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Original Article

Sensitization to *Bjerkandera adusta* Enhances Severity of Cough Symptom in Patients with Fungus-Associated Chronic Cough (FACC)

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ABSTRACT

[Objective] The aim of the present study was to clarify the influence of sensitization to *Bjerkandera adusta* (*B. adusta*) on the clinical manifestation in patients with fungus-associated chronic cough (FACC). [Methods] Seventeen patients with FACC who underwent bronchoprovocation tests using an antigenic solution of *B. adusta* were selected from among 21 FACC patients. We compared the allergological findings and clinical characteristics of the FACC patients who showed a positive reaction to the bronchoprovocation test (Allergic fungal cough sensitized to *B. adusta*; AFC-Bj) with the remaining FACC patients (non AFC-Bj) retrospectively. [Results] The eleven patients with AFC-Bj had a median age of 52 (range, 22-70) years, and 45.5% were female. The respective values for six patients with non AFC-Bj were 47.5 (range, 36-60) years of age, and 33.3% were female. The positive ratios for an immediate cutaneous reaction (45.5%; $p<0.05$) and the lymphocyte stimulation test (63.6%; $p<0.05$) to *B. adusta* were found to be significantly higher in the AFC-Bj group than in the non AFC-Bj group. The total time required for complete remission of cough symptoms was longer (median 20, range 12-43 weeks; $p=0.0009$), and the recurrence ratio of coughing was more frequent in the AFC-Bj group in comparison to those in non AFC-Bj group (2, range 1-3 times and 0.5, range 0-1 times, respectively). [Conclusions] This study demonstrated that *B. adusta*, a basidiomycetous is an environmental fungus attracting attention because of its possible role in enhancing the cough severity of FACC patients via the sensitization to this fungus.

Key words : allergic fungal cough, basidiomycetous fungi, *Bjerkandera adusta*, chronic idiopathic cough, fungus-associated chronic cough

Introduction

Studies on the pathophysiology of chronic cough have progressed, but there has been little progress in elucidating the etiological agents and the exacerbating factors. Despite extensive diagnostic evaluation and numerous treatment guidelines[1-4], a number of patients remain troubled by chronic idiopathic cough (CIC)[5,6], a chronic, uncontrollable cough that is difficult to treat. Because diagnosing patients with an intractable cough such as CIC limits management of the disease, it is important to appropriately address this problem and reduce the number of diagnoses of CIC, even though the major causes of chronic cough exhibit geographical variations[7-9].

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Recently the presence of basidiomycetous (BM) fungi in induced-sputum has been revealed to be an important factor in distinguishing the clinical manifestations of CIC from those of non-CIC[10]. Therefore, identification of the fungi that are associated with chronic cough among the approximately 30,000 species of BM fungi is important for our understanding of this disease.

We have recently encountered a cluster of patients with allergic fungal cough (AFC) which is intractable and is characterized by sensitization to *Bjerkandera adusta* (*B. adusta*), one of the BM fungi[11], within a new clinical disease concept termed fungus-associated chronic cough (FACC)[12].

To clarify the influence of sensitization with *B. adusta* on the clinical manifestation in the patients with FACC, we compared the allergological findings and clinical characteristics in FACC patients who had a positive reaction to a bronchoprovocation test using an antigenic solution of *B. adusta* (AFC sensitized to *B. adusta*) with those in other FACC patients, who had a negative reaction to this test.

Methods

Seventeen patients with FACC who underwent bronchoprovocation tests using the antigenic solution of *B. adusta* were selected from among 21 FACC patients enrolled in the previous control study (reference number R000000432; Umin 000000114), and their medical records were reviewed. All 17 patients in this study were referred to Saiseikai Kanazawa Hospital between April 2005 and May 2006. This retrospective study was approved by the Institutional Review Board (reference number 2009003), and informed consent was obtained from each of the 17 patients.

Patient examinations such as blood tests, chest and sinus radiographs, induced-sputum examination, pulmonary function tests[13], a test for cough reflex sensitivity to capsaicin[14], bronchial reversibility in response to bronchodilators and bronchial responsiveness to methacholine[15], were performed in accordance with diagnostic criteria as recommended by the Japanese Cough Research Society[1] and Japanese Respiratory Society[2].

After the trial period of the previous control study[12], FACC patients were given an additional low dose of itraconazole (ITCZ) (50 mg/day) when their cough was not adequately controlled by the standard therapy recommended by the guidelines.

When the first complete remission of cough symptoms was achieved, the patients were then subjected to the bronchoprovocation test using the antigenic solution[16] (*B. adusta*: 2 m*l* of culture-filtrate antigen, 1 mg/m*l*), through a jet nebulizer. The responses were assessed as positive when the patients developed coughing attacks with a significant increase in the cough reflex sensitivity to inhaled capsaicin, and were then diagnosed as having AFC sensitized to *B. adusta* (AFC-Bj).

Allergological tests, such as an immediate-type skin test, serological test, and lymphocyte stimulation test using the antigenic solution of *B. adusta*, were conducted to perform stratified analysis of 17 patients with FACC. For the purposes of this analysis, the other FACC patients who showed a negative reaction to the bronchoprovocation test were described as the non AFC-Bj group.

This study examined the clinical course: duration of the for required complete remission of cough symptoms; total quantity of anti-fungal drugs administered; duration of the period until the first cough recurrence; frequency of the cough recurrence defined as an isolated non-productive cough lasting more than 3 weeks despite the presence of an upper respiratory tract infection; and duration until the development of asthma.

We then compared these factors between the two FACC groups (AFC-Bj and non AFC-Bj). The outcomes of patients who were in the care of their local physicians or who had discontinued treatment were assessed by direct telephone interviews.

Mycological study

Sputum samples obtained from the patients with FACC were cultured on Sabouraud's dextrose agar (SDA) containing chloramphenicol. The morphological features of the strains were observed by the slide culture method (30℃ for 2～3 weeks). When the white colonies had grown widely on SDA the resulting colonies were moved onto CHROMagar® *Candida* spread with micafungin sodium (Funguard®, 30 μ/plate)[17].

Allergological Study

Preparation of the antigenic solution

One liter of Sabouraud dextrose broth in 3 liter flasks was sterilized by autoclaving at 121℃ for 20 min. Five m*l* of *B adusta* (NBRC 4983) spore suspension (10^5 spores per m*l*) in sterile physiological saline from 14day-old Sabouraud dextrose agar culture were used to inoculate the flask. The flasks were shaken at 150 rpm in a 25℃ rotary shaker incubator. The supernatant was then dialyzed against 5 mM ammonium bicarbonate and lyophilized.

Reactions to the *Bjerkandera adusta* antigen

An antigenic solution (polysaccharide) was injected intradermally with a tuberculin syringe (0.02 m*l*, 1 mg /m*l*) to assess the skin response to the solution.The result was judged to be positive when the longer axis of the flare exceeded 9 mm 15 minutes after the injection[18].

B. adusta-specific IgE was measured by a previously described AlaSTAT-MP technique[19], and was defined as positive when greater than 0.35 U/m*l*.

Lymphocyte stimulation test

The lymphocyte stimulation test[20] was performed using the antigenic solution with the Lymphoprep system. The results were considered positive when the magnitude of the response to *B. adusta* was beyond 200% in comparison to the controls using PHA.

Statistical analysis

Statistical analysis of quantitative data was performed using the Mann-Whitney *U* test. Dichotomous data were analyzed using a χ^2 test. A P value of less than 0.05 was considered statistically significant.

Results

Information was gathered from a total of 17 FACC patients, seven of whom were females. Median patient age at referral was 49 years (range, 22 to 70 years). Chest and sinus radiographs were normal in all patients; only one was a current smoker.

Eleven of the 17 patients (64.7%) who showed positive reactions to the bronchoprovocation test were diagnosed as having AFC-Bj. The AFC-Bj patients had a median age of 52 (range, 22-70) years, and 45.5% were female. The respective values for the remaining six patients with non AFC-Bj were 48 (range, 36-60) years, and 33.3% were female. The age distribution and gender population did not differ substantially between the two groups.

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 bronchial reversibility in response to bronchodilators were observed in only one patient while in two patients, respectively, in the non AFC-Bj group.

Bronchial responsiveness to methacholine[15] was heightened in 2 patients (18.2%) in the AFC-Bj group and one patient in the non AFC-Bj group. There was no significant difference between the two groups. Cough reflex sensitivity as assessed by the estimation of the capsaicin cough threshold[14] was increased in 5 patients (45.5%) in the AFC-Bj group and in 3 understood (50.0%) in the non AFC-Bj group (Table 1). In addition, no significant difference was observed in the cough reflex sensitivity between the two groups.

The allergological findings of 17 patients are summarized in Table 2. No significant difference in either the WBC count or the eosinophil percentage in the peripheral blood (%) was observed between the 2 groups. The total serum IgE levels were elevated in 3 patients (17.6%) in the AFC-Bj group. The value of IgE was 155.7 ± 257.4 (mean ± SEM) in the AFC-Bj and 112.8 ± 75.2 IU/m*l* in the non AFC-Bj group. Eosinophilia in the induced sputum was observed in 2 AFC-Bj patients and in 2 non AFC-Bj patients. There were no significant differences in the sputum eosinophil percentage between the groups.

The results of an investigation on the serum *B. adusta*-specific IgE were available in 8 AFC-Bj patients and 4 non AFC-Bj patients, and the value was elevated in none of them.

Positive results for the immediate cutaneous reaction to *B. adusta* were observed in 5 of 11 AFC-Bj patients (45.5%) and in 0 of 6 non AFC-Bj patients, and the positive ratio was found to be significantly different ($p<0.05$). Positive results for the lymphocyte stimulation test were observed in 7 of 11 AFC-Bj patients (63.6%) and in 0 of 4 non AFC-Bj patients, and the positive ratio

Table 1. Characteristics of subjects

	Age	Gender	Smoking	FEV1 (l)	FEV1/FVC ratio (%)	Bronchial reversibility (%)	RT-Meth (μg/ml)	Cough threshold (μM/l)
AFC-Bj								
1	69	F	non	1.87	80.3	1.6	20,000	0.98
2	30	M	non	4.68	86.6	4.7	20,000	3.91
3	22	M	non	5.24	88.8	3.2	20,000	3.91
4	47	F	non	2.80	93.0	2.1	20,000	3.91
5	68	F	non	2.38	77.0	4.7	20,000	0.49
6	70	M	non	2.33	76.9	-0.4	20,000	31.3
7	41	M	non	3.61	86.4	-1.4	1,250	31.3
8	52	M	non	3.20	83.1	-2.3	20,000	1.95
9	60	F	non	2.73	88.1	0.4	5,000	0.49
10	48	M	non	4.08	83.3	2.0	20,000	0.98
11	70	F	non	2.92	80.2	2.7	20,000	1.95
Non AFC-Bj								
12	42	M	non	2.79	74.6	2.2	20,000	31.3
13	36	M	non	4.19	93.1	2.1	20,000	0.98
14	60	F	non	2.41	72.6	7.8	5,000	3.91
15	49	F	non	2.94	77.2	2.7	20,000	3.91
16	60	M	20 x 35	3.54	66.9	-0.6	20,000	1.95
17	46	M	non	3.67	77.9	7.6	20,000	0.98

FEV1 : forced expiratory volume in 1 sec
FEV1% : the ratio of forced expiratory volume in 1 sec (FEV1) to forced vital capacity (FVC)
RT-Meth : respiratory threshold of methacholine (mg/ml) required to cause a 20% or more fall in FEV1 from the baseline value.

Table 2. Allergological findings

	WBC (/μl)	Eosinophil (%)	IgE (IU/ml)	Sputum-eosinophil (%)	Skin reaction Immediate	Late	BJ-specific IgE (U/ml)	LST for BJ
AFC-Bj								
1	7,400	0.7	8	0	2 × 3 / 0 × 0	0 × 0 / 0 × 0	0.35>	positive
2	4,300	2.2	48	0	5 × 5 / 28 × 30	4 × 5 / 24 × 20	0.35>	negative
3	5,100	1.5	893	1	6 × 7 / 32 × 52	0 × 0 / 0 × 0	0.35>	negative
4	4,000	1.2	56	0	8 × 8 / 0 × 0	NC	0.35>	negative
5	4,800	2.7	94.3	17	4 × 4 / 0 × 0	0 × 0 / 0 × 0	0.35>	positive
6	5,800	0.9	229	2	7 × 7 / 30 × 30	0 × 0 / 0 × 0	0.35>	positive
7	4,800	1.0	238	0	10 × 10 / 24 × 22	0 × 0 / 0 × 0	0.35>	positive
8	5,400	2.0	68	0	0 × 0 / 0 × 0	NC	ND	negative
9	3,800	1.5	15	7	6 × 6 / 26 × 24	0 × 0 / 0 × 0	0.35>	positive
10	5,200	1.4	55	3	8 × 6 / 0 × 0	0 × 0 / 0 × 0	ND	positive
11	5,600	3.4	8	0	2 × 2 / 0 × 0	NC	ND	positive
Non AFC-Bj								
12	7,100	4.6	228	ND	0 × 0 / 0 × 0	0 × 0 / 0 × 0	0.35>	ND
13	3,900	1.3	34	0	8 × 6 / 0 × 0	NC	0.35>	negative
14	6,800	3.8	145	54	0 × 0 / 0 × 0	0 × 0 / 0 × 0	ND	negative
15	5,600	2.5	153	3	3 × 3 / 3 × 3	NC	0.35>	ND
16	4,100	4.6	70	2	2 × 2 / 2 × 2	NC	0.35>	negative
17	6,600	4.3	47	37	0 × 0 / 0 × 0	6 × 6 / 0 × 0	ND	negative

Eo : Eosinophil
Sp-Eo : Eosinophilia in the induced sputum
LST : The lymphocyte stimulation test
BJ : *Bjerkandera adusta*

Table 3. The clinical manifestations

	Duration of time for the first CR (w)	Total amount of ITCZ for the first CR (mg)	Recurrence time	Period until the first cough recurrence (w)	Period until BA onset (w)	Eradication	Sputum culture on relapse
AFC-Bj							
1	28	4,250	2	22	16	successful	BM
2	17	4,350	1	17		successful	*Aspergillus niger*
3	12	2,050	3	47		successful	BM
4	15	2,800	2	24	124	successful	BM
5	28	1,250	2	42	57	successful	BM
6	20	1,050	2	34		n.o.	*Penicillium*
7	20	250	1	10	10	n.o.	n.o.
8	25	3,900	2	16		successful	n.o.
9	43	3,550	1	31		successful	BM / *Aspergillus niger*
10	26	1,400	2	22		n.o.	BM
11	19	1,200	2	34		successful	*Aspergillus fumigatus*
Non AFC-Bj							
12	6	700	0			n.o.	
13	6	700	1	32		successful	
14	7	600	0			n.o.	
15	10	1,050	0			n.o.	
16	5	2,250	1	64		n.o.	
17	7	700	1	24	36	successful	

CR: complete remission n.o.: not obtained BM: basidiomycetous fungi

was also found to be significantly different ($p < 0.05$).

The clinical manifestations of the FACC patients are summarized in Table 3. The duration of time required to achieve a complete remission of the cough symptoms in AFC-Bj patients (median 20, range 12-43 weeks) was significantly longer than that in non AFC-Bj patients (median 6.5, range 6-10 weeks; $p=0.0009$). The total quantity of anti-fungal drugs (ITCZ) was $2,368.2 \pm 1,458.6$ mg in the AFC-Bj group and 1000.0 ± 631.7 mg in the non AFC-Bj group; there was significant difference in the total quantity of ITCZ between the two groups ($p=0.031$). The period until the first cough recurrence was 27.2 ± 11.4 weeks in the AFC-Bj group and 40.0 ± 21.2 weeks in the non AFC-Bj group, and thus was no significant difference in this factor between the two groups.

The median recurrence time after the first complete remission was twice (range 1-3) in the AFC-Bj group, which was significantly more frequent than the median of 0.5 (range, 0-1) in the non AFC-Bj group ($p=0.0042$).

The BM fungi of sputum samples were successfully eradicated in 8 patients (72.7%) in the AFC-Bj group and in 2 (33.3%) in the non AFC-Bj group. When we were unable to obtain a sputum sample from a patient because of an improvement in symptoms, the results regarding fungal eradication were not evaluated.

The BM fungi were cultured again for 6 patients (54.5%) who had relapses of coughing attacks.

The onset of typical asthma occurred in 4 of 11 patients (36.4%) with AFC-Bj and in 1 of 6 patients (16.7%) with non AFC-Bj, thus not differing substantially between the 2 groups.

Discussion

It is important to elucidate the etiological agents and the exacerbating factors in allergic fungal diseases. Based on studies[21] of patients with atopic cough (AC)[22,23] associated with environmental fungi[24-27], our recent research has focused on the possible role of BM fungi[28] as a fungal aeroallergen.

A new clinical disease concept termed FACC[12] includes the following manifestations: (1) chronic cough; (2) the presence of environmental fungi, particularly BM fungi, in sputum; and (3) a good clinical response to antifungal drugs. The proposal of this new disease concept suggests that antifungal drugs may be efficacious in a subset of chronic coughers.

Recently the presence of BM fungi in induced-sputum has been shown to be an important factor in distinguishing the clinical manifestations of CIC from those of non-CIC[10]. From these results, it is essential to identify those fungi that are associated with chronic cough among the approximately 30,000 known species of BM fungi.

Lately, 8 patients with FACC whose sputum yielded *B. adusta*, one of the BM fungi[11] and who was sensitized to the fungus were reported to be diagnosed as having AFC caused by *B. adusta* (Yakeirotake cough). That report also demonstrated that the FACC patients who yielded *B. adusta* in their sputum were not always intractable. It is therefore considered that sensitization to the fungus is more important for the clinical manifestation of chronic coughers than the colonization of the fungus.

In the present study, 11 patients were allocated to the AFC-Bj group and 6 to the non AFC-Bj group. Although the latter 6 may have been sensitized to BM fungi other than *B. adusta* or to none of the BM fungi, for the purposes of the present stratified analysis the latter group was described as the non AFC-Bj group.

From the results of the allergological findings, the positive ratios for the immediate cutaneous reaction and the lymphocyte stimulation test to *B. adusta* were found to be significantly higher in the AFC-Bj group.

Because the inhalation provocation test is not commonly used in most clinics, it is suggested that the skin test and/or the LST to *B. adusta* may currently be a useful substitute to identify those with AFC-Bj among FACC patients.

The time required for complete remission of cough symptoms was longer, and the recurrence ratio of coughing was more frequent in the AFC-Bj group than in the non AFC-Bj group. The results of the clinical manifestations showed clearly that AFC-Bj is involved in a more intractable type of FACC.

Case reports have demonstrated the efficacy of antifungal drug therapies in AC in which sputum examinations yielded BM fungi[29,30]. Most airborne fungal structures are spores or conidia. There are robust, hydrophobic structures essentially devoid of allergenic proteins. Repeated exposure to viable hyphal fragments, which contain numerous diffusible allergens, is sufficient to directly drive an ongoing allergic response, and exposure to fungal spores is irrelevant. Gonzalez et al. also reported that *B. adusta* was susceptible *in vitro* to ITCZ, amphotericin B, and voriconazole, with MIC values of 0.125 μg/ml, 0.5 μg/ml and 0.5 μg/ml, respectively[31]. Therefore, such antifungal therapy may have some advantages in either reducing or eradicating this type of antigen exposure[32].

Although ITCZ therapy demonstrated some efficacy in all FACC patients, we were not able to fully manage the symptoms in those with AFC-Bj; therefore, the total quantity of anti-fungal drugs (ITCZ) was significantly higher in this group. To decrease the total number of anti-fungal drugs, a new strategy for ITCZ therapy will thus be required that can be planned and examined prospectively in the near future.

Though the asthma onset frequency did not differ substantially between the two groups, we should follow the FACC patients carefully to estimate whether sensitization to *B. adusta* may become one of the risk factors for onset of asthma.

To simplify the isolation of the BM fungi among other environmental fungi cultured from the sputum samples obtained from FACC patients, we prepared CHROMagar® *Candida* spread with micafungin sodium (Funguard®, 30 μ/plate)[17]. Micafungin is active against species of *Candida* and *Aspergillus* as well as *Penicillium* spp., *Scedosporium apiospernum*, and *Acremonium* spp., it was recently revealed to be inactive for species of Basidiomycota and for multiresistant species such as those of *Fusarium*[33].

First, the sputum samples obtained from the patients with FACC were cultured on SDA containing chloramphenicol. The morphological features of the strains were observed for about 10～14 days. When white lot grew widely on SDA; the yielded colonies were than moved onto a prepared plate spread with micafungin sodium. Because *Fusarium* is easily distinguished macroscopically, the cultured colonies of the fungi were identified as BM fungi.

The developed plates were useful to follow up on the presence of BM in the obtained sputum specimens. The successful treatment with ITCZ for FACC seems to be associated with the eradication of BM fungi. Furthermore, with a relapse of coughing attacks, BM fungi were again detected in many of the AFC-Bj cases. Therefore, not only the administration of antifungal drugs, but also environmental maintenance may play an important role in successfully managing these

patients.

B. adusta is a BM fungus that colonizes rotting wood, and it is distributed throughout the world. Recently, we found that the growth of the filamentous *B. adusta* was observed at 4℃ to 37℃ on SDA and asexual spores, arthroconidia, which are produced from the hyphae in abundance (A detailed report will follow in another manuscript.). The year-long sensitization caused by *B. adusta* may therefore be part of the reason why this fungus, which is a mushroom growing primarily in fields, acts as an allergen in winter as well as in summertime.

By comparing the allergological findings and the clinical characteristics in the AFC-Bj patients with non AFC-Bj patients, we concluded that *B. adusta*, a BM fungus, was one of the environmental fungi deserving attention because of its possible role in enhancing the cough severity of FACC patients via sensitization to this fungus. The utility of anti-fungal drugs may therefore provide a new treatment strategy for CIC.

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