Aspergillus fumigatus: Identification of 16, 18, and 45 kd antigens recognized by human IgG and IgE antibodies and murine monoclonal antibodies

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The immunochemical properties of antigens produced by Aspergillus fumigatus *were investigated with biochemical purification techniques in conjunction with the production of murine monoclonal antibodies (MAbs) and binding studies with human IgG and IgE antibodies.* A. fumigatus *antigens were partially purified by gel filtration and hydrophobic interaction chromatography on phenyl-Sepharose. Two fractions that eluted with either 2 mol/L or 0.15 mol/L of NaCl demonstrated strong binding to human IgG and IgE antibodies. Immunoprecipitation analysis with IgG antibodies from six patients with different* Aspergillus*-related diseases demonstrated that the 2M and 0.15M fractions contained major antigens of molecular weight 18 kd (*Asp f *1) and 45 kd, respectively. The ^{125}I-labeled 2M fraction was used to compare IgG antibodies to* A. fumigatus *in sera from 25 patients with* Aspergillus*-related diseases. IgG antibodies were significantly higher in patients with allergic bronchopulmonary aspergillosis (geometric mean, 437 U/ml) than in patients with asthma (geometric mean, 14 U/ml; $p < 0.001$), but undetectable (<5 U/ml) in 43/48 control subjects. A good correlation was found between levels of IgG antibodies to the ^{125}I-labeled 0.15M fraction and the ^{125}I-labeled 2M fraction in sera from 106 patients with cystic fibrosis ($r = 0.77$; $p < 0.001$). Five murine IgG MAbs and two IgM MAbs were raised against the 2M fraction, and immunoprecipitation with the IgG MAb demonstrated two distinct antigens within the 2M fraction,* Asp f *1, and a 16 kd antigen. The results of a solid-phase RIA with IgG MAb 4A6 demonstrated that ~85% of* A. fumigatus*-allergic patients with allergic bonchopulmonary aspergillosis had IgE antibodies to* Asp f *1. The three protein antigens defined in these studies are useful probes for investigating the immunopathogenesis of diseases associated with colonization by* A. fumigatus. *(J* ALLERGY CLIN IMMUNOL *1992;89:1166-76.)*

Key words: Aspergillus fumigatus, *aspergillosis, allergic bronchopulmonary aspergillosis, asthma, fungal allergens, monoclonal antibodies*

Fungi of the genus *Aspergillus* are associated with a spectrum of human diseases, including ABPA, invasive aspergillosis, aspergilloma, allergic asthma, and CF.[1-5] An increase in the incidence of systemic aspergillosis among immunosuppressed patients with leukemia and other hematologic malignancies has also been reported in the United States; however, *Aspergillus* infection is uncommon in patients with acquired immune deficiency syndrome.[6, 7]

Aspergillus fumigatus accounts for most *Aspergillus*

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isolates from infected patients, and there is good evidence that the different pathologic effects of *A. fumigatus* infection are mediated by different forms of the host immune response, as well as by virulence factors produced by the fungus.[1, 2] *A. fumigatus*-specific IgE and IgG antibodies have been detected in sera from patients presenting with different subsets of disease, and elevated IgE antibodies are believed to contribute to the pathogenesis of ABPA and asthma.[2] Precipitating IgG antibodies are also present in sera from patients with ABPA, and in a primate model of this disease, both precipitating IgG antibodies and passively transferred human IgE antibodies were necessary for the development of lung lesions after inhalation of *A. fumigatus* extract.[8] The highest levels of precipitating IgG antibodies have been reported in patients with aspergilloma; however, the role of these antibodies in pathogenesis of the disease is not clear.[2, 3]

Detection of specific antibodies to *Aspergillus* has been accomplished by several techniques, including double immunodiffusion, crossed immunoelectrophoresis, ELISA, RIA, and immunoblotting.[9-16] Multiple *A. fumigatus* antigens/allergens have been demonstrated to elicit human IgG and IgE antibody responses.[17-22] However, the relationship between these antigens has not been clearly established, and sequence data have not been available. *A. fumigatus* and other fungal allergens remain poorly defined by comparison with allergens derived from pollens, dust mites, and cat dander, many of which have now been cloned and sequenced.[23-26]

The aim of the present study was to use both biochemical separation techniques and production of MAbs to identify and define *A. fumigatus* antigens. We isolated two distinct *A. fumigatus* antigens, an 18 kd antigen, which we have previously defined as *A. fumigatus* allergen I (*Asp f* I),[27] and a 45 kd antigen that eluted from phenyl-Sepharose in 0.15 mol/L of NaCl. Both antigens reacted with human IgE and IgG antibodies. An additional 16 kd antigen was also identified with murine MAbs. The 18 and 45 kd antigens provide useful markers for clinical and immunologic studies of *A. fumigatus*.

MATERIAL AND METHODS

Aspergillus antigens

A. fumigatus mycelium and spore components from a long-term, stationary culture were kindly provided by Dr. Robert Esch (Greer Laboratories, Lenoir, N.C.). Forty grams of freeze-dried defatted culture containing spores and mycelia was extracted overnight in 400 ml of BBS, pH 8.0. After centrifugation (20,200 *g* for 30 minutes), the supernatant was precipitated with 100% SAS.[17] The protein concentration of this extract, according to an assay by Bradford,[28] was 20 mg/ml.

Abbreviations used

ABPA:	Allergic bronchopulmonary aspergillosis
CF:	Cystic fibrosis
Asp f I:	*Aspergillus fumigatus* allergen I
BBS:	Borate-buffered saline, pH 8
HIC:	Hydrophobic interaction chromatography
GM:	Geometric mean
MAb:	Monoclonal antibody
MW:	Molecular weight
PBS-T:	PBS, pH 7.4, containing 0.05% Tween 20
SAS:	Saturated ammonium sulfate
2M fraction:	A gel-filtration fraction eluted from phenyl-Sepharose with 2 mol/L of NaCl
0.15M fraction:	A gel-filtration fraction eluted from phenyl-Sepharose with 0.15 mol/L of NaCl
A_{280}:	Absorbance at 280 nm
SDS–PAGE:	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Human sera

Sera were obtained from 12 patients who fulfilled the clinical and immunologic criteria of ABPA,[10] 12 patients with asthma and with positive immediate skin tests to *A. fumigatus*, but without other features of ABPA, one patient with aspergilloma,[29] and 106 patients with CF. Controls included sera from 40 patients from the University of Virginia Allergy Clinic with negative skin tests to *Aspergillus* mix (26 patients with diagnoses of asthma, nine with rhinitis/sinusitis, three with chronic urticaria, one with atopic dermatitis, and one with uveitis), and eight healthy nonatopic individuals. A positive skin test was defined as a wheal size ≥8 by 8 mm in diameter after intradermal injection of 0.03 ml of 1:200 dilution of either *Aspergillus* mix (Hollister-Stier Laboratories, Spokane, Wash.) or *A. fumigatus* extract (Greer Laboratories, Lenoir, N.C.). Nine of the 12 sera were from patients with ABPA observed at the Cardiothoracic Institute, Brompton Hospital, London, U.K.).[10] Sera from patients with CF were obtained from patients observed at the Children's Hospital National Medical Center (Washington, D.C.) and University of Virginia Children's Medical Center (Charlottesville, Va.). Collection of human sera for use in these studies was approved by the Human Investigation Committee of the University of Virginia.

Purification of *A. fumigatus* antigens

One hundred twenty milligrams of *A. fumigatus* extract was applied to a 2.6 by 90 cm Sephacryl S200 column

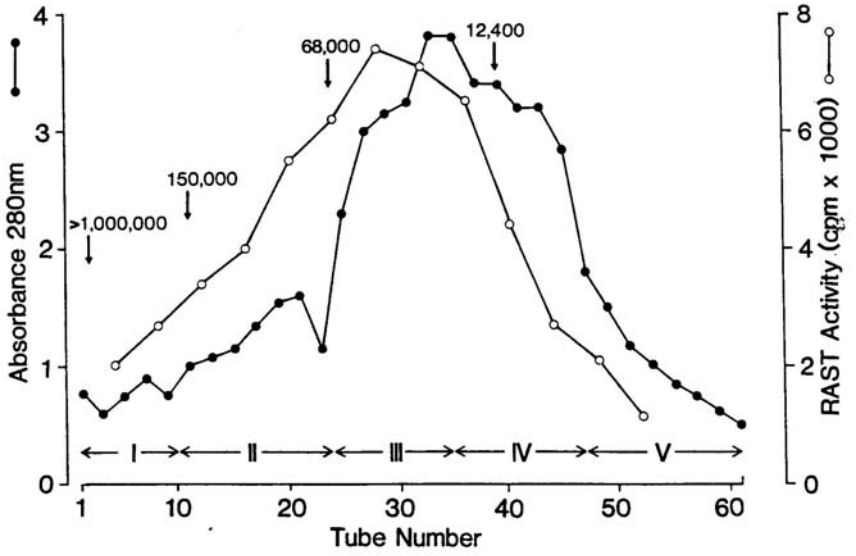

FIG. 1. Elution profile of *A. fumigatus* extract on gel filtration (Sephacryl S-200); A_{280} of *A. fumigatus* fractions (●) and binding of IgE antibodies to eluted fractions by RAST (○). Column calibrated with blue dextran (MW, >1,000,000), IgG (150,000), bovine serum albumin (68,000), and cytochrome c (12,000). RAST results, mean of each five consecutive tubes. Fractions pooled as indicated (I to V).

equilibrated in BBS at 4° C and at a flow rate of 24 ml/hr. Fractions of 4.8 ml were collected and pooled, based on optical density (A_{280}) and binding to IgE antibodies from a patient (J. H.) with ABPA (Fig. 1). After concentration, the allergenic activity of the five pooled fractions (I to V) was compared by micro-RAST with sera from eight patients with ABPA and serum from an *A. fumigatus*-allergic patient with asthma. Gel-filtration fraction III (MW 20 to 70 kd) was further purified by HIC with phenyl-Sepharose CL 4B (Pharmacia Diagnostics, Piscataway, N.J.). An 0.9 by 34 cm phenyl-Sepharose column was equilibrated with 0.05 mol/L of Tris HCl buffer, pH 7.4, containing 4 mol/L of NaCl at 4° C, and 4.5 ml of fraction III (18 mg) was applied at a flow rate of 30 ml/hr. Fractions of 1.6 ml were collected and monitored at A_{280}. The column was eluted stepwise with Tris buffer and decreasing concentrations of NaCl, 4, 2, 1, and 0.15 mol/L, and distilled water. Finally, 20% ethylene glycol was used to elute any remaining protein. The protein peak that eluted at each salt concentration was pooled, dialyzed against distilled water for 48 hours, freeze-dried, resuspended in a volume of 0.5 ml, and stored at −20° C. Concentrated fractions were tested against serum of J. H. and serum from an *A. fumigatus*-allergic patients with asthma (E. C.) by micro-RAST, as described by Kemeny and Richards.[30] Briefly, 2 to 10 μg of protein (*A. fumigatus* extract, gel filtration, or HIC fraction) in a volume of 8 μl was coupled to CNBr-activated cellulose disks. The disks were incubated with 8 μl of serum (6 hours), followed by 8 μl of ^{125}I-labeled goat antihuman IgE (2 to 5 ng, ~100,000 cpm overnight), and then counted in a gamma counter. Between each step, disks were washed 10 times with PBS-T. Fractions obtained by HIC were also used for skin testing a selected patient (W. L.) who had asthma and who had a positive immediate skin test to *A. fumigatus* extract. Intradermal tests were performed with serial tenfold dilutions of each fraction, and results were expressed as the dilution that elicited a >8 by 8 mm diameter wheal at 20 minutes after intradermal injection.

Measurement of IgG antibodies to *A. fumigatus*

The 2M and 0.15M HIC fractions were radiolabeled with 0.5 mCi ^{125}I using the chloramine-T technique.[31] The ^{125}I-labeled 2M and ^{125}I-labeled 0.15M fractions were used in an antigen-binding RIA to measure IgG antibodies, as described previously.[32] Briefly, 12 ng (0.1 ml) of ^{125}I-labeled 2M fraction or 8.2 ng (0.1 ml) of ^{125}I-labeled 0.15M fraction (~100,000 cpm, specific activity 5 and 13 μCi/μg, respectively) was incubated with 0.1 ml of serum diluted 1:12.5, 1:50, and 1:200 for 4 hours at room temperature. IgG antibodies were precipitated overnight at 4° C with 0.1 ml of sheep antihuman IgG Fc fragment (The Binding Site, San Diego, Calif.). The precipitates were washed with BBS, and the residual radioactivity was counted in a gamma

counter. When serum dilutions ≥1:200 were assayed, IgG myeloma protein (0.1 ml of 1:100 dilution) was added as a carrier. The assay was quantitated with serum from a patient with aspergilloma to form a control curve. This serum elicited a titer of 1:20,000 and was arbitrarily allotted 20,000 U/ml of *A. fumigatus*-specific IgG antibodies. Control curves were established with serial twofold dilutions of serum (from 1/50 to 1/100,000) in each assay.

Radioimmunoprecipitation

Fifty microliters of human serum diluted 1:10 or mouse MAb ascites diluted 1:100 was incubated for 1 hour with 50 μl of ^{125}I-labeled 2M or ^{125}I-labeled 0.15M HIC fraction (~100,000 cpm) in a 1.5 ml microfuge tube. Fifty microliters of 50% vol/vol protein A Sepharose in Tris buffer (50 mmol/L of Tris, 5 mmol/L of EDTA, 150 mmol/L NaCl, 0.02% Na azide, and 0.5% nonidet 40, pH 7.5) was added to each tube and incubated for 1 hour on ice. The beads were washed with Tris, Tris containing 500 mmol/L of NaCl, and Tris containing 0.05% of nonidet 40 and boiled for 2 minutes in 50 μl of SDS-reducing buffer (6% SDS, 30% glycerol, 220 mmol/L of Tris HCl, pH 6.8, 0.015% of bromophenol blue, and 15% of 2-mercaptoethanol). Dissociated antigen-antibody complexes were analyzed by SDS-PAGE with a 15% acrylamide-resolving gel and a 5% stacking gel.[33] Gels were stained with Coomassie blue R-250, dried, and autoradiographed by exposure to Kodak X-OMAT AR (Eastman Kodak, Rochester, N.Y.) film for 48 hours at −70° C.

MAbs

Five BALB/c mice, 14 to 16 weeks of age, were immunized, intraperitoneally, with 50 μg *A. fumigatus* 2M antigen in complete Freund's adjuvant, and boosted three times at 10-day intervals. Fourteen days after the last injection, mice were bled, and IgG and IgM antibodies were measured by ELISA, with microtiter plates coated with ~2 μg 2M antigen per well and peroxidase conjugated, isotype-specific goat antibodies (TAGO Inc., Bulingame, Calif.). A mouse with an IgG antibody titer of 1:25,000 was boosted intrasplenically with 40 μg of 2M antigen. Four days later, spleen cells of the mouse were fused with Sp2/0 myeloma cells at a ratio of 5:1 with polyethylene glycol, as described previously.[34] Hybrids were screened for antibody production by ELISA approximately 2 weeks after fusion. Supernatants from antibody-producing hybrids were also screened for IgG antibodies to the 2M antigen by antigen-binding RIA.[34] Four IgG1- and two IgM-producing hybrids were cloned by limiting dilution. Ascites was produced from each clone by injecting 2×10^6 cells, intraperitoneally, into pristane-primed BALB/c mice.[34] The use of animals in this study was approved by the Animal Research Committee (University of Virginia) under guidelines for the use and care of animals formulated by the National Council for Medical Research.

Binding of human IgE antibodies to *Asp f* I

A modified two-site RIA was used to compare the binding of human IgE antibodies to *Asp f* I.[27] Briefly, a 50% SAS fraction of MAb 4A6 B5 ascites was coated overnight on plastic Removawells (Dynatech Laboratories, Alexandria, Va.) (~5 μg MAbs per well). Wells were subsequently incubated with ~1 μg per well of 2M fraction, followed by 50 μl of human serum diluted 50% in horse serum, and ^{125}I-labeled goat antihuman IgE (2 to 5 ng, ~100,000 cpm) diluted in 50% horse serum in 1% bovine serum albumin-PBS-T and then counted in a gamma counter. Between each incubation step, plates were washed with PBS-T.

TABLE I. IgE antibody binding to Sephacryl S200 fractions of *A. fumigatus*

Fraction	Protein concentration (mg/ml)*	RAST activity (%†) (x ± 1 SD)	ABPA patients' RAST‡ (cpm) No. 1 (T. N.)	No. 2 (J. H.)
I	2.3	51 ± 10	15,122	7,655
II	8.3	68 ± 12	18,102	15,369
III	4.0	96 ± 17	29,280	16,888
IV	1.2	94 ± 23	30,340	10,838
V	0.2	76 ± 7	15,879	11,395
Crude *Asp* extract	20.0	100	29,437	16,572

Asp, A. fumigatus.
*Protein concentration was measured according to an assay by Bradford[28] with gamma globulin as standard.
†Eight patients with ABPA were tested. Values are expressed as a percentage of the ^{125}I-labeled anti-IgE bound (counts per minute) with *A. fumigatus* extract (mean of two control subjects, 925 cpm).
‡Examples of IgE antibodies to gel-filtration fractions measured by RAST in sera from two patients with ABPA (T. N. and J. H.).

Statistical analysis

Levels of IgG and IgE antibodies to *A. fumigatus* antigens in sera from patients with ABPA or asthma were compared by Wilcoxson's rank-sum test, and p values <0.05 were considered significant.

RESULTS

Partial purification and immunochemical analysis of *A. fumigatus* antigens

A. fumigatus proteins eluted from a Sephacryl S200 gel-filtration column in a broad peak, MW 10 to 70 kd. Human IgE antibodies in serum from a patient with ABPA were tested against each tube eluate (Fig. 1). In addition, five pooled fractions (I to V) were tested for binding to IgE antibodies with sera from eight patients with ABPA. Fractions III and IV retained 95% of the binding to IgE antibodies compared with that of *A. fumigatus* extract (Table I). Fraction III was purified by HIC with phenyl-Sepharose and elution with decreasing concentrations of NaCl (4, 2,

TABLE II. IgE antibody binding to *A. fumigatus* fractions isolated by HIC

A. Sephacryl S200 fraction III Fraction	Protein* (mg/ml)	Carbohydrate† (mg/ml)	Protein Carbohydrate ratio	IgE antibody bound‡ (cpm) J. H.	 E. C.
4M	9.6	2.4	3.9	8,184	4,027
2M	2.8	0.16	18.0	14,229	4,111
1M	<0.6	<0.08	—	13,076	2,329
0.15M	0.6	<0.08	>7.7	9,873	2,796
dH_2O	1.6	0.19	8.3	10,620	2,228
A. fumigatus extract	20.0	5	4	16,899	3,088

B. *A. fumigatus* extract Fraction	Protein* (mg/ml)	Carbohydrate† (mg/ml)	RAST activity (%§) (x ± 1 SD)	IgE antibody bound‡ (cpm) J. H.	 S. L.	End point skin test titer‖
4M	11.4	20.0	54 ± 8	11,138	16,101	10^{-7}
2M	17.2	5.2	64 ± 7	14,511	19,306	10^{-6}
1M	7.8	1.5	51 ± 12	10,068	11,133	10^{-4}
0.15M	5.2	0.5	51 ± 9	9,262	13,372	10^{-3}
dH_2O	6.0	0.8	56 ± 25	9,188	15,486	10^{-4}
A. fumigatus	13.0	6.6	100	19,866	30,024	+ +

dH_2O, Distilled water.

*Bradford[28] assay with gamma globulin as standard.

†Phenol-sulfuric acid reaction with dextran as standard.

‡Examples of IgE antibodies to phenyl-Sepharose fractions measured by RAST in sera from patients with ABPA (J. H. and S. L.) or asthma (E. C.). Control values with serum from a skin test negative donor ranged from 1200 to 1500 cpm bound.

§Nine patients with ABPA were tested. Values are expressed as a percentage of the ^{125}I-labeled anti-IgE bound (counts per minute) with *A. fumigatus* extract. Control sera against each fraction and crude extract, 1215 cpm.

‖Intradermal skin tests on patient W. L. who has asthma and a positive immediate skin test to *A. fumigatus* (6 by 6 mm in diameter wheal on prick test, + +). Results are expressed as the dilution that elicited an 8 by 8 mm diameter wheal 15 minutes after injection.

1, and 0.15 mol/L and distilled water). All five HIC fractions were active on RAST, but the highest IgE antibody binding was observed with the fraction that eluted with 2 mol/L of NaCl (the 2M fraction) (Table II, *A*). In a second experiment, a 100% SAS precipitate of *A. fumigatus* extract was separated over a phenyl-Sepharose column. The 2M NaCl fraction of this extract was also most active on RAST and elicited a positive intradermal skin test at 10^{-6} dilution in an *A. fumigatus*-sensitive patient with asthma (Table II, *B*). Carbohydrate was predominantly found in the fraction eluted with 4 mol/L of NaCl (Table II, *A* and *B*). The results revealed that the 2M fraction had a low carbohydrate content and contained proteins that bound to human IgE antibodies.

The 2M fraction and the 0.15M fraction (i.e., Sephacryl S200 fraction III that eluted with 0.15 mol/L of NaCl from phenyl-Sepharose, Table II, *A*) were radiolabeled with ^{125}I and analyzed by immunoprecipitation and SDS-PAGE (Fig. 2). The ^{125}I-labeled antigens were clearly distinct. The 2M antigen demonstrated a broad band of MW ~18 kd, whereas the ^{125}I-labeled 0.15 M antigen demonstrated a major band with MW 45 kd. Although some lesser-stained bands were also detected, the 18 and 45 kd proteins appeared to represent at least 80% of the radiolabeled protein in each fraction. The other HIC fractions, that is, 4M, 1M, and distilled water fractions, were also radiolabeled; however, immunoreactivity was greater for the 2M and 0.15M fractions (20% to 30% radioactivity bound to human IgG antibody). Binding of human IgG antibodies to ^{125}I-labeled 2M and 0.15M fractions was completely inhibited by *A. fumigatus* extract (>90%); however extracts of four other clinically relevant *Aspergillus* species (*A. niger, A. terreus, A. nidulans,* and *A. glaucus*) demonstrated <20% inhibition (data not presented).

Serum IgG antibodies to ^{125}I-labeled 2M and 0.15M fractions

IgG antibodies to the 2M fraction were measured in 24 patients with positive skin tests to *A. fumigatus* and 48 skin test–negative control subjects by antigen-binding RIA. The highest level of specific IgG antibodies were detected in serum from a patient with aspergilloma, which was arbitrarily defined as con-

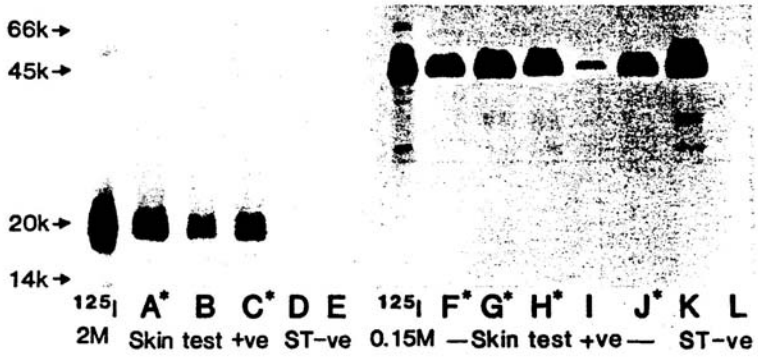

FIG. 2. Immunoprecipitation of radiolabeled *A. fumigatus* antigens with human IgG antibodies. ^{125}I-labeled 2M fraction *(left)* or 0.15M fraction *(right)* immunoprecipitated with IgG antibodies in sera from patients J. H. (*lanes A* and *F*), O. S. (*lanes B* and *I*), M. F. (*lanes C* and *J*), B. L. (*lane G*), C. V. (*lane H*), J. R. (*lane K*), and from control subjects T. M. (*lanes D* and *L*) and K. A. (*lane E*); patients with ABPA (*). Patient O. S. had chronic fungal sinusitis; patient J. R. had an aspergilloma.

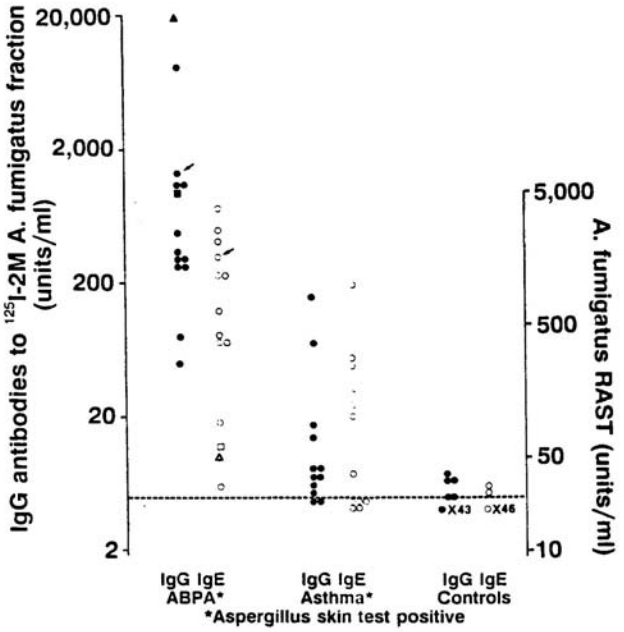

FIG. 3. Quantitative measurements of IgG antibodies to *A. fumigatus* 2M fraction by antigen-binding RIA (●) and IgE antibodies to *A. fumigatus* by RAST (○) in sera from allergic patients and control subjects. Levels obtained from a patient with aspergilloma (▲) and a patient with *A. fumigatus* sinusitis (■) are in *ABPA* column. Patient J. H. *(arrow)* had levels of IgG antibodies 20-fold lower than patient with aspergilloma; J. H. IgE antibody levels were 40-fold higher.

taining 20,000 units of IgG antibody per milliliter. Sera from skin test–positive patients with ABPA contained significantly higher IgG antibody levels (GM, 437 U/ml) than patients with asthma (GM, 14 U/ml; $p < 0.001$) (Fig. 3). Most patients (43/48) with negative skin tests to *Aspergillus* had undetectable IgG antibody levels (<5 U/ml). The antigen-binding RIA was also used to measure *A. fumigatus*-specific IgG

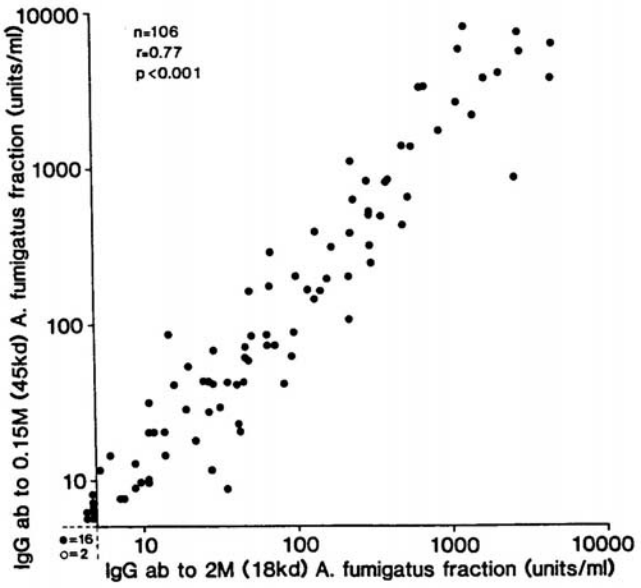

FIG. 4. Linear regression analysis of IgG antibody binding to ^{125}I-labeled 2M and ^{125}I-labeled 0.15M *A. fumigatus* fractions in sera from patients with CF (●) or control subjects (○), measured by antigen-binding assay.

antibodies in nasal washings from a patient (O. S.) with chronic sinusitis who had a positive skin test to *A. fumigatus* and repeatedly grew *A. fumigatus* in cultures of nasal secretions. This patient had 40 U/ml and 120 U/ml of IgG antibody to the 2M fraction in nasal washings collected on two separate occasions. IgE antibodies to *A. fumigatus* measured by RAST were also significantly higher in sera from patients with ABPA compared with that in sera from patients with asthma (GM, RAST, 646 U/ml and 68 U/ml, respectively; $p = 0.002$) (Fig. 3). Levels of IgG antibodies specific to the ^{125}I-labeled 0.15M fraction were compared with levels of IgG to the ^{125}I-labeled 2M fraction in sera from 106 patients with CF. There was an excellent quantitative correlation between binding of IgG antibodies to either radiolabeled fraction as demonstrated by linear regression analysis ($r = 0.77$; $p < 0.001$) (Fig. 4).

IgG MAb to the 2M *A. fumigatus* fraction define two antigens, MW 18 and 16 kd

Five IgG MAbs and two IgM MAbs were generated from a fusion with spleen cells from a BALB/c mouse immunized with the 2M fraction. The five IgG1 clones demonstrated significant binding on the RIA, particularly MAb 4A6, and no reactivity on the IgM ELISA. The two IgM clones bound strongly in the IgM ELISA but demonstrated no reactivity on binding assay and no reactivity on the IgG ELISA (Table III). Immunoprecipitation analysis demonstrated that the MAb distinguished two different antigens within the 2M fraction with MW of 18 kd (MAbs 4A6, 7A5, and 2E2) and 16 kd (MAb 10G7) (Fig. 5). We previously reported the purification of the 18 kd antigen, termed *Asp f* I, using the MAb 4A6.[27] IgE-antibody binding to *Asp f* I was compared in 24 patients with ABPA or asthma (Table IV). Of the 13 patients with high levels of *A. fumigatus*-specific IgE (RAST, >200 U/ml), 11 had IgE antibodies to *Asp f* I by solid-phase RIA. Most patients with asthma had very low levels of IgE antibodies to *A. fumigatus* by RAST (<150 U/ml). Those patients also had undetectable IgE to *Asp f* I by the RIA and low levels of IgG to the 2M fraction. Serum from one of the patients with asthma (W. L.), allergic to both *A. fumigatus* and *Trichophyton*, was used in the two-site RIA that was performed, including either *Trichophyton* or *Alternaria* extracts, in addition to *A. fumigatus* extract and the 2M fraction. In this experiment, binding of IgE antibodies in the RIA was demonstrated only when *A. fumigatus* or partially purified 2M fraction were presented by MAb 4A6, but not when *Trichophyton* or *Alternaria* extracts were

TABLE III. IgG and IgM monoclonal antibodies to *A. fumigatus* 2M fraction

Hybrid or clone*†	IgG ELISA (OD_{405} nm)	IgM ELISA (OD_{405} nm)	^{125}I-labeled 2M fraction (cpm bound)‡
IgG			
4A6	2.410	0.073	11,230
4A6G2*	2.218	ND	8,475
4A6B5*	2.219	ND	8,264
7A5	2.085	0.066	5,032
7A5B1*	1.952	ND	5,387
10G7	0.826	0.067	4,279
10G7E2*	0.735	ND	3,514
2E2	2.068	0.060	4,998
2E2A5*	1.878	ND	1,448
IgM			
2F1	0.068	2.509	790
2F1B6*	ND	2.577	462
1A8	0.064	2.666	695
1A8A4*	ND	2.489	525
Mouse polyclonal (1:100)			23,703
MAb 5H8 anti-*Der p* I§			485
MAb 6A8 anti-*Der f* I§			456
Normal mouse serum (1:100)			465

OD_{405} nm, Optical density at 405 nm; *ND*, not done; *Der p I*, major allergen from *Dermatophagoides pteronyssinus*; *Der f I*, major allergen from *D. farinae*.
*Clones derived from respective parental hybrids.
†Supernatants were assayed undiluted.
‡Radioactivity added ~150,000 cpm of ^{125}I-labeled 2M fraction.
§MAbs 5H8 and 6A8[34] were assayed at concentration of 10 μg/ml.

TABLE IV. Comparison of the binding of IgG or IgE antibodies to *A. fumigatus* antigens in sera from patients with ABPA and asthma

Patients	IgG Ab* (U/ml)	*A. fumigatus* RAST (U/ml)	IgE Ab to *Asp f* I† (cpm bound)
ABPA			
C. V.	480	1270	7613
H. D.	340	3700	7287
S. L.	260	1225	6720
B. L.	1100	2630	4916
C. H.	80	460	2587
J. H.	1350	2085	1135
T. N.	270	1580	606
C. M.	350	600	567
S. M.	270	25	359
M. F.	1100	84	296
D. L.	50	445	288
S. W.	8650	423	278
Asthma			
W. L.	162	1020	4936
C. H.	72	280	2429
O. A.	16.8	215	996
RAST <150 U/ml‡ (N = 9)	6.04 (2.5-13.7)	31.3 (12-147)	167 (128-189)
Control subjects (N = 3)	<2.5	<12	180

Ab, Antibody.
*IgG antibodies to ^{125}I-labeled 2M fraction measured by antigen-binding RIA. Results are expressed in units per milliliter relative to a control curve with serum from a patient with aspergilloma (20,000 U/ml).
†Measured by two-site RIA with microtiter plates coated with MAb 4A6 (5 μg per well) and incubated with 2M fraction (1 μg per well). Sera were assayed at 1/2 dilution, and IgE antibody bound to the antigen presented by MAb 4A6 was detected with ^{125}I-labeled goat antihuman IgE (~120,000 cpm added).
‡Nine of the patients with asthma had an *A. fumigatus* RAST of <150 U/ml, and most of these patients had low or undetectable IgG and IgE antibody levels. These data are presented as GM values with the range of results presented in parenthesis.

added to the assay (data not presented). These results suggested that MAb 4A6 was *A. fumigatus* specific.

DISCUSSION

In the present study, we report the identification of *A. fumigatus* antigens that bind IgG and IgE antibodies in sera from patients with aspergilloma, ABPA, asthma, and CF. The partially purified 2M and 0.15M antigens were physicochemically and antigenically distinct and included major components of MW 18 (*Asp f* I) and 45 kd, respectively. The 2M fraction was used as a marker for *A. fumigatus* to develop a sensitive and quantitative RIA for specific IgG antibodies, which could be used to compare antibody levels among different groups of patients with *A. fumigatus*-related diseases. The results suggest that accurate quantitation of IgG antibodies to the 2M antigen was of diagnostic value. Elevated levels of specific IgG were detected in allergic patients with asthma and ABPA and in patients with aspergilloma, in ascending order, and the quantitative differences in antibody levels between patients with ABPA and asthma were highly significant. During the past year, the RIA has also been used to follow up a patient who had aspergilloma and was treated with amphotericin B, intravenously. After treatment, his IgG antibody levels have slowly decreased by 40%, and this decrease correlated with clinical improvement.[29] Although this is of diagnostic value, the role of IgG antibodies in pathogenesis of *A. fumigatus* diseases is unclear. Precipitating IgG is believed to participate in type III reactions that could lead to lung-tissue damage during the course of ABPA. However, it has been reported that patients with ABPA develop poor precipitating

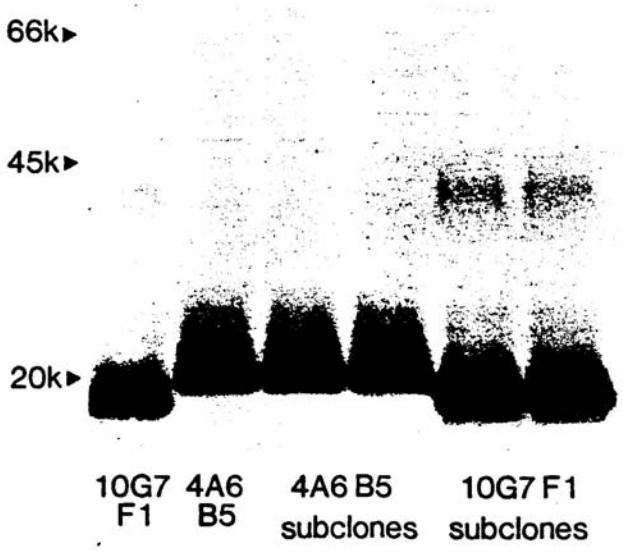

FIG. 5. Immunoprecipitation of ^{125}I-labeled 2M *A. fumigatus* antigen with MAbs. In addition to 18 kd protein recognized by MAb 4A6, a 16 kd protein was immunoprecipitated by MAb 10G7.

IgG response to low MW *A. fumigatus* allergens.[10] Although we found high levels of IgG antibodies in sera from patients with aspergilloma and ABPA by RIA, precipitins were directed against high MW fractions, that is, fractions I and II from the gel-filtration column, rather than against the low MW (<50 kd) fractions.[29]

Patients with CF are particularly susceptible to respiratory tract colonization with *A. fumigatus*, and the prevalence of ABPA among those patients has been reported to be up to 11%.[4, 5] We recently measured IgG antibodies to the 2M fraction in 147 patients with CF and detected specific IgG levels of >10 U/ml by RIA in 84% of the cases, as opposed to only 6% in that of control children.[35] Assays for either the 0.15M or 2M fractions demonstrated a much higher prevalence of IgG antibodies to *A. fumigatus* than would be expected with conventional immunodiffusion-based techniques (20% to 30%[2, 5]) and suggest that colonization by *A. fumigatus* is much more common among patients with CF. Although the RIA we have described is unlikely to be of routine diagnostic use, it should be possible to modify the assay as an ELISA, and the use of partially purified 2M or 0.15M fractions should eliminate the technical problems associated with the use of crude extracts in assays for IgG antibodies to *A. fumigatus*.[2]

Previous studies have described *A. fumigatus* antigens of MW 18 to 24 kd,[18, 19, 22] as well as higher MW antigens[15, 17, 21]; however, it is not clear how they relate to the 16, 18, and 45 kd antigens defined here. Although the 2M fraction appeared to be relatively pure on immunoprecipitation, murine MAb raised against this fraction identified an additional 16 kd antigen. Selected fractions containing low carbohydrate concentration were used to immunize mice to enhance the chances of obtaining MAb of the IgG isotype, as opposed to IgM. The availability of the MAb will make it possible to establish unequivocally the relationships between the *A. fumigatus* antigens that we have identified and antigens described by other groups.[18, 19, 22] Although the 45 kd antigen induced strong IgG and IgE antibody responses in patients with aspergilloma, ABPA, asthma, or CF, it was very weakly immunogenic in BALB/c mice, and even after prolonged high-dose immunization of mice with the 0.15M fraction in complete Freund's adjuvant, the murine IgG antibody response was very poor (<5% ^{125}I-labeled antigen bound in the RIA). The production of MAbs to the 45 kd antigen will probably require immunization of different mouse strains and/or the use of different adjuvants to generate high-titer IgG antibody responses for fusion purposes. Both *Asp f* I and the 45 kd protein appeared to be important causes

of IgE antibody responses in patients with positive immediate skin tests (or serum IgE antibodies) to *A. fumigatus*. In preliminary studies, we have detected low levels of IgG antibodies, and no IgE antibodies, to the 16 kd antigen in 15 sera from patients with ABPA.

In conclusion, we have identified three *A. fumigatus* antigens, MW 16, 18, (*Asp f* I) and 45 kd, using human IgG or IgE antibodies or murine IgG MAb. Quantitation of antibody levels to the 2M fraction (or *Asp f* I) could be useful in clinical medicine as an additional diagnostic criterion for ABPA. These antigens should also be useful probes for studying T cell responses to *A. fumigatus*, and preliminary results demonstrating proliferative responses to *Asp f* I in patients with ABPA have been reported.[36] Measurements of both humoral and cellular responses to purified *A. fumigatus* antigens should lead to a better understanding of the immunopathogenesis of *Aspergillus*-related diseases, including ABPA, asthma, aspergilloma, and CF.

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