

Disease-specific recombinant allergens for the diagnosis of allergic bronchopulmonary aspergillosis

Reto Crameri, Stefanie Hemmann, Chaim Ismail¹, Günter Menz¹ and Kurt Blaser

Swiss Institute of Allergy and Asthma Research (SIAF), Obere Strasse 22, 7270 Davos, Switzerland

¹Hochgebirgsklinik, 7265 Davos-Wolfgang, Switzerland

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Abstract

Allergic bronchopulmonary aspergillosis (ABPA), a severe pulmonary complication caused by *Aspergillus fumigatus*, is considered a complex clinical syndrome with defined serological, pathological radiological and clinical features. The diagnosis of ABPA is often difficult because of several overlapping clinical and laboratory findings shared between asthma with sensitization to *A. fumigatus* and ABPA, but essential for treatment to prevent severe deterioration of pulmonary function. We have cloned *A. fumigatus* allergens from a cDNA library displayed on phage surface, sequenced the inserts and produced recombinant proteins in *Escherichia coli*. The single recombinant allergens were used to assess the immunological response in representative groups of *A. fumigatus*-sensitized asthmatics with or without ABPA and healthy controls. The allergens rAsp f 1, a 16.9 kDa ribotoxin, rAsp f 3, a 18.5 kDa peroxisomal protein, and rAsp f 6, a 23 kDa manganese superoxide dismutase, were identified as proteins with known biological function and rAsp f 4, a 30 kDa allergen, lacks sequence homology to known proteins. The secreted ribotoxin rAsp f 1 and rAsp f 3 are recognized by serum IgE of *A. fumigatus*-sensitized asthmatics with or without ABPA, whereas the non-secreted manganese superoxide dismutase rAsp f 6 and the rAsp f 4 allergen are exclusively recognized by serum IgE of ABPA patients. The dissection of IgE-mediated immune responses to single recombinant *A. fumigatus* allergens in asthmatic patients allow a discrimination between ABPA and *A. fumigatus* sensitization with high specificity (100%) and sensitivity (90%).

Introduction

Aspergillus fumigatus, an ubiquitous mould with a natural habitat normally confined to soil and decaying matter (1), is considered as an opportunistic pathogen (2) associated with an impressive list of pulmonary complications in humans (3) and animals (4). Although the genus *Aspergillus* includes 132 species and 18 variants thereof (5), only a few are recognized as human pathogens. The most important member of this group is *A. fumigatus*, which is the etiologic agent identified in 80% of *Aspergillus*-related diseases (6). The spectrum of conditions related to the fungus ranges from mild forms such as saprophytic colonization of the lung or aspergilloma (7), intermediate forms like IgE-mediated asthma (8), allergic sinusitis or allergic rhinitis (9), to life-threatening diseases such as invasive systemic aspergillosis (10) or allergic bronchopulmonary aspergillosis (ABPA) (11). Originally con-

sidered as a rarity (12), ABPA is currently diagnosed with much greater frequency (13). The disease with varying clinical presentations has been reported to occur mainly in asthmatic individuals and in patients suffering from cystic fibrosis with incidences ranging from 1 to 23% (14,15). ABPA is a hypersensitivity disease of the lung resulting in a chronic inflammatory response of proximal bronchi and distal air space associated with an array of immune responses to *A. fumigatus* (16). For two reasons ABPA has still to be considered as a clinical syndrome difficult to diagnose (17). First, because clinical and laboratory parameters are highly variable depending on the different stages of the disease (18), and, second, because the laboratory findings largely depend on the quality of the fungal extracts used for serological assessments and skin challenges (19,20). Criteria for the

diagnosis of ABPA (16,18) are chest roentgenographic infiltrates, peripheral blood eosinophilia, an array of immunological reactions such as elevated total serum IgE, immediate cutaneous reactivity to *A. fumigatus* extracts, serum precipitins to *A. fumigatus* antigens, elevated serum IgE and IgG antibodies to *A. fumigatus* (compared to individuals with allergic sensitization to *A. fumigatus* without ABPA), and proximal bronchiectasis. When all criteria are present, the diagnosis can be readily made (21); however, all criteria are rarely present simultaneously even in classical ABPA patients. With the exception of proximal bronchiectasis in the absence of cystic fibrosis and to some extent elevated specific serum IgE and IgG to *A. fumigatus* antigens, the diagnostic criteria are not specific for ABPA (21). A clear serological differentiation between ABPA and *A. fumigatus* sensitization in asthmatic patients with the use of fungal extracts is difficult, because the immunological background of both diseases is related to polyclonal IgE-mediated responses to fungal exposure (16,17). However, the clinical evaluation of the recombinant *A. fumigatus* allergen I (rAsp f 1/a), a major allergen of the fungus (22), showed that patients suffering from ABPA elicit stronger IgE and IgG responses to this allergen than asthmatic sensitized to *A. fumigatus* (23,24). However, Western blot analyses with fungal extracts using sera of individuals suffering from ABPA or *A. fumigatus* sensitization showed significant differences in the patterns of IgE-binding proteins recognized by the two categories of patients (25). These observations indicate that a specific sensitization to single *A. fumigatus* allergens can occur and could potentially be exploited to improve the diagnosis of ABPA (26) and/or to characterize the stages of the disease (27). We have cloned, characterized and produced a panel of *A. fumigatus* allergens (28) using a new cloning system based on phage surface display technology (28–30) to study the differential IgE antibody responses of *A. fumigatus*-sensitized asthmatics to single allergens and to evaluate the possibility of using recombinant allergens to confirm a clinical suspicion of ABPA.

Methods

Patients, control subjects and routine assessments

Sera from groups of asthmatic individuals sensitized to *A. fumigatus* according to clinical history and immediate skin reactivity to *A. fumigatus* extracts with ($n = 60$) or without ($n = 40$) ABPA, and from 20 healthy control individuals were analyzed. Patients characteristics including age, sex, total IgE level and RAST class to *A. fumigatus* are summarized in Table 1. All patients enrolled in this study met the guidelines for the diagnosis and management of asthma (31). The *A. fumigatus*-sensitized asthmatics had a history of sensitization to the fungus and ABPA was excluded according to the clinical history. The patients suffering from ABPA fulfilled at least six of the seven diagnostic criteria proposed by Rosenberg *et al.* (16) and Patterson *et al.* (18). The healthy control subjects had no history of allergy or asthma and normal IgE levels. Total serum IgE levels were determined with the Pharmacia CAP System (Pharmacia, Uppsala, Sweden) (32). The titration of *A. fumigatus*-specific IgE was performed using Phadebas RAST (Pharmacia) according to the package insert and

Table 1. Characteristics of patients and control individuals

	Asthmatics sensitized to <i>A. fumigatus</i>		Healthy controls
	Without ABPA	With ABPA	
Number	40	60	20
Age (years)	45 ± 14	51 ± 12	34 ± 8
Sex ratio (F:M)	20 : 20	29 : 32	8 : 12
Total IgE (kU/l)	788 ± 955	1727 ± 2076	43 ± 45
RAST ^b	2.7 ± 0.7	3.7 ± 0.5	0
Skin reaction ^c	40	60	0
Eosinophils ^d	0.25 ± 0.21	0.93 ± 0.72	0.16 ± 0.08
Pulmonary infiltrates	6	49	0
IgG antibodies (EU/ml) ^e	8 ± 12	98 ± 84	0.3 ± 0.4
Bronchiectasis	0	59	0

^aValues are means ± SD.

^bSpecific IgE to *A. fumigatus* extract determined with the Pharmacia CAP system.

^cHistory of a positive skin prick test against *A. fumigatus* extracts.

^dEosinophil count × 10³ cells/mm³.

^eSpecific IgG to *A. fumigatus* extract determined by ELISA (23).

results converted into RAST classes as described by the manufacturer. Skin-prick tests were performed with commercial preparations from Allergopharma (Hamburg, Germany) and Bencard (SmithKline Beecham, Neuss, Germany) using standard techniques (23,24). The study was carried out according to a clinical protocol approved by the appropriate ethical committee. A full explanation of the procedure was given to all participants before testing and subsequently a written consent was obtained.

Cloning and production of recombinant *A. fumigatus* allergens

The ribotoxin rAsp f 1 (22,23), rAsp f 3, a protein homologous to peroxisomal proteins of *Candida boidinii* (33), rAsp f 4, an allergen with unknown biological function, and manganese dependent superoxide dismutase (rAsp f 6) (34) were cloned from an *A. fumigatus* cDNA library displayed on phage surface (28). The regions coding for the mature proteins (22,28,33,34) were subcloned into the p[His]₆-DHFR high level expression vector, constructs verified by nucleotide sequence determinations and used to produce hexahistidine-tagged recombinant proteins in *Escherichia coli* as described (22,34). The fusion proteins were purified in a single step by Ni²⁺-chelate affinity (22,33). Purity and molecular size of the proteins were analyzed by polyacrylamide gradient gels (12.5–20%) and by Western blot analyses using standard procedures (22,33). In each of the preparations only one band, representing the relevant protein, was visible with silver staining of SDS gels and after Western blot analysis with sera from ABPA patients. Samples of 1 mg of the pure proteins were lyophilized for long-term storage (32).

Titration of allergen-specific serum IgE

The binding of allergen-specific IgE was analyzed by a standard direct solid-phase ELISA using mouse mAb TN-142 anti-human IgE (23) and results were expressed as ELISA

units (EU)/ml calibrated against the absorbency ($OD_{405\text{ nm}}$) of an in-house reference standard arbitrarily defined as 100 EU/ml for each allergen tested (23). rAsp f 6 ImmunoCAPs were produced and evaluated as described (32).

Skin tests with recombinant allergens

Intradermal skin tests were used to define the cut-off values for the recombinant allergens in ELISA and performed as described (23). The ELISA cut-off value for each single allergen was set as the highest EU/ml value obtained from sera of *A. fumigatus*-sensitized individuals giving negative skin results (23). Patients were regarded as being sensitized to a given allergen if the size of the wheal induced by intradermal skin challenge reached half the size of the skin reaction induced by the histamine control (23) and at least a wheal of >5 mm in diameter with erythema at an allergen concentration of 1 µg/ml.

Statistical analysis

Statistical analysis was performed with the two-tailed Mann-Whitney *U*-test. Correlation coefficients and statistical significance were determined with Pearson's linear regression analysis.

Results

Cloning, characterization and production of *A. fumigatus* allergens

Cloning of cDNA libraries in phagemid pJuFo and display of the expression products on the surface of filamentous phage allows efficient isolation of cDNAs encoding proteins with affinity for the ligand used for selective enrichment of phage (28,30). We have generated a cDNA library displayed on the surface of the filamentous phage M13 using mRNA from *A. fumigatus* (28), which was enriched for phage displaying proteins able to bind human serum IgE from patients suffering from ABPA. The phage population obtained after enrichment contained phage encoding different allergenic proteins as shown by sequence determinations. Four of these cDNAs coding for the mature proteins have been sequenced, produced as recombinant N-terminal [His]₆-fusion proteins formally termed rAsp f 1, rAsp f 3, rAsp f 4 and rAsp f 6 (35), and used for serological investigations involving sera from representative numbers of *A. fumigatus*-sensitized asthmatics with or without ABPA and healthy controls. GenBank accession numbers, physico-chemical and biochemical properties of the four allergens are reported in Table 2. There are no consensus sites for *N*-glycosylation in the sequences of Asp f 1 (37), Asp f 3 and Asp f 4, whereas the intracellular Asp f 6 allergen (34) as non-secreted protein is unlikely to be glycosylated. Therefore glycosylation is not expected to play a role for antibody binding to these proteins. The recombinant allergens rAsp f 1 and rAsp f 6 exhibit enzymatic activities comparable to those of the native proteins indicating that the fusion tag allows a correct folding of the proteins (22,34). Moreover, the IgE binding properties of rAsp f 1 are comparable to those of the native Asp f 1 allergen isolated from fungal cultures (22). For these reasons we can assume that

Table 2. Physiochemical and biochemical characteristics of the allergens

	Allergen			
	rAsp f 1	rAsp f 3	rAsp f 4	rAsp f 6
GenBank accession no.	S339330	U58050	AJ001732	U53561
Mol. wt (kDa) ^a	16.86	18.45	30.04	23.03
Amino acid residues ^a	149	168	286	207
Isoelectric point ^a	9.60	5.34	4.45	8.02
Biochemical function	ribotoxin	peroxisomal protein	unknown	MnSOD
Production yield (mg/l) ^b	150	38	26	220
Purity (%) ^c	>98	>98	>98	>98
References	22,23,36	33	28	34

^aValues calculated from the deduced amino acid sequences without [His]₆-tag.

^bQuantified after Ni²⁺-NTA affinity chromatography purification.

^cEstimated from lack of contaminating bands in SDS-PAGE and Western blots.

the [His]₆-N-terminal fusions exhibit allergenic properties comparable to those of the native proteins.

Characterization of the patient and control groups

Skin test reactivity to commercial *A. fumigatus* extracts, total serum IgE and RAST were used as criteria to classify individuals with respect to sensitization against *A. fumigatus*. Discrimination between *A. fumigatus*-sensitized asthmatics with or without ABPA was according to their clinical history. All patients assigned to the ABPA group fulfilled at least six of the seven criteria proposed by Rosenberg *et al.* (16) and Patterson *et al.* (18) which are: blood eosinophilia (>1000/mm³), immediate cutaneous reactivity and precipitating antibodies to *A. fumigatus*, elevated total serum IgE levels, history of roentgenographic infiltrates, bronchiectasis, and elevated serum IgE and IgG levels to *A. fumigatus* extracts (Table 1). The presence of at least six of the diagnostic criteria is a commonly accepted rule for the diagnosis of ABPA (16,18,21), which is therefore regarded as established for each patient assigned to this group. 26 ABPA patients were assigned to stage III (exacerbation) and 34 to the corticosteroid-dependent stage IV according to the classification of Patterson *et al.* (18). Subjects assigned to the *A. fumigatus*-allergic group had a history of *A. fumigatus*-associated asthma defined by skin test reactivity to *A. fumigatus* extracts, moderately elevated total serum IgE and a positive RAST to *A. fumigatus* (Table 1). ABPA was excluded by repeated assessment of the clinical, radiologic and immunologic criteria (8). The healthy control subjects had no history of allergy or asthma and normal IgE levels (Table 1).

Differential serological responses to recombinant allergens

rAsp f 1 and rAsp f 3, two major allergens of the fungus, elicited IgE responses in subjects suffering from ABPA as well as in *A. fumigatus*-allergic asthmatics (Fig. 1). From the 40 *A. fumigatus*-sensitized asthmatics investigated, 18 (45%)

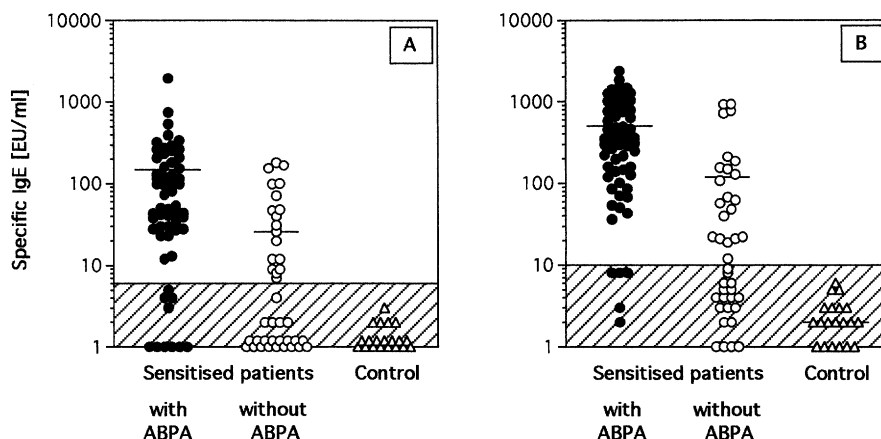


Fig. 1. Specific IgE antibodies to rAsp f 1 (A) and rAsp f 3 (B) in sera from 20 healthy control individuals (Controls), 40 *A. fumigatus*-sensitized asthmatics without ABPA and 60 *A. fumigatus*-sensitized asthmatics suffering from ABPA. IgE binding to the allergens was determined with an antigen-specific ELISA and compared to the binding of a reference serum arbitrarily assigned to 100 EU/ml (23). Mean values are indicated by lines. The hatched area represents the cut-off value determined by skin tests.

elicited an IgE response to rAsp f 1, 21 (52.5%) to rAsp f 3 and 30 (75%) at least to one of these allergens. The cut-off values defining the *in vivo* relevance of the measured allergen-specific serum IgE were determined by intradermal skin tests as described (23). According to the skin test, outcome cut-off values were set at 6 EU/ml for rAsp f 1 and 10 EU/ml for rAsp f 3. Applying the cut-off values, 50 (83%) out of the 60 patients with ABPA were sensitized to rAsp f 1, 55 (88%) to rAsp f 3 and 58 (96%) revealed an immune IgE response to at least one of these allergens with positive and negative predictive values of 65.9 and 93.7% for the disease. In spite of the fact that the ABPA-group showed statistically relevant increased responses to both rAsp f 1 (mean EU/ml value 148 ± 147 , $P < 0.001$) and rAsp f 3 (mean EU/ml value 502 ± 511 , $P < 0.001$) compared to the mean values of 26 ± 47 and 119 ± 247 EU/ml for rAsp f 1 and rAsp f 3 respectively, obtained for the group of sensitized asthmatics without ABPA, these allergens cannot be used to diagnose ABPA due to the strong overlap of the data (Fig. 1). However, the serologic data obtained with rAsp f 1 and rAsp f 3 reach a sensitivity of 88%, and positive and negative predictive values of 100 and 62.5% respectively, with respect to an overall diagnosis of sensitization to the fungus. All control subjects showed IgE levels below the cut-off values for both allergens (Fig. 1).

In contrast, the serological investigations involving rAsp f 4 and rAsp f 6 showed that allergen-specific IgE raised against these proteins could be detected exclusively in sera of patients suffering from ABPA (Fig. 2). The cut-off values determined by intradermal skin tests were set to 4 and 7 EU/ml for rAsp f 4 and rAsp f 6 respectively. According to these cut-off values, 48 (80%) subjects with ABPA elicited an IgE response to rAsp f 4, 33 (55%) to rAsp f 6 and 54 (90%) recognized at least one of these allergens. Therefore, the rAsp f 4 and rAsp f 6-based serology allowed a highly specific identification of patients suffering from ABPA with a sensitivity of 90%, and positive and negative predictive values of 100 and 90.9% respectively. Only six (10%) of the investigated sera from patients with a clinical diagnosis of ABPA lacked

IgE raised against both allergens. All *A. fumigatus*-allergic asthmatics without ABPA and all healthy controls showed rAsp f 4 and rAsp f 6 serum IgE levels below the cut-off values (Fig. 2). Automated, quantitative IgE antibody determination using the allergens rAsp f 1 (32) and rAsp f 6 immobilized to ImmunoCAPs, confirmed the results of ELISA determinations. Sera from patients with allergen-specific IgE levels below the cut-off values defined by skin test scored below the cut-off value of the ImmunoCAPs (<0.35 kU_A/l) (32). The data obtained with rAsp f 6 immobilized to ImmunoCAPs correlated closely with those obtained with the rAsp f 6-specific IgE ELISA ($r = 0.996$), demonstrating that the allergen can be used for quantitative determinations of allergen-specific IgE (32).

Elevated IgE levels to single allergens were associated with increased levels of allergen-specific IgG1 and IgG4 antibodies (not shown), indicating an IgG subclass-restricted immune response to *A. fumigatus* allergens in naturally exposed asthmatic patients (38).

Discussion

Aspergillosis is a disease complex with many faces which cause a wide spectrum of complications in humans and animals ranging from benign colonization of the lung to life-threatening diseases (2–4). Among the most severe pulmonary complications related to fungi, ABPA, an intense allergic inflammatory reaction to *Aspergillus* in the lung (8,11), has still to be considered a clinical syndrome difficult to be diagnosed (16–18) which should be ruled out in all patients with chronic asthma (8). The disease mainly affects individuals suffering from asthma (8,14,16,18) or cystic fibrosis (15,24) and is considered in patients sensitized to *A. fumigatus* when total serum IgE levels exceed 400 kU/l concomitantly with deterioration in lung function (11). From the seven criteria proposed for the diagnosis of ABPA (8,16,18), determination of blood eosinophilia and elevated levels of serum IgE, detection of pulmonary infiltrates or bronchiectasis and dia-

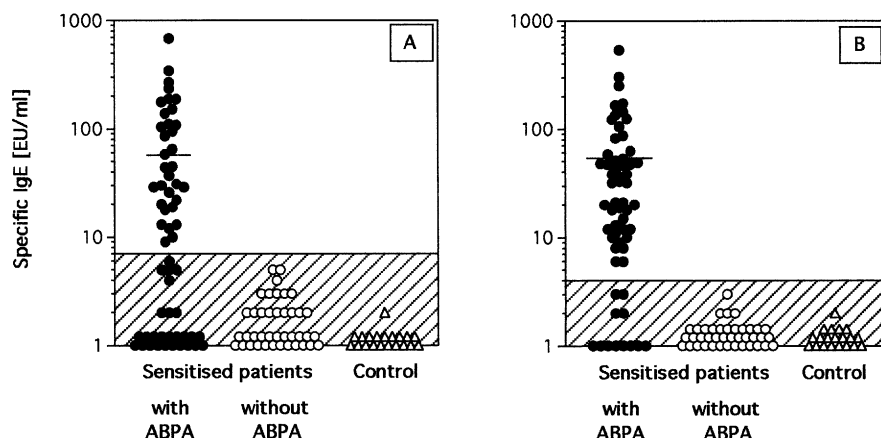


Fig. 2. Specific IgE antibodies to rAsp f 6 (A) and rAsp f 4 (B) in sera from healthy control individuals and *A. fumigatus*-sensitized asthmatics with or without ABPA. For further explanations see Fig. 1. The data show that these two allergens are highly specific for sera of patients with ABPA. From the 60 patients with ABPA, 48 (80%) elicited an IgE response to rAsp f 4, 33 (55%) to rAsp f 6 and 54 (90%) recognized at least one of these allergens. Accordingly, the sensitivity of the diagnosis reach 90% and the specificity defined by the lack of positive responses in *A. fumigatus*-sensitized asthmatics without ABPA corresponds to 100%.

gnosis of asthma pose little problems to the experienced clinician. More difficult are interpretations of immediate cutaneous reactivity to *A. fumigatus* extracts, and determination of elevated serum IgE and IgG levels to *A. fumigatus* allergens which may lead to false diagnoses. The outcome of these diagnostic criteria directly depends on the quality of the fungal extract used (17,20) and interpretation is complicated by the fact that *A. fumigatus*-sensitized asthmatics without ABPA show positive reactions to skin challenges with (16,18,22–24)—as well as immune responses to—*A. fumigatus* extracts (23,32).

Antigen/allergen extracts of *A. fumigatus* are complex mixtures containing up to 200 different proteins, glycoproteins and low mol. wt compounds (19,25,26). The fungus has been reported to be able to produce >40 IgE-binding components (25) which generate complex immunopatterns when extracts are assayed for their IgE-binding capacity with sera from allergic individuals in Western blots (27). Moreover, the quality of allergenic extracts from *A. fumigatus* depends on the pattern of allergens produced by different clinical isolates, batch to batch variations, time-dependent liberation of IgE-binding components during fungal growth, instability of the extracts due to protease content and culture conditions used to grow the fungus (17,19,25).

We have cloned and produced recombinant *A. fumigatus* allergens (28) to generate standardized reagents in order to dissect the antibody immune response of single patients to single allergens (23,33). In this study we present IgE determinations involving four recombinant allergens of *A. fumigatus* and a representative number of *A. fumigatus*-sensitized individuals with or without ABPA. The results show that the allergens rAsp f 1 and rAsp f 3 are recognized by sera of both groups of patients with high incidence (Fig. 1). In contrast, rAsp f 4 and rAsp f 6 are recognized exclusively by sera of patients suffering from ABPA (Fig. 2). Therefore, rAsp f 4 and rAsp f 6 can be used as specific markers to confirm a clinical suspicion of ABPA and represent a first step to explain at the molecular level the differences observed

between allergen 'fingerprints' occurring in Western blots developed with sera of patients with or without ABPA (25). The rAsp f 4- and rAsp f 6-based serological diagnosis of ABPA has a specificity of 100% and reaches a sensitivity of 90%. We cannot exclude that some of the six ABPA patients lacking IgE against rAsp f 4 and rAsp f 6 might suffer from allergic bronchopulmonary mycoses caused by species of *Aspergillus* other than *A. fumigatus* and therefore fulfil the classical diagnostic criteria for ABPA. However, the serology based on these two recombinant allergens allows a differential diagnosis of ABPA with a sensitivity never reached with other serological methods. The identification of the gene products provide a rational explanation for the different immunological responses in ABPA and *A. fumigatus* sensitization. rAsp f 6, a manganese superoxide dismutase (34), represents a strictly intracellular protein. Preliminary experiments devoted to elucidate the cellular distribution of rAsp f 4 with mAb indicate that the protein is not secreted by the fungus. Therefore, both proteins are unlikely to be present as aeroallergens, explaining the lack of specific IgE raised against these proteins in *A. fumigatus*-sensitized asthmatics. In contrast, patients suffering from ABPA have or had the fungus growing in the lung (8,26) and, as a result of fungal damage related to cellular defence mechanisms, become exposed also to non-secreted proteins. These patients may be therefore able to mount an immune response also to intracellular *A. fumigatus* proteins. In contrast, allergens recognized by *A. fumigatus*-sensitized asthmatics are secreted proteins produced shortly after spore germination (37) able to elicit IgE immune responses in both, allergic individuals and ABPA patients (Fig. 1). This important conclusion dictates the screening strategy for the isolation of disease-specific allergens. We are currently evaluating a panel of intracellular *A. fumigatus* proteins as candidates potentially corresponding to additional ABPA-specific allergens so far shown only in Western blot analyses (27). Production, characterization and evaluation of additional *A. fumigatus* allergens is likely to allow further improvement of the differen-

tial diagnosis of ABPA substantially contributing to the solution of an old diagnostic problem.

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Abbreviations

ABPA allergic bronchopulmonary aspergillosis

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