

Specific IgG subclass antibody pattern to *Aspergillus fumigatus* in patients with cystic fibrosis with allergic bronchopulmonary aspergillosis (ABPA)

M Skov, T Pressler, H E Jensen, N Høiby, C Koch

Abstract

Background—IgG and IgG subclass antibodies to *Aspergillus fumigatus* (*A. fumigatus*) were measured in a large population of patients with cystic fibrosis to elucidate a putative antibody pattern specific for allergic bronchopulmonary aspergillosis (ABPA).

Methods—An ELISA technique using water soluble somatic hyphal (WSSH) *A. fumigatus* antigens and subclass specific monoclonal antibodies was used for cross sectional quantification of IgG and IgG₁₋₄ subclass antibody levels in the serum of 238 patients with cystic fibrosis and 107 healthy controls.

Results—In patients with cystic fibrosis persistently colonised with *A. fumigatus* the subclass antibody levels were significantly increased compared with patients with cystic fibrosis never or rarely colonised ($p < 0.001$). The group of patients persistently colonised with *A. fumigatus* with ABPA (+Af+ABPA) had significantly increased levels of IgG antibodies to *A. fumigatus* (Af-IgG) (median 69 ELISA units (EU) versus 31) and of subclasses Af-IgG₁ (91 versus 27), Af-IgG₂ (143 versus 56), and Af-IgG₄ antibodies (72 versus 20), but not of IgG₃ (17 versus 15), compared with the colonised patients without ABPA (+Af-ABPA). Patients with cystic fibrosis with no or only rare isolates of *A. fumigatus* without ABPA (-Af-ABPA) also had significantly increased subclass antibody levels (Af-IgG₁ 9 versus 3, Af-IgG₂ 28 versus 5, Af-IgG₄ 16 versus 4; $p < 0.001$) compared with healthy controls. Low, although detectable, levels of antibodies were demonstrated in healthy controls. ABPA seemed to occur independently of *Pseudomonas aeruginosa* infection. Using diagnostic cut off levels for ABPA, sensitivity and specificity were calculated. The highest specificity was found for IgG₁ (88%); sensitivity was between 65% and 73%. The positive predictive values (PPV) were moderate, whereas the negative predictive values (NPV) were high (96% in all subclasses except IgG₃ with 94%). PPV increased to 50% if IgG₁ as well as IgG₂ and IgG₄ were included.

Conclusions—In a large number of unselected patients with cystic fibrosis significantly increased levels of Af-specific antibodies belonging to total IgG and all

four subclasses were found in all groups of patients compared with healthy controls. In patients persistently colonised with *A. fumigatus* these levels were significantly higher than in non-colonised patients, and the significantly highest levels (with the exception of IgG₃) were found in patients with ABPA. Using a sensitive ELISA technique, measurements of IgG and IgG subclass antibodies to *A. fumigatus* might be of importance in the management of ABPA, especially as a screening test to exclude the presence of ABPA; other tests are needed to confirm the diagnosis.

(Thorax 1999;54:44–50)

Keywords: cystic fibrosis; allergic bronchopulmonary aspergillosis; IgG subclass antibodies

Colonisation of the lower respiratory tract with *A. fumigatus* is particularly frequent in patients with cystic fibrosis in whom an incidence of 57%¹ and a prevalence of 40%² have been reported. A sizeable minority, ranging from 1% to 11% in various reports, develop allergic bronchopulmonary aspergillosis (ABPA).^{1,3} The diagnosis of ABPA in patients with cystic fibrosis is more difficult than in other patients because several clinical and paraclinical findings are similar to those caused by the more frequent bacterial infections, and by atopy which is present in up to 27% of patients with cystic fibrosis.¹ It would therefore be valuable to obtain improved diagnostic tools for the early and specific diagnosis of ABPA in patients with cystic fibrosis. Interaction of specific IgG and IgE antibodies with *A. fumigatus* antigens in the bronchial tree leads to activation of complement and mast cells resulting in mediator release and cytokine production, contributing to lung damage.³ We have previously demonstrated that the distribution of anti-*Pseudomonas aeruginosa* antibodies among the four IgG subclasses is related to the degree of lung damage in patients with cystic fibrosis and chronic *P. aeruginosa* infection.⁴ As an initial step we therefore undertook the present comprehensive study of specific total IgG and subclasses IgG₁₋₄ antibodies to *A. fumigatus* in 238 unselected patients with cystic fibrosis and related the findings to the degree of colonisation with *A. fumigatus* and to the presence and absence of ABPA.

Department of Pediatrics

M Skov
T Pressler
C Koch

Department of Clinical Microbiology

N Høiby

National University Hospital, Copenhagen, Denmark

Department of Pharmacology and Pathobiology, The Royal Veterinary and Agricultural University, Copenhagen, Denmark
H E Jensen

Correspondence to:
Dr M Skov, Center of Cystic Fibrosis 5003, Department of Pediatrics GGK, Juliane Marie Centre, National University Hospital, Blegdamsvej 9, DK-2100 Copenhagen Ø, Denmark.

Received 26 January 1998
Returned to author
15 April 1998
Revised manuscript received
6 July 1998
Accepted for publication
17 September 1998

Methods

PATIENTS

The diagnosis of cystic fibrosis was based on abnormal sweat electrolytes, cystic fibrosis specific mutations, and typical clinical appearance. All patients are seen on a regular monthly basis in the Danish Cystic Fibrosis Centre. At each visit the clinical condition, pulmonary function, and sputum microbiology including plating on Sabaraud maltose agar to detect fungal growth are assessed. Serological parameters such as eosinophil count, IgE, Af-IgE (Radio Allergo Sorbent Test, RAST), precipitating antibodies to *A fumigatus*, and immunoglobulins G, A and M are measured at least once a year. Data have thus been recorded prospectively since 1970.

Based on data obtained over a five year period (1990–1994), all patients with cystic fibrosis were divided into three groups. Group 1: ABPA (+Af+ABPA), patients with cystic fibrosis persistently colonised with *A fumigatus* and with ABPA (median age 16.5 years (range 9–37), $n = 26$); group 2: non-ABPA (+Af-ABPA), patients with cystic fibrosis persistently colonised but without ABPA (median age 19 years (range 9–44), $n = 35$); and group 3: non-*A fumigatus* (-Af-ABPA), patients with cystic fibrosis with no or only rare isolates of *A fumigatus* (median age 18 years (range 1–45), $n = 177$). The results were compared with healthy controls (group 4), a normal healthy control population aged 1–75 years ($n = 107$) from the serum bank at the Department of Clinical Microbiology, National University Hospital.

All serum samples from the cystic fibrosis patients were obtained within one year (1995) and with a few exceptions during the winter and stored at -20°C until use.

DIAGNOSIS OF ABPA

The “golden standard” diagnostic criteria for ABPA are, according to Nelson¹: (1) radiological pulmonary infiltrates, (2) wheezing, (3) positive *Aspergillus* cultures, (4) positive immediate type reaction by epidermal skin test with *A fumigatus*, (5) increased serum levels of total IgE, (6) increased serum levels of *A fumigatus* specific IgE, and (7) increased serum levels of *A fumigatus* precipitating antibodies. Pulmonary infiltrates and wheezing are, however, extremely common in cystic fibrosis, and atopy, including presence of *A fumigatus* specific IgE, is more frequent than in the background population. The specificity of these criteria is therefore low in patients with cystic fibrosis. In the present study we therefore defined ABPA as the persistent presence of *A fumigatus* in lower tract secretions, combined with persistent abnormally raised titres of *A fumigatus* specific IgE and precipitating antibodies over the five year observation period. *A fumigatus* specific precipitating antibodies, expressed as titre, were measured at the Department of Clinical Microbiology, University Hospital, Aarhus (normal value $\leq 2^5$) and specific *A fumigatus* IgE antibodies were measured at the Laboratory of Medical Allergy,

National University Hospital, Copenhagen (normal value, RAST class 0⁶).

Diagnostic cut off levels for the diagnosis of ABPA were determined for IgG and IgG subclass antibodies.

PREPARATION OF *A FUMIGATUS* ANTIGEN

Following the disintegration of mycelial mats of *A fumigatus* strain ATCC 42202 in an X-press operated at a maximal force of 200 MPa at -30°C , preparation of water soluble somatic hyphal (WSSH) antigens were prepared as described previously.⁷ The protein concentration was measured using a refractometer (TS meter, AO Scientific Instruments, Alabama, USA). The antigen preparation was stored (from 1992 until use (1996)) at -80°C .

ELISA

ELISA assays similar to those used previously in our laboratory were established.⁸ Irradiated 96-well polystyrene microtitre plates (Nunc-Immuno-Plate, Maxisorb, Nunc, Denmark) and reagent volumes of 100 μl were used. All incubation steps were carried out at 22°C . Between each step the plates were washed in phosphate buffer saline (PBS) with 0.1% Tween 20 (Bie & Berntsen, Denmark), pH 7.2. Antigen was diluted 1:10 000 for IgG and 1:1000 for IgG subclasses in PBS and incubated for one hour (0.5 μg of protein per well for IgG and 5.1 μg per well for IgG subclasses) at room temperature (22°C). The samples for IgG measurements reacted overnight (18 hours) at 5°C after coating. Additional binding sites in the wells were blocked with the PBS, 0.1% Tween 20. Serum samples were diluted 1:4000 for IgG measurements. IgG subclass samples were incubated with antigen for an hour and blocked with PBS and serum in a dilution of 1:400 before they were allowed to react overnight at 22°C . Peroxidase-conjugated rabbit anti-human IgG specific for gamma chains (Dako, Denmark) and mouse monoclonal anti-human IgG₁₋₄ conjugated to horse radish peroxidase (HRP) (Central Laboratory of the Red Cross, The Netherlands) were added and incubated for one hour. The specific antibodies were diluted 1:10 000 for IgG, 1:60 000 for IgG₁, 1:3000 for IgG₂, 1:2000 for IgG₃, and 1:20 000 for IgG₄. To each well 1,2-phenylenediamine solution (0.1 M citric acid-phosphate buffer, pH 5.0; Dako, Denmark) containing 5 μl H_2O_2 per plate was added. After incubation for one hour the reaction was stopped by adding H_2SO_4 (1 M). The optical density (OD) was measured using an ELISA plate reader (492 nm, Bio-rad, Japan). All measurements were performed in duplicate. The antigen concentration and serum and antibody dilutions used in the present assays were chosen from serial dilutions carried out in pilot studies. A standard pool of serum from six cystic fibrosis patients colonised with *A fumigatus* was used for calibration for each plate. A logarithmic curve was drawn from the results of the standard serum in each plate. The values were determined in relation to the curve and expressed in ELISA units (EU).⁸

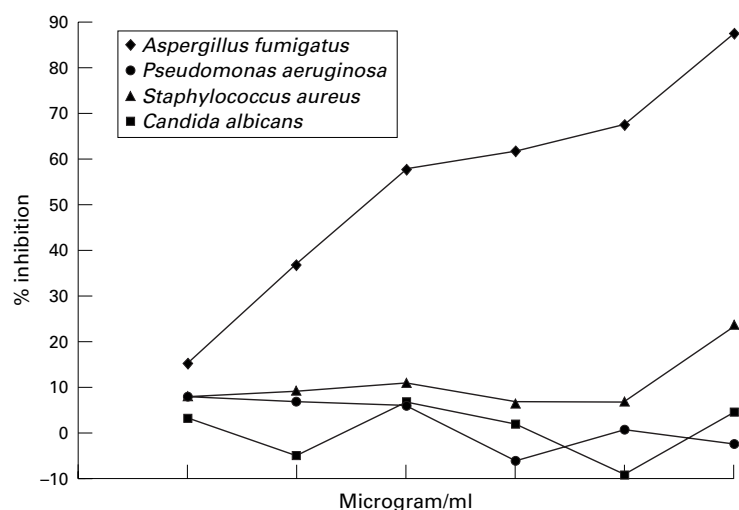


Figure 1 Antigenic specificity of the assay expressed as increasing % inhibition (decreasing antibody activity) after absorption of the standard serum with increasing amounts of antigen preparations of *A. fumigatus*, *P. aeruginosa*, *S. aureus*, and *C. albicans*.

ASSESSMENT OF ANTIGENIC SPECIFICITY OF THE ASSAY

P. aeruginosa and *Staphylococcus aureus* are common causes of pulmonary infection in patients with cystic fibrosis and most have

increased specific antibody titres against these organisms. The influence of antibodies to these bacteria on specific *Aspergillus* antibodies was assessed. Another fungus, *Candida albicans*, which is known to be present in the lungs of patients with cystic fibrosis, was also absorbed. Standard serum samples diluted 1:10 000 for IgG and 1:1000 for IgG subclasses were absorbed with increasing amounts of sonicated preparations of *P. aeruginosa*, *S. aureus*⁸ (protein A-deficient strain to avoid reaction with the Fc part of IgG)⁹ and *C. albicans*. The absorption was compared with similar absorption performed with *A. fumigatus* antigen.

REPRODUCIBILITY OF THE ASSAY

Twelve serum samples from cystic fibrosis patients colonised with *A. fumigatus* (samples covering the spectrum from low to high levels) were tested in duplicate in two different plates on two different days. The intraplate, plate-to-plate, and day-to-day variations were determined using the formula: standard deviation (SD) = ($\Sigma d^2/2n$), where Σd^2 = the sum of the squared differences of double determinations of the same sample, and n = the number of observations.

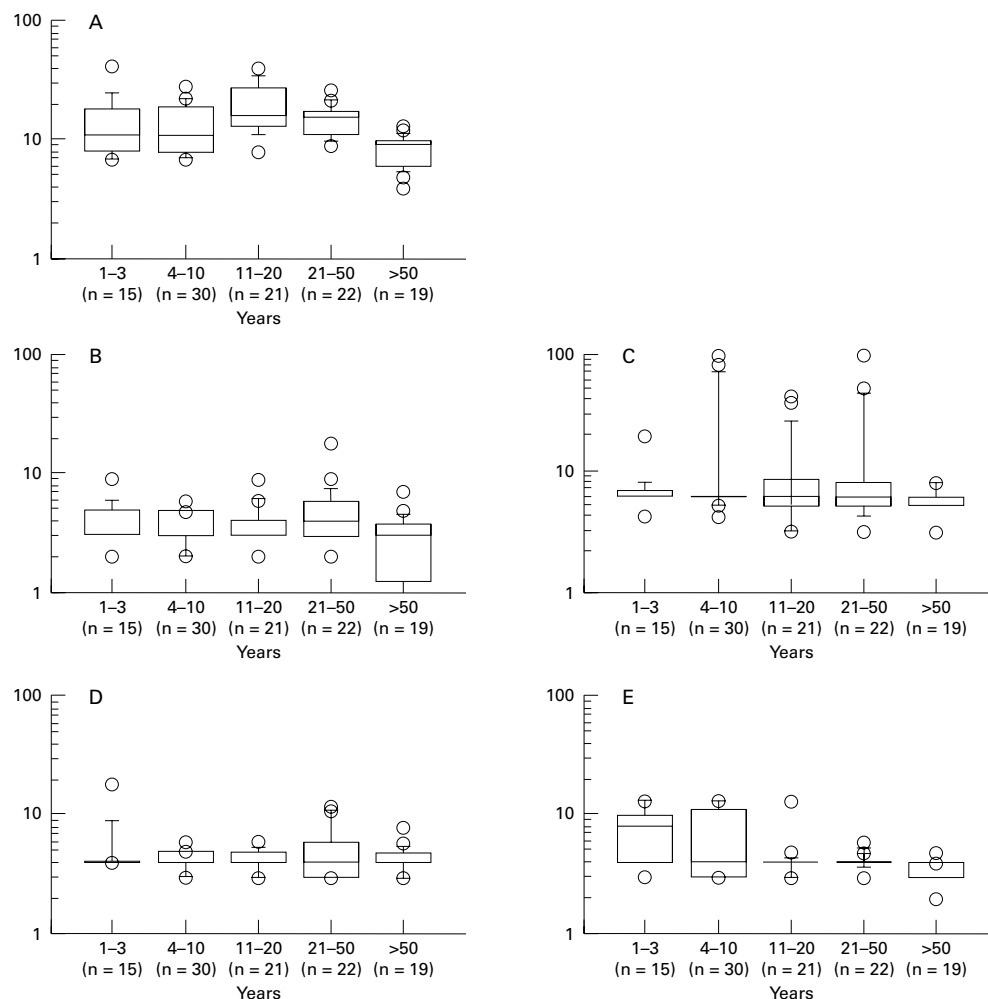


Figure 2 *A. fumigatus*-IgG and IgG subclass antibody levels in 107 healthy controls. A = IgG, B = IgG₁, C = IgG₂, D = IgG₃, E = IgG₄. Values are given in ELISA units (EU). The box plots show the 10th, 25th, 50th, 75th, and 90th percentiles. Values above and below are shown separately.

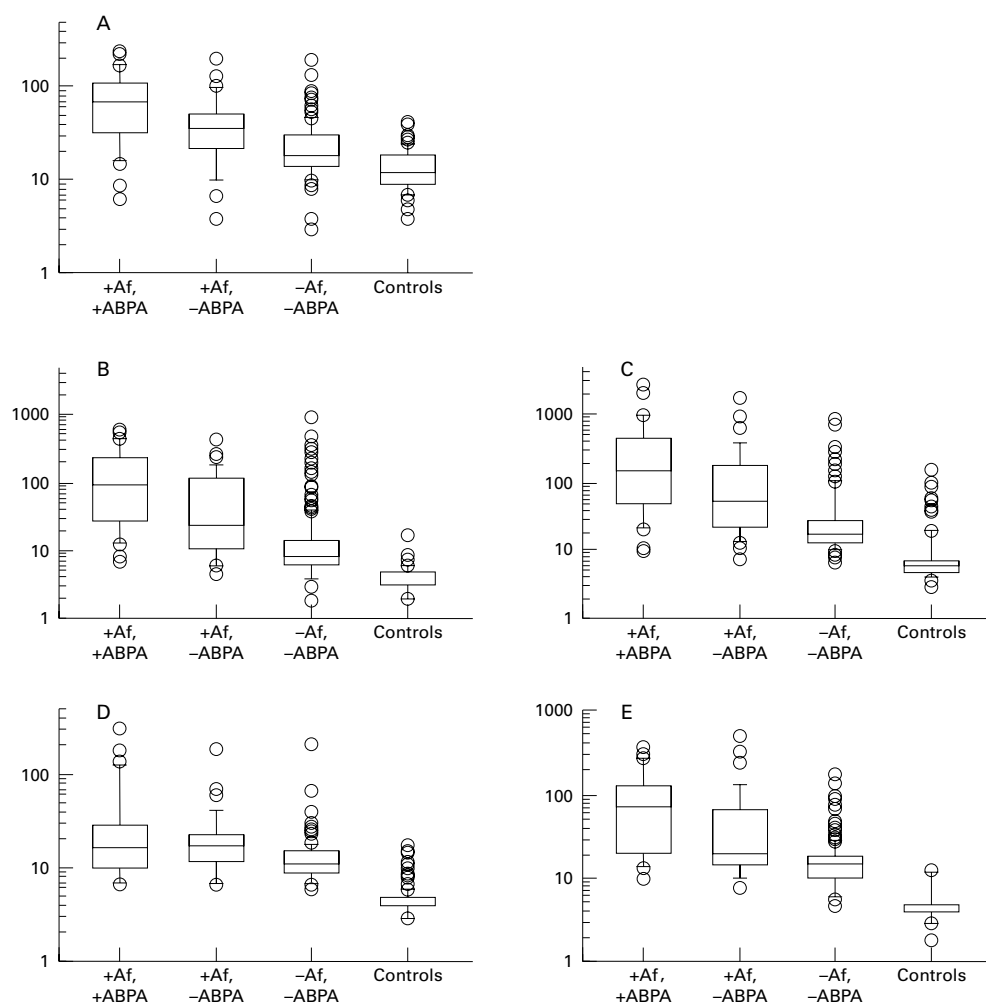


Figure 3 *A fumigatus*-IgG and IgG subclass antibody levels in ABPA (+Af+ABPA) patients, non-ABPA (+Af-ABPA) patients, non-*A fumigatus* (-Af-ABPA) patients, and controls. A = IgG, B = IgG₁, C = IgG₂, D = IgG₃, E = IgG₄. Values are given in Elisa units (EU). The box plots show the 10th, 25th, 50th, 75th, and 90th percentiles. Values above and below these percentiles are shown separately. +Af+ABPA (n = 26, median age 18.5 years (range 9–37)); +Af-ABPA (n = 35, age 19 years (9–44)); -Af-ABPA (n = 177, age 18 years (1–45)); controls (n = 107, age 21 years (1–75)). The p value between two adjoining groups in each box plot is p < 0.001 in IgG and all the subclasses except in IgG₃ (panel D); p = 0.02 between +Af-ABPA and -Af-ABPA; p = 0.8 between +Af+ABPA and +Af-ABPA.

ANALYSIS OF DATA

Sensitivity (SE), specificity (SP), positive predictive value (PPV), and negative predictive value (NPV) were calculated according to the guidelines from the Workshop on Experimental Methodology.¹⁰ An optimal diagnostic cut off level for ABPA was determined, giving maximum improvements for diagnostic accuracy, which means improving SE without losing SP, or increasing SP without decreasing SE.

To compare all four groups the non-parametric equivalent of an analysis of variance (the Kruskal Wallis test) was performed. The Mann-Whitney U test for non-parametric unpaired data was used within the groups whenever the Kruskal Wallis test was significant. p values of <0.05 were considered significant. The values given are medians (range). For independence the χ^2 test was used.

Results

SPECIFICITY AND REPRODUCIBILITY OF THE ASSAYS

Absorption with increasing amounts of *A fumigatus* antigen resulted in decreased antibody

activity (increased inhibition) in IgG as well as IgG₁₋₄ subclasses. Absorption with either *P aeruginosa*, *S aureus*, or *C albicans* antigen resulted in only a minor reduction in the antibody activity (fig 1).

The intraplate variation was 15%, 10%, 17%, 11%, and 17%, respectively, for IgG, IgG₁, IgG₂, IgG₃, and IgG₄, the interplate variation was 17%, 9%, 13%, 16%, and 14% and the day-to-day variation was 8%, 27%, 15%, 10%, and 17%, respectively.

IGG AND IGG SUBCLASS ANTIBODIES TO *A FUMIGATUS* IN SERUM FROM HEALTHY CONTROLS
Low levels of IgG and IgG subclass antibodies to *A fumigatus* were seen in healthy controls (fig 2).

IGG AND IGG SUBCLASS ANTIBODIES TO *A FUMIGATUS* IN SERUM FROM PATIENTS WITH CYSTIC FIBROSIS AND HEALTHY CONTROLS
Figure 3 shows the levels of IgG and IgG₁₋₄ subclass specific antibodies to *A fumigatus* in serum samples from patients with cystic fibrosis and controls. In cystic fibrosis patients

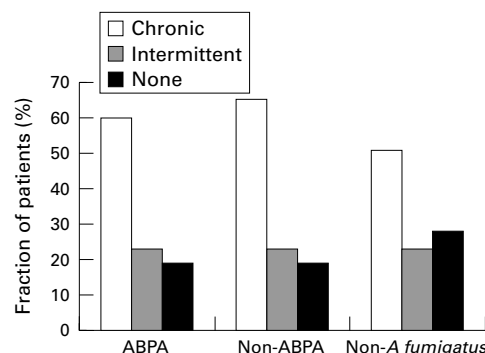


Figure 4 Distribution of chronic, intermittent, or no *P aeruginosa* infection in the three groups of patients with cystic fibrosis: ABPA (+Af+ABPA), non-ABPA (+Af-ABPA), and non-*A fumigatus* (-Af-ABPA).

rarely colonised with *A fumigatus* (group 3) significantly higher IgG ($p < 0.001$) as well as IgG₁₋₄ antibody levels ($p < 0.001$) were observed compared with controls. The most marked increases were observed in patients persistently colonised with *A fumigatus* (groups 1 and 2). Significantly higher IgG and IgG subclass antibody levels were observed for both groups compared with patients rarely colonised (group 3; $p < 0.001$). Patients persistently colonised with *A fumigatus* with ABPA (group 1) had significantly higher IgG and IgG₁, IgG₂, and IgG₄ ($p < 0.001$) antibody levels, whereas the IgG₃ antibody level was not significantly increased ($p = 0.8$) compared with persistently colonised patients without ABPA (group 2; fig 3).

SENSITIVITY, SPECIFICITY, PPV AND NPV

Distinction between cystic fibrosis patients with ABPA and those without ABPA (with or without *A fumigatus* colonisation) was made using diagnostic cut off levels in each IgG subclass antibody. The sensitivity (% of patients with ABPA with a positive test), specificity (% of patients without ABPA with a negative test), positive predictive value (PPV = % of patients with a positive test with ABPA), and negative predictive value (NPV = % of patients with a negative test without ABPA) were calculated. The diagnostic cut off levels for ABPA were ≥ 40 EU for IgG, ≥ 40 EU for IgG₁, ≥ 60 EU for IgG₂, ≥ 15 EU for IgG₃, and ≥ 35 EU for IgG₄. Sensitivity was calculated to be 69%, 73%, 73%, 65%, and 73%, respectively, for IgG, IgG₁, IgG₂, IgG₃, and IgG₄, specificity was 82%, 85%, 77%, 67%, and 88%, PPV was 32%, 37%, 28%, 19%, and 43%, and NPV was 96%, 96%, 96%, 94%, and 96%, respectively. The specificity increased to 93% and the PPV to 50% if IgG₁, IgG₂, and IgG₄ were combined.

CORRELATION BETWEEN *P AERUGINOSA* STATUS AND *A FUMIGATUS* STATUS

The three groups of cystic fibrosis patients were correlated according to their *P aeruginosa* status. A possible predisposition to colonisation with *A fumigatus* in patients chronically colonised with *P aeruginosa* was examined. The *P aeruginosa* status was either chronic, intermittent, or none at the time of the study. Chronic *P aeruginosa* means positive *P aeruginosa* culture in six succeeding months or

shorter if there is a significant increase in specific precipitating antibodies to *P aeruginosa* (≥ 2); intermittent *P aeruginosa* means positive *P aeruginosa* culture at least once with normal precipitating antibodies to *P aeruginosa* (0–1). A similar distribution of chronic colonisation with *P aeruginosa* was seen in the three groups (group 1 versus 2, $p = 0.28$; group 2 versus 3, $p = 0.26$); 60% in the ABPA group, 65% in the non-ABPA group, and 51% in the non-*Aspergillus* group. Intermittent colonisation with *P aeruginosa* was seen in 23%, 23%, and 21% and no colonisation in 19%, 14%, and 28% in the ABPA, non-ABPA and non-*Aspergillus* groups, respectively (fig 4).

Discussion

This study demonstrates a specific Af-IgG subclass pattern in cystic fibrosis patients with ABPA (+Af+ABPA), with significantly increased levels of Af-IgG₁, Af-IgG₂ and Af-IgG₄, but not Af-IgG₃ compared with non-ABPA (+Af-ABPA) cystic fibrosis patients. Both of these patient groups had significantly increased levels of all Af-IgG subclasses compared with the non-*Aspergillus* colonised patients with cystic fibrosis (-Af-ABPA). Furthermore, in the latter group significantly higher levels in all four IgG subclasses were found compared with healthy controls. Thus, generally higher levels of IgG and IgG subclass antibodies to *A fumigatus* were seen in all patients with cystic fibrosis compared with healthy controls of comparable age. Low but detectable levels of all four subclasses of antibodies were found in all the healthy controls.

The highest specificity for the diagnosis of ABPA was reported in subclass IgG₄ (88%), which increased to 92% if IgG₁ and IgG₂ were also included. The PPV was moderate with IgG and the IgG subclasses while the NPV was high (96%, except 94% in IgG₃). With 82% specificity of total IgG and 96% NPV, measurements of total IgG alone may be as useful as measurement of the subclasses. Determination of IgG and IgG subclass antibody levels may be particularly valuable as a screening test to exclude the presence of ABPA. Other tests still have to be used to confirm the diagnosis of ABPA.

The assays presently used have high antigen specificity with regard to IgG and the IgG subclasses. With an analytical variation similar to other assays used for the determination of *A fumigatus* IgG antibodies¹¹ or *P aeruginosa* subclass antibodies,^{8 12 13} the reproducibility was acceptable. The ELISA assays were calibrated with pooled high titre serum samples and therefore the precision of the measurement may decrease slightly with very low levels of antibodies. Most of the serum samples were obtained in the winter time to avoid a possible seasonal variation in exposure to *A fumigatus*.

In various studies patients with cystic fibrosis have been divided into groups according to increasing positivity to different *A fumigatus* specific tests.^{14–16} All these studies have shown the same gradual increase of all the IgG subclasses with increasingly positive *A fumigatus* specific tests, much like the grouping in this

study. However, our study is a cross sectional study using a much larger number of unselected cystic fibrosis patients. Furthermore, it seems to be the only study in which Af-IgG₃ does not increase significantly from +Af-ABPA patients to +Af-+ABPA patients compared with the other subclasses. The reason for this is unclear.

Most studies of ABPA have been performed in non-cystic fibrosis patients. Comparing non-cystic fibrosis ABPA patients with patients with aspergillomas, asthmatics, and healthy controls, Kurup *et al* found significantly increased levels of all four subclasses in the ABPA group but lower levels of Af-IgG₃ than the other isotypes.¹⁷ Similar findings were presented in a study comparing patients with serological signs of ABPA (ABPA-S) and patients with serological ABPA including central bronchiectasis (ABPA-CB).¹⁸ Af-IgG₁, Af-IgG₂, and Af-IgG₄ levels were significantly increased in ABPA-CB patients compared with ABPA-S patients while Af-IgG₃ levels were similar.¹⁸

Two smaller studies have suggested that increased levels of Af-IgG₄¹⁹ or Af-IgG₁ and Af-IgG₄²⁰ are characteristic of ABPA. Even though we found ABPA to be characterised by increased Af-IgG₁, Af-IgG₂, and Af-IgG₄, it was confirmed by the present study that Af-IgG₄ showed highest specificity and that specificity increased if Af-IgG₁ and Af-IgG₄ were both increased.

Interestingly, using the recombinant *A fumigatus* protein antigen 1/a (rAsp f 1/a) Nikolaizik *et al*¹⁴ recently found significantly increased levels of IgG₁ and IgG₄ in patients with cystic fibrosis with ABPA compared with patients with cystic fibrosis allergic only to *Aspergillus*. Specific IgG₂ and IgG₃ levels were, however, not measured.

Demonstration of precipitating antibodies to *A fumigatus* is the most widely used method for serodiagnosis of ABPA. Thus, only two criteria have been reported to be highly specific for ABPA—namely, raised IgG and IgE antibodies to *A fumigatus* combined with central bronchiectasis.³ The sensitivity of ELISA has the clinical advantage that the presumed pre-injury phase of ABPA may be diagnosed by using IgG and IgE ELISAs and, furthermore, it has been proved to be superior to immunoblotting.^{4, 21, 22} Using the current standard criteria ABPA may not be diagnosed until considerable loss of lung function is present. The prevalence of detectable IgG antibodies to *A fumigatus* has been reported to increase with age in patients with cystic fibrosis.^{15, 23} In one study 35 of 50 patients with cystic fibrosis aged 2.5–29 years had measurable IgG antibodies to *A fumigatus*; all patients over 12 years and none younger than four years had measurable IgG antibodies to *A fumigatus*.²³ Accordingly, 84% of 147 patients with cystic fibrosis aged 5–43 years had serum IgG antibodies to *A fumigatus* allergen 1/a (Asp f 1), increasing to 98% in patients over 10 years.¹⁶ The low levels of Af-IgG antibodies in healthy controls in the present study are probably due to the ubiquitous presence of the fungus.

An association between *P aeruginosa* colonisation and the development of ABPA was reported by Nikolaizik *et al*.¹⁴ This is in contrast to a previous report,² as well as the present findings, showing that ABPA occurs independently of *P aeruginosa* infection.

In conclusion, measurements of IgG and IgG subclass antibodies to *A fumigatus*, using a multivalent antigen extract and ELISA techniques, may be valuable in the diagnosis of ABPA, especially as a screening test to exclude the diagnosis. Confirmation of the diagnosis still requires other tests. We have shown a specific pattern of significantly increased Af-IgG₁, Af-IgG₂, and Af-IgG₄, but not Af-IgG₃, antibody levels in patients with ABPA. Measurement of the IgG₄ antibody was most specific in establishing the diagnosis of ABPA. The NPV was high in IgG as well as in all subclasses. Further longitudinal studies of IgG subclass antibodies to follow fluctuations in the levels due to changes in disease activity are needed for appropriate medication. Early diagnosis and treatment of ABPA may be of importance in the prevention of serious and potentially irreversible lung damage.

This study was supported by The Ville Heise Foundation and The Danish Research Councils.

- 1 Nelson LA, Callera ML, Schwartz RH. Aspergillosis and atopy in cystic fibrosis. *Am Rev Respir Dis* 1979;20:863–73.
- 2 Schönheyder H, Jensen T, Høiby N, *et al*. Frequency of *Aspergillus fumigatus* isolates and antibodies to *Aspergillus* antigens in cystic fibrosis. *Acta Pathol Microbiol Immunol Scand Sect B* 1985;93:105–12.
- 3 Greenberger PA, Miller T, Roberts M, *et al*. Allergic bronchopulmonary aspergillosis in patients with and without evidence of bronchiectasis. *Ann Allergy* 1993;70:333–8.
- 4 Pressler T, Mansa B, Jensen T, *et al*. Increased IgG₂ and IgG₃ concentration is associated with advanced *Pseudomonas aeruginosa* and poor pulmonary function in cystic fibrosis. *Acta Paediatr Scand* 1988;77:576–82.
- 5 Schönheyder H, Andersen P, Stenderup A. Serum antibodies to *Aspergillus fumigatus* in patients with pulmonary aspergillosis detected by immunofluorescence. *Acta Pathol Microbiol Immunol Scand Sect B* 1982;90:273–9.
- 6 Wide L, Bennich H, Johansson SG. Diagnosis of allergy by an in-vitro test for allergen antibodies. *Lancet* 1967;ii:1105–7.
- 7 Jensen HE, Aalbak B, Lind P, *et al*. Development of murine monoclonal antibodies for the immunohistochemical diagnosis of systemic bovine aspergillosis. *J Vet Diagn Invest* 1996;8:68–75.
- 8 Pressler T, Pedersen SS, Espensen F, *et al*. IgG subclass antibodies to *Pseudomonas aeruginosa* in sera from patients with chronic *P. aeruginosa* infection investigated by enzyme linked immunosorbent assay. *Clin Exp Immunol* 1990;81:428–34.
- 9 Forsgren A, Sjöquist J. "Protein A" from *Staphylococcus aureus*. I. Pseudoimmune reaction with human gamma-globulin. *J Immunol* 1966;99:822.
- 10 Metcalfe DD, Sampson HA. Workshop on experimental methodology for clinical studies of adverse reactions to food and food additives, Washington DC, November 1988. *J Allergy Clin Immunol* 1990;86:421–42.
- 11 Wilson EV, Hearn VM. Use of *Aspergillus fumigatus* mycelial antigens in enzyme-linked immunosorbent assay and counter-immunoelectrophoresis. *J Med Microbiol* 1983;16:97–105.
- 12 Høiby N. *Pseudomonas aeruginosa* infection in cystic fibrosis. Diagnostic and prognostic significance of *Pseudomonas aeruginosa* precipitins determined by means of crossed immunoelectrophoresis. *Acta Pathol Microbiol Scand Sect C* 1977;262(Suppl):1.
- 13 Pedersen SS, Espersen F, Høiby N. Diagnosis of chronic *Pseudomonas aeruginosa* infection in cystic fibrosis by enzyme-linked immunosorbent assay. *J Clin Microbiol* 1987;25:1830–6.
- 14 Nikolaizik WH, Moser M, Cramer R, *et al*. Identification of allergic bronchopulmonary aspergillosis in cystic fibrosis patients by recombinant *Aspergillus fumigatus* 1/a-specific serology. *Am J Respir Crit Care Med* 1995;152:634–9.
- 15 Knutsen AP, Mueller KR, Hutcheson PS, *et al*. Serum anti-*Aspergillus fumigatus* antibodies by immunoblot and ELISA in cystic fibrosis with allergic bronchopulmonary aspergillosis. *J Allergy Clin Immunol* 1994;93:926–31.

- 16 El-Dahr JM, Fink R, Selden R, et al. Development of immune responses to *Aspergillus* at an early age in children with cystic fibrosis. *Am J Respir Crit Care Med* 1994;**150**: 1513–8.
- 17 Kurup VP, Resnick A, Kalbfleish J, et al. Antibody isotype responses in *Aspergillus*-induced diseases. *J Lab Clin Med* 1990;**115**:298–303.
- 18 Greenberger PA, Patterson R. Application of enzyme-linked immunosorbent assay (ELISA) in diagnosis of allergic bronchopulmonary aspergillosis. *J Lab Clin Med* 1982;**99**: 288–93.
- 19 Trompell J, Becker WM, Schlaak M. Analysis of IgG subclass and IgE response in allergic disease caused by *Aspergillus fumigatus* by immunoblotting techniques. *Int Arch Allergy Immunol* 1994;**104**:390–8.
- 20 Tomee JFC, Dubois AE, Koeter GH, et al. Specific IgG₄ responses during chronic and transient antigen exposure in aspergillosis. *Am J Respir Crit Care Med* 1996;**153**:1952–7.
- 21 Leser C, Kauffman HF, Virchow C, et al. Specific serum immunopattern in clinical phases of allergic bronchopulmonary aspergillosis. *J Allergy Clin Immunol* 1992;**90**:589–99.
- 22 Leung PSC, Gerswin ME, Coppel R, et al. Localization, molecular weight and immunoglobulin subclass response to *Aspergillus fumigatus* allergens in acute bronchopulmonary aspergillosis. *Int Arch Allergy Appl Immunol* 1988;**85**: 416–21.
- 23 Forsyth KD, Hohmann AW, Martin AJ, et al. IgG antibodies to *Aspergillus fumigatus* in cystic fibrosis: a laboratory correlate of disease activity. *Arch Dis Child* 1988;**63**:953–7.