Ⅳ. 研究成果の刊行物・別刷

講　座　ピットフォール

アレルギー性気管支肺アスペルギルス症

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要　旨　アレルギー性気管支肺アスペルギルス症（ABPA）は，アトピー素因を有する患者において，*Aspergillus fumigatus* を原因として生じるアレルギー性呼吸器疾患である。末梢血好酸球増多を伴い，肺に浸潤影または/および中枢性気管支拡張像を認める症例において，血清総IgE値が高ければABPAを疑うべきである。固形成分を有する痰の喀出の既往をきき出すことは重要である。診断に際しては，気管支喘息を合併しない症例，肺癌や肺結核との鑑別を要する症例，感染病型を合併する症例，*Af* 以外を原因とするアレルギー性気管支肺真菌症などの非典型例の存在に注意する。治療の中心は全身性ステロイド投与であり，不可逆的な肺の破壊が進行する前に早期に投与することが重要である。

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キーワード：真菌　喘息　アレルギー性気管支肺真菌症

Ⅰ．はじめに

地球上にはいまだ同定されていないものも含めて，無数の真菌が存在し，人間の気道には呼吸により毎日多くの真菌が吸入されている。これらの真菌のなかでも，特に *Aspergillus* は，呼吸器疾患の原因真菌として重要視されている。正常の免疫能を有する健常人では気道に到達した *Aspergillus* は容易に排除されるが，免疫能に問題がある場合は様々な呼吸器疾患が発生する可能性がある。免疫不全状態の患者においては感染症である菌球型アスペルギルス症や慢性壊死性肺アスペルギルス症，侵襲性肺アスペルギルス症などが発症する。一方，アトピー素因を有する喘息患者において発症するのが，アレルギー性呼吸器疾患のアレルギー性気管支肺アスペルギルス症（allergic bronchopulmonary aspergillosis：ABPA）である。*Aspergillus* にも多くの種類が存在するが，なかでも *Aspergillus fumigatus*（*Af*）が本症の原因となることが最も多いとされてきた。ABPAは欧米においてはアレルギーの診療において忘れてはならない疾患とされており，わが国においても疾患概念の普及に伴い近年報告症例が増加している。以前，本誌『呼吸』において典型的なABPA症例を中心に概説したので[1]，今回はそれを一部引用しながら，診断のピットフォールに陥りがちな非典型的な症例についても述べてみたい。

Ⅱ．病態と発症機序

本症の発症機序に関しては，従来より以下のような説が考えられてきた。喘息患者に特有の粘稠な喀痰と閉塞した気道中に *Af* が定着，増殖し，患者がアトピー素因を有するため *Af* 特異的IgE抗体が産生されⅠ型アレルギー機序により喘息が増悪する。またIgG抗体も産生され，Ⅲ型，一部Ⅳ型アレルギーも関与し，組織破壊が特に菌糸が充満しやすい中枢気道に起こるため，末梢気管支が正常の中枢性気管支拡張が発症する。拡張した気管支の末梢では中枢

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部に定着した*Af*が吸気の陰圧によって末梢肺野に到達し，そこで好酸球性肺炎による浸潤影を呈する。しかし，環境中に普遍的に存在し，健常人の気道からは容易に排除される*Af*が，なぜ一部の喘息患者の気道にだけ定着するのかはいまだに完全には解明されていない。

Ⅲ．疫　学

真菌はいたる所に存在する普遍的な吸入アレルゲンであり，環境中の定量・同定が困難なため，真菌関連アレルギー疾患の正確な頻度は明らかではないが，欧米においては，全人口の約1％が真菌の吸入により呼吸器症状を呈すると考えられている。ABPA に限っていえば，喘息患者全体の1から2％を占め，発作を繰り返す重症喘息に限るとその頻度はさらに増加することが報告されている[2]。

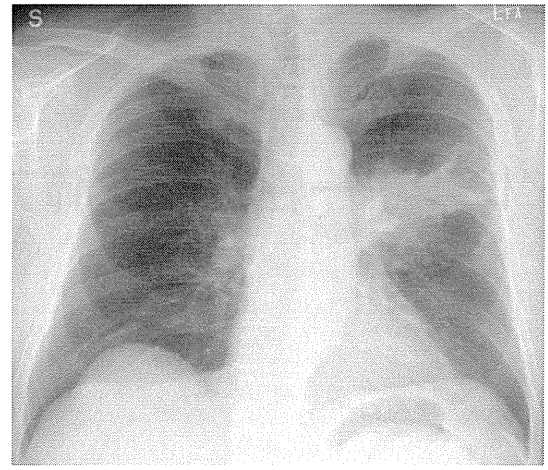


図1　ABPA 患者の胸部 X 線像
左肺門から連続する粘液栓による無気肺像(mucoid impaction)が認められる。

Ⅳ．臨 床 像

1．症　状

発症に男女差はなく，好発年齢は30～40代である。頻度の高い症状としては，38度を超えない微熱，喘鳴，咳嗽，喀痰などがあり，血痰を伴うこともある。粘液栓子の喀出は，注意して問診すればかなりの頻度で認められ，本症を示唆する重要な症状である。患者には，“明らかに固形物と認識できる成分を有する喀痰”というきき方をするとよい。

2．検査成績

検査所見としては後述する診断基準に挙げられているように，末梢血好酸球増加，血清総 IgE 値の増加，*Af*に対するⅠ型・Ⅲ型アレルギー陽性反応(即時型・Arthus 型皮膚反応，特異的 IgE 抗体や沈降抗体)，喀痰からの*Af*培養などの結果が得られるが，これらの検査の陽性率はステロイド治療の有無や病期によって異なるため，各検査項目の病期別の陽性率をよく理解しておく必要がある。

3．画像診断

喘息のみでは胸部 X 線で肺野の異常陰影は原則として認められず，喘息患者の胸部 X 線で異常陰影を認めた場合には先ず ABPA を念頭に置く必要がある。ABPA の胸部異常陰影は，中枢性気管支拡張とそこに充満する粘液栓による mucoid impaction，および末梢の好酸球性肺炎からなる。胸部 X 線では，肺門側から連続する練り歯磨き状，グローブ状といわれる mucoid impaction の像が認められ，肺野には移動する好酸球性肺炎による浸潤影が認められることがある(**図1**)。胸部 CT は中枢性気管支拡張の診断に有用であり，縦隔条件では高濃度領域を有する粘液栓(high attenuation mucoid impaction)が認められることがある(**図2**)。喘息の状態が許せば，気管支鏡も積極的に行うべき検査であり，区域気管支以下を閉塞する粘液栓子が肉眼的に観察可能で(**図3**)，同部からの気管支洗浄で，好酸球，シャルコーライデン結晶，菌糸が確認できれば診断的価値は高い。

Ⅴ．診断におけるピットフォール

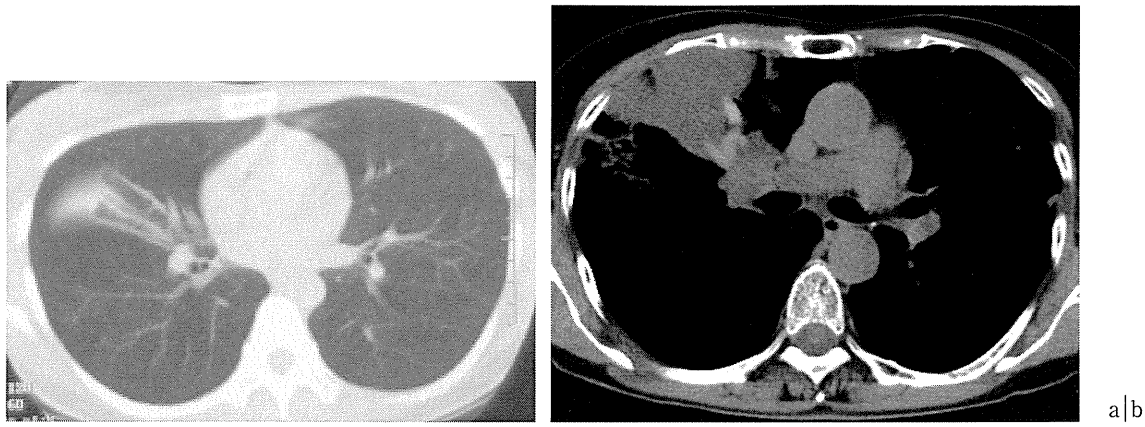
表1にわが国で頻用される Rosenberg-Patterson による ABPA の診断基準[3]を示す。この診断基準によって確定診断される典型例の診断は必ずしも困難ではないが，そのような症例は決して多くはない。ここでは診断のピットフォールに陥る可能性のある幾つかの非典型例を提示する。

1．喘息非合併例

図4の症例は，喘息の既往を有さず，胸痛のために撮影された胸部 X 線で舌区の無気肺を指摘され，気管支造影で中枢性気管支拡張が認められた。本例は，気管支喘息以外のすべての診断基準を満たす ABPA の症例であったが，喘息症状がないのみでなく，気道過敏性も認められなかった。喘息を合併しない ABPA は従来から認識されており，喘息の存在は ABPA を強く疑う所見ではあるが必須ではないことを理解すべきである。

2．肺癌や肺結核との鑑別を要する症例

機序は不明であるが，mucoid impaction を有する ABPA では血清中の CEA が上昇することがある。**図5**の症例は多発する肺の結節影と高 CEA 血症のため当初肺癌を疑われたが，ステロイド治療により陰影の改善と血清 CEA 値の低下が認められた。肺癌以外にも本症は，陰影が上葉優位で，時に空洞を合併するため，肺結核と誤診さ



a|b

図2 ABPA 患者の胸部 CT 像
a：肺野条件で右肺に末梢が正常の中枢性気管支拡張がみられる。
b：別の症例の胸部 CT 縦隔条件像(造影なし)であるが，無気肺像のなかに内部に高吸収域を伴う粘液栓が確認できる。

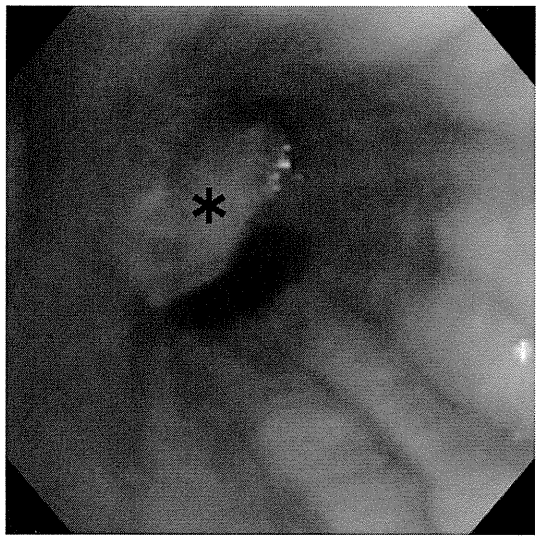


図3 ABPA 患者の気管支鏡所見
気管支鏡で区域気管支の入口部に粘液栓(＊)が認められる。

れやすいことが知られている。

3．感染病型の合併例

図6は重症喘息でフォロー中に胸部異常陰影を呈した症例である。胸部 CT では上肺野の空洞内に菌球型アスペルギルス症がみられ，下肺野には中枢性気管支拡張と浸潤影からなる ABPA の所見が認められる。このように同一症例内にアレルギー型と感染型の病型が合併することがある。*Aspergillus* 以外の肺感染症を合併することもあり，**図7**は ABPA にびまん性汎細気管支炎を合併した症例で，ABPA の所見に加えて全肺野に小葉中心性の粒状影が認められる。喀痰からは *Af* に加えて緑膿菌が持続的に検出され，ABPA の治療にマクロライドの併用が有効であった。

4．*Af* 以外の真菌によるアレルギー性気管支肺真菌症

ABPA は喀痰培養を行わなくても，血清学的，放射線学的に診断できるため，気道中の *Af* の存在なしに診断されることも多い。一方，血清学的，放射線学的には典型的な ABPA であっても，喀痰から *Af* 以外の真菌が検出されることがあり，これらはアレルギー性気管支肺真菌症(allergic bronchopulmonary mycosis：ABPM)と総称される。当科では臨床的に ABPA が強く疑われる症例に対しては，積極的に気管支鏡を行って粘液栓子を採取し，検鏡に加えて遺伝子診断も使って原因真菌の培養と同定を行ってきた。**表2**は，血清学的，放射線学的に ABPA と診断された症例の気道中の定着真菌と皮膚テストまたは/および血清 IgE 測定により同定された感作真菌を示している。全例がアスペルギルスに感作されていたが，他の真菌にも同時に感作されており，気道中の定着真菌と感作真菌は解離していた。定着真菌として最も多いのは *Aspergillus* 属であったが，*Af* よりも他の *Aspergillus* の頻度が高く，担子菌である *Schizophillum commune*(スエヒロタケ)も高率に同定された[4]。喀痰培養以外の Rosenberg 診断基準を満たす典型的と思われる ABPA 症例のなかに *Af* 以外の真菌や担子菌を原因とする ABPM が含まれている可能性があり，これは治療薬として抗真菌薬の併用を考える場合に重要である。

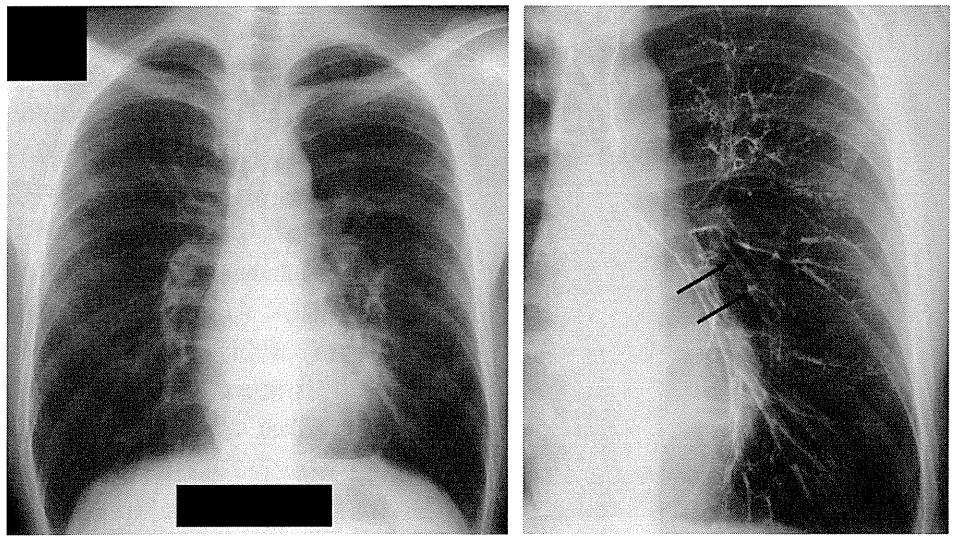
5．実臨床における ABPA 診断のポイント

末梢血好酸球増多を伴い，肺に浸潤影または/および中枢性気管支拡張像を認める症例において，血清総 IgE 値が高ければ ABPA を疑うべきである。その際，気管支喘息の存在は ABPA を強く示唆するが診断に必須ではない。固形成分を有する痰の喀出の既往をきき出すことは重要で

表1　ABPA 診断基準(Rosenberg-Patterson)

主要所見
1．発作性の気管支の閉塞(喘息)
2．末梢血好酸球増多
3．アスペルギルス抗原に対する即時型皮膚反応陽性
4．アスペルギルス抗原に対する沈降抗体の存在
5．血清 IgE 値上昇
6．(胸部 X 線上)肺浸潤影の既往(一過性もしくは固定性)
7．中枢性の気管支拡張症
二次的所見
1．喀痰中に *Aspergillus fumigatus* を証明
(培養を繰り返し行うか，あるいは顕微鏡検査による)
2．褐色の粘液栓あるいは喀痰を喀出した既往症
3．アスペルギルス抗原に対する Arthus 型皮膚反応陽性

主要所見の 6 つを満たせばほぼ確実，7 つすべてを満たせば確実。さらに 2 次的所見を満たせば確実性が増す。



a|b

図4　気管支喘息を伴わない ABPA
胸部 X 線正面像では舌区の無気肺が認められ(a)，気管支造影では中枢性気管支拡張像が確認された(b，矢印)。

ある。確定診断に近づくためには，喀痰真菌培養，各種真菌に対する血清特異的 IgE 抗体および皮膚反応，*Af* に対する沈降抗体測定を行い，喀痰中に同定された真菌に対して免疫学的反応が陽性であれば診断が確定する。しかし，実際には真菌が培養されても同定困難であることも多く，その際には原因真菌に対する免疫学的な検査は行えない。逆にある真菌に対して免疫反応が陽性であってもその真菌が喀痰から培養できるとは限らない。本症は基本的には予後良好な疾患であるが，治療の遅れにより不可逆的な肺機能異常が進行するため，確診にいたることができない場合でも，臨床的に ABPA と診断すれば現状では副腎皮質ステロイド薬の全身投与を開始し，自覚症状，胸部 X 線所見などの反応をみるべきである。

Ⅵ．治　　療

ABPA は本来，*Af* を原因とするアレルギー性呼吸器疾患であり，その治療の基本は抗真菌薬ではなく，全身性のステロイドホルモン投与である。通常，プレドニゾロン換算で 0.5 mg/kg/日の経口ステロイドを漸減投与する。漸減中に，喀痰の増加を伴う咳嗽，喘鳴，呼吸困難の増悪，肺機能(ピークフロー値)の低下，血清総 IgE 値の増加，胸部画像診断により新たな陰影が出現した場合には急性増悪を疑い，ステロイドを増量する。多くの症例がこの治療に

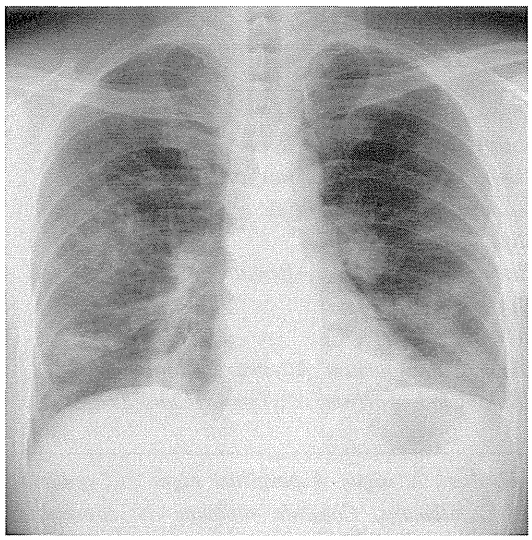
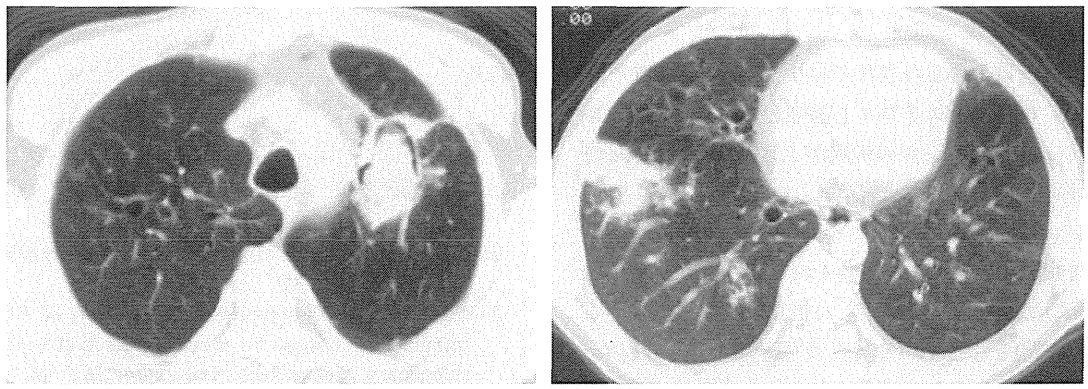


図5 高CEA血症を呈したABPA
胸部X線正面像で多発性結節影を認め，血清CEAが24.6 ng/m*l*であったため当初肺癌が疑われた。ステロイド投与により陰影の改善とともにCEAは1.9 ng/m*l*まで低下した。



a|b

図6 菌球型アスペルギルス症を合併したABPA
胸部CTで左上肺野の空洞内に菌球(a)，下肺野にABPAの像が認められる(b)。

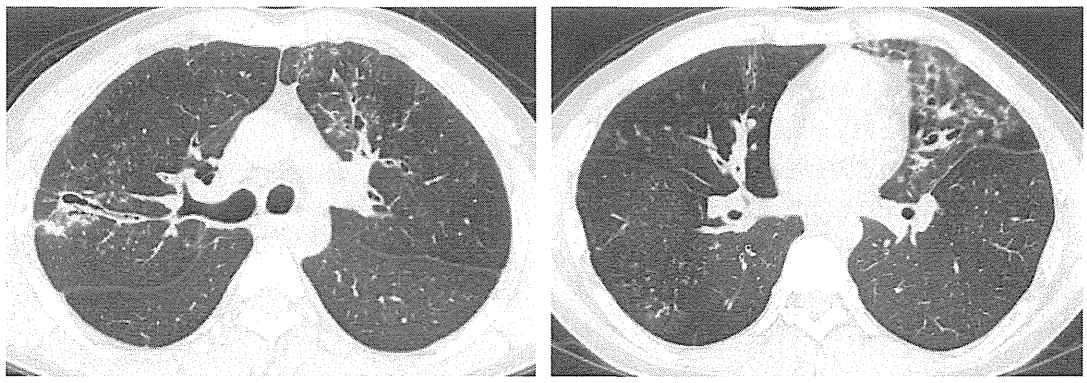


図7 びまん性汎細気管支炎を合併したABPA
胸部CTではABPAの像に加えて小葉中心性の粒状影が認められる。喀痰からは*Af*に加えて緑膿菌が培養され，ステロイドとマクロライドの併用により改善した。

反応し，生命予後は良好であるが，なかには糖尿病などの合併症のためにステロイドの全身投与ができない症例や，再発を繰り返し，ステロイドの減量ができない症例も経験される。最近本症の病態形成にIgE抗体が深く関与する点に着目し，抗IgE抗体投与が有効であった症例が報告されており興味深い[5]。本性の治療にどの時点から抗真菌薬を併用すべきかどうかの結論は出ていない。ステロイド依存性難治性ABPAの治療にステロイドと抗真菌薬の併

表2　ABPA患者の気道定着真菌と感作真菌の比較

No	定着真菌	感作真菌
1	*A. niger, C. albicans*	*Asp., Penicillium, Alternaria, Candida*
2	*S. commune, A. sydowii*	*Asp., Penicillium, Cladosporium, Alternaria, Candida*
3	*A. fumigatus, A. niger*	*Asp., Penicillium, Cladosporium, Alternaria, Candida*
4	*S. commune*	*Asp., Penicillium, Alternaria, Candida*
5	*Penicillium spp.*	*Asp., Penicillium*
6	*A. fumigatus, Rhizopus oryzae*	*Asp., Penicillium, Cladosporium, Alternaria, Candida, Mucor*
7	*A. terreus*	*Asp., Penicillium*
8	*A. niger*	*Asp.*
9	*S. commune*	*Asp., Penicillium, Cladosporium, Alternaria, Candida*
10	*C. albicans*	*Asp., Penicillium, Cladosporium, Alternaria, Candida*
11	*A. terreus, S. commune*	*Asp.*

略号　*Asp. Aspergillus*；*A. fumigatus, Aspergillus fumigatus*；*A. niger, Aspergillus niger*；*A. sydowii, Aspergillus sydowii*；*A. terreus, Aspergillus terreus*；*C. albicans, Candida albicans*；*S. commune, Schizophyllum commune.*

(Matsuse H, *et al*[4]. *Ann Allergy Asthma immunol*　111(3)：2013より引用)

用を行った報告は幾つかなされており，最近のメタアナライシスでは，標準的なステロイド治療とアゾール系抗真菌薬であるイトラコナゾールの併用がABPAの一部の症例に有効である可能性があると結論づけられている[6]。一方で，臨床的にABPAと診断される症例のなかに，先述した*Af*以外が原因のABPMが含まれる可能性があり，原因真菌の同定なしに*Af*を想定した抗真菌薬を併用することは，近年問題となっている抗真菌薬耐性の*Aspergillus*を助長する可能性もあり，真菌培養を行わずに，はじめから抗真菌薬を投与する姿勢には疑問が残る。

Ⅶ. 結　　語

ABPAは，決して頻度の高い疾患ではないが，診断の遅れが不可逆的な肺機能障害を招く早期診断が重要な疾患である。臨床医は，アレルギー素因を有する胸部異常陰影の患者を診療する場合にはABPAの可能性を念頭に置く必要がある。ステロイド以外の治療薬の意義については，欧米における投与期間が短い臨床研究が主体の議論となっており，今後わが国における大規模な臨床研究の成果が待たれる。

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Dissociation between sensitizing and colonizing fungi in patients with allergic bronchopulmonary aspergillosis

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ABSTRACT

Background: Because allergic bronchopulmonary aspergillosis (ABPA) does not require the presence of *Aspergillus fumigatus* for diagnosis, serological and radiological findings without cultures usually confirm this condition.

Objective: To determine which fungi colonize the airways of patients with definitive ABPA.

Methods: We enrolled 11 patients (ages 57.5 ± 17.1 years; male: female, 4:7) with ABPA diagnosed by serological and radiological criteria. Fungi colonizing the airway were identified from mucous plugs that were naturally expectorated or obtained by fiberoptic bronchoscopy.

Results: *Aspergillus* spp. ($n = 8$) was the most frequently isolated, followed by *Schizophyllum commune* ($n = 4$), *Candida albicans* ($n = 2$), *Rhizopus oryzae* ($n = 1$), and *Penicillium* spp. ($n = 1$). Among the *Aspergillus* spp., *A. niger*, *A. terreus*, and *A. sydowii* were more frequently isolated (total, $n = 6$) than *A. fumigatus* ($n = 2$). Many patients were sensitized with several fungi in addition to *Aspergillus*, which were dissociated with airway-colonizing fungi.

Conclusion: Multiple fungal species can colonize the airway, and dissociation between colonizing and sensitizing species frequently occurs in definitive ABPA. Considering the increased prevalence of azole-resistant *Aspergillus* spp., administering antifungal drugs that target *A. fumigatus* without identifying which fungal species colonize the airway might be problematic.

Introduction

Aspergillus species are ubiquitous environmental fungi that can cause several types of pulmonary diseases in humans. Clinical features differ depending on whether the condition is saprophytic infestation, invasive, or allergic and include *Aspergillus*-induced asthma, hypersensitivity pneumonia, and allergic bronchopulmonary aspergillosis (ABPA). The latter was identified by Hinson et al[1] in patients with asthma with recurrent pulmonary infiltration, peripheral blood eosinophilia, and *Aspergillus fumigatus* in sputum. Allergic bronchopulmonary aspergillosis occurs in 1% to 2% of patients with persistent asthma and in 2% to 15% of patients with cystic fibrosis,[2] although cystic fibrosis is extremely rare in Japan. Often, ABPA can be diagnosed based on clinical symptoms, radiology, and serology. Cultured *A. fumigatus* derived from sputum is supportive but not diagnostic for ABPA. The fungus can also grow in patients with other pulmonary diseases because of its ubiquitous nature. Physicians rarely culture sputum to diagnose ABPA,[3] although the causative organisms of ABPA are mainly fungal, especially *A. fumigatus*, but they can also be *Aspergillus flavus*, *Aspergillus niger*, *Candida albicans*, *Penicillium*, and *Schizophyllum commune*.[4–7] Thus, ABPA has recently been referred to as allergic bronchopulmonary mycosis (ABPM). International guidelines[8] recommend systemic corticosteroid combined with the antifungal agent itraconazole as the primary treatment for ABPA. Based on the fact that fungi other than *A. fumigatus* can also cause similar syndromes and that antifungal agents targeting *A. fumigatus* might be primarily administered, we believe that understanding which fungi colonize the airways of patients with ABPA is critical. The present study determined fungi colonizing the airways of patients with definitive ABPA irrespective of sputum culture.

Methods

Patients

We included patients with definitive ABPA based on the Rosenberg-Patterson diagnostic criteria (Table 1), in which 6 or more of the primary criteria were positive.[9] Positive findings for secondary criteria further confirmed the diagnosis but were not essential. Other diseases were excluded. The medical records of the

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Table 1
Diagnostic criteria for allergic bronchopulmonary aspergillosis[9]

Primary criteria
1. Episodic bronchial obstruction (asthma)
2. Eosinophilia
3. Immediate skin reactivity (type I) to *Aspergillus* antigen
4. Precipitating antibodies against *Aspergillus* antigen
5. Elevated serum IgE concentrations
6. History of pulmonary infiltrates (transient or fixed)
7. Central bronchiectasis
Secondary criteria
1. *Aspergillus fumigatus* in sputum
2. History of expectoration of brown plugs or flecks
3. Arthus skin reactivity (type III) to Aspergillus antigen

Being positive for 6 of 7 primary criteria renders a diagnosis of allergic bronchopulmonary aspergillosis almost certain.

patients and radiological findings of definitive ABPA were retrospectively analyzed. The Special Committee of Nagasaki University Hospital provided ethical approval for the study (Project registration number 12062549), and written informed consent was obtained from all patients.

Diagnostic Methods

Routine blood tests, including white blood cell counts and differentiation and serum total immunoglobulin E (IgE) as well as post bronchodilator pulmonary function tests, were performed at the central laboratory of Nagasaki University Hospital. When a history of asthma was unknown, airway hyperresponsiveness to inhaled methacholine was determined using a standard procedure. Allergen-specific serum IgE levels against 33 common allergens were determined using enzyme-linked immunosorbent assays at a commercial laboratory (SRL Inc, Tokyo, Japan). *Aspergillus fumigatus* antigens were used for serum *Aspergillus*-specific IgE. Serum *A. fumigatus* precipitins were detected using the Aspergillus Immunodiffusion System (Microgen Bioproducts Ltd, Camberley, United Kingdom). Sensitivity to *A. fumigatus*, *Cladosporium cladosporioides*, *Alternaria kikuchiana*, *Penicillium luteum*, *Candida albicans*, and house dust was assessed by intradermal skin tests that involved injecting 20 μL (1:10,000 w/v) of fungal allergens and 1:1,000 (w/v) of house dust allergens (Torii Pharmaceutical Co. Ltd, Tokyo, Japan). Test results were read every 15 minutes for 1 hour, and then at 6 and 8 hours. The reactions were classified as type I if a wheal and erythema developed within 15 minutes. After the type I reaction subsided, erythema and edema that started at the skin test site and peaked at 6 to 8 hours were considered type III reactions. We defined positive central bronchiectasis (CB) when bronchi were dilated compared with an adjacent bronchial artery in the inner two thirds of the lung field determined by high-resolution computed tomography.

Identification of Fungal Isolates

Airway-colonizing fungi isolated from mucous plugs that were spontaneously expectorated or obtained by fiberoptic bronchoscopy (Fig 1) were identified according to macroscopic colony morphological and micromorphological characteristics. If species could not be identified using microbiological methods, they were determined using polymerase chain reaction and direct sequencing targeting the internal transcribed spacer region and the D1/D2 region of the 28S subunit at the National Institute of Infectious Diseases (Tokyo, Japan).

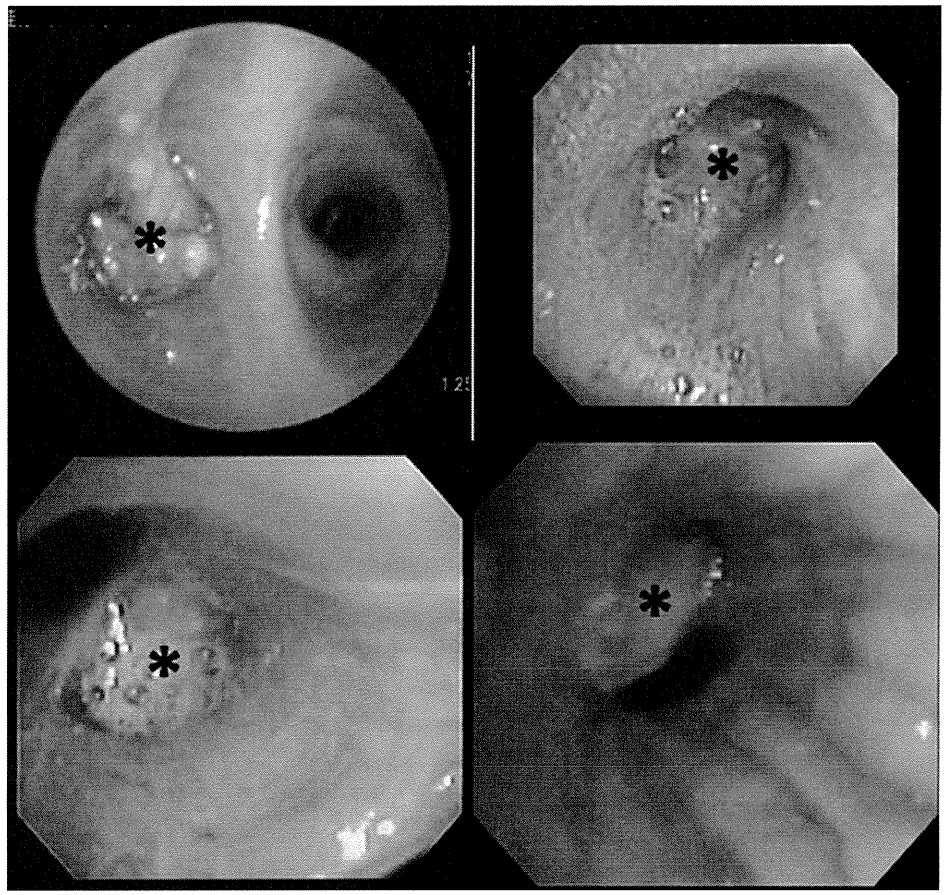

Figure 1. Representative fiberoptic bronchoscopy images of definitive ABPA. Mucous plugs in airways of patients with ABPA. *Mucous plugs collected via bronchoscopy and cultured for fungi.

Table 2
Nonspecific diagnostic criteria for *Aspergillus*

No.	Sex/age (years)	Asthma	Post BD % FEV_1	Peripheral eosinophils (/μL)	Total IgE (IU/L)	Chest infiltration	CB
1	F/31	Y	122.0	567	724.7	Y	Y
2	F/61	Y	126.1	730	2,357.0	Y	Y
3	M/38	Y	48.9	1,335	9,021.6	Y	Y
4	F/69	N	94.3	806	863.1	Y	Y
5	F/46	Y	97.2	1,204	1,109.7	Y	Y
6	F/56	Y	76.6	413	3,442.0	Y	Y
7	M/88	Y	101.9	854	745.7	Y	Y
8	F/63	Y	108.4	1,092	1,396.7	Y	Y
9	F/56	Y	89.6	940	9,630.0	Y	Y
10	M/79	Y	124.0	1,730	3,395.0	Y	Y
11	M/46	Y	43.8	2,914	722.6	Y	Y

BD, bronchodilator; FEV_1, forced expiratory volume in 1 second; IgE, immunoglobulin E; CB, central bronchiectasis; N, no; Y, yes.

Definition of Sensitizing and Colonizing Fungi

Sensitization was defined as elevated serum-specific IgE or positive type I skin reaction to fungi. Patients were considered to have airway colonization if any fungi were identified in airway samples by morphological or molecular biological methods.

Results

General Characteristics of Patients

Four male and seven female patients aged (mean ± SD) 57.5 ± 17.1 years were diagnosed with definitive ABPA at then Second Department of Internal Medicine, Nagasaki University Hospital, Nagasaki, Japan, between January 2010 and March 2012. All patients were symptomatic and had chest infiltration with CB at the time of ABPA diagnosis. None had used inhaled or systemic corticosteroids (CS), and the results shown in the tables were obtained before starting systemic CS therapy.

Nonspecific Clinical Features of Aspergillus

Table 2 shows the findings based on the nonspecific diagnostic criteria for *Aspergillus*. All except 1 patient (patient 4) had a history of asthma or airway hyperresponsiveness. Postbronchodilator pulmonary function tests revealed obstructive defects of varying severity. All patients had peripheral blood eosinophilia (1,144.1 ± 692.7/μL; mean ± SD) and increased serum total IgE (3,055.1 ± 3,268.4 IU/L; mean ± SD). High-resolution computed tomography showed CB in all patients, confirming that they all had the representative clinical features of ABPA.

Specific Clinical Features of Aspergillus

Table 3 shows the specific diagnostic criteria for *Aspergillus*. All patients were positive for serum-specific IgE and the type I skin test against *Aspergillus* antigen. Type III skin reactions and serum precipitins against *Aspergillus* antigen were found in 40% and 36.4% of the patients, respectively. Fungal cultures derived from respiratory samples revealed that *Aspergillus* spp. ($n = 8$) were the most prevalent followed by *Schizophyllum commune* ($n = 4$), *Candida albicans* ($n = 2$), *Rhizopus oryzae* ($n = 1$), and *Penicillium* spp. ($n = 1$). Among the *Aspergillus* spp, *Aspergillus niger*, *Aspergillus terreus*, and *Aspergillus sydowii* (total, $n = 6$) were more prevalent than *A. fumigatus* ($n = 2$).

Comparison of Colonizing and Sensitizing Fungi

Table 4 compares colonizing and sensitizing fungi in the patients. Patient 8 was colonized with *A. niger* and sensitized only to *Aspergillus*. Patient 11 was colonized with *A. terreus* and *S. commune* and sensitized to *Aspergillus*. Because we did not determine serum *S. commune*–specific IgE, whether patient 11 was sensitized to *S. commune* remains unknown. The remaining 9 patients were sensitized to several fungal allergens besides the fungi that colonized their lungs. Thus, colonizing and sensitizing fungi are frequently dissociated.

Discussion

The major finding of the present study was that sensitizing and airway colonizing fungi were dissociated in patients diagnosed with definitive ABPA according to serological and radiological criteria. Even in such patients, *A. fumigatus* was less frequently isolated than other *Aspergillus* spp. The cause of this dissociation might be explained as follows. First, *A. fumigatus* and other fungi might simultaneously colonize the airways of patients with ABPA, and current culture methods might not be sensitive enough to identify *A. fumigatus*. Second, causative fungi might cross-react with *A. fumigatus* antigen, because crude extracts of *A. fumigatus* frequently cross-react with other antigens.[10] This critical issue might be clarified using specific but not crude *A. fumigatus* antigen[11] for immunological testing and to establish diagnostic criteria for ABPM caused by fungi other than *A. fumigatus*.

Table 3
Specific diagnostic criteria for *Aspergillus*

No	Sex/age (years)	Sample	Colonizing fungi	*Aspergillus* IgE	*Aspergillus* skin test (Type I)	*Aspergillus* skin test (Type III)	*Aspergillus* precipitins
1	F/31	BF	*A. niger* *C. albicans*	+	+	+	-
2	F/61	BF	*S. commune* *A. sydowii*	+	+	+	-
3	M/38	BF	*A. fumigatus* *A. niger*	+	+	-	+
4	F/69	BF	*S. commune*	+	+	-	-
5	F/46	Exp	*Penicillium spp.*	+	+	-	+
6	F/56	BF	*A. fumigatus* *Rhizopus oryzae*	+	+	-	+
7	M/88	BF	*A. terreus*	+	+	-	-
8	F/63	Exp	*A. niger*	+	+	ND	-
9	F/56	BF	*S. commune*	+	+	+	-
10	M/79	Exp	*C. albicans*	+	+	+	-
11	M/46	Exp	*A. terreus* *S. commune*	+	+	-	+

BF, mucous plug obtained by bronchofibroscopy; Exp, naturally expectorated mucous plug; ND, not done; +, positive; -, negative. *A. fumigatus*, *Aspergillus fumigatus*; *A. niger*, *Aspergillus niger*; *A. sydowii*, *Aspergillus sydowii*; *A. terreus*, *Aspergillus terreus*; *C. albicans*, *Candida albicans*; *S. commune*, *Schizophyllum commune*.

Table 4
Comparison of colonizing and sensitizing fungi

No	Sex/Age (years)	Colonizing fungi	Sensitizing fungi
1	F/31	*A. niger, C. albicans*	*Asp., Penicillium, Alternaria, Candida*
2	F/61	*S. commune, A. sydowii*	*Asp., Penicillium, Cladosporium, Alternaria, Candida*
3	M/38	*A. fumigatus, A. niger*	*Asp., Penicillium, Cladosporium, Alternaria, Candida*
4	F/69	*S. commune*	*Asp., Penicillium, Alternaria, Candida*
5	F/46	*Penicillium spp.*	*Asp., Penicillium*
6	F/56	*A. fumigatus, Rhizopus oryzae*	*Asp., Penicillium, Cladosporium, Alternaria, Candida, Mucor*
7	M/88	*A. terreus*	*Asp., Penicillium*
8	F/63	*A. niger*	*Asp.*
9	F/56	*S. commune*	*Asp., Penicillium, Cladosporium, Alternaria, Candida*
10	M79	*C. albicans*	*Asp., Penicillium, Cladosporium, Alternaria, Candida*
11	M/46	*A. terreus, S. commune*	*Asp.*

Asp. Aspergillus; A. fumigatus, Aspergillus fumigatus; A. niger, Aspergillus niger; A. sydowii, Aspergillus sydowii; A. terreus, Aspergillus terreus C. albicans, Candida albicans; S. commune, Schizophyllum commune.

Although the pathogenesis of ABPA remains unknown, the activation of Th2 cells by *Aspergillus* antigen seems to play a critical role.[12] Having asthma is not an essential indicator of ABPA,[13] and patients can produce specific IgE antibodies not only against *Aspergillus* but also against other allergens associated with interleukin (IL)-4 and IL-13 production. The present study also identified serum-specific IgE antibodies against fungal, inhaled, and food allergens in patients with ABPA. Thus, because serum IgE antibody against *Aspergillus* can be produced in patients with ABPM as a reaction against other fungi, measuring serum IgE antibodies against *Aspergillus* per se cannot definitively diagnose ABPA.

We cultured and identified fungi isolated from mucous plugs that were naturally expectorated or obtained by bronchoscopy to avoid environmental contamination. We applied molecular biological methods when conventional morphological methods could not identify fungal species. *Schizophyllum commune* is one such organism that we identified using molecular biological methods, and it is common in Japan, where it is thought to cause ABPM.[7]

Although ABPA is typically not fatal and responds well to drug therapy, a delay of therapy can cause bronchiectasis and irreversible pulmonary fibrosis. The therapeutic modality for ABPA mainly consists of early and systemic CS to suppress immune reactions against *Aspergillus* spp. A subset of patients develops recurrent exacerbations even after systemic CS, and several reports have described that antifungal drugs in addition to CS can help attenuate the fungal burden.[14,15] However, none of these studies describes long-term outcomes. Nonetheless, international guidelines indicate that CS should be combined with itraconazole to treat ABPA.[8] The prevalence of azole-resistant *A. fumigatus* is increasing in several countries.[16,17] We previously analyzed the relationship between a history of triazole treatment and triazole minimum inhibitory concentrations for clinically isolated *A. fumigatus* and found that azole-resistant *A. fumigatus* might emerge after extended itraconazole administration.[18] Thus, considering the limited number of clinically available antifungal drugs, treating patients with serologically and radiologically diagnosed ABPA with an initial combination of itraconazole and CS without identifying other colonizing fungi might induce azole-resistant *A. fumigatus*.

Another problem associated with antifungal therapy for ABPA is whether the causative fungi are alive or dead. The present study describes the culture results of respiratory samples, indicating that live fungi can colonize airways. Antifungal therapy might be effective for such patients. However, dead fungi could also be involved in the development of allergies. We previously reported the differential effects of live and dead *A. fumigatus* on the function of dendritic cells in the development of asthma in a murine model.[19] From this viewpoint, dead *A. fumigatus* might play a dominant role in patients with ABPA who respond to omalizumab, an anti-IgE antibody.[20]

In conclusion, fungi that colonize airways are dissociated from sensitizing fungi in patients with ABPA. Fungal cultures of respiratory samples are not required for a diagnosis of ABPA, and the present study did not prohibit the early administration of systemic CS. Physicians should identify fungi that colonize airways in addition to sensitizing fungi, especially when they plan to treat ABPA with antifungal drugs.

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ORIGINAL ARTICLE Allergens

Glucoamylase is a major allergen of *Schizophyllum commune*

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Summary

Background *Schizophyllum commune* is one of the causative agents of basidiomycosis including disorders such as allergic bronchopulmonary mycosis, allergic fungal sinusitis, and mucoid impaction of bronchi, the incidence of those of which has been increasing. These mycoses are difficult to diagnose because only a limited number of diagnostic tools are currently available. The biggest problem is that no specific antigens of *S. commune* have been identified to enable serodiagnosis of the disease.

Objective In this study, we attempted to identify a major antigen of *S. commune* to establish a reliable serodiagnostic method.

Methods We used mass spectrometry to identify an antigen that reacted with the serum of a patient with allergic bronchopulmonary mycosis caused by *S. commune*. The protein was expressed in *Escherichia coli*, highly purified, and the patient sera IgG and IgE titres against the protein were determined by enzyme-linked immunosorbent assay.

Results The protein identified as a major antigen of *S. commune* was named Sch c 1; it was a homolog of glucoamylase. The IgG and IgE titres against Sch c 1 in patient sera were significantly higher than those in healthy volunteer sera ($P < 0.01$).

Conclusions and Clinical Relevance Sch c 1 is recognized by the host immune system of patients as an antigen/allergen. The purified glucoamylase Sch c 1 is a promising candidate antigen for the serodiagnosis of *S. commune*-induced mycosis.

Keywords allergic bronchopulmonary mycosis, basidiomycosis, glucoamylase, Sch c 1, *Schizophyllum commune*
Submitted 22 March 2013; revised 31 October 2013; accepted 8 December 2013

Introduction

Schizophyllum commune is a basidiomycete that is ubiquitous in the environment. It was not recognized as a human pathogen until the 1950s when several studies suggested that it could be a causative agent of human infectious diseases [1–3]. However, the detailed pathogenicity of the fungus remains unclear. In the 1980s, many cases of *S. commune*-induced mycosis were reported worldwide, particularly in developed countries. At present, *S. commune* is a well-known causative agent of human diseases including allergic bronchopulmonary mycosis (ABPM), allergic fungal sinusitis, mucoid impaction of bronchi (MIB), and basidiomycosis [4].

Although the number of patients with allergic mycosis is increasing, diagnosis remains a challenge for physicians. There are no characteristic signs and symptoms of *S. commune*-induced ABPM or MIB, which are the most common forms of the disease in Japan. It is also rather uncommon to successfully culture this fungus from sputum. Even if the fungus is isolated from clinical samples, its morphology is quite common and non-specific, and therefore, it is often regarded as a contaminant. Serological examination, which is a very powerful tool for diagnosing allergic bronchopulmonary aspergillosis (ABPA), cannot be used for these diseases because there is no known specific antigen against the fungus. A novel diagnostic system such as a serodiagnostic method that uses a specific antigen has long been awaited.

In this study, we identified and named an antigenic protein, Sch c 1, in the culture supernatant of *S. commune*. The recombinant protein purified from *Escherichia coli* reacted strongly with most of the sera samples from

patients with *S. commune*-induced ABPM/MIB, but not with the sera of controls. These findings indicate that Sch c 1 is a novel antigen/allergen of *S. commune* and that the purified protein could be a useful tool for diagnosing diseases caused by this pathogen.

Methods

Fungus, bacteria, and growth conditions

The dikaryotic strain used in this study, *S. commune* IFM47458, was originally isolated from a patient with lung and brain abscess [5] and was stored in the culture collection of the Medical Mycology Research Center, Chiba University. It was routinely transferred to potato dextrose agar and cultured at 25°C before use. *E. coli* DH5α was used for genetic manipulations and protein preparations. The *E. coli* strain was routinely grown in Lysogeny (L) broth with or without the appropriate antibiotics.

Preparation of culture filtrate

Hyphae of *S. commune* IFM47458 on slants were inoculated in a liquid medium containing 0.5% yeast nitrogen base without amino acids (BD, Franklin Lakes, NJ, USA) and 1% glucose and were statically cultured at 35°C for 5 weeks. After filtration, the culture supernatant was subjected to protein precipitation with trichloroacetic acid and acetone. The resultant precipitant was solved in a buffer for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Western blotting

The proteins that developed on the gel were visualized by Coomassie Brilliant Blue R-250 (CBB) staining (Quick-CBB, Wako Pure Chemical Industries, Osaka, Japan) or were transferred onto a polyvinylidene fluoride membrane. Immunoblot analysis was performed using common protocols. For the detection of antigenic proteins in concentrated culture filtrates using Western blotting, Protein-L (Pierce, Rockford, IL, USA) that can bind a wider range of Ig classes including human IgG and IgE was used as a secondary antibody. For the examination of the reactivity of IgE with rSch c 1 in patients' sera, a mouse anti-human IgE monoclonal antibody conjugated with biotin (clone no. f0822, produced and supplied by Biomatrix Research, Inc., Chiba, Japan) and streptavidin–horseradish peroxidase (HRP) (ThermoFisher Scientific Inc., Waltham, MA, USA) were used.

Mass spectrometry

A protein band that reacted with an antibody in a patient's serum sample was excised from the gel stained with CBB. The antigenic protein was identified by in-gel tryptic digestion followed by liquid chromatography (LC)–mass spectrometry (MS). In-gel tryptic digestion was performed as described previously [6]. The digested peptides were injected into a 0.3 × 5 mm L-trap column (Chemicals Evaluation and Research Institute, Saitama, Japan) and a 0.1 × 50 mm monolith analytical column (AMR, Tokyo, Japan) attached to a high-performance liquid chromatography (HPLC) system (Nanospace SI-2; Shiseido Fine Chemicals, Tokyo, Japan). The flow rate of the mobile phase was 1 μL/min. The solvent composition of the mobile phase was programmed to change in 35 min cycles with varying mixing ratios of solvent A (2% v/v CH_3CN and 0.1% v/v HCOOH) to solvent B (90% v/v CH_3CN and 0.1% v/v HCOOH): 5–50% B 20 min, 50–95% B 1 min, 95% B 3 min, 95–5% B 1 min, and 5% B 10 min. Peptides purified by HPLC were introduced into an LTQ-XL ion trap mass spectrometer (Thermo Scientific, CA, USA) via an attached Pico Tip (New Objective, MA, USA). MS and tandem MS (MS/MS) peptide spectra were measured in a data-dependent manner according to the manufacturer's operating specifications. The MASCOT search engine (Matrix Science, London, UK) was used to identify proteins from the mass and tandem mass spectra of the peptides by searching the NCBInr database (Jan 2011, 12 747 899 entries). The database search parameters included the following: taxonomy, fungi (781 053 entries); peptide mass tolerance, 1.2 Da; fragment tolerance, 0.6 Da; enzyme trypsin, allowing up to one missed cleavage; variable modifications, methionine oxidation. The minimum criteria for protein identification were set at a false discovery rate (FDR) of < 1%. FDR was estimated by searching against a randomized decoy database created by the MASCOT Perl program supplied by Matrix Science.

Cloning of the sch c 1 *gene and the expression of recombinant* Sch c 1 *in* E. coli

Based on the sequence of *sch c 1* indentified on the *S. commune* genome project page (http://genome.jgi-psf.org/Schco1/Schco1.home.html) and on our 5′ rapid amplification of cDNA end (5′-RACE; data not shown) to obtain the 5′ end sequence of the RNA transcript, we cloned *sch c 1* with an extra upstream region coding N-MATLGDGVPQFVNNMSAAIWEYKERLVLEW-CGNSTS-LSNTQGLGA. The codon usage was optimized by GeneOptimizer, and the optimized gene registered in DNA Data Bank Japan (Accession No. AB736191) was synthesized by GENEART AG (Regensburg, Germany). The gene was cloned into pQE-80L (Qiagen, Hilden, Germany) to express the His-tagged protein and transformed into *E. coli* DH5α. Transformants were cultured at 25°C for 24 h. After cultivation, the bacteria were suspended in buffer A (50 mM Tris–HCl, 500 mM NaCl,

20 mM imidazole, pH 7.5) and disrupted with a Bioruptor sonicator (Cosmo Bio Co., Ltd., Tokyo, Japan). The lysate was separated by centrifugation, and the pellet containing the inclusion body of the recombinant protein was washed with buffer A and then gently suspended in buffer A supplemented with 8 M urea. The suspension was separated by centrifugation, and the supernatant was transferred to Ni Sepharose 6 Fast Flow slurry (GE Healthcare UK Ltd., Buckinghamshire, UK) for binding at room temperature for 15 min. The slurry was then washed with buffer A supplemented with 8 M urea and eluted three times with buffer B (50 mM Tris–HCl, 500 mM NaCl, 500 mM imidazole, pH 7.5) supplemented with 8 M urea. The purified protein was used as the recombinant antigen rSch c 1 for further analysis.

Enzyme-linked immunosorbent assay to determine IgG or IgE titres against rSch c 1 or glucoamylases from Aspergillus niger *or* Rhizopus *sp. in patient sera*

Glucoamylases from *A. niger* and *Rhizopus* sp. were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) was used to determine the titres of IgG or IgE against glucoamylases in human sera, as described below. A total of 200 ng of a glucoamylase was diluted with 200 μL of 0.05 M carbonate buffer (pH 9.6) and applied to coat the wells of a Nunc-Immuno plate with a MaxiSorp surface (ThermoFisher Scientific Inc.). After being washed three times with Tris-buffered saline (TBS), the wells were blocked with protein-free blocking buffer at 25°C for 1 h. They were then washed three times with TBS supplemented with 0.1% Tween-20 (TBS-T) using ImmunoWash model 1575 (Bio-Rad Laboratories, Hercules, CA, USA) and then incubated with sera from patients or healthy volunteers diluted 100-fold with TBS-T at 25°C for 2 h. Thereafter, the wells were washed three times with TBS-T, and goat anti-human IgG antibody (MP Biomedicals, LLC, Solon, OH, USA) conjugated with HRP was added. The plates were then incubated at 37°C for 1 h, after which they were washed three times with TBS-T. Detection using HRP substrates (TMB Peroxidase EIA Substrate Kit, Bio-Rad Laboratories) was performed in accordance with the directions provided by the manufacturer. After the reaction, the plates were read at 450 nm using a Sunrise Rainbow Thermo microplate reader (Tecan Group Ltd., Männedorf, Switzerland). The absorbance of wells not coated with protein was subtracted from that of the coated wells.

To determine IgE titres, the sera were diluted tenfold with TBS-T. Mouse anti-human IgE monoclonal antibody conjugated with biotin (Clone No. f0822) and streptavidin-HRP was used to determine IgE titres instead of anti-IgG antibody conjugated with HRP as described above. The plates were incubated at 25°C for 1 h.

Data from the patient and healthy volunteer groups were analysed by the Mann–Whitney *U*-test. For correlation analysis, we calculated Spearman's rank correlation coefficient.

Analysis of sera from patients with S. commune*-induced ABPM/MIB, aspergilloma, or chronic pulmonary aspergillosis and healthy volunteers*

We analysed the sera of 13 patients with *S. commune*-induced ABPM/MIB (Table 1), four patients with aspergilloma (five sera samples), five patients with chronic pulmonary aspergillosis (CPA, five sera samples), and 20 (or 21 in Fig. 4) healthy controls. *Schizophyllum commune* was isolated from all patients with *S. commune*-induced ABPM/MIB. The serum from each patient was separated from blood and stored at − 80°C until use. This protocol was approved by the ethics committee of the Medical Mycology Research Center, Chiba University (No. Chibadai-I-Sou-519).

Results

Identification of a major antigenic protein, a homolog of glucoamylase, from S. commune

To screen for antigenic proteins from *S. commune*, we prepared a culture supernatant of *S. commune*-containing antigenic proteins [7]. A limited number of proteins in the culture supernatant were detected by CBB staining, and two bands were strongly visualized by Western blot analysis of patient serum (Fig. 1a). Because the corresponding bands could not be excised

Table 1. Major clinical characteristics of patients whose sera were used in this study

Patient	Asthma	*Schizophyllum commune*	Note
1	No	Isolated	Mucoid impaction of bronchi
2	Yes	Isolated	
3	No	Isolated	Organized pneumonia
4	No	Isolated	
5	No	Isolated	Coughing, atelectasis, mucoid impaction of bronchi
6	No	Not isolated	Hyphae detected in the histopathological specimen, mucoid impaction of bronchi
7	n/a	Isolated	
8	n/a	Isolated	
9	Yes	Isolated	
10	No	Isolated	Rhinosinusitis
11	n/a	Isolated	
12	No	Isolated	
13	No	Isolated	Coughing

n/a, not available.

separately, they were excised together from the gel and analysed by LC-MS/MS. We used a database search to identify the bands as glycoside hydrolase family 15 and carbohydrate-binding module family 20 (glucoamylase), a novel major antigen of *S. commune* (Table 2). Although we have no data whether the bands are varieties of glycosylated forms of the same protein or of different proteins, the MASCOT score indicates that the *S. commune* glucoamylase is the major protein in the two bands. We named the gene *sch c 1* and the protein Sch c 1 in accordance with the naming convention set by the IUIS Allergen Nomenclature Subcommittee [8].

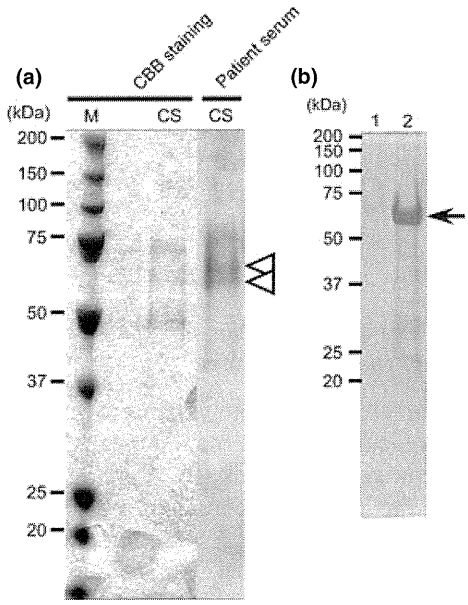

Fig. 1. (a) Coomassie Brilliant Blue R-250 (CBB) staining of proteins in the culture supernatant and Western blotting of the serum of a patient with allergic bronchopulmonary mycosis. Arrowheads indicate the bands excised for mass spectrometry analysis. M: molecular weight maker; CS: culture supernatant. (b) Purified rSch c 1. Lane 1: proteins purified from the *E. coli*-transformed vector, pQE-80L; lane 2: proteins purified from *E. coli* expressing rSch c 1. Arrow indicates purified rSch c 1.

IgG titres against rSch c 1 in patient sera

We used ELISA to examine IgG titres against the recombinant Sch c 1 (rSch c 1) of *S. commune* (Fig. 1b) in the sera of 20 healthy volunteers and 13 patients. The absorbance of the patient and volunteer groups was 1.235 ± 0.6318 and 0.1470 ± 0.0743, respectively (Fig. 2a). The IgG titre against rSch c 1 in the patient group was significantly higher than that in the volunteer group ($P < 0.01$). This finding suggests that rSch c 1 retains antigenicity and reacts strongly and specifically with the sera of patients infected with *S. commune* in whom specific IgG against the antigen is present.

IgE titres against rSch c 1 in patient sera

To examine whether specific IgE against Sch c 1 from *S. commune* was detected in patient sera, we measured IgE titres by ELISA. As shown in Fig. 2b, the IgE titres against rSch c 1 among patients with *S. commune*-induced ABPM were significantly higher than those of healthy volunteers (1.812 ± 1.118 vs. 0.04425 ± 0.07937; $P < 0.01$). Using Western blotting, we confirmed the reaction of IgE with rSch c 1 in four of five patients' sera (Figure S1). The serum in the 5th lane strongly reacted with rSch c 1 on ELISA; however, Western blotting did not detect an obvious band (Figure S1). These suggest that in most patients with *S. commune*-induced ABPM, both specific IgG and specific IgE against Sch c 1 are produced in significant amounts.

Cross-reactivity of rSch c 1 to sera from patients with aspergilloma or chronic pulmonary aspergillosis

Aspergillus spp. are the major causative agents of ABPM, which is also known as ABPA. Homology between Sch c 1 and the glucoamylase from *Aspergillus* spp. is high (identity around 50%, Fig. 3). To investigate the cross-reactivity of rSch c 1, the IgG and IgE titres in sera from patients with aspergilloma or CPA

Table 2. Mass spectrometric identification of the protein reacted with antibody in a patient's serum

	Candidate name	NCBInr accession No.	MASCOT score	Seq. cov.[1] (%)	MS/MS[2] (total)	MS/MS[3] (unique)
Top-ranked candidate	Glycoside hydrolase family 15 and carbohydrate-binding module family 20	gi|302681819	1159	18	27	7
2nd-ranked candidate	Glycoside hydrolase family 31 protein	gi|302693302	139	2	4	2

[1]Sequence coverage.

[2]Number of peptide fragments of a protein that yielded informative MS/MS data. The significant threshold *P*-value was set as false discovery rate (FDR) <1%.

[3]Number of unique peptide fragments of a protein.

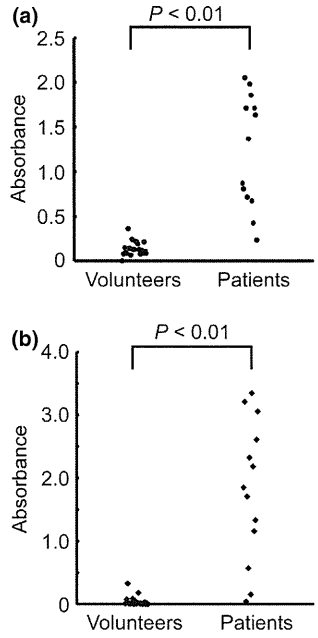

Fig. 2. IgG (a) and IgE (b) titres against rSch c 1 determined by ELISA. Data from 20 sera samples from healthy volunteers and 13 sera samples from patients were plotted on this graph. The *P*-value was calculated by Mann–Whitney *U*-test.

were determined. Antibodies against *Aspergillus* were detected in all of these sera (data not shown). As shown in Fig. 4a, IgG in 2 of 10 sera samples from patients reacted significantly with rSch c 1. The 2 sera samples were from CPA patients, suggesting that the IgG titre against glucoamylase is elevated in some patients with CPA. On the other hand, no elevation of IgE titres was detected in the sera of patients with aspergilloma or CPA (Fig. 4b). These findings indicate that some patients with CPA produced IgG, but not IgE, when recognizing Sch c 1.

Cross-reactivity of glucoamylase from A. niger *and* Rhizopus *sp. to sera from patients with* S. commune *ABPM*

As described above and shown in Fig. 3, Sch c 1 has a high identity with glucoamylases of fungi including *Aspergillus* spp. To investigate the cross-reactivity of *S. commune*-induced ABPM from patients' sera with *A. niger* glucoamylase (*An*GA) and *Rhizopus* sp. glucoamylase (*Rh*GA), the IgG and IgE titres against *An*GA and *Rh*GA in the patients' sera were determined. As shown in Fig. 5a and b, in majority of the patients' sera, IgG and IgE reacted with *An*GA. Although the IgG titre to *An*GA was significantly related with the titre to rSch c 1 ($P = 0.048$, Figure S2a), the markers were dispersedly distributed in the scatter plot. Although the IgE titres against *An*GA and rSch c 1 were significantly correlated ($P = 0.014$, Figure S2b), in almost all of these sera the IgE titres against *An*GA were lower than those against rSch c 1. Although the titre against *Rh*GA in the patient group was significantly higher than that of the healthy volunteer group ($P < 0.01$), IgG in sera not only from patients but also from volunteers strongly reacted with *Rh*GA (Fig. 6a), and the correlation between the IgG titres against *Rh*GA and rSch c 1 was not significant ($P = 0.148$, Figure S2c). In addition, although the IgE titre against *Rh*GA in patients' sera was significantly higher than that detected in healthy volunteers ($P < 0.01$, Fig. 6b), the correlation between the IgE titres against *Rh*GA and rSch c 1 was not significant ($P = 0.068$, Figure S2d). Moreover, in patients' sera, the IgE titre against *Rh*GA was lower than that against rSch c 1. These data suggest that *An*GA and *Rh*GA are also recognized by IgG and IgE in patients with *S. commune*-induced ABPM. However, these data also suggest that the correlations of IgG titres are weak or not significant, and the IgE affinities to *An*GA and *Rh*GA are lower than the affinity to rSch c 1.

Discussion

We have previously reported that the culture supernatant of *S. commune* contains antigenic molecules that react strongly with patient sera [7], but the antigenic substance was not identified. Recently, several antigens from *Coprinus comatus* and *Psilocybe cubensis* were identified and cloned by Helbling et al. [9–11]. However, the antigens of most basidiomycetes have not been identified, and *S. commune* is not an exception. To the best of our knowledge, this is the first report to identify a major antigen of *S. commune*.

Our data indicate that the glucoamylase homolog identified in this study and named Sch c 1 is a major antigen of *S. commune*. As indicated in Fig. 2b, in patients with *S. commune*-induced diseases, specific IgE against Sch c 1 was abundantly produced, which indicates that the protein is a novel allergen of *S. commune*. Glucoamylase is a member of a well-known group of enzymes that cleave the α-1,4 linkage of starch from the non-reducing end and produce glucose; it is widely used in the food industry [12]. *Schizophyllum commune* has 2 glucoamylase genes in its genome, and these are similar to each other, but the alternative glucoamylase does not possess a C-terminal carbohydrate-binding module [13]. Recently, Ohm et al. [13] reported the expression of the predicted genes of *S. commune*, and the second glucoamylase was scarcely expressed at any growth stage [13]. These data suggest that the glucoamylase gene identified in this study is actually expressed and recognized as an antigen.

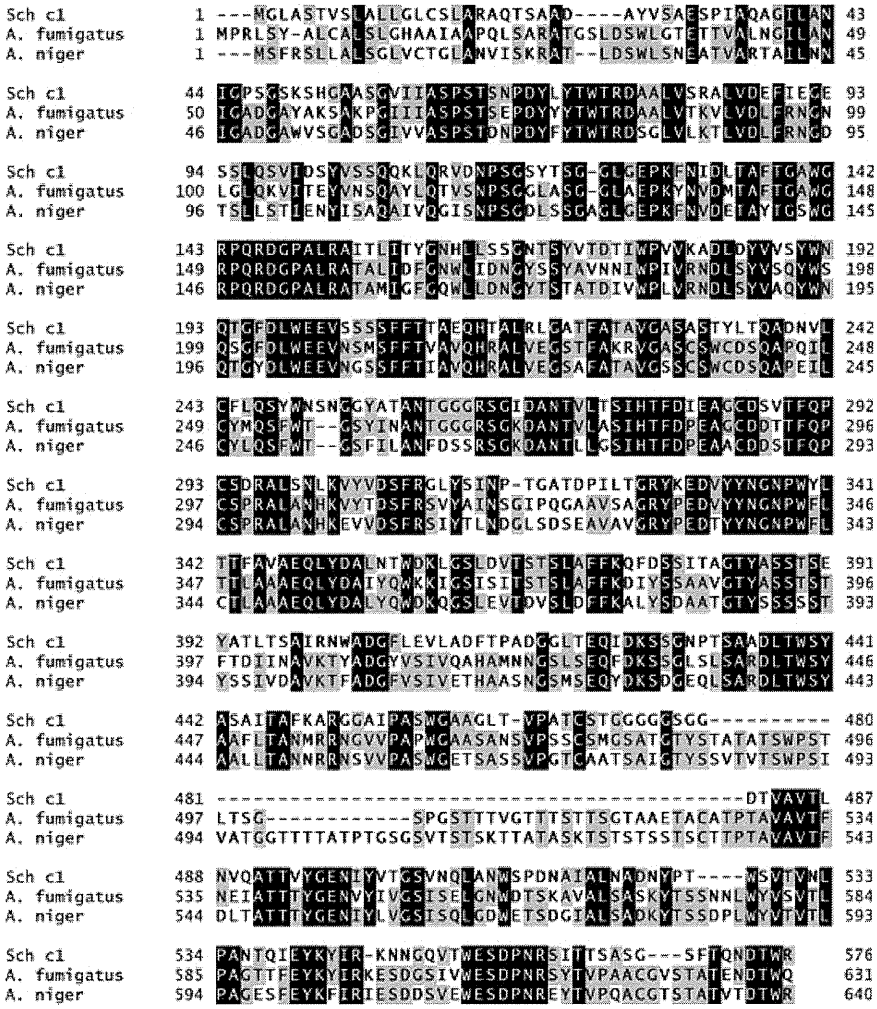

Fig. 3. Alignment between Sch c 1 and glucoamylases of *A. fumigatus* (NCBI Reference Sequence: XP_749206.1) and *A. niger* (NCBI Reference Sequence: XP_001390530). Hyphens indicate gaps. Black-shaded and grey-shaded characters indicate completely and partially identical residues, respectively.

Some of the glucoamylase enzymes of other fungi function as antigenic molecules. Quirce et al. [14] reported that patients with baker's asthma reacted positively in a skin prick test to glucoamylase taken from *Aspergillus niger,* which is widely used as a baking additive, and produced IgE antibodies against glucoamylase. Recently, Luo et al. [15] reported that the glucoamylase of *Penicillium chrysogenum* was identified as an exoantigen. Although no glucoamylase enzymes from basidiomycetes have been described as antigens or allergens to date, our study shows that the protein is recognized as an antigen or allergen in a number of human diseases. In terms of the cross-reactivity of other mycoses to Sch c 1, IgG in sera samples from 2 of 10 patients with aspergilloma or CPA reacted significantly with the glucoamylase from *S. commune* (Fig. 4a). The 2 sera samples also reacted strongly to glucoamylase from *A. niger* (Figure S3), suggesting that although the possibility that these patients have been infected or sensitized with *S. commune* cannot be excluded, in some cases with aspergillosis Sch c 1 glucoamylase is also recognized because of the high homology to *Aspergillus* glucoamylase. On the other hand, the elevation of IgE titres against Sch c 1 was not observed in patients with CPA or aspergilloma. In patients with CPA or aspergilloma, the titre against the *Aspergillus* antigen frequently increases, whereas the amount of IgE rarely increases. On the other hand, in majority patients with ABPM including ABPA, the IgE level is elevated in the serum. We are currently investigating the reactivity to Sch c 1 of sera samples from patients with ABPA. Further analyses including the cross-reactivity of other mycoses to Sch c 1, particularly allergic diseases, and the antigenicity of the glucoamylase enzymes of other fungi are warranted.

In conclusion, we identified Sch c 1, a homolog of glucoamylase, as a major antigen/allergen of *S.*

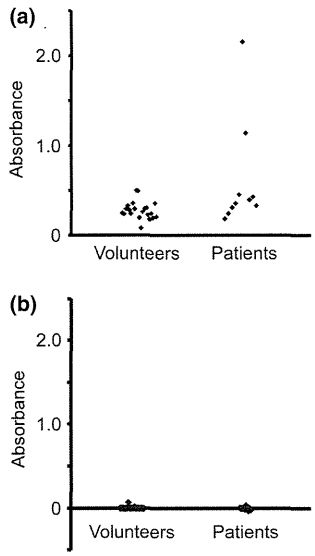

Fig. 4. IgG (a) and IgE (b) titres against rSch c 1 in sera from patients with aspergilloma or CPA. 21 healthy controls, and 10 sera with aspergilloma or CPA were used.

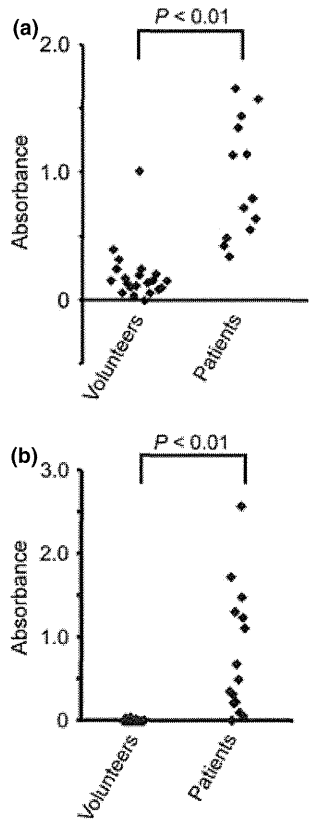

Fig. 5. The reaction of sera from healthy volunteers and individuals with allergic bronchopulmonary mycosis induced by *Schizophyllum commune* with *A. niger* glucoamylase (*An*GA). The IgG and IgE titres against *An*GA in sera are indicated in (a) and (b), respectively.

commune. The titres of IgG and IgE against rSch c 1 in patient sera were significantly higher than those in healthy volunteers. We believe that this protein could

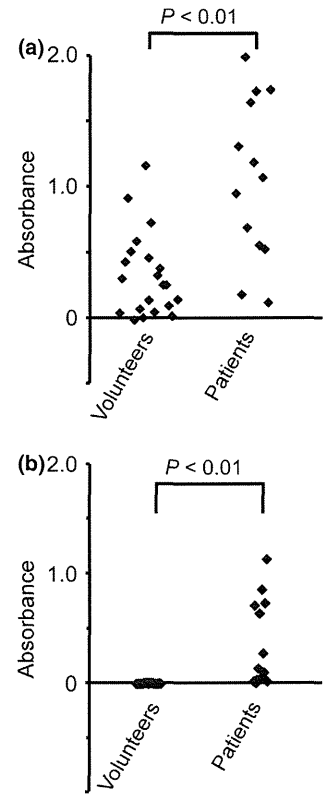

Fig. 6. The reaction of sera from healthy volunteers and individuals with allergic bronchopulmonary mycosis induced by *Schizophyllum commune* with *Rhizopus* glucoamylase (*Rh*GA). The IgG and IgE titres against *Rh*GA in sera are indicated in (a) and (b), respectively.

pave the way for a rapid and handy diagnosis of *S. commune*-induced ABPM/MIB.

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Conflict of interest

The authors have no conflicting financial interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Detection of anti-Sch c 1 IgE in each patient's serum by Western blotting. Among the five sera selected, all sera except one strongly reacted with Sch c 1 (arrow). Patient IDs listed in Table 1 of the used serum are shown beneath each strip.

Figure S2. Dot plots indicating the correlation between the IgG (a and c) or IgE (b and d) titres of patients with *S. commune*-induced ABPM against rSch c 1 and *An*GA or *Rh*GA. In each plot, the x-axis indicates the titre against rSch c 1, and the y-axis indicates the titre against *An*GA (a and b) or *Rh*GA (c and d). The lines in these plots indicate the regression line. ρ- and P-values are shown in the figure.

Figure S3. The dot plot indicates the titres in the sera of patients with CPA and aspergilloma against rSch c 1 and *An*GA. The x-axis and the y-axis indicate the titre against rSch c 1 and *An*GA, respectively.

