

Standardization of *Alternaria alternata*: Extraction and quantification of Alt a 1 by using an mAb-based 2-site binding assay

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Background: *Alternaria alternata* is recognized as an important cause of allergic disease. As with other molds, the extracts of *A. alternata* used for diagnosis and therapy are highly heterogeneous, and there is a need for improved standardization. The major allergen Alt a 1 is well characterized and has been produced as a recombinant protein, but very few data are available on the Alt a 1 content in extracts.

Objective: An assay for the quantification of Alt a 1 was developed and used for monitoring batch-to-batch consistency of *A. alternata* extracts, and the correlation between skin prick test responses and Alt a 1 concentrations was studied.

Methods: A 2-site binding assay based on an Alt a 1-specific mAb was developed and used for the quantification of Alt a 1 in allergen extracts. Quantitative skin prick tests were performed on 16 *A. alternata*-sensitive patients and correlated with the Alt a 1 concentration.

Results: The Alt a 1-specific mAb was found to be suitable for affinity purification, as well as for a 2-site binding quantification assay. In allergen extracts the Alt a 1 content was estimated as 2% to 4.7% of total protein. Quantitative skin prick tests showed an Alt a 1 concentration-dependent response.

Conclusion: Quantification of Alt a 1 in *A. alternata* extracts reflects their batch-to-batch consistency. Skin prick test responses to a standardized *A. alternata* extract correlate with the Alt a 1 contents. An extract containing 3.7 µg/mL Alt a 1 caused a response equal to that of a 1% histamine dihydrochloride solution. (*J Allergy Clin Immunol* 1999;103:128-35.)

Key words: *Alternaria alternata*, monoclonal antibody, Alt a 1, ELISA, skin prick test, allergens, fungi

Allergens derived from fungi, especially *Alternaria alternata*, *Cladosporium herbarum*, *Aspergillus* spp, and *Penicillium* spp, are well-known causes of type I allergy and cause sensitization in about 20% of all allergic patients.

Abbreviation used

PBS-T: PBS containing 0.1% Tween 20

Allergen extracts produced from molds show considerable heterogeneity as a result of different culture conditions and extraction procedures.¹ For these reasons, attempts to establish an international standard for *A. alternata* failed, as did the determination of an allergen spectrum considered as a "best fit" for diagnosis and therapy.² Recent attempts to improve standardization and characterization of mold extracts for diagnosis and specific immunotherapy have focused on cloning of important allergens and increased knowledge of their molecular structures.³⁻⁷

Improved standardization can also be achieved by the development of assays to quantify specific allergens. Monoclonal antibody-based assays can be particularly useful for standardization procedures,^{8,9} as well as for the determination of specific allergen concentrations in the environment.¹⁰

Alt a 1 is the major allergen in *A. alternata* extracts, reacting with serum IgE of more than 80% of *A. alternata*-sensitized patients. The allergen exists as a dimer with a molecular weight of 29 kD.⁶

Although some mold allergen-specific antibodies have been described,^{3,5} to date there are no data available for the quantification of Alt a 1 in environmental samples and only a few data of Alt a 1 contents in allergen extracts.¹¹ In this study we describe the purification of Alt a 1, the development of Alt a 1-specific mAbs and a 2-site binding assay based on one of these antibodies, and the use of Alt a 1 for improved standardization of *A. alternata* extracts for in vivo diagnosis.

METHODS

A. alternata extracts

A. alternata extracts were produced by Allergopharma (Reinbek, Germany). Raw material containing spores and mycelia of *A. alternata* (CBS no. 103.33) was purchased from Allergon (Engelholm, Sweden). Extraction was performed in Coca's solution overnight at 4°C. After centrifugation (10,000g for 1 hour), the supernatant was filtered

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and subjected to diafiltration (cut off, 5000 d), lyophilized, and stored at -20°C . Protein content was measured according to a modified Lowry method,¹² using human serum albumin as a reference.

Monoclonal antibodies and polyclonal antisera

BALB/c mice were immunized with *A alternata* extract or HPLC-purified Alt a 1. Hybridoma cell lines were generated according to the method of Köhler and Milstein.¹³ Supernatants were screened for specific antibodies by an ELISA with either HPLC-purified Alt a 1 or *A alternata* extract. Specific mAbs producing cell lines were subcloned to ensure monoclonal hybridomas. Monoclonal antibodies were purified from a hybridoma supernatant by ammonium sulphate precipitation and subsequent affinity chromatography with Protein G Sepharose. The subtype of mAb 121 was determined to be IgG₁ by using a hybridoma subtyping kit (Calbiochem). Purity was checked by SDS-PAGE and isoelectric focusing. Biotinylation of purified antibodies was performed by using a biotinylation kit (Boehringer Mannheim). Epitope mapping was achieved by an ELISA with microtiter plates coated with *A alternata* extract and an unlabeled mAb as inhibitor for the binding of a biotinylated mAb.

Rabbits were immunized with *A alternata* extract (1 mg of protein on days 0, 14, 28, and 42) in NaCl (0.9% wt/vol) mixed with either complete Freund's adjuvant (Gibco; injections on day 0) or incomplete Freund's adjuvant (Gibco; injections on days 14, 28, and 42) at 6 subcutaneous injection sites on the animals' backs. Sera were collected 10 days after the last injection. All procedures, including the use of animals, were conducted in strict accordance with the requirements of the Federal German Authority.

Two-site binding assay and quantification of Alt a 1

Microtiter plates were coated with the purified mAb 121 (1 $\mu\text{g}/\text{mL}$ in carbonate-bicarbonate buffer [pH 9.6] overnight at 4°C ; 100 $\mu\text{L}/\text{well}$), washed with PBS (pH 7.5) containing 0.1% Tween 20 (PBS-T), and blocked with PBS-T plus 1% BSA for 30 minutes. Serial 2-fold dilutions of affinity-purified Alt a 1 were used to form a standard curve starting with 100 ng Alt a 1/mL. Mass units were assigned by using the extinction coefficient of Alt a 1 in stock solutions with 100 μg Alt a 1/mL. The extinction coefficient at 280 nm (0.1%, 1 cm) for purified Alt a 1 is 4.06, as determined by a combination of estimated ODs of 205 nm and 280 nm.¹⁴ Standard and samples were incubated for 1 hour, and, after washing, the biotinylated mAb 121 was added and incubated for 1 hour. Bound biotinylated mAb was detected by using streptavidin peroxidase and 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid (Sigma) as a substrate.

Detection of bound Alt a 1 by a polyclonal rabbit anti-*A alternata* extract antiserum was performed as follows. The rabbit antiserum was diluted 1:500 in PBS-T, and samples were incubated for 1 hour at ambient temperature. Bound rabbit antibodies were detected by using an alkaline phosphatase-conjugated anti-rabbit IgG (Sigma) with *p*-nitrophenyl phosphate (Sigma) as a substrate, and OD at 405 nm was measured.

Skin prick testing

A group of 16 patients with suspected sensitivity to *A alternata* underwent skin prick tests with a series of 3-fold dilutions of an extract of the mold in triplicate in a double-blind, randomized format. The study protocol was approved by the local and regional ethics committees.

A skin response was considered positive if it elicited a wheal reaction greater than 7 mm² (3 mm diameter). All test solutions were prepared with 50% glycerol and 0.4% phenol. All wheal reactions were delineated with a ballpoint pen, copied with transparent adhesive tape, and placed in the patients' records. The areas of the wheal responses

were determined by using computerized planimetry. *A alternata*-specific IgE was determined by RAST (Allergopharma) and CAP (Pharmacia) systems, according to the manufacturers' instructions.

SDS-PAGE and immunoblotting

SDS-PAGE was performed according to the method of Laemmli¹⁵ in a slab gel apparatus (Phase, Lübeck, Germany). Proteins were stained with Coomassie blue or transferred to a polyvinylidene difluoride membrane (BioRad) by electroblotting, according to the method of Kyhse-Andersen.¹⁶ After blocking with PBS-T plus 1% BSA (wt/vol) for 15 minutes, blots were incubated with either human sera (1:5 dilution in PBS-T), a specific mAb (10 ng/mL PBS-T), or polyclonal rabbit anti-*A alternata* extract antiserum (1:1000 in PBS-T) overnight at ambient temperature. Bound IgE was detected with an alkaline phosphatase-conjugated monoclonal anti-human IgE antibody (Allergopharma), and the blot was developed by the chemiluminescence substrate CSPD (Tropix, Bedford, Mass) with Kodak X-Omat film. Bound mAbs were detected by using alkaline phosphatase-conjugated anti-mouse IgG (Jackson Immuno Research; 1:10,000 dilution in PBS-T), and bound rabbit antibodies were detected by using alkaline phosphatase-conjugated anti-rabbit IgG (Sigma; 1:2000 in PBS-T) and developed by chemiluminescence.

Purification of Alt a 1 by size exclusion and ion exchange chromatography

A alternata extract was subjected to size exclusion HPLC by using a TSK 2000 column (Beckmann, Munich, Germany) and 0.5 mol/L ammonium acetate as eluent (flow, 1 mL/min; detection UV, 280 nm). Fractions were collected, dialyzed against double-distilled water, and freeze-dried. After analysis by SDS-PAGE and immunoblot, Alt a 1-containing fractions were pooled and subjected to anion-exchange HPLC (Fractogel EMD TMAE 650 (S); Merck, Darmstadt, Germany) by using 0 to 1 mol/L NaCl in 20 mmol/L Tris/HCl buffer (pH 8.0). Fractions were collected and analyzed by SDS-PAGE and immunoblot after dialysis and lyophilization.

Affinity chromatography with an Alt a 1-specific mAb

Approximately 2 mg of mAb 121 was covalently linked onto protein G Sepharose (Pharmacia, Uppsala) by using dimethyl pimelimidate as a cross-linker, according to the method of Harlow and Lane.¹⁷ The affinity column was incubated with up to 4 mg of *A alternata* extract in 8 mL of PBS for 4 hours at ambient temperature or overnight at 4°C . The column was washed with PBS, and bound Alt a 1 was eluted with 0.15 mmol/L triethanolamine (pH 12.0). Alt a 1 fractions were collected, dialyzed, and freeze-dried.

Reduction and alkylation of Alt a 1

Alt a 1 was dissolved in 1 mL of 0.5 mol/L Tris, 2 mmol/L EDTA, and 6 mol/L guanidine hydrochloride (pH 8.1) and heated for 30 minutes at 50°C . Dithiothreitol was added (5 mmol/L), and samples were incubated for 4 hours at 50°C . After cooling to ambient temperature, iodoacetamide (5 mmol/L) was added, pH was checked (pH 8), and the sample was kept in the dark for 20 minutes at ambient temperature. As a control, Alt a 1 was treated in the same way but without dithiothreitol and iodoacetamide. After concentration and buffer exchange (PBS-T), Alt a 1 was subjected to quantification and SDS-PAGE/immunoblotting.

N-terminal amino acid sequencing

N-terminal amino acid sequencing was performed by automated Edman degradation on an Applied Biosystems 476 sequencer. Samples were prepared by running purified Alt a 1 on SDS-PAGE and subsequent transfer of the protein to a polyvinylidene difluoride

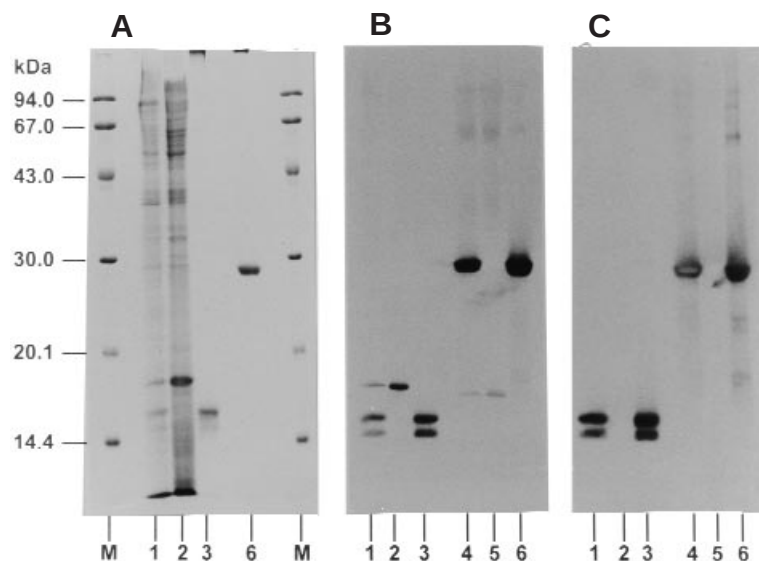


FIG 1. Analysis of *A alternata* extracts and affinity-purified Alt a 1 by SDS-PAGE and immunoblot. *A alternata* extract was subjected to affinity chromatography by using mAb 121. The extract, Alt a 1-depleted extract, and Alt a 1 were analyzed by SDS-PAGE and immunoblot by using mAb 121 and a pool of human sera. **A**, Coomassie stain; **B**, immunoblot with a serum pool of 15 human sera from patients allergic to *A alternata*; **C**, immunoblot with mAb 121. M, Molecular weight marker proteins; lanes 1, *A alternata* extract (40 μ g) separated under reducing conditions; lanes 2, Alt a 1-depleted extract (40 μ g) separated under reducing conditions; lanes 3, Alt a 1 (4 μ g) separated under reducing conditions; lanes 4, *A alternata* extract (40 μ g) separated under nonreducing conditions; lanes 5, Alt a 1-depleted extract (40 μ g) separated under nonreducing conditions; lanes 6, Alt a 1 (4 μ g) separated under nonreducing conditions.

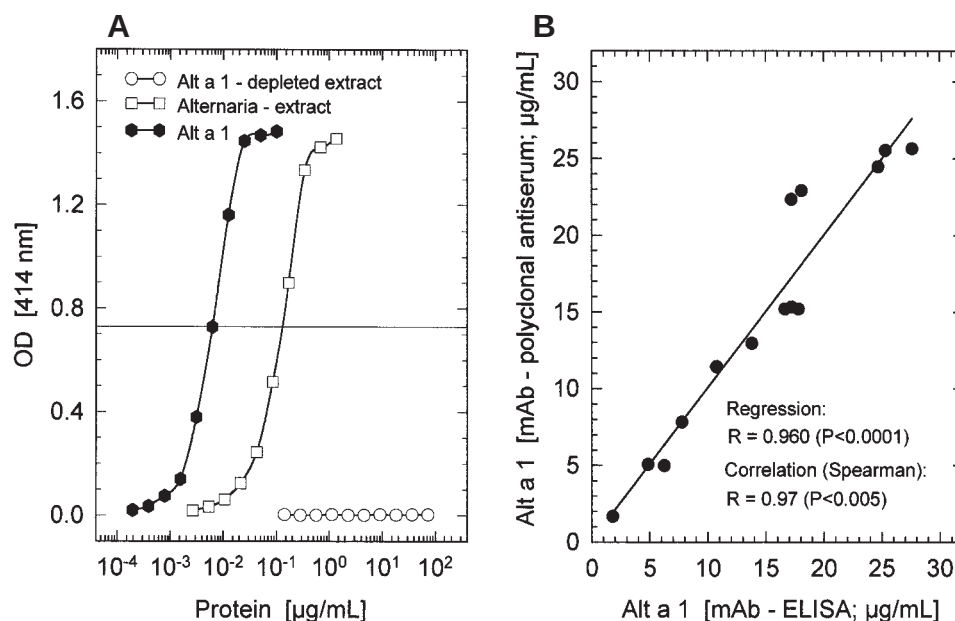


FIG 2. Quantification of Alt a 1 by 2-site binding assays. *A alternata* extract, purified Alt a 1, and Alt a 1-depleted *A alternata* extract were applied to the Alt a 1 2-site binding assay. The reference line is equivalent to 6.13 ng Alt a 1/mL (**A**). The extract contains 45.9 ng Alt a 1/ μ g protein, and the Alt a 1-depleted extract contains less than 0.02 ng Alt a 1/ μ g protein (see Table III). Alt a 1 was quantified in 14 extracts by the mAb-based assay and by an assay with mAb 121 for capturing and a polyclonal antiserum for detection of bound Alt a 1. Results were correlated in **B**.

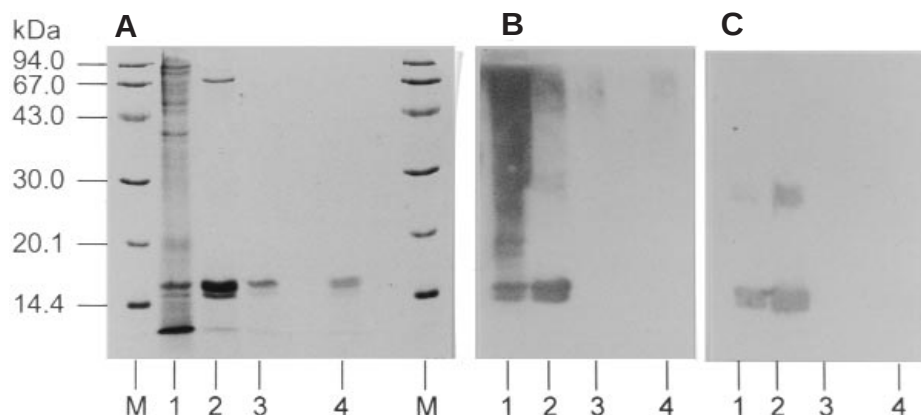


FIG 3. SDS-PAGE and immunoblot of reduced and alkylated Alt a 1. Affinity-purified Alt a 1 was reduced and alkylated. Samples were subjected to SDS-PAGE and immunoblotting. Immunoblots were developed with a polyclonal anti-*A alternata* antiserum or the Alt a 1-specific mAb 121. **A**, Protein stain (Coomassie blue); **B**, immunoblot with the polyclonal anti-*A alternata* antiserum; **C**, immunoblot with the Alt a 1-specific mAb 121. *M*, molecular weight marker proteins; *lanes 1*, *A alternata* extract (30 μ g); *lanes 2*, affinity-purified Alt a 1 (3 μ g); *lanes 3*, affinity-purified Alt a 1 (3 μ g) reduced, alkylated, and treated with dithiothreitol before separation; *lanes 4*, affinity-purified Alt a 1 (3 μ g) reduced, alkylated, and separated under nonreducing conditions.

TABLE I. Batch-to-batch consistency: Alt a 1 content in *A alternata* extracts

Extract	Protein (μ g/mL*)	Alt a 1		n†
		μ g/mL*	ng/ μ g protein	
A	53	1.05 $\pm$ 0.16	19.8	4
B	160	4.75 $\pm$ 0.86	29.7	4
C	140	5.12 $\pm$ 0.42	36.6	4
D	249	7.72 $\pm$ 1.30	31.0	5
E	175	5.20 $\pm$ 0.45	29.7	3
F	576	21.72 $\pm$ 1.84	37.7	6
G	100	4.22 $\pm$ 0.86	42.2	6
H	1329	62.50 $\pm$ 5.74	47.0	6

*Differences of protein and Alt a 1 contents per milliliter are due to different amounts of lyophilisate dissolved per milliliter. This procedure was chosen for checking variability of the assay depending on dilution steps before the measurements, and these values do not mirror contents in any preparations for diagnostic use.

†Number of determinations.

membrane. The proteins were stained with Coomassie blue, and the protein bands were cut out of the membrane and sequenced.

RESULTS

Two-site binding assay for the quantification of Alt a 1

The dimeric structure of Alt a 1 suggested that it might be possible to establish a 2-site binding assay with only one antibody for capture and detection, assuming that the same epitope was present in each monomer. Immunoblots showed that mAb 121 bound to the nonreduced dimer, as well as to both subunits (Fig 1, C), which was also shown for IgE binding. Dose-reponse curves obtained by using mAb 121 (Fig 2, A) were comparable with those revealed by using mAb 121 for capture and a

TABLE II. Skin prick tests: Specific IgE and dose response

Patient	Specific IgE (kUA/L)	Alt a 1* (ng/mL)	Dose response (<i>A alternata</i> extract)
1	3.63	280	+
2	1.77	93.3	+
3	5.76	93.3	+
4	5.88	93.3	+
5	0.75	93.3	+
6	1.03	93.3	—
7	8.05	93.3	+
8	<0.35	—	—
9	0.52	2520	—
10	9.28	93.3	+
11	4.06	840	—
12	12.60	280	+
13	8.86	93.3	+
14	<0.35	—	—
15	<0.35	—	—
16	5.67	93.3	+

*Alt a 1 content causing a positive skin prick test response.

TABLE III. Affinity purification of Alt a 1

	Protein (mg)	Alt a 1	
		μ g	Percent of protein
Extract	11.7	464	4.6
Fraction not retained	7.4	$\leq$ 0.15	$\leq$ 0.002
Alt a 1	0.32	314	98

polyclonal anti-*A alternata* antiserum for detection. Results obtained with this system correlate well with those obtained with the mAb as detecting antibody (Fig 2, B), indicating that the detection of bound Alt a 1 by

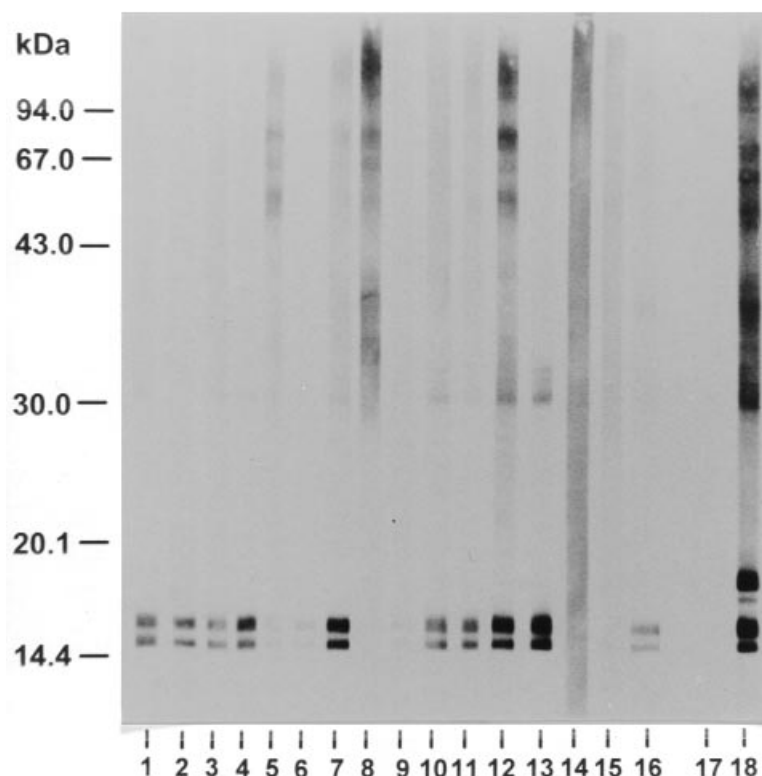


FIG 4. Analysis of IgE binding of human sera with *A alternata* extract proteins by immunoblot. *A alternata* extract was separated under reducing conditions by SDS-PAGE. Separated proteins (50 μ g per lane) were transferred to a polyvinylidene difluoride membrane. Strips were incubated with human sera, and bound IgE was detected. Lanes 1 to 16 refer to the sera listed in Table II. Lane 17, incubated with a serum pool from non-allergic individuals ($n = 10$; negative control); lane 18, incubated with a serum pool of sera from patients allergic to *A alternata* ($n = 15$; positive control).

mAb 121 is not restricted or influenced by irregularly distributed epitopes.

Alt a 1 was reduced and alkylated to investigate the binding of mAb 121 to the Alt a 1 subunits. Reduced and alkylated Alt a 1 did not react in the mAb-based assay or in the mAb polyclonal assay combination, indicating that only the intact dimer can be quantified in the assay and not the reduced and alkylated Alt a 1 monomers. Immunoblots with alkylated and reduced Alt a 1 monomers in comparison with reduced Alt a 1 confirm these results (Fig 3), revealing no binding of either the polyclonal antiserum or mAb 121 to reduced and alkylated Alt a 1 monomers.

Alt a 1 contents in 8 batches of *A alternata* extracts were determined by monitoring batch-to-batch consistency. These extracts contained 19.8 to 47.0 ng Alt a 1 per microgram of protein (Table I; mean = 34.2 ± 8.5 ng Alt a 1/ μ g protein). The coefficient of variation is equivalent to 25%. The intraassay precision expressed as coefficient of variation was estimated as 8% ($n = 18$) or less, the interassay precision as 15% (coefficient of variation, $n = 24$) or less, and the lower detection limit as 3.1 ng Alt a 1/mL.

Skin prick tests with a standardized *A alternata* extract

Sixteen patients with suspected allergy to *A alternata* were investigated by means of skin prick testing with

several dilutions of an extract with Alt a 1 concentrations from 0.09 to 22.7 μ g/mL. Three patients had negative skin prick test responses, and serologic investigations revealed negative ELISA and CAP results (Table II) and no detectable IgE binding on immunoblots (Fig 4). The remaining 13 patients had both skin prick test and ELISA/CAP positive responses (Table II), but only 10 of these patients showed a dose-dependent skin test response (Fig 5). The concentration of *A alternata* extract causing a mean skin test response equal to that of 1.0% histamine dihydrochloride contained the equivalent of 3.73 μ g/mL Alt a 1.

Alt a 1-specific IgE antibodies were detected in all 13 ELISA/CAP-positive sera. From these sera, 9 showed a strong reaction and 4 showed a weak reaction, indicating differences of Alt a 1-specific IgE titers (Fig 4). Extracts were separated under reducing conditions, which resulted in 2 bands representing Alt a 1, both of which retained their IgE-binding capacity.

Purification of Alt a 1

Affinity-purified Alt a 1 revealed a single band with an apparent molecular weight of 28 kd when analyzed by SDS-PAGE under nonreducing conditions and, when analyzed under reducing conditions, a distinct band with an apparent molecular weight of 16 kd and a weak band

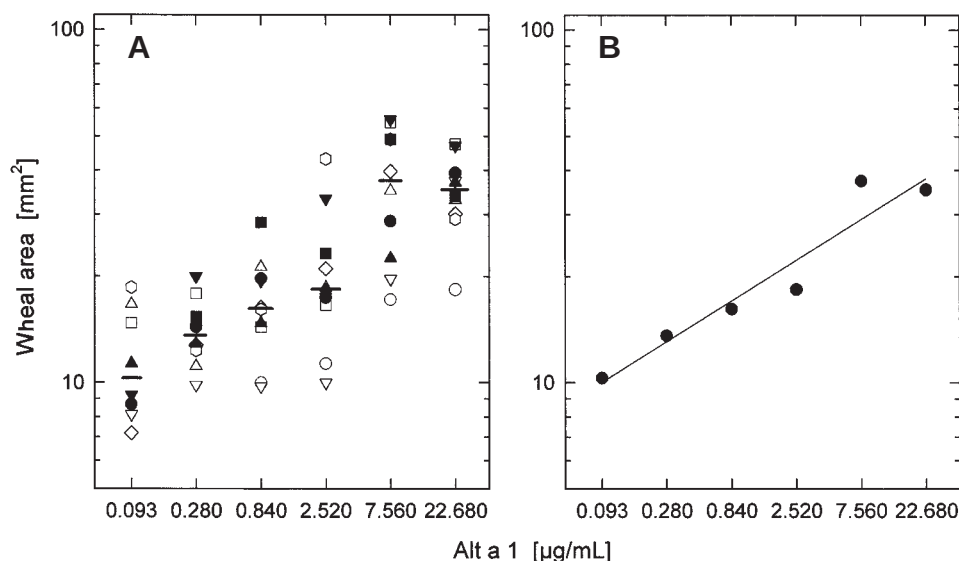


FIG 5. Dose response of *A alternata* extract in skin prick testing. **A**, Median values of wheal area obtained with 10 patients and the Alt a 1 concentrations are indicated (symbols). Median values for each concentration are also indicated (lines). **B**, Median values of wheal responses of all patients tested with *A alternata* extract are shown.

TABLE IV. N-terminal amino acid sequences of Alt a 1 preparations

		1					5					10					15					
N-terminal Alt a 16 G	20																					
		A	D	P	V	T		T	E	G	D	Y	V	V	K	I	S	E	F	Y		
	R																					
	26																					
	48																					
Clone RB51.AA6 G	D	T	A	S	C	P	V	T		T	E	G	D	Y	V	W	K	I	S	E	F	Y
	R																					
	1																					
15-kd subunit	D	T	—	—	P	P	V	T		X	E	G	D	Y	V	K	K	I				
	1																					
16-kd subunit	D	T	A	S	X	P	V	T		T	E	G	D	Y	V	G	K	I	R			

Positions of sequenced amino acids as indicated.

X, Not identified; —, gaps in the 15-kd subunit because of homology screening.

with an apparent molecular weight of 15 kd (Fig 1, A). Affinity purification with the Alt a 1-specific mAb 121 yields a nonretained fraction, which was almost depleted of Alt a 1, as shown by SDS-PAGE and quantification of Alt a 1 (Fig 1, A and Table III).

Affinity-purified Alt a 1 eluted under alkaline conditions retained its IgE-binding activity in ELISA inhibition, as well as in immunoblots (Fig 1, B), and was still able to bind to mAb 121. Identity was confirmed by N-terminal amino acid sequencing. Analyzing the subunits obtained by reduction, we found differences in positions 3 and 4, which were missed in the 15-kd subunit (Table IV).

DISCUSSION

The quantification of Alt a 1 is an important method for standardization of extracts, but it is essential to apply all available methods, starting with an effective quality

control of the raw material followed by reproducible extraction procedures and detailed comparison of batches with a well-characterized biologic standardized in-house reference extract. These analytic procedures include the comparison of protein and allergen patterns and measurement of allergenic activity by using a well-characterized RAST inhibition system.

The 2-site binding assay was established with only 1 mAb for capture and detection, assuming that Alt a 1 exists as a dimer in its native form. To confirm this, we compared the mAb-based assay and a combination of the mAb for capture and a polyclonal antiserum for detection to establish whether Alt a 1 monomers were present in several different *A alternata* extracts. The results show a very good correlation between the assays, suggesting that there are no detectable amounts of Alt a 1 monomers or any other fragments in the extracts. This conclusion is based on the assumption that the polyclonal antiserum

would likely recognize more than one epitope per monomer. The apparent absence of Alt a 1 monomers in extracts, also supported by measurements with either combinations of mAbs specific for different epitopes or a polyclonal antiserum-based RIA (results not shown), should best be complemented by measuring monomers. Generating Alt a 1 monomers without alkylation fails, indicating that the capability to form dimers is very strong. This finding is supported by the fact that recombinant Alt a 1 also forms dimers spontaneously⁶ (M. Breitenbach, personal communication). Although there is a remarkable difference between reduced, nonalkylated Alt a 1 and the nonreduced molecule, there are no detectable differences in the binding capacity with the mAb, the polyclonal antiserum, or specific IgE in immunoblots. Reduced and alkylated Alt a 1 monomers do not bind with the mAb or the polyclonal antiserum either in the ELISA systems or in immunoblotting, a result that we considered particularly surprising in the case of the polyclonal antiserum. IgE-binding activity with reduced and alkylated Alt a 1 is also reduced, as was reported by de Vouge et al⁶ for the recombinant Alt a 1. Therefore binding sites of the reduced nonalkylated monomers on a membrane might be formed either by intramolecular mechanisms (ie, rebuilding of 3-dimensional structures by disulfide bonds) or by interactions of the monomers during the transfer process resulting in formation of homodimers. Both mechanisms might be possible because the reducing agent is only present in trace amounts during the transfer process. Further investigations are necessary to define binding sites and molecular features of Alt a 1, probably based on the recombinant proteins.

The method used for purification of Alt a 1 was different from that published by Paris et al¹⁸ for the purification of Alt a 1₁₅₆₃ and comparable with the method published by Curran et al.¹⁹ The molecular characteristics of the purified Alt a 1 were similar in those reported earlier,⁶ giving a single band with an apparent molecular weight of 28 kd (nonreduced) and 2 bands with apparent molecular weights of 15 and 16 kd (reduced). Affinity purification with the Alt a 1-specific mAb 121 proved to be very effective in that this 1-step purification yielded Alt a 1 with more than 90% purity. The alkaline conditions used for elution caused the highest yield and were found to have no apparent detrimental effect on the activity of the purified protein either with respect to IgG binding or to IgE binding.

The N-terminal amino acid sequence of the 16-kd subunit was found to be identical with the sequence of the recombinant Alt a 1⁶ from position 26 to 39 (numbers according to clone rb51⁶), starting at position 26, and showed variations at positions 40 and 43, whereas the N-terminus of the 15-kd subunit exhibited greater variations, with gaps in positions 28 and 29 and differences in positions 30, 34, and 40. Differences between the recombinant Alt a 1 and native Alt a 1 in position 40 have been reported.^{6,19} Microheterogeneity for Alt a 1 in isoelectric focusing was reported, which was not affected by removal

of carbohydrate moieties.¹⁹ Therefore it is likely that microheterogeneity is due to the exchange of single amino acids, as detected in N-terminuses of native Alt a 1.

Our findings indicate that a well-established extraction protocol achieves a high batch-to-batch consistency, reflected by a narrow range of Alt a 1 contents in different batches. Monitoring batch-to-batch consistency of intermediate bulk extracts, which are produced as lyophilisates, the Alt a 1 contents were estimated as nanograms of Alt a 1 per microgram of protein. This is different from determinations of allergen contents in final products (ie, skin prick test solutions), which are usually given as weight (ie, micrograms) per volume (ie, milliliters). For quality control, the specific allergen values are more reliable than volume-based values because variations of protein contents can occur as a result of variations of raw materials or extraction procedures. Bearing in mind the heterogeneity and complexity of *A alternata* extracts, the ratio of the contents of 2 allergens might be essential for extract quality. This would be analogous to the quantification of the major allergens Der p 1 and Der p 2 in commercial house dust mite extracts, in which the ratios of the allergens vary between 20:1 and 1:1 because of different raw materials used for production.²⁰⁻²² However, *A alternata* extracts were shown to contain Alt a 1 as the only major allergen, and allergens studied by molecular cloning techniques are minor allergens.⁴ This view might change through use of molecular cloning techniques and effective purification schemes for native allergens, when other *A alternata* allergens may be assessed as major allergens.²³

The lower detection limit of the quantification assay was determined to be 3.1 ng Alt a 1/mL, which is more than 60-fold lower than the sensitivity of 0.2 µg Alt a 1/mL reported by Portnoy et al.¹¹ These differences are most likely due to differences in protein levels of the standards used. Our Alt a 1 standard was characterized by several methods thus checking for purity, identity, and protein content, including the determination of the Alt a 1 extinction coefficient. However, it should be borne in mind that there is neither an international standard available nor a method for characterizing primary standards for Alt a 1 quantification assays. These differences will be best overcome by establishing international standards, possibly on the basis of recombinant proteins. However, it is clear that much still has to be done to standardize assay systems before the definition of major allergen content can be adopted with confidence as a basis for comparing extracts from different sources. Nevertheless, our results of quantitative skin prick testing demonstrate that the quantification of Alt a 1 could be used to define effective diagnostic and therapeutic levels of the allergen. The status of Alt a 1 as a major allergen was confirmed by the fact that Alt a 1-specific IgE antibodies were detected by immunoblotting in all ELISA/CAP-positive sera.

The Alt a 1 quantification assay will be useful for further studies (ie, evaluation of biologic activity of Alt a 1 and determination of Alt a 1 in environmental samples).

Furthermore, affinity purification with mAb 121 provides highly purified native Alt a 1, a prerequisite for further studies on molecular features of this molecule.

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