

CLONING AND EXPRESSION OF RECOMBINANT *Aspergillus fumigatus* ALLERGEN I/a (rAsp f I/a) WITH IgE BINDING AND TYPE I SKIN TEST ACTIVITY¹

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Aspergillus fumigatus secretes an 18-kDa nonglycosylated IgE-binding protein. This protein was previously shown to be a ribotoxin, like α -sarcin and mitogillin. A 686-bp long *A. fumigatus* cDNA encoding an 18-kDa ribotoxin was cloned and expressed in *Escherichia coli* as a fusion protein with six adjacent histidines (rAsp f I/a). rAsp f I/a was purified to homogeneity by Ni²⁺-chelate affinity chromatography and refolded. The recombinant protein was enzymatically active resulting in the cleavage of 28S rRNA within a universally conserved region. rAsp f I/a was cytotoxic for EBV immortalized or PHA stimulated human PBMC. Furthermore, rAsp f I/a was recognized by murine mAb made against an 18-kDa ribotoxin. IgE of individuals allergic to *A. fumigatus* bound to rAsp f I/a as shown by ELISA, dot blots, and Western blots. rAsp f I/a elicited positive immediate type I skin reactions in individuals allergic to *A. fumigatus* but not in healthy control individuals. The results show that rAsp f I/a has similar functional characteristics when compared to the native 18-kDa ribotoxin. rAsp f I/a expressed in *E. coli* can therefore be used as a standardized Ag/allergen for serologic and clinical diagnosis of *A. fumigatus*-associated diseases.

Aspergillus fumigatus is considered to be the most pathogenic *Aspergillus* species for humans. This fungus is found ubiquitously and is responsible for a variety of allergic or invasive diseases (1). *A. fumigatus* affects mainly three groups of patients: immunocompromised individuals (2), patients with cystic fibrosis (3, 4), and *A. fumigatus* allergic individuals (5, 6). Decreased immunocompetence may be induced by drugs necessary to tolerate transplanted organs (7). In contrast, allergies are believed to be overreactions of the immune system leading to asthma or other immunopathologic complications.

ABPA³ is the most severe form of *A. fumigatus*-induced asthma (6, 8).

There are a number of diagnostic tools to confirm a clinical suspicion of *A. fumigatus*-induced allergy or ABPA. However, the reproducibility of the results largely depends on the availability of standardized extracts or purified allergens from *A. fumigatus* (9, 10).

In serologic studies it was shown that different Ag/allergens can induce IgG and/or IgE when allergic or nonallergic individuals were exposed to *A. fumigatus* (11). *A. fumigatus* Ag can be divided into groups based on gel sizing and Western blotting. The Ag range in size from 15 to 25 kDa, 30 to 40 kDa, 50 to 60 kDa, and of more than 80 kDa (12). Individual Ag of such groups have been isolated using biochemical and immunochemical methods (13). An 18-kDa *A. fumigatus* protein was found to be present in urine of patients suffering from invasive aspergillosis (14). Recently the partial amino acid sequence of another but similar 18-kDa protein shown to be a major *A. fumigatus* allergen was reported (15). All these 18-kDa proteins are related to ribotoxins such as α -sarcin (16), mitogillin (17), and restrictocin (18). These ribotoxins are structurally similar and are all produced by members of the genus *Aspergillus* (19, 20). Ribotoxins inhibit protein translation by cleaving a conserved region of 28S rRNA (21) and hence were used as antitumor agents (22–25).

We report the cloning and expression of rAsp f I/a from *A. fumigatus*. The recombinant protein was purified to homogeneity and shown to have enzymatic and cytotoxic activity. rAsp f I/a bound IgE and elicited positive skin reactions in individuals allergic to *A. fumigatus*. Hence, rAsp f I/a is an allergen that has biologic activity and can be obtained reproducibly in large amounts.

MATERIALS AND METHODS

Strains, bacteriophages, and plasmids. The *A. fumigatus* strain (AF 102; American Type Culture Collection 42202 (Bethesda, MD)) was isolated from sputum of a patient with aspergilloma and kindly supplied by Dr. V. P. Kurup (Medical College of Wisconsin, Milwaukee, WI). The following *E. coli* strains were used: XL1-Blue (26); M15 (27) and SG13009 (28). The Uni-ZAP II XR vector system (Stratagene, La Jolla, CA) was used for the construction of unidirectional cDNA expression libraries. In vivo excision of phagemids was performed

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³ Abbreviations used in this paper: ABPA, allergic bronchopulmonary aspergillosis; asp f I/a, gene encoding the 18-kDa ribotoxin Asp f I/a; Asp f I/a, 18-kDa ribotoxin; American Type Culture Collection, American Type Culture Collection; PCR, polymerase chain reaction; rAsp f I/a, recombinant 18-kDa ribotoxin expressed as fusion protein with six adjacent histidines residues.

using the R408 helper phage (Stratagene). Plasmid p6xHis-DHFRS (0) (29) was used for high level production of rAsp f 1/a allowing a one-step purification using immobilized metal ion affinity chromatography (30, 31).

Enzymes and reagents. DNA-modifying enzymes and restriction endonucleases were obtained from Stratagene or Boehringer (Mannheim, FRG) and used as recommended by the suppliers. All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO), Merck (Darmstadt, FRG), or Fluka (Buchs, Switzerland) if not otherwise specified. Vent DNA polymerase used for PCR was from New England Biolabs (Schwalbach/Taunus, FRG). A native *A. fumigatus* 18-kDa ribotoxin (14) was generously provided by Dr. J. P. Latgé (Institut Pasteur, Paris, France).

Antibodies. The following antibodies were used in ELISA or Western blotting experiments: alkaline-phosphatase labeled goat anti-human Ig α -, γ -, μ -, and λ -chains, alkaline phosphatase-labeled goat anti-mouse Ig γ and λ chains, and horseradish peroxidase-labeled goat anti-mouse Ig γ - and λ -chains all from TAGO (Burlingame, CA). Mouse monoclonal TN-142 anti-human IgE was generously provided by Dr. C. H. Heusser (Ciba Geigy, Basel, Switzerland). The murine mAb 4A6 and 10G7 directed against an 18-kDa ribotoxin (15) were kindly supplied by Dres. L. K. Arruda and T. A. E. Platts-Mills (University of Virginia, Charlottesville, VI).

Isolation of poly(A)⁺ RNA, synthesis of cDNA and construction of expression libraries. For the isolation of mRNA, a 4-day-old culture of *A. fumigatus* grown according to Kurup et al. (32) was used. Total RNA was isolated according to Teeri et al. (33). The isolation of poly(A)⁺ RNA was performed according to Sambrook et al. (34) or using the Poly(A) Quick mRNA isolation kit (Stratagene). Five μ g poly(A)⁺ RNA were used to synthesize cDNA with the ZAP-cDNA synthesis kit (Stratagene). The cDNA was inserted into the Lambda ZAP II vector and packaged in vitro using the Gigapack II Gold packaging extracts (Stratagene). The protocols provided by the manufacturer were followed.

Library screening and DNA sequencing. *E. coli* XL1-Blue cells were infected with $\sim 10^6$ recombinant phage particles and plated at 3 to 4×10^4 pfu/150-mm petri dish. The plaques obtained were lifted on Hybond-ECL nitrocellulose filters (Amersham, Buckinghamshire, UK) and screened using different probes (Table I). The N-probe was derived from the published N-terminal amino acid sequence of the 18-kDa ribotoxin (15). A second oligonucleotide (C-primer; Table I) corresponding to the Leu-Cys-Ser-His sequence element at the C-terminus of mitogillin was defined (15, 17). The cDNA library was first screened with the digoxigenin-labeled N-probe using standard conditions as described by the manufacturer with a peroxidase-labeled detection system (Boehringer) and visualization by chemiluminescence (ECL, Amersham). Positive phage clones were subsequently reprobated using a ribotoxin-specific PCR fragment. The PCR fragment was obtained by amplifying 1 ng of cDNA ligated to Lambda ZAP II DNA using the 5' λ ZAP II and C primers (Table I). The conditions for PCR are described below. Primers were allowed to anneal at 28°C. Double-positive phage clones were purified, phage-mids excised in vivo (35) and the DNA electrophoretically analyzed after *Bam*HI/*Xho*I digestion. The largest DNA insert among the positive clones was subcloned into the M13 mp19 phage vector and sequenced by the dideoxy chain termination method (36).

PCR amplification of asp f 1/a gene and subcloning into high level expression vectors. The analysis of the sequencing data allowed the synthesis of specific PCR primers to amplify the coding region of mature Asp f 1/a³ (Fig. 1; Table I) (15) to facilitate the subcloning into an expression vector (Fig. 2) (29). PCR was performed in a volume of 100 μ l. The sample contained the appropriate buffer, 200 μ M of each deoxy nucleotide triphosphate, 0.5 μ M each of the asp f 1/a cDNA primers, 1 ng of asp f 1/a cDNA, and 1 U of Vent DNA polymerase. The samples were subjected to 30 cycles of amplification consisting of the following steps: 1 min at 94°C, 2 min at 55°C and 1 min at 72°C, followed by a final incubation at 72°C for 10 min. The PCR products were double digested with *Bam*HI and *Hind*III, subjected to electrophoresis in a 2% agarose gel, purified by using the double GeneClean procedure (Bio 101 Inc., La Jolla, CA) and ligated into *Bam*HI/*Hind*III cleaved and dephosphorylated plasmid p6xHis-DHFRS (0). The plasmids were introduced into *E. coli* M15 cells by electroporation using a Gene Pulser (Bio-Rad, Richmond, CA). Transformants were selected on LB-plates containing ampicillin ($100 \mu\text{g} \times \text{ml}^{-1}$) and kanamycin ($25 \mu\text{g} \times \text{ml}^{-1}$) (34).

Production and purification of rAsp f 1/a. *E. coli* cells containing the repressor plasmid pRep4 and the plasmid p6xHis-DHFRS (0) with the cloned asp f 1/a insert were grown in 2x TY-medium as described (29). The rAsp f 1/a was affinity purified to homogeneity as described (29). For the refolding procedure, fractions containing rAsp f 1/a were dialyzed against PBS, pH 7.4, at 4°C for 2 days using a molecularporous membrane with a 3.5-kDa cut off (SPECTRA/POR, Houston, TX). The second dialysis was against distilled H₂O.

Determination of protein concentration was performed using the Bio-Rad protein assay with BSA as standard.

Enzymatic activity measurements. Cleavage of the α -fragment from 28S rRNA by rAsp f 1/a was assayed as described (37) using 50 μ l of rabbit reticulocyte lysate (Promega Biotec, Madison, WI) and 100 ng of rAsp f 1/a.

Isolation of PBMC and EBV transformation. Human PBMC were isolated using Ficoll-Paque (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden) from venous blood of an ABPA-patient as described (38). For EBV transformation (39), 20×10^6 PBMC were suspended in 5 ml RPMI 1640 supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μ g streptomycin, 0.05 mM 2-ME all from GIBCO (Basel, Switzerland) containing 30% supernatant from an EBV-infected B95-8 marmoset cell line (40), 20% FCS and 800 ng $\times \text{ml}^{-1}$ of cyclosporin A (Sandoz, Basel, Switzerland). After 24 h the cells were washed, resuspended, and cultivated in supplemented RPMI 1640, 20% FCS, and 800 ng $\times \text{ml}^{-1}$ cyclosporin A for 1 mo. Further cultivation was in supplemented RPMI 1640 medium containing 10% FCS.

Toxicity assay and measurement of [³H]TdR incorporation. rAsp f 1/a and the 18-kDa ribotoxin were diluted in complete RPMI 1640 medium, containing 10% FCS and filtered through a 0.2- μ m prewetted filter. Different dilutions were applied in triplicates to a flat-bottom 96-well tissue culture plate (Costar, Cambridge, MA). The EBV transformed PBMC were resuspended at 2×10^5 cells/well. After 48 h, 1 μ Ci of [³H]TdR (Du Pont, NEN products, Regensburg, Switzerland) was added to each well. The cells were harvested onto glass fibre filters (Pharmacia) 16 h later using a semiautomatic harvester and the [³H]TdR incorporation was measured by a β -plate liquid scintillation counter (Pharmacia).

PAGE, Western blot analysis, and dot blots. PAGE for proteins (41, 42) was performed under denaturing and reducing conditions using a Midget Electrophoresis Unit (Pharmacia). The staining with Coomassie brilliant blue G250 (Merck) was as described (43).

For Western blot analysis, proteins were separated on a 12.5% PAGE using denaturing and reducing conditions. The proteins were then transferred to a Hybond-ECL nitrocellulose membrane (Amersham) using a Multiphor II Electrophoretic Transfer Unit (Pharmacia) at 60 mA for 2 h (44). Western blots were developed using the ECL system (Amersham) according to the manufacturer's recommendations. Patients sera were diluted 1/100.

For the dot blot analysis, different amounts of rAsp f 1/a or native 18-kDa ribotoxin were spotted onto Hybond-ECL nitrocellulose membrane (Amersham) and processed as described for the Western blotting.

Ag-specific ELISA. The binding of anti-rAsp f 1/a antibodies was analyzed by a direct solid phase ELISA (45). Briefly, polystyrene microtiter plates Maxisorp (Nunc, Roskilde, Denmark) were coated overnight at 4°C with $10 \mu\text{g} \times \text{ml}^{-1}$ rAsp f 1/a in PBS, pH 8, as described (45). The remaining free sites were blocked with PBS, pH 7.4, containing 5% (w/v) nonfat dry milk powder at 37°C for 1 h (blocking buffer). The wells were then washed three times with PBS, pH 7.4. Serially diluted primary antibody or sera (100 μ l) were added to each well, and the plates were incubated in blocking buffer including 5% Tween 20 (dilution buffer) at 37°C for 2 h. The secondary Ab used are described above. After washing, the wells were incubated with substrate (100 μ l) (45).

Inhibition ELISA. Coating of rAsp f 1/a to solid phase and blocking of the plates was performed as described above. Sera diluted 1/8 (100 μ l) were preincubated in dilution buffer in a separate untreated 96-well plate at 37°C for 2 h containing various amounts of rAsp f 1/a or native 18-kDa ribotoxin. Thereafter, the Ag preincubated sera were transferred to the washed rAsp f 1/a-coated plates, incubated, and developed as described for the Ag-specific ELISA.

Skin testing. Immediate type I skin reactions were performed using skin prick testing or intracutaneous testing (46). Allergen extracts were applied to the forearm, pricked, and blotted (47). The reaction was read after 15 min measuring the maximal longitudinal (D₁) and transversal (D₂) diameter of the wheal and vascular flare reaction. The surface of the wheal skin was calculated using the formula $[(D_1 + D_2)/2]^2$. Patients were regarded as being sensitized to a particular allergen if the wheal had a surface of $>9 \text{ mm}^2$. Histamine and physiologic NaCl solution were used as positive and negative controls, respectively. The immediate type I skin reaction of rAsp f 1/a was compared to two commercial and two crude extracts from *A. fumigatus*. The commercial *A. fumigatus* extracts were used as recommended by the supplier. The crude extracts and rAsp f 1/a were used at protein concentrations of $100 \mu\text{g} \times \text{ml}^{-1}$.

Patients. An ethical approval for skin testing human subjects using recombinant allergens was obtained. Selected patients from the Hochbergsklinik Davos-Wolfgang were tested. A full explanation of the procedure was given to all individuals before testing and subsequently a written consent was obtained.

RESULTS

Isolation of *A. fumigatus asp f I/a* gene. Messenger RNA from a 4-day-old culture of a clinical isolate of *A. fumigatus* (AF 102; American Type Culture Collection 42202) was used to construct cDNA expression libraries using the cloning vector Lambda Zap II. A typical library consisted of inserts larger than 400 bp and with an estimated unamplified titer of 7×10^6 recombinant plaque-forming units. The background of nonrecombinant phage was less than 0.5%. For the identification of recombinant phage clones containing mature *Asp f I/a*-encoding DNA fragments, labeled oligonucleotides and probes amplified by PCR were used (Table I). Approximately 10^5 plaques were first screened with the digoxigenin labeled N-probe. Positive plaques were rescreened using an digoxigenin labeled PCR product obtained by amplifying nonpackaged cDNA as target and the C-primer and 5' λ ZAP II for priming (Table I). The hybridization double positive plaques were picked, purified and DNA from *in vivo* excised phagemids isolated. The largest insert was nucleotide sequenced (Fig. 1).

Analysis of nucleotide sequence of cDNA clone *asp f I/a*. The possible reading frames predicted from the cDNA sequence of *asp f I/a* (GenBank accession number 000) were compared to the published partial or deduced amino acid sequence of three 18-kDa ribotoxins (14, 15, 48). Based on this comparison *asp f I/a* contained the entire coding sequence of a mature protein similar to the 18-kDa ribotoxins (Fig. 1). The comparison of the cDNA clone *asp f I/a* with the genomic DNA sequence (48) indicated that the leader sequence of *Asp f I/a* was incomplete. The comparison predicts that *Asp f I/a* contained 72 of the 81-bp long leader sequence assuming identity of the signal peptide.

Expression of *asp f I/a* gene in *E. coli*. DNA from the cDNA clone *asp f I/a* was amplified using PCR and subcloned into a high level expression vector p6xHis-DHFRS (0) to produce the mature protein (Fig. 2; Table I). The construct was verified, for frame and sequence confirmation. *E. coli* M15 transformants produced about $50 \text{ mg} \times \text{l}^{-1}$ rAsp f I/a.

Protein analysis and enzymatic activity of rAsp f I/a. Affinity chromatography purified and refolded rAsp f I/a was analyzed by SDS-PAGE (Fig. 3). rAsp f I/a appeared as one band with a molecular mass of approximately 19 kDa (predicted size 18.5 kDa). Native 18-kDa ribotoxin (14) had a lower m.w. due to the lack of the 6 histidine affinity tail (Figs. 2 and 3).

The enzymatic activity of rAsp f I/a was compared to restrictocin and mitogillin using rabbit reticulocyte lysates as substrate. The experiment revealed specific cleavage of the α -fragment from the 28S rRNA (37) (data

not shown). We also tested the ability of rAsp f I/a and native 18-kDa ribotoxin (14) to inhibit growth of EBV-transformed PBMC measured by [^3H]TdR incorporation (Fig. 4). The assay resulted in a dose-dependent inhibition of growth. The 50% inhibition was reached at $90 \mu\text{g} \times \text{ml}^{-1}$ rAsp f I/a or $9 \mu\text{g} \times \text{ml}^{-1}$ native 18-kDa ribotoxin. In addition, rAsp f I/a also inhibited the growth of PHA-stimulated PBMC (data not shown).

Antigenic/allergenic characterization of rAsp f I/a by Western blot. The IgE binding of rAsp f I/a and native 18-kDa ribotoxin (14) was compared in Western blot assays (Fig. 5). Serum IgE from ABPA patients E.D. or L.M. strongly reacted with the monomeric and dimeric form of both rAsp f I/a and 18-kDa ribotoxin. An additional band of ~ 27 kDa was visualized using the 18-kDa ribotoxin preparation (Fig. 5, lane 2). In parallel Western blots rAsp f I/a or 18-kDa ribotoxin were reacted with murine mAb specific to a ribotoxin (15). The monomeric and dimeric forms of the ribotoxins were recognized by the murine mAb but not the 27-kDa protein (data not shown). No IgE-specific binding to ribotoxins was detected in Western blots using sera from healthy control individuals or a nonallergic chronically *Aspergillus*-infected patient (data not shown).

Dot blot experiments. Different amounts of rAsp f I/a or the native 18-kDa ribotoxin (14) were spotted directly onto nitrocellulose membranes (Fig. 6). The filters were incubated with serum from ABPA patients E.D. or L.M.. Depending on the serum used, 25 to 100 ng of rAsp f I/a but 12 to 50 ng of the 18-kDa ribotoxin could be detected by IgE antibodies. The data provide further evidence that serum IgE from patient L.M. preferentially detects the 27-kDa contaminant allergen present in the native ribotoxin preparation (Fig. 5B and 6B). This was further shown by inhibition ELISA (data not shown).

Ag-specific ELISA. For serologic studies, a rAsp f I/a-specific ELISA was developed. rAsp f I/a was therefore coated to solid phase followed by addition of murine mAb or human sera and finally by the appropriate detection reagent. Murine mAb reacted to solid phase-bound rAsp f I/a (data not shown). rAsp f I/a-specific IgE from ABPA patients E.D. or L.M. could be detected in ELISA (Fig. 7). In contrast, individuals allergic to bee venom or a chronically *Aspergillus*-infected person had no detectable IgE against solid phase-bound rAsp f I/a. Further, we analyzed rAsp f I/a-specific IgE and IgG among four members of a family with comparable exposure to *Aspergillus*. In this family M.R. was diagnosed as an ABPA patient. The data show that all members of the family have rAsp f I/a-specific IgG but only M.R. suffering from ABPA has IgE specific to rAsp f I/a (Fig. 8).

Inhibition ELISA. To demonstrate the specificity of the ELISA results further an inhibition ELISA was per-

TABLE I
List of PCR-primers^a and oligonucleotide probe^b

Primers/Probe	Amino Acid Sequence	Sequence Used
C-primer	Leu Cys Ser His	5' (A/G)TG I(G/C)(A/T) (A/G)CA I(A/G/C) 3'
N-probe	Ala Thr Trp Thr Cys Ile Asn Gln Gln	5' GCI ACI TGG ACI TG(C/T) ATI AA(C/T) CA(A/G) CA(A/G) 3'
5' λ ZAP II		5' GAA CTA GTG GAT CCC CCG AGC TCC AGG 3'
5' <i>asp f I/a</i> cDNA	Ala Thr Trp Thr Cys Ile Asn	5' CC GGA TCC GCG ACC TGG ACA TGC ATC AAC 3'
3' <i>asp f I/a</i> cDNA	Gly Asp Leu Arg Leu Cys Ser His	5' CC AAG CTT CTA ATG AGA ACA CAG TCT CAA GTC TCC 3'

^a C-primer, C-terminal end of mitogillin (14, 17); 5' λ ZAP II, region upstream the polyclonal site in the plasmid vector λ ZAP II (Stratagene); 5' *asp f I/a* cDNA and 3' *asp f I/a* cDNA, 5' and 3' end respectively of the cDNA clone *asp f I/a* (Fig. 1).

^b N-probe, N-terminal end of *A. fumigatus* allergen I (15).

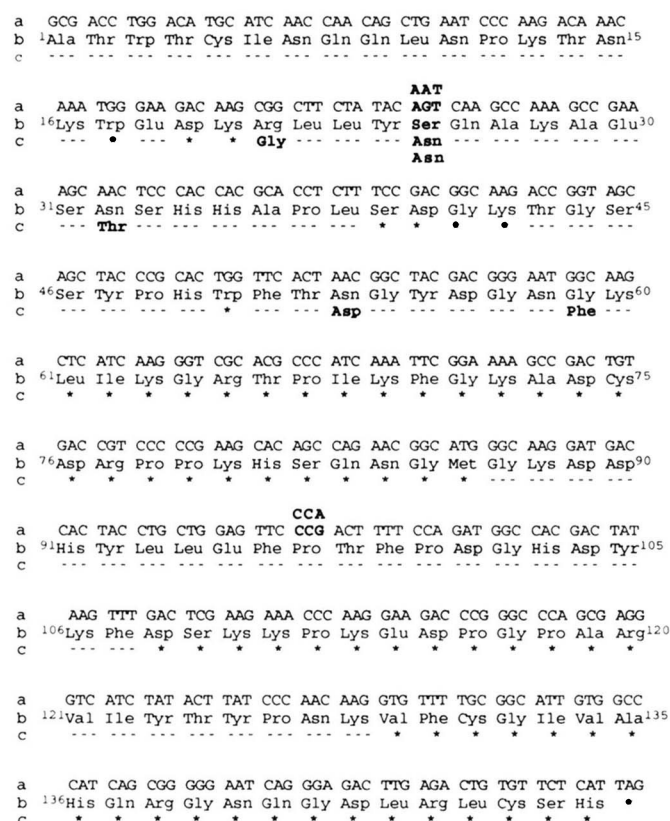


Figure 1. Alignment of the nucleotide sequence (a) encoding for three mature ribotoxins, the deduced amino acid sequences (b), and the partial amino acid sequence (c) of a similar 18-kDa ribotoxin reported by Arruda et al. (15) is shown; asterisk, no data available; (a) *asp f I/a* and the identical nucleotide sequence of restrictocin reported by Lamy and Davies (48). In the gene encoding for native 18-kDa restrictocin (14) the amino acid at position 25, Asn is encoded by AAT and at position 97, Pro is encoded by CCA.

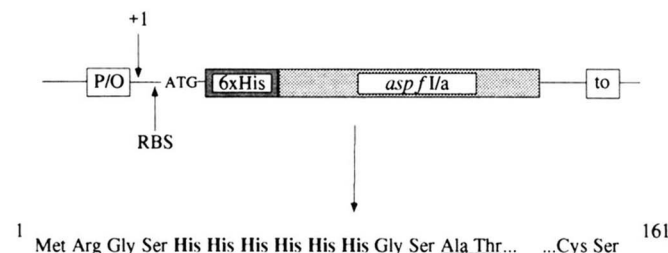


Figure 2. Partial amino acid sequence of *rAsp f I/a* fusion protein expressed using plasmid p6xHis-DHFRS (29). P/O, promoter/operator; RBS, synthetic ribosomal binding site; to, the transcriptional terminator of phage lambda. The first two amino acids of the mature ribotoxin encoded by *asp f I/a* are underlined.

formed. The binding of serum IgE from patient E.D. to solid phase bound *rAsp f I/a* was inhibited by increasing amounts of either *rAsp f I/a* or native ribotoxin (Fig. 9). Although both proteins are able to fully inhibit the binding of IgE to solid phase bound *rAsp f I/a* approximately 10 times more of the *rAsp f I/a* preparation was needed to achieve the same inhibitory effect.

Skin test. The biologic relevance of *rAsp f I/a* was evaluated in immediate type I skin tests and compared with two commercial extracts and two crude extracts from *A. fumigatus*. Allergic, ABPA, or control individuals were grouped as listed in Table II. The results of one representative member of each group is given. All healthy control individuals ($n = 7$) and all allergic but non-*A. fumigatus* allergic individuals ($n = 6$) were negative on

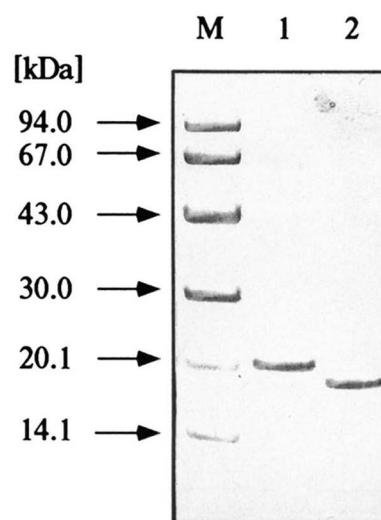


Figure 3. SDS-PAGE of *rAsp f I/a* or native 18-kDa ribotoxin analyzed under reducing conditions. A total of 2.5 μ g of affinity chromatography purified *rAsp f I/a* (lane 1) or native 18-kDa ribotoxin (lane 2) were analyzed on a 12.5% SDS-PAGE, and visualized by Coomassie blue staining. The positions of the M_r markers (M) are indicated.

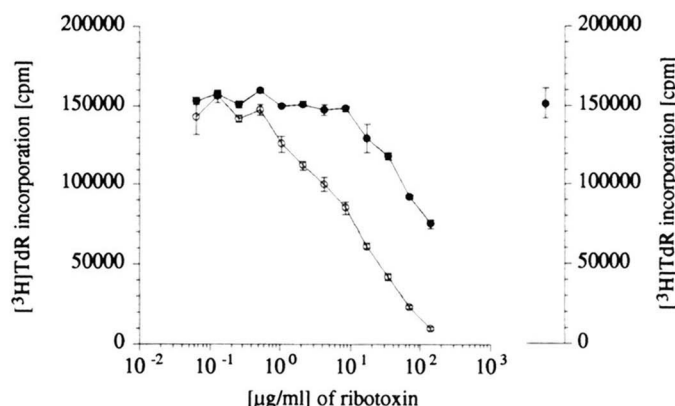


Figure 4. $[^3\text{H}]\text{TdR}$ incorporation of EBV-transformed PBMC at different ribotoxin concentrations. Triplicate samples of EBV transformed PBMC were cultivated for 48 h in the presence of *rAsp f I/a* (●) or native 18-kDa ribotoxin (○). Thereafter the cultures were labeled and the $[^3\text{H}]\text{TdR}$ incorporation was graphed as a function of ribotoxin concentration. Triplicate samples of cultivated EBV-transformed PBMC without ribotoxin were used as control (right graph).

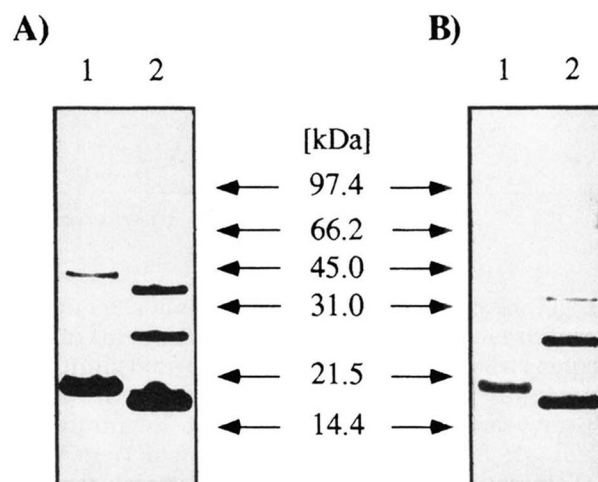


Figure 5. IgE immunoblot of ribotoxins. Two μ g *rAsp f I/a* (lane 1) or 18-kDa ribotoxin (lane 2) were separated on a 12.5% SDS-PAGE and transferred to a nitrocellulose membrane. Serum IgE of ABPA-patients E.D. (A) or L.M. (B) was analyzed by Western blotting.

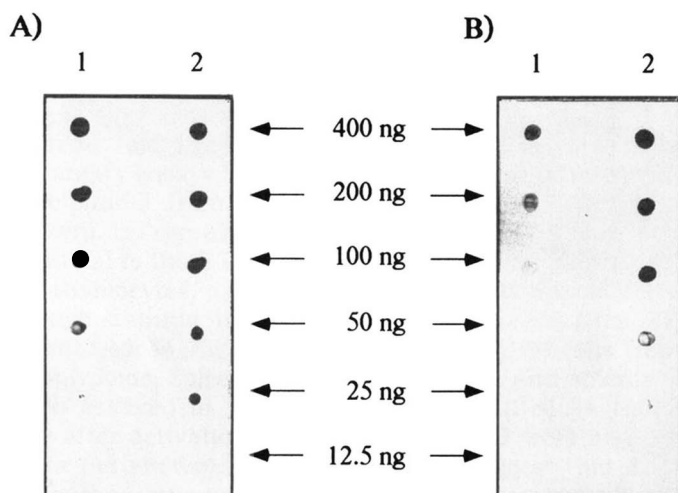


Figure 6. IgE detection sensitivity of ribotoxins. Different amounts rAsp f I/a (lane 1) or native 18-kDa ribotoxin (lane 2) were spotted onto a nitrocellulose membrane. The binding of serum IgE of the ABPA patients E.D. (A) and L.M. (B) was tested (see Fig. 5).

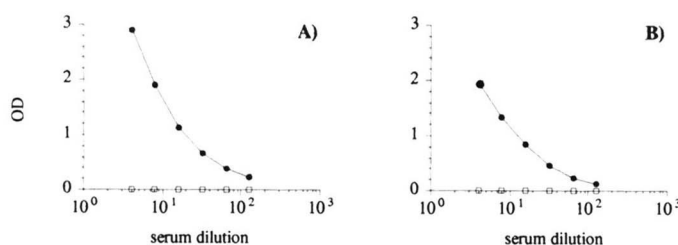


Figure 7. Ag-specific ELISA. IgE response against rAsp f I/a of different patients sera. A. ABPA patient E.D. (●) compared to an allergic person to bee venom (□); B. ABPA patient L.M. (●) compared to a chronically *Aspergillus*-infected patient (□).

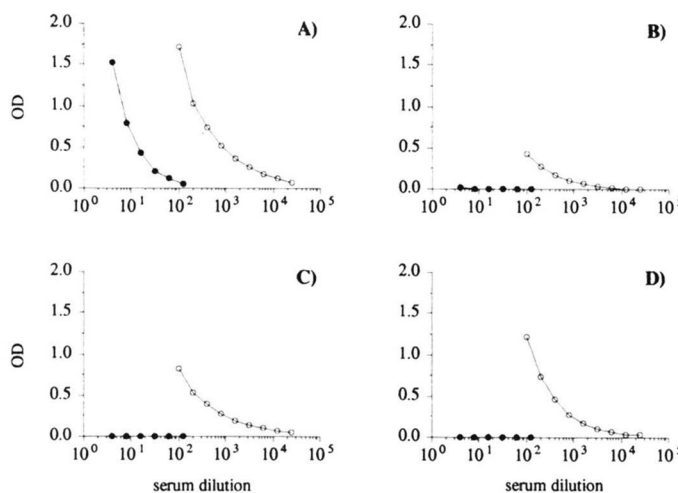


Figure 8. Ag-specific ELISA. IgG (○) and IgE (●) response against rAsp f I/a of family members with comparable exposure to *A. fumigatus*. ABPA patient M.R. (A); sister (B); mother (C); father (D).

skin testing with rAsp f I/a or preparations of *A. fumigatus* extracts. All allergic individuals to *A. fumigatus* ($n = 5$) showed positive skin test reactions to at least one *A. fumigatus* extract and two had positive reactions against rAsp f I/a. Five ABPA-patients ($n = 9$) showed a type I skin reaction against rAsp f I/a. Interestingly, ABPA patient E.B. reacted stronger against rAsp f I/a compared to either of the *A. fumigatus* extracts. In contrast, ABPA patient G.H. did not react at all against rAsp f I/a in skin tests although the reaction against extracts

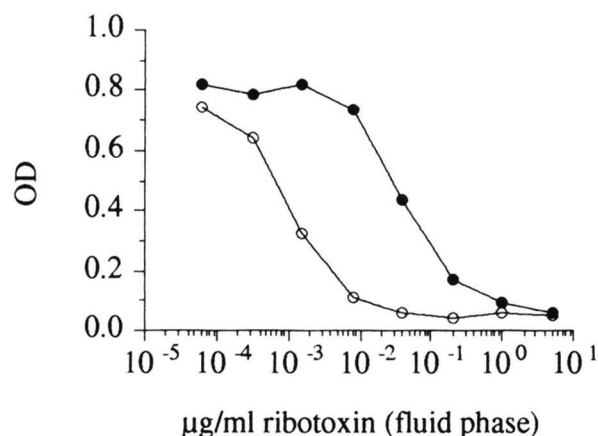


Figure 9. Inhibition of IgE binding to solid phase-coated rAsp f I/a. Serum from E.D. was preincubated with different amounts of native 18-kDa ribotoxin (○) or rAsp f I/a (●). Preincubated serum samples were transferred to rAsp f I/a-coated wells and IgE bound was analyzed by Ag-specific ELISA.

were strong. Skin test results from patient G.H. correlated well with serologic analyses. There was no rAsp f I/a-specific IgE in serum from patient G.H. when analyzed in ELISA or dot blots (data not shown).

DISCUSSION

We report cDNA cloning and production of rAsp f I/a in *E. coli* as a functional molecule. This recombinant *A. fumigatus* allergen binds to human IgE and elicits positive type I skin reactions.

The comparison of the amino acid sequence from three ribotoxins as predicted by the deduced nucleotide sequence revealed more than 99% similarity (Fig. 1). In fact there is only one amino acid difference between restrictocin from a strain designated *Aspergillus restrictus* (48) and another strain designated *A. fumigatus* (14). No difference in coding sequence could be detected between cDNA clone *asp f I/a* derived from a clinical *A. fumigatus* isolate and the gene encoding for restrictocin cloned from an *A. restrictus* strain (48). A partial amino acid sequence of a ribotoxin (15) differed in at least five amino acids when compared to the predicted sequence derived from cDNA clone *asp f I/a* (Fig. 1). The data therefore show that ribotoxins produced by *A. fumigatus* or *A. restrictus* are not encoded by a single gene. However, the small differences in nucleotide sequence between the genes encoding the different 18-kDa ribotoxins suggest allelic forms of one gene. The heterogeneity of this putative gene locus encoding for this type of ribotoxin in various *Aspergillus* isolates could easily be analyzed using PCR and nucleotide sequencing. Such studies will be aided by data available from detailed analysis of the locus encoding *asp f I/a* (48). We expect that the genes similar to rAsp f I/a amplified by PCR from various *A. fumigatus* isolates can be expressed in *E. coli* as described in this report. The recombinant proteins will allow to analyze the antigenic or allergenic potential.

rAsp f I/a and native 18-kDa ribotoxin (14) were compared in various assays to evaluate structural similarities. Both proteins have the ability to form dimers. These dimers are resistant to reduction and alkylation as reported (21). Further, rAsp f I/a or native 18-kDa ribotoxin were recognized by two murine mAb specific to a related ribotoxin (Fig. 1) (15). mAb were shown to be powerful

TABLE II
 Wheal and flare reaction from immediate skin tests^a

Patient	NaCl	Histamine	Com 1 ^b	Com 2 ^b	rAsp f I/a	CF ^c	EX ^c	Remarks
M.S.	0	+	0	0	0	0	0	Healthy control
A.E.	0	+	1	1	1	0	1	Allergic: Asp. f. neg
H.B.	0	+	10.6	42.2	14	36	10.6	Allergic: Asp. f. pos
G.H.	0	+	18	25	0	14	12.3	ABPA
L.M.	0	+	6.3	16.0	16.0	56.3	14.1	ABPA
E.B.	0	+	12.25	20.25	27.6	22.6	22.6	ABPA

^a Prick tests were performed and analysed as described in *Material and Methods*. The surface of the reaction is given in mm².

^b Two different commercial extracts from *A. fumigatus*.

^c Two different crude extracts from *A. fumigatus*.

reagents to provide information on the three-dimensional structure of native or refolded proteins (49). In addition, rAsp f I/a had the same substrate specificity and similar enzymatic activity as restrictocin and mitogillin. These observations can be used as indications but not proof that rAsp f I/a is correctly folded. Ongoing studies comparing x-ray structures of mitogillin (50) and rAsp f I/a combined with NMR studies will resolve these questions.

Highly purified native 18-kDa ribotoxin appeared homogeneous when analyzed by PAGE (Fig. 3). Western blot and dot blot experiments however revealed a 27-kDa IgE binding protein that was recognized by patient sera E.D. or L.M. (Fig. 5) but not by murine mAb directed against an 18-kDa ribotoxin (15). The data illustrate the difficulties in obtaining pure native allergens. This impeded conclusive quantitative comparisons between this preparation of native 18-kDa ribotoxin and rAsp f I/a. The apparent 10-fold functional difference between rAsp f I/a and this preparation of native 18-kDa ribotoxin (Figs. 4 and 9) may be explained by unknown influences on shape or function exerted by proteins tightly associated with ribotoxins such as the 27-kDa protein. Alternatively, rAsp f I/a may not be folded completely due to the fusion peptide at the N-terminus. This possibility will be analyzed in more detail by enzymatically removing the fusion peptide from the N-terminus after chelate affinity chromatography.

Limited serologic analyses showed that only *A. fumigatus* allergic individuals had serum IgE to rAsp f I/a. Individuals not allergic to *A. fumigatus* but exposed to this fungus may express IgG or IgA against rAsp f I/a (Fig. 8). An ongoing study will evaluate the usefulness of rAsp f I/a as a diagnostic tool to help diagnosing immediate hypersensitivity to *A. fumigatus*.

Serum antibodies against rAsp f I/a and skin test responses were compared. It is noteworthy that a single recombinant allergen could induce a stronger type I skin reaction when compared to four *A. fumigatus* extracts used at the same concentration (Table II). In contrast, some ABPA patients showed no response to rAsp f I/a in skin test. Data from previous publications (14, 15) indicated that although the 18-kDa ribotoxin was a major allergen it did not have an allergenic potential for all *A. fumigatus* allergic individuals. Alternatively, it is possible that Asp f I/a may have lost its allergenic potential, due to a switch of the patients immune system to an Ig isotype other than ϵ . Further, we cannot entirely rule out the possibility that a family of ribotoxins antigenically different from rAsp f I/a may exist, inducing IgE that does not bind to rAsp f I/a in vitro nor in vivo.

The availability of rAsp f I/a with IgE binding and skin test activity shows the feasibility of recombinant proteins

for diagnosing *A. fumigatus*-associated allergies. Ultimately, the cloning, production and characterization of recombinant allergens will permit the dissection of the molecular basis of the allergic immune response.

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