

Immunodeficiency and other clinical immunology

Serum anti-*Aspergillus fumigatus* antibodies by immunoblot and ELISA in cystic fibrosis with allergic bronchopulmonary aspergillosis

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Allergic bronchopulmonary aspergillosis (ABPA) occurs with a prevalence of 5% to 15% in patients with cystic fibrosis (CF). Because of the frequent colonization with Aspergillus fumigatus (Af) in CF, the causative agent of ABPA, antibody reactivity to Af proteins is frequently observed, which obscures the diagnosis of ABPA. Patients with CF are also categorized according to the presence of positive skin test responses to Af and/or the presence of positive precipitins. In this study we used ELISA and immunoblot assay to detect IgE and IgG anti-Af antibodies in patients with CF and ABPA (n = 13) compared with other groups of patients with CF: those with positive skin test and positive precipitin results (n = 18), those with positive skin test and negative precipitin results (n = 14), those with negative skin test and positive precipitin results (n = 10), and those with negative skin test and negative precipitin results (n = 35). IgE and IgG anti-Af antibodies were significantly elevated in patients with ABPA as determined by both immunoblot assay (p < 0.01) and ELISA (p < 0.01). However, detection of Af antibodies by ELISA was more sensitive in discriminating patients with CF and ABPA from patients with CF who had positive skin test and positive precipitin results but lacked radiographic and clinical evidence of ABPA. In patients with CF and ABPA the immunoblot assays demonstrated a multitude of IgE, IgG, and IgA antibody responses to Af proteins, which ranged in molecular weight from 14 kd to greater than 106 kd. The level of IgE anti-Af antibody to individual proteins decreased during remissions of ABPA. IgG and IgA anti-Af antibodies, however, once developed did not decrease nearly as much. (J ALLERGY CLIN IMMUNOL 1994;93:926-31.)

Key words: Cystic fibrosis, allergic bronchopulmonary aspergillosis, anti-*Aspergillus fumigatus* antibodies, ELISA, immunoblot

Allergic bronchopulmonary aspergillosis (ABPA) is a hypersensitivity lung disease occurring as a complication of asthma and cystic fibrosis (CF) in patients colonized with *Aspergillus fumigatus* (Af).¹⁻⁶ The diagnosis of ABPA in CF, as in asthma, is based on clinical criteria of wheezing and pulmonary infiltrates associated with the laboratory criteria of elevated serum IgE,

Abbreviations used

ABPA:	Allergic bronchopulmonary aspergillosis
Af:	<i>Aspergillus fumigatus</i>
ANOVA:	Analysis of variance
CF:	Cystic fibrosis
NCM:	Nitrocellulose membrane
OD:	Optical density
PPT+:	Positive precipitin
PPT-:	Negative precipitin
ST+:	Positive skin test
ST-:	Negative skin test

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eosinophilia, and increased IgE and IgG anti-Af antibodies. However, ABPA is especially difficult to diagnose in patients with CF because of the presence of recurrent or chronic infiltrates,

wheezing, and frequent colonization with Af.^{3, 7} The colonization of Af provokes immunologic reactivity characterized by anti-Af antibodies but not necessarily manifested as symptoms of ABPA. Because ABPA that is left untreated progresses to bronchiectasis and pulmonary fibrosis, investigators have explored more sensitive in vitro techniques for the detection of anti-Af antibodies to distinguish ABPA in patients with CF. Our group⁷ and others⁸ have reported that patients with CF and ABPA have quantitatively increased IgE and IgG anti-Af antibodies as determined by ELISA, which was a helpful adjunctive criteria in the diagnosis of ABPA. Recently, several groups have reported that immunoblotting was helpful in the diagnosis of ABPA in subjects with asthma.⁹⁻¹¹ Immunoblotting was found to semiquantitate serum IgE and IgG antibodies to specific Af antigens that correlated with disease activity. In the present study we compared immunoblotting and ELISA to measure serum IgE and IgG anti-Af antibodies to determine whether these assays were helpful to distinguish patients with CF and ABPA from patients with CF and only previous colonization with Af (i.e., without ABPA).

METHODS

Patients

Sixty-five patients with CF from St. Louis University Cystic Fibrosis Clinic were longitudinally studied prospectively one to two times yearly over a 3- to 6-year period. Seven patients fulfilled criteria for the diagnosis of ABPA as outlined by Laufer et al.¹² Namely, in these patients with CF ABPA was diagnosed by presence of recurrent wheezing, history of new chest radiographic infiltrates, immediate skin reactivity to an Af extract, positive precipitating antibody against Af antigens measured by Ouchterlony double gel diffusion, elevated serum IgE greater than 1000 ng/ml, and peripheral blood eosinophilia greater than 500 cells/cm. Bronchiectasis was not used as a criterion because of its high frequency in patients with CF as a result of repeated bacterial infections. In addition to the diagnosis of ABPA, patients with CF were categorized as to the immunoreactivity to *Aspergillus* skin tests (STs) and/or precipitins. One patient with ABPA (LB) was not treated with corticosteroids, and elevated total IgE and IgE and IgG anti-Af antibodies spontaneously diminished. This study was approved by the St. Louis University Institutional Review Board.

Serum IgE concentrations

The total serum IgE concentration was determined by paper radioimmunosorbent test assay and results are expressed as international units per milliliter.¹³

Aspergillus precipitins

Serum precipitating antibodies to *Aspergillus* antigens were measured by the double gel diffusion technique of Ouchterlony, as previously described.¹³

Skin tests. Intracutaneous skin tests were performed with an Af extract (Greer Laboratories, Lenoir, N.C.) of a 1:20 wt/vol concentration.

ELISA. Quantitation of serum IgE and IgG anti-*Aspergillus* antibodies was performed by an ELISA, as previously described.⁷ Af antigen, either 2.5 µg/ml for IgG or 100 µg/ml for IgE assays in carbonate-bicarbonate buffer (pH 9.4), was placed in wells of microtiter plates (Immulon II, Flow Laboratories, Rockville, Md.) overnight at 4° C. After the wells were washed with normal saline solution containing 1% bovine serum albumin and 0.05% Tween, bovine serum albumin was added to each well to block unbound sites. Then, after washing, 100 µl of diluted serum was incubated for 2 hours at 37° C. The wells were again washed, and 100 µl of 1:1000 goat anti-human IgG (affinity-purified γ-chain-specific) conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.) was added to each well and incubated for 2 hours at 37° C. After washing each well, 200 µl of substrate in 10% diethanolamine buffer was added and incubated at room temperature for 30 minutes. The reaction was stopped with 50 µl of 5N NaOH, and optical density (OD) was read at 405 nm on a Bio-Tek microplate reader (Bio-Tek Instruments, Inc., Winooski, Vt.). For IgE anti-Af assays, rabbit anti-human IgE, 1:1000 (affinity-purified ε-chain-specific) (Calbiochem, San Diego, Calif.), was added, incubated for 1.5 hours, and washed as described above. The remainder of the assay was the same as that described for IgG. Serum dilutions for IgG anti-Af were 1:500 and 1:1000, and for IgE anti-Af dilutions were 1:50 and 1:100; only the results for the lower dilution are presented. Serum samples were assayed in triplicate, and the results are presented as the OD of patient minus control values (net OD) and as the OD of patient divided by control values (times).

Immunoblot. Measurement of serum IgE and IgG antibodies to individual *Aspergillus* proteins was semiquantitated by immunoblot assays.¹⁴ Af proteins were separated with an Af extract (Greer Laboratories) by sodium dodecylsulfate-polyacrylamide gel electrophoresis with a discontinuous buffer system as described by Laemmli.¹⁵ The separating gel was 13% bis-acrylamide (Bio-Rad Laboratories, Chemical Div., Richmond, Calif.), and the stacking gel was 3%. Two hundred fifty microliters of Af protein (0.25 mg/ml) was mixed and reduced with Tris-sodium dodecylsulfate buffer containing 2-mercaptoethanol and heated at 100° C for 5 minutes. Af protein (400 µg) was loaded to the well of a preparative comb such that each subsequently cut lane received approximately 40 µg of protein per lane, and simultaneous low-molecular weight prestained standard (Bio-Rad Laboratories, Chemical Div.) was loaded to the reference well.

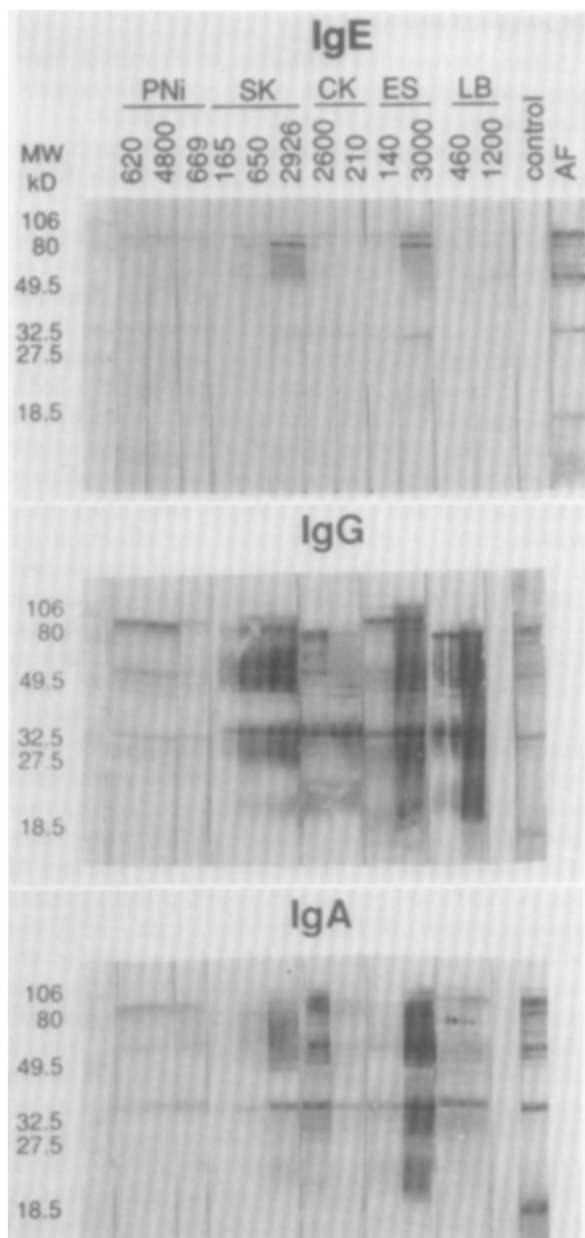


FIG. 1. IgE, IgG, and IgA immunoblots to Af proteins in five patients with CF and ABPA. IgE, IgG, and IgA reactivity to a number of Af antigens is demonstrated. In patients *PNi*, *SL*, and *LB* ABPA developed with increasing total and specific serum IgE levels; patients *PNi*, *CK*, and *ES* show a decrease in total and specific IgE with treatment. The values below the patients' initials correspond to the serum IgE values (in international units per milliliter). IgG and IgA anti-Af immunoblots demonstrated similar antibody reactivity to *Aspergillus* proteins. Though new and dense bands appeared with the onset of ABPA, they remained dense even after remission. Molecular weight standards are in lane 1, lane 14 (control), and lane 15 (Aurodyne stain of Af proteins).

The gels were run in a Mini-Protein II electrophoresis unit (Bio-Rad Laboratories, Chemical Div., Richmond, Calif.) at 100 V for approximately 2 hours. The Af proteins were then electrophoretically transferred to

0.20 μ m nitrocellulose paper (Schleicher and Schuell, Keene, NH) with an electroblotting apparatus (Bio-Rad Laboratories) at 50 V for 1 hour. A sandwich cassette was assembled submerged in transfer buffer in the following order: sponge, filter paper, nitrocellulose membrane (NCM), gel, NCM filter paper, and sponge. The cassettes were vertically placed in the electrophoretic chamber immersed in transfer buffer. One lane was stained with Aurodyne Forte (Amersham International, Amersham, U.K.) to confirm transfer of the proteins.

Immunoblotting was performed initially by blocking with phosphate-buffered saline containing 5% nonfat milk. Then, the NCM strips were incubated with serum diluted in phosphate-buffered saline containing 2% fetal calf serum and 0.05% Tween at 1:25 for IgE and at 1:100 for IgG at room temperature on a rocking platform. The NCM strips were washed for 1 hour with phosphate-buffered saline containing 0.05% Tween. Then, the NCM strips were sequentially incubated with biotin-conjugated anti-IgE 1:500, anti-IgG 1:500 (Tago, Inc., Burlingame, Calif.), or anti-IgA 1:500 (Tago, Inc.) for 1 hour, then avidin-alkaline phosphatase 1:500 for 30 minutes, then nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate substrate for 20 minutes. The NCM strips were washed between the various reagent steps. The reaction was stopped with 0.5 mol/L ethylenediaminetetraacetic acid. The density of the individual bands was graded from 0+ (no band) to 4+ (dense band) with the use of the scale previously reported by Bernstein et al.⁹ The grading of individual bands at particular molecular weights was recorded. In addition, the number of positive bands and the sum of densities were recorded.

Statistics

Statistical evaluation was performed by analysis of variance (ANOVA), and significant differences within the group were measured by post hoc analyses with the Sheffé test at a *p* value of less than 0.05.

RESULTS

The protein-stained gel banding pattern of the Af antigens used demonstrated multiple bands with molecular weights ranging from 14 kd to greater than 106 kd, as seen in Fig. 1. The sera from patients with CF and ABPA revealed heterogeneous IgE, IgG, and IgA antibody responses to 12 major bands at molecular weights of 14, 18, 20, 34, 47, 50, 55, 61, 70, 81, 93, and greater than 106 kd. Because a 13% acrylamide gel was used, resolution of bands at higher molecular weights was incomplete. During acute flare-ups (stage 3) of ABPA, multiple dense bands were observed for IgE, IgG, and IgA anti-Af antibodies (Fig. 1). The density of these anti-Af IgE bands were absent before development of ABPA and diminished during remissions (stage 2). When patients with ABPA were compared with patients who had

TABLE I. IgE, IgG, and IgA immunoblots in patients with CF and ABPA

Group	Serum IgE (IU/ml)	IgE		IgG		IgA	
		No. of bands	Sum	No. of bands	Sum	No. of bands	Sum
ABPA (<i>n</i> = 13)	2699	6.2 ± 2.1	13.8 ± 8.9	8.7 ± 1.3	25.3 ± 8.8	8.3 ± 1.6	24.4 ± 9.3
ST+/PPT+ (<i>n</i> = 18)	749	4.8 ± 2.9	9.4 ± 8.4	9.2 ± 1.5	26.8 ± 9.4	7.6 ± 2.3	21.2 ± 8.4
ST+/PPT- (<i>n</i> = 14)	380	2.6 ± 1.9	4.0 ± 3.3	8.2 ± 1.1	19.4 ± 4.5	7.2 ± 3.5	19.8 ± 11.0
ST-/PPT+ (<i>n</i> = 10)	42	1.2 ± 0.9	1.3 ± 1.0	5.3 ± 2.9	11.6 ± 7.9	5.9 ± 1.9	11.9 ± 5.6
ST-/PPT- (<i>n</i> = 35)	85	0.9 ± 1.2	1.2 ± 1.8	5.7 ± 2.8	12.9 ± 9.3	4.2 ± 3.1	8.7 ± 8.2
<i>p</i> Value*		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Data are presented as means ± SD.

*Determined by ANOVA.

TABLE II. Serum IgE and IgG anti-*Aspergillus fumigatus* antibodies determined by ELISA in patients with CF and ABPA

CF group	IgE (IU/ml)	Af-E		Af-G	
		OD	Times	OD	Times
ABPA (<i>n</i> = 13)	2699 ± 2161	0.74 ± 0.30	2.6 ± 1.0	1.08 ± 0.54	18.5 ± 25.9
ST+/PPT+ (<i>n</i> = 18)	749 ± 691	0.45 ± 0.20	2.0 ± 0.8	0.65 ± 0.63	8.8 ± 9.8
ST+/PPT- (<i>n</i> = 14)	380 ± 476	0.21 ± 0.10	1.0 ± 0.7	0.15 ± 0.14	21.4 ± 66.9
ST-/PPT+ (<i>n</i> = 10)	42 ± 25	0.38 ± 0.17	2.2 ± 1.8	0.22 ± 0.25	7.2 ± 11.1
ST-/PPT- (<i>n</i> = 35)	85 ± 196	0.26 ± 0.14	1.1 ± 0.6	0.08 ± 0.12	1.5 ± 3.3
<i>p</i> Value*	<0.01	<0.01	<0.01	<0.01	NS

Data are expressed as means ± SD.

OD, Net optical density of patient minus control values; *Times*, OD of patient divided by control values;

NS, not significant.

*Determined by ANOVA.

positive Af skin test (ST+) and positive precipitin (PPT+) results, the frequency of IgE to individual Af proteins was very similar (14 kd, 15.4% vs 16.7%; 20 kd, 15.4% vs 11.1%; 34 kd, 100% vs 100%; 47 kd, 84.6% vs 61.1%; 50 kd, 92.3% vs 61.1%; 55 kd, 61.5% vs 55.6%; 61 kd, 46.2% vs 27.8%; 70 kd, 46.2% vs 22.2%; 81 kd, 46.2% vs 22.2%; 93 kd, 100% vs 88.9%; and > 106 kd, 7.7% vs 5.6%). Thus there was a tendency to develop IgE antibodies more frequently to proteins at molecular weights of 47, 50, 61, 70, and 81 kd in patients with ABPA compared with patients with CF with ST+ and PPT+ results. On the other hand, once IgG and IgA antibodies to Af proteins appeared, they tended to persist during remissions of ABPA, though there was increased intensity of the banding during flare-ups. In addition, there was no increased frequency of IgG and IgA antibodies to individual protein bands observed in patients with ABPA compared with patients with CF and ST-/PPT+ results.

In attempts to quantitate the immunoblot assays, the number of bands and the sum of densities of individual bands were tabulated (Table I).

Patients with CF and ABPA had significantly increased number and total (sum) density of bands for IgE, IgG, and IgA anti-Af antibodies compared with other patients with CF (ANOVA; *p* < 0.01, <0.01, <0.01, respectively). However, though there tended to be increased number and density of IgE, IgG, and IgA bands for patients with ABPA compared with patients with ST+/PPT+ results, this was not statistically significant as determined by post hoc analysis.

Serum IgE and serum IgG anti-Af determined by ELISA are presented in Table II. The mean net IgE anti-Af OD value of serum samples was significantly increased in patients with CF and ABPA when compared with subjects with CF and ST+/PPT+, ST+/PPT-, ST-/PPT+, or ST-/PPT- results (ANOVA, *p* < 0.01). Furthermore, the IgE anti-Af OD was significantly increased in patients with ABPA compared with patients with CF and ST+/PPT+ results (post hoc analysis with Scheffé test, *p* < 0.05). Similar results were observed when the data were expressed as a ratio to a control serum (times). Similarly, the mean IgG anti-Af OD recording was

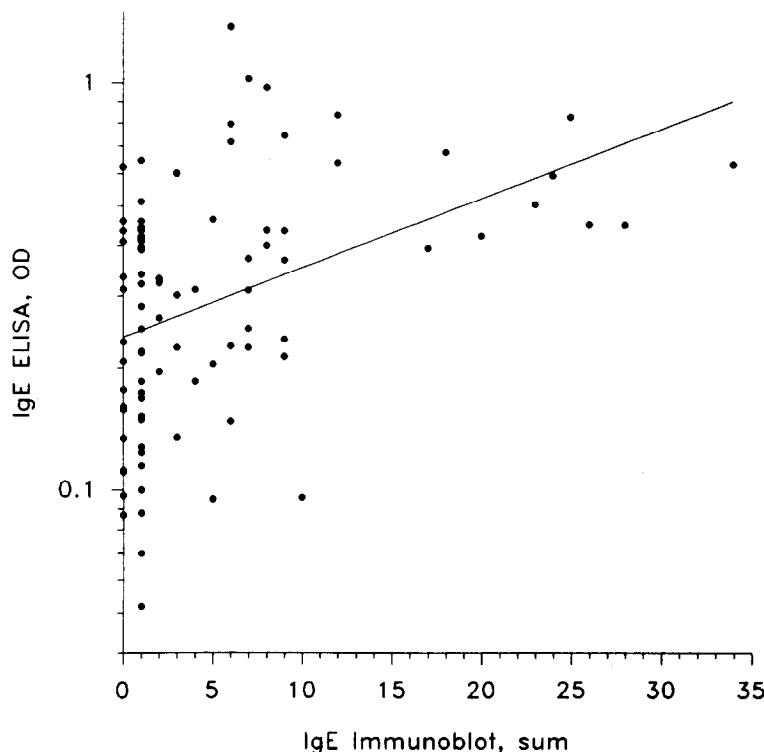


FIG. 2. Regression analysis comparing detection of anti-Af antibodies by immunoblot assay and ELISA. The two techniques were significantly comparable ($r = 0.414$, $p < 0.01$).

significantly increased in ABPA when compared with all other CF groups ($p < 0.01$; ANOVA).

Comparison of IgE anti-Af antibodies, determined by either immunoblot or ELISA assays, revealed a significant correlation ($r = 0.41$, $p < 0.01$), as seen in Fig. 2.

DISCUSSION

The pathophysiology of ABPA is hypothesized to be similar to a chronic late-phase allergic response to persistent allergen exposure in the bronchial tree.¹⁶ A T-cell response, predominated by CD4+ T_{H2}-like cells, secreting such cytokines as interleukin-4 and interleukin-5, drive IgE synthesis and eosinophilia. This proposed immune mechanism would explain the increased total and specific IgE and eosinophilia that are observed during development and exacerbations of ABPA. It might be expected, then, that measurement of IgE antibodies to specific Af proteins would be helpful in the diagnosis of ABPA in patients with CF.

In this study increased number and density of IgE bands were observed in patients with CF and ABPA. The density and number of bands fluctuated with disease activity, as observed in patients

with asthma and ABPA.^{9, 10} Uniformly, patients with CF and ABPA had IgE antibodies to Af proteins at 34, 47, 50, and 93 kd and approximately one half the time at 55, 61, 70, and 81 kd. These findings are different from those reported by Bernstein et al.⁹ in patients with asthma and ABPA, who reported major bands at 24, 33 to 35, and 36 to 40 kd for IgE and at 47 to 50, 70 to 83, 84 to 90, and greater than 110 kd for IgG and by Leung et al.,¹¹ who reported major IgE bands only at 70 kd. Baur et al.¹⁰ reported IgE antibodies to Af proteins at molecular weights similar to those in our findings. Those groups that found few bands may have used less immunoreactive Af extracts. IgE anti-Af antibodies quantitated by ELISA were significantly elevated in patients with CF and ABPA, similar to those reported in patients with asthma and ABPA.^{2, 17, 18} The two techniques for detecting IgE anti-Af antibodies correlated significantly. However, our method of recording the immunoblots was only semi-quantitative; improved correlation might have even been better by densitometric measurement of the immunoblot bands.

Because colonization with *A. fumigatus* is frequent in patients with CF, immunologic reactivity

was frequently observed. In patients with CF who had ST+/PPT+ results, IgE, IgG, and IgA antibodies to Af proteins were frequently observed. Though patients with CF who had ST+/PPT+ results had less IgE antibody to Af proteins, immunoblot assays did not distinguish this group from patients with CF and ABPA, whereas ELISA did. However, even in the ELISA there was some individual overlap. Thus the ELISA was more sensitive than immunoblots in quantitating IgE antibodies in patients with CF, as reported in patients with asthma.¹⁸ Improved quantitation of the immunoblot data might improve the distinction between ABPA and ST+/PPT+ result in patients with CF.

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