

Sensitization to *Zygophyllum fabago* pollen. A clinical and immunologic study

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Zygophyllum fabago is a herbaceous plant found widely in the Mediterranean area. There are no previous reports of its allergenicity. An aerobiologic and clinical survey was conducted in Murcia, southern Spain, to determine the quantity of airborne pollen and establish the possible role of this pollen as a cause of allergic symptoms. With a Hirst volumetric trap, we determined the atmospheric concentrations of this pollen in 1993, 1994, 1995, and 1996. Of 1180 patients tested, 181 (15.34%) had a positive skin test. To determine its allergenicity, we divided 47 patients into three groups: in group 1, all the patients had symptoms of rhinoconjunctivitis plus asthma; in groups 2 and 3, rhinoconjunctivitis. In group 1, we performed a bronchial provocation test (BPT); in groups 2 and 3, we performed nasal provocation (NPT) and conjunctival provocation (CPT) tests, respectively. SDS–PAGE was used to characterize the antigenic fractions and RAST inhibition to determine cross-reactivity with other pollens. The pollen dispersion period is from May to September (445 grains/m³). BPT was positive in 13 of 15 patients, NPT in 14 of 16 patients, and CPT in 13 of 16 patients. RAST inhibition revealed cross-reactivity with *Mercurialis*, *Ricinus*, *Olea*, and *Betula*. SDS–PAGE identified 25 IgE antibody-binding components, five of which (60, 65, 41, 38, and 15.5/14.7 kDa) were recognized by 40% of the sera. By SDS–PAGE immunoblotting with sunflower antiprofilin rabbit serum and affinity chromatography we established that the *Z. fabago* extract has profilin. This study shows that this pollen becomes airborne and elicits an IgE response which triggers respiratory symptoms in allergic subjects.

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The palinologic study of a geographic area aids correct diagnosis of hypersensitivity to pollens (10). In daily practice, we encounter patients with symptoms suggesting pollinosis and negative skin tests, and it is also not uncommon for us to treat patients with a poor response to immunotherapy with pollens. Various authors have reported unusual sensitization as a cause of pollinosis (1, 5, 8, 9, 16). We therefore consider it necessary to study the allergenicity of the airborne pollen found even in small concentrations (less than 10% of the total pollen) on spore-trap slides.

In the atmosphere of our geographic area (Murcia, southern Spain), we have found that *Zygophyllum fabago* pollen constitutes 6.06% of the total pollen. No previous data show that it is allergenic. Only

Small & Smell, in 1946, mentioned the *Zygo-*phyllaceae family as a possible cause of allergic disease (12). It is a herbaceous plant found widely in the Mediterranean area, belongs to the *Zygo-*phyllaceae family (order Sapindaceae), measures 60–80 cm in height, and grows both on the coast and inland (4). It is principally entomophilous, with a flowering period lasting from May to August. The pollen grain averages 15–17 µm in size, and is isopolar, suboblated, and tricolporated. The presence of this pollen in the atmosphere may be related to the exertive disposition of the stamen and the abundant biomass of this plant locally. The image of this pollen under an optic microscope has often been confused with *Tamarix* and sometimes *Mercurialis* pollen.



Fig. 1. *Z. fabago* pollen grains ($\times 500$).

In a previous article, we reported that 17.59% of our patients were sensitized to *Z. fabago* pollen (2), and in the present study we aim to clarify:

- 1) the ability to cause sensitization in the Murcia region
- 2) the cross-reactivity with other common pollens in Murcia
- 3) the characteristics of the main allergens involved.

Material and methods

Patients

The patients, all with a clinical history compatible with rhinoconjunctivitis (RC) and/or pollen asthma, were selected between September 1994 and January 1996. Diagnosis was established from clinical history and skin tests. Routine skin prick tests were performed with mites (*Dermatophagoides pteronyssinus* and *D. farinae*), molds (*Alternaria*, *Aspergillus*, *Cladosporium*, *Penicillium*), danders (dog, cat, rabbit), and pollens (*Olea*, Gramineae, *Plantago*, *Parietaria*, *Artemisia*, *Chenopodium*, *Salsola*, *Zygophyllum*, *Mercurialis*, *Platanus*, *Robinia*, *Quercus*, *Pinus*, *Cupressus*, *Helianthus*, *Rumex*, *Taraxacum*, and *Chrysanthemum*). All the extracts used were commercial products except *Z. fabago* extract, which was prepared by us.

The patients sensitized only to *Zygophyllum* kept a diary with a score of their symptoms from February to October (the pollination period in our area).

The patients were enrolled after providing written informed consent, and we performed serologic and *in vivo* tests only in 47 patients who gave their consent and allowed us to report significant findings.

Pollen count

The *Zygophyllum* pollen was identified and counted by an expert palinologist (Fig. 1). Airborne pollen was collected by a Hirst 7-day recording volumetric trap (Lanzoni VPPA 2000, Italy) between January 1993 and December 1996. The trap was installed, exposed to all winds, on the roof of the Cartagena railway station (Murcia), located at latitude 37°36'N, longitude 0°59'W, at a height of 10 m, with no nearby gardens or parks.

Pollen extract

During the spring of 1994, we collected *Zygophyllum* pollen and by optic microscopy determined a purity of 99%. We prepared an extract by the standard w/v method, which includes defatting, extraction in buffered saline solution, filtration, and sterility testing, to obtain dry extract. The protein content was determined by the Bradford method.

In 40 sensitized patients, we performed skin prick tests at concentrations of 0.1, 1, 5, 10, and 50 mg/ml. The mean wheal area produced by each allergen concentration was plotted as a function of the allergen concentration in a log-log system, and

linear regression was performed. The allergen concentration eliciting a wheal equal to the wheal produced by 10 mg/ml histamine was defined as 1 SPT (skin prick test) unit (13). We used these units to perform skin tests, and 10-fold dilutions were used for the bronchial provocation test (BPT), conjunctival provocation test (CPT), and nasal provocation test (NPT).

Twenty nonallergic patients, who served as a control group, were tested with this extract (1 SPT unit/ml) to ensure that this concentration was not irritative.

In vivo immunoallergic study

This study aimed to determine whether *Zygophyllum* extract is a cause of allergic symptoms (15, 17). Of the 181 patients sensitized to *Zygophyllum*, 47 consented to the provocation and serologic tests and were divided into three groups: in group 1, all the patients had symptoms of RC plus asthma (RCA); in groups 2 and 3, the patients had symptoms of RC. In group 1, we performed a BPT; in groups 2 and 3, we performed an NPT and a CPT, respectively.

Bronchial provocation test

First, the patient inhaled buffered saline solution for 2 min through a DeVilbiss 646 nebulizer, after which the FVC and FEV₁ values were measured; if the differences were no greater than 10% compared to the baseline values, the patient after 5 min underwent antigen challenge by the graded dose technique (0.0001, 0.001, 0.01, 0.1, and 1 SPT/ml), until a 20% drop in baseline FEV₁ occurred or the patient showed signs of respiratory discomfort. After each BPT, patients were observed for any late-phase reactions over a period of 8 h (7).

Nasal provocation test

The antigen was administered by a spray with a normalized valve, which released 0.1±0.05 ml in each jet. The seated patient was administered two jets of diluent (physiologic serum), and the peak nasal inspiratory flow (PNIF) rate was recorded at 5 min (Youlten PNIF meter, Airmed SL, London, UK); if the decrease was no greater than 10% compared to the baseline measurement, two jets of allergen were administered every 15 min in progressively larger concentrations (0.0001, 0.001, 0.01, 0.1, and 1 SPT/ml). We considered the test positive if the patient had more than five sneezes or a volume of secretion of ≥0.5 ml, or if the PNIF rate fell by more than 50% (22).

Conjunctival provocation test

This test was performed according to Moller et al. (14) by administering one drop of allergen dilution (0.0001, 0.001, 0.01, 0.1, and 1 SPT/ml) in the left conjunctival sac. If no reaction was observed within 20 min, the next concentration of aqueous extract was applied. The criterion for a positive test was congestion of the conjunctival mucosa, itching, and watering of the eye and nose. A negative control was carried out by administering one drop of diluent in the right conjunctival sac.

As a control, we tested 10 patients presenting with respiratory allergy to *Olea* pollen by the same technique as for bronchial, nasal, and conjunctival provocation tests.

Serum specific IgE assay

Solid-phase antigen was obtained by coupling the extract solution (10 mg/ml) to 6-mm-diameter CNBr-activated paper disks, according to Ceska & Lundqvist (3). RAST was performed by the method of Wide et al. (21) with phadezym reagents (Kabi Pharmacia Diagnostics AB), according to the manufacturer's instructions.

Isoelectric focusing (IEF) immunoblotting

IEF immunoblotting was done on a support of agarose in Isogel gels (FMC Bioproducts), in a pH range of 10.2–3.6. Electrophoresis was performed in an LKB 2117 Multiphor II system of horizontal electrophoresis, at a temperature of 8°C. In parallel with this, we applied an isoelectric point pattern (FMC): cytochrome (10.2), myoglobin, major band (7.4), minor band (7.0), carbon anhydrase (6.1), β-lactoglobulin (A, B [A, 5.3; B, 5.1]), ovalbumin (4.8), glucose oxidase (4.2), and aminoglucosidase (3.6). Once separated according to their isoelectric point, the proteins were transferred to PVDF membranes (Immobilon-P), which were incubated with a pool of patient serum. The IgE-binding bands were revealed by an enzyme system with an anti-IgE-peroxidase conjugate (diluted 1:25).

SDS-PAGE immunoblotting

SDS-PAGE was performed by Laemmli's discontinuous method on 12.5% acrylamide gel, with proteins stained with Coomassie brilliant blue R-250, with the simultaneous application of a pattern of proteins of known molecular weights (Pharmacia Biotech): phosphorilase b (94.0 kDa), bovine seroalbumin (67.0 kDa), ovalbumin (43.0 kDa), carbon anhydrase (30.0 kDa), trypsin inhibitor

Table 1. *Zygophyllum fabago* pollen counts in pollen season

Year	Day 1	Last day	Maximum	Grains/m ³	Duration (days)
1993	25 May	7 Aug	2 June	399	75
1994	5 May	9 Aug	1 June	405	97
1995	4 May	13 July	3 June	440	71
1996	6 May	15 Sept	27 May	538	133

(20.1 kDa), and α -lactalbumin (14.4 kDa). We added 10 μ l/lane of pattern and samples, containing 20 μ g of protein (Bradford) each. The proteins separated in SDS-PAGE were electrotransferred to PVDF membranes (Immobilon-P) and incubated with the patients' sera. The IgE-binding bands were revealed with an enzyme system using an anti-IgE-peroxidase conjugate (diluted 1:25). The pattern of molecular weights was revealed with amido black.

RAST inhibition

This was done on Ifidesa-Aristegui disks, with allergen coupled to CNBr-activated disks (2 mg/disk). Serum pools were incubated overnight with 10-fold serial dilutions of pollen extract, ranging in protein concentration from 100 to 0.001 mg/ml. Supernatants were centrifuged, and RASTs were performed on cyanogen-fixed *Zygophyllum*-extract disks. In each experiment, the correlation coefficient, dose-response curves, and regression lines were calculated. Parallel-line bioassay was used to analyze the linearity and parallelism of the RAST-inhibition curves. The antigen concentration necessary to obtain a 50% inhibition was also calculated.

Study of the presence of profilins in the extract

Since the molecular weight of some of the allergens present in the extract coincided with the molecular weight reported for the profilins of various vegetable species, it was worth investigating the possible presence of profilins. For this, and because of the cross-reactivity of the profilins of different species, we performed SDS-PAGE immunoblotting of the *Z. fabago* pollen extract, and, with rabbit serum, revealed antiprolin (IgG) of *Helianthus annuus*. The profilin present in the *Zygophyllum* extract was purified by affinity chromatography in a poly-L-proline Sepharose column, as described by Lindberg. After purification, it was subjected to electrophoresis in denaturing conditions (18, 19).

Results

From September 1994 to January 1996, we diagnosed 1180 patients with pollinosis. We found a total of 181 patients sensitized to *Z. fabago*. This is

Table 2. Patient characteristics, specific IgE values, and bronchial, nasal, and conjunctival provocation test results

Patient no.	Age/sex (years)	Symptoms	RAST PRU/class	NPT	
				LR	
1	16/M	RC	>17.5/4	0.01	No
2	23/F	RC	8.91/3	0.001	No
3	17/M	RC	0.57/1	0.01	Yes
4	28/F	RC	<0.35/0	—	—
5	16/M	RCA	6.95/3	0.001	No
6	32/M	RC	2.47/2	0.1	No
7	15/F	RC	0.54/1	—	—
8	18/F	RC	14.5/3	0.01	Yes
9	16/M	RCA	>17.5/4	0.0001	No
10	16/M	RC	<0.35/0	1	No
11	23/F	RC	0.68/1	0.1	No
12	28/M	RC	2.70/2	—	—
13	24/F	RC	3.83/3	0.01	No
14	36/F	RC	1.02/2	0.1	No
15	45/F	RCA	7.09/3	0.001	Yes
16	18/M	RC	4.42/3	0.001	No
CPT					
17	40/M	RC	0.69/1	1	
18	28/M	RCA	1.53/2	0.01	
19	14/M	RC	>17.5/4	0.001	
20	26/F	RC	>17.5/4	0.01	
21	22/F	RCA	0.69/1	0.1	
22	35/F	RCA	2.21/2	0.01	
23	15/M	RC	0.47/1	—	
24	38/M	RC	2.06/2	0.1	
25	23/F	RC	<0.35/0	1	
26	24/M	RC	1.94/2	0.01	
27	28/M	RCA	1.16/2	0.1	
28	29/M	RCA	0.82/2	0.01	
29	14/M	RC	<0.35/0	—	
30	38/F	RC	1.9/2	0.001	
31	20/F	RC	1.07/2	0.1	
32	14/M	RC	0.78/2	0.01	
BPT					
				PC ₂₀	
				LR	
33	14/F	RCA	3.24/2	0.006	No
34	22/F	RCA	2.24/2	0.043	Yes
35	42/F	RCA	1.5/2	0.089	No
36	26/F	RCA	1.19/2	0.12	Yes
37	22/F	RCA	1.49/2	0.03	No
38	18/M	RCA	0.61/1	0.41	No
39	31/M	RCA	<0.35/0	—	—
40	38/F	RCA	1.35/2	0.07	No
41	21/M	RCA	5.77/3	0.003	Yes
42	15/M	RCA	1.42/2	0.05	No
43	23/M	RCA	>17.5/4	0.0007	No
44	35/F	RCA	2.03/2	0.009	No
45	41/F	RCA	8.77/3	0.24	No
46	25/M	RCA	1.85/2	—	—
47	13/M	RCA	15.39/3	0.03	No

NPT, CPT, and BPT values are expressed in SPT units. RC: rhinoconjunctivitis; RCA: rhinoconjunctivitis plus asthma; LT: late reaction.

an incidence of 15.34%. Some 102 patients had RC, and 79 RCA. Ninety-seven were male and 84



Fig. 2. IgE-binding fractions to *Z. fabago* pollen extract in 24 patients by molecular weight. Patient sera with RAST $\geq$ class 2 were used (M: molecular weight pattern; C: pool sera of nonatopic patients).

female, with a mean age of 24.49 ± 9.31 years. Some 62% lived in rural areas, and 38% in urban districts. Three of the 181 (1.65%) *Zygophyllum*-sensitized patients were skin-reactive only to *Zygophyllum*, and 178 (98.35%) were skin-reactive additionally to other pollens: *Olea*, 122 (67.40%); Gramineae, 91 (50.27%); Chenopodiaceae, 126 (69.61%); *Artemisia*, 31 (17.12%); *Parietaria*, 73 (40.33%); *Plantago*, 28 (15.46%); *Mercurialis*, 27 (14.91%); *Helianthus*, 33 (18.33%); and *Chrysanthemum*, 28 (15.46%). None of the 178 patients were sensitized to *Platanus*, *Robinia*, *Pinus*, *Cupressus*, or *Taraxacum*.

The three monosensitized patients had allergic symptoms only in June and July, remaining asymptomatic for the rest of the year, but, in 1996, they had symptoms until September.

The palinologic study shows that the pollination period began on 6 May and extended to 15 September. The pollen concentration corresponding to the mean of the years 1993 to 1996 was 445 grains/m³ of pollen (Table 1). Other pollens counted during the same period in which this pollen is found in our atmosphere were Chenopodiaceae, 655 (11.05%); *Olea*, 1229 (20.74%); Poaceae, 576 (9.72%); Urticaceae, 2226 (37.56%); *Quercus*, 639 (10.78%); *Ligustrum*, 158 (2.66%); and Asteraceae, 80 (1.35%).

The NPT was positive in 13 of 16 patients, and late reaction was recorded in patients 3, 8, and 15. Patient 9 had a positive test with the lowest concentration administered. All the patients presented with a PNIF rate decrease of more than 50%, and sneezing, itching, and nose running occurred in all the patients at some time during the provocation test (Table 2).

Of 16 patients, 14 presented with congestion of the conjunctival mucosa, pruritus, and eye watering, with the CPT considered positive. No patient had a delayed response (Table 2).

The BPT was positive in 13 of 15 patients. Two patients did not respond to the BPT, even with an

allergen concentration as high as 1 SPT unit/ml. We observed late-phase reactions after challenge in patients 34, 36, and 41 (Table 2).

There were no adverse reactions noted by any patients to the *in vivo* tests. None of the subjects in the control groups had a positive CPT, BPT, or NPT response.

Serum-specific IgE levels ranged from 0.35 to >17.5 PRU/ml. RAST class 0 was found in five sera, whereas seven demonstrated RAST class 1, 21 RAST class 2, nine RAST class 3, and five RAST class 4. In general, the highest RAST scores occurred in patients with more positive skin tests (Table 2).

The isoelectric point of the most relevant IgE-binding bands, according to the IEF-immunoblotting results, was between 4.8 and 5.5; the most important had isoelectric points of 4.5, 4.8, and 4.9.

Employment of sera from 47 patients sensitized to *Zygophyllum* pollen with nitrocellulose blots of the pollen protein revealed 25 IgE-binding bands. More than 40% of the sera that proved positive in the blotting study reacted to at least five of the protein bands of approximate molecular weight 60/65 (doublet) (46%), 41 (50%), 38 (46%), and 15.5/14.7 (45%) kDa. These components generally also bound the greatest quantity of IgE, as evidenced by the depth of staining, although some of the bands were only faintly visible with a few of the sera. The most frequent finding of these bands reinforced the conclusion that these components are some of the dominant allergenic proteins. No binding of anti-IgE was observed when normal sera from nonatopic subjects were employed in the protein blotting studies (Fig. 2).

RAST-inhibition experiments with sera from subjects sensitized to *Zygophyllum* pollen clearly showed that extracts of this pollen inhibited IgE antibody binding to solid phases prepared from *Zygophyllum* pollen extract. When the free phase was constituted by *Mercurialis*, *Ricinus*, *Olea*, *Betula*, *Parietaria*, *Helianthus*, and *Lolium*, IgE anti-

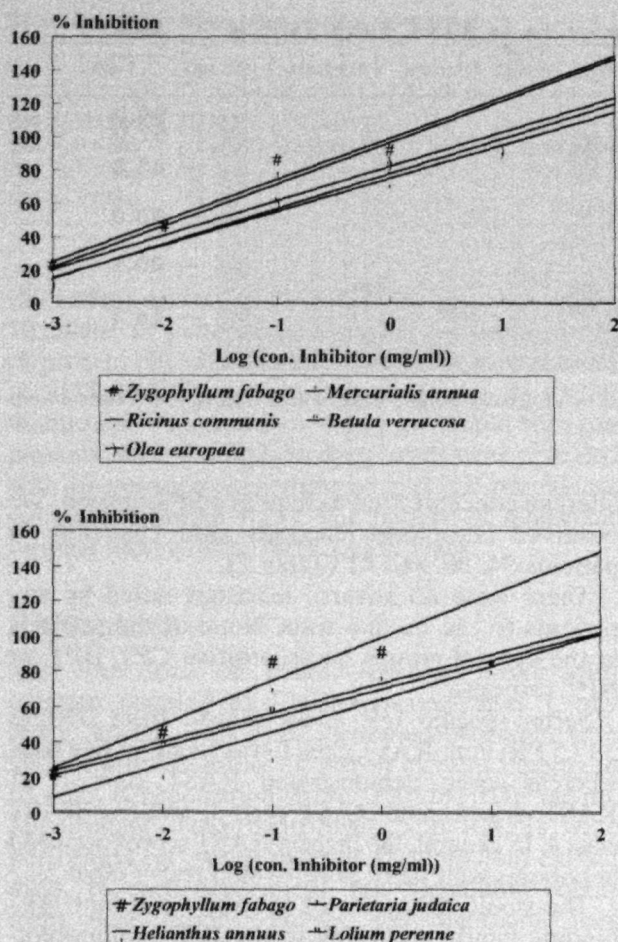


Fig. 3. *Z. fabago* RAST inhibition.

bodies were found to be inhibited by these pollen extracts and showed inhibition profiles in which all of the pollens tested inhibited IgE antibody binding to *Zygophyllum* pollen protein solid phase in various degrees. The values of the antigen concentrations producing an inhibition of 50% were *Z. fabago*, 3.2; *Mercurialis*, 16.3; *Ricinus*, 8.1; *Olea*, 7.7; *Betula*, 8.7; *Parietaria*, 29; *Helianthus*, 47; and *Lolium*, 57 μ g of protein/ml (Fig. 3).

With sunflower antiproliferin rabbit serum, SDS-PAGE immunoblotting revealed an IgG-binding band of 15.7 kDa (Fig. 4). By affinity chromatography, we established that the *Z. fabago* extract had profilin because it remained coupled to the poly-L-proline column, and when it was electrophoresed in denaturing conditions, we observed the presence of a protein doublet of 15.5/14.7 kDa.

Discussion

We frequently observe pollens that are not present in the atmosphere in large quantities, but which do contribute to increasing the total allergenic load

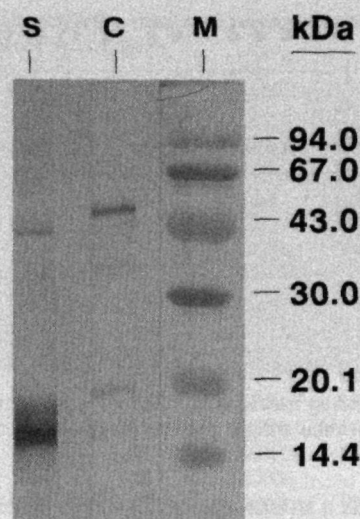


Fig. 4. SDS-PAGE immunoblotting with sunflower antiproliferin rabbit serum showed band of 15.7 kDa (M: molecular weight pattern; S: antiproliferin rabbit serum; C: nonatopic serum).

(5, 8, 9, 11, 16). Studying the allergenicity of these pollens gives us information on the real allergenic pressure to which these pollinic patients are subjected (23). Likewise, it is important for allergists to be acquainted with the local flora and allergens in their area to be able to test and treat with specific extracts. New agricultural technologies can cause imbalances in the local flora that affect pollinosis, and local aerobiologic studies detect palinologic alterations which can clarify the relation between aerobiology and pollinosis (6).

Despite the wide distribution of the *Zygophyllaceae* family, we have no information to confirm its allergenicity. *Z. fabago*, a very common herbaceous plant widely distributed throughout the Mediterranean area, causes symptoms of an IgE-mediated inhalant allergy diagnosed by clinical history, skin prick test, challenge test results, and findings of increased specific IgE antibodies (15).

We established that 15.34% of the study population was sensitized to this pollen. We found only three monosensitized patients, who presented with symptoms of RC coinciding with the pollination period. The remaining patients (98.35%) were sensitized also to other pollens, the pollination periods of which are longer. We therefore believe that the allergenic action consists of an increased load of total allergenic pollen in most exposed patients (23). Furthermore, a threshold level to provoke symptoms may be affected by a priming effect of other allergens just before or during the season. We would need symptoms scores of patients with single and multiple sensitivities to develop levels of threshold counts of this allergen.

Since atmospheric levels are not very high (6.06% of the total pollen), we could question the clinical significance of hypersensitivity to this entomophilous pollen, but we have shown by provocation that this pollen causes allergy symptoms. Various authors have reported that levels of 7–15 grains/m³ or 20 grains/m³ are enough to cause symptoms (20), although it is difficult to establish the mean pollen count required to elicit allergic symptoms.

Only three of 16, two of 15, and two of 16 patients had negative NPT, BPT, and CPT, respectively. These patients probably had a much higher symptom-inducing threshold level or were not sensitized to this pollen and had a positive skin test and RAST due to cross-reactivity with other pollens.

It is known that the reproducibility of provocation tests with pollens is high. For ethical reasons, we did not study intrasubject difference in the response to antigen.

The *Zygophyllum* extract was standardized by the biologic method, by skin prick test, to establish a clinical relevant potency for future research, although we know that a single method is not sufficient to ensure the reproduction of allergen extract both qualitatively and quantitatively.

We have been able to define several IgE-binding bands by *in vitro* studies. Each of them was recognized with different frequency and intensity by the sera of sensitized patients. However, each patient had a specific model of IgE-binding protein bands. The importance of these IgE-binding bands was variable, the 60/65-, 47-, 41-, 38-, and 15.5/14.7-kDa bands being bound by 40% of the patients, whereas others had minor allergenic capacity. Cross-reactivity was high with *Ricinus*, *Olea*, and *Betula*, lower with *Mercurialis* and *Parietaria*, and low with *Lolium* and *Helianthus*. Various authors have shown cross-reactivity between pollens of different species (1), and the presence of cross-reactivity with other species could not result in a short and well-defined symptomatic or pollinic season. It is difficult to explain these results clearly: in some cases, we could explain them by phylogenetic remoteness; in others, we could explain them by the presence of profilins common to various pollen types, or by our patients' high degree of polysensitization (18, 19). There obviously is a high degree of cross-reactivity to other pollen species. Because of this, we believe it of interest to pursue RAST-inhibition and immunoblotting inhibition studies with sera from monosensitized patients.

These results showed close correlation between clinical symptoms, cutaneous response, serum specific IgE levels, and provocation test results.

Z. fabago pollen becomes airborne and elicits an IgE response which triggers respiratory symptoms in allergic patients. These clinical findings, backed up by laboratory studies, suggest that further research may be required to ascertain the epidemiologic relevance of *Zygophyllum* pollen in people living in the Mediterranean areas where this plant grows widely.

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