Precipitins and specific IgG antibody to *Aspergillus fumigatus* in a chest unit population

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Abstract

Background The enzyme linked immunosorbent assay (ELISA) for detecting IgG antibodies to *Aspergillus fumigatus* is more sensitive than the measurement of *Aspergillus* precipitins. The relation of the results from both techniques to the clinical pattern of disease in a large unselected group of patients from a large referral centre is unknown.

Methods The clinical relation of precipitins to *Aspergillus fumigatus* to clinical disease was determined retrospectively in 98 patients attending a primary referral centre. Precipitin results were compared with the specific IgG antibody to *A fumigatus* in 88 of the sera. Precipitins were determined by the agar gel double diffusion test and specific IgG antibody to *A fumigatus* by a quantitative ELISA.

Results Precipitins were detected in the unconcentrated serum of 51 patients. Thirty nine of these had a mycetoma or allergic bronchopulmonary aspergillosis, 34 having specific IgG antibody to *A fumigatus* more than the control range. Forty seven patients had precipitins only after threefold concentration of serum or to only one of the four *A fumigatus* antigen extracts. Most of these had specific IgG in or near the control range. Thirty of these had *A fumigatus* skin test negative asthma or bronchiectasis, in which aspergillus was probably not pathogenic. There was a close relation between the level of antibody detected by the ELISA and the number of precipitin lines.

Conclusions This study reaffirmed the supportive role of aspergillus precipitins in the diagnosis of pulmonary aspergillosis. No additional benefit in the routine use of the ELISA was seen. It also showed that care should be taken in interpreting positive precipitin results from concentrated serum and that using several rather than one *A fumigatus* antigen extract is helpful for identifying allergic aspergillosis.

Aspergillus fumigatus is associated with mycetoma and allergic bronchopulmonary aspergillosis, the major non-invasive forms of pulmonary aspergillosis.^{1,2} Precipitating

antibody to *A fumigatus* may be detected in serum in both disorders.³⁻⁵

Allergic bronchopulmonary aspergillosis, the most common cause of pulmonary eosinophilia, may cause irreversible fibrosis.⁶⁻⁸ Diagnosis is made on clinical and radiological criteria and supported by the finding of immediate skin reactivity, circulating specific IgE antibody to *A fumigatus*, and precipitin lines on agar gel double diffusion in unconcentrated or concentrated serum.^{4,6} In these disorders precipitin results are useful and their interpretation straightforward. In routine practice, however, a precipitin test may be requested because of a suspicion of *A fumigatus* involvement (as in difficult asthma), a positive skinprick test response to *A fumigatus*, suspicious radiological appearances, or peripheral blood eosinophilia. It is in these circumstances that precipitin results with varying degrees of positivity occur, which prove difficult to interpret in the context of the supposed or eventual diagnosis.

The agar gel double diffusion method is the standard method for detecting *Aspergillus* precipitins, but it is a qualitative test of low sensitivity. Enzyme linked immunosorbent assay (ELISA) methods for detecting specific IgG antibodies to *A fumigatus* have greater sensitivity.⁹⁻¹⁵ Many comparisons have been made between ELISA and agar gel double diffusion tests, but these have usually been limited to small numbers of highly selected patients. None has compared the clinical value of the two methods in routine practice.

We undertook this study to determine the meaning of a positive result in the precipitin test, to compare the agar gel double diffusion and ELISA methods in a primary referral population, and to evaluate the ELISA as a routine method for detecting IgG antibody to *A fumigatus*.

Methods

PATIENTS

Precipitins to *A fumigatus* were detected in serum samples from 98 patients who attended a primary referral centre during seven years. The first positive serum precipitin sample was used in this study. On the basis of the precipitin results the following four groups were defined: group 1—patients with three or more precipitin lines in unconcentrated serum; group 2—patients with one or two precipitin lines in unconcentrated serum; group 3—patients with precipitins detectable

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only after threefold concentration of serum; group 4—patients with precipitins to only one or two of the four antigen extracts tested even after threefold concentration of serum and not to the same extract.

A group of 18 randomly selected precipitin negative patients attending the chest clinic served as controls for the ELISA data.

Case notes were reviewed to determine the diagnosis and collate results of clinic investigations. In particular, we recorded blood eosinophilia ($>0.5 \times 10^9/l$); positive skin-prick test responses to *A. fumigatus* and other common allergens; *A. fumigatus* specific IgE (radioallergosorbent test (RAST), Phadenzym Pharmacia), sputum production, positive cultures, or microscopic appearances characteristic of *A. fumigatus* and lung function tests (either forced expiratory volume in one second (FEV₁), forced vital capacity (FVC), or peak expiratory flow (PEF). Chest radiographs were reviewed and subjects allocated to clinical groups by DJS without knowledge of their precipitin grouping. Mycetoma was diagnosed from the characteristic radiological appearances, consisting of the presence of a radio-opaque mass in a lung cavity with an air crescent. Allergic bronchopulmonary aspergillosis was diagnosed in accordance with accepted criteria: chest radiographic abnormalities, immediate cutaneous reactivity to *Aspergillus*, increased total and specific IgE antibodies to *Aspergillus*, peripheral blood eosinophilia, asthma, and proximal bronchiectasis^{4,5} (though bronchography had not been performed on all patients). Bronchiectasis, not associated with allergic bronchopulmonary aspergillosis, was diagnosed from a history of chronic (over three years) purulent sputum production, bronchographs showing distal bronchiectasis, and an absence of immediate skin reactivity to *A. fumigatus*. Asthma was defined on the basis of a history of reversible symptoms of wheeze, chest tightness, and dyspnoea with a documented 15% reversibility of airflow obstruction (FEV₁ or PEF) in response to inhaled salbutamol (200 µg).

AGAR GEL DOUBLE DIFFUSION

Each serum sample was tested against four extracts of *A. fumigatus*, two from Bencard, one from the Mycology Reference Laboratory, and one produced from a local strain.

The local extract was produced from surface culture on a dialysable medium at 37°C for four to five weeks.¹ The mycelial matt was homogenised in the culture filtrate, frozen, thawed, and sedimented at 3000 rev/min. The supernatant was filtered through a membrane filter (0.45 µm), dialysed against running tap water and then against two changes of distilled water at 4°C, freeze dried, and used at a concentration of 20 mg/ml. Four extracts were used to include as many antigens of *A. fumigatus* as possible. After staining and drying, precipitin lines were counted (JAF) and recorded as the greatest number reacting to any extract.¹⁶ Serum samples were stored at 4°C with 0.1% sodium azide until assayed in the ELISA.

ELISA FOR IgG ANTIBODIES TO A FUMIGATUS

An antibody capture assay was developed with an antigen extract from a locally isolated strain of *A. fumigatus*. The intra-assay and interassay coefficient of variation for the local strain was below 10%. Results were expressed in terms of a specific binding index for each specimen with reference to a highly positive laboratory standard.¹³ This index was derived as follows:

$$\frac{OD_x - OD_{lc}}{OD_{hc} - OD_{lc}} \times 100,$$

where OD_x is the optical density of unknown serum, OD_{hc} the mean optical density of highly positive serum, and OD_{lc} the mean optical density of negative control serum.

STATISTICAL METHODS

Data were analysed by precipitin group and clinical group. The ELISA data were skewed, so the significance of differences between groups was assessed by the Mann-Whitney U test. Exact critical values and p values below 0.05 were accepted as significant and are quoted as such to simplify presentation. Results are expressed as medians with corrected 25% and 75% interquartile ranges (Q1c and Q3c).

Results

The most frequent diagnosis was asthma, which occurred in 57 of the 98 patients and was evenly distributed between the four precipitin groups (table 1). The proportion of patients with allergic bronchopulmonary aspergillosis, all of whom had asthma, was much less in group 4—1/10 versus 12/12, 16/20, and 8/15 in groups 1, 2, and 3 respectively. Mycetoma was found in 11 patients and bronchiectasis in 15 patients. Of the remaining 15 subjects, seven had chronic irreversible airways obstruction without features of asthma; four had pulmonary fibrosis related to tuberculosis, sarcoidosis, and ankylosing spondylitis; and the remainder may have been colonised by aspergillus because of altered lung clearance mechanisms due to carcinoma (2) and severe pneumonia leading to chronic necrotising pulmonary aspergillosis (2).

Table 1 Distribution of diagnoses in the four precipitin groups (number of samples available for the enzyme linked immunosorbent assay in each group in parentheses)

Diagnosis	Precipitin group				Total
	1	2	3	4	
Mycetoma	11 (11)	—	—	—	11 (11)
ABPA	12 (12)	16 (15)	7 (7)	1 (0)	36 (34)
Asthma skin test negative	—	4 (3)	8 (7)	9 (7)	21 (17)
Bronchiectasis	—	2 (1)	5 (5)	8 (7)	15 (13)
Other diagnoses	—	6 (6)	5 (3)	4 (4)	15 (13)
Total	23	28	25	22	98 (88)

ABPA—allergic bronchopulmonary aspergillosis.

Table 2 Specific binding indices for each precipitin group and the control group

	Precipitin lines	Specific binding index	
		Median	Interquartile range
Group 1	>2	81*	75–103
Group 2	1–2	28*	25.8–34.7
Group 3	Concentrated serum†	19‡	16.6–25.7
Group 4	Variable	4‡	2.3–6.8
Controls	Zero	3	0–4.5

* $p < 0.05$ between these groups.

†Detectable only after threefold concentration of serum.

‡ $p < 0.05$ between these groups.

CLINICAL ASSESSMENTS

Apart from sputum culture and microscopy for *A. fumigatus*, 83% of patients had skin tests, 96% tests for blood eosinophils, and 71% tests for sputum eosinophils. All asthmatic patients with a negative skin prick test response to *A. fumigatus* had specific IgE to *A. fumigatus* determined and one third had a positive result. A positive skin test response or specific IgE antibody to *A. fumigatus* was recorded in all the patients with allergic bronchopulmonary aspergillosis and in a quarter of the patients with a mycetoma, but in no other patients. Three of the aspergillus skin test negative patients with asthma had specific IgE antibody to one or more common allergens and blood eosinophilia of similar magnitude to that seen in the patients with allergic bronchopulmonary aspergillosis. Sputum culture for *A. fumigatus* was performed for only 31 subjects in groups 1 and 2 and 19 in groups 3 and 4. Positive cultures occurred twice as often in groups 1 and 2 as in groups 3 and 4.

RELATION BETWEEN PRECIPITIN AND ELISA RESULTS

The median specific binding index was greatest for group 1 and lowest in group 4. There were

Comparison of specific IgG antibody to *Aspergillus fumigatus* (specific binding index) and precipitins (groups 1–4) for the major diagnostic groups. ABPA—allergic bronchopulmonary aspergillosis; SPT—skinprick test negative.

Table 3 Specific binding indices for the major diagnostic groups and the precipitin negative control serum samples

Diagnosis	Specific binding index		n†
	Median	Interquartile range	
Mycetoma	100*	82.5–140.3	11/11
ABPA	43.5*	36.1–59.6	34/36
Asthma aspergillus negative	12	3.6–17.8	17/21
Bronchiectasis	10	6.1–15.4	13/15
Others	25	4–39	13/15
Controls	3	0–4.5	18

* $p < 0.05$ in the comparison with all following groups.

†Number of serum samples available for the enzyme linked immunosorbent assay.

ABPA—allergic bronchopulmonary aspergillosis.

significant differences between groups 1 and 2 and groups 3 and 4, but not between groups 2 and 3 or between group 4 and precipitin negative controls. Groups 1, 2, and 3 had significantly greater specific binding index values than group 4 and controls, who had a range of 1–18 (table 2). The specific binding index and precipitin line results of groups 1 and 2 (positive in unconcentrated serum) were significantly related ($r = 0.08$, $p < 0.001$, $df = 45$).

CLINICAL GROUPS

Mycetoma

All patients with a mycetoma had more than two precipitin lines in unconcentrated serum. The specific binding index values were higher than those found in the other clinical groups ($p < 0.05$; table 3).

Allergic bronchopulmonary aspergillosis

Most patients with allergic bronchopulmonary aspergillosis (28/36) had precipitins in unconcentrated serum (groups 1 and 2); the remainder were in group 3, apart from one patient in group 4. A wide range of antibody levels was seen by the ELISA. The specific binding index values were significantly lower than those seen in the patients with mycetoma (though individual results overlapped), but the values were significantly greater than those seen in the other clinical groups (figure, table 3). The subjects with allergic bronchopulmonary aspergillosis in group 3 had significantly lower values than those in groups 1 and 2 (median 30, Q1c 18.3, Q3c 34.8, $n = 7$, versus median 50, Q1c 39.8, Q3c 61.3, $n = 27$; $p = 0.05$). The patients in group 3 were also separated significantly from patients with no precipitins and from patients with skin test negative asthma, though individual results overlapped. Nine patients with allergic bronchopulmonary aspergillosis had no radiographic evidence of disease at the time of assessment.^{17–19} Their median specific binding index was less than that of the remainder of the group (those with no radiological evidence: median 24, Q1c 23.8, Q3c 40.3, $n = 9$; those with radiological evidence: median 48, Q1c 37.5, Q3c 62.5, $n = 23$; $p < 0.05$).

Aspergillus skin test negative asthma

Of the 21 patients with aspergillus skin test

negative asthma, only three had positive skinprick test responses to other antigens, though 13 had a recorded absolute peripheral blood eosinophilia. Four patients were in group 2, eight were in group 3, and nine were in group 4. The ELISA showed a low level of specific antibody to *A. fumigatus* in the serum of 17 of these patients. The specific binding index values in this group did not differ from those of the controls and the patients with bronchiectasis and were lower than those of the group with allergic bronchopulmonary aspergillosis ($p < 0.05$; table 3).

Bronchiectasis

All the patients with bronchiectasis had airways obstruction (FEV_1/FVC below 70%) and a negative skinprick test response to *A. fumigatus*. Most were in precipitin groups 3 and 4. The ELISA results showed antibody levels that were insignificantly different from those of the patients with skin test negative asthma and the precipitin negative control group.

Other diagnoses

Patients in this group had negative skinprick test responses and no IgE antibody to *A. fumigatus*. Of the 15 patients, six had one or two precipitin lines and five had precipitins after concentration of the serum, but none was considered to have aspergillosis. The ELISA results showed moderate antibody levels, though the median specific binding index of 25 was less than that in the mycetoma group ($p < 0.05$).

Discussion

This study reviewed the clinical significance of precipitins for positive *A. fumigatus* determined by the agar gel double diffusion test in patients attending a primary referral chest clinic and compared these findings with those obtained by an ELISA method.

Precipitin groups 1–4 on the agar gel double diffusion test compared closely with the results of ELISA. More than 90% of individuals with precipitins in unconcentrated serum had a specific binding index significantly above control values. Previous comparisons between agar gel double diffusion and ELISA have been based on small numbers of subjects with well defined diseases by ELISA results. Separation between serum from control subjects, serum from non-atopic subjects, and serum with one or two precipitin lines has not always been possible.^{9–12} In a comparison based on 758 serum samples collected from patients attending a chest clinic 81% (615) were negative by both agar-gel double diffusion and ELISA methods and, of the 127 (19%) positive by ELISA, only 39 were positive by agar gel double diffusion. Only 46 serum samples were from patients with aspergillosis, however.²⁰ It was concluded that the ELISA could detect antibodies against non-precipitating antigenic components of *A. fumigatus* in addition to the antibodies detected by agar gel double diffusion.²⁰ Using an optimised ELISA,¹³ we have found a clear relation between the results of the two methods. The ELISA also separated the different

precipitin groups, although it did not completely distinguish the mycetoma and allergic bronchopulmonary aspergillosis groups from the non-aspergillosis groups.

In this study 43 of the 51 patients with precipitins in unconcentrated serum had a mycetoma or asthma and 28 of the 32 with asthma had allergic bronchopulmonary aspergillosis. Of the eight patients with neither mycetoma nor asthma in groups 1 and 2, four died soon after assessment and the development of antibody to *A. fumigatus* was probably a terminal event related to impaired lung clearance mechanisms. Of the remainder, one had cystic fibrosis, one was repeatedly precipitin positive, and a further patient, with a specific binding index of 100, had extrinsic allergic alveolitis, probably related to exposure to aspergillus while working in a stable.

The clinical relevance of precipitins detected only after threefold concentration of serum may be difficult to determine. In this study only a third of patients in group 3 had aspergillosis. In patients with allergic bronchopulmonary aspergillosis such results have always been considered to be positive. The proportion of precipitin positive patients with allergic bronchopulmonary aspergillosis increased from 60% to 84% when precipitins in concentrated serum were considered to represent a positive result.⁴ The group 3 patients with allergic bronchopulmonary aspergillosis had significantly greater amounts of specific IgG according to ELISA than did the precipitin negative controls, suggesting that their precipitin results are clinically relevant. Among the patients with allergic bronchopulmonary aspergillosis, those in group 3 had specific binding index values significantly below those in groups 1 and 2, though the separation of most of the seven patients in group 3 from the precipitin negative controls and the asthmatic patients with a negative skin test response to *A. fumigatus* suggests that precipitin results in concentrated serum should be considered to be positive when other criteria of allergic bronchopulmonary aspergillosis are met. The ELISA suggests that care is needed in interpreting the group 4 precipitin results. If precipitin lines are obtained, especially if weak and in response to only one extract in a patient without evidence of mycetoma or allergic bronchopulmonary aspergillosis, further samples may be helpful.

Patients with skin test negative asthma had a lower median specific binding index than the patients with allergic bronchopulmonary aspergillosis and, although two thirds had blood eosinophilia, none had radiological changes of bronchopulmonary aspergillosis. This supports the suggestion that specific IgE in addition to specific IgG is needed to produce the chronic inflammatory changes seen in the bronchi with allergic bronchopulmonary aspergillosis.^{6,7} The patients with bronchiectasis had been reported to have precipitins, but none had a positive skinprick test response and the ELISA confirmed low concentrations of specific IgG in this group. Poor tracheobronchial clearance mechanisms may have made

these patients more exposed to aspergillus antigen than are normal subjects, leading to the production of precipitating antibody.

The ELISA had no major advantages over the routine determination of precipitins in the diagnosis of non-invasive pulmonary aspergillosis, unless the use of expanded diagnostic criteria for allergic bronchopulmonary aspergillosis are adopted.^{5,6} A compatible history and radiological appearances and a positive response to the *A fumigatus* skinprick test or specific IgE in patients with precipitins in unconcentrated or threefold concentrated serum may be taken to indicate the presence of allergic bronchopulmonary aspergillosis. Weak precipitin reactions were not a feature of mycetoma and their presence in patients not meeting other criteria of allergic bronchopulmonary aspergillosis rules out any important degree of pulmonary aspergillosis. The ELISA used in a quantitative form allows within and between patient comparisons,¹³ whereas the agar gel double diffusion test is semiquantitative, subjective, and too insensitive for monitoring the progress of allergic bronchopulmonary aspergillosis.^{4,9} The use of the ELISA may have a further clinical advantage in that the presumed pre-injury phase of allergic bronchopulmonary aspergillosis may be diagnosed by using ELISAs for *A fumigatus* specific IgG and IgE antibody. This might be of value as allergic bronchopulmonary aspergillosis may not be diagnosed according to current standard criteria until appreciable loss of lung function has occurred,^{4,6,7} though there is no evidence that treatment at this stage reduces lung destruction.

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