakeirotake in Japanese; *B. adusta*), we assumed that *B. adusta* is one of the important potential etiological agents of FACS.

We this time selected 8 patients with chronic cough in which sputum examination revealed to yield *B. adusta* beforehand, and conducted

allergological tests by using the antigenic solution of *B. adusta* (polysaccharide). Though this study had not been performed prospectively and is not beyond a level of case report, this report will be helpful to consider the association between exposure to environmental fungi and appearance of the cough attacks.

Materials and methods

Patients

Eight patients who were referred to our hospital for the diagnosis and treatment of chronic cough that was defined as isolated persistent cough lasting for 8 weeks or more during a 10-month period (from April 2006 to April 2007) and whose sputum examination yielded *B. adusta* were selected in this study. This group consisted of 5 men and 3 women with ages ranging from 20 to 68 years. Based on the history, the duration of cough was 2 to 12 months. None of the patients complained of shortness of breath or wheezing and had a post-nasal drip or sinobronchial syndrome [14] to attribute to the cough. All patients were life-time non-smokers. A carefully noted history, physical examination, complete blood count with differential, and chest and sinus roentgenograms were normal. This case study was approved by the institutional review boards and informed consent was obtained from the 8 patients.

Mycological study when selecting the patients and recruiting this study

Strains and DNA preparation

The sputum samples obtained from the patients with chronic intractable cough were cultured on Sabouraud' s dextrose agar (SDA) containing

chloramphenicol. The morphological features of the strains were observed with the slide culture method (30 ° C for 2-3 weeks). When white lot grew widely on SDA, the DNAs from the strains were prepared according to a previously described method [15]. One microliter of the DNA solution was used as the template for the polymerase chain reaction (PCR).

PCR and sequencing

PCR was performed with the primers for the 28 SrDNA partial sequence (D1/D2 region) [16], NL-1 (5' -GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5′ -GGTCCGTGTTTCAAGACGG-3′), under the following conditions: 25 cycles of 94 ° C, 1 min; 60 ° C, 15 s; and 72 ° C, 15 s. The products were detected as a single band of 0.5 kbp by agarose gel analysis. The products were detected as a single band of 0.5 kbp by agarose gel analysis. Both strands of the PCR products were directly sequenced using a DNA sequencing kit (Applied Biosystems) and an automatic sequencer (Genetic Analyzer 310; Applied Biosystems) according to the manufacturer' s instructions. The DDBJ/EMBL/GenBank nucleotide sequence databases were searched for matches of the confirmed sequences by using BLAST programs [17].

Diagnoses and therapy for chronic cough

The causes of chronic cough in the 8 patients were diagnosed according to the diagnostic criteria for causes of chronic cough recommended by both the Japanese Cough Research Society guidelines for diagnosis and treatment of chronic cough [2] and the Japanese Respiratory Society guidelines for management of cough [3]; a questionnaire, blood

examination (WBC (/・1), CRP (mg/dl), cell differential of peripheral blood, IgE (U/ml), specific IgE for house dust or mite), chest and sinus X-rays, induced sputum examination, pulmonary function tests [18], bronchial reversibility to inhaled ・2-agonists, cough reflex sensitivity to inhaled capsaicin [19], bronchial responsiveness to methacholine [20], and the efficacy of individual causes-specific treatments.

Capsaicin cough threshold was measured as an index of airway cough reflex sensitivity according to the method reported by us [19] at the first visit and after each therapy. The capsaicin cough threshold was defined as the lowest concentration of inhaled capsaicin eliciting five or more coughs. Positive bronchial reversibility was defined as percent increase in FEV1 > 12% and absolute increase in FEV1 > 200 mL. The non-specific bronchial responsiveness to methacholine was assessed according to the method described by Cockcroft et al [20]. The results were expressed as the provocation concentration (mg/ml) required to cause a 20% or more fall from the baseline FEV1 (respiratory threshold of methacholine; RT-Meth).

Suspected cough-variant asthma (CVA) [21] was first treated with β2-agonists (a combination of clenbuterol 40 μg/day given orally and salbutamol inhalation 200 μg at bedtime and on demand). If this proved effective but insufficient, the treatment was stepped up in accordance with the guidelines on the treatment of asthma. Suspected atopic cough (AC) [22, 23] - bronchodilator-resistant non-productive cough associated with atopic constitution - was treated with histamine H1 antagonists and inhaled corticosteroids (a combination of 10 mg/day

cetrizine hydrochloride and 400-800 ・g/day fluticasone propionate). Suspected sinobronchial syndrome (SBS) [14] was treated with clarithromycin (200 mg/day orally). Suspected gastroesophageal reflux disease (GERD) [4] was treated with a high dose of proton-pump inhibitors. The duration of each treatment was a minimum of 2 months.

In case the definite diagnoses could not be established because of the lack of complete relief of cough, according to our experience of successful treatment for atopic cough caused by BM antigens [12, 13], inhalation of amphotericin B (1 ml of 2.5 mg/ml solution, once or twice a week) [24] or oral administration of low-dose antifungal drug (ITZ 50 mg/day, 14-21 days) were given.

Assessment of efficacy of treatment on cough symptom

Efficacy of treatment on cough was assessed using subjective cough symptom scale (ACSS). It was assumed that sum sensation concerning intensity and frequency of cough during one week before the first visit was 10 cm and complete absence of cough was 0 cm. Each patient was asked what cm on the cough scale remained at his/her each visit. The efficacy of the treatment was evaluated based on the change in the cough scale before and after the treatment (Δ cough scale): 10-8 cm; excellent, 7-5 cm; good, 4-3 cm; fairly good, 2-0 cm; unsatisfactory.

Cough was considered recurrent when an isolated non-productive cough lasted for more than 2 weeks despite upper respiratory infection. The onset of typical asthma was defined as wheezing and/or a dyspnoeic attack responding to inhaled β 2-agonists.

Allergological Study

Induced sputum

Sputum was induced by inhalation of 10 ml of 5% saline solution for 20 min through a jet nebulizer (MMN-20S, Millicon-S; Sin-Ei Industries, Inc., Tokyo, Japan). If spontaneous cough did not occur 10 min after the start of inhalation, the patient was asked to induce a deep cough. Patients who were not able to bring up sputum at the end of the 20-min inhalation period were released. The specimens were considered adequate if alveolar macrophages were detected. A part of the sputum was used for fungal culture. Another part of the sputum was transferred to slides in small amounts and finely distributed over 2 microscopic slides. The smear was air-dried and stained by the May-Grunwald-Giemsa method. A total of 400 cells were counted from randomly selected fields under oil immersion objectives. The results were expressed in percentages and recorded. Sputum eosinophilia was defined when beyond 5%.

Preparation of the antigenic solution

One liter of Sabouraud' s dextrose broth in 3 liter flasks were sterilized by autoclaving at 121℃ for 20 min. Five ml of *Bjerkandera adusta* (NBRC 4983)' s spore suspension (10^5 spores/ml) in sterile physiological saline from 14 day-old Sabouraud' s dextrose agar culture was used to inoculate the flask. The flask were shaken at 25 ℃ at 150 rpm in a rotary shaker incubator. The flask was removed after 14 days. The mycelium was separated by filtration and centrifuged. The supernatant were dialyzed against 5 mM ammonium bicarbonate and lyophilized.

Immediate-type skin test and serological test

Reactions to the Bjerkandera adusta antigen

The antigenic solution (polysaccharide) was injected intradermally with a tuberculin syringe (0.02 ml, 1 mg /ml) to assess the skin response to the solution. The result was judged to be positive in a case of the longer axis of the flare beyond 10 mm at 15 minutes after the injection.

Bjerkandera adusta-specific IgG was measured by a modified ELISA inhibition technique described previously [25]. For inhibition, 25 μL of sera either from control patients or *B. adusta* cases were incubated with either *B. adusta* or irrelevant antigen and 200 μL of ovalbumin diluent in polystyrene tubes overnight at 4 ° C. Microtiter plates were coated with 100 μL of *B. adusta* regand and incubated for 12 hours at high humidity. Plates were then washed and blocked and 200 μL of inhibited sera were added to each well. Alkaline-phosphatase-coupled developing antibody was added, followed by enzyme substrate amplifier solution. Plates were observed for color development, at which point absorbance was measured at 650 nm per 10 seconds in 5 minutes with an automated microliter plate reader. RAST and ELISA mean values from the samples were calculated. The positive *B. adusta* -specific IgG was defined when grater than 0.41 mg/L.

B. adusta-specific IgE was measured by an AlaSTAT-MP technique that have been previously described [26]. In brief, soluble *B. adusta* was first coupled to insoluble cyanogens-bromide-activated immunosorbent disks by incubation for 48 hours on a rotating device. Next, for inhibition, 50 μL of serum from *B. adusta* cases or controls were incubated with 50 μL of *B. adusta* antigen or irrelevant antigen (human serum albumin) for 6 hours at 4 ℃ in polystyrene tubes. *B. adusta*-coupled disks were then added to

each tube for 12 to 16 hours. The disks were then washed four times with PBS-Tween and subsequently incubated with I^{125}-radiolabeled antihuman IgE for another 12 hours. Plates were observed for color development as same as *B. adusta*-specific IgG. The positive *B. adusta*-specific IgE was defined when grater than 0.35 U/mL.

Bronchoprovocation test

When cough symptom scale (ACSS) improved to be under 4 after the standard therapy, patients were subjected to bronchoprovocation test using the antigenic solution [27]; *B. adusta* and *Penicillium thomii* (as control) (2 ml of culture-filtrate antigen, 1 mg/ml) through a jet nebulizer. The responses were assessed to be positive when laboratory findings such as WBC and CRP elevated significantly and/or patients developed cough attacks with a significant increase in cough reflex sensitivity to inhaled capsaicin which was measured before and 24 hours after the provocation test.

Lymphocyte stimulation test

The lymphocyte stimulation test using the antigenic solution was performed by using Lymphoprep system. The results were assessed to be positive when the magnitude of the response to *B. adusta* was beyond 200% when compared with these controls using PHA [28].

Results

Estimation of chronic cough

Eight patients (5 males and 3 females) complaining of chronic cough, with a mean age of 50.4 ± 17.1 (SD) years, were examined (Table 1). A carefully

noted questionnaire revealed that all patients complained cough with "tickle and stuck" in their throat. Chest and sinus X-rays were normal in all the patients. Atopic tendency were recognized in 4 patients (Patient 3, 4, 5, and 7) (Table 2).

Chronic airflow limitation, defined as the ratio of the forced expiratory volume in 1 sec (FEV1) to the forced vital capacity (FVC), i.e., FEV1/FVC < 0.7 and FEV1 < 80% of the predicted value, and positive bronchial reversibility was not observed in any of the 8 patients. RT-Meth was heightened in 3 patients (Patient 3, 5, and 6). Cough reflex hypersensitivity as assessed by the estimation of the capsaicin cough threshold [19] was observed to be increased in 4 patients (Patient 2, 6, 7, and 8) (Table1).

Efficacy of each treatment and diagnosis of 8 patients

With the exception of 2 patients (Patient 3, and 5) in whom the response was fairly good, bronchodilator therapy failed to produce any response. Further, histamine H1 antagonists and inhaled corticosteroids failed to produce any response except in 2 patients (Patient 7, and 8) (Table 4).

Based on these results, the probable diagnoses for the causes of chronic cough in the 8 patients were as follows: atopic cough [23] in 2 patients (Patients 7, and 8) with cough reflex hypersensitivity; cough variant asthma [21] in 2 patients (Patients 3, and 5) with heightened bronchial responsiveness to methacholine [20]; however, even a probable diagnosis could not be achieved in the remaining 4 patients (Patient 1, 2, 4 and 6).

Thus, we were unable to establish the definite diagnoses due to lack of complete relief of cough with individual standard therapies in all of the

8 cases; however it was remarkable that the addition of antifungal drugs; ITZ 50 mg/day 14-41 days and/or inhalation of amphotericin B [24] (1 ml of 2.5 mg/ml solution, once or twice a week, a total of 2-7 times), or amphotericin B syrup [13] (2 ml/day for 12 days) proved efficacious with significant decrease of subjective cough symptom scale in all patients including the 4 patients given probable diagnosis (Table 4).

From these results we diagnosed all 8 patients as FACS.

Allergological tests for 8 patients of FACS

Peripheral blood eosinophilia was noticed in Patient 3. The total serum IgE levels were found to be elevated in 4 patients (Patient 3, 4, 5, and 7). Positive AlaSTAT for house dust and mite was observed in two patients (Patient 3, and 7), and 3 patients (Patient 3, 5, and 7), respectively.

Out of the 6 patients who underwent the induced sputum examination, sputum eosinophilia was observed in 2 patients (Patient 6 and 8).

Five of the 8 patients demonstrated immediate cutaneous response to the *B. adusta* antigenic solution. The positive *B. adusta*-specific IgG was observed in 6 patients, whereas *B. adusta*-specific IgE was not observed in any one (Table 2).

The bronchoprovocation test and the lymphocyte stimulation test

Five of 7 patients (Patient 2, 4, 5, 7 and 8) demonstrated positive reaction to the bronchoprovocation test with appearance of some cough attacks. Four of the 5 patients (Patient 2, 4, 5, and 8) revealed positive reaction to the lymphocyte stimulation test (Table 3). All patients who demonstrated positive reaction to LST revealed positive results for bronchoprovocation

tests.

Clinical course of 8 patients of FACS

The eradication of BM from the sputum was confirmed only in 2 patients, because most patients became non-productive in cough with success of the itraconazole therapy.

The mean duration for apparent relief of cough symptom which revealed significant decrease of subjective cough symptom scale after initiation of anti-fungal drugs was 2.8 ± 1.0(Median 2.0, ranged from 2 to 4 weeks), however the mean duration required for the first complete remission after initiation of anti-fungal drugs was 12.4 ± 7.4 (Median 11.5, ranged from 4 to 24 weeks). The duration required for the complete remission with the antifungal drugs was significantly longer in the sensitized patients (median 4 weeks, Patient 2, 4, 5, 7, and 8) than the non-sensitized patients (median 15 weeks, Patient 1, 3, and 6) (p=0.0339 by Mann-Whitney U test).

The recurrence of chronic cough was noticed in 5 patients and in 3 patients of them fungal culture of sputum revealed BM fungi again. The onset of typical asthma was recognized in 3 patients (Table 5).

Discussion

Despite extensive diagnostic evaluation and numerous treatment guidelines [1-4], a number of patients remain troubled by chronic and uncontrollable cough.

Haque et al. have described a subgroup of chronic idiopathic cough (CIC) patients for whom systematic investigation did not yield a specific diagnosis and in whom trials of specific therapy did not improve their

symptoms [29-30]. CIC is not rare and is a serious problem worldwide, so it is important to investigate how to deal with CIC and how to minimize assigning patients with this condition to CIC.

We have previously reported cases of atopic cough (AC) caused by a hypersensitivity to *Trichosporon asahii* [5], *Pichia guilliermondii* [6], *Streptomyces albus* [7], and also reported the first case of nonasthmatic sputum eosinophilia caused by allergic reaction to a BM fungus (originally mislabeled as *Humicola fuscoatra*) in which the increase of eosinophils in the induced sputum was established by repeated environmental surveys to be closely related to the appearance of BM fungi in the patient' s house [8]. Furthermore, we have reported several cases of AC which were sensitized by BM fungi and successfully eradicated and treated with low-dose itaraconazole [12] or oral cleansing with amphotericin B [13]. From our series of clinical studies on fungus-associated allergic cough, we have focused on the possible role of BM as a fungal aeroallergen [9].

Initially we performed pharyngeal swab cultures for the detection of fungi in 141 patients with chronic nonproductive cough and identified *Candida* and BM in 10.6% and 6.4% of all the examined patients, respectively [10]. Since BM fungi are rarely detected in the culture of pharyngeal swabs taken from non-coughers [9], furthermore the positive ratio of the immediate subcutaneous reaction for BM fungi in allergic airway diseases such as AC (18.8%), cough variant asthma (26.9%), and cough predominant asthma (22.4%) are significantly higher than that in physically unimpaired persons (3.4%) (Sequentially, p=0.049, p=0.014, p=0.021) (unpublished data), we suspect that these colonization were not caused by an environmental fungal contamination. Therefore we have proposed that BM fungi colonizing in the

pharynx or lower respiratory tract play an important role in allergic airway disorders [10]. We use Sabouraud' s dextrose agar (SDA) mainly, but also PDA is useful. There is the point that we should be careful to; though it needs only about two days to detect *Aspergillus* which is well-known as a cause of the disease eumycetes, it needs more about 10days to culture BM. Surprizingly it became clear at the meeting of the 10 t h Japan cough research society that many laboratories are storing the nutrient medium only for about two days. Such a disadvantageous recognition of culture may have made the detection of BM difficult in other many institutions.

Although only a limited number of reports have documented infectious diseases caused by BM fungi, such as *Schizophyllum commune* [31] and *Coprinus* species [32], there are many reports concerning the possible role of basidiospores as airborne allergens [33-35]. The phylum of Basidiomycetes (mushrooms, puffballs, rusts, smuts, and bracket fungi) deserves special attention because of the high atmospheric concentration of basidiospores during the fall season. Such spores are generally small (3 to 7 ・m in diameter); this size enables easy deposition within the airway [36].

According to our previous examination using molecular biological analyses described in the introduction, we assumed that *B. adusta* (100% homology to DDBJ/EMBL/GenBank DNA database accession number AB096738) represent one of the important potential etiological agents of chronic cough among species of BM phylum, consisting of approximately 30,000 species.

B. adusta is well-known in the field of agricultural science because it secretes multiple lignin peroxidase isozymes [37, 38]. Gloria et al [39] studied a total of 44 clinical isolates of BM fungi, namely, *S. commune* (n=5), the *Coprinus species* (n=8), *B. adusta* (n=14), and sterile, uncharacterized

Basidiomycetes (n=17). In their report, the white-rot Basidiomycetes – *B. adusta* – were detected from bronchial washes (n=8), sputum (n=3), skin, pleural fluid, and lung tissue (n=1 for each); however, the clinical significance of each isolate was not clarified.

The participation in planning of the antifungal drugs in the cough treatment strategy based on understanding of FACS will be useful and the possibility of the clinical application will spread. However, it is difficult to ascertain whether such an exposure of environmental fungi causes a true allergy [34], an infection, or a combination of both an allergy and an infection.

In this study, we selected 8 patients with chronic cough in which sputum culture yielded *B. adusta* beforehand, and conducted allergological tests using the antigenic solution of *B. adusta* (polysaccharide). We described clinical manifestations of the 8 patients from the medical reports.

Even a probable diagnosis for the causes of chronic cough in the 8 patients was obtained in only 4 patients, and we were unable to establish the definite diagnoses because of lack of complete relief of cough with individual standard therapies in all of the 8 patients. However, according to our experience of successful treatment for atopic cough caused by BM antigens [12, 13], inhaled amphotericin B [24] or oral low-dose antifungal drug therapy successfully abolished the cough symptoms in the 8 patients. Thus 8 patients recruited in this study were diagnosed all as FACS.

From the results of allergological test, such as the skin test, bronchoprovocation test, and lymphocyte stimulation test, we found a subgroup which is sensitized by *B. adusta* among the 8 patients of FACS.

Clinical features in the 5 patients of so-called allergic fungal

cough(AFC) (Patient 2, 4, 5, 7, and 8) are (1) chronic intractable cough with "tickle and stuck" in their throat lasting for more than 8 weeks; (2) absence of wheezing, dyspnea, hemoptysis, or pleurisy, and no adventitious lung sounds on physical examination; (3) environmental fungi cultured from the sputum, basidiomycetous fungus particularly *B. adusta*; (4) at least one of positive reactions to immediate cutaneous reactivity and/or inhalation bronchoprovocation test and/or lymphocyte stimulation test to the fungus; (5) good clinical response to anti-fungal drugs for which long duration (more than about 10 weeks) required for complete remission of cough symptom; (6) frequent recurrence of cough; (7) peripheral blood eosinophilia, elevated total IgE level in the serum, and eosinophilia in the induced sputum, which are not necessarily seen; (8) normal FEV1, FVC and FEV1/FVC ratio; (9) normal ～slightly increased airway cough reflex sensitivity; (10) bronchial responsiveness within normal limits.

On the other hand, interestingly other 3 patients who were not sensitized with *B. adusta* revealed excellent clinical response to antifungal drugs and no recurrence of cough.

Despite the remarkable fact that antifungal drug therapy was efficacious for disappeararance of both cough symptoms and sense of stuck in the throat and successful eradication of BM fungi from specimen of respiratory tract, 5 patients sensitized with *B. adusta* were apparently intractable on treating and managing their symptoms. According to these findings, *B. adusta* seems to show various relations on chronic cough and different clinical manifestations depend upon whether each patient of FACS is sensitized with the fungus or not.

Because the major species of BM fungi relating to FACS may change their

profile or increase in amount by natural environmental factors such as global warning or geographical characteristic, the epidemiological investigation concerning BM fungi will be required.

Thus it is suggested that so-called allergic fungal cough (AFC), which is sensitized with various kinds of BM fungi as well as *B. adusta*, may be included in chronic intractable cough as a part of FACS in future. Further study is waited for whether crossing-over reactivity in BM fungi except *B. adusta* is observed, and whether this reaction is peculiar to *B. adusta*.

As an intractable allergic disease and the connection of the environmental fungi are not problems limited to department of both otolaryngology [40] and dermatology [41] anymore, an allergological study with using such as *B. adusta* against chronic intractable cough patients should be planned prospectively by many institutions.

We hope that the recognition of this new disease concept AFC will bring advantage for a study of chronic intractable cough.

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(Table 1) **Characteristics of patients**

	Age	Gender	Pulmonary function tests %FVC	FEV1	%FEV1	FEV1%	Bronchial reversibility (%)	RT-Meth mg/ml	Cough threshold (μM)
1	20	M	135.6	5.24	123.9	88.8	2.2	20	31.3
2	46	M	126.9	4.08	120.7	83.3	2.0	20	0.98
3	58	F	113.2	1.86	109.4	72.1	8.6	5	1.95
4	66	F	94.7	2.33	103.1	76.9	-0.4	20	31.3
5	40	M	106.9	3.61	103.4	86.4	-1.4	1.25	31.3
6	68	M	135.5	2.41	120.5	72.6	7.8	5	3.91
7	39	M	135.6	5.24	123.9	88.8	3.2	20	3.91
8	66	F	128.7	2.38	126.6	77.0	4.7	20	0.49

(Table 2) **Allergological findings(I)**

	WBC	Eo (%)	CRP	IgE (U/ml)	Eo in SP (%)	Specific IgE House dust 1	Specific IgE Mite	With *Bjerkandera adusta* antigen Skin test Immidiate	Skin test Late	Specific-IgE (U/ml)	Specific-IgG (mg/ml)
1	7100	4.6	0.12	228	NA	ND	ND	0x0/0x0	0x0/0x0	0.35>	0.92
2	5200	1.4	0.06	55	3	ND	ND	4x4/0x0	0x0/0x0	0.35>	<0.1
3	5000	11.3	0	628	NA	0.8(+)	0.47(+)	4x4/15x10	4X3/10X7	0.35>	4.35
4	5800	0.9	0.12	229	2	(-)	ND	7x7/30x30	0x0/0x0	0.35>	0.41
5	4800	1.0	0.12	238	0	(-)	2.35(+)	10x10/24x22	0x0/0x0	0.35>	16.76
6	6800	3.8	0.04	145	54	(-)	(-)	0X0/0X0	0X0/0X0	0.35>	16.38
7	5100	1.5	0.02	893	1	0.47(+)	2.28(+)	6X7/32X52	0x0/0x0	0.35>	6.93
8	4800	2.7	0.01	94.3	17	(-)	(-)	5x6/30x28	0x0/0x0	0.35>	1.48

(Table 3) **Allergological findings (II)**

	Bronchoprovocation test Cough threshold (before)	(after)	Cough attack	Result	Lymphocyte stimulation test S.I	Result
1	15.6	31.3	no	Negative		ND
2	1.95	1.95	8 hr	Positive	702	Positive
3	ND	ND	ND	ND		ND
4	125	62.5	5 hr	Positive	205	Positive
5	1.95	1.95	8 hr	Positive	312	Positive
6	31.3	125	no	Negative		ND
7	125	62.5	9 and 10 hr	Positive	132	Negative
8	15.6	1.95	7 and 11 hr	Positive	888	Positive

(Table 4)

Clinical manifestations of 8 patients (I)

	subjective cough symptom scale on each therapy			Total use of anti-fungal drugs for
	β_2-stimulant	Histamine H1 antagonists and inhaled corticosteroids	Anti-fungal drugs	complete remission of cough symptoms
1	10	10	1	ITZ 50mg x 14d
2	9	8	3	ITZ 50mg x 28d, APTB 7 times
3	7	ND	4	ITZ 50mg x 14d, APTB 1 time
4	9	8	0	ITZ 50mg x 21d, APTB 5 times
5	7	6	0	APTB 5 times
6	10	8	3	APTB syrup 2ml x 12d
7	10	7	1	ITZ 50mg x 41d, APTB 6 times
8	10	7	4	ITZ 50mg x 25d, APTB 2 times

(Table 5)

Clinical manifestation of 8 patients(II)

	Duration for apparent relief of cough after initiation of anti-fungal drugs (weeks)	Duration required for first complete remission after initiation of anti-fungal drugs (weeks)	Duration required for first complete remission after first visit (weeks)	Eradication of BM in sputum	Relapse of cough	fungal culture of sputum on relapse	onset of BA
1	2	4	6	Not obtained	(-)	ND	(-)
2	4	21	26	Not obtained	(+)	BM	(-)
3	2	8	36	ND	(-)	ND	(+)
4	2	15	20	Not obtained	(+)	Peni	(-)
5	2	13	17	Not obtained	(+)	ND	(+)
6	4	4	4	Not obtained	(-)	ND	(-)
7	2	10	11	Successful	(+)	BM	(-)
8	4	24	25	Successful	(+)	BM	(+)

V. FUNGUS-ASSOCIATED COUGH SYNDROME (FACS): The efficacy of itraconazole in the treatment of patients with chronic cough whose sputa yield basidiomycetous fungi

Abstract

[Background] Although we have previously reported the efficacy of antifungal drug therapies in atopic cough in which sputum examination yielded basidiomycetous (BM) fungi, there are few trial data to support this recommendation. **Objectives**: This controlled study was performed to clarify the therapeutic benefit of itaraconazole in treating BM-colonized patients with chronic cough.

[Methods] BM was detected in the sputum of 39 out of 171 patients who visited our hospital for the diagnosis and treatment of chronic cough. Informed consent was obtained from 21 of these 39 patients, and the 21 patients were enrolled in this trial. After the administration of the standard therapy, all the patients were enrolled into a randomized placebo-controlled study on 2-weeks treatment with a low dose itraconazole (50 mg/day) (n=10) in comparison with a corresponding period treatment with matched placebo (ambroxol hydrochloride 45 mg/day) (n=11). Cough was assessed using subjective cough symptom score, and capsaicin cough challenging.

[Results] The treatment with itraconazole, but not placebo (P=0.17), was associated with a significant improvement in the cough score (P=0.0051); moreover, the improvement achieved with itraconazole was significant (P<0.001) when compared with that of the placebo.

[Conclusions] Low dose itraconazole has been revealed to be an

effective antitussive in patients with chronic cough in whom sputum examination yielded BM fungi. The clinical features observed in our patients constitute a unique disease entity; we therefore propose a new clinical concept "FACS" as an acronym for fungus-associated cough syndrome.

Introduction

Many studies have reported the pathophysiological features of allergic airway disorders [1-3], which present as chronic cough; however, only few studies, including our case reports [4, 5], have demonstrated the etiological agents involved in these disorders. Following our previous report of a typical case of chronic cough with sputum eosinophilia caused by an allergic reaction to basidiomycetous fungi (BM) [6] (it was mislabeled as *Humicola fuscoatra* at that time of publishing) [7], we have focused on the possible role of BM as a fungal aeroallergen [8]. Initially we performed pharyngeal swab cultures for the detection of fungi in 141 patients with chronic nonproductive cough and identified *Candida* and BM in 10.6% and 6.4% of all the examined patients, respectively [9]. Since BM fungi are rarely detected in the culture of pharyngeal swabs taken from non-coughers [10], and the positive ratio of the immediate subcutaneous reaction for BM fungi in allergic airway diseases such as atopic cough (AC) [11-13], cough-variant asthma (CVA) [14], and cough-predominant asthma was significantly higher than that in non-coughers (unpublished data), we suspect that the positive culture results were not caused by an environmental fungal contamination.

Therefore we have emphasized that BM fungi colonizing in the pharynx or lower respiratory tract play an important role in allergic airway disorders.

Although we have previously reported the efficacy of antifungal drug therapies in atopic cough in which sputum examination yielded BM fungi [15, 16], there are few trial data to support this recommendation. The present controlled study was performed to clarify the therapeutic benefits of itraconazole in patients with fungus-associated chronic cough.

Methods

Patients

From April 2005 to May 2006, 171 patients visited our hospital for the diagnosis and treatment of chronic cough, which was defined as isolated persistent cough lasting for 8 weeks or more. BM fungi were detected in the sputa from 39 of the 171 patients (22.8%). Of these 39 patients, informed consent was obtained from 21 patients, and subsequently the 21 patients were enrolled in this study. This randomized, single-blinded, placebo-controlled, parallel-group trial was approved by the institutional review boards (reference number 2005005) and approval for the study was obtained from UMIN-CTR (reference number R000000432; Umin000000114) .

Diagnosis of chronic cough

The cause of chronic cough in each patient was diagnosed based on a questionnaire, blood examination findings, chest and sinus X-rays,

induced-sputum examination, pulmonary function tests [17], test for cough reflex sensitivity to inhaled capsaicin [18], bronchial reversibility in response to bronchodilators, bronchial responsiveness to methacholine [19], and the efficacy of individual causes-specific treatments.

Capsaicin cough threshold was measured as an index of airway cough reflex sensitivity according to the method reported by us [18] at the first visit and after each therapy. The capsaicin cough threshold was defined as the lowest concentration of inhaled capsaicin eliciting five or more coughs. Positive bronchial reversibility was defined as percent increase in FEV1 > 12% and absolute increase in FEV1 > 200 mL. The non-specific bronchial responsiveness to methacholine was assessed according to the method described by Cockcroft et al [19]. The results were expressed as the provocation concentration (mg/ml) required to cause a 20% or more fall from the baseline FEV1 (respiratory threshold of methacholine; RT-Meth).

The examinations were performed in accordance with the diagnostic criteria for each cause, as recommended by the Japanese Cough Research Society [20] and Japanese Respiratory Society [21]. The clinical features of AC [11] were considered to be as follows: (1) chronic bronchodilator-resistant nonproductive cough with "tickle" in the throat lasting for more than 8 weeks; (2) absence of wheezing, dyspnea, hemoptysis, or pleurisy, and no adventitious lung sounds on physical examination; (3) presence of one or more global atopic findings, including past history and/or complication of allergic diseases except for bronchial asthma, family history of allergic diseases,

peripheral blood eosinophilia, elevated total IgE level in the serum, positive specific IgE antibody to common aeroallergens, and positive allergen skin test; (4) existence of eosinophils in hypertonic saline-induced sputum and/or submucosa of biopsied trachea and/or bronchi; (5) normal FEV1, FVC and FEV1/FVC ratio; (6) no bronchial reversibility defined as less than a 5% increase in FEV1 after inhalation of 300 μg salbutamol following 250 mg aminophylline injection; (7) bronchial responsiveness within normal limits; (8) increased airway cough reflex sensitivity; and (9) complete relief of the cough on treatment with histamine H1-antagonists and/or corticosteroid therapy.

CVA was diagnosed based on the following diagnostic criteria [14]: (1) isolated chronic non-productive cough lasting more than 8 weeks; (2) absence of a history of wheezing or dyspnea, and no adventitious lung sounds on physical examination; (3) absence of PND to account for the cough; (4) FEV1, FVC, and FEV1/FVC ratio within normal limits; (5) presence of bronchial hyper-responsiveness (PC20 < 10 mg/mL), (6) cough reflex sensitivity within normal limits (C5 > 3.9 μm), (7) no abnormal findings indicative of cough aetiology on chest radiograph; and (8) relief of cough with bronchodilator therapy. When all criteria were satisfied, a definite diagnosis of CVA was made.

Sinobronchial syndrome (SBS) [22] was diagnosed according to the following diagnostic criteria [14]: (1) productive cough without wheezing lasting 8 weeks or more, (2) one or more of the following findings: (i) symptoms such as post-nasal drip (PND) and throat clearing; (ii) signs such as mucus or mucopurulent secretion in the

upper and middle pharynx and cobble stone appearance of the mucosa; (iii) fluid retention or mucosal thickening on sinus X-ray or computed tomographic (CT) scan; and (iv) increased neutrophils without eosinophils in nasal secretions and spontaneous sputum; (3) no atopic findings; (4) no bronchial reversibility; (5) bronchial responsiveness within normal limits; (6) cough reflex sensitivity within normal limits; and (7) relief of cough on treatment with 14- or 15-member macrolides.

Assessment of efficacy of treatment on cough symptom

Efficacy of treatment on cough was assessed using subjective cough symptom scale (ACSS). It was assumed that sum sensation concerning intensity and frequency of cough during one week before the first visit was 10 cm and complete absence of cough was 0 cm. Each patient was asked what cm on the cough scale remained at his/her each visit. The efficacy of the treatment was evaluated based on the change in the cough scale before and after the treatment (Δ cough scale): 10 ~ 8 cm; excellent, 7~5 cm; good, 4~3 cm; fairly good, 2~0 cm; unsatisfactory.

Study design for the treatment of chronic cough

All patients were administered the standard therapy, which involved administration of bronchodilators for the first 7 days (a combination of oral 40 ・g/day clenbuterol and salbutamol inhalation at bedtime and/or when required) and administration of histamine H1 antagonists and inhaled corticosteroids (a combination of 10 mg/day cetirizine

hydrochloride and 400 ・g/day fluticasone propionate) for the next 7 days. Subsequently, they were randomly assigned to receive either 50 mg/day (low dose) itraconazole or 45 mg/day ambroxol hydrochloride (placebo) for 2 weeks (trial phase).

Patients who completed the above mentioned study were given the opportunity to take part in 2-weeks extension study and were given itraconazole when their cough had not been adequately controlled during the core study.

Strain isolations and morphological observations

Sputum samples taken from the patients were inoculated on Sabouraud' s dextrose agar (SDA) containing chloramphenicol. These cultures were incubated at 30° C for 2 weeks. The colonial characteristics of the strains were observed by culture on SDA plates at 30° C for 14 days. The morphological features of the strains were observed by using the slide culture method (30° C for 2 weeks) and lactophenol cotton blue staining by a mycological specialist.

Statistical analysis

The subjective cough symptom scale was compared between the itraconazole and the placebo groups by using Mann-Whitney *U* test. The change in the cough scale within the group was analyzed using Wilcoxon signed-rank test. A P value less than 0.05 was considered statistically significant.

Results

Clinical findings and diagnosis of chronic cough

The patients studied comprised 11 males and 10 females with chronic cough (4 males and 6 females in itraconazole group, and 7 males and 4 females in control group) with mean age of 51.9 ± 14.2 (SD) years (49.6 ± 18.4 (SD) years in itraconazole group, and 54.0 ± 9.5 (SD) years in control group). The gender and age population were not significantly different between the two groups. Chest and sinus X-rays were normal for all the patients. Atopic tendency such as pollinosis was seen in 5 patients (patient 1, 4, 11, 15, and 21). Three patients were current-smokers in control group, however only one patient was ex-smoker in itraconazole group. There was no significant difference in the mean duration of cough symptom before the first visit between the two groups (8.2 ± 18.2 (SD) months in the itraconazole group and 4.7 ± 4.7 (SD) months in the control group).

The total serum IgE levels were found to be elevated in 3 patients (one in the itraconazole group and 2 in the control group). Out of the 15 patients in whom adequate induced sputum was obtained by inhalation of hypertonic saline, sputum eosinophilia was observed in 5 patients (one in the itraconazole group and 4 in the control group).

Chronic airflow limitation was considered present when the ratio of forced expiratory volume in 1 s (FEV1) to forced vital capacity (FVC) (FEV1/FVC ratio) was <0.7. Further, FEV1 of <80% of the predicted value was found in 2 of the 21 patients. Positive bronchial reversibility was not observed in any patients. Cough reflex

sensitivity [18] was increased in 8 patients. Bronchial responsiveness to methacholine [19] was exaggerated in 4 patients. Of the 21 patients enrolled in this study, 10 were randomly assigned to the itraconazole group and 11 to the active placebo group (control group). No clinically important demographic differences were observed between the 2 groups. No patient withdrew from the study (Table 1).

Effect of each therapy

During the first treatment phase, the bronchodilator therapy showed a partial effect in 3 patients (patients 1, 12, and 21). During the second treatment phase, histamine H1 antagonists and inhaled corticosteroids produced a partial response in 4 patients (patients 6, 10, 14, and 15). There were no patients in whom the cough completely relieved on these treatments. During the trial phase, no patient in the control group experienced relief from cough. However, the antifungal therapy was remarkably efficacious in relieving cough in 10 patients in the itraconazole group; the result was excellent in 3 patients (patients 3, 6, and 7) and good in 7 patients (patients 1, 2, 4, 5, 8, 9, and 10).

No significant difference was observed in the subjective cough symptom scale between the 2 groups before the trial phase. The treatment with itraconazole, but not placebo (P=0.17), was associated with a significant improvement in the cough scale (P=0.0051); moreover, the improvement achieved with itraconazole was significant

(P<0.001) when compared with that achieved with the placebo (Figure 1). Individual changes in the cough scale before and after the tested treatments are shown in Figure 2.

Except patient 2, all patients in the itraconazole group did not reveal deterioration in airway cough reflex sensitivity to capsaicin after the 2-weeks itraconazole trial, while only 1 patient out of 11 patients in the control group demonstrated improvement in the cough threshold (Figure 3). As a group, the cough threshold tended to increase in the itraconazole group (p=0.0747) but did not in the control group (p=0.1380) as shown in figure 3.

After the trial period, all the patients in the control group were administered 50 mg/day of itraconazole for 14 days as an extension study, following which they exhibited a clinical response with significant decrease in the subjective cough symptom scale. Consequently, no patient was treated with a high dose of proton-pomp inhibitors for suspected GER [23]. When we finished itraconazole treatment with the result of the improvement of cough symptoms, we confirmed successful eradication of BM fungi in all 11 patients in whom the expectoration was able to be obtained.

Discussion

Although only a limited number of reports have documented infectious diseases caused by BM fungi, such as *Schizophyllum commune* [24], the *Coprinus* species [25], and allergic bronchopulmonary mycosis [26], there are many reports about the possible role of basidiospores as airborne allergens [8]. From the results of series of our studies,

we have emphasized that BM fungi colonizing in the pharynx or lower respiratory tract play an important role in chronic cough. We have encountered several patients who presented with chronic intractable cough; their sputum examination yielded environmental fungi (particularly BM fungi) and they clinically responded to anti-fungal drug therapies [15, 16]. The present controlled study was performed to clarify the therapeutic benefits of itraconazole in patients with fungus-associated chronic cough.

Concerning study design, at first, based on the response to bronchodilators, we evaluated asthmatic component, and next based on the responses to histamine H1 antagonists and inhaled corticosteroids we evaluated component of atopic cough. Because it is well-known that GER-associated cough and PND-induced cough are very rare in Japan [27, 28], we decided to perform exclusion of GER-associated cough in the final stage of treatment. In other words we decided to use proton-pomp inhibitors when the itraconazole treatment failed.

Although 21 patients enrolled in this study were randomly assigned to two groups according to the ID number of each patient, the patients in the control group demonstrated more severe airway obstruction compared with those in the itraconazole group did. This result may be explained because there were many smokers in the control group; however no significant difference was seen in mean age and FEV1/FVC ratio between the two groups. A definite diagnosis could not be established in any of the 21 patients at the previous stage before the trial phase started, because the standard therapies for cough variant asthma and atopic cough did not relieve the cough enough in

any patients. When we tried to apply them to an existing diagnosis according to extensive diagnostic evaluation and numerous treatment guidelines [20,21,23], we had barely achieved diagnosis of probable cough variant asthma [14] in 2 patients (Patients 1, and 12); based on the result of partial response to bronchodilators with heightened bronchial responsiveness to methacholine, and probable atopic cough [11] in 2 patient (patient 10, and 15); based on the partial response to the combination of histamine H1 antagonists and inhaled corticosteroids, increased cough reflex sensitivity and some atopic tendency. Thus, the remaining 17 patients were diagnosed as unknown etiology.

There is no standard therapeutic plan concerning an amount and a period of the antifungal drugs for chronic cough. We decided to carry out a low-dose use of anti-fungal drugs and fixed a treatment period at two weeks, based on our previous treatment experience [15, 16]. In the present study, in order to evaluate change in cough symptom on treatments, we used subjective cough symptom scale as described in the methods section. This scale is simpler and easier in daily outpatient practice. As for the primary outcome of this study, we used both the cough scale as a subjective index and capsaicin cough threshold as an objective one.

The cough scale was not related with capsaicin cough threshold at the start of the itraconazole trial. In addition, the change in the cough scale after the treatment (itraconazole or placebo) was not related with the change in the capsaicin cough threshold. This will be explained because some patients without cough hypersensitivity

similar to cough variant asthma [11, 20] were included in the 21 patients.

It was remarkable that the low-dose itraconazole was efficacious after the trial phase in all the 21 patients who did not have an existing diagnosis. As a result of this, the treatment with proton-pomp inhibitors for suspected GER-associated cough was not necessary for anyone in this study after administration of itraconazole.

Here, we consider mechanism of cough based on the effect of each drug which was used before the trial phase. Histamine H1 antagonists and inhaled corticosteroids produced a partial response in 4 patients. If BM fungi cause allergic inflammation in the respiratory tract, we can explain the fact that anti-allergic drugs demonstrated availability in the 4 fungus-associated coughers whether it is diagnosed as probable AC or not. On the other hand, during the first treatment phase, the bronchodilator therapy showed a partial effect in 3 patients. Among the 3 patients, 2 patients with heightened bronchial responsiveness to methacholine were assigned to probably cough variant asthma. Similarly, we are able to explain the efficacy of the bronchodilators in the 3 patients whether they are diagnosed as probable CVA or not, if the colonization of BM fungi in the respiratory tract exacerbates the potentially heightened bronchomotor tone in such patients. From such a standpoint, we carried out maintenance therapy in the 3 patients against the cough variant asthma component behind even after a cough was improved by successful eradication of BM fungi. Thus, it was suggested that the patients in

whom itraconazole was effective alone and the patients in whom addition of itraconazole was effective coexisted.

Therefore, we decided to propose a new clinical concept "FACS" as an acronym for fungus-associated cough syndrome; (1) chronic cough, (2) environmental fungi in the sputum, particularly BM fungi, and (3) clinical response to antifungal drugs.

As FACS is not an independent disease concept removing other diseases, it is difficult to establish a constant pathophysiologic tendency such as airway cough reflex hypersensitivity and bronchial hyperresponsiveness. But this doesn't confuse the researchers of the field of the cough study. Even if the basic condition of patients in which BM fungi were detected in their sputum was different, the possibility that antifungal drugs may be effective for them will be brought by this proposal of FACS.

Although the fact that a cough symptom was not improved satisfactorily unless BM fungi are eradicated from the respiratory tract is another important result in this study, the association between exposure to environmental fungi and appearance of the cough attacks has not been understood clearly yet. Further studies [29, 30] are required to elucidate the mechanism by which the low-dose of itraconazole is efficacious for FACS; low antifungal susceptibility of itraconazole [31] or the other potential immunological action of the drug as an immunomodulator.

Thus the participation in planning of the antifungal drugs in the cough treatment strategy is epoch-making, and the possibility of the clinical application spreads. We hope that the recognition of FACS

and the further investigation concerning appropriate uses of the anti-fungal drugs may provide an alternative useful avenue for the treatment of these otherwise difficult to manage patients.

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Characteristics of the subjects

		Age	Gender	FEV1	FEV1%	Bronchial reversibility(%)	RT-Meth (μg/ml)	Cough threshold (solution number)
ITZ	1	66	F	1.86	72.1	**8.6**	**5000**	4
	2	64	F	2.10	73.9	1.9	ND	4
	3	40	M	2.79	74.6	2.2	20000	8
	4	67	F	1.87	80.3	1.6	20000	**3**
	5	28	M	4.68	86.6	4.7	20000	5
	6	20	M	5.24	88.8	3.2	20000	5
	7	66	F	2.09	90.1	2.2	20000	**3**
	8	34	M	4.19	93.1	2.1	20000	**3**
	9	45	F	2.80	93.0	2.1	20000	5
	10	66	F	2.38	77.0	4.7	20000	**2**
MUC	11	68	M	2.33	76.9	-0.4	20000	8
	12	39	M	3.61	86.4	-1.4	**1250**	8
	13	58	F	2.41	72.6	**7.8**	**5000**	5
	14	47	F	2.94	77.2	2.7	20000	5
	15	58	M	3.54	**66.9**	-0.6	20000	**4**
	16	58	M	2.50	**66.3**	**6.8**	20000	**2**
	17	50	M	3.20	83.1	-2.3	20000	**4**
	18	44	M	3.67	77.9	**7.6**	20000	**3**
	19	58	F	2.73	88.1	0.4	**5000**	**2**
	20	46	M	4.08	83.3	2.0	20000	**3**
	21	68	F	2.92	80.2	2.7	20000	4

FEV1% ; the ratio of forced expiratory volume in 1 s (FEV1) to forced vital capacity (FVC) (FEV1/FVC)
The capsaicin cough threshold (C5); was defined as the lowest concentration of inhaled capsaicin eliciting five or more coughs.
Bronchial reversibility(%) ; was assessed as percent increase in FEV1.
RT-Meth(μ g/ml); The results of the non-specific bronchial resposiveness to methacholine were expressed as the provocation concentration(mg/ml) required to cause a 20% fall from the baseline FEV1(PC20).

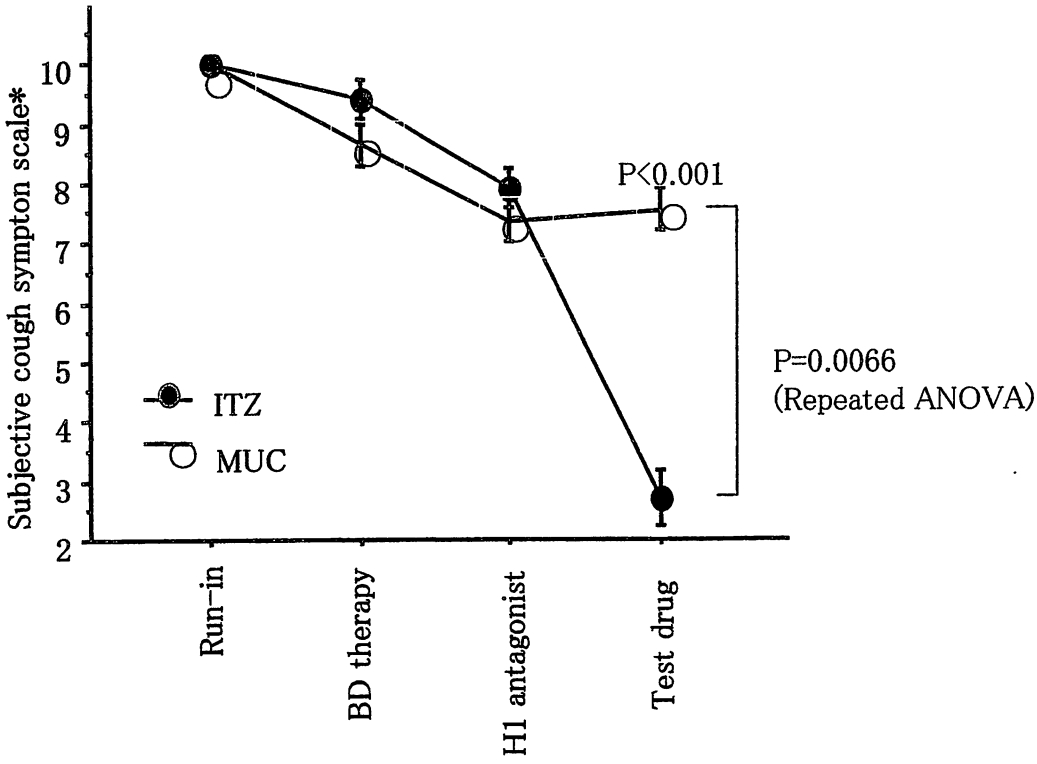

Figure 1 Change in subjective cough symptom scale on each treatment cough
*It was assumed that sum sensation concerning intensity and frequency of cough during one week before the first visit was 10 cm and absence of cough was 0 cm. Each patient was asked what cm on the cough scale remained at each visit following treatments.

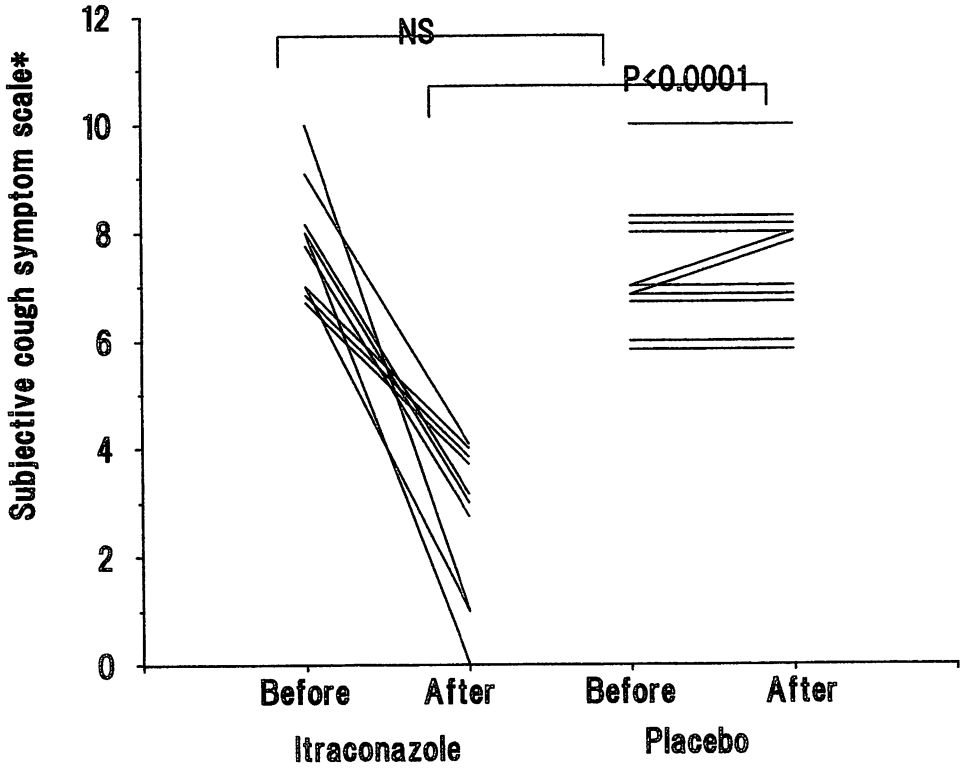

Figure 2 Individual changes in subjective cough symptom scale before and after the tested treatments
*It was assumed that sum sensation concerning intensity and frequency of cough during one week before the first visit was 10 cm and absence of cough was 0 cm. Each patient was asked what cm on the cough scale remained at each visit following treatments.

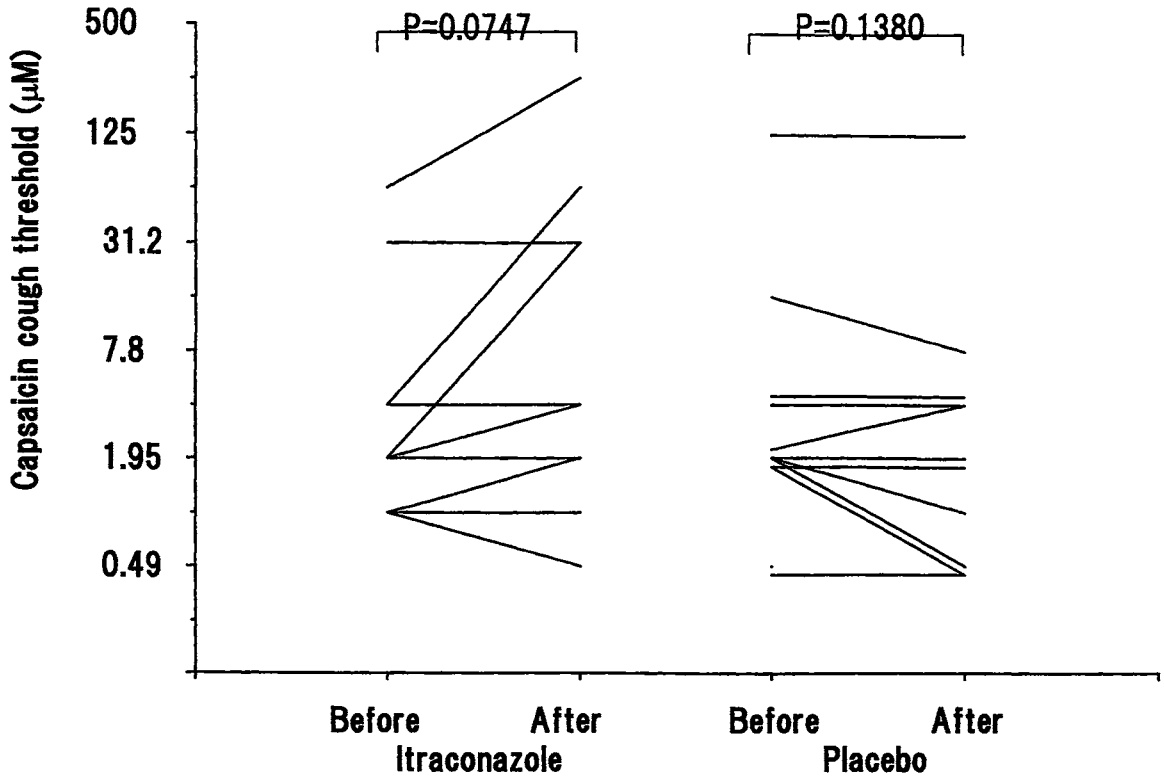

Figure 3 Cough reflex sensitivity to inhaled capsaicin before and after the tested treatments

VI Exhaled nitric oxide (NO) levels in patients with atopic cough and cough variant asthma

Abstract

[Background] Atopic cough (AC) is an established clinical entity presenting with chronic persistent non-productive cough in Japan. Exhaled NO level is a biomarker of eosinophilic airway inflammation. The present study was conducted to elucidate whether exhaled NO levels were increased in AC in comparison with cough variant asthma (CVA) and bronchial asthma (BA).

[Methods] Consecutive patients complaining of isolated cough lasting at least 8 weeks who visited our respiratory medicine clinic and gave informed consent for participating in this study were enrolled. Causes of chronic cough were diagnosed according to the Japanese Respiratory Society guidelines for management of cough. Exhaled NO, capsaicin cough sensitivity (C5) and bronchial reversibility were measured at their first visit. Bronchial responsiveness (PC20) was measured at their second visit following 6-day bronchodilator therapy.

[Results] Out of 58 enrolled and sufficiently assessed patients, 9 and 11 patients were strictly diagnosed with AC and CVA as a single cause of chronic cough. Ten patients with BA did not undergo inhaled corticosteroid therapy at their first visit. Exhaled NO levels in patients with AC were significantly lower than those in patients with CVA and BA. There was no significant difference in the exhaled NO levels between patients with CVA and BA.

[Conclusions] These results confirm that pathogenesis in AC is

different from those in CVA and suggest that exhaled NO may reflect eosinophilic inflammation of peripheral airways. Exhaled NO measurement may be useful in differentiating CVA from AC and other causes of chronic non-productive cough.

Introduction

Cough variant asthma (CVA) and atopic cough (AC) are major causes of chronic non-productive cough in Japan [1]. AC is a new clinical entity presenting with bronchodilator-resistant non-productive cough, which has been established in Japan [2]. It is the most important that bronchodilator therapy has anti-tussive efficacy only in CVA. The fundamental feature of AC includes eosinophilic inflammation of central airway and increased cough reflex sensitivity [3] while that of CVA consists of eosinophilic inflammation of central to peripheral airway [4] and mildly increased bronchial responsiveness [5]. CVA is recognized as a precursor of asthma because asthma onset develops in nearly 30% of patients with CVA [6, 7]. On the other hand, atopic cough does not transform to asthma [7].

Exhaled nitric oxide (NO) levels have been recognized as an index of eosinophilic airway inflammation [8]. De Diego A et al. [9] reported that exhaled NO levels were same between CVA and BA patients. Nevertheless, exhaled NO levels have not been reported in AC. The present study was conduced to elucidate whether exhaled NO levels are increased in AC in comparison with CVA and BA.

Subjects and methods

Study design

The design was a cross-sectional observational study comparing patients with AC, CVA and BA. Consecutive patients complaining of isolated cough lasting at least 8 weeks, who visited our respiratory medicine clinic for the diagnosis and treatment from 1 October 2004 to 30 September 2006 and gave informed consent for participating in this study, were enrolled. Measurements of exhaled NO concentration, capsaicin cough sensitivity and bronchial reversibility, sputum induction using inhalation of 5% saline solution, and assessment of atopic constitution were performed in this order at their first visit. Then, each patient underwent bronchodilator therapy (oral clenbuterol 40 μg/day + inhaled salbutamol sulfate (200 μg) on demand) for 6 days until the day before his or her second visit. Efficacy of the bronchodilator therapy on cough was assessed according to the underwritten method for assessment of efficacy of treatment on cough and bronchial responsiveness to methacholine was measured at the second visit. After then, each patient received suitable treatment according to the Japanese Respiratory Society (JRS) guidelines for the management of cough [2]. This study protocol was approved by the ethics committee of the Kanazawa University Hospital.

Exhaled NO levels in patients, who initially visited during the study period, had not undergone inhaled or systemic corticosteroid therapy during at least 4 weeks previously, and were diagnosed to have typical bronchial asthma (BA), were used as disease control values. Diagnosis of BA was made based on the following three criteria: 1) a history of recurrent episodes of wheezing, 2) reversible airway obstruction

documented by a physician, and 3) an improvement of 12% or more and 200 ml or more in forced expiratory volume in 1 s (FEV1) after inhalation of 300 μg salbutamol sulfate.

Assessment of efficacy of treatment on cough

Efficacy of treatment on cough was assessed using visual analogue scale (VAS). It was assumed that sum sensation concerning intensity and frequency of cough during several days at first visit was 10 cm and absence of cough was 0 cm. Each patient was asked what cm on the cough scale remained at each visit. Efficacy of bronchodilator therapy, which is the most important criterion for the diagnosis of CVA, was judged to be effective when the VAS at the second visit (after 6-day bronchodilator therapy) was 7 cm or less.

Diagnosis of atopic cough (AC) and cough variant asthma (CVA)

Atopic cough (AC) and cough variant asthma (CVA) as a single cause of chronic cough were strictly diagnosed according to the following diagnostic criteria for selection of subjects in clinical studies recommended by JRS [2]. In other words, patients with probable diagnosis of AC or CVA based on the brief diagnostic criteria prepared by JRS [2] were excluded from this study so that everybody accepts the selection of studied patients with AC or CVA.

The diagnosis of AC was made according to the following criteria [2]:

1) Non-productive cough lasting more than 8 weeks without wheezing or dyspnea
2) Presence of one or more findings indicative of an atopic

constitution, including a past history and/or complications of allergic diseases excluding asthma, a peripheral blood eosinophilia (>5% or >400 cells/μl), elevated total IgE level in the serum (>150 IU/ml), positive specific IgE antibody to aeroallergens and/or induced sputum eosinophilia (≥2.5%)

3) No bronchial reversibility, defined as less than a 10% increase in FEV1 after inhalation of 300 μg salbutamol sulphate
4) Bronchial responsiveness within normal limits (provocative concentration of methacholine causing a 20% fall in FEV1 (PC20) ≥10 mg/ml)
5) Increased cough reflex sensitivity (capsaicin concentration eliciting 5 or more coughs (C5) ≤3.9 μM)
6) Cough resistant to bronchodilator therapy (oral clenbuterol 40 μg/day plus inhaled salbutamol 200 μg at bedtime and on demand)
7) No abnormal findings indicative of cough aetiology on chest radiograph
8) Normal FEV1 (≥80% of predicted value), FVC (≥80% of predicted value), and FEV1/FVC ratio (≥70%)

When all criteria were satisfied, a definite diagnosis of atopic cough was given. All patients diagnosed with AC were successfully treated with histamine H1-antagonists alone and/or with inhaled and/or oral corticosteroids. The diagnosis of CVA was made according to the following criteria [2]:

1) Isolated chronic non-productive cough lasting more than 8 weeks
2) Absence of a history of wheezing or dyspnea, and no adventitious lung sounds on physical examination

3) Absence of post-nasal drip to account for the cough

4) FEV1, FVC, and FEV1/FVC ratio within normal limits

5) Presence of bronchial hyperresponsiveness (PC20 <10 mg/mL)

6) Relief of cough with bronchodilator therapy

7) No abnormal findings indicative of cough aetiology on chest roentgenogram

When all criteria were satisfied, a definite diagnosis of CVA was given. All patients with CVA had been successfully treated with bronchodilators, the leukotriene antagonist montelukast and/or inhaled corticosteroids.

Measurement of exhaled nitric oxide (NO) concentration

Exhaled NO concentration was measured by the online method using a chemiluminescence analyzer (model 280, Sievers Instruments, Boulder, CO, USA) according to the American Thoracic Society (ATS) guidelines [10], between 9 a.m. and 1 p.m. at his (her) first visit. Expiratory flow rate was 0.05 L/sec as recommended by the guidelines and exhalation pressure was 16 cmH2O. Measurement of exhaled NO was repeated until reproducible three NO plateau values were achieved. Exhaled NO was then calculated as the mean of these three values.

Pulmonary function testing

Routine pulmonary function, cough reflex sensitivity and bronchial reversibility were measured in this order at the first visit, and bronchial responsiveness was determined at the second visit (one week after the first visit). FVC, FEV1 and flow-volume curves were measured

using a dry wedge spirometer (Chestac 11, Chest Co., Ltd., Tokyo, Japan). Spirometry was performed and evaluated according to the ATS criteria [11]. Capsaicin cough threshold (C5), a concentration of capsaicin solution eliciting 5 or more coughs, was measured as an index of cough reflex sensitivity [12,13]. To assess bronchial reversibility, spirometry was performed before and 30 minutes after inhalation of 300 μg salbutamol sulfate. A provocative concentration of methacholine causing a 20% fall in FEV1 from prechallenge values (PC20) was measured as an index of non-specific bronchial responsiveness [14].

Statistical analysis

Data values for exhaled NO, capsaicin cough threshold (C5) and bronchial responsiveness (PC20) were expressed as geometric mean with geometric standard error of the mean (GSEM). Other data values were expressed as mean and standard deviation. Differences in data values between patients with AC, CVA and BA were analyzed by one-way analysis of variance (ANOVA) followed by Fisher' s PLSD. In the case, data for exhaled NO, PC20 and C5 were calculated using logarithmically transformed values. P values of less than 0.05 were considered statistically significant.

Results

Diagnostic procedure was completed in 58 of 80 patients with chronic cough who were enrolled during the 2-year study period. Out of the 58 patients, 9 and 11 patients were strictly diagnosed with AC and CVA as a single cause of chronic cough. Exhaled NO values in 10 patients with

BA, who had not undergone inhaled or systemic corticosteroid therapy at their first visit, were available during the study period. Patients' characteristics are summarized in Table 1. Duration of cough symptom before the initial visit, successful treatment for relief of cough and time after the first visit required to relieve cough on the successful treatment are summarized for individual patients with AC and CVA in Table 2. Findings showing atopic constitution for individual patients with AC and CVA are shown in Table 3.

Exhaled NO levels in patients with AC (13.0 ppb, GSEM 1.14) were significantly lower than those in patients with CVA (39.4 ppb, GSEM 1.25, p=0.0007) and BA (36.1 ppb, GSEM 1.26, p=0.0020) (Fig. 1). There was no significant difference in the exhaled NO levels between patients with CVA and BA (p=0.7618).

Discussion

The present study clearly showed that exhaled NO levels were significantly lower in AC compared with CVA and BA. We could not represent normal values of exhaled NO in this study because it was difficult to collect gender- and age-matched, non-atopic, never smoking normal subjects. It has been established that exhaled NO is a non-invasive biomarker of eosinophilic airway inflammation because it is increased in steroid-naive asthmatic patients [8]. Accordingly, it is concluded that exhaled NO in AC is lower compared with that in CVA, which is as same as that in steroid-naïve BA.

Two diagnostic criteria for each cause of chronic cough are recommended by Japanese Respiratory Society (JRS) [2]. One is

diagnostic criteria for selection of subjects in clinical studies (research use) and another is brief criteria for use in general clinical practice (clinical use). In the present study, only patients with AC and CVA, which were strictly diagnosed based on the diagnostic criteria for the research use [2], were included. As the diagnostic criteria have been made for selection of subjects in clinical studies so that data are available commonly among world wide researchers and research groups, it is hard even for cough specialists like us to complete all the listed measurements. In addition, it is also difficult that all the criteria are satisfied. For example, as a woman in whom chronic cough was completely relieved by bronchodilator therapy and bronchial responsiveness was within normal limits was diagnosed to have probable CVA, but not definite CVA, she was not included in this study. Thus, it is a reason why only small proportion of patients who visited our pulmonary medicine clinic for the diagnosis and treatment of chronic cough could be included in the present study. Further studies using the brief diagnostic criteria for AC and CVA to elucidate whether the same results are investigated may be worthy for determining that measurement of exhaled NO is useful for making diagnosis of CVA in general practice.

Chronic cough is defined as isolated persistent cough lasting 8 or more weeks. CVA is strictly diagnosed when isolated chronic cough, which we can not diagnose as bronchial asthma because of no history of wheezing or dyspnea indicative of bronchial asthma and no wheezes audible on lung auscultation, responds to bronchodilator therapy [5]. Although non-specific bronchial responsiveness is mildly increased as a group of such patients whose cough responds to bronchodilator therapy [5,7],

its considerable proportion is overlapped with those of normal subjects [15]. In addition, Irwin and co-workers have clearly shown that measurement of bronchial responsiveness can not predict efficacy of bronchodilator therapy on isolated chronic cough [16]. Taking together, it should be recognized that efficacy of bronchodilator therapy on cough is the most important criterion for the diagnosis of CVA while increased bronchial responsiveness is the second. Nevertheless, some chest physicians believe that chronic cough with increased bronchial responsiveness is CVA. Thus, we strictly selected patients with CVA, whose cough responded to bronchodilator therapy and bronchial responsiveness was increased, in this study.

AC is a newly established clinical entity presenting with chronic isolated bronchodilator-resistant non-productive cough in Japan [2]. The fundamental features are increased cough reflex sensitivity [3,7,15] with normal bronchial responsiveness physiologically and eosinophilic inflammation of central airway pathologically [3]. These are different from those of CVA: increased bronchial responsiveness [5,15] and eosinophilic inflammation of central to peripheral airway [4]. CVA is a prelude of typical asthma but AC is not [7] (Table 4).

In many countries non-asthmatic eosinophilic bronchitis (EB) is being accepted for a clinical entity presenting with chronic cough responding to corticosteroid therapy [17]. The diagnostic criteria consist of sputum eosinophilia and normal bronchial responsiveness [17]. In such patients with EB, it has been reported that cough reflex sensitivity is increased [18], eosinophils are increased in bronchoalveolar lavage fluid [19,20], and typical asthma onset develops

[21]. Two studies [20, 22] have shown that exhaled NO levels in patients with EB are increased to the same levels as those in patients with BA. Thus, some features are same but others are different between AC and EB as summarized in Table 4. At present, it may be concluded that AC is different from EB. The findings that exhaled NO and eosinophils in bronchoalveolar lavage fluid are increased in BA and EB, but not in AC, suggest that exhaled NO reflects eosinophilic inflammation of peripheral airway, of which surface area is much larger than that of central airway.

De Diego et al [9] compared airway inflammatory markers between typical asthma and CVA, which was strictly diagnosed based on both increased bronchial responsiveness and relief of cough with bronchodilator therapy as same as in our present study. The authors investigated that exhaled NO as well as cough reflex sensitivity and bronchial responsiveness did not differ between typical asthma and CVA. The same levels of exhaled NO and cough reflex sensitivity were confirmed in the present study, while bronchial responsiveness was more increased in typical asthma compared with CVA. It is likely that severity of typical asthma is severer in our study compared with De Diego and coworkers' study. Taking together, it is concluded that exhaled NO levels are increased in CVA to the same levels seen in steroid-naive typical asthma.

In conclusion, the results of this study confirm that pathogenesis in AC is different from that in CVA or EB, and suggest that exhaled NO may reflect eosinophilic inflammation of peripheral airways. Exhaled NO measurement may be useful in differentiating CVA and EB from AC and

other causes of chronic non-productive cough, in which exhaled NO has been shown to be lower than in asthma [23].

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Table 1 Characteristics of patients with atopic cough (AC), cough variant asthma (CVA) and typical bronchial asthma (BA)

	AC	CVA	BA
Number of subjects	9	11	10
Gender (male : female)	2:7	2:9	3:7
Age (years)	42.8 ± 13.1	44.8 ± 16.1	43.0 ± 18.9
Height (cm)	164.3 ± 7.1	160.0 ± 8.8	157.9 ± 5.2
Body weight (kg)	62.7 ± 13.1	55.7 ± 8.4	55.1 ± 10.8
FVC, % predicted	119.2 ± 9.0	111.3 ± 19.6	102.4 ± 14.2
FEV_1, % predicted	103.3 ± 10.1	98.1 ± 10.7	83.6 ± 12.5*** ss
FEV_1 / FVC (%)	80.1 ± 7.6	79.4 ± 9.9	72.1 ± 13.6
C5 (μM)	1.0 (1.20)	18.9 (1.4)****	7.8 (2.2)**
PC20 (mg/mL)	39.7 (1.52)	2.4 (1.22)****	0.81 (1.60)**** s

Data are presented as mean (± SD)
Data in C5 is presented as geometric mean (geometric standard error of the mean)
**$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ compared with AC
s $p < 0.05$, and ss $p < 0.01$ compared with CVA
C5 capsaicin cough sensitivity

Table 2 Results of treatment in patients with atopic cough (AC) and cough variant asthma (CVA)

Patient	Age (years)	Sex	Symptom duration (months)	Successful treatment	Time[s] for relief of cough (weeks)
Atopic cough					
1	52	F	8	Azelastine, FP, PSL	9
2	39	F	2	Azelastine	4
3	63	F	2	Azelastine	4
4	41	F	7	Azelastine, FP	5
5	21	M	2	FP, PSL	10
6	40	F	2	Azelastine	4
7	51	F	2	Azelastine, FP	4
8	51	M	24	Azelastine, FP	6
9	27	F	2	Azelastine	3
Cough variant asthma					
1	37	F	4	Clenbuterol	1
2	41	F	2	Clenbuterol	8
3	52	F	2	Clenbuterol, montelukast	4
4	69	F	120	Procaterol, theophylline	3
5	46	F	4	Clenbuterol, salbutamol inhaled	4
6	17	F	2	Clenbuterol	6
7	30	F	5	Clenbuterol, montelukast, salmeterol inhaled	2
8	55	M	3	BDP	4
9	64	M	2	Clenbuterol, BUD, salmeterol inhaled	4
10	27	F	3	Montelukast, BUD	5
11	55	F	5	Clenbuterol, montelukast, BUD	8

FP, fluticasone dipropionate inhalation (200-400 μg twice daily); BUD, budesonide inhalation (200-400 μg twice daily); BDP, beclomethasone dipropionate inhalation (200-400 μg twice daily); PSL, oral prednisolone (20 mg once daily) for less than 3 weeks. [s]time required to relieve cough after the first visit.

Table 3 Characteristics of atopic constitution in patients with atopic cough (AC) and cough variant asthma (CVA)

Case Number	Past history	Compli-cation	Family history	Eosinophils in peripheral blood (%)	Eosinophils in peripheral blood (/μL)	Eosinophils in sputum (%)*	Total IgE in serum (IU/mL)	Specific IgE in serum
Atopic cough								
1	–	–	–	7.6	251	NG	11	–
2	–	–	–	1.0	42	0	32	JC
3	PO	UR	–	3.7	218	0	138	–
4	UR	–	–	0.7	43	NG	94	–
5	CBA	PO	–	3.7	283	NG	2200	HD, D, RW, JC
6	–	–	AR	2.0	138	NG	220	HD
7	–	–	–	3.3	158	NG	68	RW, JC
8	–	UR	–	4.0	176	NG	86	HD, D
9	–	PO, AD	–	8.2	558	19.5	175	JC, HD,D, CD
Cough variant asthma								
1	UR	–	–	1.5	72	0	40	–
2	–	–	–	3.0	255	NG	0	–
3	–	–	–	1.0	63	NG	56	HD, RW
4	–	–	–	1.5	60	NG	58	–
5	–	AR	–	0	0	1.0	9	–
6	–	AR	BA	3.0	177	NG	0	–
7	–	–	–	6.0	360	NG	320	HD, D
8	–	–	–	10.8	756	3.6	187	JC, RW
9	AR, UR	–	–	1.0	69	NG	32	–
10	CBA	PO	AR	4.8	262	0.8	504	HD, D
11	–	–	–	1.9	110	NG	61	JC

(%)*, percent of nucleated cells, NG, inadequate sputum sample; AR, allergic rhinitis; PO, pollinosis; UR, urticaria; AD, atopic dermatitis; CBA, childhood bronchial asthma; HD, house dust; JC, Japanese cedar; D, dermatophagoides; RW, ragweed; CD, cat dander.

Table 4 Comparison between atopic cough (AC), nonasthmatic eosinophilic bronchitis (EB) and cough variant asthma (CVA)

	Cough variant asthma	Atopic cough	Eosinophilic bronchitis without asthma
Physiology			
Cough reflex sensitivity	Not increased	Increased	Increased
Bronchial responsiveness	Increased but less than asthma	Not increased	Not increased
Pathology			
Eosinophils in			
induced sputum	Increased	Increased	Increased
biopsied bronchi	Increased same as asthma	Increased but less than asthma	Increased same as asthma
BAL fluid	Increased same as asthma	***Not increased***	***Increased same as asthma****
Inflammatory markers			
Exhaled NO	Increased	***Not increased***	***Increased***
Outcome			
Asthma onset without ICS	30%	***No***	Not investigated
with ICS	6%	Not investigated	***9%***

Underlined findings were from the present study
*Asthmatics treated with inhaled corticosteroids

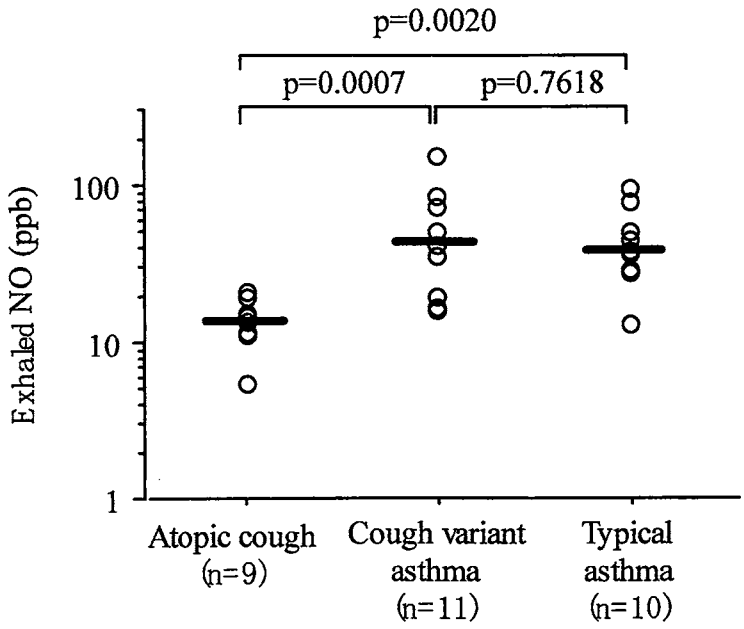

Figure 1 Exhaled nitric oxide (NO) levels in patients with atopic cough (AC), cough variant asthma (CVA) and typical bronchial asthma (BA)
Horizontal bars represent geometric mean values. P values analyzed by Fisher's PLSD are shown.

VII The effect of pressure stress to airway on cough reflex sensitivity in guinea pigs

Abstract

Rationale: We hypothesized that cough stress to airway wall makes a self-perpetuating cough-reflex cycle, in which antigen-induced increase in cough reflex sensitivity results in pathologic cough, and the cough in turn further amplifies cough reflex sensitivity.

Objectives and Methods: Our aim was to develop an experimental guinea pig model, in which airway collapse seen at coughing was induced by rapid negative pressure stress to airway of artificially ventilated animals, and to examine influence of this stress on cough reflex sensitivity to inhaled capsaicin and bronchoalveolar lavage (BAL) cell component. After the completion of artificial ventilation, number of coughs due to capsaicin was measured and then BAL was performed.

Results: Both capsaicin cough sensitivity and BAL neutrophils were increased 6h after the stress and disappeared within 24h. In animals without mechanical stress, the cough sensitivity or BAL cell components were not changed. BAL neutrophils were significantly correlated to the number of coughs. Hydroxyurea inhibited the stress-induced increase in the number of coughs and airway neutrophil accumulation.

Conclusions: Our studies suggest that cough itself is traumatic as mechanical stress to airway wall and may induce the neutrophilic airway inflammation and cough reflex hypersensitivity. Thus, the cough stress to airway wall may make a self-perpetuating cough-reflex cycle.

Introduction

Cough is one of the most common symptoms of respiratory diseases. Patients with chronic non-productive cough usually have an enhanced cough reflex sensitivity to inhaled tussive agents such as capsaicin and citric acid (1, 2). Little is known about the underlying mechanisms of the increased cough response. Based on our clinical experience, we hypothesized that cough stress to airway wall makes a self-perpetuating cough-reflex cycle, in which antigen-induced increase in cough reflex sensitivity results in pathologic cough, and the cough in turn further amplifies cough reflex sensitivity. We developed an experimental guinea pig model, in which airway collapse seen at coughing was induced by rapid negative pressure stress to airway of artificially ventilated animals and examine influence of the rapid negative pressure stress on cough reflex sensitivity to inhaled capsaicin, bronchoalveolar lavage cell components and histological changes of airway wall.

Materials and Methods

Animals

Male, albino, Hartley-strain guinea pigs (body weight 550 to 600 g), which were obtained from Sankyou Laboratory Service (Toyama, Japan), were used. They were quarantined in the Animal Research Center of Kanazawa University. All the animal procedures in this study complied with the standards set out in the Guidelines for the Care and Use of Laboratory Animals at the Takara - machi Campus of Kanazawa University.

Study design

Experimental protocol 1

We divided the animals without pretreatment with hydroxyurea (HU) into four groups as follows: normal control (NA) group, negative control (NE) group and positive control (PO10 or PO20) groups (n = 8 or 9 for each group). Guinea pigs were anesthetized by an intraperitoneal injection of 30 mg/kg of sodium pentobarbital and placed in a supine position. Animals in NA group were tracheotomized only. After the trachea was cannulated with a polyethylene tube (outside diameter; 2.5 mm, inside diameter; 2.1 mm), the animals in PO and NE groups were artificially ventilated using a small animal respirator (model 1680, Harvard Apparatus Co., Inc., South Natick, MA) adjusted to a tidal volume of 10 ml/kg at a rate of 60 strokes/min for 1 min. And then animals in PO groups were exposed to the mechanical negative pressure stress. Animals in PO10 group were exposed to negative pressure of 10 cmH_2O and animals in PO20 group were exposed to negative pressure of 20 cmH_2O. Animals in NE group were only ventilated artificially without negative pressure stress. After the ventilation, the trachea and skin were sutured, and then animals awoke from the anesthesia. Cough reflex sensitivity to inhaled capsaicin was measured 6, 12 and 24 hours after the completion of artificial ventilation and then animals were anesthetized and bronchoalveolar lavage was performed at each time. Increases in cough reflex sensitivity to inhaled capsaicin and neutrophil count in BALF were significant in PO20 group, but not in PO10 group. Therefore, we examined the time course of cough reflex sensitivity and BAL fluid examinations in PO20 group in comparison with those in NE group.

Three additional groups of guinea pigs (NA, NE and PO20) were prepared for histologic examination (n = 5 or 6).

Experimental protocol 2

Naïve guinea pigs pretreated with HU or saline (Sal) were divided to six groups: normal control with HU (NA-HU) group and with Sal (NA-Sal) group, negative control with HU (NE-HU) group and negative control with Sal (NE-Sal) group, positive control with HU (PO20-HU) group and positive control with Sal (PO20-Sal) group (n = 8 or 9 for each group). Guinea pigs pretreated with Sal were given 1.0 mL/kg of 0.15 mM NaCl daily for 5 days via intraperitoneal injection and those pretreated with HU were intraperitoneally given 1.0 mL/kg of 800 mg/mL HU solution daily for 5 days. Cough reflex sensitivity to inhaled capsaicin was measured 6 hours after the completion of artificial ventilation and then animals were anesthetized and bronchoalveolar lavage was carried out.

Experimental apparatus for mechanical negative pressure stress

Experimental apparatus for exposing mechanical negative pressure stress to airway of artificially ventilated animals is shown in Figure 1. The electromagnetic valve (model USG3-6-1-H, CKD Corporation, Aichi, Japan) opened linked with ventilation and this valve opened for 20 times during the 60 seconds artificial ventilation. This valve kept opened for 0.2 second. During the 0.2 second negative pressure caused by a large aspirator (model MERA MS-005, Izumi Koika Kogyo Co., Ltd., Tokyo, Japan) was exposed to the animals in PO groups. The changes in resistance to lung inflation, the lateral pressure of the tracheal tube

(pressure at the airway opening: Pao, cmH_2O), were measured with a pressure transducer (Model TP-603T, Nihon Koden Kogyo Co., Ltd., Tokyo, Japan). Figure 2A and Figure 2B show the examples of Pao measurement in NE and PO group.

Cough reflex sensitivity

Cough reflex sensitivity was measured 6, 12 and 24 hours after the one-minute artificial ventilation with or without exposing negative pressure stress to the airway. Each conscious guinea pig was placed in an airtight custom-built transparent plastic box consisting of a head chamber (1600 ml volume) isolated from a body chamber, and pressure in the body chamber was recorded. Coughs were detected as a change in the pressure showing a rapid inspiration followed by rapid expiration. To disregard motion- and sneezing-related changes in the pressure, movements of the guinea pigs were visually monitored. Coughs were counted by a trained observer and recognized by the characteristic animal posture and the pressure transducer recordings. Increasing concentrations of capsaicin solution (10^{-6}, 10^{-4} M) were inhaled for 2 minutes from a Devilbiss 646 nebulizer (Devilbiss Co., Somerset, PA) operated by compressed air at 1.6 l/min (Iwaki Air Pump AP-115AN, Iwaki Co., Ltd., Tokyo, Japan). The nebulizer output was 37 µl /min. The number of coughs was counted during a 2 minutes inhalation of each capsaicin solution and for additional 1 minute. The total number of coughs during the 3 - minute period was recorded on the inhalation of each concentration of capsaicin. Different animals were used for each time measurement to avoid the possibility of tachyphylaxis to repeated

inhalation of capsaicin.

Bronchoalveolar lavage (BAL)

After the measurement of cough reflex sensitivity to capsaicin, animals were anesthetized by an intraperitoneal injection of 75 mg/kg of sodium pentobarbital and placed in a supine position. After the trachea was cannulated with a polyethylene tube (outside diameter; 2.5 mm, inside diameter; 2.1 mm), through the tracheal cannula bronchoalveolar lavage was performed with 10 ml of 0.15 mM NaCl 2 times (total: 20 ml). The cells in BAL fluid were stained with Turk solution and counted in duplicate in a hemocytometer (in a Burker chamber). Differential cell counts were made on a smear prepared by cytocentrifuge and stained with Wright-Giemsa.

Time course of cough reflex sensitivity, BAL cell components and concentration of substance P in BAL fluid after mechanical stress

The analysis of BAL fluid was carried out 1, 3, 6, 12 and 24 hours after the 1-minute artificial ventilation in both animals in PO20 and NE groups (n=8 or 9 for each groups). Because animals 1 and 3 hours after the ventilation were not fully awakened from the anesthesia, only inhalation of increasing concentration of capsaicin solution to animals was performed, but number of coughs elicited by capsaicin was not measured. After the examination of cough sensitivity to capsaicin, BAL was performed. Different animals were used for each measurement to avoid possibility of tachyphylaxis to repeated inhalation of capsaicin.

The concentration of substance P in BAL fluid was measured using a commercial enzyme immunoassay (EIA) kit (Cayman Chemical Company, USA). This kit is a competitive assay that provides accurate measurements of substance P with a working range of 3.9 to 500 pg/ml.

Histological examination

Animals were exsanguinated from abdominal aorta 6 hours after the mechanical negative pressure stress under deep anesthesia. The airway and lungs of each animal were excised *en bloc* by opening the chest. After the excision, the trachea was cannulated and inflated with 10% formalin via the tracheal cannula. The airway and lungs were fixed for at least 48 hours and embedded in paraffin. The trachea and main bronchus up to 5 mm past the main carina, the lower lobes of the left lung were sectioned and stained with hematoxylin and eosin. The number of neutrophils in the epithelium of the left main bronchus was counted by a light microscope at magnification x400 and expressed as the number of cells mm^{-1} length of the airway basement membrane, which was measured with an objective micrometer.

We also counted the number of neutrophils in the airway epithelium in the cartilaginous and non- cartilaginous airways in the lower lobe of the left lung separately using Win ROOF, image analysis software (Mitani Co., Ltd., Fukui, Japan) as the number of cells/ dimension of airway mucosa mm^2.

Preparation of drugs

The following chemicals were used: sodium pentobarbital (Abbott

Laboratories, North Chicago, IL), 0.15 mM NaCl (Otsuka Pharmaceutical Co., Ltd., Osaka, Japan), capsaicin (Sigma, St. Louis, MO) and hydroxyurea (Wako Pure Chemical Ind., Osaka, Japan).

Statistical analysis

All data are shown as mean ± standard error of the mean (SEM). Statistical differences were determined by analysis of variance (ANOVA) followed by Fisher's protected test significant differences (Statview; SAS Institute, Cary, NC, USA), excluding the concentration of substance P in BAL fluid. Differences in the concentration of substance P in BAL fluid were analyzed by Mann-Whitney's *U* test. The relationship between the number of BAL neutrophils and the number of cough caused by inhaled capsaicin was analyzed using simple regression analysis. A *P* value less than 0.05 was considered statistically significant.

Results

Cough reflex sensitivity 6 hours after negative pressure stress

Figure 3 shows the number of coughs induced by inhaled capsaicin in guinea pigs. Animals in P010 group were exposed to negative pressure of 10 cmH_2O and animals in P020 group were exposed to negative pressure of 20 cmH_2O. Animals in NE group were only ventilated artificially without negative pressure stress. The number of coughs elicited by inhaled capsaicin was significantly increased in a negative pressure intensity-dependent fashion (Figure 3). No significant differences in the cough reflex sensitivity were observed between normal control (NA)

and NE groups (Figure 3).

Pretreatment with hydroxyurea (HU) significantly and completely inhibited the mechanical negative pressure stress -induced increase in the number of capsaicin-induced coughs (Figure 4).

BAL fluid analysis 6 hours after the negative pressure stress

The numbers of total cells and neutrophils and the percentage of neutrophils in BAL fluid were significantly increased in a negative pressure intensity-dependent manner (Figure 5). There was no significant difference in the percentage of neutrophils or the number of total cells or neutrophils between NE and NA groups (Figure 5). There was also significant increase in the number of macrophages in a negative pressure intensity-dependent fashion. No significant differences in the percentage of macrophages, lymphocytes or eosinophils or the number of lymphocytes or eosinophils were observed among four groups (data not shown).

Effects of HU on BAL cell counts and cell differentiation are shown in Figure 6. HU inhibited the mechanical negative pressure stress-induced increase in the number and percentage of neutrophils. And HU decreased the number of total cells and macrophages of NA, NE and P020 groups (data not shown). No significant influence of HU was observed in number of BAL lymphocytes or eosinophils, or in the percentage of macrophages, lymphocytes or eosinophils (data not shown).

Time course of cough reflex sensitivity after negative pressure stress

The time courses of cough reflex sensitivity to inhaled capsaicin after the mechanical negative pressure stress in PO20 and NE groups are shown in Figure 7. Although the number of 10^{-4} capsaicin induced-coughs was significantly increased in PO group 6 and 12 hours after the pressure stress compared with NE group, the cough number was much greater 6 hours after the stress. The increase in capsaicin-induced cough number decreased nearly to the level of NE group within 24 hours. There were no changes in the cough reflex sensitivity in NE group during the 24 hours.

Time course of BAL cell components after mechanical stress

The time courses of number of total cells and neutrophils in BAL fluid collected from PO20 and NE groups are shown in Figure 8A and Figure 8B. The time courses of percentage of neutrophils in BAL fluid collected from PO20 and NE groups are shown in Figure 8C. The number of total cells and neutrophils and the percentage of neutrophils in BAL fluid were much increased in PO20 group 6 hours after the negative pressure stress and decreased to the level of NE group within 24 hours. Neither number nor percentage of neutrophils in NE group did change during the 24-hour period.

Significant increase in number of macrophages was observed in PO20 group 6 hours after the negative pressure stress compared with NE group. There were no significant differences in the number of lymphocytes and eosinophils between these two groups during the 24-hour period. The percentage of macrophages, lymphocytes or eosinophils was not significantly different between the two groups during the period (data

not shown).

Time course of concentration of substance P in BAL fluid after mechanical negative pressure stress

The time courses of the concentration of substance P in BAL fluid collected from PO20, NE and NA groups are shown in Figure 9. Significant increase of substance P concentration in BAL fluid was observed 6 hours after the pressure stress in PO20 group. There were no significant differences in the concentration of substance P between NE and NO groups during 24 hours following the surgical procedure.

Relationship between number of coughs induced by inhaled capsaicin and absolute number of BAL neutrophils

The BAL neutrophil number was significantly correlated to the number of coughs caused by both the low and the high concentration of capsaicin (Figure 10).

Histology

The number of neutrophils in the epithelium is shown in Table 1. The number of neutrophils in the epithelium collected from PO20 group was significantly greater than those from NA and NE groups.

Discussion

The present study investigated whether mechanical negative pressure stress to the airway could influence cough reflex sensitivity to capsaicin, and could induce airway inflammation. The mechanical stress to airway enhanced cough reflex sensitivity to capsaicin, which was

accompanied by airway neutrophil accumulation. In addition, BAL neutrophil number was significantly correlated to the number of coughs caused by inhaled capsaicin. Furthermore, HU completely inhibited the mechanical stress -induced increase in the cough reflex sensitivity and airway neutrophil accumulation. We failed to investigate the relationship between neutrophil counts in bronchial tissue and cough reflex sensitivity to inhaled capsaicin in the present study.

In studies on cough, many investigators have devised system to induce cough. In animal studies, the inhalation of citric acid or capsaicin has been often used to evaluate the cough reflex sensitivity. Cough and bronchoconstriction are distinct, but interrelated reflexes. It is speculated that inhalation of higher concentrations of capsaicin solution more than 10^{-4} M, when delivered at high flow rate, may cause bronchoconstriction as well as coughing (3) (4). The number and intensity of coughs elicited by aerosolized 10^{-4} M capsaicin in naïve guinea pigs are considered to be smaller and weaker as compared with those of cough attacks in patients with chronic cough. Therefore, we developed the present experimental guinea pig model, in which cough like reaction was induced by mechanical negative pressure stress. In experimental animals, the pattern of cough varies depending on which part of the respiratory tract is stimulated (5). It is considered that cough consists of forced rapid expiration after deep inspiration (6, 7). The bronchi and intra-thoracic trachea are compressed and narrowed during forced rapid expiration of coughing. The narrowing results from a transmural pressure gradient between the extraluminal and the intraluminal pressure (7). Immediately before the start of expiration

phase of coughing when larynx is closed, both extraluminal and intraluminal pressure of trachea and bronchi rises to positive, and then in expiration phase of coughing when the larynx is opened, intraluminal pressure is suddenly decreased to the ambient pressure, resulting in the huge transmural pressure to compress tracheal and bronchial mucosa. In our experimental model, sudden negative pressure was exposed to the airway to simulate the expiratory phase of coughing after positive airway pressure. This simulation method has been used by several research groups (8-10).

In human, it has been reported that patients with non-asthmatic dry cough and idiopathic cough (11) and those with chronic cough (12, 13) have a significantly higher proportion of neutrophils in induced sputum than normal controls. Those patients also have cough hypersensitivity to capsaicin compared with normal controls (14). Reversely, Niimi *et al.* reported that submucosal neutrophils were not correlated to the cough reflex sensitivity in non-asthmatic cough (15). Although it is unknown about the interaction of cough reflex sensitivity and neutrophilic inflammation, we speculate that the act of mechanical stress, that is, repeated cough itself could induce the neutrophilic airway inflammation. It was demonstrated that cyclic stretch of airway induced several cytokines and chemokines, macrophage inflammatory protein (MIP)-2 in BALF from rats (16), MIP-2, IL-6 and tumor necrosis factor (TNF)-alpha in isolated perfused lungs from mice (17), TNF-alpha, IL-1-beta, IL-6, MIP-2, and IFN-gamma in isolated lung from rats (18), and IL-8 from human bronchial epithelial cells (19, 20). It has also been shown that positive end-expiratory pressure (PEEP) causes

neutrophil adhesion and recruitment mediated by P-selectin, endothelin and intercellular adhesion molecule (ICAM)-1 in the tracheal vasculature from rats and mice (21, 22). TNF-alpha and IL-8 were significantly increased in induced sputum of non-asthmatic patients with chronic dry cough (11). Nightingale *et al.* reported that the percentage of neutrophils in induced sputum of healthy subjects rose at 8 and 24 hours compared with the baseline value (23). Coughing, hypertonic saline and/or sputum itself might cause the increase in neutrophils in induced sputum. Jatakanon *et al.* speculated that the act of coughing itself may contribute to induction of release of proinflammatory cytokines such as TNF-alpha and IL-8 based on their findings that neutrophilia in induced sputum was found in all causes of cough (11). It is unknown whether inflammatory cytokines, such as TNF-alpha and IL-8, have influence on the cough reflex sensitivity. It is likely that other mediators, such as prostaglandins and bradykinin, produced directly through the action of these cytokines could enhance the cough reflex sensitivity. In our study, cough reflex sensitivity remained increased although BALF neutrophils were back to normal after 12 and 24 hours. The discrepancy of time course between increase in cough reflex sensitivity and BALF neutrophilia suggested that cytokines, chemokines and chemical mediators produced from neutrophils, but not accumulated neutrophils per se, could increase the cough reflex sensitivity.

HU is representative of a group of compounds that inhibit the enzyme ribonucleoside diphosphate reductase and are specific for the S phase of the cell cycle. HU has been employed in several numbers of studies

on circulating leukocyte-depleted animals (24-26). Thompson JE and coworkers reported that HU did not decrease the neutrophils in the lamina propria of trachea (24). O' Byrne and coworkers reported that HU tended to decrease the neutrophils in airway epithelium, but not neutrophils in BALF (26).Our BALF findings of animals treated with HU are consistent with those reported. HU inhibited both of the mechanical stress -induced increase in the cough reflex sensitivity and airway neutrophil accumulation. BAL neutrophil number was significantly correlated to the number of coughs caused by inhaled capsaicin. As the direct influence of HU on the cough reflex sensitivity is neglected based on our results in the control groups, there may be possibility that neutrophilic airway inflammation can develop increase in cough reflex sensitivity.

Previous reports demonstrated that an inactivation of neutral endopeptidase (NEP) was caused by airway epithelium damage (27-29). In the airway, NEP is recognized as the major enzyme degrading substance P (30). In this respect, the inactivation of NEP activity may cause the cough reflex hypersensitivity to capsaicin in our guinea pig model. Substance P has been demonstrated to induce the chemotaxis of human neutrophils (31), to release IL-8 from human polymorphonuclear leukocytes and to release TNF-alpha from human monocytes (32). Furthermore, non-asthmatic patients with sputum neutrophilia have high level of substance P (33). Therefore, in our guinea pig model, substance P may play an important role in release of cytokines and accumulation of neutrophils into the airway.

The receptor for capsaicin, termed vanilloid receptor-1 (VR-1), is

expressed in guinea pigs. VR-1 mediates cough induced by capsaicin (34). An increased expression of VR-1 has also been reported in humans with chronic cough (35). Therefore, in the guinea pig airways exposed to mechanical negative pressure stress, the epithelial damage might induce an increase in the number of VR-1 contributing to the enhanced cough reflex sensitivity. This possibility should be examined in future studies.

Vagal afferent nerves are responsible for initiating cough reflex. Afferent neuronal subtypes are identified based on their physicochemical sensitivity, adaptation to sustained lung inflation, neurochemistry, origin, myelination, conductive velocity, and sites of termination in the airways, that is, C fiber, rapidly-adapting receptors (RARs) and slowly adapting stretch receptors (SARs) (36). RARs are activated by the dynamic mechanical forces accompanying lung inflation and deflation, and become more active as the rate and volume of lung inflation increase (37-39). It is considered that RAR activity is higher in guinea pigs, rats, and newborns of all species (36).

The present study demonstrated that mechanical negative pressure stress to the airway induced increase in the cough reflex sensitivity at 6 hour after the stress. It is unknown whether the natural or induced cough could induce cough hypersensitivity after 6 hours similar to our present study. Induced cough is considered to be weaker and less frequent compared with mechanical stress. The natural cough is considered to be a strong stress to the airway. Generally, the natural cough is accompanied with airway inflammation. There is no method of suppressing only the cough without suppressing the inflammation.

Therefore, we cannot investigate the change of cough reflex sensitivity between the subjects having airway inflammation with natural cough and those without natural cough. We also cannot investigate the continuous change of cough reflex sensitivity in healthy subject with extremely strong induced cough causing the muscle pain, incontinence of urine or rib fracture.

Both of the natural cough and induced cough continue in some period and disappeared. This principle of cough disappearance is uncertain as well as the sneeze. We demonstrated that mechanical stress to the airway induced increase in the neutrophilic airway inflammation and the cough reflex sensitivity. It is an interesting question whether the neutrophilic airway inflammation and cough reflex sensitivity reinforce further or weaken when mechanical stimulation is repeated, but it is not possible to examine this question in our experimental model.

In conclusion, the present study clearly demonstrated that mechanical negative pressure stress exposed to airway, which simulated cough attack, induced neutrophilic airway inflammation resulting in increase in cough reflex sensitivity in guinea pigs. It is possible that the mechanical stress to airway may make a self-perpetuating cough-reflex cycle. To elucidate the possibility influence of repeated mechanical stress to airway should be examined in future studies.

Acknowledgements

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Table 1
Neutrophil number in bronchial mucosa (number / mm^2 epithelium)

	Left main bronchus	Cartilaginous bronchus	Non-cartilaginous bronchus
	Number of neutrophils (number /mm epithelium)	Number of neutrophils (number / mm^2 epithelium)	Number of neutrophils (number / mm^2 epithelium)
Normal control	0.4±0.25	0±0	0±0
Negative control (Ventilation alone)	1.6±0.72	0±0	0±0
Positive control20 (Ventilation and negative pressure stress)	18±6.3**	7.9±3.3*	10.0±4.0*

**$P < 0.01$ compared with negative control group. *$P < 0.05$ compared with negative control group, n = 5 or 6 in each group.

Figure 1

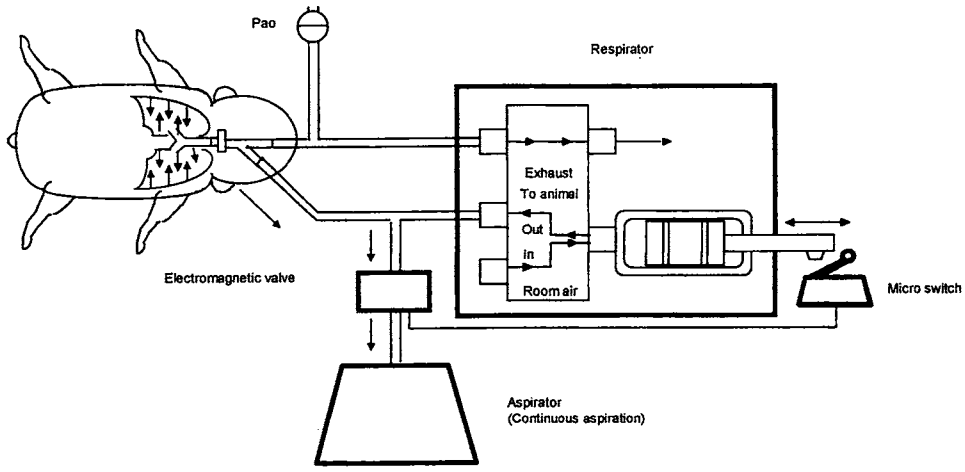

Figure 1 Experimental apparatus for loading mechanical negative pressure stress to airway wall of artificially ventilated animals

The electromagnetic valve opened linked with ventilation and this valve opened 20 times during the 60-second artificial ventilation. This valve kept opened for 0.2 second. During the 0.2-second period negative pressure caused by a large aspirator was exposed to the airway in PO group. The changes in resistance to lung inflation, the lateral pressure of the tracheal tube (pressure at the airway opening: Pao, cmH2O), were measured with a pressure transducer.

Figure 2A

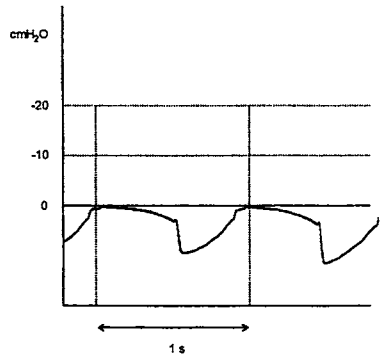

Figure 2B

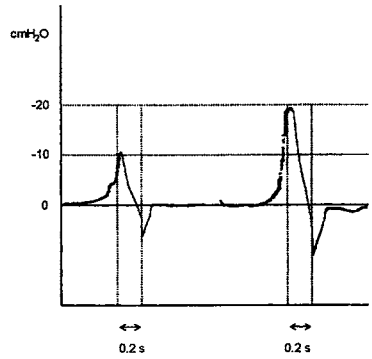

Figure 2 Examples for change in Pao in guinea pigs exposed (PO group) (B) and not exposed (NE group) (A) to mechanical negative pressure stress

Figure 3

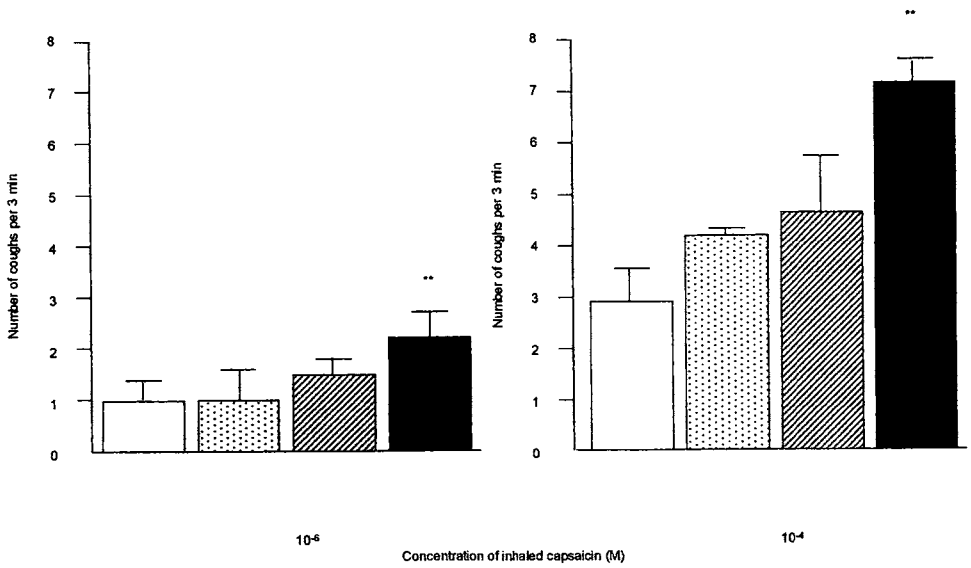

Figure 3
Cough reflex sensitivity to inhaled capsaicin 6 hours after the artificial ventilation with or without negative pressure stress in guinea pigs.
Open bars; normal control group (only tracheotomy), dotted bars; negative control group (tracheotomy and ventilation), hatched bars; positive control 10 group (exposed to −10 cmH2O mechanical negative pressure stress), solid bars; positive control 20 group (exposed to −20 cmH2O mechanical negative pressure stress). $**P < 0.01$ compared with negative control group. n = 8 or 9 in each group.

Figure 4

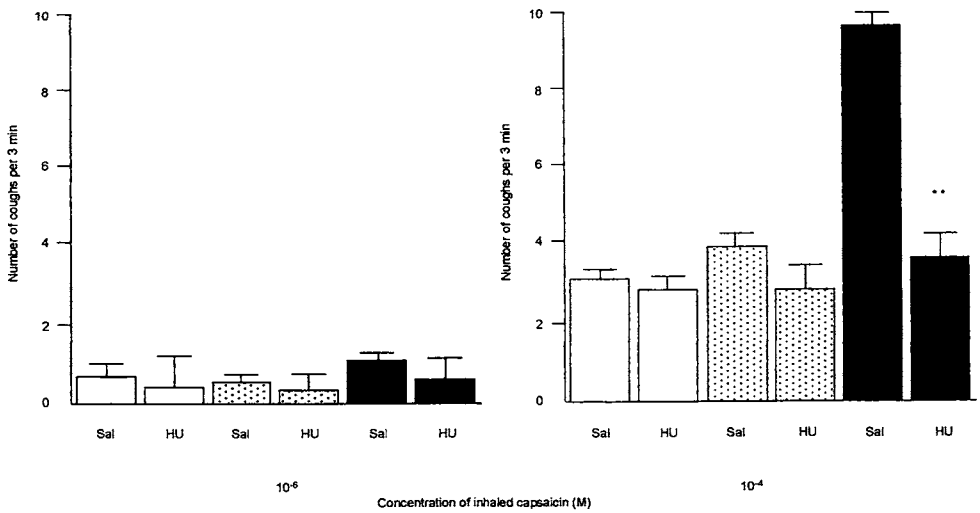

Figure 4
Cough reflex sensitivity to inhaled capsaicin 6 hours after the artificial ventilation with negative pressure stress in guinea pigs pretreated with 0.15 mM NaCl (Sal) or hydroxyurea (HU).
Open bars; normal control group (only tracheotomy), dotted bars; negative control group (tracheotomy and ventilation), solid bars; positive control 20 group (exposed to −20 cmH2O mechanical negative pressure stress). $**P < 0.01$ compared with positive control 20 group pretreated with 0.15 mM NaCl. n = 8 or 9 in each group.

Figure 5

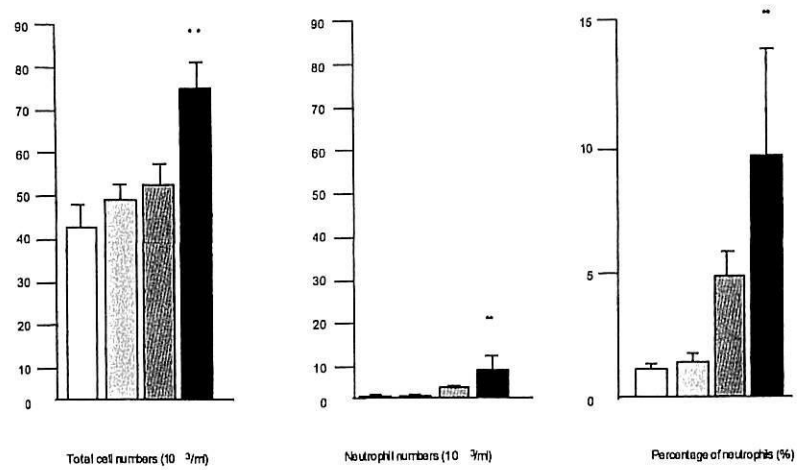

Figure 5
Bronchoalveolar lavage cell findings 6 hours after the artificial ventilation with or without negative pressure stress
Open bars; normal control group (only tracheotomy), dotted bars; negative control group (tracheotomy and ventilation), hatched bars; positive control 10 group (exposed to −10 cmH2O mechanical negative pressure stress), solid bars; positive control 20 group (exposed to −20 cmH2O mechanical negative pressure stress). $**P < 0.01$ compared with negative control group. n = 8 or 9 in each group.

Figure 6

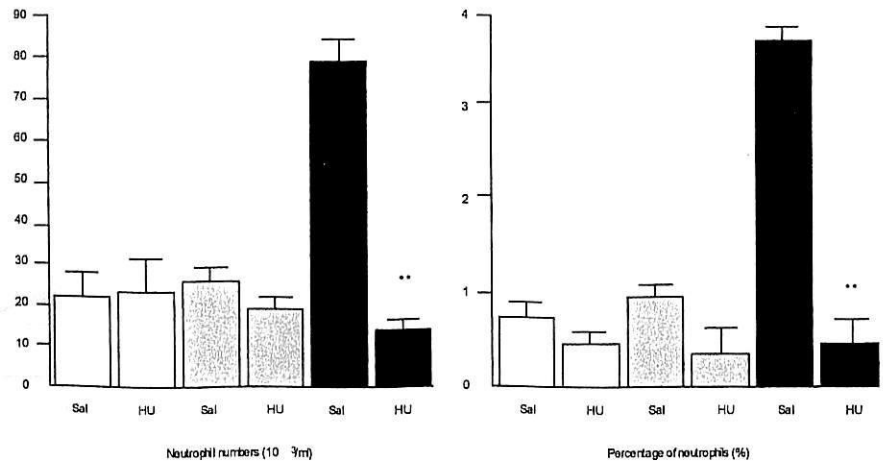

Figure 6
Bronchoalveolar lavage cell findings 6 hours after the artificial ventilation with negative pressure stress in guinea pigs pretreated with 0.15 mM NaCl (Sal) or hydroxyurea (HU).
Open bars; normal control group (only tracheotomy), dotted bars; negative control group (tracheotomy and ventilation), solid bars; positive control 20 group (exposed to −20 cmH2O mechanical negative pressure stress). $**P < 0.01$ compared with positive control 20 group pretreated with 0.15 mM NaCl, n = 8 or 9 in each group.

Figure 7

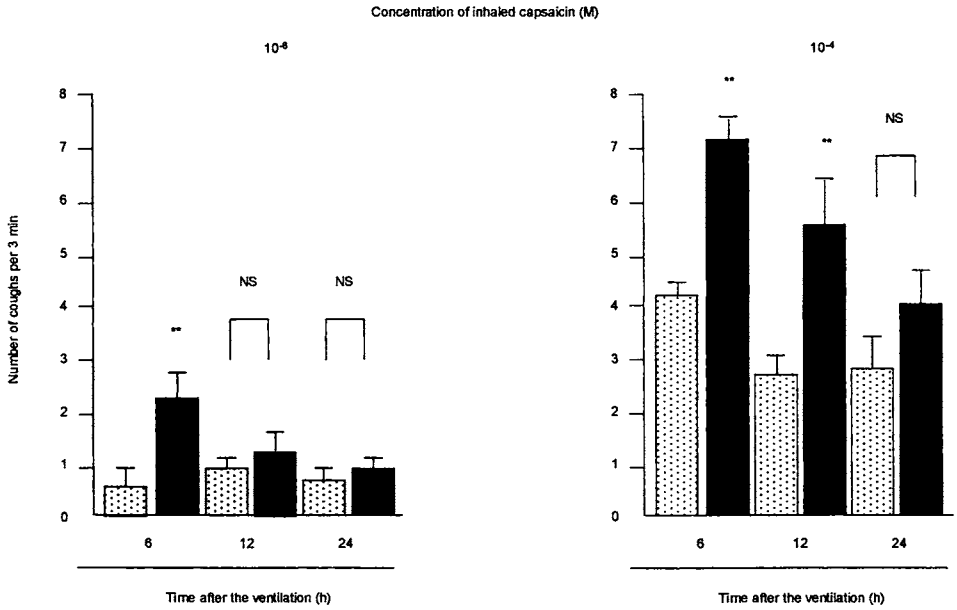

Figure 7
Time course of cough reflex sensitivity to inhaled capsaicin after the artificial ventilation with or without negative pressure stress
Dotted bars; negative control group (tracheotomy and ventilation), solid bars; positive control 20 group (exposed to −20 cmH2O mechanical negative pressure stress). $**P < 0.01$ compared with negative control group at each times. n = 8 or 9 in each group.

Figure 8A

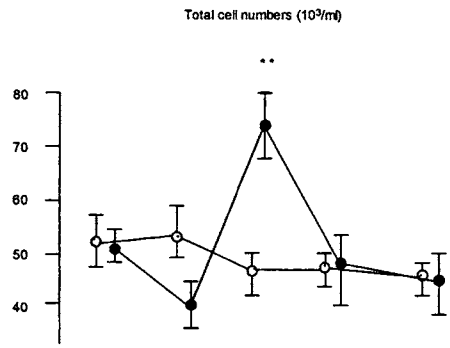

Figure 8B

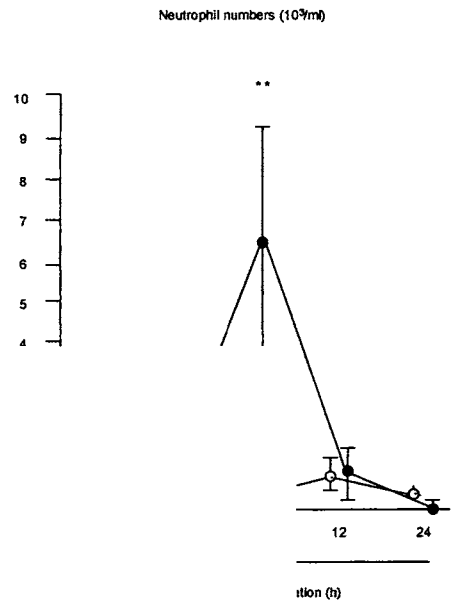

Figure 8C

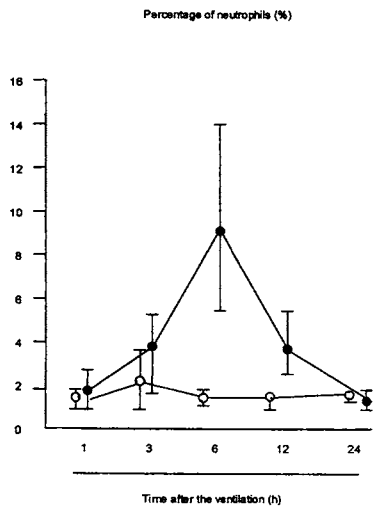

Figure 8
Time course of numbers of total cells (Figure 8A) and neutrophils (Figure 8B) and percentage of neutrophils (Figure 8C) in bronchoalveolar lavage (BAL) fluid
Dotted circles; negative control group (tracheotomy and ventilation), solid circles; positive control 20 group (exposed to −20 cmH2O mechanical negative pressure stress). $**P < 0.01$ compared with negative control group at each times, n = 8 or 9 in each group.

Figure 9

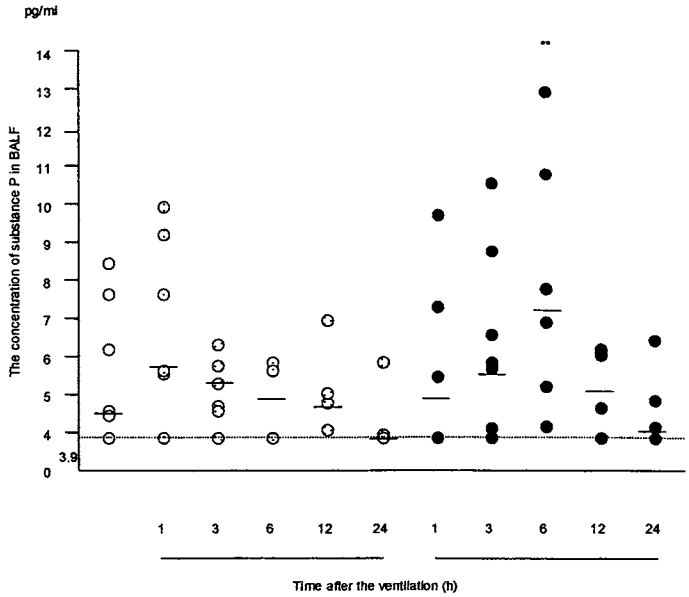

Figure 9
Time course of concentration of substance P in bronchoalveolar lavage (BAL) fluid.
Open circle; normal control, dotted circles; negative control group (tracheotomy and ventilation), solid circles; positive control 20 group (exposed to −20 cmH2O mechanical negative pressure stress). Horizontal bars indicate the median values of each group. $**P < 0.01$ compared with negative control group at each times, n = 5 to 8 in each group.

Figure 10

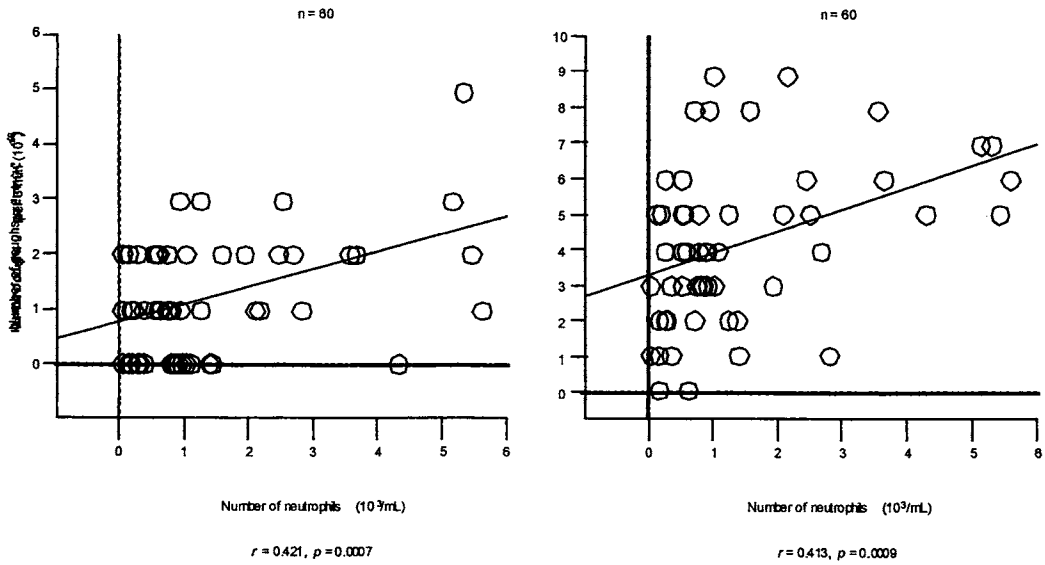

Figure 10
Relationship between number of BAL neutrophils and number of coughs elicited by inhaled capsaicin
r; Spearman's rank correlation coefficient