Original Article

Biological standardization and maximum tolerated dose estimation of an *Alternaria alternata* allergenic extract

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Abstract. *Background:* The manufacture of allergenic extracts from the mold *Alternaria alternata* is influenced by factors such as strain variability, allergenic origin, culturing conditions and extraction process, which affect the reproducibility of the preparations intended for diagnostic and therapeutic use.
Objectives: To select the most adequate antigenic source of *A. alternata* extracts and determine its maximum tolerated dose (MTD) to be used in a subsequent immunotherapy efficacy clinical trial.
Methods: Twenty-one patients monosensitized to *A. alternata* were involved in a biological standardization process of *A. alternata* extracts. Four different mold strains were cultured and used to produce extracts by three different methods, each incorporating proteins from different origins: culture filtrate, buffer extractable fraction and cellular antigens. The selected extract, characterized as in-house reference (IHR) preparation was used in a MTD finding immunotherapy study. Serum IgE, IgG, IgG1 and IgG4 specific of complete extract and purified natural and recombinant forms of Alt a 1 were determined by different EIA methods.
Results: Culture filtrate extract containing the allergens secreted to the spent medium was shown to be the most adequate option for establishing an IHR preparation for *A. alternata* extract manufacturing. A maximum dose of 1670 UBE, equivalent to 0.1 µg Alt a 1, was determined as MTD for immunotherapy. One year of administration of such a dose at monthly intervals elicited pronounced immunological changes with statistically significant decreases in IgE and increases in IgG4, both estimated with whole extract or purified Alt a 1.
Conclusion: A high quality natural *A. alternata* extract has been developed and preliminarily tested to define its MTD for subsequent determination of the optimal dose in an immunotherapy efficacy clinical trial.

Keywords: Mold allergy, mold immunotherapy, specific antibodies, in-house reference, Alt a 1, recombinant allergens.

Resumen. La fabricación de extractos alergénicos del hongo *Alternaria alternata* se ve influida por distintos factores, como la variabilidad de la cepa, el origen del alérgeno, las condiciones de cultivo y el proceso de extracción, que afectan a la reproducibilidad de las preparaciones destinadas para uso diagnóstico y terapéutico.
Objetivos: Seleccionar la fuente antigénica más adecuada de los extractos de *A. alternata* y determinar la dosis máxima tolerada (DMT) que deberá utilizarse en los posteriores ensayos clínicos de eficacia de la inmunoterapia.
Métodos: Se incluyeron 21 pacientes monosensibilizados a *A. alternata* para participar en un proceso de estandarización biológica de extractos de *A. alternata*. Se cultivaron cuatro cepas de hongos diferentes y se utilizaron para producir extractos mediante tres métodos distintos, cada uno de los cuales incorporó proteínas de orígenes diversos: filtrado de cultivo, fracción extraíble con solución amortiguadora y antígenos celulares. El extracto seleccionado, caracterizado como preparación de referencia interna, se ha utilizado en un estudio de determinación

de DMT en inmunoterapia. Las IgE, IgG, IgG1 e IgG4 séricas específicas del extracto completo y las formas recombinantes y naturales purificadas de Alt a 1 se determinaron mediante distintos métodos de enzimo-inmunoensayo.
Resultados: El extracto de filtrado de cultivo que contenía los alérgenos secretados en el medio de crecimiento demostró ser la opción más adecuada para establecer una preparación de referencia interna para la fabricación del extracto de *A. alternata*. Se determinó una dosis máxima de 1.670 UBE, equivalente a 0,1 µg de Alt a 1, como DMT para inmunoterapia. Un año de administración con esta dosis a intervalos mensuales puso de manifiesto cambios inmunológicos destacados con reducciones de IgE y aumentos de IgG4 estadísticamente significativos, ambos estimados con el extracto completo o Alt a 1 purificado.
Conclusión: Se ha desarrollado un extracto de *A. alternata* natural y de alta calidad, que fue probado previamente para definir su DMT para una posterior determinación de la dosis óptima en un ensayo clínico de eficacia de la inmunoterapia.

Palabras clave: alergia a hongos, inmunoterapia con hongos, anticuerpos específicos, referencia interna, Alt a 1, alérgenos recombinantes.

Introduction

Alternaria alternata is one of the molds most frequently found as causative agent of type I allergies in indoor and outdoor environments, particularly in regions with a warm climate. Sensitivity to *A. alternata* is a common cause of asthma [1, 2], but also upper respiratory tract symptoms can be developed when high spore levels are present in environmental air [3]. Sensitization to the fungus *A. alternata* has been recognized by a number of studies. Thus in a large epidemiological study performed in a population of the United States, it was found that 3.6% of the assayed patients were sensitized to this mold [4]. A similar figure (4%) was reached in Northern Europe, as described in a Scandinavian study [5]. In Southern Europe, the level of sensitization to *A. alternata*, evaluated by skin prick test, has been reported as variable, but clearly relevant, varying from approximately 3% of the patients in Portugal (positive to either *Alternaria* or *Cladosporium*) to 20% in Spain [6]. Recently, an epidemiological study in a large sample of patients with respiratory tract symptoms suggestive of allergic disease (from Italy and Austria) showed that 19% of patients were sensitized to fungi and 66% of them were positive to *A. alternata* [7]. The great variability in prevalence could be due to many factors such as exposure conditions, geoclimatic areas, type of population, but also to different characteristics of diagnostic extracts [8].

The quality of allergenic extracts intended for diagnosis and treatment of allergic patients are always affected by the lack of reproducibility of biological raw materials, this drawback being dramatically increased in fungal sources, were selection of antigenic origin, strain variability, and extraction process play a key role.

In a previous paper [9] we preliminarily studied different obtainment methods which were not validated through the activity of the produced extracts by *in vivo* reactivity in allergic patients. The aim of the present work was to select the most appropriate antigenic source of *A. alternata* by means of a biological standarization program, and to estimate the MTD in order to obtain a safe preparation for immunotherapy.

Abbreviations:

ALT:	whole *A. alternata* allergenic extract
ASIT:	Allergen specific immunotherapy
BEA:	buffer extractable antigens
CA:	cellular antigens
CBrN:	cyanogen bromide
CF:	culture filtrate
CPT:	conjunctival provocation test
DBPC:	double blind placebo controlled
EAACI:	European Academy of Allergology and Clinical Immunology
EAST:	enzyme allergosorbent test
ELISA:	enzyme linked immunoassay
IHR:	in-house reference
LSR:	late systemic reaction
MTD:	maximum tolerated dose
PBS:	phosphate-buffered saline
PBS-B-T:	PBS-1% BSA-0.05% Tween 20
RC:	rhinoconjunctivitis
SPT:	skin prick test
SR:	systemic reaction
TBS:	tris-buffered saline
UBE:	equivalent biological units

Material and Methods

Patients

The study was carried out in the section of Allergy of Hospital Virgen del Camino in Pamplona (Spain). The protocol was approved by the hospital Committee of Investigation and Ethics, and all subjects gave informed consent.

For the biological standardization program, twenty-one allergic patients (age over 14) monosensitized to *A. alternata* with rhinitis and/or bronchial asthma were included for cutaneous test evaluation (Prick test end point titration), and 5 ml serum of each were collected for immunochemical characterization. Patients were recruited

consecutively from those attending our outpatient clinic and fulfilling the inclusion criteria for biological standardization as outlined by S. Dreborg [10].

For MTD determination, ten out of the twenty-one patients who participated in the biological standardization program were randomly selected to follow a 1-year immunotherapy period with the *A. alternata* extract. Patients not disposed to regular attendance at our ASIT (Allergen Specific Immunotherapy) unit and those with contraindications for ASIT [11] were excluded from the study.

Source materials and extract production

Four different strains of *A. alternata* were grown on a synthetic medium (Czapek) and used for allergen extraction. Three of them were purchased from Centraalbureau voor Schimmelcultures (CBS), and the fourth was supplied by Dr. José María Torres, Instituto Municipal de Investigación Médica (IMIM, Barcelona, Spain). Extraction was carried out, basically, according to the procedure previously described [8]. Three different allergenic sources were used in order to compare their respective extracts: culture filtrate (CF) containing metabolic antigens, unbroken mycelium-spores with periplasmatic antigens extracted with ammonium bicarbonate, namely buffer extractable fraction (BEA) and broken mycelium-spores containing somatic proteins or cellular antigens (CA). The overall production flow-chart is depicted in Figure 1. Briefly, stationary liquid cultures in Czapek medium from the different *A. alternata* strains were left to grow at 25ºC for 21 days. After the incubation time, the liquid medium was filtered (Whatman Nº 1 followed by clarifying glass fiber filters), concentrated and dialyzed by ultrafiltration (Pellicon system, Millipore, Bedford, MA, USA) with a 5,000 Da cut-off point. Afterwards, the extract was subjected to sterilizing filtration (0.22 mm pore diameter membrane, Millipore) and lyophilization, giving rise to the CF extract. BEA was obtained from the mycelium-spores retained by the initial filtration step. The math was cut into small pieces and then stirred and suspended in 0.125 M ammonium bicarbonate, pH 7.5 at 4ºC overnight. Afterwards, the solubilized proteins were separated by centrifuging for 30 min at 12,000 xg. Finally, the crude extract was filtered, dialyzed, freezed and lyophilized as described above. CA was prepared from the myceliar sediment remaining after BEA extraction by homogeneization in a blade shear (VirTishear-25, VirTis Inc, Gardiner, NY, USA) at 25,000 rpm for 5 min cycles and at 4ºC. The mixture was then subjected to ultrasounds for 5 min at 90% (Ultrasonador Branson, MO, USA). Afterwards, the suspension was magnetically stirred at 4ºC for 30 min and centrifuged at 12,000 xg, 4ºC for 30 min. The resulting pellet was discarded and the supernatant further processed as in CF and BEA.

Biological standardization program

Quantitation of biologic activity of the *A. alternata* extracts was done by cutaneous testing. The twenty-one enrolled patients, showing wheal diameter >3 mm and specific IgE titre >0.7 kU/L to *A. alternata* extract were subjected to skin end point titration through the skin prick test (SPT) method, performed according to the guidelines of the Subcommittee on Skin Test of the EAACI [12]. All drugs that might have modified the results were carefully avoided. Skin tests were carried out in duplicate with four concentrations of *A. alternata* extracts (0.5, 1, 2 and 5 mg/ml of freeze-dried material). Extracts were reconstituted in a phenolated saline solution (0.9% NaCl, 0.4% phenol) containing 50% glycerol (v/v). Negative (saline) and positive (histamine dihydrochloride, 10 mg/ml) controls were also used. Wheal areas were recorded after 15 min, transferred to a translucent tape, and later measured by digitalization by means of computer-aided design software (AutoCAD v.11).

The dose response curve, i.e., the relationship between allergen concentrations and the wheal area produced was calculated using the following model: Log Y = k + m Log X, where "Y" is the geometric mean of the wheal area size, "X" is the concentration of allergenic extract, constant "K" is the intercept with the Y-axis, and "m" is the slope of the line. The logarithmic transformation of the skin response normalizes the distribution of wheal areas and homogenizes their variances over the response interval.

Ten atopic patients without allergy to molds and ten non-atopic patients were tested as controls. Sera were collected from selected patients and pooled to be used in all the IgE based standardization analytical techniques. After biologic activity quantitation, allergenic extracts were kept in freeze-dried aliquots and labelled as IHR. From the dose response curves a SPT value (in mg/ml) was interpolated and defined as the extract concentration eliciting a wheal area equal to that produced by the histamine reference in the same population. In our system, this figure is arbitrarily multiplied by 10,000 to obtain the unitage of biological activity of the IHR, i.e., UBE/ml (Equivalent Biological Units per milliliter), which is used to label the products equilibrated with respect to the internal reference by IgE-based methods [13].

SDS-PAGE Immunoblotting

SDS-PAGE was carried out by the method of Laemmli [14].

Respectively, 12.5% and 3% polyacrylamide concentrations were used for separating and stacking gels. Proteins dissolved in 0.125 M HCl-Tris, pH 6.8 were dissociated with 0.1% SDS and 5% β-mercaptoethanol by treatment at 100ºC during 5 min. Twenty mg protein, estimated by the Bradford [15] method, was applied per lane. After electrophoresis, gels were stained by diffusion in 0.1% Coomassie Brilliant Blue R-250 dissolved in methanol/acetic acid/distilled water (4:1:5). Destaining was also performed by diffusion in the same mixture without dye. Separated protein bands were electrophoretically transferred to Immobilon-P® membranes (Millipore,

Billerica, MA, USA), essentially by the method of Towbin et al. [16].

Immunochemical staining was performed by first blocking the Immobilon-P® membrane with 9% defatted dry milk in Tris-buffered saline (TBS) (1h at 37ºC). After three washes with TBS, the blots were incubated with 2.5 ml of undiluted human serum (16 h at 4ºC). The blots were washed 3 times with TBS and incubated with 2.5 ml of 1:25 peroxidase-conjugated rabbit immunoglobulins to human e-chains (Dako, Glostrup, Denmark). Bound peroxidase was detected by the addition of 3 ml of freshly prepared 0.06% w/v of 4-chloro-1-naphthol solution (Bio-Rad Laboratories, Richmond, Calif., USA) and 0.01% H_2O_2 in TBS. After 30 min, the blots were rinsed in distilled water and air-dried. Antigen-antibody binding was identified by purple stain deposition [17].

Alt a 1 determination

The content of Alt a 1 allergen in the CF extract used as in-house reference extract for MTD estimation was measured by a monoclonal antibody based ELISA (INDOOR Biotechnologies Inc, Charlottesville, VA, USA) as described in Vailes *et al.* [18].

Purified allergens for ELISA (nAlt a 1, rAlt a 1)

Natural and recombinant forms of the major allergen Alt a 1 were purified by liquid chromatographic methods according to previously published procedures [19].

Specific immunotheraphy: Maximum tolerated dose (MTD)

The study was designed as an open-dose titration trial. Ten adult patients (6 male, 4 female) (mean age 17.4 years, range 14-23 years) with a positive history of rhinitis (2 of them) or rhinitis plus asthma (8 of them) due to sensitization to *A. alternata* proved by positive SPT and specific serum IgE, were enrolled.

MTD was defined as the highest allergenic dose not inducing a systemic reaction, or the one just before that inducing three consecutive large local reactions [20]. The treatment induction phase schedule was a 14-week conventional depot administration. This dose increment schedule is presented in Table 1. Patients continued ASIT for one year with 1 ml administration of the MTD at monthly intervals.

A. alternata vaccines for immunotherapy, which were supplied by Bial-Arístegui (Bilbao, Spain), contained CF extract in depot presentation, i.e., adsorbed in aluminium hydroxide gel (Allergovac® depot). Maximum dose to be tested was initially the concentration of extract giving rise to a wheal equivalent to 10 mg/ml histamine (1 SPT).

The safety of ASIT was monitored according to EAACI guidelines [11]. All doses of ASIT were administered in the ASIT Unit. After allergen injections, the patients remained in the clinic for 30 min, and any sign or symptom of systemic or large local reactions were carefully registered and treated. Side effects were registered and graded according to the EAACI scale (11).

In vivo parameters, like SPT and conjunctival provocation tests (CPT) were followed up at different times during ASIT of this sample of patients: T0 (before ASIT), T1 (after the build-up dose phase), T2 (after six months of ASIT at maintenance dose=MTD) and T3 (after one year of ASIT at maintenance dose). SPT were carried out at three different extract concentrations (3.3, 10 and 30 mg/ml) and CPT at three concentrations: 0.0209, 0.209 and 2.09 mg/ml.

Antibody determinations

Antibody determinations were done before initiating immunotherapy and after one year of maintenance dose administration. Serum specific IgE to *A. alternata* extract

Table 1. Schedule for dose increase phase.

Nº	Week	Vial	Volume (ml)	Dose (UBE)	Dose Alt a 1 (μg)
1	1	0 (20.9 UBE/ml)	0.5	10.5	0.0006
2	2	1 (209 UBE/ml)	0.1	20.9	0.001
3	3		0.2	41.8	0.002
4	4		0.4	83.6	0.005
5	5		0.8	167.2	0.01
6	6	2 (2.090 UBE/ml)	0.1	209	0.01
7	7		0.2	418	0.02
8	8		0.4	836	0.05
9	9		0.8	1.672	0.10
10	10	3 (20.900 UBE/ml)	0.1	2.090	0.13
11	11		0.2	4.180	0.25
12	12		0.4	8.360	0.50
13	13		0.6	12.540	0.75
14	14		0.8	16.720	1.00
15	15		1.0	20.900	1.25
16	17		1.0	20.900	1.25
17	21		1.0	20.900	1.25

was determined by enzyme allergosorbent test (EAST). *A. alternata* extract was coupled to CBrN-activated paper disks as described by Ceska *et al*. [21]. Fifty-microliter aliquots of sera were added to the allergen discs in tubes and incubated at 37ºC for 1 hour. After washing, discs were incubated for 30 min at 37ºC with 50 ml of alkaline phosphatase-labeled mouse anti-human IgE and developed following the instructions of Hytec-specific IgE enzyme immunoassay (Hycor Biomedical Inc, Kasel, Germany). Specific IgE calibrators allowed the estimation of IgE titer in IU/ml.

Serum specific total IgG to *A. alternata* extract was determined by fluoroenzyme immunoassay (UniCAP®, Pharmacia & UpJohn, Uppsala, Sweden) according to manufacturer instructions. Specific IgG calibrators allowed the estimation of IgG concentrations in mg/l.

Serum IgG subclasses 1 and 4 to *A. alternata* extract and IgG (total), IgG1 and IgG4 to nAlt a 1 and rAlt a 1 were determined by ELISA according to the following procedure: Microwell plates (Greiner, Frickenhausen, Germany) were coated overnight with 100 µl/well of *A. alternata* extract (2.5 µg/ml) or Alt a 1 (1 µg/ml) in 0.1 M bicarbonate buffer pH 9.6. Next, plates were blocked by adding 200 µl/well of PBS-1% BSA-0.05% Tween 20 (PBS-B-T) and incubated 1 h at 37ºC. Afterwards, plates were incubated with 100 µl/well of patient sera (diluted in PBS-B-T from 1:10 to 1:1000 according to patient titers) followed by a second incubation with a HRP-conjugated anti-human IgG (Dako, Glostrup, Denmark), IgG1 or IgG4 (both from Southern Biotech, Birmingham Al, USA) diluted 1:2000 in PBS-B-T. All incubations were carried out at 37ºC for 90 min and with 3 washes of 200 µl/well of PBS-T between incubations. Color development was performed at room temperature and in darkness with a solution of o-phenylenediamine (Sigma-Fast o-phenylenediamine dihidrochloride Tablet Sets; Sigma, St. Louis, Mo, USA). The reaction was stopped at 30 min with 50 ml/well of 3 M H_2SO_4, and the optical density was measured at 492 nm.

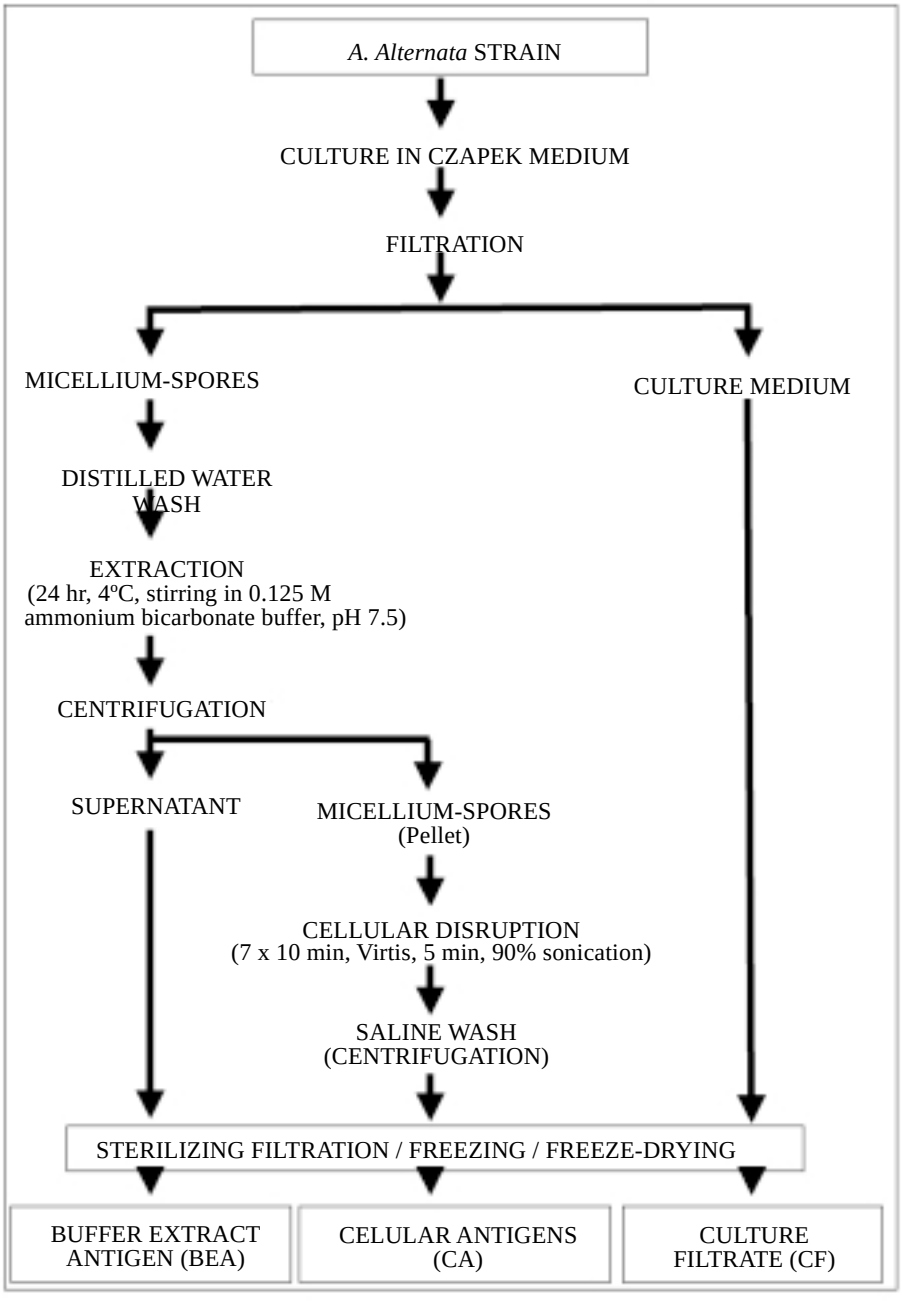

Figure 1. Flow chart of *A. alternata* extract production.

Statistical analysis

Statistical analysis was performed using the Wilcoxon signed ranks test. A P-value < 0.05 was regarded as statistically significant.

Results

Extract selection and biological standardization

Three different *A. alternata* extracts were produced by the methods depicted in Figure 1. This was done separately with four different mold strains in order to minimize antigenic variability. The four different strains, identified in Table 2, from international collections (CBS) o local isolates (IMIM) were selected on the basis of their

Table 2. Allergen yield (mg freeze-dried extract/l culture) of *A. alternata* strains used.

Strain	Origin	CF	BEA	CA
106.24	CBS	226	154	118
60.378	CBS	253	250	50
93.039	CBS	1.102	64	335
MR-3.292	IMIM	415	115	160

stability on culturing, their efficient growing under culture conditions, the lack of production of mycotoxins and a good yield of the extraction process. The yields obtained after extraction process are shown on Table 2.

The four extracts of each allergenic source (CF, BEA and CA) were pooled for further analysis. *A. alternata* extracts showed different degrees of cutaneous reactivity. The most reactive one was the CF extract, which gave rise to a SPT value of 2.09 mg/ml (Figure 2), while the BEA and CA extracts showed SPT (extrapolated) values of 28.84 and 2754 mg/ml, respectively. CF extract contained also the highest amount of Alt a 1 major allergen: 2.1% of total proteins, as estimated by a monoclonal based ELISA, and the most diverse and intense IgE binding pattern by western blot (Figure 3). SDS-PAGE immunoblotting revealed that CF extract containing metabolic antigens not only presented a pattern with the most intense IgE binding at 14.5 and 16.0 kDa (which accounted for the major Alt a 1 allergen), but also in the overall blotted lane extract incubated with the pool of *A. alternata* allergic patients.

Maximum tolerated dose

Considering the previously shown results, CF *A. alternata* extract was established as IHR for the MTD finding in specific immunotherapy. Initially, maximal dose to be tested was 2.09 mg or 20,900 UBE. However, in any case, doses higher than 8,360 UBE (containing 0.5

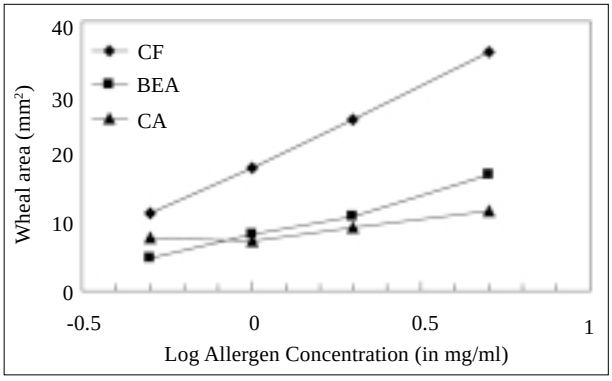

Figure 2. Biological activity of *A. alternata* extracts. Average values of 21 allergic patients.

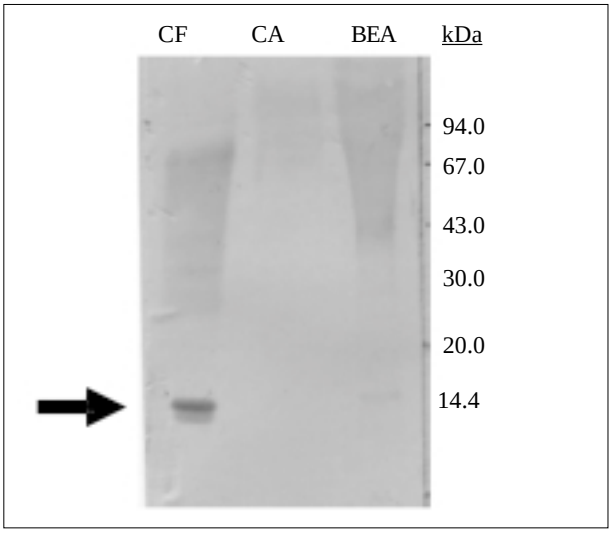

Figure 3. SDS-PAGE immunoblotting of *A. alternaria* extracts from different origins incubated with a pool of sera from 21 allergic patients. (CF: culture filtrate. CA: cellular antigens. BEA: buffer extractable antigens). Arrow indicates Alt a 1 bands.

Table 3. Side effects during the dose increase phase of immunotherapy.

	UBE (µg Alt a 1)					
Patient Nº	1670 (0.1)	2090 (0.125)	4180 (0.25)	8360 (0.5)	12540 (0.75)	16700 (1)
1	T	T	T	T		
2	T	T	T	T	SR-3	
3	T	T	T	LSR-RC		
4	T	T	T	T	SR-2	
5	T	T	T			
6	T	T	LSR-RC			
7	T	T	T	SR-2 LSR-RC+A		
8	T	T	SR-1			
9	T	LSR-RC				
10	T	T	T	T		

T: Tolerated administration. SR: Immediate Systemic Reaction. LSR: Late Systemic Reaction. RC: Rhinoconjunctivitis. A: Asthma.

µg of Alt a 1) could not be administered. MTD, i.e., the allergen concentration which was tolerated by all the patients, was 1,670 UBE given in a 1 ml volume. Doses higher than 1,670 UBE elicited systemic reactions in seven of the tested patients (Table 3).

The first patient to reach the dose of 12,540 UBE experienced a grade 3 systemic reaction (SR) with an episode of urticaria with concomitant asthma fifteen minutes after allergen injection, the reaction being severe enough to require adrenaline, corticosteroids and antihistamines. The next patient reaching 12,540 UBE had an immediate SR graded 2 presented as a mild asthmatic reaction 25 min after injection, which was alleviated with $ß_2$-agonist inhalation. At 8,360 UBE dose, a patient experienced a late systemic reaction (LSR) presented as an episode of mild rhinoconjunctivitis 45 minutes after allergen administration. Another patient presenting a SR-2 as RC 30 min after 8,360 UBE dose was alleviated with antihistamines, but again experienced episodes of RC and asthma one hour after leaving the clinic. At 4,180 UBE one of the patients presented a RC episode 12 hours after allergen administration, which was treated with antihistamines. At the same dose another patient had a SR graded 1 presented within 30 min as a sensation of pharyngeal hindrance with normal physical examination, and which ceased spontaneously. Finally, one patient suffered a LSR with RC within 60 min after receiving the 2,090 UBE dose which was treated with antihistamines. Some patients suffering positive side effects could tolerate the dose after stopping the increment dose for one or more administrations.

According to these findings, MTD corresponded to 1,670 UBE (Figure 4), i.e., 0.167 mg of freeze-dried extract, corresponding to 5 µg of protein and 0.1 µg of Alt a 1.

Immunotherapy monitorization

The patients receiving immunotherapy to determine MTD in an open titration study continued the treatment for at least one year. *In vivo* reactivity by cutaneous test and *in vitro* immunological induction was monitored before (T0) and after immunotherapy (T3). No decrease was observed in SPT response at the end of the treatment period with respect to baseline. Conjunctival reactivity measured by CPT showed a decreasing tendency at any monitored time (T1, T2 and T3), although with no statistical significance. However, specific serum antibodies significantly changed with the treatment. Specific antibodies of IgE and IgG classes and IgG1 and IgG4 subclasses were determined against *A. alternata* extract (ALT), nAlt a 1 and rAlt a 1 (Figure 5). Serum specific IgE decreased and specific IgG4 subclass increased significantly after ASIT in all assays when using ALT or the isolated major allergen forms (nAlt a 1, rAlt a 1) in solid phase. Total IgG increased significantly with respect to ALT, but not regarding Alt a 1. Furthermore, specific IgG1 subclass increased also in the three cases, though statistical significance was not reached.

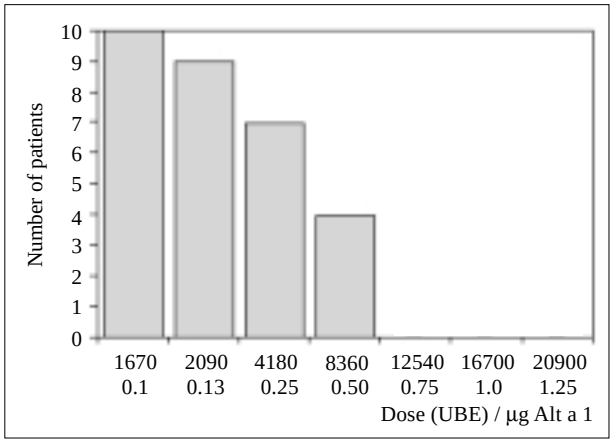

Figure 4. Maximum dose administered per number of patients.

Discussion

Allergenic vaccines for ASIT are still manufactured with natural allergenic extracts, with a different degree of purification. Allergenic sources used as raw materials for vaccine production are consequently of biological nature and subjected to inherent variability. In the case of fungal extracts some additional factors derived from the industrial preparation of the raw materials are affecting the reproducibility of the product, such us the inter-strain variability, the precise origin of extracted antigens (mycelium, spores, spent medium), and the growth conditions of mold cultures. These factors together with those derived from the extractive process, could explain the difficulty to achieve inter-batch lot-to-lot consistency in the mold extract manufacturing process. Also, there are highly significant differences among *A. alternata* extracts commercialized by different laboratories, a fact to be considered regarding their potential interchangeability [22, 23].

Strain variability and its effect on the antigenicity and allergenicity of mold extracts in general, and of *A. alternata* preparations in particular has been previously documented [24]. As a result, the use of several strains has been encouraged [25]. The basic culture conditions, such as temperature, growth period and static liquid cultures on synthetic allergen-free (Czapek) medium were previously established [8] and adopted because of their advantages for industrial production-being superior in yield to solid media. Culturing in solid media maximizes sporulation, but complicates the collection of extracellular components secreted into the growth media, as in the case of CF. This allergen fraction provided by far the best yield in terms of extract, protein or allergen recovery (Table 2) in comparison to the other two tested fractions, according to the production procedure outlined in Figure 1. BEA accounted for the readily and aqueously protein extractable fraction, that is protein from the external parts

of mycelium and spores and CA contained cytoplasmic proteins only released after mechanical homogeneization of the collected math of mycelium and spores. This CA extract thus would correspond to the same antigenic source as that employed in the 1980s international collaborative study aimed at developing a reference standard of *A. alternata* under the auspice of the World Heath Organization/ International Union of Immunological Societies allergen standardization Committee [26] which was left aside for various reasons.

The three allergenic extracts (CF, BEA and CA) obtained for each cultured *A. alternata* strain were combined and comparatively evaluated. CF extract containing the metabolic antigen fraction, i.e., the protein secreted into the growth medium, was revealed as more allergenic than the other two both *in vivo* (SDS-PAGE immunoblotting) and *in vitro* (cutaneous test) assays.

The superior allergenic reactivity of CF extract was demonstrated *in vivo* by SPT (Fig. 2), since this extract gave rise to wheal areas of around two-fold the size of those produced by the other compared preparations at all tested concentrations. BEA was shown to be slightly more reactive than CA fraction. The allergenic predominance of the culture filtrate fraction containing the secreted antigens agrees with a previously published paper [27] and is also ratified by recent findings demonstrating, by a 2-site monoclonal antibody sandwich ELISA, the quantitative predominance of Alt a 1 in CF [28].

Examination of the western blot patterns obtained after incubation of the three extracts with a pool of sera from *A. alternata* patients showed that CF extract gave rise to a higher band intensities and, specifically, at 14.5-16 kDa, the characteristic doublet band corresponding to the major *A. alternaria* allergen, Alt a 1, electrophoresed under reduced conditions (Fig. 3).

Accordingly, a CF extract was set up as in-house reference preparation (IHR), that is as a prototype to which to adjust the successive *A. alternata* manufactured batches. It can be argued that it is not necessary to use several strains for the routinely produced extracts if they are going to be compared with the multiple-strained IHR within the frame of a biological standardization program, and therefore under defined quality control specifications. The number of produced extracts fulfilling the validated specifications will provide the answer to that question.

Cutaneous test of *A. alternaria* IHR within the sample of patients recruited in the biological standardization program yielded an SPT value of 2.09 mg/ml, which was arbitrarily transformed into biological unitage as 20,900

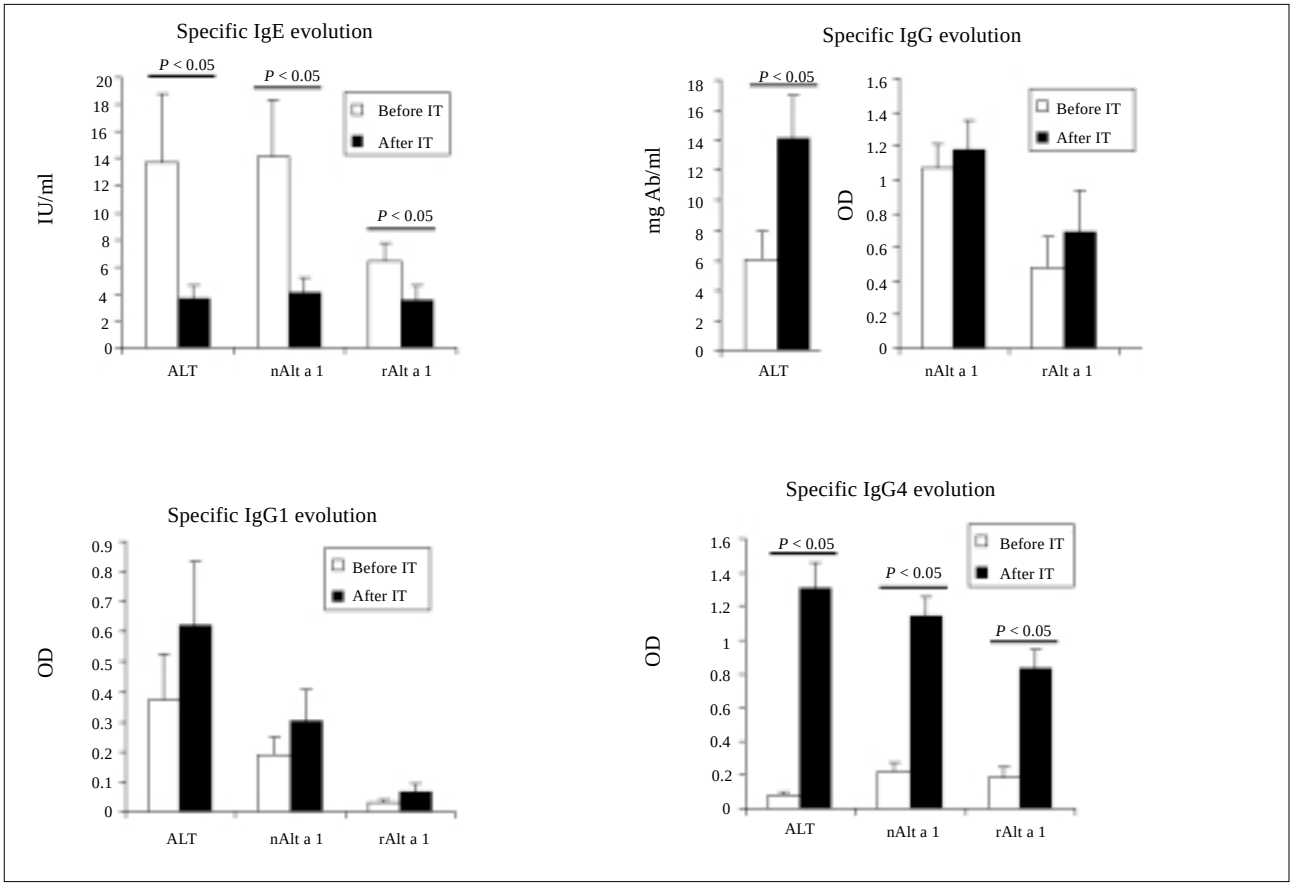

Figure 5. Evolution of antibodies to *A. alternata* extract (ALT), nAlt a 1 and rAlt a 1 before and after one year of ASIT (maintenance phase). Bars represent mean values ± standard error of the means of the 10 patients assayed in the MTD determination. P-values refer to between group comparisons by Wilcoxon signed rank tests. OD: Optical Density.

UBE/ml [13]. Sera from the mentioned patients were pooled to be used in the IgE-based immunochemical techniques involved in the biological standardization procedure. The encountered dose of 20,900 UBE was established as a reference of maximal concentration to be applied in MTD finding immunotherapy assay to be carried out according to a conventional depot administration schedule of induction phase in 14-weeks (Table 1).

It is remarkable that there is a very scarce number of published papers addressing the definition of the maximum tolerated dose (MTD), this being a crucial matter for the safety and efficacy of the allergenic vaccine under development. In this study, we have followed a previously reported definition (20), according to which MTD was considered "the highest dose not inducing a systemic reaction or the one just before that inducing three consecutive large local reactions". As one of the patients experienced an SR (delayed and referred as a rhinoconjunctivitis episode) at 2,090 UBE dose (0,125 μg Alt a 1) (Table 3), the immediate lower dose of 1,670 UBE was established as MTD, i.e., as the dose for the maintenance phase of immunotherapy. As it is not industrially viable to manufacture MTD-taylored allergenic vaccines for ASIT, our first approach to optimal dose finding should be restricted by fixing a MTD under the mentioned definition in order to provide a treatment with an acceptable number and grade of side-effects, to be validated later through a double blind placebo controlled assay of efficacy.

The estimated Alt a 1 content of 2.1% of total proteins in our established IHR extract lies within the interval of concentrations found in the series of *A. alternata* extract batches analyzed by Aden et al. [29]. The determined MTD in our study corresponds to a 0.1 μg Alt a 1 dose, which is within the range of maintenance concentrations of commercialized vaccines as reported by Vailes et al [18] and Esch [22], but perhaps somewhat in the lower side of the ranges.

Although the immunotherapy assay presented in this work was merely intended as a safety study for DMT finding, some *in vivo* and *in vitro* parameters were monitored until one year of immunotherapy. *In vivo* parameters (skin prick and conjunctival challenge tests) did not vary significantly. This fact is not uncommon in other DBPC or open ASIT studies in which the efficacy parameters improved significantly [30].

In contrast, specific serum IgE decreased and specific IgG4 increased both significantly and independently of the solid phase used for serum titration (Fig. 5), either the natural conventional extract used for ASIT (ALT) or the purified natural or recombinant Alt a 1. The behavior of the response (not the intensity) and the significance were the same using the recombinant or the natural form. The detected immunological changes in IgE and IgG4 with the course or immunotherapy clearly demonstrated the immunological capacity of the extract used.

The objective of this work was to establish an adequate *A. alternata* natural complete extract for efficient diagnostic and safe treatment purposes. It is possible that for a number of *A. alternata* sensitized patients, treatment based on the use of only its mayor allergen, Alt a 1, either as natural or recombinant form, would be sufficiently effective. The study of this latter possibility is currently underway; first clinical results, related to diagnosis, have been obtained [31] and seem to be promising. For other patients reacting to several *A. alternata* allergens, the treatment of their pathologies will still require, at least over the middle term, the existence of high quality commercialized natural extracts.

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References

1. Halomen M, Stern DA, Wright AL, Taussig LM, Martinez FD. *Alternaria* as a major allergen for asthma in children raised in a desert enviroment. Am J Respir Crit Care 1997;155:1356-61.
2. Gergen PJ, Turkeltaub PC. The association of individual allergen reactivity with respiratory disease in a national sample. Data from de second National Health and Nutrition Examination Survey, 1976-80 (NHANES II). J Allergy Clin Immunol 1992;90:579-88.
3. Andersson M, Downs S, Mitakakis T, Leuppi J, Marks G. Natural exposure to *Alternaria* spores induces allergic rhinitis symptoms in sensitised children. Pediatr Allergy Immunol 2003;4:100-5.
4. Germen PJ, Turkeltaub PC, Kovar MG. The prevalence of skin test reactivity to eight common aeroallergens in the US population: results from the second National Health and Nutrition Examination Survey. J Allergy Clin Immunol 1987;80:669-79.
5. López M, Salvaggio JE. Mold sensitive asthma. Clin Rev Allergy 1985:3:183-96.
6. D´Amato G, Chatzrgeorgiou G, Corsico R, Giuolekas D, Jager L, Jager S, Kontou-Fili K, Kouridakis S, Liccardi G, Meriggi A, Palma-Carlos A, Palma-Carlos ML, Pagan Aleman A, Parmiani S, Puccinelli P, Russo M, Spieksma FT, Torricelli R, Wuthrich B. Evaluation of the prevalence of prick skin test positively to *Alternaria* and *Cladosporium* in patients with suspected respiratory allergy. Allergy 1994;49:460-65.
7. Mari A. Schneider P, Wally V, Breitenbach M, Simon-Nobbe B. Sensitization to fungi: epidemiology, comparative skin tests, and IgE reactivity of fungal extracts. Clin Exp Allergy 2003;33:1429-38.
8. D´Amato G, Spieksma FThM. Aerobiologic and clinical aspects of mould allergy in Europe. Allergy 1995;50:870-7.
9. Martínez J, Martínez A, Gutiérrez G, Llamazares A, Palacios R, Sáenz de Santamaría. Influencia del proceso de obtención en la actividad alergénica y rendimiento de extractos de *Alternaria alternata*. Rev Iberoam Micol 1994;11:10-3.

10. Dreborg S, Grimmer O. Biological standardization of allergen extracts/preparations. Arb Paul Ehrlich Inst Georg Speyer haus Ferdinand Blum Inst Frankf A.M. 1983;8:77-82.
11. Malling HJ, Weeke B. Position paper: Immunotherapy. Allergy 1993; 48(Suppl. 14):9-35.
12. EAACI Subcommittee of skin tests and allergen standardization. Skin tests used in type I allergy testing. Allergy 1989;44(suppl.10):13-60.
13. Dreborg S, Frew A. Allergen standardization and skin tests. EAACI Position Paper. Allergy 1993; 48(Suppl.14):49-82.
14. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 1970;277:680-5.
15. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principles of protein-dye binding. Anal Biochem 1976;72:248-54.
16. Towbin H, Staehelin I, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 1979;76:4350-4.
17. Shen HD, Wang SR, Tang RB, Chang ZN, Su SN, Han SH. Identification of allergens and antigens of Bermuda-grass pollen by immunoblot analysis. Clin Allergy 1988;18:401-10.
18. Vailes L, Shridara S, Cromwel O, Weber B, Breitenbach M, Chapman M. Quantitation of the major fungal allergens, Alt a 1 and Asp f 1, in commercial allergenic products. J Allergy Clin Immunol 2001;107:641-6.
19. Asturias JA, Arilla MC, Ibarrola I, Eraso E, González R, Martínez A. A sensitive two-site enzyme-linked immunosorbent assay for measurement of the major *Alternaria alternata* allergen Alt a 1. Ann Allergy Clin Immunol 2003;90:529-35.
20. Olaguibel JM, Tabar AI, García Figueroa BE, Cortés C. Immunotherapy with standardized extract of *Dermatophagoides pteronyssinus* in bronchial asthma: a dose-titration study. Allergy 1997;52:168-78.
21. Ceska M, Lundqvist U. A new and simple radioimmunoassay method for the determination of IgE. Immunochemistry 1972;9:1021-30.
22. Esch RE. Manufacturing and standardizing fungal allergen products. J Allergy Clin Immunol 2004;113:210-5.
23. Gastaminza G, Algorta J, Audicana M, Etxenagusia M, Fernández E, Muñoz D. Systemic reactions to immunotherapy: influence of composition and manufacturer. Clin Exp Allergy 2003;33:3-5.
24. Steringer I, Aukrust L, Einarsson R. Variability of antigenicity and allergenicity in different strains of *Alternaria alternata*. Int Arch Allergy Appl Immunol 1987;84:190-7.
25. Helm RM, Squillace DL, Aukrust L, Borch SM, Baer H, Bush RK, Lowenstein H, Znamirowski R, Nitchuk W, Yunginger JW. Production of an international reference standard *Alternaria* extract I. Testing of candidate extracts. Int Arch Allergy appl Immun 1987;82:178-89.
26. Helm RM, Squillace DL, Yunginger JW, Members of the international collaborative trial. Production of a proposed international reference standard *Alternaria* extract. II. Results of collaborative trial. J Allergy Clin Immunol 1998;81:651-63.
27. Portnoy J, Pacheco F, Barnes C, Upadrashta B, Crenshaw R, Esch R. Selection of representative Alternaria strain groups on the basis of morphology, enzyme profile, and allergen content. J Allergy Clin Immunol 1993;91:773-82.
28. Ibarrola I, Suárez-Cervera M, Arilla C, Martínez A, Monteseirín J, Conde J, Asturias JA. Production profile of the major allergen Alt a 1 in *Alternaria alternata* cultures. Ann Allergy Asthma Immunol 2004;93:589-93.
29. Aden E, Weber B, Bossert J, Teppke M, Frank E, Whal R, Fiebig H, Cromwell O. Standardization of *Alternaria alternata*: extraction and quantification of Alt a 1 by using an mAb-based 2-site binding assay. J Allergy Clin Immunol 1999;103:128-35.
30. Smith H, White P, Annila I, Poole J, Andre C, Frew A. Randomized controlled trial of high-dose sublingual immunotherapy to treat seasonal allergic rhinitis. J Allergy Clin Immunol 2004;114:831-7.
31. Asturias JA, Ibarrola I, Ferrer A, Andreu C, López-Pascual E, Quiralte J, Florido F, Martinez A. Diagnosis of *Alternaria alternata* sensitization with natural and recombinant Alt a 1 allergens. J Allergy Clin Immunol 2005;115:1210-7.

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