

# Improved diagnosis of allergic bronchopulmonary aspergillosis with gp66 (formerly antigen 7) of *Aspergillus fumigatus* for specific IgE detection

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**Background:** In cystic fibrosis (CF) serologic tests assume increased significance for discriminating the symptoms of allergic bronchopulmonary aspergillosis (ABPA) from those of bacterial infections. Elevated specific IgE is a sensitive indicator of ABPA, but the high prevalence of patients with CF who have simple allergy to *Aspergillus fumigatus* means that it lacks specificity.

**Objective:** The aim of this study was to develop an assay to detect IgE to a single allergenic protein of *A. fumigatus* to increase the discrimination between ABPA and uncomplicated *A. fumigatus* allergy.

**Methods:** To identify a more selective allergen, serum samples from nine patients with ABPA and five patients with allergy were used to screen *A. fumigatus* proteins for allergenic activity by crossed radioimmuno-electrophoresis. Molecular weights were determined by electrophoresis and immunoblotting, and carbohydrate content was analyzed by concanavalin A chromatography and endoglycosidase digestion.

**Results:** The allergen identified was found to be a glycoprotein with a molecular weight of 66 kd, referred to as gp66. An ELISA was developed to measure specific IgE to gp66, and the efficacy was compared with IgE detection by using the unfractionated *A. fumigatus* extract on serum samples from 14 patients with CF and ABPA, 42 patients with CF and allergy but not ABPA, and 23 patients with CF without ABPA or allergy.

**Conclusion:** Use of gp66 increased the specificity of IgE analysis from 58.5% to 78.5%, whereas the sensitivity of 92.9% was maintained. (*J Allergy Clin Immunol* 1996;98:55-63.)

**Key words:** *Aspergillus fumigatus*, allergic bronchopulmonary aspergillosis, cystic fibrosis, ELISA, IgE

The presence of IgE-mediated hypersensitivity to *Aspergillus fumigatus* may be associated with asymptomatic atopy, atopic asthma, or in a minority of patients (<1% with asthma), allergic bronchopulmonary aspergillosis (ABPA). The latter condition arises as a complication of a hypersensitive immune response directed against noninvasive airway colonization by *A. fumigatus*. The quoted prevalence of both uncomplicated allergy (ele-

## Abbreviations used

|           |                                                          |
|-----------|----------------------------------------------------------|
| ABPA:     | Allergic bronchopulmonary aspergillosis                  |
| BSA:      | Bovine serum albumin                                     |
| CF:       | Cystic fibrosis                                          |
| CIE:      | Crossed immunoelectrophoresis                            |
| CRIE:     | Crossed radioimmuno-electrophoresis                      |
| SDS-PAGE: | Sodium dodecylsulfate-polyacrylamide gel electrophoresis |

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vated serum IgE) to *A. fumigatus*, 22% to 25%<sup>1-3</sup> and ABPA 6.5% to 10.7%,<sup>2-4</sup> is relatively high in patients with cystic fibrosis (CF). In patients with CF serologic tests assume increased significance for discriminating the clinical symptoms and signs

of ABPA from those of bacterial infections. Chest roentgenograms of children with CF are frequently abnormal and therefore may mask the abnormalities characteristic of ABPA. However, with approximately 77% of patients exhibiting at least one immunologic response to *A. fumigatus*,<sup>2</sup> the distinction between classical ABPA and simple allergy is problematic. Both *A. fumigatus*-specific IgE and IgG titers are diagnostic criteria<sup>5</sup> and have been reported to show a relationship with pulmonary disease.<sup>6,7</sup> Specific IgE has been observed to have greater specificity for ABPA than IgG analysis, and concentrations show greater fluctuations with disease progression,<sup>8</sup> whereas IgG and IgA concentrations persist during remissions of ABPA.<sup>9</sup> *A. fumigatus* antigen extracts contain a heterogeneous mixture of proteins and glycoproteins, the composition of which varies with the growth conditions of the source organism.<sup>10</sup> Pulmonary colonization by *A. fumigatus* is part of the etiology of ABPA, patients being exposed to the organism growing at 37° C in the lung secretions. Patients with simple allergy are only exposed to inhaled particles, which is likely to result in exposure to a different antigen repertoire. The immune responses to individual allergenic proteins of *A. fumigatus* may therefore show greater differentiation between the disease states than responses to the crude extract.

The aim of this study was to identify an allergen of *A. fumigatus* with potential to improve the specificity of the diagnosis of ABPA when compared with IgE measurement in which the crude extract is used.

## METHODS

### Patients

All patients in this study were diagnosed as having CF, which was verified by a positive sweat iontophoresis result in the presence of classical symptoms and signs. Serum samples were obtained from patients attending the pediatric CF clinic for annual assessment. Patients were classified into five clinical groups as assessed by an independent clinician.

Patients in group 1 (ABPA,  $n = 14$ ) had active ABPA within 3 months of the time samples were obtained. Criteria used for diagnosis of ABPA included the presence of roentgenographic changes in the lungs, reversible airway obstruction, peripheral blood eosinophilia, and a positive skin prick test response to *A. fumigatus*. Patients with ABPA also had elevated total and specific IgE and IgG antibodies<sup>5</sup> and required steroid treatment. Patients in group 2 (pre ABPA,  $n = 3$ ) had no history of ABPA. Samples from these patients were obtained 3 months before the diagnosis of ABPA was made. Patients in group 3 (atopic,  $n = 27$ ) had IgE sensitivity to

*A. fumigatus* ( $>15\%$ , as determined by RAST) and did not exhibit clinical features of ABPA. Patients in group 4 (weakly allergic,  $n = 15$ ) had weak sensitivity to *A. fumigatus* (5% to 14%, as determined by RAST) and did not exhibit features of ABPA. Patients in group 5 (nonallergic,  $n = 23$ ) had normal total IgE and specific IgE antibodies and no history of ABPA.

A pool of ABPA serum was prepared with samples from the 14 patients with diagnosed current ABPA (group 1).

All patients in the ABPA group were given oral steroids in the form of prednisolone between 1 and 2 mg/kg per day for 4 weeks after symptoms and physical signs had resolved. The dose was then reduced within the next 4 to 6 weeks to 1 to 2 mg/kg on alternate days. Subsequently, dosing was reduced over a period of 3 months with the aim of withdrawing therapy if symptoms and signs permitted.

### Allergen identification

*Crossed radioimmuno-electrophoresis (CRIE).* Native *A. fumigatus* proteins were separated to high resolution by crossed immunoelectrophoresis (CIE).<sup>11</sup> A water-soluble extract of the culture filtrate of six pooled batches of *A. fumigatus* (freshly isolated from patients with CF) after 4 weeks of growth at 37° C was used in CRIE. Briefly, glass plates (8 × 8 cm) were coated with agar (1%) (Electran; Merck Ltd., Diagnostics Div., Dorset, U.K.) in barbital buffer (1:1 dilution of stock: barbital sodium, 20.6 gm; barbital, 3.7 gm; distilled water, 1 L; pH, 8.65). The crude extract (3  $\mu$ L, 30 mg/ml wt/vol) was electrophoresed in the first dimension (20 mA, 90 minutes) and in the second dimension (10 mA, 18 hours) into an agarose window containing pooled reference hyperimmune rabbit antisera against *A. fumigatus* (11.5  $\mu$ L/cm<sup>2</sup>) in the main gel window and no antisera in the intermediate gel window. Monospecific antisera in the intermediate gel was used to cross-reference precipitin peaks with those already described; these included antigens 5, 7, and 13.<sup>12-14</sup> Antigen 5 is a minor antigen and allergen of *A. fumigatus*, which is located on the surface of germinating conidia. Antigen 7 has been described as a major IgG-binding component for patients with ABPA<sup>15</sup> and is an intracellular cytoplasmic component for the mycelium, which is also released into the medium after 6 days of growth in vitro at 37° C.<sup>10</sup> Antigen 13 forms a precipitin with IgG from ABPA sera and is a mycelial intracellular component.<sup>14</sup> A standard template was stained with Coomassie brilliant blue R250 (0.5%) (Sigma Chemical Co., St. Louis, Mo.). Autoradiography was used to identify allergenic peaks. The washed CIE plates were overlaid with serum (2.5 ml) diluted (1:2) in phosphate-buffered saline with bovine serum albumin (BSA) (3%) overnight, washed, then incubated with anti-human IgE conjugated to iodine 125 (Phadebas; Kabi-Pharmacia, Uppsala, Sweden). CIE plates were washed again (three times), followed by direct exposure on x-ray film (Kodak X-

Omat; Eastman Kodak Co., Rochester, N.Y.) for 7 days at room temperature. Allergenic peaks were identified by comparing the autoradiographs with the standard template. Serum samples from nine patients in group 1, two patients in group 2, and five patients in group 3 were analyzed in this way.

### Antiserum

*Pooled reference antisera for use in CRIE.* Dutch lop rabbits were immunized with an "in house" water-soluble antigen extract prepared from the culture filtrate of four different isolates of *A. fumigatus*, grown at 25° C for 4 weeks. Antigen (3 mg of protein per milliliter), in Freund's incomplete adjuvant with saline solution (1:2), was used to immunize rabbits every 4 weeks, and rabbits were bled 2 weeks later.

*Monospecific antisera.* Precipitating monospecific rabbit antisera was used to identify previously described protein peaks (or antigens) in CIE. Antisera to antigens 5 and 7 were provided by Dr. J. L. Longbottom, and antisera to antigen 7 for immunoblotting was prepared as described previously. IgG was purified by precipitation with 70% ammonium sulfate after precipitation of serum impurities with caprylic acid (1:40); purity was assessed by crossed-line immunoelectrophoresis and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (results not shown).<sup>16</sup>

### *A. fumigatus* antigen extract

The filtrates of *A. fumigatus* cultures after 28 days of static incubation at 37° C were prepared as described previously.<sup>10</sup>

*Molecular weight determination.* Monospecific antisera to antigen 7 was used to precipitate *A. fumigatus* proteins by rocket immunoelectrophoresis. The immunoprecipitate was eluted from the agarose with Tris (10 mmol/L) SDS (0.1%) buffer, centrifuged, dialyzed, and freeze-dried before SDS-PAGE.<sup>17</sup> The reduced proteins were run on 10% to 20% linear gradient acrylamide gels containing 0.1% SDS with a Hoefer mighty small II Se-250 slab gel electrophoresis system (Hoefer Scientific Instruments, San Francisco, Calif.). For antigen identification the separated proteins were transferred to nitrocellulose sheets (Hybond super; Amersham International Ltd., Little Chalfont, Bucks, U.K.) in a transblotting chamber (Hoefer) at 0.07 A over 12 hours. The sheets were then blocked with 1% BSA for 2 hours at 24° C. Immunolabeling was carried out for 2 hours at room temperature with the primary antibody, which was either rabbit polyclonal antisera against peak 7 (1:100) in antibody buffer (NaCl, 0.9%; Tris, 50 mol/L; BSA, 0.25%; Tween-20, 0.25% at pH 7) or pooled ABPA serum (1:10). After washing the sheets for 2 hours, bound antibody was detected by using goat anti-rabbit IgG (1:5000) (Sigma) or monoclonal anti-human IgE (1:100) conjugated to peroxidase with diaminobenzidine (0.1%; Sigma) as chromogen and peroxidase as substrate (0.144%; Sigma).

### Allergen purification

The crude extract was fractionated by ammonium sulfate precipitation, and the dialyzed supernatant from the 70% ammonium sulfate fraction was used as the first step of purification. Purified antibody against peak 7 (10 mg) was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia) (1 gm dry weight) according to the manufacturer's instructions. The antigen in antibody buffer (Tris, 10 mol/L; ethylenediaminetetraacetic acid, 1 mol/L; phenylmethylsulfonyl fluoride, 0.2 mol/L; glycerol, 5%) (250 µl, 30 mg/ml) was incubated with the Sepharose-antibody complex (2 ml) for 1 hour before being loaded to the glass column (0.5 × 10 cm). After washing in antibody buffer, the bound antigen was removed with eluting buffer (glycine 0.1 mol/L at pH 3.2). The protein content of the eluents were measured with a UV monitor (Uvicord S II; Pharmacia Biotech, Herts, U.K.). The fractions were dialyzed overnight against ammonium hydrogen carbonate (10 mol/L at pH 7.2, 10 L) and then placed in universal tubes and freeze-dried.

### Carbohydrate analysis

*Concanavalin A column chromatography.* Five milliliters of antigen (70% AS-S fraction, 4 mg/ml) in assay buffer (Tris, 10 mol/L; NaCl, 0.15 mol/L; calcium chloride, 2 mol/L; mangan chloride, 2 mol/L; Tween-20, 0.05%; pH 7) was incubated with the concanavalin A-Sepharose (1 gm wet weight) (Sigma) at room temperature for 2 hours. The Sepharose-antigen mixture was loaded on a glass column (0.5 × 10 cm) and washed with 50 ml of the assay buffer. Once protein was not detectable in the eluents, indicating that the unbound proteins were removed, 10 ml of assay buffer with α-D-mannopyranose (0.1 to 1 mol/L) was used to displace bound glycoprotein. The fractions were dialyzed and freeze-dried before being resuspended in equal volumes of distilled water and stored at -20° C in aliquots. The fractions were analyzed by SDS-PAGE and Western blotting.

*Enzymatic deglycosylation of antigen 7.* The purified protein (50 µg) was resuspended in distilled water (100 µl) and added to digestion buffer (SDS, 0.01%; Triton X-100, 0.2%; sodium acetate, 50 mol/L; 2-mercaptoethanol 0.1 mol/L; pH 5.5) to bring the volume to 500 µl and a final concentration of 100 µg/ml. The mixture was then heated to 100° C for 3 minutes to linearize the protein and was frozen immediately.

The endoglycosidase H (EC 3.2.1.96; Boehringer Mannheim Biochemica, East Sussex, U.K.) (10 mU) was added to the linearized protein (100 µl) to produce a ratio of approximately 10 mU of enzyme to 10 µg of the protein. The protein and enzyme were incubated overnight at 37° C; the enzyme activity was then supplemented (10 mU), and the reaction mixture was incubated for an additional 14 hours. The reaction was stopped by boiling for 3 minutes and frozen immediately. The digested protein was then analyzed by concanavalin A chromatography as described previously.

**TABLE I.** Intensity of serum IgE binding to six protein peaks in CRIE

| Peak      | Group 1* (ABPA) |   |   | Group 3† (allergic without ABPA) |   |   | Group 2‡ (pre ABPA) |   |   |
|-----------|-----------------|---|---|----------------------------------|---|---|---------------------|---|---|
|           | ++              | + | – | ++                               | + | – | ++                  | + | – |
| Antigen 7 | 8               | 0 | 1 | 1                                | 3 | 1 | 2                   | 0 | 0 |
| Antigen 5 | 3               | 5 | 0 | 1                                | 1 | 4 | 0                   | 0 | 2 |
| a         | 3               | 5 | 1 | 3                                | 1 | 1 | 0                   | 0 | 2 |
| b         | 4               | 1 | 4 | 3                                | 1 | 1 | 0                   | 0 | 2 |
| c         | 5               | 2 | 2 | 2                                | 1 | 2 | 0                   | 1 | 1 |
| d         | 1               | 4 | 4 | 0                                | 0 | 5 | 0                   | 0 | 2 |

Serum samples were obtained from nine patients in group 1, five patients in group 3, and two patients in group 2.

\**A. fumigatus* RAST range, 16% to 41% (median, 28%).

†*A. fumigatus* RAST range, 16% to 31% (median, 21%).

‡*A. fumigatus* RAST range, 11% to 16% (median, 13.5%).

### IgE detection

*IgE to A. fumigatus whole extract.* The freeze-dried crude extract (3.9 mg) (as used in CRIE) was conjugated to cyanogen bromide-activated Sepharose (1 gm) to form the antigen particle complex. The antigen particle complex (100  $\mu$ l at 4%) was incubated (12 hours) with sera (50  $\mu$ l), washed, and incubated (12 hours) with anti human IgE  $^{125}$ I conjugate (50  $\mu$ l) (Phadebas, Pharmacia), after washing. The percent uptake of  $^{125}$ I by the antigen particle complex was calculated.

*IgE to antigen 7.* ELISA plates (Immunoplates; Nunc GmbH, Wiesbaden, Germany) were coated with 100  $\mu$ l of monospecific purified IgG to peak 7 (2  $\mu$ g/ml) overnight, washed, blocked for 2 hours with 1% BSA before being incubated with freeze-dried antigen extract 70% ammonium sulfate (0.4 mg/ml) for 2 hours at room temperature. The washed plates were incubated with serum diluted 1:1. Bound IgE was detected with mouse monoclonal anti-human IgE, followed by anti-mouse IgG horse-radish peroxidase conjugate. The optical density of control wells with capture antibody, positive patient sera, detector antibodies (but no antigen) was less than 0.1 optical density units (background), which confirmed that there was no cross-reactivity with the rabbit capture immunoglobulins and human IgE. To decrease intraassay variability, a reference standard curve consisting of ABPA pooled sera at dilutions of 1:1, 1:9, 1:49, and 1:99 was run and plotted in each assay. In this way test optical densities of the standard curve could be read and results could be given as equivalence units; thus, readings from one plate could be compared with results from another plate.

### ELISA equivalence units

$$= \frac{\text{Optical density of test extrapolated from the graph}}{\text{Dilution of serum sample}}$$

### Statistics

Analysis between groups was done with the Mann-Whitney U test (two-tailed), and correlations were determined with Spearman's rank correlation.

## RESULTS

### Identification of allergen

*CRIE.* Serum IgE from individual patients bound between two and 16 protein peaks. Over 20 protein peaks in CIE were bound by IgE from at least one patient; five of these proteins were consistently labeled intensely by sera from at least three patients. The graded intensity of binding of serum IgE to six of the peaks in CRIE is shown in Table I. These include two peaks identified as antigen 7<sup>13</sup> and antigen 5<sup>12</sup> and four additional peaks arbitrarily labeled *a*, *b*, *c*, and *d*. The number of peaks labeled by IgE from an individual patient correlated with the RAST values for each patient ( $p = 0.02$ ,  $r = 0.547$ ,  $n = 17$ ). The number of peaks bound by IgE from the nine patients in group 1 and the five patients in group 3 were not significantly different (median values: 9 and 5, respectively). Peaks *a*, *b*, and *c* were potent allergens for both patients with ABPA (group 1) and atopic patients (group 3) and thus resulted in high sensitivity but low specificity for ABPA. Peak *d* was weakly allergenic for the ABPA group and was not labeled by sera from patients in group 3, hence producing high specificity but low sensitivity. Antigen 7 showed the greatest specificity while retaining sensitivity. Serum samples from two patients in the pre-ABPA group with a low total IgE had strong IgE binding to peak 7 but no binding to the other protein peaks. Control patients showed no IgE

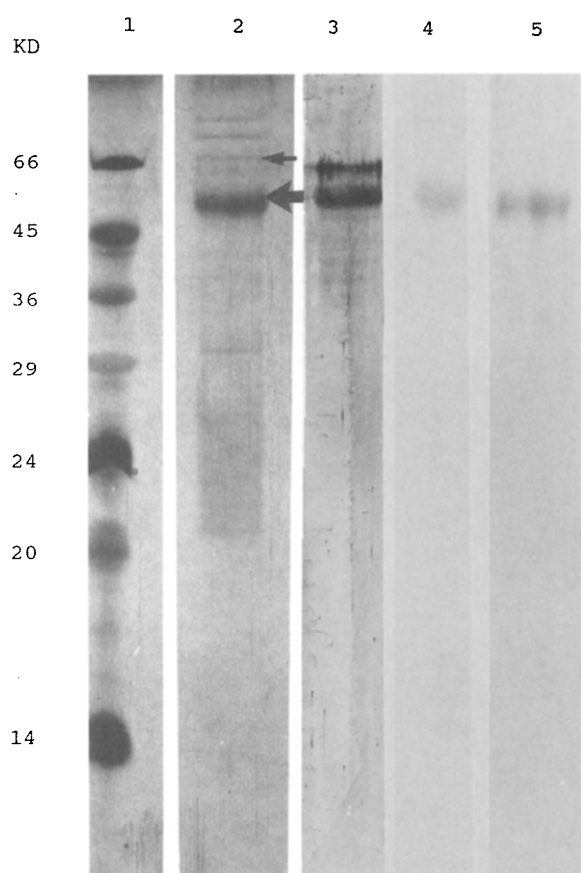


**FIG. 1.** Silver stain of SDS-PAGE (tracks 1 and 2) and immunoblot analysis (tracks 3 to 5) of the immunoprecipitate. *Lane 1*, Molecular weight standards; *lane 2*, peak 7 reduced (10 µg); *lane 3*, peak 7 reduced (10 µg); *lane 4*, peak 7 reduced (10 µg); *lane 5*, partially purified rabbit IgG (5 µg). Tracks 1 and 2 were silver-stained, and tracks 3 and 5 were immunolabeled with polyclonal rabbit IgG to peak 7 as primary antibody and anti-rabbit IgG peroxidase conjugate as secondary antibody. Track 4 was labeled with secondary antibody only. *Small arrow* denotes antigenic component of peak 7, and *large arrow* denotes the rabbit immunoglobulin heavy chain from the precipitating antibody.

binding to *A. fumigatus* proteins in CRIE (results not shown).

**SDS-PAGE and Western blotting.** The molecular weights of the proteins precipitated by antisera to peak 7 are shown in Fig. 1. Five bands with the following relative molecular weights were visualized: two at approximately 70 kD, one at 66 kD, one at 58 kD, one at 30 kD, and several minor bands at 20 to 25 kD. The bands between 20 and 25 kD and at 58 kD were thought to correspond to the dissociated heavy and light chains of IgG by virtue of their known molecular weight. Western blotting and labeling with the monospecific antiserum to

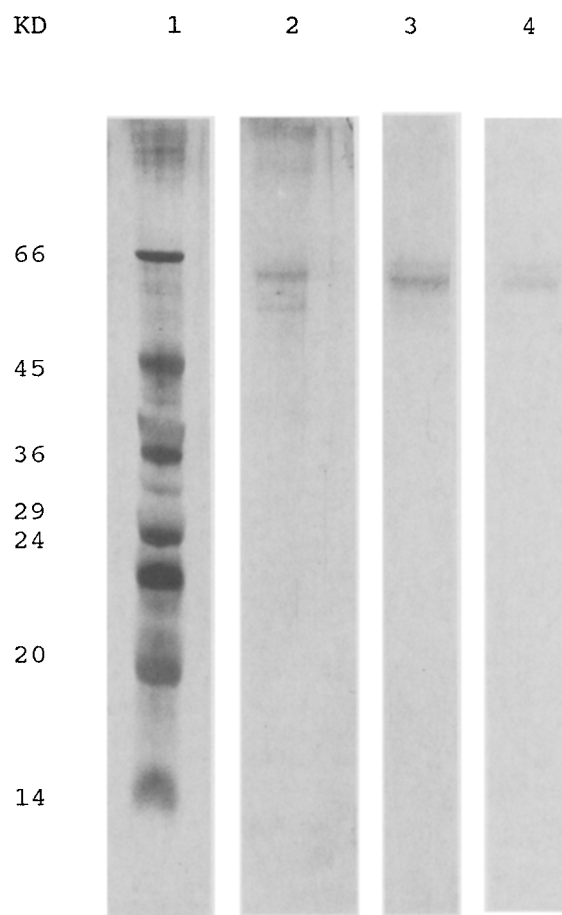


**FIG. 2.** Silver stain of SDS-PAGE (tracks 1 and 2) and immunoblot (tracks 3 and 4) of purification of gp66 by immunoaffinity column chromatography. *Lane 1*, Molecular weight markers; *lane 2*, gp66 (1 µg); *lane 3*, gp66 (1 µg) immunolabeled with polyclonal rabbit IgG to peak 7 as primary antibody and anti-rabbit IgG peroxidase conjugate as secondary antibody; *lane 4*, gp66 (1 µg) immunolabeled with pooled ABPA serum as primary antibody and anti-human IgE peroxidase conjugate as secondary antibody.

antigen 7 (Fig. 1) revealed two peroxidase-stained bands at 66 kD and 58 kD. The control tracks of immunoprecipitate labeled with only the anti-rabbit conjugate and of purified IgG labeled with primary and secondary antibody (Fig. 1) confirmed that the 58 kD band was the heavy chain of rabbit IgG. The band at 66 kD was therefore identified as the major antigenic protein component in the peak 7 immunoprecipitate.

#### Purification of the 66 kD allergen

Analysis by SDS-PAGE with silver staining revealed that the major proteins eluted from the immunoaffinity column in the glycine fraction were

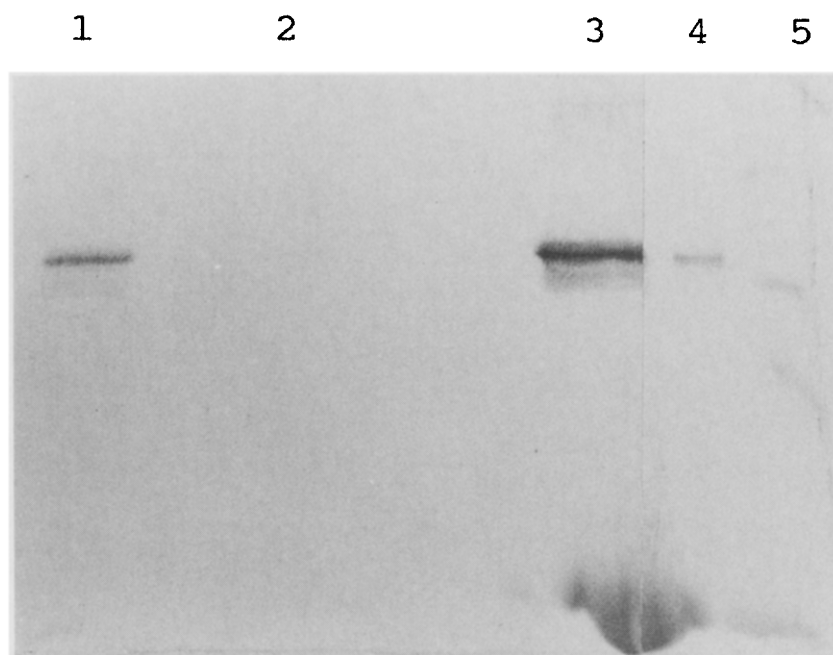


**FIG. 3.** Analysis of endoglycosidase digestion of purified gp66. Immunoblot analysis of eluents of concanavalin A chromatography. *Lane 1*, Column buffer fraction of endoglycosidase H digest of gp66; *lane 2*, 1 mol/L mannose fraction of endoglycosidase H digest; *lane 3*, column buffer fraction of gp66; *lane 4*, 1 mol/L mannose fraction of gp66; *lane 5*, molecular weight markers.

at approximately 64 and 66 kD (Fig. 2). The main IgG binding band was visualized at approximately 66 kD, (Fig. 2). The transferred proteins were also probed with patient serum and anti-IgE conjugate to identify the major allergen, which was again identified as the 66 kD protein (Fig. 2).

### Carbohydrate content

The 66 kD protein bound to concanavalin A in lectin chromatography and was displaced by the addition of 0.1 mol/L and 1 mol/L  $\alpha$ -D-mannopyranose (mannose). The purified linearized protein treated with endoglycosidase F was eluted in the assay buffer fraction and did not bind to concanavalin A. The control of undigested linearized gp66 (50  $\mu$ g/ml) was eluted in the mannose fraction (Fig. 3). The small decrease in molecular weight after digestion revealed that the carbohydrate moiety was a small proportion of the total molecule. The antigenic component of peak 7 was therefore identified as a glycoprotein component with a molecular weight of 66 kD and is therefore referred to as gp66.

### IgE to gp66 as an indicator of ABPA

The diagnostic potential of IgE measurement for diagnosis of ABPA with gp66 or the crude extract were compared. The serum IgE concentrations of patients from groups 1 and 3 specific for

the crude extract and antigen 7 are shown (Fig. 4). A greater distinction was seen between the patients in group 1 and those in group 3 when the gp66 extract ( $p = 0.004$ ) compared with the crude extract ( $p = 0.03$ ) was used. In both assays 13 of the 14 patients with ABPA showed elevated IgE responses, whereas other patients had false-negative results when the different assays were used.

The efficacy of the two assays for diagnosis of ABPA in groups 1, 3, 4, and 5 is shown in Table II. IgE specific for gp66 maintained the sensitivity of the RAST assay and increased the overall specificity from 58.5% to 78.5%. When only those patients with IgE sensitivity to *A. fumigatus* and no diagnosed ABPA (i.e., groups 3 and 4) were analyzed, only 14 of the 42 patients had a positive IgE to gp66, therefore effectively increasing the specificity from 36% to 67%.

### DISCUSSION

By using CRIE and RAST we found a correlation between detection of specific IgE to individual proteins and total *A. fumigatus*-specific IgE. This is in agreement with a previous study<sup>18</sup> but is in contrast with specific IgE detection by SDS-PAGE which does not correlate with the total IgE to *A. fumigatus* (RAST).<sup>8,19</sup> This may be attributed in part to changes in protein conformation that occur in SDS-PAGE because the anionic detergents and

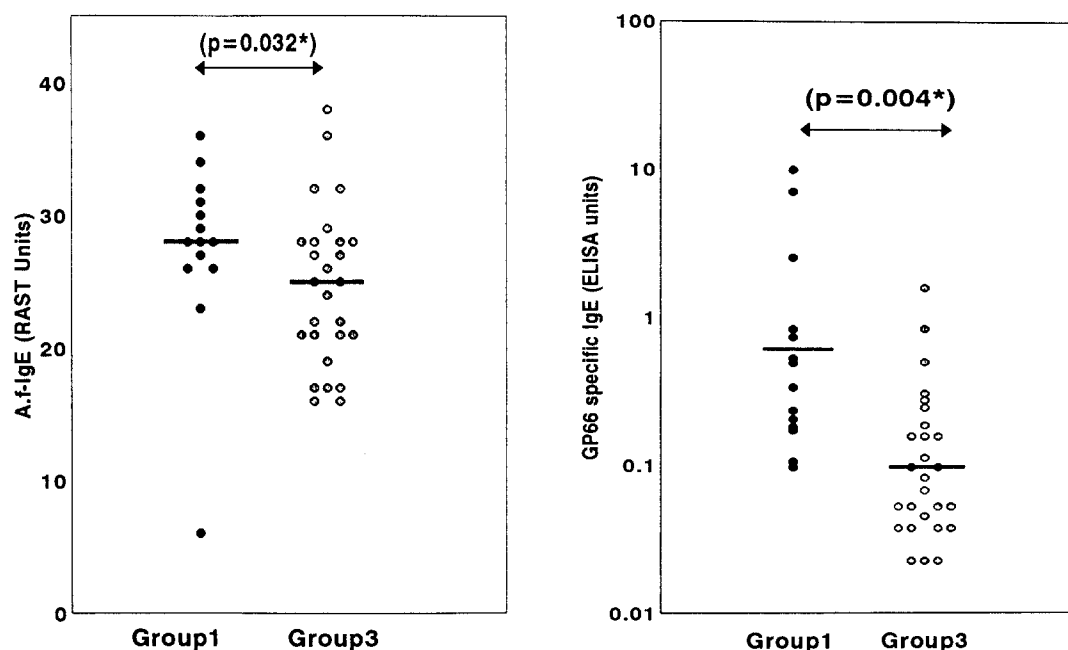


**FIG. 4.** Comparison of IgE measurement with crude extract or gp66 for distinguishing between patients with ABPA (group 1) and patients allergic to *A. fumigatus* without ABPA (group 3).  
\*Mann-Whitney U test.

reducing agents used alter IgE binding to certain proteins.<sup>20</sup>

The protein gp66, originally identified as peak 7 (antigen 7) in CRIE, improved the specificity of IgE detection for the diagnosis of ABPA in the *Aspergillus*-sensitive CF population. Previously, it was shown by Harvey and Longbottom<sup>12</sup> that the protein forming a precipitate with antisera to peak 7 was eluted from a Sepharose column in the fraction containing 150 to 200 kd proteins. This fraction, on analysis by SDS-PAGE, contained many low molecular weight bands, including one at approximately 66 kd. However, Harvey and Longbottom<sup>12</sup> identified the major protein in this fraction as a 36 kd band, which they found to have antigenic activity. This 36 kd protein was not found to be enriched in the peak 7 precipitate in our study or in the glycine eluents from the immunoaffinity column chromatography. In the presence of SDS and reducing agents, the major allergenic or antigenic protein of peak 7 was identified as a 66 kd protein; this was determined by direct analysis of peak 7 precipitate formed in CIE and by isolation of the protein by immunoaffinity column chromatography. We are not able to fully explain the discrepancies between our results and the results published previously. These results suggested that the native protein of 150 to 200 kd had a quaternary structure of homologous or heterologous subunits of approximately 66 kd held together with noncovalent bonds and/or disulfide bridges. The

**TABLE II.** Comparison of the efficacy of IgE concentration for ABPA diagnosis with the ELISA for detecting IgE to gp66 (IgE-gp66) and the RAST for detecting IgE to the crude *A. fumigatus* extract (IgE-Af)

| IgE-gp66                                       | ABPA<br>(n = 14) | Allergic<br>(n = 42) | Nonallergic<br>(n = 23) |
|------------------------------------------------|------------------|----------------------|-------------------------|
| Positive ( $\geq 1$ ELISA U)                   | 13               | 14                   | 0                       |
| Negative ( $< 1$ ELISA U)                      | 1                | 28                   | 23                      |
| Efficacy                                       |                  |                      |                         |
| Sensitivity (%)                                | 92.9             | —                    | —                       |
| Specificity (%)*                               | —                | 67                   | 100                     |
| IgE-Af                                         | ABPA<br>(n = 14) | Allergic<br>(n = 42) | Nonallergic<br>(n = 23) |
| Positive ( $\geq 15\%$ $^{125}\text{I}$ bound) | 13               | 27                   | 0                       |
| Negative ( $< 15\%$ $^{125}\text{I}$ bound)    | 1                | 15                   | 23                      |
| Efficacy                                       |                  |                      |                         |
| Sensitivity (%)                                | 92.9             | —                    | —                       |
| Specificity (%)†                               | —                | 36                   | 100                     |

\*Overall specificity = 78.5%.

†Overall specificity = 58.5%.

protein nature of 66 kd band was evident because it was visualized with silver stain. Both the native protein and the 66 kd subunits were demonstrated to be glycoproteins, with a minor carbohydrate

moiety containing  $\alpha$ -D-mannopyranose or  $\alpha$ -D-glucopyranose terminal residues. The protein gp66 was found to be only a very minor component of *A. fumigatus* extracts in SDS-PAGE; it was not detected in the crude extract with Coomassie brilliant blue stain, and the yield in immunoaffinity column chromatography was low. The low concentration may explain why it was not described as a major antigenic band in earlier articles. Previously,<sup>10</sup> it was shown that release of this immunoreactive protein peaked after 24 days of incubation and was only present consistently after 14 days of incubation. This is in contrast to Asp f 1, a potent allergen of *A. fumigatus* for both patients with ABPA and asthmatic patients, which peaks after 3 days of growth.<sup>21</sup> This may account for the more restricted antibody response to gp66 because only patients with established colonies will be exposed to and become sensitized to the antigen. By comparing CIE plates with archive plates provided by Joan Longbottom, peak *a* in this study was thought to correspond to antigen 3.<sup>16</sup> Antigen 3 has been shown to have a molecular weight of 18 kd<sup>22</sup> and is thought to correspond to Asp f 1. In CRIE, peak *a* was a potent allergen for both the group of patients with allergy and the group of patients with ABPA. The majority of allergens described for atopy and ABPA have been low molecular weight proteins: less than 14 kd,<sup>17</sup> 18 kd,<sup>20</sup> and 18 kd.<sup>23</sup> Recently, an allergenic or antigenic 65 kd fusion protein expressed by complementary DNA of *A. fumigatus* has been described,<sup>24</sup> and it is thought to be a member of the heat shock protein family. This protein may correspond to the 66 kd protein described here. The nature of fusion proteins does allow for comparisons of posttranscriptional changes that lead to quaternary structure and glycosylation. It is hoped that any homology can be examined further by using available antisera. Another high molecular weight antigenic glycoprotein has been described,<sup>25</sup> which had a molecular weight of 55 kd in the reduced state and 450 kd in the native state. The differences in molecular weight indicate that the glycoprotein is not the same as the gp66 described here.

The ELISA developed to measure IgE to gp66 proved to be a useful tool in the diagnosis of ABPA. The specificity of the IgE assay was greatly improved compared with the RAST in which the crude extract was used. Surprisingly, this was not at the expense of sensitivity. This assay would prove useful whether used alone or in conjunction with the traditional assay in which unpurified extract is used. Diagnosis of ABPA in patients with CF is

problematic, with variations in the classical indicators being described, such as normal total IgE<sup>2</sup> and no precipitating antibody.<sup>4</sup> Asymptomatic episodes of ABPA have been described when specific antibody concentrations become as high as those during symptomatic phases, which if they remain untreated, may develop into symptomatic episodes.<sup>26</sup> Colonization by *A. fumigatus*, although critical in the development of the disease, is not often used for diagnostic purposes. False-positive results often occur,<sup>27</sup> and repeated samples are required. Antibody levels to gp66 represent an indication of the potential to develop an inflammatory response to this organism and may also prove to be a marker of colonization of the airways. We believe that longitudinal analysis with this assay will prove to be useful in detecting ABPA in the early stages, because we detected antibodies to gp66 in two patients 3 months before the development of the clinical symptoms of ABPA. Early diagnosis will aid intervention with antifungal agents and steroid treatment, which may prevent complications, such as proximal bronchiectasis.

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