

# Skin-test reactivity and isotype-specific immune responses to recombinant Asp f 3, a major allergen of *Aspergillus fumigatus*

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## Summary

**Background** The *in vitro* diagnostic value of recombinant allergens depends on the correlation between allergen specific IgE in serum and ability of the protein to elicit immediate type allergic reactions in sensitized individuals.

**Objective** The study was carried out to evaluate the reliability of recombinant Asp f 3 (rAsp f 3)-based serological determinations in ELISA and ImmunoCAP compared to skin-test responses in *A. fumigatus* sensitized subjects.

**Methods** Patients suffering from allergic bronchopulmonary aspergillosis (ABPA,  $n = 11$ ) or allergic asthma with *A. fumigatus* sensitization ( $n = 8$ ) and healthy control subjects ( $n = 4$ ) were investigated. Intradermal skin tests (IDT) and allergen-specific serology were performed using rAsp f 3 protein.

**Results** All 11 patients with ABPA and five out of eight *A. fumigatus*-sensitized asthmatics without ABPA exhibited a type I skin reaction. All rAsp f 3 skin test positive subjects showed relevant rAsp f 3-specific serum IgE levels compared with IDT-negative individuals who scored below the rAsp f 3-ImmunoCAP cut-off values of  $< 0.35 \text{ kU}_A/\text{L}$ . Analyses of rAsp f 3-specific immunoglobulins revealed significantly higher serum levels of IgG, IgG1, IgG4 and IgE antibodies in patients with ABPA compared with *A. fumigatus*-sensitized asthmatics and healthy controls.

**Conclusion** rAsp f 3 represents a major allergen of *A. fumigatus*. It is recognized by 84% of asthmatic individuals sensitized to the fungus and elicits specific type I skin reactions. The skin test outcome strictly depends on the presence of rAsp f 3-specific IgE and the lack of false positive or false negative results comparing skin test outcome with specific serum IgE levels determined by ELISA or rAsp f 3-ImmunoCAP suggests the possibility to rely on serological data obtained with recombinant allergens to diagnose sensitization to *A. fumigatus*.

**Keywords:** *Aspergillus fumigatus*, recombinant allergens, serology, ABPA.

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## Introduction

The assessment of sensitization against allergens is based on clinical history and skin-prick or intradermal skin tests with allergen extracts, corroborated by various serological methods [1]. Skin tests and *in vitro* determinations of allergen-specific IgE antibodies are of well established value in the clinical evaluation of allergic diseases [1,2].

However, both, *in vivo* and *in vitro* diagnostic methods depend on the quality of the employed allergen preparation [3]. In the case of the opportunistic pathogenic fungus *A. fumigatus*, which represents a complex allergenic system [4,5], extracts are difficult to standardize. As a consequence thereof the diagnosis of *A. fumigatus* sensitization based on *in vivo* or *in vitro* tests with commercial extracts is not always satisfactory [6]. Genetic engineering and biotechnological procedures allow production of recombinant allergens of consistent quality and purity in virtually unlimited amounts [7] and may help to solve many

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problems associated with the standardization of allergenic extracts. Moreover, the availability of single *A. fumigatus* allergens may allow the generation of patients-specific reactivity patterns against single allergens and to correlate these reactivity patterns with different diseases related to the fungus [8].

Despite the considerable knowledge about primary allergen structures and IgE-binding properties of recombinant allergens demonstrated *in vitro*, only a few of them have been systematically investigated for their reactivity *in vivo*. However, the final demonstration that a protein preparation acts as allergen and therefore possesses biological activity *in vivo* is its ability to elicit type I skin reactions in sensitized individuals. The demonstration of allergenicity *in vivo* is required for recombinant proteins expressed in heterologous hosts which may have to be refolded *in vitro* to generate biologically active allergens [9]. Skin-test reactivity has so far been investigated for PLA2, the major allergen of honeybee venom [10], the birch pollen allergens Bet v 1 and Bet v 2 [11,12], some mite [13–15] and grass allergens [16] and for a major allergen of *A. fumigatus*, rAsp f 1 [6,17].

A further allergen of *A. fumigatus*, rAsp f 3, has so far been shown to be homologous and crossreactive regarding IgE-binding with two peroxisomal membrane proteins (PMP) of *Candida boidinii* [18]. *In vitro* IgE-binding studies showed that 72% of the sera from *A. fumigatus* sensitized individuals tested reacted against rAsp f 3, demonstrating that the proteins represents a major allergen of the fungus [18]. In this study we report the clinical and serological evaluation of rAsp f 3 in allergic asthmatics sensitized to *A. fumigatus* with or without ABPA.

## Materials and methods

### Subjects

*A. fumigatus* sensitized patients suffering from allergic asthma with ( $n = 11$ ) or without ( $n = 8$ ) ABPA and four healthy control individuals were involved in this study. Patients' characteristics including age, sex, total IgE and RAST against *A. fumigatus* are shown in Table 1. None of the subjects received immunotherapy, antihistamine or other medications that might interfere with the outcome of the tests before and during the investigations.

All *A. fumigatus* sensitized patients had stable bronchial asthma with no evidence of chest infections and met the guidelines for the diagnosis and management of asthma [19]. Patients suffering from ABPA fulfilled at least six of the seven diagnostic criteria proposed by Rosenberg *et al.* [20] and Patterson *et al.* [21]. The healthy control subjects had not history of atopy or asthma and normal total serum IgE levels.

### Total and specific serum IgE determinations

Total serum IgE and specific serum IgE against rAsp f 3 and *A. fumigatus* extract were measured with a Pharmacia UniCAP device (Pharmacia, Uppsala, Sweden). rAsp f 3 ImmunoCAPs were produced as described [22]. Specific IgE was converted into RAST classes according to the manufacturer's instructions and values  $<0.35 \text{ kU}_A/\text{L}$  were set as  $0.34 \text{ kU}_A/\text{L}$  for tables and graphics.

### Cytotoxic activity of rAsp f 3

Recombinant Asp f 3 or Asp f 1 protein, produced as described [18,23], was diluted in RPMI 1640 medium (GibcoBRL, Life Technologies, Paisley, UK), containing L-Glutamine, 10% heat-inactivated FCS, 0.1% 2-ME (GibcoBRL) and antibiotics (cRPMI). Different allergen dilutions ranging from  $0.1 \mu\text{g/mL}$  to  $900 \mu\text{g/mL}$  were applied in triplicates to a flat-bottom 96-well tissue culture plate (Costar, Cambridge, MA, USA). After addition of IL-2-dependent mouse cytotoxic T cell line cells (CTLL,  $10^4$  cells/well) resuspended in cRPMI supplemented with  $8 \text{ U/mL}$  IL-2 (Sandoz, Basel, Switzerland), plates were incubated for 24 h at  $37^\circ\text{C}$  in humidified atmosphere with 5%  $\text{CO}_2$ . Cells were pulsed with [ $^3\text{H}$ ]TdR ( $1 \mu\text{Ci/well}$ , DuPont, NEN Products, Boston, MA, USA) for further 8 h and then harvested onto glass fibre filters (Wallac, Turku, Finland) using a semiautomatic harvester. [ $^3\text{H}$ ]TdR incorporation was measured by a  $\beta$ -plate liquid scintillation counter (Wallac).

### Intradermal skin tests

An approval for skin testing human subjects with recombinant allergens was obtained by the responsible ethical committee. A full explanation of the procedure was given to all participants before testing, and subsequently a written consent was obtained.

Intradermal skin test (IDT) was performed on the patients' back by injecting  $100 \mu\text{L}$ -test solution containing  $1 \text{ ng}$  rAsp f 3 as described [17]. If no positive reaction could be observed after 20 min, testing was continued by injecting an always 10-fold higher amount of rAsp f 3. The test was stopped when the weal surface reached at least half of the histamine weal size or when no reaction could be observed with  $10 \mu\text{g}$  rAsp f 3.

Injection of physiological saline solution (Sintetica, Mendrisio, Switzerland) served as negative, histamine dihydrochloride solution (0.01%, Streuli & Co., Uznach, Switzerland) as positive control. All skin tests were done by the same investigator. Results were recorded by outlining skin reactions with a fine-tipped felt pen and transferred to paper with transparent tape. The maximal longitudinal ( $D_1$ )

**Table 1.** Data of investigated *A. fumigatus* sensitized asthmatics with (ABPA) or without ABPA (sensitized) and healthy control individuals (control)

| Patient | Diagnosis  | Sex | Age (years) | Total IgE (kU/L) | RAST to <i>A. f</i> (class) | rAsp f 3 specific IgG (EU/mL) | rAsp f 3 specific IgE (EU/mL) | rASP f 3 specific IgE (kUA/L) | IDT reactivity |
|---------|------------|-----|-------------|------------------|-----------------------------|-------------------------------|-------------------------------|-------------------------------|----------------|
| 1       | ABPA       | F   | 68          | 413              | 4                           | 129                           | 311                           | 8.10                          | +              |
| 2       | ABPA       | M   | 58          | 332              | 3                           | 190                           | 70                            | 1.49                          | +              |
| 3       | ABPA       | M   | 59          | 1003             | 5                           | 129                           | 1411                          | 44.00                         | +              |
| 4       | ABPA       | M   | 65          | 1882             | 5                           | 347                           | 1208                          | 21.20                         | +              |
| 5       | ABPA       | M   | 52          | 1205             | 5                           | 332                           | 867                           | 9.62                          | +              |
| 6       | ABPA       | M   | 37          | 905              | 5                           | 158                           | 222                           | 6.53                          | +              |
| 7       | ABPA       | M   | 56          | 3151             | 4                           | 95                            | 358                           | 8.20                          | +              |
| 8       | ABPA       | M   | 32          | 4018             | 6                           | 70                            | 1277                          | 36.40                         | +              |
| 9       | ABPA       | M   | 45          | 1508             | 4                           | 389                           | 119                           | 2.18                          | +              |
| 10      | ABPA       | F   | 50          | 1433             | 4                           | 216                           | 657                           | 11.20                         | +              |
| 11      | ABPA       | F   | 58          | 542              | 3                           | 148                           | 296                           | 6.28                          | +              |
| Mean    |            |     | 53          | 1490             | 4                           | 200                           | 618                           | 14.12                         |                |
| SD      |            |     | 11          | 1156             | 1                           | 108                           | 495                           | 14.01                         |                |
| 12      | Sensitized | F   | 51          | 118              | 3                           | 11                            | 9                             | 0.34                          | –              |
| 13      | Sensitized | M   | 43          | 315              | 4                           | 60                            | 618                           | 15.60                         | +              |
| 14      | Sensitized | M   | 34          | 322              | 3                           | 39                            | 40                            | 0.74                          | +              |
| 15      | Sensitized | F   | 61          | 565              | 4                           | 197                           | 299                           | 6.24                          | +              |
| 16      | Sensitized | F   | 48          | 1171             | 2                           | 24                            | 5                             | 0.34                          | –              |
| 17      | Sensitized | M   | 64          | 130              | 2                           | 28                            | 22                            | 0.36                          | +              |
| 18      | Sensitized | M   | 60          | 1061             | 2                           | 23                            | 1                             | 0.34                          | –              |
| 19      | Sensitized | M   | 56          | 237              | 2                           | 24                            | 57                            | 1.39                          | +              |
| Mean    |            |     | 52          | 490              | 3                           | 51                            | 131                           | 3.17                          |                |
| SD      |            |     | 10          | 412              | 1                           | 61                            | 220                           | 5.41                          |                |
| 20      | Control    | M   | 32          | 25               | 0                           | 3                             | 1                             | 0.34                          | –              |
| 21      | Control    | F   | 30          | 39               | 0                           | 3                             | 0                             | 0.34                          | –              |
| 22      | Control    | M   | 30          | 15               | 0                           | 6                             | 2                             | 0.34                          | –              |
| 23      | Control    | M   | 23          | 26               | 0                           | 24                            | 2                             | 0.34                          | –              |
| Mean    |            |     | 29          | 26               | 0                           | 9                             | 1                             | 0.34                          |                |
| SD      |            |     | 4           | 10               | 0                           | 10                            | 1                             | 0.00                          |                |

and transversal ( $D_2$ ) diameters of the wheals were measured and the surface calculated with the formula  $[(D_1 + D_2)/2]^2$  [17].

#### Determination of rAsp f 3-specific IgA, IgG, IgG1, IgG2, IgG3, IgG4 and IgE ELISA

Sera for ELISA analyses and ImmunoCAP-determinations were obtained directly before skin testing and stored at  $-20^\circ\text{C}$  until use. For ELISA, maxisorp polystyrene microtitre plates (Nunc, Roskilde, Denmark) were coated over night at  $4^\circ\text{C}$  with  $10\text{ }\mu\text{g/mL}$  rAsp f 3 [17] in PBS pH 8.0, washed three times with PBS pH 7.4 containing 0.05% Tween 20 (as after all following incubation steps) and blocked for 1 h at  $37^\circ\text{C}$  with 2% (w/v) skimmed milk powder and 5% (v/v)

Tween 20 in PBS pH 7.4. Sera were diluted in blocking buffer (1:2 for IgG2 and IgG3, 1:5 for IgG4 and IgE, 1:10 for IgA, 1:20 for IgG1 and 1:50 for IgG determinations), serially twofold diluted in the coated plate and incubated for 2 h at  $37^\circ\text{C}$ . Subsequently, secondary antibodies specific for the different human immunoglobulin isotypes diluted in blocking buffer were added for 2 h at  $37^\circ\text{C}$  (peroxidase (PO)-conjugated rabbit antihuman IgA, DAKO, Glostrup, Denmark,  $125\text{ ng/mL}$ ; alkaline phosphatase (AP)-conjugated goat antihuman IgG, TAGO, Burlingame, CA, USA,  $1:10'000$ ; NL16 monoclonal mouse antihuman IgG1, OXOID, Hampshire, UK,  $500\text{ ng/mL}$ ; PO-conjugated mouse antihuman IgG2, Zymed, South San Francisco, CA, USA,  $1:3'000$ ; PO-conjugated mouse antihuman IgG3, Zymed,  $1:3'000$ ; RJ4 monoclonal mouse antihuman

IgG4, OXOID, 143 ng/mL; TN-142 monoclonal mouse IgG1 antihuman IgE, 182 ng/mL). The detection of IgG1, IgG4 and IgE required an additional incubation step with AP-conjugated goat antimouse IgG, H + L chains (Pierce, Rockford, IL, USA, 109 ng/mL) for 1 h at 37°C. ELISA plates with AP-conjugated antibodies were developed with 1.5 mg/mL 4-nitrophenyl phosphate disodium salt hexahydrate (NPP, Merck, Darmstadt, Germany) in Diethanolaminebuffer pH 9.8 for 45 min and the reaction stopped by adding 50 µL 2 M NaOH. Plates with PO-conjugated antibodies were developed with 1.5 mg/mL *o*-phenylenediamine dihydrochloride (OPD, Sigma, St Louis, MO, USA) in citrate buffer pH 5.0 for 45 min and stopped with 50 µL 12.5% (v/v) H<sub>2</sub>SO<sub>4</sub>.

Absorbance was measured at 405 nm (NPP) and 490 nm (OPD) with a Molecular Devices Reader (Menlo Park, CA, USA) and converted to arbitrary ELISA units per ml (EU/mL) as described [17,18]. Values less than 1 EU/mL were set as 1 EU/mL for graphics. The detection limit for IgG, IgG1, IgG4 and IgA ELISAs was considered to be three times the blank value, corresponding to 5 EU/mL for IgG, IgG1 and IgG4 and 25 EU/mL for IgA. The cut-off for relevant IgE titres was determined by skin testing.

#### Statistical analyses

Comparison of different groups of patients was done by the nonparametric Mann–Whitney *U*-test. *P*-values < 0.05 were considered to be significant. Correlation coefficients were determined with Pearson's linear regression analysis.

## Results

#### Reactivity to rAsp f 3 in IDT

Prior to the skin tests we investigated whether rAsp f 3 might be toxic for cells since nothing was known about its function and properties *in vivo*. Therefore, CTLs were incubated with different amounts of rAsp f 3 or rAsp f 1 as positive control. Proliferation was measured by [<sup>3</sup>H]TdR incorporation and compared with the data obtained from the incubation in cRPMI alone (Fig. 1). Using allergen concentrations up to ≈ 1 mg/mL no dose-dependent cytotoxic effect of rAsp f 3 could be observed in contrast to the results obtained for rAsp f 1, a ribotoxin able to inhibit protein synthesis and described as being cytotoxic [17,23].

Intradermal skin tests were performed in order to assess the ability of rAsp f 3 to elicit type I reactions in *A. fumigatus* sensitized patients and therefore demonstrate that rAsp f 3 can act as allergen *in vivo*.

Figure 2 shows the relation between intradermal reactivity to rAsp f 3 and specific IgE-levels in serum. Among the whole group of patients sensitized to *A. fumigatus* 16 of 19

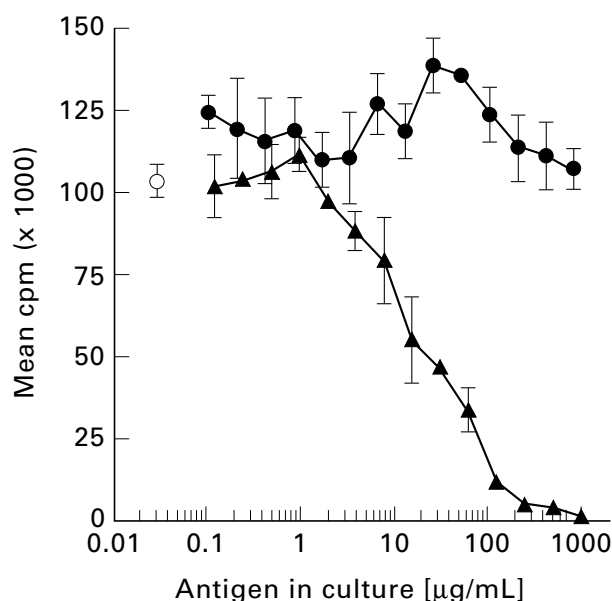


**Fig. 1.** Cytotoxic effect of rAsp f 3. 10<sup>4</sup> CTLs were incubated in triplicates with different allergen concentrations (●) rAsp f 3, ▲ rAsp f 1 (positive control) or with medium alone (○ negative control). Proliferation was measured by [<sup>3</sup>H]TdR incorporation. Symbols represent mean values ± SD.

(84%) showed a weal-and-flare skin reaction against rAsp f 3. All 11 ABPA patients (100%) were positive in IDT and serology, whereas in the group of *A. fumigatus* sensitized allergic asthmatics with five of eight patients (63%) scored positive.

From the total of 23 investigated individuals 16 showed rAsp f 3-specific IgE values > 10 EU/mL in ELISA and responded in IDT to rAsp f 3, whereas the seven individuals with IgE values < 10 EU/mL were consistently negative in skin test (Fig. 2). ELISA determinations of specific IgE, however, do not allow quantification of the results in an absolute scale. In contrast, a system such as the automated Pharmacia CAP system, which is calibrated against the World Health Organization (WHO) standard for IgE, allows quantitative expression of total or allergen-specific IgE [22]. rAsp f 3 ImmunoCAPs were produced and used to corroborate the ELISA results by quantifying the amounts of rAsp f 3-specific IgE (Fig. 3). The comparison of IgE values obtained by ELISA with those of rAsp f 3 ImmunoCAP determinations showed that all sera with < 10 EU/mL rAsp f 3-specific serum IgE scored below the cut-off value of the Pharmacia CAP system of < 0.35 kU<sub>A</sub>/L. IgE ImmunoCAP determinations in sera of IDT-positive individuals varied between 0.36 and 44.0 kU<sub>A</sub>/L. All CAP values correlated excellently with the results of the ELISA determinations (*r* = 0.941, *P* < 0.001, Fig. 3) showing that rAsp f 3 can be immobilized in ImmunoCAPs with retained

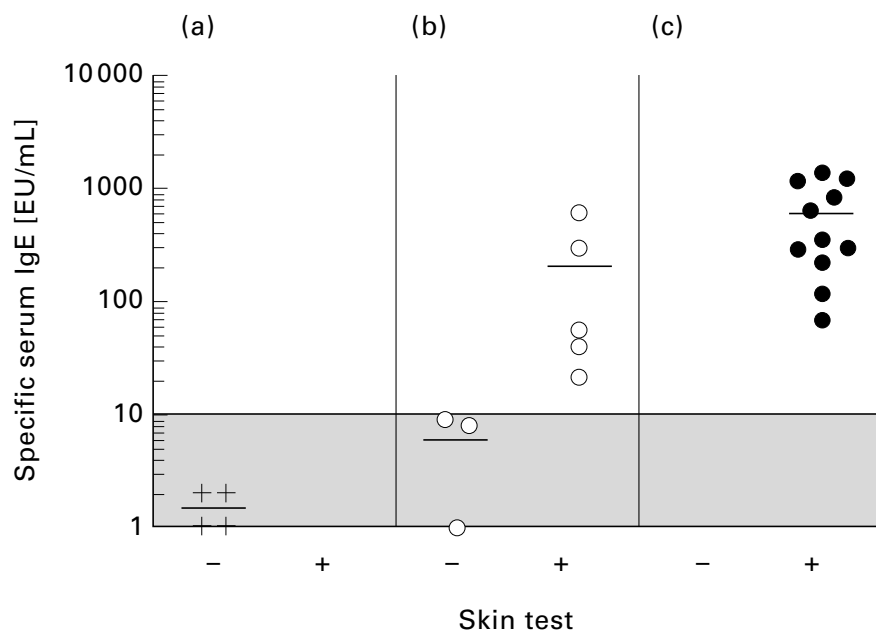


**Fig. 2.** Intradermal skin-test result with rAsp f 3 in distinct groups of individuals; 100  $\mu$ L physiological saline solution containing different amounts of rAsp f 3 were injected in the dermis of *A. fumigatus* sensitized asthmatic patients with (●) or without (○) ABPA and healthy controls (⊕). Patients were grouped according to disease and the skin-test outcome. Mean values are represented by bars. The shadowed area corresponds to the cut-off value for a positive skin test. (a) Healthy controls; (b) sensitized asthmatics; (c) ABPA.

antigenic activity allowing a fully automated serological application of this particular allergen.

Consequently, the sensitivity of the rAsp f 3-based assessment of sensitization to *A. fumigatus* reached 84%, whereas the specificity, defined by the lack of weal-and-

flare reactions in patients with rAsp f 3-specific IgE levels < 10 EU/mL or < 0.35 kU<sub>A</sub>/L, is 100%.

#### Determination of rAsp f 3-specific serum-immunoglobulins by ELISA

Sera from all subjects listed in Table 1 were analysed for antigen-specific serum IgE, IgA, IgG, IgG1, IgG2, IgG3 and IgG4 antibodies. rAsp f 3-specific IgG2 was not detectable in any subject, IgG3 only in one ABPA patient (No. 5, Table 1).

All subjects except two controls showed detectable levels of rAsp f 3-specific IgG in serum (Table 1). IgG antibody levels were raised in patients suffering from ABPA compared to *A. fumigatus* sensitized asthmatic subjects ( $P < 0.005$ ) and in both patient groups compared with healthy individuals ( $P < 0.005$  and  $P < 0.05$ ). Investigation of IgG subclasses showed that specific IgG1 was detectable in all individuals eliciting an IgG response against rAsp f 3. The titres showed significant differences between patients with ABPA and healthy control subjects ( $P < 0.005$ ) or *A. fumigatus*-sensitized subjects ( $P < 0.005$ ) but not between the last two groups ( $P > 0.05$ , Fig. 4). Patients suffering from ABPA exhibited elevated specific serum IgG4 antibody titres compared with *A. fumigatus* sensitized asthmatics ( $P < 0.01$ ) and both groups compared with healthy individuals ( $P < 0.005$  and  $P < 0.05$ ). In sera from four individuals with detectable specific IgG titres, IgG4 was not detectable (patient nos. 12, 18, 22, 23).

rAsp f 3-specific IgE levels above 10 EU/mL could be detected only in sera from *A. fumigatus*-sensitized

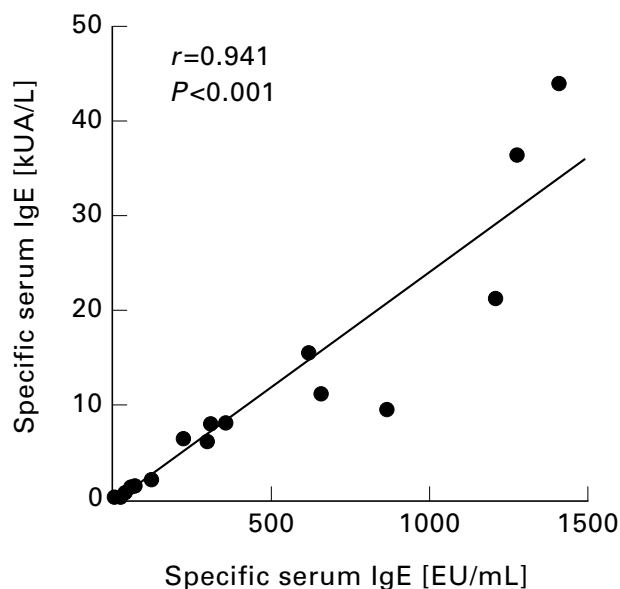


**Fig. 3.** Correlation of specific serum IgE values determined by ELISA and Pharmacia CAP system. Sera of all individuals were analysed for rAsp f 3-specific IgE by the Pharmacia CAP system and by ELISA. Values of both methods were plotted against each other.

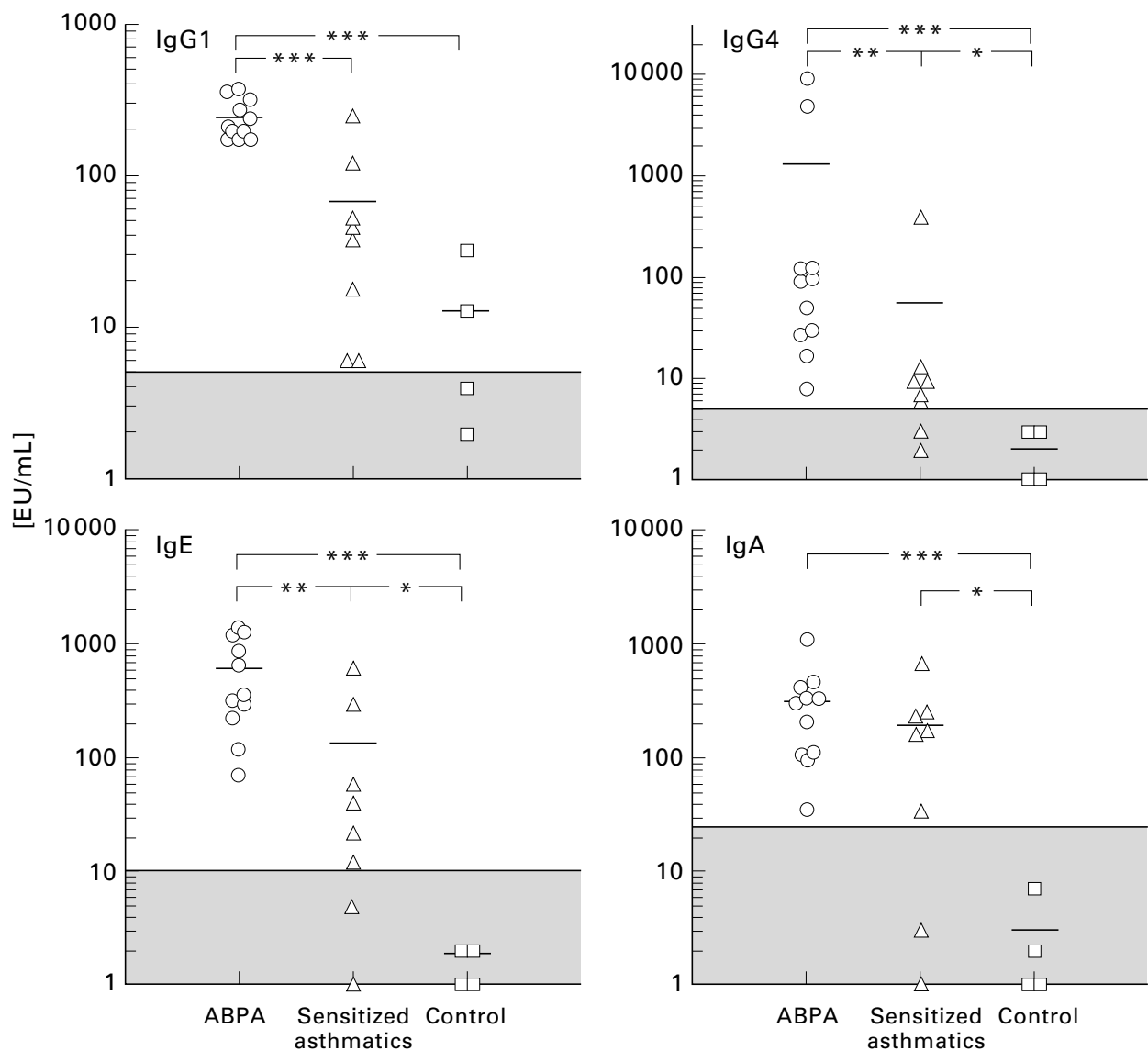


**Fig. 4.** rAsp f 3-specific immunoglobulin isotypes in serum of asthmatic patients with or without ABPA and healthy control individuals. Sera of asthmatic patients with (○) or without ABPA (△) and of control subjects (□) were analysed for the presence with rAsp f 3-specific immunoglobulins in serum. ELISA units (EU) were calculated as described in Materials and methods. Mean values are represented by bars and *P*-values abbreviated as follows: \* ( $P < 0.05$ ), \*\* ( $P < 0.01$ ), \*\*\* ( $P < 0.005$ ). The shadowed area corresponds to the detection limit of the assay (IgG, IgG1, IgG4, IgA) or to the cut-off value for a positive skin test (IgE).

asthmatics with or without ABPA who responded in IDT to rAsp f 3 (Fig. 2). Comparison of the IgE-levels between the groups of patients with or without ABPA revealed significant differences ( $P < 0.01$ , Fig. 4) with a tendency to develop stronger IgE responses to rAsp f 3 in ABPA patients. Antibody responses of the IgA isotype were comparable between the two patient groups but significantly raised compared to the levels obtained for control subjects ( $P < 0.005$  and  $P < 0.05$ ).

Linear regression analyses of the serum immunoglobulin

data from sensitized patients to detect possible correlations between different rAsp f 3-specific immunoglobulin isotypes and subclasses showed that none of the data sets were significantly correlated with each other (Table 2). Elevated IgG levels were mostly associated with raised titres of rAsp f 3-specific IgE and IgA, whereas elevated IgE serum levels were in most cases associated with increased IgG1 and IgG4. However, the correlation between IgE and IgG1 or IgG4 (each  $P = 0.075$ ) did not reach a clear statistical significance.

**Table 2.** Correlation between different serum immunoglobulins

|      | IgG4  | IgE   | IgA   |
|------|-------|-------|-------|
| IgG  | ND    | 0.127 | 0.189 |
| IgG1 | 0.356 | 0.075 | 0.244 |
| IgG4 | –     | 0.075 | 0.796 |
| IgA  | –     | 0.548 | –     |

ND, not determined.

## Discussion

The application and further development of molecular biological techniques aimed to clone, produce and characterize IgE-binding molecules has resulted in the fast elucidation of the primary structure of an impressive list of allergens [7]. The knowledge of the primary structure of antigens from different sources has improved our understanding of crossreactivity detectable in sera of atopic individuals sensitized to different, apparently unrelated sources [24–27]. Furthermore, the production of recombinant allergens is of particular importance for organisms whose extracts are difficult to standardize like ragweed [28] or fungi [5,27]. Recombinant allergens represent standardized allergen preparations and might be useful for diagnostic purposes after having been evaluated in clinical studies with well diagnosed patients.

In this study, we report the evaluation of rAsp f 3, for its performance in skin test and serology. The investigations entailed IDT and serological analyses of 23 individuals: 11 patients with ABPA, eight individuals with allergic asthma sensitized to *A. fumigatus* and four healthy controls. All patients with ABPA and five of the eight subjects with allergic asthma responded positively to skin challenges with rAsp f 3 (Fig. 2) resulting in an overall sensitization to rAsp f 3 of 84% among all analysed *A. fumigatus* sensitized individuals. The incidence confirms that rAsp f 3 is a major allergen of the fungus as postulated in an earlier study based on serology [18]. The more important finding of this study is that specific serum IgE titres were of reliable, predictive value for immediate type I skin reactions after challenge with rAsp f 3, in contrast to discrepancies observed when fungal extracts are used [29]. The variable, subject dependent skin reactivity to histamine (mean  $237 \text{ mm}^2 \pm \text{SD } 139 \text{ mm}^2$ ) was compensated by relating the rAsp f 3-induced weal size to the histamine control [2]. Besides specific IgE, other factors influencing skin reactivity did not seem to play a decisive role, since exclusively patients with rAsp f 3-specific IgE ImmunoCAP values  $> 0.35 \text{ kU}_A/\text{L}$  (corresponding to  $> 10 \text{ EU/mL}$  in ELISA), were positive in skin tests with rAsp f 3 (Table 1). The close correlation between the

rAsp f 3-specific values obtained with ELISA and ImmunoCAP determinations ( $r = 0.941$ ) show that rAsp f 3 protein retains similar immunological properties after immobilization to different solid phases. Patients as well as clinicians may benefit from the use of a quantitative, automated method for the determination of specific serum IgE levels, thus avoiding potentially harmful skin challenges.

It has been shown that, depending on the allergen source, certain IgG subclasses predominate the IgG response in naturally exposed individuals [30]. In this study the extension of the rAsp f 3-specific serology to different immunoglobulin isotypes (Fig. 4) showed that IgG1 as well as IgG4, but basically no IgG2 or IgG3, was detectable in sera containing IgG against rAsp f 3, confirming earlier findings with a major antigen (Ag 7) of *A. fumigatus* [31]. The measured ELISA units indicate that the IgG response was mainly of the IgG1 subclass, except in two ABPA patients (Nos. 4 and 5 in Table 1) where IgG4 exceeded the IgG1 titres. The IgG immune response to allergens is usually predominated by IgG1 antibodies and only after chronic allergen exposure or immunotherapy IgG4 levels increase [32,33].

One single allergen is of course not sufficient to identify all subjects sensitized to *A. fumigatus*, since the fungus can produce more than 40 IgE-binding components and elicits a highly variable pattern of IgE reactivity depending on the status of sensitization of the investigated individuals [5]. However, the high diagnostic value of single recombinant allergens results from the perfect standardization of the preparations which allows serological and skin test investigations basically free of false positive and false negative results as demonstrated for rAsp f 1 [6,17,22] and for rAsp f 3, in this study. The relatively low sensitivity of the single allergen-based diagnosis will be substantially increased by determining the IgE responses against additional recombinant allergens of the fungus which have already been cloned from a phage surface display library [8].

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