

Original article

IgE antibody to *Aspergillus fumigatus* recombinant allergens in cystic fibrosis patients with allergic bronchopulmonary aspergillosis

Background: Allergic bronchopulmonary aspergillosis (ABPA) in cystic fibrosis (CF) is characterized by a heightened Th2 CD4⁺ T-cell response to *Aspergillus fumigatus* (Af) allergens and a hyper-immunoglobulin E (IgE) state compared with cystic fibrosis patients without ABPA. The IgE serologic differentiation of ABPA from atopic CF patients can be difficult. We propose as the reactivity with purified antigens varies qualitatively and quantitatively and that the antibody response is more specific than with crude Af antigen extract, the IgE responses to purified recombinant Af allergens may differentiate ABPA from atopic CF patients.

Methods: Serum IgE reactivity to seven recombinant purified allergens and to a crude extract of Af was measured in 15 ABPA, in 23 Af skin test positive (ST+), and in 19 Af skin test negative (ST−) CF patients. Four of the ABPA CF patients were studied before and after developing ABPA. Nine ABPA patients were studied during flares and remissions of ABPA.

Results: Allergic bronchopulmonary aspergillosis patients had significantly increased IgE reactivity to Asp f2, f3, f4, f6, and f16 compared with the Af ST+ and ST− non-ABPA CF patients. In the ABPA patients studied before and after developing ABPA, IgE reactivity also increased to Asp f2, f3, f4, and f6, and to the crude extract. In ABPA CF patients, IgE reactivity to Asp f1, f2, f3, and f6 significantly increased during periods of ABPA flares compared with periods of remission. Analysis of the receiver operating curve demonstrated that IgE reactivity to Asp f3 and f4 gave the best sensitivity and specificity and were better than IgE reactivity to a crude extract of *Aspergillus*. Furthermore, in ABPA patients studied during periods of remission the IgE reactivity to Asp f3 and f4 remained significantly elevated compared with Af ST+ non-ABPA patients. The IgE responses when considered either to be positive or negative to Asp f3 and f4 significantly differentiated ABPA from Af ST+ and ST− non-ABPA CF patients. In contrast, IgE reactivity was considered positive to the crude extract in 89% of ABPA, 61% of Af ST+, and 0% of Af ST− non-ABPA CF patients.

Conclusions: Immunoglobulin E reactivity to a panel of purified Af allergens, especially to Asp f3 and f4, differentiates ABPA from atopic Af ST+ non-ABPA CF patients. Serial determinations of IgE reactivity to individual purified *Aspergillus* antigens, especially Asp f3, demonstrates that increases in IgE reactivity may provide improved distinction between stages of flares and remission compared with changes in IgE reactivity to a crude *Aspergillus* extract.

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Allergic bronchopulmonary aspergillosis (ABPA) occurs in approximately 7–10% of patients with cystic fibrosis (CF) and can contribute to worsening of their pulmonary

disease (1). ABPA is an allergic late-phase inflammatory response to *Aspergillus fumigatus* characterized by skewed CD4⁺ Th2 T-cell responses, hyperimmunoglobulin immunoglobulin E (IgE) and bronchial eosinophilia that leads to pulmonary infiltrates, bronchiectasis and fibrosis (1). Sensitization to *A. fumigatus* in CF has been reported to increase with age and to be as high as 56% (2). In

Abbreviations: ABPA, allergic bronchopulmonary aspergillosis; Af, *Aspergillus fumigatus*; CF, cystic fibrosis; Ppn, precipitin; ST, skin test.

nonatopic patients, immunologic responses to *A. fumigatus* is predominantly a Th1 CD4⁺ T cell and IgG antibody response; whereas in atopic patients, a Th2 CD4⁺ T-cell response and IgE and IgG antibody responses prevail with a clinical spectrum of allergic sensitivity to ABPA (1).

Furthermore, there is overlap of clinical symptoms of pulmonary infections in CF and ABPA, namely pulmonary infiltrates, wheezing, and bronchiectasis. As sensitization to *A. fumigatus* in CF is frequent with elevated IgE and IgG anti-*Aspergillus* antibodies and elevated total IgE levels are frequent in CF, it is not clear whether ABPA is contributing to the pulmonary disease in CF patients or is it only adding to severity of the response. Recently, Cramer et al. reported that *Aspergillus*-sensitized non-ABPA patients develop IgE antibodies to limited number of *Aspergillus* allergens, Asp f1 and f3; whereas, ABPA patients develop antibodies to these allergens plus others including Asp f4 and f6 (3–5). In addition, Kurup et al. reported on the specificity of Asp f2 and Asp f16 IgE antibody in the sera of patients with ABPA (6–10). The evaluation of IgE responses to recombinant purified allergens from *A. fumigatus* demonstrates that the results may yield improved immunodiagnosis of ABPA. The need for a dependable diagnostic assay is essential in the differentiation of ABPA and atopy in CF. In the present study, we examined IgE reactivity to a number of purified recombinant *A. fumigatus* (Af) allergens, including Asp f1, f2, f3, f4, f6, f12, and f16 in ABPA and in non-ABPA CF patients and compared the results obtained with a crude *Aspergillus* extract.

Material and methods

Patients

Cystic fibrosis patients were enrolled in the study from the Cardinal Glennon Children's Hospital Cystic Fibrosis Clinic. Studies were approved by the Institutional Review Board at St Louis University, and informed consent was obtained. The diagnosis of ABPA was based on the presence of the following criteria recommended by the Cystic Fibrosis Society (2):

- Asthma (recurrent wheezing).
- Pulmonary infiltrates unresponsive to antibiotics and resolved with corticosteroids.
- Elevated total serum IgE > 1000 IU/ml.
- Positive *A. fumigatus* immediate prick skin test.
- Positive precipitating antibodies to *A. fumigatus*.
- Elevated IgE and IgG anti-*A. fumigatus* antibodies by enzyme-linked immunosorbent assay (ELISA) or UniCAP (Pharmacia Diagnostics Division, Kalamazoo, MI).

Bronchiectasis and eosinophilia were not required for a diagnosis. This is a retrospective study utilizing banked sera from these patients. Sera were stored in 2 ml cryogenic vials (Nalgene Company, Rochester, NY) at –70°C. The study was approved by the St Louis University Institutional Review Board. Patients were grouped as having ABPA and as to being Af ST+ and Af ST– non-ABPA. This classification of groups was performed as reactivity to

A. fumigatus skin test is a useful initial diagnostic study in evaluating for ABPA.

Recombinant Af allergens

Seven major recombinant allergens (Asp f1, f2, f3, f4, f6, f12, and f16) from *A. fumigatus* were used in the study. A cDNA library was constructed from *A. fumigatus* in λ ZAP II vector and the protein expressed in a pET 23b (+) vector as described before (6, 8). After isopropyl thiogalactoside induction, the cells containing the expressed proteins were sonicated and the recombinant proteins were purified using nickel charged N-(5-amino-1-carboxypentyl)-amino-diacetic acid (Ni-NTA) agarose column (Qiagen Inc, Valencia, CA). The purity and molecular size of the allergens were studied by sodium dodecyl sulfate-polyacrylamide gel electrophoresis while the reactivity of the allergens was evaluated with known sera as described (6, 8). All the recombinant allergens were stored at –70°C.

IgE levels

Total serum IgE levels and IgE antibodies to unfractionated *A. fumigatus* extract and to purified recombinant Af allergens were measured by ELISA according to previously described method (6, 8). Purified Asp f allergens (5 μ g/ml) were coated on the ELISA plates. Serum dilutions were allowed to react with the antigen and the binding of IgE with antigen was demonstrated by color reaction as described before (6). The color was read as O.D. values and the data evaluated by comparing the different groups.

Statistics

The data was expressed as the geometric mean $\pm$ SE. Statistical analysis using one way analysis of variance (ANOVA) was performed on the three groups, followed by a postprocedure, Scheffe test to determine difference between pairs. Comparison of a group at different times was performed by paired Student's *t*-test. To analyze sensitivity and specificity for each of the *Aspergillus* antigen preparations, analysis of the receiver operating curve (ROC) was performed for each.

Results

The skin-test reactivity to *A. fumigatus* of all ABPA and Af ST+ and ST– non-ABPA CF patients are shown in Table 1. There were 15 patients with ABPA and 23 Af ST+, and 19 Af ST– patients who did not have ABPA.

Table 1. Total IgE and antibodies specific for *A. fumigatus* in CF patients

Study	ABPA (n = 15)	Af ST+ (n = 23)	Af ST– (n = 19)	P
Number of studies	28	23	19	
IgE (IU/ml)	2323 $\pm$ 389	263 $\pm$ 41	34 $\pm$ 5	<0.001
Af ST+	28	23	0	
Af precipitin+	25	12	6	

Data presented as mean $\pm$ SE.

Statistical analysis was performed by anova; comparison between groups was performed by *post hoc* Scheffe procedure comparing ABPA vs Af ST+ and ABPA vs Af ST–. CF, cystic fibrosis; ABPA, allergic bronchopulmonary aspergillosis; ST, skin test; Af, *Aspergillus fumigatus*.

The total serum IgE levels were significantly elevated in ABPA patients compared with Af ST+ and Af ST- CF patients, 2323 ± 389 vs 263 ± 41 vs 34 ± 5 IU/ml ($P < 0.001$ and $P < 0.001$). In the four ABPA patients evaluated before and after developing ABPA, the IgE level significantly rose following development of ABPA compared with prior levels, 3804 ± 1423 IU/ml vs 221 ± 40 IU/ml ($P < 0.05$). Nine of the ABPA patients were studied at different time points ranging from 1 to 4 years. In these nine ABPA patients serially evaluated, the mean serum IgE level was significantly elevated during periods of flare compared with periods of remission, 4104 ± 867 IU/ml vs 1023 ± 236 IU/ml ($P < 0.01$).

The results of the IgE reactivity from ABPA patients during flares and remission compared with Af ST+ and Af ST- CF patients to different Af allergens are shown in Fig. 1. ABPA patients during flares compared with ABPA patients during remission had significantly elevated IgE reactivity to the crude *Aspergillus* extract ($P < 0.01$) and to Asp f3 ($P < 0.02$). The IgE antibodies to *A. fumigatus* crude extract was significantly elevated in ABPA patients during flares vs Af ST+ and Af ST- patients, geometric mean 0.2377 vs 0.0957 vs 0.002 ($P < 0.01$ and $P < 0.01$), respectively. When purified

recombinant Af allergens were studied, ABPA patients during flares had significantly elevated IgE antibodies compared with Af ST+ CF patients to Asp f1 ($P < 0.01$), Asp f2 ($P < 0.01$), Asp f3 ($P < 0.01$), Asp f4 ($P < 0.01$), Asp f6 ($P < 0.02$), and Asp f16 ($P < 0.04$). The IgE antibodies were also elevated substantially against Asp f12 in ABPA patients, but did not reach significance compared with Af ST+ CF patients. ABPA patient during remission also had significantly elevated IgE reactivity compared with Af ST+ CF patients to Asp f3, geometric mean 0.2009 vs 0.0277 ($P < 0.03$), and to Asp f4, 0.2048 vs 0.0270 ($P = 0.06$). Analysis of the ROC characteristics of each *Aspergillus* antigen preparation comparing all samples from ABPA patients compared with Af ST+ patients demonstrated that IgE reactivity to Asp f3 and Asp f4 gave the best sensitivity and specificity and were superior to IgE reactivity to a crude *A. fumigatus* extract (Table 2).

Of the four ABPA CF patients who were studied prior and after development of ABPA, IgE antibody levels against Asp f2, f3, f4, f6, and f16 increased dramatically (Fig. 2). We examined changes in IgE reactivity to purified *Aspergillus* allergens in nine ABPA patients during stages of flares and during remissions. The serum

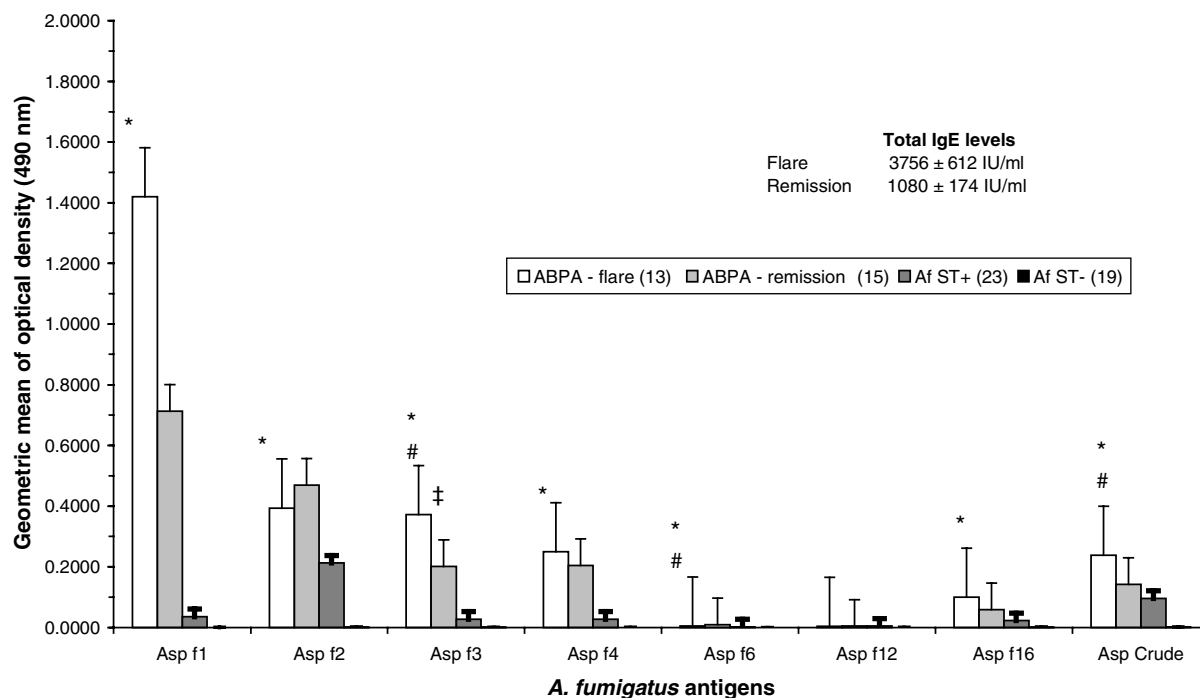


Figure 1. Immunoglobulin E (IgE) antibodies to purified recombinant *Aspergillus fumigatus* (Af) allergens in cystic fibrosis (CF) patients. Allergic bronchopulmonary aspergillosis (ABPA) CF patients during flares and during remission compared with Af skin test negative (ST-) CF patients had significantly elevated IgE antibodies to Asp f1, f2, f3, f4, f6, f16, and crude *Aspergillus* extract. ABPA patients during flares compared with remission had significantly increased IgE antibodies to Asp f3. ABPA patients during flares had significantly increased IgE antibodies to Asp f1, f2, f3, f4, f16 and crude *Aspergillus* extract; whereas, ABPA patients during remission had significantly elevated IgE antibodies to Asp f3 and f4. Data presented as geometric mean $\pm$ SE. Statistical analysis was performed by ANOVA; comparison between groups was performed by *post hoc* Sheffe procedure: (#) comparing ABPA-flare vs ABPA-remission; (*) comparing ABPA-flare vs Af ST+ and Af ST-; (‡) comparing ABPA-remission vs Af ST+.

Table 2. Analysis of sensitivity and specificity of IgE antibodies to recombinant *Aspergillus fumigatus* allergens in ABPA CF patients

Antigen	Optimal O.D. cut-off Value	Sensitivity (%)	Specificity (%)	AUC	P
Asp f1	0.153	82.1	52.2	0.660	0.038
Asp f2	0.261	82.1	43.5	0.735	0.004
Asp f3	0.178	82.1	30.4	0.845	<0.001
Asp f4	0.126	82.1	34.8	0.773	<0.001
Asp f6	0.249	42.9	13.0	0.590	0.009
Asp f12	0.003	81.5	69.6	0.540	0.457
Asp f16	0.0701	59.3	8.7	0.734	0.005
Crude <i>Aspergillus</i> extract	0.300	75.0	34.8	0.725	0.006

Ig E, immunoglobulin E; O.D., optical density; AUC area under the curve; CF, cystic fibrosis; ABPA, allergic bronchopulmonary aspergillosis.

Statistics performed by evaluating analysis of the receiver operating curve (ROC) for each antigen comparing ABPA vs Af ST+ non-ABPA patients.

IgE level was significantly elevated during stages of flare compared with stages of remission. This was reflected by significantly elevated IgE levels to Asp f1, f2, f3, and f6 comparing periods of flares vs remission ($P < 0.03$, <0.02 , <0.02 , and <0.04 , respectively). IgE reactivity to Asp f4 and the crude *Aspergillus* extract also increased but were not significant. It was observed that IgE reactivity to Asp 3 and f4 remained significantly elevated in ABPA patients during remission compared with Af ST+ non-ABPA patients ($P < 0.02$, <0.05).

We next examined whether the IgE response was positive. For the purified recombinant *Aspergillus* allergens this was taken from the cut-off value determined from ROC analysis. The pattern of IgE positive reactivity to recombinant *Aspergillus* allergens demonstrated that ABPA patients had significant increased IgE-positive reactivity compared with Af ST+ patients to Asp f1 (86% vs 52%, $P < 0.02$), Asp f2 (75% vs 44%, $P < 0.03$), Asp f3 (79% vs 30%, $P < 0.01$), Asp f4 (82% vs 35%, $P < 0.01$), Asp f6 (25% vs 0%, $P < 0.01$), and Asp f16 (41% vs 0%, $P < 0.01$) (Table 3). The average number of positive reactions to Asp f3 and Asp f4 was significantly greater in ABPA compared with Af ST+ non-ABPA CF patients, 1.6 ± 0.1 vs 0.7 ± 0.2 ($P < 0.001$), as seen in Fig. 3. Furthermore, this pattern of reactivity was significantly greater in the subgroup of ABPA patients during remission compared with Af ST+ non-ABPA patients, 1.3 ± 0.3 vs 0.7 ± 0.2 ($P < 0.04$). By comparison, positive reactivity to the crude *Aspergillus* extract was 89% in ABPA vs 61% in non-ABPA patients ($P < 0.03$).

Discussion

In this preliminary study of ABPA and *A. fumigatus* sensitive CF patients, IgE antibody titers were significantly increased to *Aspergillus* allergens especially to Asp f2, f3, f4, f6, and f16 after development of ABPA. Other

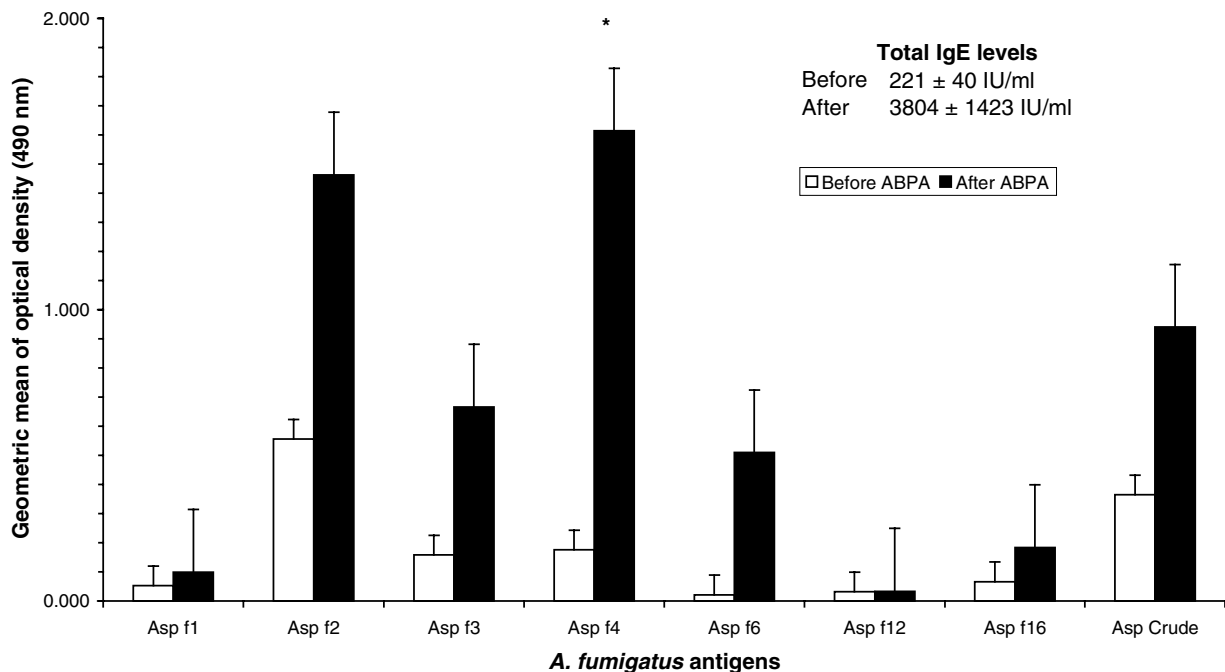


Figure 2. Immunoglobulin E (IgE) antibodies to purified recombinant *Aspergillus fumigatus* allergens in four allergic bronchopulmonary aspergillosis (ABPA) cystic fibrosis (CF) patients before and after developing ABPA. In four ABPA CF patients IgE anti-Asp f2, f3, f4, and f6 antibodies increased following development of ABPA. Data presented as geometric mean $\pm$ SE. Statistical analysis was performed by paired Student's *t*-test comparing patients before and after development of ABPA (*).

Table 3. IgE antibodies to recombinant *Aspergillus fumigatus* allergens in CF patients

Study	ABPA (28)	Af ST+ (23)	Af ST- (19)
Asp f1	24 (86)	12 (52)	1 (5)
Asp f2	21 (75)	10 (44)	0 (0)
Asp f3	22 (79)	7 (30)	0 (0)
Asp f4	23 (82)	8 (35)	1 (5)
Asp f6	7 (25)	0 (0)	0 (0)
Asp f12	2 (7)	0 (0)	0 (0)
Asp f16	11 (41)	0 (0)	0 (0)
Crude Af extract	25 (89)	14 (61)	0 (0)

Data presented as number of samples with positive IgE antibody and in parentheses the percentage of patients with positive serology. Cut-off optical densities were obtained from receiver operating curve analysis (Table 2).

CF, cystic fibrosis; ABPA, allergic bronchopulmonary aspergillosis; ST, skin test; Af, *Aspergillus fumigatus*.

investigators have also reported that IgE reactivity to Asp f2 and f4 differentiated ABPA patients from allergic sensitization (3–9). Significantly, we observed that IgE reactivity to Asp 3 and Asp f4 remained elevated in ABPA patients even during period of remission and helped distinguish from Af ST+ non-ABPA patients. We found that IgE reactivity (positive/negative) to Asp f1, f2,

f3, f4, f6, and f16 were increased in ABPA patients compared with Af ST+ non-ABPA patients. However, significant levels of IgE antibodies to Asp f1, f2, f3, and f4 were present in Af ST+ non-ABPA patients. However, ABPA patients had positive reactivity to Asp f3 and f4 compared with Af ST+ patients. Thus, this pattern of reactivity may distinguish ABPA from Af ST+ CF patients.

Furthermore, serial evaluation of IgE reactivity to purified *Aspergillus* allergens may be a better tool to evaluate disease activity than IgE reactivity to an *Aspergillus* crude extract. This was demonstrated in the nine patients who were serially evaluated. Whereas the IgE reactivity to a crude *Aspergillus* extract increased during flares, it was not as dramatic as the significant increases to Asp 1, f2, f3, f4, and f6. The reactivity to Asp f3 remained significantly elevated even during periods of remission in ABPA CF patients. In addition, in interpreting our studies with others reported, it becomes clear that it is also important realize that IgE reactivity to individual purified recombinant allergens changes with different stages of ABPA and that this need to be clarified.

In previous studies, we have examined *in vitro* T-cell responses to purified Af allergens in ABPA and non-

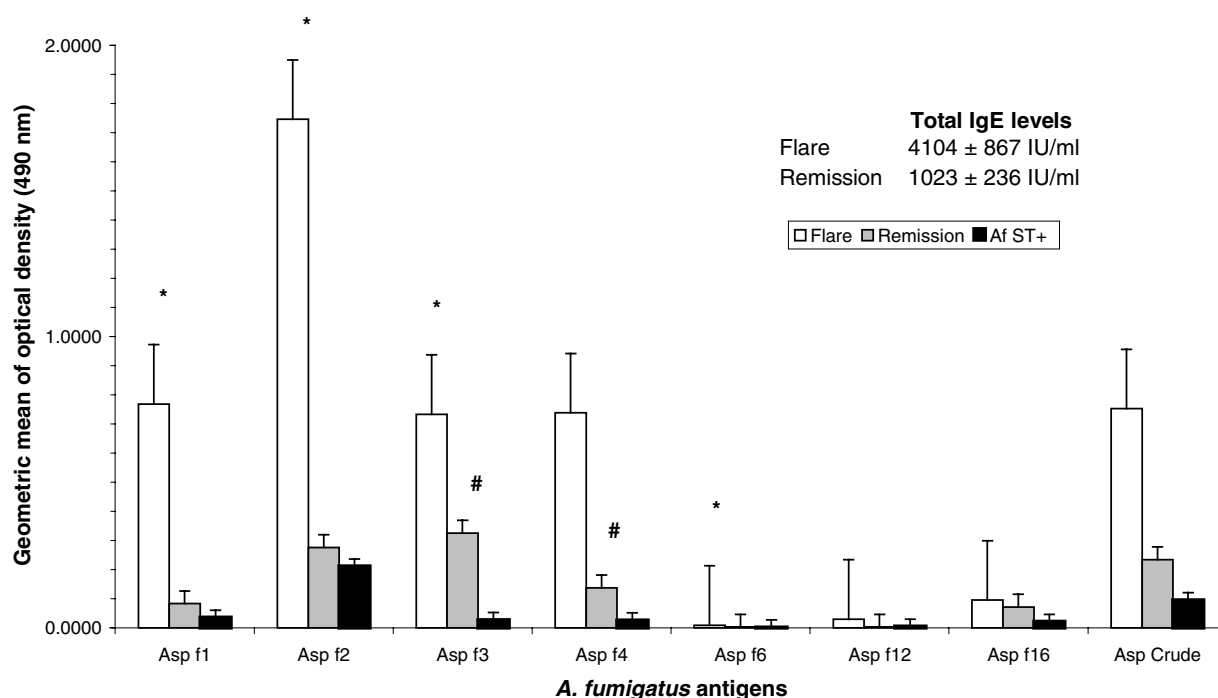


Figure 3. Immunoglobulin E (IgE) antibodies to purified recombinant *Aspergillus fumigatus* (Af) allergens in nine allergic bronchopulmonary aspergillosis (ABPA) cystic fibrosis (CF) patients serially evaluated during stages of flare compared with remission. For comparison, IgE reactivity is also shown for Af skin test positive (ST+) non-ABPA patients. Data presented as geometric mean ± SE. Statistical analysis was performed by paired Student's *t*-test comparing ABPA patients during remission and flare (*). Comparison of ABPA patients during remission and Af ST+ non-ABPA patients performed by Student's *t*-test (#). During flares, IgE reactivity was significantly elevated for Asp f1, f2, f3, and f6. During remission, IgE reactivity was significantly elevated compared with Af ST+ non-ABPA patients for Asp f3 and f4.

ABPA patients (11, 12). In ABPA patients, increased frequency of *Aspergillus*-specific Th2 T-cells and skewed Th2 cytokine synthesis is observed to Asp f1, f2, f3, and f4. Furthermore, Asp f2, f3, f4 induce increased synthesis of interleukin-10 (IL-10) in ABPA and non-ABPA patients. IL-10 down-regulates both Th1 and Th2 T-cells with preferential skewing of Th2 T-cell responses. Thus, the major *Aspergillus* allergens further promote a Th2 T-cell response and the development of ABPA in genetically susceptible patients.

In summary, the clinical and serologic diagnosis of ABPA in CF patients may be difficult. This study demonstrates that IgE antibodies to *A. fumigatus* purified

allergens are elevated in particular to Asp f3 and f4 in ABPA patients. IgE reactivity to a number of purified Af allergens would help distinguish ABPA from non-ABPA CF patients.

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