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Skin Test Reactivity to Recombinant *Aspergillus fumigatus* Allergen I/a in Patients with Cystic Fibrosis

Key Words

Recombinant *Aspergillus fumigatus*
allergen I/a
Allergic bronchopulmonary
aspergillosis
Cystic fibrosis
Recombinant allergen
Skin prick test

Abstract

We evaluated the diagnostic value of recombinant *Aspergillus fumigatus* allergen I/a (rAsp f I/a) for skin testing in cystic fibrosis (CF) patients with allergic bronchopulmonary aspergillosis (ABPA), allergy to *A. fumigatus* and in CF controls not allergic to *A. fumigatus*. Skin prick test reactions to rAsp f I/a were 8.5-fold stronger in *A. fumigatus*-allergic and 3.3-fold stronger in ABPA patients compared to the CF controls. Three out of 6 well-characterized ABPA patients had positive skin prick test reactions to rAsp f I/a; the same number reacted to crude *A. fumigatus* extracts. The skin prick test results to rAsp f I/a were significantly correlated with those to crude and commercial preparations. According to these results, rAsp f I/a represents a defined allergen that provides the possibility to perform skin prick tests in CF patients in a highly standardized way. In future, a larger panel of recombinant allergens might also pick up those CF patients with ABPA and allergy to *A. fumigatus* who did not react to this single allergen.

Introduction

Allergy to *Aspergillus fumigatus* has been reported in up to 59% of patients with cystic fibrosis (CF) [1], while only few patients develop allergic bronchopulmonary aspergillosis (ABPA) [2]. The differentiation between ABPA and allergy to *A. fumigatus* is difficult but clinically relevant, since untreated ABPA may lead to substantial lung damage including proximal bronchiectasis or total lung collapse [3, 4]. The importance of IgE and IgG antibodies to *A. fumigatus* has been emphasized in ABPA [5], but both antibody classes can also be detected in 20–30% of *A. fumigatus*-allergic patients suffering from CF [2]. The reliability of diagnostic procedures such as skin tests depends mostly on

the quality of the antigen preparations. In commercial preparations, the content of *A. fumigatus* allergens usually varies considerably among different isolates [6] and may be due to rapid changes in IgE-binding components expressed during culture [7] or different sources used to prepare allergen extracts such as mycelia, spores or culture filtrates [8]. To overcome the problems of impurity of allergen preparations, attempts have been made to standardize the production of *A. fumigatus* extracts [7, 9] or to purify various allergens biochemically [10, 11]. Even these apparently homogeneous allergens may be contaminated by degradation products or other allergenic components in trace amounts but are of similar immune reactivity.

The production of purified recombinant allergens of defined biological activity has been a major advance [12]. Recently, recombinant *A. fumigatus* antigen I/a (rAsp f I/a), a major allergenic protein of *A. fumigatus*, has been cloned and expressed [13]. rAsp f I/a expressed in *Escherichia coli*

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and purified by absorption chromatography was shown to express the same functional characteristics as the native 18-kD ribotoxin and can be used as a defined allergen or antigen for the diagnosis of *A. fumigatus*-associated diseases [13]. In a recently published study, we have demonstrated that *rAsp-f-I*-specific serology is a highly sensitive and specific test to reliably identify ABPA in CF patients [14]. The serologic assays revealed a 10-fold increase in *rAsp-f-I*-specific IgE, a 5-fold increase in *rAsp-f-I*-specific IgG1 and a 4-fold increase in *rAsp-f-I*-specific IgG4 antibodies in ABPA patients compared with the *A. fumigatus* allergy and CF control group. Now we have set up a study to evaluate the diagnostic value of *rAsp-f-I* for skin testing in CF patients with ABPA, allergy to *A. fumigatus* and CF controls not allergic to *A. fumigatus*.

Patients and Methods

Subjects

Forty-four patients, 26 males and 18 females with CF, admitted to the Alpine Children's Hospital in Davos, Switzerland, were entered into the study. All patients had the same routine assessment for CF and allergy. The mean age of the patients was 21.1 ± 7.5 years. The diagnosis of CF had been substantiated by the sweat iontophoresis test with sweat sodium concentrations of > 70 mmol/l in at least 2 specimens of > 100 mg of sweat [15]. All patients enrolled in the study were informed verbally and in writing about the purpose and the procedure of the study. Approval for skin testing human subjects with recombinant allergens was obtained from the official ethics committee of Davos before starting the study.

The CF patients were classified into three groups according to the following criteria:

(1) ABPA patients: the diagnosis of ABPA was based on a minimum of 6 out of 7 criteria described by Nelson et al. [16]: (1) wheezing, (2) pulmonary infiltrate, (3) positive sputum culture of *A. fumigatus*, (4) positive immediate reaction to *A. fumigatus* extracts in skin prick testing (i.e. weal ≥ 3 mm in diameter) [17], (5) increased total IgE levels (i.e. IgE more than 2 SD greater than normal values for age) [18], (6) increased specific IgE to *A. fumigatus* (RAST score ≥ 2), (7) increased level of specific IgG to *A. fumigatus* (ELISA > 40 EU/ml) [14, 19].

(2) *Aspergillus*-allergic CF patients: CF patients were assigned to the group with *Aspergillus* allergy if routine skin prick tests with a commercial allergen preparation (Bencard, Smithkline Beecham, Neuss, Germany) were positive for *A. fumigatus* (weal ≥ 3 mm in diameter) [17] or the RAST to *A. fumigatus* was ≥ 2 .

(3) CF control group: CF patients were assigned to the CF control group if routine skin prick tests with a commercial allergen preparation (Bencard) were negative for *A. fumigatus* (weal < 3 mm in diameter) [17] and the RAST score to *A. fumigatus* was < 2 .

Routine Assessment

Each patient had a routine assessment when admitted to the hospital including sputum cultures for bacteria and fungi, chest radiography, lung function test and skin prick testing to *A. fumigatus* and a range of

other allergens (Bencard). None of the patients received antihistamine medications at the time of the study. The skin test reactions were recorded after 15 min by measuring the diameter of the weal, and the results were considered positive with a weal of ≥ 3 mm and no reaction to the saline control solution [17]. A positive control was included with 0.1% histamine.

Blood samples were taken for routine blood cell count, IgE and IgG antibody determination, RAST for IgE antibodies to *A. fumigatus* (Pharmacia, Uppsala, Sweden) using the allergen-specific CAP system and converting the results into RAST classes from 0-4 as described by the manufacturer (RAST class 0: < 0.35 IU/ml, class 1: 0.35-0.69 IU/ml, class 2: 0.70-3.49 IU/ml, class 3: 3.50-17.49 IU/ml, class 4: ≥ 17.50 IU/ml). IgG antibodies to *A. fumigatus* were analyzed by ELISA using standardized DPC antigens (Diagnostic Products Corporation, Los Angeles, Calif., USA) bound to solid phase [19].

Skin Prick Tests or *rAsp-f-I*, Crude and Commercial Extracts

The patients included into the study had extra skin prick tests to *rAsp-f-I*, two crude antigens, DPC and LP-CF (late-phase culture filtrate, kindly provided by Dr. Kautzman, Groningen, The Netherlands), and three commercially available antigens, Bencard, ALK (Horsholm, Denmark) and Allergopharma (Hamburg, Germany). *rAsp-f-I* and the two crude extracts were used at protein concentrations of 0.1 mg/ml [13], the three commercial extracts as recommended by the supplier. The results were recorded after 15 min by measuring the maximal longitudinal (D1) and transversal (D2) diameter of the weal. The surface (S) of the weal was calculated according to the following formula: $S = [(D1 + D2)/2]^2$. The results were considered positive with a surface of the weal ≥ 9 mm² [17]. Positive and negative control solutions were the same as in the routine skin prick test.

Statistics

Data were analyzed with the Systat® computing system for statistics (Systat Inc., Evanston Ill., USA). The paired Wilcoxon test was used for comparison of repeated values within the same subject and the Mann-Whitney U test for comparison of data between different subjects. Categorical data were compared by the Pearson χ^2 test with Yates' correction for small numbers. Correlation coefficients were determined using Pearson's linear-regression analysis. Differences associated with probabilities of $p < 0.05$ were considered significant.

Results

Six of the 44 CF patients were diagnosed as having ABPA, 3 of them with 7 and 3 with 6 of Nelson's criteria (table 1). According to the inclusion criteria, 25 CF patients were assigned to the group with allergy to *A. fumigatus*: 23 had positive skin prick tests to *A. fumigatus*, and 2 other patients had RAST scores higher than 2 despite negative skin prick tests. Thirteen CF patients were assigned to the control group. The mean Shwachman score, assessing the severity degree of the disease [20], from all patients was 60.9 ± 15.2 , indicating that the patients were moderately to severely affected. Five patients (11%) including 2 ABPA patients (patients 1 and 2, table 1) used oral corticosteroids in

Table 1. Nelson's criteria in CF patients with ABPA

Patient	Age years	Sex	Wheezing	Infiltrates	<i>A. fumigatus</i> sputum	<i>A. fumigatus</i> prick, mm	IgE IU/ml	<i>A. fumigatus</i> RAST (0-4)	<i>A. fumigatus</i> IgG, EU/ml
1	11	f	+	+	+	3	2,400	4	162
2	12	f	+	+	-	5	5,230	4	147
3	7	m	+	+	+	3	3,800	4	50
4	17	m	+	+	-	4	5,420	4	40.6
5	22	f	+	+	-	4	1,530	4	50.2
6	15	f	+	+	+	3	1,020	4	82.2

low doses (prednisolone 5–10 mg/day), and 15 patients (34%) inhaled corticosteroids. There was no significant difference between the study groups regarding the severity of the disease ($p > 0.05$, Mann-Whitney U test) or the distribution of patients with steroid therapy ($p > 0.05$, Pearson's χ^2 test).

Table 2 compiles the data from the *routine clinical assessment* and compares the results between the study groups. Skin prick test reactions to *A. fumigatus* were significantly stronger in ABPA and *Aspergillus*-allergic patients compared to the CF control group ($p < 0.0001$, Mann-Whitney U test). Total IgE was significantly raised in ABPA compared to CF controls and *Aspergillus*-allergic patients ($p < 0.005$). *Aspergillus*-allergic patients had significantly higher IgE levels compared to the CF controls ($p < 0.005$). ABPA patients had significantly higher *Aspergillus* RAST scores than *Aspergillus*-allergic patients or CF controls ($p < 0.005$), and *Aspergillus*-allergic patients had significantly higher RAST scores than CF controls ($p < 0.0001$). IgG antibodies to *A. fumigatus* were significantly higher in ABPA patients compared to *Aspergillus*-allergic patients ($p < 0.05$).

Table 3 shows the single patient results of the *extra skin prick test* to *A. fumigatus*. Skin prick test reactions to rAsp f I/a significantly correlated with DPC ($r = 0.64$, $p < 0.0001$), Bencard ($r = 0.45$, $p < 0.005$) and ALK ($r = 0.42$, $p < 0.005$). There was a tendency towards significance with LP-CF ($r = 0.27$, $p = 0.076$) and Allergopharma ($r = 0.27$, $p = 0.082$). Skin prick test reactions between both crude and the three commercial preparations significantly correlated with each other. The best correlation was found between ALK and Allergopharma ($r = 0.85$, $p < 0.0001$). The correlation coefficient between ALK and Bencard was $r = 0.71$ ($p < 0.0001$) and between Allergopharma and Bencard $r = 0.64$ ($p < 0.0001$). The correlation coefficient between the crude preparations LP-CF and DPC was $r = 0.74$ ($p < 0.0001$), similar to the correlation coefficients between LP-CF and the

Table 2. *A. fumigatus*-related routine assessment in CF patients

	CF controls (n = 13)	<i>A. fumigatus</i> allergy (n = 25)	ABPA (n = 6)
<i>A. fumigatus</i> prick, mm	0.0 ± 0.0	4.8 ± 2.3 ^b	3.7 ± 0.8 ^c
Eosinophil count × 10 ⁹ cells/l	0.26 ± 0.25	0.33 ± 0.21	0.87 ± 0.85
IgE, IU/ml	41 ± 69	290 ± 274 ^a	3,230 ± 1,880 ^{a,d}
<i>A. fumigatus</i> RAST score (0-4)	0.26 ± 0.50	2.73 ± 1.19 ^b	4.00 ± 0.00 ^{a,d}
<i>A. fumigatus</i> IgG antibodies, EU/ml	39.1 ± 39.8	43.9 ± 49.1	88.7 ± 53.1 ^c

^a $p < 0.005$, ^b $p < 0.0001$, versus CF controls; ^c $p < 0.05$, ^d $p < 0.005$, versus *A. fumigatus* allergy.

three commercial preparations ($r = 0.71$ – 0.72 , $p < 0.0001$). The correlation between skin prick test reactions with DPC and the three commercial preparations was slightly lower ($r = 0.51$ – 0.53 , $p < 0.0005$).

Compared to the classification of the routine assessment there was a considerable number of divergent results in the extra skin prick test, since 4 of the 6 ABPA patients were skin prick test negative with LP-CF allergens, 3 with rAsp f I/a, 2 with Bencard and DPC, and 1 with Allergopharma. Thirteen of the 25 CF patients, previously classified as allergic to *A. fumigatus*, were skin prick test negative with rAsp f I/a, 7 with LP-CF and DPC, 5 with Bencard, 4 with ALK and 3 with Allergopharma. Three of the 13 CF control patients, previously not allergic to *A. fumigatus*, were skin prick test positive with ALK allergens, 2 with Allergopharma and 1 with rAsp f I/a.

Table 4 summarizes the results of the *extra skin prick test* to *A. fumigatus*. The size of the wheal was significantly larger with ALK preparations compared to Allergopharma

Table 3. Skin prick tests (mm²) to *rAsp f* I/a and other *A. fumigatus* preparations in CF patients with ABPA and *A. fumigatus* allergy

Patient	Diagnosis	<i>rAsp f</i> I/a	ALK	Allergo- pharma	Bencard	DPC	LP-CF
1	ABPA	0	20	36	25	0	0
2	ABPA	9	36	36	18	14	4
3	ABPA	12	30	23	16	12	9
4	ABPA	16	16	25	14	12	9
5	ABPA	2	12	4	1	16	0
6	ABPA	3	12	16	2	0	0
		3 6 pos.	6 6 pos.	5 6 pos.	4 6 pos.	4 6 pos.	2 6 pos.
7	<i>A. fumigatus</i> allergy	64	121	105	64	23	18
8	<i>A. fumigatus</i> allergy	0	116	49	28	16	18
9	<i>A. fumigatus</i> allergy	4	12	56	9	5	5
10	<i>A. fumigatus</i> allergy	2	5	6	9	8	9
11	<i>A. fumigatus</i> allergy	16	60	42	25	28	28
12	<i>A. fumigatus</i> allergy	3	53	28	12	25	16
13	<i>A. fumigatus</i> allergy	0	42	46	6	4	1
14	<i>A. fumigatus</i> allergy	11	42	42	42	33	30
15	<i>A. fumigatus</i> allergy	14	81	56	46	100	53
16	<i>A. fumigatus</i> allergy	20	110	72	56	28	46
17	<i>A. fumigatus</i> allergy	68	64	56	20	150	39
18	<i>A. fumigatus</i> allergy	6	28	20	12	23	2
19	<i>A. fumigatus</i> allergy	4	4	4	4	4	4
20	<i>A. fumigatus</i> allergy	156	196	77	56	100	16
21	<i>A. fumigatus</i> allergy	12	72	39	11	20	16
22	<i>A. fumigatus</i> allergy	0	324	169	46	36	46
23	<i>A. fumigatus</i> allergy	0	6	10	9	2	0
24	<i>A. fumigatus</i> allergy	12	72	30	53	16	20
25	<i>A. fumigatus</i> allergy	2	46	36	20	33	23
26	<i>A. fumigatus</i> allergy†	11	25	23	9	16	9
27	<i>A. fumigatus</i> allergy	16	138	90	100	39	28
28	<i>A. fumigatus</i> allergy	2	56	64	4	12	9
29	<i>A. fumigatus</i> allergy	9	4	4	2	0	2
30	<i>A. fumigatus</i> allergy	3	156	218	30	64	36
31	<i>A. fumigatus</i> allergy	8	56	9	5	4	6
		12 25 pos.	21 25 pos.	22 25 pos.	20 25 pos.	18 25 pos.	18 25 pos.
32-44	CF controls	12 13 neg.	10 13 neg.	11 13 neg.	13 13 neg.	13 13 neg.	13 13 neg.

($p < 0.05$, Wilcoxon test) and the other preparations ($p < 0.0001$). Skin prick test reactions with Allergopharma were significantly stronger compared to DPC, Bencard, *rAsp f* I/a and LP-CF ($p < 0.0005$). Skin prick test reactions with DPC and Bencard did not differ significantly and were both stronger compared to *rAsp f* I/a and LP-CF ($p < 0.05$). There was no significant difference between the latter two.

CF patients who by definition had been assigned to the group with *Aspergillus* allergy again had significantly stronger reactions than CF control patients with Bencard

allergens ($p < 0.0001$, Mann-Whitney U test) but also with ALK, Allergopharma, DPC, LP-CF ($p < 0.0001$) and *rAsp f* I/a ($p < 0.005$). ABPA patients had significantly stronger reactions than CF controls ($p < 0.05$) with *rAsp f* I/a ($p < 0.05$) and the other five preparations ($p < 0.005$). Skin prick test reactions to ALK, DPC and LP-CF allergens were significantly stronger in patients with *Aspergillus* allergy than ABPA ($p < 0.05$). Skin prick test reactions to histamine were not significantly different between the three patient groups.

Table 4. Skin prick tests (mm²) to *A. fumigatus* in CF patients

	CF controls (n = 13)	<i>A. fumigatus</i> allergy (n = 25)	ABPA (n = 6)
r <i>Asp f</i> I a	2.1±6.3	17.8±33.7 ^b	7.0±2.6 ^a
ALK	3.5±5.3	75.6±72.2 ^{c,d}	21.1±9.9 ^b
Allergopharma	2.6±3.8	54.1±50.2 ^c	23.3±12.3 ^b
Bencard	0.6±1.6	27.1±24.7 ^c	12.7±9.4 ^b
DPC	0.1±0.4	31.5±36.2 ^{c,d}	9.1±7.2 ^b
LP-CF	0.0±0.0	19.1±15.4 ^{c,d}	3.7±4.4 ^b
Histamine 0.1%	38.6±30.5	25.8±12.0	24.0±8.1

^ap<0.05, ^bp<0.005, ^cp<0.0001, versus CF controls; ^dp<0.05, versus ABPA.

Discussion

We have evaluated the diagnostic value of r*Asp f* I/a for skin prick testing in three groups of CF patients and compared the results with two crude and three commercial *A. fumigatus* extracts. Skin prick test reactions to r*Asp f* I/a significantly correlated with those to the crude extract from DPC and the commercial preparations from Bencard and ALK. There was a tendency towards significant correlations with LP-CF and Allergopharma allergens. r*Asp f* I/a, which has the same functional characteristics as the native 18-kD ribotoxin [13], has already been proven to be of diagnostic value for skin testing in adult asthmatics [21] and patients with atopic dermatitis [22]. In a previous study, we have shown that r*Asp f*-I a-specific serology allowed a reliable diagnosis of ABPA in CF patients even without knowledge of any clinical data [14]. From this study, we can confirm that r*Asp f* I/a represents a defined allergen that provides the possibility to perform skin prick testing in CF patients in a highly defined way.

Comparing single patients results there was a considerable number of divergent results between the different *Aspergillus* preparations used in the study. We found a discrepancy between the results of the routine and the extra skin prick tests, both performed with the same extract. We believe that this was due to the variable allergen content in the preparations [23] especially since different charges were used for the routine and the extra skin prick test. Furthermore, the LP-CF antigens are generally used for IgG ELISA and the double immunodiffusion test in the diagnosis of different manifestations of aspergillosis. LP-CF have been shown to contain large numbers of precipitating antigens [9]. For high IgE binding generally mycelial and/or

spore-derived antigenic extracts are used [9]. Commercial allergen preparations are usually heterogeneous mixtures of proteins and glycoproteins and may also contain components such as lectins, endotoxins, hemolytic toxins and histamine [10, 24, 25]. Even standardized extracts and biochemically purified *A. fumigatus* allergens may be contaminated by degradation products of other allergenic components [26]. This emphasizes the need for standardized reagents for routine tests in the clinical practice. This requirement is perfectly fulfilled by recombinant allergens which can be produced in large amounts as highly pure proteins [12, 13].

Allergy to *A. fumigatus* is common in CF [1, 2] but must clearly be distinguished from ABPA which has a low prevalence rate of 0.5–11% [2, 16, 27, 28]. The wide range of prevalences might be explained by differences in defining the condition but might also be due to the quality of *A. fumigatus* allergens used for testing [29]. In our study skin prick test reactions to r*Asp f* I/a were 8.5-fold stronger in *Aspergillus*-allergic and 3.3-fold stronger in ABPA patients compared to the CF controls. The routine assessment showed significantly higher total IgE and RAST to *A. fumigatus* in ABPA patients compared to *Aspergillus*-allergic patients. However, there was no significant difference between skin test results obtained from ABPA and *Aspergillus*-allergic patients using the highly pure, biologically active r*Asp f* I/a. However, several other major antigens of *A. fumigatus* have been described that can be released and provoke immunologic reactions [26, 29]. Recently the availability of a whole panel of recombinant *A. fumigatus* allergens has been reported [30]. Preliminary skin test results with selected allergens distinct from r*Asp f* I/a showed that *A. fumigatus*-allergic or ABPA patients with negative skin tests to r*Asp f* I/a can react to different recombinant allergens from *A. fumigatus* (data not shown). This panel of allergens will provide an ideal test system for further studies which might help to better understand the immunologic mechanisms involved in ABPA and allergy to *A. fumigatus* and perhaps allow a differential diagnosis of ABPA.

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