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Relative reactivity of *Aspergillus* allergens used in serological tests

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Aspergillus is a common disease-causing agent, both as an allergen causing ABPA and severe asthma with fungal sensitization (SAFS) and as a pathogen causing invasive aspergillosis in immunocompromised individuals and chronic cavitating disease (CCPA) in apparently immune competent individuals. Currently detection of *Aspergillus* is problematic and some of the most useful tests rely on detection of antibody response to *Aspergillus* allergens. Here we examine the IgE antibody response to crude and recombinant allergen tests (Asp f 1, Asp f 2, Asp f 4 and Asp f 6) in individuals with allergic conditions ABPA, SAFS and in individuals with CCPA. Additionally we use recently obtained genomic information to examine the possibility of cross reaction to these allergens and show that possible cross reactive epitopes occur in several species of *Aspergillus*.

Keywords *Aspergillus*, allergen, ImmunoCAP

Introduction

Aspergillus fumigatus is a ubiquitous fungus capable of causing invasive disease in immunocompromised individuals as well as cavitating disease and allergic reactions in immune competent individuals [1–4]. Hypersensitivity to *Aspergillus* allergens may result in IgE-mediated asthma, allergic sinusitis or rhinitis, or most severely, allergic bronchopulmonary aspergillosis (ABPA) [5]. Patients who have persistent severe or brittle asthma (despite standard treatment) and evidence of fungal sensitisation, as defined by positive prick testing, or fungus or fungal antigen-specific blood IgE testing, and do not meet the criteria for ABPA are considered to have severe allergy with fungal sensitisation (SAFS). Invasive pulmonary aspergillosis (IPA) affects immunocompromised persons, with increasing incidence [6]. In the immunocompetent host, where pre-existing disease, such as tuberculosis, has resulted in the formation of intrathoracic cavities, opportunistic colonization and the formation of an expanding cavity termed chronic cavitating pulmonary aspergillosis (CCPA) may occur [7]. A common diagnostic test used to establish the presence of *Aspergillus* in these conditions is based on detection of IgE binding to immobilised *A. fumigatus* antigens [8–12]. A major problem with older investigations that use crude *Aspergillus* extracts is the variability and impurity of the antigens used for antibody detection. Culture filtrate and mycelial extracts showed considerable differences in their antigenic components. These crude antigens prevented quantifiable results from sensitive methods such as the enzyme-linked immunosorbent assay (ELISA) and radioallergosorbent test (RAST). In addition, little could be understood about the immunopathogenesis of *Aspergillus*-disease as long as the antigens remained unidentified. A more detailed assessment of the immunological response is therefore of clinical importance.

The fungus is reported to be able to produce more than 40 IgE-binding components, however, and the complex immunopatterns generated by Western blots in past studies are difficult to interpret [11]. The development of cDNA cloning systems has since allowed identification and characterization of a panel of antigens of *A. fumigatus*, which are pure and standardized, and may be produced in large quantities. Importantly, recombinant antigens of *A. fumigatus* are comparable in their antigenicity to their native molecules [8,9]. Specific serum IgE level testing to recombinant *A. fumigatus* antigens has the potential to provide

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a useful diagnostic tool to distinguish between these closely related conditions, particularly amongst the hyperimmune group [12,13]. The ImmunoCAP system (Pharmacia Diagnostics) provides a standard laboratory technique for measurement of specific IgE to recombinant allergen proteins. Briefly this comprises a fluoroenzyme sandwich immunoassay where immobilised antigen protein is probed with serum then bound IgE is detected using an anti IgE antibody coupled to a fluorogenic enzyme. The clinical sensitivity and specificity of ImmunoCAP™ mold has been documented in several studies. Four different single allergen components from *A. fumigatus* have been produced with recombinant technique for use with the ImmunoCAP™ system: rAsp f 1, rAsp f 2, rAsp f 4, and rAsp f 6. Asp f 1, is a ribotoxin restricted to *Aspergillus spp* encoded by gene Afu5g02330 (Cadre nomenclature; www.cadre.man.ac.uk). Along with Asp f 3, it can be regarded as an indicator of sensitization to the fungus, but neither can be used to discriminate between asthmatic patients with *A. fumigatus* sensitization and ABPA by their IgE binding [14,15]. Asp f 2, encoded by gene Afu4g09580, has also been shown to be a major allergen, and studies have demonstrated its usefulness in the diagnosis of ABPA [16]. It has significant sequence homology to a protein from *Aspergillus nidulans*, Asp nd 1, pH regulatory protein from *Candida albicans*, and fibrinogen binding protein [17]. Asp f 4, encoded by Afu2g03830, is an intracellular protein allergen with unknown biochemical function, has shown strong reactivity with IgE from sera of CF patients with asthma or ABPA [18]. Asp f 6, encoded by Afu1g14550, represents a strictly intracellular enzyme, manganese superoxide dismutase (MnSOD). Although less sensitive than Asp f 4, it too has exhibited diagnostic value for ABPA, particularly in combination with Asp f 4 [18].

Here we conduct a retrospective, open, non-comparative study of patients suffering from *Aspergillus*-related disease in order to determine the value of serological testing for recombinant *A. fumigatus* antigens (allergens) rAsp f 1, rAsp f 2, rAsp f 4, and rAsp f 6 in the immunodiagnosis of CCPA in comparison to ABPA and SAFS. The issue of cross reactivity of IgE to related allergen-like proteins from other fungi has been addressed in a few studies; however the true range of cross-reactivity to these allergens has not been determined. Therefore we also assess the utility of the immobilised allergen tests in distinguishing between types of fungal disease and use recently published genomic sequences to determine possible range of cross-reactivity.

Materials and methods

Inclusion criteria for patients

A retrospective study was undertaken of 43 patients attending Wythenshawe Hospital (Manchester, UK) for *Aspergillus*-related disease who had been diagnosed having at one time fulfilled criteria for either CCPA ($n = 22$), ABPA ($n = 16$), or severe asthma with fungal sensitization ($n = 5$), given below.

Criteria for CCPA [7]: 1—Chronic pulmonary or systemic symptoms for more than 3 months, compatible with CPA, including at least one of weight loss, productive cough or haemoptysis. 2—Cavitary pulmonary lesion with evidence of paracavitary infiltrates or expansion of cavity size over time. 3—Either positive serum *Aspergillus* precipitins test or isolation of *Aspergillus* from pulmonary or pleural cavity. 4—Elevated inflammatory markers (C-reactive protein, plasma viscosity or erythrocyte sedimentation rate). 5—Exclusion of other pulmonary pathogens by appropriate cultures and serology that are associated with a similar disease presentation including *Mycobacteria* and endemic fungi. 6—No overt immunocompromising conditions (e.g., HIV infection, leukaemia, chronic granulomatous disease).

Criteria for ABPA [19–22]: 1—Asthma. 2—Peripheral blood eosinophilia ($>1000\ mm^{-3}$). 3—Immediate skin reactivity to *A. fumigatus* antigenic extracts within 15 ± 5 mins. 4—Precipitating (IgG) and IgE antibodies against *A. fumigatus*. 5—Elevated levels of total IgE in serum (>1000 kIU/L). 6—History of pulmonary infiltrates. It should be noted that a number of ABPA patients had not ever been tested for immediate skin reactivity, but were considered to fulfil sufficient criteria, given a raised serum specific IgE would equate to a similar immunopathology. Furthermore, some had required long-term steroid treatment and so eosinophil count would have been low. Patients who have suffered from ABPA for a number of years have also been recognised not to maintain levels of precipitating antibodies. Most patients had CT scan evidence of central bronchiectasis, although the quality of the CT scan and CT scanner age determined the sensitivity of this criterion.

Proposed criteria [23] for severe asthma with fungal sensitization (SAFS) are: 1—severe (poorly controlled) asthma requiring a combination of inhaled steroid, long-acting bronchodilator, and occasional oral steroid treatment, and 2—either a positive skin test or raised specific IgE to any fungus, and a total serum IgE of $<1{,}000$ kIU/L. In this study, we only included those with evidence of sensitization to *Aspergillus*.

Patients were identified from outpatient clinic attendance lists over the previous six months, and patient notes were retrieved from medical records. Diagnosis was compared to the above criteria and confirmed by the physician. Information was manually extracted from patient notes. Patient characteristics including age, gender, ethnicity, and weight were recorded. Date of diagnosis, any pre-existing or secondary disease, radiological findings and current treatment were noted.

Laboratory analysis

Eosinophil count, total and *Aspergillus*-specific serum IgE levels, including those to the *A. fumigatus* antigens rAsp f 1, rAsp f 2, rAsp f 4, and rAsp f 6, Eosinophil count and erythrocyte sedimentation rate (ESR) were measured as part of the standard laboratory blood differential analysis. The ESR was measured after collection and before refrigeration. All other hematological estimations were performed within 24 h of collection. Total white blood cell counts were obtained in an automatic counter (Beckman Instruments, Inc.). Peripheral blood smears were stained with May–Grünwald–Giemsa and differential white blood cell count determined under oil immersion ($\times 1000$). At least 200 cells were counted and results were expressed as the number of leucocytes, neutrophils or eosinophils per millilitre of blood. C-reactive protein (CRP) was measured using the iCHROMA CRP assay according to manufacturer's instructions. Total and specific IgE levels were measured using the ImmunoCAP™ system (Sweden Diagnostics) according to the manufacturer's protocol.

Demographics of the patient population used in this study

Of the patient cohort ($n=43$), 55.8% were male ($n=24$) and 44.2% were female ($n=19$). All were of British (white) ethnic origin. Mean age was 55 years. 51.2% were diagnosed with CCPA ($n=22$), 37.2% were diagnosed with ABPA ($n=16$), and 11.6% were diagnosed with SAFS with *Aspergillus* sensitization ($n=5$).

Statistical analysis

Once data was collated, the quantitative data was analysed statistically. Normal values and raised values of total, *Aspergillus*-specific and recombinant antigen IgE, eosinophil count, and inflammatory markers were compared between the diseases in order to determine any statistically significant difference, using unpaired - Student's t and Mann Whitney tests. Normal levels for total IgE were considered as being less than 200 kIU/l. Normal levels for *Aspergillus*-specific and recombinant antigen IgE levels were considered as being less than 0.4 kUa/l and those that were recorded as 0. A normal CRP level of less than 5 mg/l was also recorded as 0. No detectable precipitating antibodies were recorded as 0, and when positive, the denominator of the dilution at which levels were still detectable was used as a value, in order to represent the increasing levels of antibody.

Results

Levels of IgE reactivity to the various Aspergillus allergens in patients with ABPA, CCPA and SAFS

Patients from the CCPA and severe asthma with fungal sensitization groups for whom results had not been received had all previously demonstrated negative *Aspergillus*-specific IgE levels (<0.4 kUa/l), so were considered to have a result of 0 for the recombinant tests as well and were included in the data analysis. Patient characteristics including age, sex, *Aspergillus* precipitins, total IgE level, RAST class to *A. fumigatus*, and eosinophil count, according to diagnosis, are summarized in Table 1. This table clearly demonstrates the significant allergic component of ABPA, with median total serum IgE and *Aspergillus*-specific IgE and mean eosinophil count significantly higher than levels for SAFS and CCPA. Median was considered a better measure of the average IgE level as such levels are prone to extreme values, particularly in ABPA, where the discrepancy between the mean (2704 kIU/l) and median (1800 kIU/l) values was considerable. Statistical analysis of this difference between ABPA and CCPA is confirmatory. Unpaired t tests on total IgE, *Aspergillus*-specific IgE and eosinophil levels all give two sided P values of <0.05. However, due to the variability in the two groups, this result could not be considered valid; Mann Whitney tests also gave a two sided P value of <0.05. Statistically, there is therefore a significant difference between total IgE, *Aspergillus*-specific IgE and eosinophil levels in ABPA and CCPA. An insufficient number of SAFS patients had been recruited to perform statistical analysis on this group. Fig. 1 shows mean levels of serum reactivity to the four allergens in patients with each condition. ABPA patients exhibited >10 fold greater levels of serum IgE than patients with CCPA who in turn showed >10 fold greater levels of serum IgE than patients with SAFS. The extremely low mean levels of reactivity to Asp f1, Asp f 2 and Asp f 6 shown in SAFS patients are hard to distinguish from zero in Fig. 1. No SAFS patient showed any reactivity with Asp f 4. We note that all SAFS patients showed raised RAST responses to total *A. fumigatus* protein mixture

Table 1 Patient characteristics

	CCPA	ABPA	SAFS
Number	22/43 (51.2%)	16/43 (37.2%)	5/43 (11.66%)
Age (years)	57 ± 20.5	53 ± 18.5	55 ± 18
Sex ratio (M:F)	14:8	8:8	3:2
Aspergillus precipitins raised N	22/22 (100%)	9/16 (56.3%)	0/5 (0%)
Raised IgE (>200 kIU/l) N	6/22 (27.2%)	16/16 (100%)	3/5 (60%)
Total IgE (kIU/l)* – all pts – raised	82.6 ± 233	1800 ± 2659	220 ± 183
	420 ± 230	1800 ± 2659	290 ± 140
Raised RAST to *A. fumigatus* (>0.4 kUa/l) N	12/22 (54.5%)	16/16 (100%)	5/5 (100%)
RAST to *A. fumigatus* – all pts (kUa/l)* – raised	0.5 ± 5.9	60.9 ± 121	1.8 ± 2.8
	1.6 ± 7.6	60.9 ± 121	1.8 ± 2.8
Raised eosinophils ($>0.4 \times 10^9$/l) N	2/22 (9.1%)	6/16 (37.5%)	2/5(40%)
Eosinophils – all pts – raised	0.23 ± 0.16	0.52 ± 0.46	0.46 ± 0.42
	0.65 ± 0.09	0.94 ± 0.52	0.90 ± 0.16

*Median used as a measure of average as IgE levels are prone to extreme values. Standard deviation values are given beside average values.

(Table 1) suggesting that these patients may react more strongly with other antigens.

Patterns of allergen reactivity vary between CCPA, ABPA and SAFS

In order to determine whether different Aspergillus related conditions produced different potentially diagnostic patterns of serum IgE reactivity to the individual allergens in the tests we performed a comparison of reactivity scores for patients who historically presented with either CCPA or ABPA and patients currently presenting at Wythenshawe Hospital with SAFS. Fig. 2 shows the percentage of patients showing reaction to individual allergens and to patterns of allergens in the three conditions. In general patients from all three groups reacted with Asp f 1 and Asp f2 whereas patients with SAFS never showed reaction to Asp f 4 and patients with CCPA never showed serum reactivity to Asp f 6. Combinations of Asp f 6 reactivity together with reactivity to any other allergen were specific to ABPA. In no case did 100% of patients with any condition react with a single allergen with the most common level of reaction being 90% of patients who reacted to Asp f 2.

No serum reactivity to any of the four allergens was seen in 14/22 (64%) patients with CCPA showed, whereas 16/16 ABPA patients showed serum reactivity to one or more allergens with 3/16 (19%) showing reactivity to all four allergens and only one of five (20%) SAFS patients showed reactivity to any of the allergens.

Discussion

The results presented here show that patterns of reactivity to individual *Aspergillus* allergens are unlikely to be useful indicators for diagnosis of different *Aspergillus* diseases. A large body of research has been published regarding the use of recombinant antigen

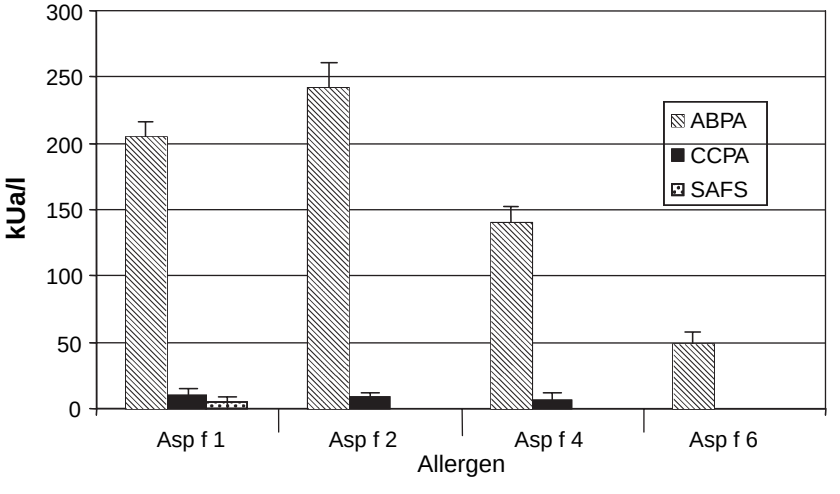

Fig. 1 Level of serum IgE reactivity in patients with *Aspergillus*-related conditions for various allergens. Levels shown are means from all patients studied and error bars represent standard error.

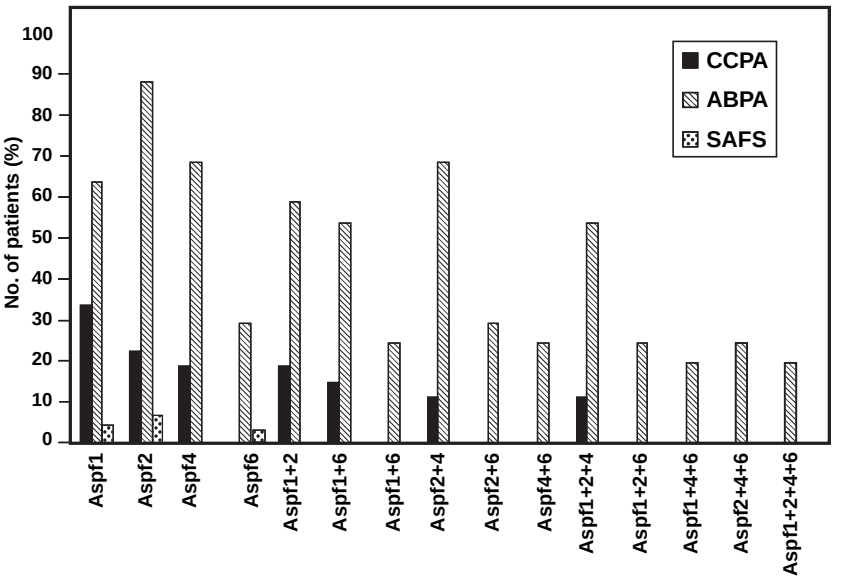

Fig. 2 Relative proportion of patients with *Aspergillus*-related conditions that reacted to the various allergens. Percentages of patients that reacted to more than one allergen are also included.

testing in ABPA. Various studies have shown that the ribotoxin Asp f 1 is not specific to ABPA and is primarily a marker of *Aspergillus* sensitization, although it is useful in the respect that it may be considered genus-specific [9]. It would therefore be unsurprising to find a response to Asp f 1 in those CCPA patients that have had positive *Aspergillus*-specific IgE serology. Two of the eight patients in the study with positive recombinant results did not have a raised IgE level to Asp f 1. Eight of the CCPA patients had positive results for Asp f 2. It has been suggested that Asp f 2 was able to differentiate ABPA from severe asthma with fungal sensitization [16]. This appears not to be the case with CCPA. One of the CCPA patients had a high positive result to Asp f 4 and yet a barely detectable IgE level for Asp f 2. Despite this result, patients with positive Asp f 4 results all had positive Asp f 2 findings. The utility of Asp f 4 may therefore be limited in the immunodiagnosis of CCPA.

Asp f 6 has been reported as having diagnostic value for ABPA [13]. This was not found to be the case in this small sample of patients. Most ABPA patients had positive results for Asp f 1, Asp f 2, and Asp f 4, but less than half were positive for Asp f 6. Its significance in CCPA would appear to be even less, with only one positive result out of the 12 who had a positive RAST class to *A. fumigatus*. It is possible that its detection might signify alternative or additional pathogenic mechanisms [30,31] but data are lacking.

The level of IgE was a helpful distinguishing feature of SAFS patients compared to ABPA patients, the former having negligible levels of IgE specific to *Aspergillus* allergens, and the latter having very high levels of total and *Aspergillus* specific IgE. In general, the number of patients with ABPA reacting with specific allergens matched the percentages found in previous studies. Crameri *et al*. [30] found that 45% of patients sensitised to fungi reacted to Asp f 1 with none in this group reacting to Asp f 4 and Asp f 6 and that 80% and 55% of patients with ABPA reacted with Asp f 4 and Asp f 6 respectively. In another study Knutsen *et al*. studied populations of patients with ABPA and showed 86% reacting to Asp f 1, 755 reacting to Asp f 2, 82% reacting to Asp f 4 and 25% reacting to Asp f 6 allergens. Here 65% of ABPA patients reacted with Asp f 1, 90% with Asp f 2, 70% with Asp f 4 and, 30% with Asp f 6. However in this study we also measured reactivity for patients with CCPA and show that a proportion react with Asp f 1 (34%), Asp f 2 (23%) and Asp f 4 (19%) although we were unable to find any CCPA patient who showed serum reactivity with Asp f 6. Thus Asp f 6 remains a possible marker for ABPA while Asp f 4 is unlikely to distinguish between ABPA and CCPA. Levels of serum reactivity and number of patients studied for SAFS were too low to draw any robust conclusion about patterns of reactivity in this condition. A large number of SAFS patients are being recruited in an antifungal trial at Wythenshawe Hospital and we will repeat this analysis at the conclusion of this trial.

Levels of IgE in the three different Aspergillus-related conditions were remarkably different with the highest levels occurring in ABPA patient followed by approximately 10-fold lower levels in CCPA patients and barely detectable levels in SAFS patients. These levels are not surprising when the nature of each

condition is considered: ABPA is caused by significant growth of *Aspergillus* in lungs of patients with pre-existing allergic conditions such as asthma, CCPA occurs in patients who may not be allergic to fungi or even atopic and SAFS may arise form transient or low level exposure to allergens arising from fragments of non-living hyphae.

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