# Skin test reactivity to 2 recombinant *Aspergillus fumigatus* allergens in *A fumigatus*–sensitized asthmatic subjects allows diagnostic separation of allergic bronchopulmonary aspergillosis from fungal sensitization

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**Background: *Aspergillus fumigatus*, an opportunistic pathogen, is associated with an impressive list of pulmonary complications. Among these, allergic bronchopulmonary aspergillosis (ABPA) represents a complex clinical syndrome that is difficult to diagnose. A clear distinction between allergic sensitization to *A fumigatus* and ABPA is essential for therapy to prevent deterioration of pulmonary function in subjects with ABPA.**

**Objective: This study was carried out to determine the specificity and sensitivity of 2 *A fumigatus* allergens for the in vivo diagnosis of ABPA.**

**Methods: Serologic investigations with recombinant *A fumigatus* allergens indicated the existence of disease-specific allergens that are useful for discrimination between ABPA and fungal sensitization. However, serologic studies fail to indicate the allergen-specific IgE levels required to elicit an allergic reaction in vivo.**

**Results: We show that the recombinant *A fumigatus* allergens rAsp f 4, a protein with unknown biologic function, and rAsp f 6 (manganese superoxide dismutase) are able to provoke immediate skin reactions exclusively in patients with ABPA. The reactions, which are elicited by a few nonograms of the allergens, strictly depend on the presence of allergen-specific serum IgE. The IgE cut-off values for positive skin reactions to rAsp f 4 and rAsp f 6 of 0.9 and 1.2 $kU_A/L$ correspond to allergen-specific serum concentrations of 2 to 3 μg/L and allow a sensitive, highly specific diagnosis of ABPA.**

**Conclusions: In contrast to fungal extracts, rAsp f 4 and rAsp f 6 allow discrimination between ABPA and sensitization to *A fumigatus*. Moreover, the allergens are suitable for an automated serologic diagnosis of ABPA, facilitating their introduction in clinical practice. (J Allergy Clin Immunol 1999;104:601-7.)**

***Key words:*** *Skin test, Aspergillus fumigatus, IgE antibodies, allergy, allergic bronchopulmonary aspergillosis, recombinant allergens, ImmunoCAP*

*Abbreviations used*
ABPA: Allergic bronchopulmonary aspergillosis
IDT: Intradermal skin test
MnSOD: Manganese superoxide dismutase

The first cases of allergic bronchopulmonary aspergillosis (ABPA), a life-threatening disease caused by the opportunistic human pathogen *Aspergillus fumigatus*, were reported by Hinson et al in 1952.[1] This disease, originally considered a rarity, is today diagnosed with increasing frequency as a result of the improvement in serologic and radiologic diagnostic methods.[2] The classical diagnostic criteria for ABPA in asthmatic patients proposed by Greenberger and Patterson[3] are peripheral blood eosinophilia, chest roentgenographic infiltrates, bronchiectasis, elevated total serum IgE, immediate cutaneous reactivity, precipitating antibodies to fungal extracts, and elevated levels of *A fumigatus*–specific serum IgE and IgG. In stages of the disease during which most of the criteria are evident, ABPA can readily be diagnosed. However, all criteria are rarely present at the same time, even in patients with classic ABPA.[3] Unfortunately, the first 4 criteria, which do not cause significant problems to the experienced clinician, are not sufficient for diagnosis of the disease. The assessment of hypersensitivity to *A fumigatus*, a prerequisite for the diagnosis of ABPA, is routinely investigated by skin testing and determination of fungus-specific IgE antibodies.[4] The lack of standardized *A fumigatus* extracts[5] may account partly for the discrepancy in the incidence of ABPA, which is reported to range from 7% to 22% among asthmatic patients sensitized to *A fumigatus*[6,7] and from 0.1% to 12% in patients with cystic fibrosis.[8,9] However, undetected ABPA has far-reaching consequences for the patient because the untreated disease leads to irreversible changes in lung function and to end-stage pulmonary fibrosis.

Many efforts to standardize *A fumigatus* extracts have been made, and these efforts have contributed substantially to the improvement of the diagnosis of ABPA.[10] *A fumigatus* represents an extremely complicated allergenic system, resulting from the ability of the fungus to

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**TABLE I.** Serologic data and skin test responses to rAsp f 4 and rAsp f 6 in a group of *A fumigatus*–sensitized asthmatic subjects without ABPA but with high RAST scores to *A fumigatus* extracts

| | | | | | | | rAsp f 4 | | | | rAsp f 6 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Specific IgE reactivity | | | | Specific IgE reactivity | | | |
| Patient No. | Sex | Age (y) | Steroids (mg/d) | Total IgE* (kU/L) | Specific IgG*† (mg/L) | RAST class*† | CAP (kU/L) | ELISA (EU/mL) | IDT | Wheal size (mm²) | CAP (kU/L) | ELISA (EU/mL) | IDT | Wheal size (mm²) |
| 1 | F | 58 | 0 | 477 | 19.70 | 4.0 | 0.34 | 0 | – | Negative | 0.34 | 0 | + | Negative |
| 2 | F | 33 | 0 | 564 | 31.50 | 3.0 | 0.34 | 2 | – | Negative | 0.41 | 4 | – | Negative |
| 3 | M | 43 | 5 | 328 | 27.20 | 4.0 | 0.36 | 1 | – | Negative | 0.34 | 3 | + | Negative |
| 4 | M | 53 | 0 | 734 | 26.40 | 4.0 | 0.34 | 3 | – | Negative | 0.47 | 1 | – | Negative |
| 5 | F | 68 | 5 | 413 | 23.10 | 4.0 | 0.38 | 2 | – | Negative | 0.36 | 2 | – | Negative |
| 6 | F | 25 | 10 | 2,321 | 28.70 | 4.0 | 0.34 | 0 | – | Negative | 0.34 | 1 | – | Negative |
| 7 | M | 41 | 0 | 275 | 43.90 | 3.0 | 0.34 | 0 | – | Negative | 0.34 | 4 | – | Negative |
| Mean | — | — | 3 | 730 | 28.64 | 3.7 | 0.35 | 1 | — | — | 0.37 | 2 | — | — |
| SD | — | — | 4 | 718 | 7.73 | 0.5 | 0.02 | 1 | — | — | 0.05 | 2 | — | — |

*Determined with a UniCAP device (Pharmacia, Uppsala, Sweden).
†Against *A fumigatus* extract.

produce more than 40 IgE-binding proteins that are variably expressed depending on growth stage and culture conditions used.[11] Consequently, commercially available *A fumigatus* extracts from different suppliers generate discordant results in skin tests and serologic examinations.[4] Moreover, the availability of standardized fungal extracts would hardly allow for discrimination between allergic sensitization to *A fumigatus* and ABPA in routine investigations because both diseases reflect polyclonal IgE responses to fungal exposure.[12] We have chosen a molecular biologic approach to dissect the specific IgE responses against single allergens in different *A fumigatus*–associated diseases.[12] Using a special phage surface display technology,[13] we have cloned a panel of recombinant *A fumigatus* allergens.[12,14] Thus far it has been shown that patients with ABPA, compared with *A fumigatus*–sensitized asthmatic subjects, exhibit significantly elevated specific IgE values against Asp f 1 and Asp f 3, 2 major allergens of *A fumigatus*.[15,16] In both cases the IgE levels in *A fumigatus*–sensitized patients with or without ABPA to these 2 allergens were strongly overlapping and do not allow a reliable discrimination between these 2 diseases. Nevertheless, a study using *A fumigatus* extracts and Western blot analyses pointed to the possibility that ABPA-specific allergens might exist because sera from patients with ABPA were able to detect some previously unidentified protein bands not detected by sera of *A fumigatus*–sensitized asthmatic subjects without ABPA.[11]

In this report we present preliminary results of a highly specific ABPA diagnosis based on skin tests with the 2 recombinant *A fumigatus* allergens rAsp f 4, a protein with unknown biologic function, and rAsp f 6, a manganese superoxide dismutase (MnSOD) described earlier as a panallergen, which is possibly involved also in autoimmune responses resulting from molecular mimicry.[17,18]

## METHODS

### Subjects

*A fumigatus*–sensitized patients with asthma who do (n = 12) or do not (n = 12) have ABPA and 5 healthy individuals were involved in this study. Additionally, a group of 7 *A fumigatus*–sensitized asthmatic subjects without ABPA but with high RAST scores to the fungus were investigated. All patients had a positive skin prick test response to commercial *A fumigatus* extracts (Allergopharma, Hamburg, Germany, and Bencard, SmithKline Beecham, Neuss, Germany), were free of chest infections, had stable bronchial asthma, and received no antihistamine medication during the time of the study. ABPA in asthmatic subjects with positive skin prick test responses was ruled out because, according to their clinical histories, they did not have sufficient criteria for ABPA (Table II). The diagnosis of ABPA was based on at least 7 of the 8 criteria proposed by Greenberger and Patterson[3] for the diagnosis of the disease in *A fumigatus*–sensitized asthmatic subjects. According to this commonly accepted rule, the diagnosis of ABPA can be regarded as established in each individual assigned to this group. Bronchiectasis was manifested in all patients with ABPA but was absent in *A fumigatus*–sensitized asthmatic subjects without ABPA. Healthy control subjects demonstrated no indication for atopy and a negative skin prick test response to *A fumigatus* extracts. Further information about the subjects is summarized in Tables I and II. The study was approved by the ethical committee of Davos. Before skin testing, a full explanation of the procedure was given to all participants, and written consent was obtained.

### Identification, subcloning, production, and purification of rAsp f 4 and rAsp f 6

The complementary (c)DNA encoding Asp f 4 and Asp f 6 (names approved by the WHO/IUIS Allergen Nomenclature Sub-Committee) were obtained from filamentous phages isolated by biopanning of an *A fumigatus* phage surface display cDNA library against human serum IgE from patients with ABPA.[13] Inserts carried by IgE-binding phages were sequenced and subcloned in expression vector pDS56 RBS II for high level expression of protein.[12] After DNA sequence verification, the deduced amino acid sequences of these cDNA clones were analyzed with the program PEPTIDESORT and compared with data bank entries by using

**TABLE II.** Data of investigated *A fumigatus*–sensitized asthmatic subjects with or without ABPA and healthy control individuals

| | | | | | | | | rAsp f 4 | | | | rAsp f 6 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Specific IgE reactivity | | | | Specific IgE reactivity | | | |
| Patient No. | Diagnosis | Sex | Age (y) | Steroids (mg/d) | Total IgE* (kU/L) | Specific IgG*† (mg/L) | RAST class*† | CAP (kU/L) | ELISA (EU/mL) | IDT | Wheal size (mm²) | CAP (kU/L) | ELISA (EU/mL) | IDT | Wheal Size (mm²) |
| 1 | ABPA | F | 63 | 6 | 475 | 78.30 | 3 | 0.34 | 0 | – | Negative | 2.30 | 15 | + | 94 |
| 2 | ABPA | F | 60 | 50 | 10,957 | 185.00 | 5 | 39.40 | 190 | + | 189 | 1.20 | 4 | – | Negative |
| 3 | ABPA | M | 65 | 4 | 1,882 | 56.60 | 5 | 9.65 | 38 | + | 100 | 1.89 | 18 | + | 117 |
| 4 | ABPA | M | 53 | 0 | 2,457 | 107.00 | 5 | 2.97 | 19 | + | 79 | 3.78 | 28 | + | 129 |
| 5 | ABPA | F | 61 | 5 | 629 | 26.30 | 4 | 14.70 | 48 | + | 110 | 0.66 | 3 | – | Negative |
| 6 | ABPA | M | 59 | 8 | 517 | 45.20 | 4 | 4.47 | 18 | + | 105 | 0.34 | 1 | – | Negative |
| 7 | ABPA | M | 57 | 0 | 637 | 48.20 | 5 | 3.62 | 11 | + | 90 | 0.34 | 4 | – | Negative |
| 8 | ABPA | M | 43 | 10 | 681 | 135.00 | 4 | 2.04 | 8 | + | 80 | 2.86 | 30 | + | 163 |
| 9 | ABPA | F | 60 | 0 | 619 | 98.80 | 4 | 10.20 | 56 | + | 132 | 0.44 | 5 | – | Negative |
| 10 | ABPA | M | 53 | 2 | 163 | 85.40 | 3 | 0.40 | 1 | – | Negative | 0.34 | 2 | – | Negative |
| 11 | ABPA | F | 57 | 12 | 1,779 | 32.50 | 5 | 9.22 | 33 | + | 116 | 11.10 | 93 | + | 129 |
| 12 | ABPA | M | 33 | 10 | 1,472 | 130.00 | 5 | 20.90 | 82 | + | 95 | 1.18 | 7 | – | Negative |
| Mean | — | — | 55 | 9 | 1,857 | 85.69 | 4 | 9.83 | 42 | — | 110 | 2.20 | 18 | — | 126 |
| SD | — | — | 9 | 14 | 2,952 | 47.85 | 1 | 11.18 | 53 | — | 32 | 3.02 | 26 | — | 25 |
| 13 | Sensitized | M | 59 | 0 | 116 | 30.10 | 2 | 0.42 | 2 | – | Negative | 0.34 | 5 | – | Negative |
| 14 | Sensitized | M | 60 | 0 | 76 | 7.17 | 3 | 0.34 | 0 | – | Negative | 0.34 | 1 | – | Negative |
| 15 | Sensitized | F | 50 | 8 | 67 | 14.00 | 3 | 0.34 | 0 | – | Negative | 0.34 | 1 | – | Negative |
| 16 | Sensitized | M | 34 | 0 | 322 | 25.50 | 3 | 0.34 | 0 | – | Negative | 0.34 | 1 | – | Negative |
| 17 | Sensitized | M | 57 | 20 | 193 | 52.50 | 2 | 0.41 | 2 | – | Negative | 0.34 | 4 | – | Negative |
| 18 | Sensitized | M | 57 | 6 | 627 | 3.80 | 2 | 0.34 | 1 | – | Negative | 0.34 | 2 | – | Negative |
| 19 | Sensitized | F | 49 | 0 | 354 | 2.10 | 3 | 0.34 | 0 | – | Negative | 0.34 | 3 | – | Negative |
| 20 | Sensitized | F | 41 | 0 | 1,759 | 28.50 | 2 | 0.34 | 0 | – | Negative | 0.51 | 6 | – | Negative |
| 21 | Sensitized | F | 46 | 0 | 494 | 22.40 | 3 | 0.41 | 1 | – | Negative | 0.34 | 3 | – | Negative |
| 22 | Sensitized | M | 43 | 0 | 5,198 | 18.70 | 4 | 0.34 | 0 | – | Negative | 0.34 | 2 | – | Negative |
| 23 | Sensitized | M | 60 | 0 | 1 | 28.20 | 2 | 0.34 | 0 | – | Negative | 0.34 | 2 | – | Negative |
| 24 | Sensitized | M | 56 | 0 | 237 | 6.87 | 2 | 0.34 | 0 | – | Negative | 0.34 | 1 | – | Negative |
| Mean | — | — | 51 | 3 | 875 | 19.99 | 3 | 0.36 | 1 | — | — | 0.35 | 3 | — | — |
| SD | — | — | 9 | 6 | 1,477 | 14.43 | 1 | 0.03 | 1 | — | — | 0.05 | 2 | — | — |
| 25 | Control | M | 44 | 0 | 148 | 13.20 | 0 | 0.34 | 0 | – | Negative | 0.34 | 2 | – | Negative |
| 26 | Control | M | 32 | 0 | 34 | 9.66 | 0 | 0.34 | 0 | – | Negative | 0.34 | 2 | – | Negative |
| 27 | Control | F | 39 | 0 | 52 | 5.68 | 0 | 0.43 | 3 | – | Negative | 0.34 | 2 | – | Negative |
| 28 | Control | F | 30 | 0 | 45 | 2.96 | 0 | 0.34 | 0 | – | Negative | 0.34 | 2 | – | Negative |
| 29 | Control | M | 31 | 0 | 130 | 14.60 | 0 | 0.34 | 0 | – | Negative | 0.34 | 1 | – | Negative |
| Mean | — | — | 35 | 0 | 82 | 9.22 | 0 | 0.36 | 1 | — | — | 0.34 | 2 | — | — |
| SD | — | — | 6 | 0 | 53 | 4.92 | 0 | 0.04 | 1 | — | — | 0.00 | 0 | — | — |

*Determined with a UniCAP device (Pharmacia, Uppsala, Sweden).
†Against *A fumigatus* extract.

FASTA (Wisconsin Sequence Analysis Package, Madison, Wis). Hexahistidine-tagged recombinant *A fumigatus* allergens were produced in *Escherichia coli* M15 as inclusion body proteins, purified by $Ni^{2+}$-chelate chromatography under denaturing conditions, and refolded by dialysis against a physiologic buffer (PBS, pH 7.4). Both allergens were found to lack toxicity, as determined by incubation with IL-2–dependent mouse cytotoxic T-cell lines as described elsewhere.[19]

## IgE-specific immunoblots

For IgE-specific immunoblot analysis, proteins were separated on 4% to 20% Tris-Glycine polyacrylamide gels (Novex, San Diego, Calif) with denaturing (2% [wt/vol] SDS) and reducing (5% [vol/vol] 2-mercaptoethanol) or denaturing and nonreducing sample buffers. Separated proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham, Buckinghamshire, UK) by using a Novex blot module (1 hour, 25 V) according to the manufacturer's instructions. Membranes were blocked and subsequently incubated overnight at 4°C either with supernatant of mAbs secreting hybridoma cells (mouse IgG anti-rAsp f 4, Cl854, 15 μg/mL, 1:3200 dilution; mouse IgG anti-rAsp f 6, Cl866-1B1, 34 μg/mL, 1:800 dilution) or with sera of patients sensitized against the 2 allergens (1:20 dilution). Membranes incubated with serum were additionally incubated with mAb TN-142 (mouse anti-human IgE, 1 mg/mL, 1:6500 dilution, 2 hours, room temperature) as a second antibody. After washing, membranes were incubated with peroxidase-conjugated goat anti-mouse IgG (Dako, 1 mg/mL, 1:5000 dilution, 3 hours, room temperature) and binding of human IgE or mAbs to the allergens visualized by SuperSignal-Substrate (Pierce, Rockford, Ill), exposing the membranes to Hyperfilm-ECL (Amersham).

## Intradermal skin tests

The rAsp f 4 or rAsp f 6 allergens were dissolved in 0.9% saline solution at a concentration of 10 μg/mL. One hundred microliters of

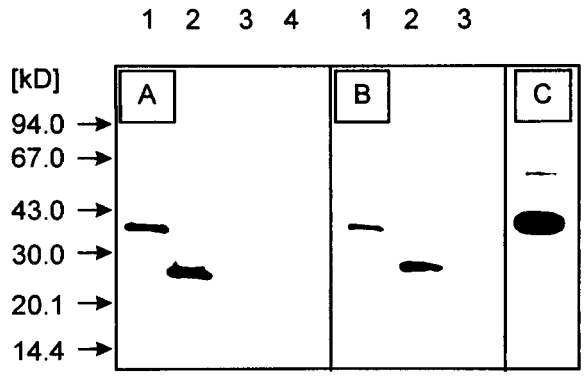


**FIG 1.** IgE Western blots with recombinant Asp f 4 and Asp f 6. One microgram of rAsp f 4 *(lanes 1)*, 1 μg of rAsp f 6 *(lanes 2)*, and 10 μg of total protein of an uninduced *E coli* culture lysate used as negative control *(lanes 3)* were separated by 4% to 20% SDS-PAGE under reducing conditions (***A*** and ***B***) and 1 μg of rAsp f 4 was separated under nonreducing conditions ***(C)***. Blots were developed for the binding of serum IgE from a patient sensitized against *A fumigatus* extract (***A*** and ***C***) or mAbs raised against both allergens ***(B)***.

serially diluted protein, starting from a concentration of 0.01 μg/mL up to 10 μg/mL, was injected into the dermis of each patient's back. The same investigator performed all tests. A skin reaction was regarded as positive, and the procedure was stopped when, after 20 minutes, the allergen-induced wheal was surrounded by an erythema and reached at least half the size of the wheal induced by the histamine positive control (0.01% histamine) in the absence of a skin reaction with the allergen diluent as negative control (0.9% NaCl solution).[15,19] Skin responses were outlined with a felt-tip pen and transferred to paper with transparent adhesive tape. After measuring the longitudinal (D1) and transversal (D2) diameters, wheal sizes were calculated according to the following formula: $[(D1 + D2)/2]^2$.[15]

### rAsp f 4– and rAsp f 6–specific serology

The patients' sera were taken before skin testing and stored at –20°C until use. Maxisorp polystyrene microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 10 μg/mL allergen in PBS (pH 8.0) and further processed as previously described.[15,19] Binding of human IgE was detected with mAb TN-142 anti-human IgE visualized by incubation with alkaline phosphatase–conjugated goat anti-mouse IgG antibody (Pierce; 0.6 mg/mL, 1:6500 dilution) and 4-nitrophenyl phosphate as substrate.[15]

Absorbence was measured at 405 nm with a Molecular Devices Reader (Menlo Park, Calif), and optical densities were converted into arbitrary ELISA units per milliliter (EU/mL) calibrated against an in-house serum pool, as previously described.[15] Values of less than 1 EU/mL were set as 1 EU/mL for graphic representation. The patients' sera were analyzed in triplicate to assess the reproducibility of the ELISA results, and the mean coefficient of variation was less than 10%. ImmunoCAPs carrying rAsp f 4 and rAsp f 6 were prepared and evaluated as previously described.[20]

### Statistical analyses

Different patient groups were compared with the nonparametric Mann-Whitney *U* test, and correlation coefficients were determined with Pearson's linear regression analysis. Probability *(P)* values of less than .05 were considered to be significant.

**TABLE III.** Allergen characteristics

| | rAsp f 4 | rAsp f 6 |
|---|---|---|
| Accession No. | AJ001732 | U53561 |
| Coding sequence (nucleotides) | 858 | 621 |
| Amino acid residues* | 286 | 207 |
| Molecular weight (kd)* | 30.041 | 23.031 |
| Isoelectric point* | 4.45 | 8.02 |
| Molar extinction coefficient* | 74,350 | 52,630 |
| Production per liter of culture (mg)† | 20 | 70 |
| Biochemical function | Unknown | MnSOD |

*Calculated from the deduced amino acid sequences without cloning tag.
†Yields produced in *E coli* cultures after affinity purification.

## RESULTS

### Sequence analysis of Asp f 4 and Asp f 6

Analysis of cDNA and deduced amino acid sequence identified Asp f 6 (GenBank accession No. U53561) as MnSOD.[17] For the allergen Asp f 4 (GenBank accession No. AJ001732), no homologies with known sequences were found in sequence banks. Physicochemical and biochemical properties of the recombinant allergens are reported in Table III.

### Purity and IgE-binding properties of rAsp f 4 and rAsp f 6

Before skin testing and serologic investigations, the purity of both recombinant allergens was analyzed. Fig 1 shows a representative Western blot. The allergens were separated in SDS-PAGE under reducing or nonreducing conditions, transferred onto nitrocellulose membranes, and developed for binding to IgE from serum of patients with ABPA or to specific mAbs against the allergens. The mAbs and serum IgE specifically detected rAsp f 4 and rAsp f 6, thereby indicating the allergenic nature of the proteins. The recombinant allergen preparations were free of IgE-binding contaminants, and IgE against *E coli* proteins was not detectable in the patients' sera (Fig 1, *A* and *B, lane 3*).

rAsp f 4 protein, which contains 4 cysteines, was further analyzed for IgE binding under nonreducing conditions (Fig 1, *C*). This blot revealed an additional band approximately the size of an rAsp f 4 dimer, which was undetectable under reducing conditions (Fig 1, *A*).

### Intradermal skin tests and allergen-specific serologic investigation

Intradermal skin tests (IDTs) were performed to determine the minimal serum IgE concentration required to elicit a specific positive skin reaction after allergen challenge. Sera were analyzed for rAsp f 4– or rAsp f 6–specific IgE content, and the results were compared with those of the skin test. Ten of twelve patients with ABPA (83%) had skin challenge responses with rAsp f 4, and 5 (42%) had positive skin responses for rAsp f 6. All but one patient (No. 10, Table II) reacted to rAsp f 4, to rAsp f 6, or to both allergens at concentrations ranging from 1

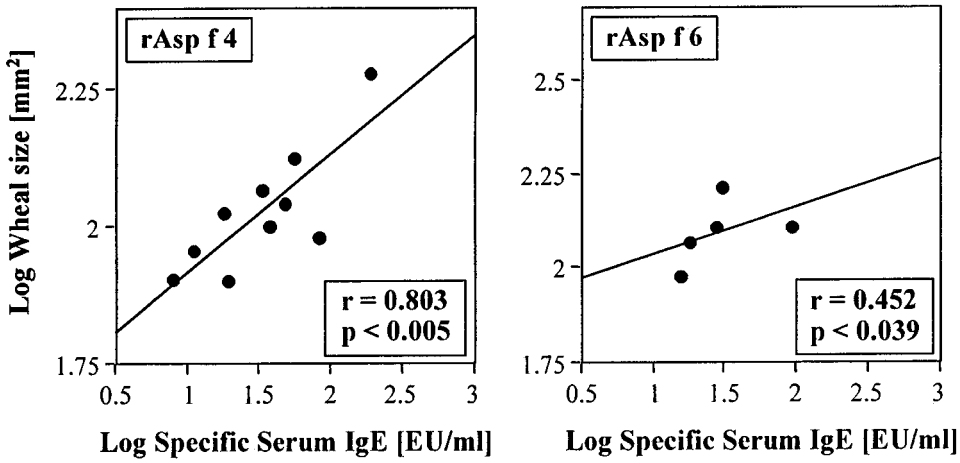


**FIG 2.** Correlation between allergen-specific IgE determined by ELISA and surface of the wheal provoked by IST. Log values for allergen-specific IgE were plotted against the log values of the wheal surface induced by intradermal allergen challenge.

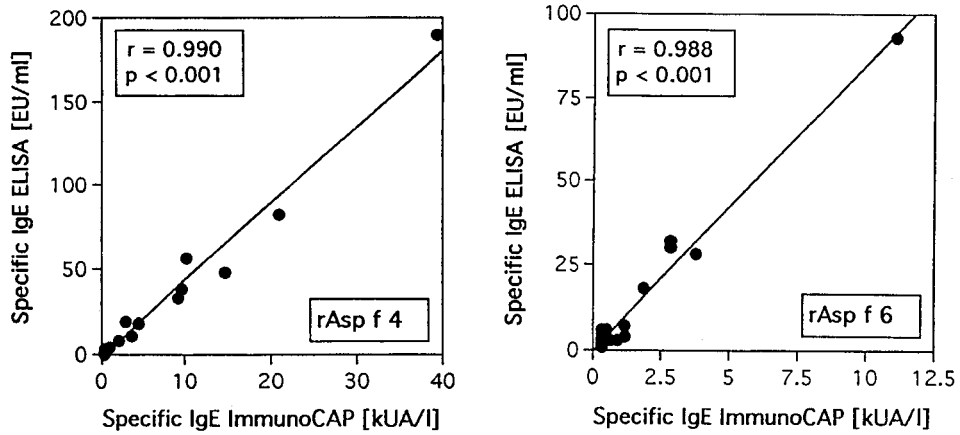


**FIG 3.** Correlation of specific serum IgE values determined by ELISA and the Pharmacia CAP system. Sera of all skin tested individuals were analyzed for rAsp f 4– or rAsp f 6–specific IgE by the Pharmacia CAP system and by ELISA. Values of both methods were plotted against each other.

to 100 ng. None of the *A fumigatus*–sensitized asthmatic subjects without ABPA or healthy control subjects showed skin reactions after challenge with either of the allergens, even at the highest concentration of 10 μg/mL (Table II). The lack of skin test reactivity to rAsp f 4 and rAsp f 6 in *A fumigatus*–sensitized asthmatic subjects is confirmed by the results obtained with 7 individuals with RAST scores comparable to those of the patients with ABPA (Table I). The skin test results strictly correlated with the presence or absence of allergen-specific IgE in serum. This correlation allows the estimation of an approximate cut-off value for a positive IDT response assumed to correspond to the highest allergen-specific serum IgE value with a negative skin test response. The allergen-specific serum IgE levels representing the minimal concentration needed to elicit a skin reaction corresponded to approximately 4 and 7 EU/mL for rAsp f 4 and rAsp f 6, respectively. A significant correlation between magnitude of the wheal surface and amount of specific IgE in serum for both allergens was observed (Fig 2). Considering rAsp f 4 and rAsp f 6 as specific markers for ABPA, 92% of the tested patients with ABPA had positive skin test responses to at least one of these allergens (Table II). Because of the lack of allergen-specific IgE and positive skin reactions in the control group, the rAsp f 4– and rAsp f 6–based diagnosis of ABPA reached 92% sensitivity and 100% specificity with predictive positive and negative values of 100% and 94%, respectively. The specificity of the rAsp f 4– and rAsp f 6–based distinction between ABPA and fungal sensitization is corroborated by the negative skin test results obtained with an additional group of 7 *A fumigatus*–sensitized asthmatic subjects with high RAST scores to *A fumigatus* (Table I).

ImmunoCAPs with immobilized rAsp f 4 and rAsp f 6 prepared as described[20] were used for quantitative deter-

mination of allergen-specific IgE by using the Pharmacia UniCAP system. Fig 3 demonstrates a highly significant correlation between the results obtained by ELISA and ImmunoCAP with rAsp f 4 ($r$ = 0.990, $P$ < .001) and rAsp f 6 ($r$ = 0.988, $P$ < .001). This allows the calculation of absolute cut-off values for a positive IDT response of 0.9 $kU_A/L$ for rAsp f 4 and 1.2 $kU_A/L$ for rAsp f 6, respectively, which corresponds to allergen-specific IgE concentrations in serum in the range of 2 to 3 μg/L.

## DISCUSSION

In spite of recent progress in the immunodiagnosis of ABPA,[10] various overlapping clinical and laboratory findings shared between *A fumigatus* sensitization and ABPA still hamper a clear-cut diagnosis of the disease.[9] An interesting emerging concept to improve both sensitivity and specificity of the diagnosis of ABPA is related to the search for disease-specific allergens.[12,23] The existence of such molecules was first postulated from Western blot analyses on the basis of *A fumigatus* extracts probed with sera from individuals with or without ABPA who were sensitized to the fungus.[11] These studies showed that from 43 IgE-binding components detected in *A fumigatus* extracts, at last 31 were shared by the 2 different pulmonary complications. However, a few of them were only recognized by sera of patients with ABPA.[11,12] The disease-specific components were not biochemically purified, and the molecular nature of these proteins remained unknown.

We used a phage surface display–based cloning system, which allows rapid cloning, sequencing, and production of IgE-binding molecules,[12-14] to clone a panel of *A fumigatus* allergens.[12] The major allergens rAsp f 1 and rAsp f 3 were investigated in large serologic and clinical studies.[15,16,19] In spite of the fact that patients with ABPA showed significantly increased IgE responses to both allergens compared with those of *A fumigatus*–sensitized asthmatic subjects without ABPA, these allergens cannot be used to discriminate between the 2 diseases because of strongly overlapping data.[12,15,16,19] On the other hand, preferential binding of IgE from the sera of patients with ABPA was reported for an *A fumigatus* allergen termed rAsp f 2.[21] Our serologic investigations show that the 2 recombinant *A fumigatus* allergens rAsp f 4 and rAsp f 6 represent highly specific markers for ABPA,[12,22,23] but these studies lacked a demonstration of the biologic relevance of the allergens in vivo. Skin test reactivity of a protein is, however, a prerequisite for allergenicity. Moreover, it is required to determine the clinically relevant cut-off values of the in vitro analyses. In our preliminary investigations we demonstrated that both rAsp f 4 and rAsp f 6 elicit type I skin reactions in sensitized individuals (Table II). A positive IDT response to the allergens strictly correlated with the presence of allergen-specific serum IgE. In agreement with previous serologic studies,[12,22,23] IgE against rAsp f 4 and rAsp f 6 was only detectable in sera of patients with ABPA (Table II). As shown in Table I, even *A fumigatus*–sensitized asthmatic subjects with RAST scores comparable with those of the patients with ABPA scored negatively in skin tests with the recombinant allergens, indicating a high specificity of the diagnostic procedure. By using allergen extracts[24] and, in particular, mold extracts,[25] discrepancies between results of skin tests and allergen-specific IgE determinations have been reported. In contrast, skin studies with rAsp f 1[15] and rAsp f 3,[19] similar to the results presented here, show that all patients with a positive wheal-and-flare reaction to a recombinant allergen also possess detectable levels of allergen-specific serum IgE antibodies. Furthermore, the levels of rAsp f 4– and rAsp f 6–specific IgE significantly correlated with the magnitude of the wheal surface (Fig 2). The excellent correlation between serologic data and skin test results suggest the possibility of relying on serologic data, thereby avoiding allergen challenges.

From a practical point of view it would be easier to directly perform skin tests with the recombinant allergens to obtain immediate results. This way does not seem feasible at the present time because in most countries ethical approval is needed to test patients with recombinant proteins and because the substances are not yet approved for clinical use. However, determination of allergen-specific IgE by ELISA is not absolutely quantifiable. Therefore ImmunoCAPs[20] carrying immobilized rAsp f 4 and rAsp f 6 were evaluated with the Pharmacia CAP System, which is calibrated against the WHO standard for IgE, allowing quantitative expression of allergen-specific IgE.[26] The IgE cut-off values for a positive skin test response were found to be approximately 0.9 $kU_A/L$ and 1.2 $kU_A/L$ for rAsp f 4 and rAsp f 6, respectively, corresponding to an absolute allergen-specific IgE serum concentration in the range of 2.5 μg/L. As depicted in Fig 3, the allergen-specific values obtained with ELISA and ImmunoCAP were closely correlated. This suggests the possibility of using the recombinant allergens for a fully automated, highly specific diagnosis of ABPA never before achieved with other diagnostic methods. Taken together, we have demonstrated that the threshold value of allergen-specific serum IgE required for a positive skin reaction is close to 2 μg/L, whereas the lowest amount of allergen able to elicit a positive skin reaction is less than 1 ng. This demonstrates the high biologic activity of the recombinant allergenic proteins used.

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