Diagnosis of *Alternaria alternata* sensitization with natural and recombinant Alt a 1 allergens

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Background: Diagnosis of *Alternaria alternata* sensitization is hampered by the variability and complexity of fungal extracts, and thus simplification of the diagnostic procedures with purified allergens should be pursued.
Objective: We sought to compare *A alternata* extract and purified natural Alt a 1 (nAlt a 1) and recombinant Alt a 1 (rAlt a 1) allergens for their diagnostic value.
Methods: Forty-two patients allergic to *A alternata*, 10 atopic patients with negative skin prick test responses to *A alternata* extract, and 10 healthy subjects were investigated. Skin prick tests and determination of specific IgE levels were performed with nAlt a 1 and 2 different types of rAlt a 1: rbAlt a 1, expressed in *Escherichia coli*, and ryAlt a 1, expressed in the yeast *Yarrowia lipolytica*.
Results: Prevalence for Alt a 1, Alt a 2, and Alt a 11 by IgE dot-blot testing was 98%, 0%, and 15%, respectively, and therefore Alt a 1 was used as a marker for *A alternata* sensitization. Immunoblotting and inhibition analysis showed no IgE-binding differences between nAlt a 1 and rAlt a 1. The whole group of patients with allergy to *A alternata* had positive skin test reactions to purified allergens at 100 μg/mL, whereas no false-positive reactions were detected. Natural or ryAlt a 1 elicited a similar response in skin tests compared with *A alternata* extract, although a reduced reactivity was observed with rbAlt a 1. Specific IgE levels to nAlt a 1 or rAlt a 1 showed significant correlation and similar sensitivity and specificity.
Conclusions: Alt a 1, either in its natural or recombinant form, is sufficient for a reliable diagnosis of *A alternata* sensitization and induces skin prick reactivity comparable with that produced by *A alternata* extract. (J Allergy Clin Immunol 2005;115:1210-7.)

Key words: *Alt a 1, diagnosis, IgE, mold allergy, recombinant allergen, skin prick test*

Abbreviations used
EAST: Enzyme allergosorbent test
RA: Recombinant allergen
rbAlt a 1: Recombinant Alt a 1 expressed in bacteria *(Escherichia coli)*
ryAlt a 1: Recombinant Alt a 1 expressed in yeast *(Yarrowia lipolytica)*
SPT: Skin prick test

Fungal allergy is a worldwide problem because fungi grow almost everywhere, and exposure to allergenic molds can lead to IgE-mediated rhinitis and asthma and atopic dermatitis.[1] Depending on geographic and climate conditions, the prevalence of allergy to molds might be as high as 30%.[2] A recent study on 4962 respiratory patients showed that 19% of the allergic population reacted to at least one fungal extract, as determined by means of skin tests, and more than 66% of these fungal sensitized patients reacted to *Alternaria alternata* extract.[3] The diagnosis of IgE-mediated allergy is based on clinical history and skin test reactivity to appropriate allergen preparations corroborated by *in vitro* allergen-specific IgE determinations. *In vivo* and *in vitro* mold allergy diagnosis are performed with fungal extracts consisting of a complex mixture of proteins, glycoproteins, polysaccharides, and other substances; these extracts show a considerable variability as a result of interstrain genomic differences, different culture conditions, and variable extraction procedures.[4] Currently, there are no standardized fungal allergen products available in the United States,[5] and the quality of commercial fungal extracts in Europe is variable.[3,6] Thus recombinant mold allergens could be of great interest because recombinant allergens (RAs) can be produced in suitable purity and batch consistency and hence are a perfectly standardized diagnostic material. Diagnostic studies with allergenic molecule–based approaches for skin testing have been performed,[7-9] and first results on *A alternata* allergens in 7 patients were promising.[6] The use of purified proteins for *in vitro* assays has been more widely used because some pollen allergens are available as commercial reagents, allowing a component-resolved diagnosis approach for some of them[10-12] or evaluation immunotherapy by means of determination of specific antibodies toward purified allergens.[13]

More than 9 allergens have been described in *A alternata* extracts, although only 2 of them are major allergens. Alt a 1, the most relevant allergen in *A alternata* extracts, is a dimer of 29 kd that dissociates into 14.5- and

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16-kd subunits under reducing conditions[14] and reacts with serum IgE in 82% to 100% of *A alternata*–sensitized patients.[14] Alt a 2 is recognized by IgE antibodies of 16 (61%) of 26 individuals allergic to *A alternata*.[15] Minor *A alternata* allergens, such as Alt a 11 (the highly conserved fungal allergen enolase), Alt a 3, Alt a 4, Alt a 6, Alt a 7, Alt a 10, Alt a 12, and nuclear transport factor 2, have been also reported.[16-18]

The great majority of RAs used thus far in skin tests have been produced in *Escherichia coli*,[7] but because of some limitations related to the lack of posttranslational modifications and protein folding, alternative hosts, such as yeasts, baculovirus, and transgenic plants, have been used.[19] *Pichia pastoris* is the eukaryotic host most widely used in RA expression for skin tests,[7,20] but other nonconventional yeasts, such as *Yarrowia lipolytica*, could be used. This nonpathogenic yeast belongs to the generally recognized as safe (''GRAS'') class, showing favorable traits, such as strong and tightly regulated promoters, high-level secretion, and low-cost growing substrates, which make *Y lipolytica* a good alternative eukaryotic host.[21]

In the present study we compare the *A alternata* extract with purified natural Alt a 1 (nAlt a 1) and 2 recombinant Alt a 1 (rAlt a 1) from bacteria and yeast expression systems with regard to their capacity to bind specific IgE and to elicit skin prick test (SPT) responses in 42 patients with *A alternata* allergy.

METHODS

Patients

Forty-two patients with *A alternata* allergy (18 female and 24 male patients; mean age, 20.5 years; age range, 10-33 years) and 20 control subjects (13 female and 7 male patients; mean age, 32.3 years; age range, 14-70 years) were included in the study. The diagnosis of *A alternata* allergy was based on clinical history of recurrent rhinitis, positive SPT response to commercially available *A alternata* extracts (Bial-Arístegui, Bilbao, Spain), and specific IgE serum level to *A alternata* extract, as determined by using the Pharmacia ImmunoCAP System (Pharmacia Diagnostics, Uppsala, Sweden). Control subjects included 10 healthy subjects and 10 allergic individuals sensitized to different allergenic sources unrelated to *A alternata*, as demonstrated by negative SPT responses and lack of *A alternata*–specific IgE.

The study was approved by the Research Ethics Committee of the Hospital ''Vega Baja'' de Orihuela and Hospital General de Jaén. All patients and control subjects provided oral and written informed consent.

Cloning and expression of rAlt a 1

E coli–expressed Alt a 1 (rbAlt a 1) was produced as previously described.[22] For cloning Alt a 1 cDNA in *Y lipolytica*, cDNA encoding mature Alt a 1 was amplified by means of RT-PCR in a standard reaction with the following primers: A21F, 5′-GCGGATCCATGTCCTGCCTGTCACCAC (for the N-terminus), and A21R, 5′-GCGGATCCTTAAGAGCTCTTGGGGAGAG (for the C-terminus; *Bam*HI restriction sites are underlined). The PCR amplification product was digested with *Bam*HI, cloned into pMMR1, and introduced into the *Y lipolytica* genome under the control of a copper-inducible promoter, as described elsewhere.[23] For production of *Y lipolytica*–expressed Alt a 1 (ryAlt a 1), a 50-mL preculture of 0.7% YNB medium (Yeast Nutrient Broth; Hispanlab, Madrid, Spain) supplemented with 1% glucose, 0.2 mM uracile, and 0.3 mM histidine was inoculated with an isolated colony from a YNB agar plate and grown overnight at 28°C with vigorous agitation (280 rpm). Cells were collected by means of centrifugation at 3000*g* for 5 minutes in sterile conditions and resuspended in 1 L of the same medium at an OD_{600} of 0.5. When the culture reached an OD_{600} of 0.8 to 1, $CuSO_4$ was added at a final concentration of 0.4 mM, and the culture was continued for 24 hours. Spent culture medium was concentrated and diafiltrated by using a 5000-kd cut-off point.

Purification of nAlt a 1 and rAlt a 1

Both nAlt a 1 and *A alternata* commercial extract were obtained from spent culture medium after 3 weeks of growing on Czapeck broth media at 25°C. Spent culture medium was concentrated and diafiltrated by using a 5000-kd cut-off point. Purification of nAlt a 1 and ryAlt a 1 was achieved by means of immunoaffinity with an immunosorbent column prepared with rabbit anti-Alt a 1 polyclonal antibodies.[22] *A alternata* and *Y lipolytica* spent culture media were passed through the column, and after extensive washing with PBS, bound protein was eluted with 100 mM citrate buffer, pH 2.7. Fractions were collected in tubes containing neutralizing buffer (1 M Tris-HCl, pH 8.0), pooled, and concentrated by means of ultrafiltration. rbAlt a 1 was purified as previously described.[22] The yield of expression of recombinant proteins was about 0.5 mg and 40 mg per liter of culture for ryAlt a 1 and rbAlt a 1, respectively.

Purified rAlt a 215 was kindly donated by Dr R. K. Bush (University of Wisconsin-Madison), and rAlt a 11^{16} was purchased from Biomay (Vienna, Austria).

Electrophoresis and immunoblotting

A alternata extracts and purified Alt a 1 were separated by means of SDS-PAGE under reducing and nonreducing conditions.[24] For immunoblot studies, the separated proteins were transferred electrophoretically to Hybond P (Amersham Biosciences, Uppsala, Sweden). After blocking, membranes were incubated at 4°C overnight with human sera, and then washed and bound antibodies were detected with 1:10,000 horseradish peroxidase–conjugated anti-human IgE (Dako, Copenhagen, Denmark). Then the blot was washed and developed with the ECL western blotting detection system (Amersham Biosciences). For IgE dot-blot testing, 200 ng in 2 μL of PBS was dotted onto nitrocellulose (Hybond C+, Amersham Biosciences). After drying, the membrane was blocked and incubated as described above.

Determination of specific IgE levels and inhibition assays

Specific IgE levels to *A alternata* extract and Alt a 1–purified allergens were evaluated by means of the enzyme allergosorbent test (EAST), following the instructions of Hytec specific IgE EIA (Hycor Biomedical, Kassel, Germany). After preliminary testing, the optimal concentrations of *A alternata* extract (400 μg/mL) and Alt a 1 (50 μg/mL) were coupled to cyanogen bromide–activated paper discs.[25] For EAST inhibition assays, 50 μL of human sera (diluted 1:4) were preincubated with 50 μL of 10-fold serial dilutions of inhibitors. Allergen discs were added to the tubes and incubated at 37°C for 1 hour, and bound IgE was detected as mentioned above. ELISA inhibition assays were carried out in microtiter plates (Greiner, Frickenhausen, Germany), which were coated with 2 μg/mL Alt a 1 in 0.1 M bicarbonate buffer, pH 9.6, and blocked with 1% BSA and 0.05% (vol/vol) Tween 20 in PBS. One hundred microliters of human sera (diluted 1:4) preincubated at 4°C overnight with each inhibitor

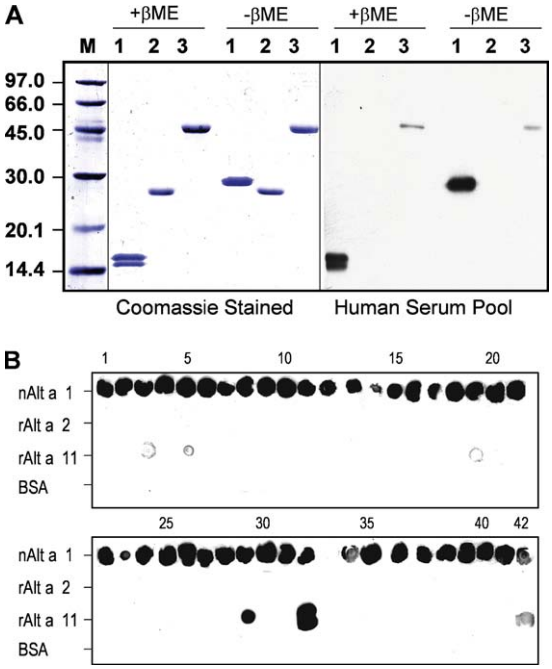

FIG 1. A, Coomassie-stained SDS-PAGE and immunoblots of *A alternata* allergens. nAlt a 1 *(lanes 1)*, rAlt a 2 *(lanes 2)*, and rAlt a 11 *(lanes 3)* were run in the presence and absence of β-mercaptoethanol *(βME)*. Immunoblots were incubated with a human serum pool of patients with *A alternata* allergy. **B**, Dot blots for nAlt a 1, rAlt a 2, rAlt a 11, and BSA spotted onto nitrocellulose membrane. Dot blots were incubated with individual sera from patients with *A alternate* allergy.

were added to the plates, incubated for 1 hour at 37°C, and developed as previously described.[26]

SPTs with *A alternata* extract, nAlt a 1, and rAlt a 1

SPTs were performed with a commercial *A alternata* extract (605 mg/mL protein, Bial-Arístegui), nAlt a 1, and 2 types of rAlt a 1 (rbAlt a 1 and ryAlt a 1). The individual proteins were diluted in NaCl 0.9% at concentrations of 1, 10, and 100 μg/mL. Histamine hydrochloride, 10 mg/mL, and NaCl 0.9% served as positive and negative controls. All skin tests were performed in duplicate on the volar forearm and applied in 2 opposite directions with a prick needle (Morrow-Brow, Derby, United Kingdom). Twenty microliters of allergen solution at each concentration was used to perform *in vivo* titrations. The sizes of the elicited wheals were determined by means of computerized planimetry. Wheal surface areas of greater than 7 mm^2 were considered positive.

Statistical analysis

Statistical analysis was performed by using the Wilcoxon test, and correlation coefficients were determined by means of linear regression. A P value of less than .05 was considered significant.

RESULTS

Alt a 1 is a marker for *A alternata* allergy

The presence of IgE against 3 major *A alternata* allergens (Alt a 1, Alt a 2, and Alt a 11) was evaluated, as a preliminary approach, by immunoblotting with a pool of sera from patients with *A alternata* allergy (Fig 1, *A*). In reducing conditions a faint IgE-reactive signal was obtained at 45 kd with Alt a 11, whereas a strong signal was found at 15 to 16 kd, corresponding to Alt a 1. No detectable IgE reactivity was observed for Alt a 2. This finding was corroborated by means of EAST inhibition, showing that Alt a 11 and Alt a 2 merely inhibited less than 12% of the IgE-binding capacity of serum to the *A alternata* extract in EAST inhibition. In contrast, Alt a 1 is able to inhibit 74% of the IgE-binding capacity of the serum pool.

The prevalence of purified allergens Alt a 1, Alt a 2, and Alt a 11 were tested by means of IgE dot-blot testing with sera from 42 patients with *A alternata* allergy. Forty-one sera reacted with Alt a 1, 6 with Alt a 11, and none with Alt a 2 (Fig 1, *B*), confirming previous data with a pool of sera. Therefore we focused on Alt a 1 as a marker for *A alternata* allergy by means of skin testing and serum-specific IgE determinations.

nAlt a 1–specific IgE from patients with *A alternata* allergy recognizes epitopes on its recombinant counterparts

Reagents used in this work were checked with SDS-PAGE and immunoblotting against a pool of sera from patients with *A alternata* allergy (Fig 2, *A*). nAlt a 1 and rAlt a 1 migrate as a 30-kd protein under nonreducing conditions, but under reducing conditions, natural protein breaks down into 16.4- and 15.3-kd subunits, and recombinant protein breaks down into 15.5-kd polypeptides. Purified proteins were subjected to periodic acid–Schiff staining for detection of glycoproteins, with the result that none of them showed a detectable amount of sugar.

The sera of the 42 patients with *A alternata* allergy, as determined by means of EAST, contained IgE antibodies against *A alternata* extract (Table I). Forty-one of these patients showed IgE antibodies to nAlt a 1 and its recombinant counterparts. EAST inhibition experiments were performed to compare the IgE-binding capacity of both forms of rAlt a 1. nAlt a 1 and rAlt a 1 were able to inhibit 69% to 74% of the IgE-binding capacity of the sera pool to *A alternata* extract in corroborating the dominant role of Alt a 1 as an IgE-binding component of *A alternata* extract (Fig 2, *B*). This result suggested that a number of conformational epitopes recognized by the patients' IgE were present in both rAlt a 1.

Specific IgE levels to *A alternata* extract and each purified allergen showed a significant correlation (see Fig E1 in the Journal's Online Repository at www.mosby.com/jaci), confirming that Alt a 1 corresponds to a major allergen. IgE-binding values for *A alternata* extract and nAlt a 1 and for both recombinant versions showed no significant differences, whereas with most of the sera, IgE levels for nAlt a 1 were slightly higher than those obtained for rAlt a 1. A significant correlation was also found between specific IgE levels to natural and recombinant allergens (see Fig E1 in the Journal's Online Repository).

IgE binding to Alt a 1 and its recombinant counterparts was quantitatively evaluated in ELISA inhibition experiments performed by coating wells with nAlt a 1 and using sera from 3 individual patients. The 3 allergens displayed the same extent of binding inhibition to IgE antibodies, indicating that they share allergenic determinants (data not showed).

rAlt a 1 allergens elicit comparable SPT reactivity than nAlt a 1 and *A alternata* extract

The whole group of patients with *A alternata* allergy showed positive skin reactions to *A alternata* extract and to 100 μg/mL of nAlt a 1 and rAlt a 1 (Table I and Fig 3). At this concentration, nAlt a 1 reactivity was not significantly different from that of rbAlt a 1 but significantly higher than that of ryAlt a 1 ($P < .01$). At a concentration of 10 μg/mL, the number of positive SPT responses with rbAlt a 1 decreased to 32 of 42, whereas all patients reacted with nAlt a 1 and ryAlt a 1. There was a slight but significantly decreased reactivity to nAlt a 1 compared with ryAlt a 1 ($P < .05$) that was markedly reduced in rbAlt a 1 ($P < .001$). Thirty-four patients had a positive SPT response at 1 μg/mL of nAlt a 1, 23 patients had a positive SPT response with ryAlt a 1, and only 11 of 42 reacted with rbAlt a 1. SPT reactivity of the extract was higher than that of purified allergens at 1 and 10 μg/mL but was the lowest at 100 μg/mL.

There was a significant statistical correlation (R value range, 0.47-0.75; $P < .01$) between the wheal surface area produced by *A alternata* extract, nAlt a 1, rbAlt a 1, and ryAlt a 1 (data not shown). The best correlation was found between nAlt a 1 and ryAlt a 1. No positive reaction was observed in unrelated allergic or healthy control subjects, either to the extract or to the purified allergens. No adverse side effects were observed in SPTs in all the subjects tested.

Additionally, the geometric mean of the wheal sizes at corresponding dilutions of nAlt a 1 and rAlt a 1 was calculated. The concentration of nAlt a 1 that induced a wheal surface area of the same size as the median wheal size produced by histamine (10 mg/mL) was 2.91 μg/mL, 5.81 μg/mL for ryAlt a 1, and 14.63 μg/mL for rbAlt a 1. The concentration of *A alternata* extract that produces the same effect was 3.27 mg of dry weight per milliliter.

High specificity and sensitivity of SPT and humoral responses with purified Alt a 1

Specificity and sensitivity results of skin test and specific IgE determinations are shown in Table II. In SPTs no false-positive results were detected with any purified Alt a 1, thus confirming the high specificity of the test. One hundred percent sensitivity was found at 10 μg/mL for nAlt a 1 and ryAlt a 1, whereas rbAlt a 1 showed a 76% sensitivity. This RA reached 100% sensitivity at 100 μg/mL.

Regarding the use of purified allergens as an *in vitro* diagnostic for *A alternata* sensitization with EAST, only one false-negative reaction was observed (Table II). On the other hand, a similar number of false-positive reactions (5/20) were detected with *A alternata* extract, as well as with purified allergens. The low amount of specific IgE bound in most of the false-positive cases stands out (Table I).

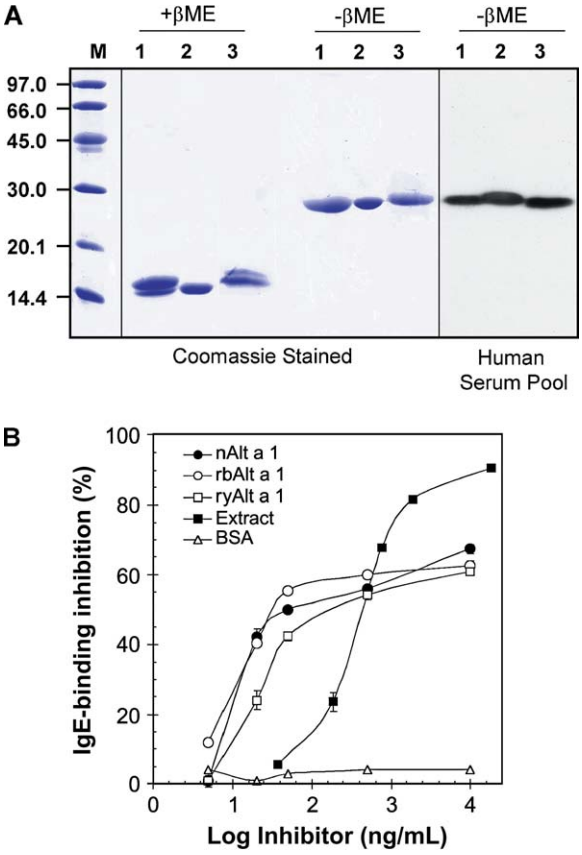

FIG 2. A, Coomassie-stained SDS-PAGE and immunoblot of *A alternata* allergens. nAlt a 1 *(lanes 1)*, rbAlt a 1 *(lanes 2)*, and ryAlt a 1 *(lanes 3)* were run in the presence and absence β-mercaptoethanol *(βME)*. Immunoblots were incubated with a human serum pool of patients with *A alternata* allergy. **B**, EAST inhibition curves. The binding of human IgE to *A alternata* extract was inhibited with the following: *A alternata* extract, nAlt a 1, rbAlt a 1, and ryAlt a 1. Values represent means ± SD.

DISCUSSION

Sensitization to *A alternata* is a risk factor for the development of wheezing and asthma and has been associated with severe cases of asthma and respiratory arrest.[1] RAs offer the possibility of component-resolved diagnosis by means of classical *in vivo* and *in vitro* diagnosis[7,19] and other more recent methods with microarrayed allergen molecules on glass chips for monitoring allergic patients' IgE reactivity profiles in a single measurement.[27] Over the last 10 years, about 15 studies have been performed with fungal RA for skin testing,[7] and only one study was done with *A alternata* allergens in 7 patients.[6] The present study is the first report where *in vivo* and *in vitro* allergenicity of 2 types of rAlt a 1 were

TABLE I. *In vivo* and *in vitro* pattern of sensitization to *A alternata* whole extract and purified allergens

Patient no.	Symptoms	Additional sensitization	*A alternata* extract		nAlt a 1		rbAlt a 1		ryAlt a 1	
			IgE (kU/L)	SPT, 605 μg/mL (mm^2)	IgE (kU/L)	SPT, 100 μg/mL (mm^2)	IgE (kU/L)	SPT, 100 μg/mL (mm^2)	IgE (kU/L)	SPT, 100 μg/mL (mm^2)
1	RA	T,M	34.2	19.5	56.7	33.6	64.7	47.6	14.3	31.8
2	RCA	–	6.7	38.9	5	168	2.9	93.6	4.1	97.3
3	RC	T,M,G,W,O	5.8	84.7	4.9	113	2	114	2.2	68.6
4	RCA	T,M,G	15.7	59.6	13.7	99.3	6.2	77.7	13.9	104
5	A	T	6	41.5	10.4	38.9	5.6	61.1	5.3	33.6
6	RC	T,M,G,W,D	13.2	41.3	15.7	58.1	10.3	104	11.1	47.2
7	RA	T,M,O	3.8	44.7	3.3	72.7	1.4	59.7	2	59.3
8	RC	W	3.3	29	4	48.1	2.6	49.4	2.7	35
9	RA	–	5.8	57	6.1	98	2.8	157	4	70.4
10	RA	T,M	9	45.8	13.4	87.4	11.2	99.8	12.9	127
11	RC	–	1.6	23.5	3.7	67.4	1.6	32.9	2.5	40.1
12	RCA	W	1.6	63.7	2.2	79.4	1	75.7	1.4	89.7
13	R	T,M,G,W,D	6.5	31.5	8.5	63.3	4.7	72	6.5	40.9
14	RC	W,D	1	48.9	1.5	61	0.6	81.3	1	75.2
15	RC	F	4.5	16.2	8.2	45.6	10.3	44.3	5.1	31.7
16	RC	O	6	24.2	23.8	32.6	70.1	61.6	13.9	55.2
17	RC	T,G,W,D	3.1	31.1	3.1	48.2	1.9	49.5	1.2	31.7
18	RCA	T,G,W,D	10.3	29.9	9.4	31.6	6.6	40.8	5.5	38.4
19	RCA	T,M,W	6.5	34	5.5	182	5	121	4.2	109
20	RA	G	13.2	90.5	11.8	149	9.5	113	6.6	160
21	RCA	T	9.4	53.2	10.2	81.3	6.9	113	7.4	88.3
22	RC	W	4.5	63.5	7.2	81.5	4.8	122	5.9	106
23	RC	G,D	4.3	34.1	2.9	73	2.1	42.9	2.5	45.6
24	RCA	T,M,W	9.7	30.9	11.2	54.9	8.1	41.3	6.8	62
25	RA	T	5.8	50.1	6.9	73.8	6.9	73.6	7.4	54.9
26	RC	T,M,W	7.7	76.5	8.8	90.3	6	35	7.2	47.3
27	RC	T,G,W	1.7	56.1	2.3	57.8	1.6	57.8	1.4	36.8
28	R	D	2.4	38.2	0.8	114	1	66.3	1.4	78.6
29	RCA	T,G,W	6.9	174	1.4	108	1.4	74.6	0.9	105
30	RCA	T,G,W	12.7	127	13.9	155	7.4	129	8.9	110
31	RA	M	5.2	69.1	10.7	33.3	5.2	79	8.4	44.5
32	RA	T,G,W	2.9	114	2	54.4	1.2	141	1.5	93.7
33	R	–	0.4	76.8	<0.35	55.6	<0.35	76.2	<0.35	32.6
34	RCA	T,G,W,D	2.2	106	2.2	170	0.8	143	1	147
35	RA	T,G,D	62.3	162	26.3	250	29.2	133	22.3	115
36	RA	T,G,D	27.2	79.8	14.7	216	9.1	121	12.8	211
37	A	T,W,M	6.7	109	9.5	187	5.8	93.7	6.2	150
38	RA	T,G,M	5.9	166	6.9	98.1	2.6	110	1.5	86.1
39	A	T	4.9	81.1	6.8	55.6	4.6	133	3.9	77.4
40	RA	T,G	8	153	8.4	87.3	5.5	146	6.1	100
41	A	T,G,W	3.2	113	3.7	190	2	119	2.4	67.5
42	R	T,G	0.6	48	0.7	60.7	0.5	41.3	0.8	39.3
43	Control	T,M,G,W	<0.35	0	<0.35	0	<0.35	0	<0.35	0
44	Control	T,W	<0.35	0	<0.35	0	<0.35	0	<0.35	0
45	Control	T,G,W,D	<0.35	0	<0.35	0	<0.35	0	<0.35	0
46	Control	T,G,W,O	<0.35	0	0.5	0	<0.35	0	0.5	0
47	Control	G,W,O	1.7	0	2.5	0	1.1	0	1.5	0
48	Control	T,M,G,W,D	<0.35	0	<0.35	0	<0.35	0	<0.35	0
49	Control	T,G	0.5	0	<0.35	0	1.4	0	0.5	0
50	Control	T,W	<0.35	0	<0.35	0	<0.35	0	<0.35	0
51	Control	M,D	<0.35	0	<0.35	0	<0.35	0	<0.35	0
52	Control	T,G,W	<0.35	0	<0.35	0	<0.35	0	<0.35	0
53	Healthy	–	<0.35	0	<0.35	0	<0.35	0	<0.35	0
54	Healthy	–	<0.35	0	<0.35	0	<0.35	0	<0.35	0
55	Healthy	–	<0.35	0	<0.35	0	<0.35	0	<0.35	0

(continued)

TABLE I. (continued)

Patient no.	Symptoms	Additional sensitization	*A alternata* extract IgE (kU/L)	*A alternata* extract SPT, 605 μg/mL (mm^2)	nAlt a 1 IgE (kU/L)	nAlt a 1 SPT, 100 μg/mL (mm^2)	rbAlt a 1 IgE (kU/L)	rbAlt a 1 SPT, 100 μg/mL (mm^2)	ryAlt a 1 IgE (kU/L)	ryAlt a 1 SPT, 100 μg/mL (mm^2)
56	Healthy	–	<0.35	0	0.5	0	<0.35	0	<0.35	0
57	Healthy	–	0.5	0	<0.35	0	<0.35	0	<0.35	0
58	Healthy	–	3.2	0	4.5	0	2.5	0	3	0
59	Healthy	–	1.2	0	1.9	0	1.2	0	1.1	0
60	Healthy	–	<0.35	0	<0.35	0	<0.35	0	<0.35	0
61	Healthy	–	<0.35	0	<0.35	0	0.5	0	<0.35	0
62	Healthy	–	<0.35	0	<0.35	0	<0.35	0	<0.35	0

RA, Rhinitis plus asthma; *T*, tree pollen; *M*, mites; *RCA*, rhinoconjunctivitis plus asthma; *RC*, rhinoconjunctivitis; *G*, grass pollen; *W*, weed pollen; *O*, other molds; *A*, asthma; *D*, animal dander; *R*, rhinitis; *F*, foods.

compared with the reactivity of the natural counterpart in 42 patients allergic to *A alternata*.

The relevance of Alt a 1 were established by performing *in vitro* tests, such as dot blots and EAST, to study the prevalence of IgE binding to the reported important *A alternata* allergens Alt a 1, Alt a 2, and Alt a 11.[15,16,28] In our cohort of 42 patients, Alt a 1, Alt a 2, and Alt a 11 were recognized by 98%, 0%, and 15% of patients with *A alternate* allergy, respectively. Previously reported prevalence values of IgE binding to Alt a 1 range from 80% to 95%,[28] and IgE reactivity of Alt a 11 in our study is close to the reported prevalence of sensitivity to this allergen by means of SPTs and *in vitro* of 28% and 22%, respectively.[6,16] In contrast, the prevalence of IgE binding to Alt a 2 in 26 patients with *A alternate* allergy from the United States was 61%,[15] whereas no patients in our study had serum IgE antibodies to this allergen. The different prevalence in both studies is currently unclear and requires further study. Alt a 2 is present in a very low amount in the *A alternata* extract,[15] and therefore the extract used in our study could be underrepresented in Alt a 2, and some patients who would react only to this allergen might not have been included in our study. On the basis of these results, we chose Alt a 1 as a marker of sensitization to *A alternata*. In a similar way, antibody responses to Alt a 1 have been used to distinguish patients with high IgE levels who are reactive to *A alternata* with asthma from those with atopic dermatitis.[29]

Reliable *in vivo* and *in vitro* diagnosis of *A alternata* allergy is possible with nAlt a 1 and rAlt a 1. All the patients with *A alternata* allergy could be detected by means of skin testing with 10 μg/mL nAlt a 1 or ryAlt a 1 and 100 μg/mL rbAlt a 1. No false-positive results were obtained with control patients, even at the highest concentration. The concentration of purified nAlt a 1 that induced a wheal surface area of the same size as the medium wheal size produced by histamine was 2.91 μg/mL, very close to the Alt a 1 content (2.78 μg/mL) of the *A alternata* extract that produces the same effect.[22] Taking into account that a skin prick diagnostic preparation has 5 times the concentration corresponding to a wheal size

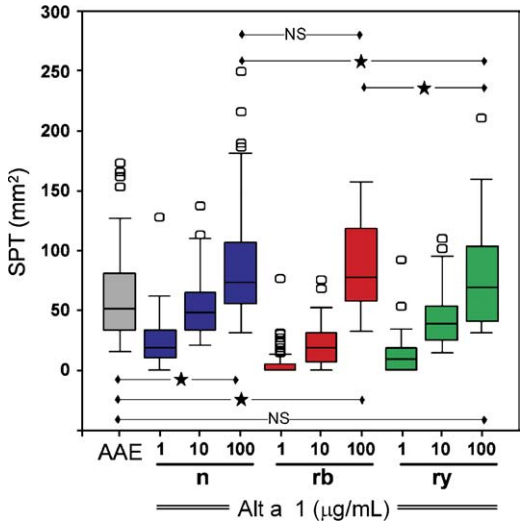

FIG 3. SPT with *A alternata* extract *(AAE)*, and 3 different concentrations of purified natural and rAlt a 1 are shown. Values are given as means of wheal surface areas. *Difference significant at $P < .05$. *NS*, Not significant.

produced by histamine, both values are near the concentration of nAlt a 1 (10 μg/mL) with highest sensibility and specificity (Table II). RAs, when produced by using appropriate strategies and systems, can be used for allergy diagnosis. RAs have been produced in a variety of different expression systems, depending of the nature of each particular allergen (protein folding and glycosylation). Some RAs, such as Ole e 1, Cyn d 1, and Der p 1, are very dependent of the expression systems used because of glycosylation and lack of native conformation of recombinant protein produced.[30] In our case both systems, prokaryotic and eukaryotic, seem to be similar as follows: (1) Alt a 1, as reported by de Vouge et al[14] and confirmed in this work, is not a glycosylated protein; (2) experiments on circular dichroism spectroscopy demonstrated that the 3 Alt a 1 proteins showed identical secondary structure (J. A. Asturias, unpublished results); and (3) no major

TABLE II. Sensitivity and specificity of *A alternata* extract and purified Alt a 1 preparations by using skin tests and specific IgE determination

	A alternata extract		SPT concentration	nAlt a 1		ryAlt a 1		rbAlt a 1	
	Sensitivity*	Specificity†	(μg/mL)	Sensitivity*	Specificity†	Sensitivity*	Specificity†	Sensitivity*	Specificity†
			1	81%	100%	55%	100%	26%	100%
SPT	100%‡	100%‡	10	100%	100%	100%	100%	79%	100%
			100	100%	100%	100%	100%	100%	100%
Specific IgE	100%	75%		98%	75%	98%	75%	98%	75%

*n = 42 allergic patients.
†n = 20 control patients.
‡Commercial *A alternata* extract preparation (16.35 mg dry weight per milliliter, Bial-Arístegui).

differences on serum IgE or skin reactivity have been shown among nAlt a 1 and rAlt a 1. Taking into account the great differences between expression yields of both systems (0.5 mg and 40 mg per liter of culture for yeast and bacterial systems, respectively), expression of Alt a 1 in *E coli* seems to be more convenient. Specific immunotherapy on the basis of RA could be particularly suitable when the number of allergens involved is limited, and vaccination with rBet v 1, the main elicitor of birch pollen allergy, is under clinical evaluation.[31]

In summary, this study has demonstrated that diagnosis of *A alternata* allergy can be simplified by using a single allergen, Alt a 1, from either natural or recombinant sources, and future specific immunotherapy on the basis of this allergen should be considered.

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