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Occupational asthma associated to the exposure to limonium tataricum flowers

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Summary

Limonium tataricum (Lt) is a plant belonging to the family of Plumbaginaceae. The role of this family and in particular, that of dried flowers (but not of the pollen) in occupational allergy has already been described. We have observed a farmer with asthma occurring in the presence of fresh flowers. Standard methacoline test demonstrated that the patient was a true asthmatic. The allergenicity of Lt pollen was thus investigated. Skin prick tests (SPT) were carried out using both standard allergens and the Lt extract and the patient's mucosal reactivity was evaluated by nasal provocation test with the pollen extract. In vitro studies were also performed on the patient's serum by evaluating routine specific anti-allergen IgE on raw extracts and on Microarray Allergen Chip (ISAC). Finally, the raw extract of the fresh Lt pollen was also used in ELISA inhibition test, immunoblotting and Basophil Activation Test (BAT). The specific sensitization was demonstrated by Skin Prick test and nasal provocation test. The sensitization was also confirmed by specific IgE and by in vitro activation of basophils in the presence of the pollen. By using RAST inhibition test, the presence of cross-reactivity with other pollens was ruled out. According to our results, Lt extracts contain an allergenic activity not only as dried flowers, but also as fresh pollen. For its role in occupational asthma, this allergen should be included in any allergy screening at least in farmers or in the flower industry employers.

Introduction

Occupational Asthma (OA) is primarily caused (or exacerbate) by inhalatory expositions in the workplace. At present, 9-15% of asthmatic episodes in adults are related to OA (1). OA is the result of many different causes, which include genetic, environmental and behavior factors. In general, an asymptomatic sensitization period, characterized by a variable length, preceedes the onset of respiratory tract related symptoms. The intensity of the exposition to sensitizer allergens is the main cause of OA. In the last few years, a number of reviews and guidelines have been published (2-6) and nowadays, more than 350 workplace sensitizers have been reported. The large majority of these sensitizers are plants. The first sensitizers to be recognized as OA causes were cereals and flowers (7) that, together with isocyanates, represent the most common cause of OA. Along this line, a large number of OA cases were reported in flower workers (8-12). Of note, some patients are sensitized against other allergens or components (not present in the workplace) that co-express cross-reacting epitopes against professional allergens. This cross reactivity may result in a significant difficulty in making an etiological diagnosis of OA. The Limonium tataricum (Lt), commonly known as German Statice, is a plant belonging to the family of Plumbagi-

naceae. The flowers are characterized by a wide range of pastel colors and they are sold as fresh and dried up flowers. The height of the plant ranges between 35 to 60 cm and plants are in bloom between May and September. Lt is also characterized by peduncled floral panicles with several very thin and divergent ramifications. Of note, this flower is one of the most diffuse cultivation in the world.
A single case of OA related to Lt has been already described in Pamplona, Spain (13). The patient was a floriculturist who showed immediate respiratory and cutaneous symptoms after handling the dried plant. In this paper, we describe another case of OA caused by Lt observed by in an ambulatory patient, who had symptoms after exposure to a plant in culture

Material and methods

Case report

A floricolturist (man, 50 years old), not smoker, showed a history of seasonal (mainly September) rhino-conjunctivitis lasting several years, related to a already diagnosed sensitization to Compositae (Artemisia absintium). The patient started to work with Lt three years before our observation and his activity consisted in cutting the stalks and preparing bunches to be sold to the public. After the first year of work with this plant, during the month of June, he started suffering from asthma and conjunctivitis, strictly related to his presence in the cultivation. In fact, during holidays, the patient never suffered from asthmatic attacks. Recently, the patient was visited in our ambulatory and, on the basis of his clinical history, we decided to deepen his sensitization status. For this reason, a number of both clinical and laboratory tests were performed and in this report, we will confirm that Lt pollen could be cause of specific allergy not only in farmers and flower industry workers as a cause of OA, but also in allergic patients living in regions where the cultivation of this flower is carried out.

Preparation of Extracts

Pollen of Lt was obtained from the flowers during the bloom season, then defatted with diethyl ether before being submitted to 5% (w/v) aqueous extraction in 0.15 M PBS for 16 h at 4 °C under stirring. The suspension was centrifuged at 20,000 x g for 1 hour at 4°C and supernatant extensively dialyzed against distilled water before being lyophilized in different vials. Lyophilized samples were reconstituted at 1/10 of the initial volume before being used and protein content determined by BioRad method was 0.25 mg/ml (14). Parietaria judaica, Ambrosia artemisifolia, Artemisia absintium and Grass extracts, used as controls, were obtained by Lofarma SpA, Milan Italy.

In vivo tests

Skin prick tests (SPT)

SPTs were performed according to the method of Aas and Belin with the use of a standardized pricker (15,16). A test was considered positive if the value calculated was ≥3 mm and controls showed adequate reactions. Solution for skin prick tests was prepared reconstituting an aliquot of lyophilized Lt sample with 0.5 mL of 0.68% NaCl, 0.275% NaHCO3, 50% glycerol, 0.4% phenol.

Methacholine test

The methacholine test (Lofarma S.p.A., Milan, Italy) was performed according to a standardized method (19).

Nasal provocation tests

The nasal provocation test was performed between 9 to 11 a.m. to avoid possible circadian variation (18). As in previous studies (19-21), the Youlten peak inspiratory flow meter (PIFRn meter) was used. It offers some advantages over

Figure 1 -

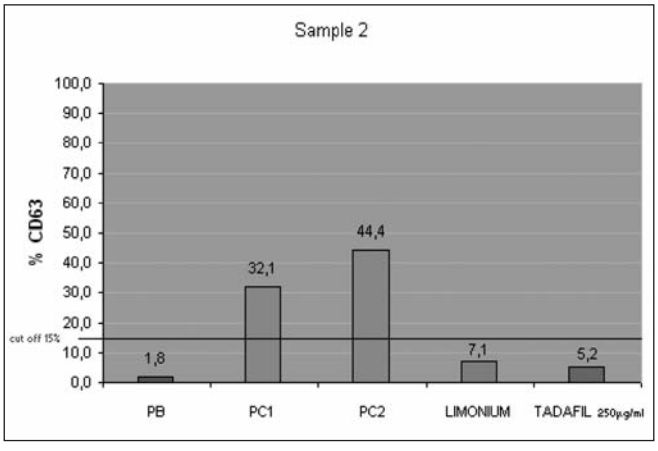
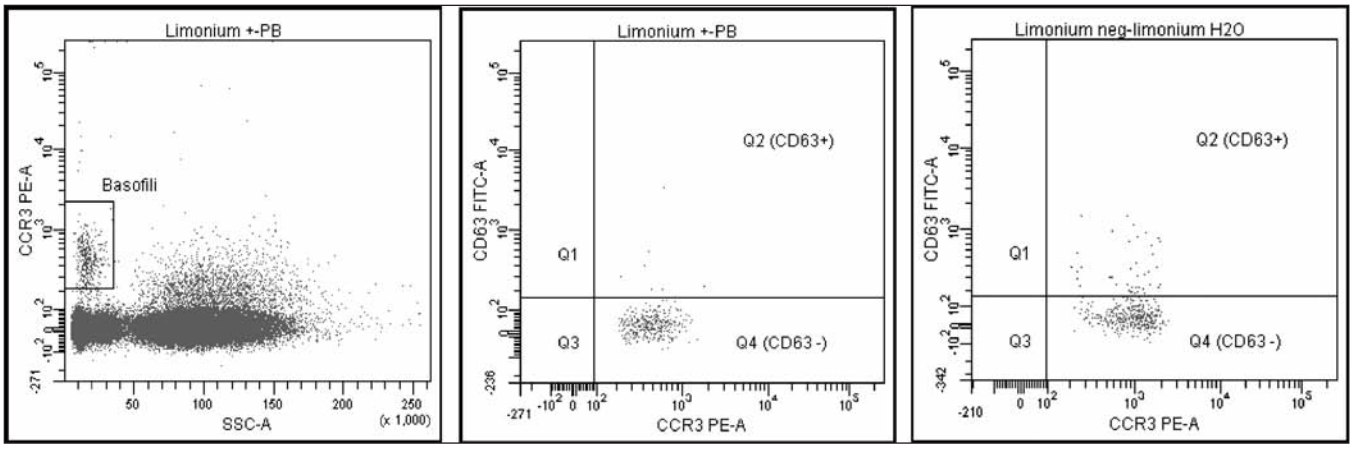


Figure 2 -



the respiratory flow rate meter, especially when used in provocation test and it is also easier to handle, compared with standard rhinomanometry. The same extract of Lt (protein content determined by BioRad method was 0.25 mg/mL) at concentration of 0,2% and 1% was used. The results were expressed as the mean values of three consecutive registrations. The test was considered positive when a drop in basic value of 20% or more after instillation of Lt solution was observed. The same test on two other healthy subjects used as controls was carried out.

In vitro tests

SDS-PAGE and immunoblotting

Electrophoresis of Lt pollen extract was carried out in a 10% polyacrilamide precast Nupage Bis-Tris gel according to manufacturer instructions (Novex, Prodotti Gianni, Milan, Italy) at 180 mA for 1 hour. Thirty µl of the extract were loaded per lane. The resolved proteins were stained with Coomassie Brillant Blue) or transferred for 1 h onto a nitrocellulose membrane according to Towbin et al. (22, 23) for immunoblotting analysis. The membrane was then saturated with 0.1 mol/L tris-buffered saline containing 5% fat-free milk powder and incubated for 16h at 4°C with serum (1:2 in saturation buffer). After 3 washings, bound specific IgE were detected by peroxidase-conjugated anti-human IgE antibodies from goat (1:6000 in saturation buffer) and using an ECL western blotting kit (Amersham, Milan, Italy) as substrate.

Total and Specific IgE determination

Total IgE were measured using the Pharmacia CAP system IgE FEIA (fluoro-enzyme-immunoassay). A standard panel of specific IgE against inhalants was carried out by using the ImmunoCAP system (Phadia, Milano, Italy). The assays were carried out according to the manufacturer's instructions (24, 25).

Immuno Solid Phase Allergen Chip (ISAC)

To deepen the IgE repertoire screening, the patient's serum was also tested with ImmunoCAP ISAC (Phadia, Milano, Italy). This microarray contained 103 allergen components derived from 47 allergen sources. The testing procedure was carried out as described, previously, in another paper (26).

ELISA and ELISA-inhibition experiments

IgE to Lt pollen extracts were measured in the patient's serum by ELISA. Parietaria judaica, Ambrosia artemisifolia, Artemisia absintium and Phleum pratense were used as controls of the assay. One hundred microliters of 1:100 diluted Limonium extract or 1 µg/0.1 mL (coating buffer: 15 mmol/L Na2CO3 and 35 mmol/L NaHCO3, pH 9.6) of the other extracts per well were used for sensitizing 96 well microtitre plates (Maxisorp Nunc, Roskilde, Denmark). IgE binding was expressed in absorbance units (AU). Values higher than the double of negative control values were considered positive.

Basophil Activation Test (BAT)

BAT is based on the basophil activation mediated by allergens or controls, detected by flow cytometric measurement of the increase of the CD63 (gp53) on the cellular surface. Both IgE and non-IgE mediated reactions can be detected. We used this test according to the standardized protocol (27).

Results

Skin prick tests

The skin prick tests towards standard allergen pollens demonstrated a weak and expected positivity to Artemisia absintium. Prick test for Lt was positive either with the crude extract or with the one prepared in laboratory.

Methacholine test

The methacoline test, carried out outside of the seasonal period of blooming, turned out positive, with a 20% decline in FEV1 at the concentrations of 10 mg/mL (64 IU).

Nasal provocation test

The test was positive. After instillation of 100 mL of 1% solution of Lt extract, a decrease in the flow rate more than 20% from baseline was observed. In the two control subjects, no decrease in the flow rate was also recorded.

SDS –PAGE and immunoblotting experiments.

Lt pollen extract showed a band at about 28 kDa and an intense smear at high molecular weights probably caused by free polysaccharides. Immunoblotting experiments using patient's serum did not demonstrate any IgE-binding band (data not shown). A possible explanation of different results observed for ELISA and immunoblotting, could be to the fact that IgE recognized epitopes can be destroyed by the reducing conditions which are commonly used for SDS-PAGE technique.

Total and specific IgE analysis using commercial allergen whole extracts (ImmunoCAP)

IgE level was 68 KU/L, while cpecific IgE titer to Artemisia absintium was under 0.10 KU/L.

ISAC results

ISAC resulted in a low positivity (2.1 ISU) for nAmb a 1 (the main component of Ambrosia artemisifolia) and 1.8 ISU for nSal k 1, the main component of Salsola kali.

ELISA and ELISA inhibition

The patient's serum showed a slight positivity for Lt (O.D.: 0.413 AU) and a moderate positivity for Artemisia (O.D.: 0.861 AU) while it was negative for Parietaria and Grass extracts when tested by ELISA method (data not shown). In addition the IgE-binding against Lt or Artemisia extracts were not inhibited by the pre-incubation of serum with Artemisia or Lt extracts respectively; on the contrary, as expected, Lt and Artemisia extracts, when used as inhibitors, were able to completely reduce the respective IgE-binding activities (Table 1). These data clearly demonstrated the absence of any cross-reactivity phenomena between the two pollens.

Table 1 - ELISA and ELISA-Inhibition

Coating Extract	Inhibiting Extract	AU patient' serum	AU negative control	% inhibition
Limonium	-	0.413	0.145	-
Limonium	Limonium	0.120		100%
Limonium	Artemisia	0.408		0%
Artemisia	-	0.813	0.120	-
Artemisia	Artemisia	0.148		100%
Artemisia	Limonium	0.820		0%

The ELISA inhibition demonstrates that not there is cross-reactivity between Lt and Compositae.

BAT results

In vitro stimulation of the patient's whole blood sample with the extract obtained from Lt solution was highly positive. Indeed, up to 76.3% of basophils expressed the CD63 surface molecule. The White Birch allergen used as control, was unable to induce a basophil activation, confirming the specificity of BAT. All clinically and serologically negative controls were below the 5% cut off, while both stimulation controls were highly positive, as a proof of the capacity of control sample basophils to be activated

Discussion

Occupational Asthma has been associated to the exposure to different plants. Causes of this asthma can be the pollen allergens or non pollinic plant allergens, which are present in other parts of the same plant. In other cases there can be reactions caused by cross reactivity between different plant species, taxonomically related (28).
Lt is a plant with an allergenicity known to cause rhinoconjunctivitis, asthma and urticaria following occupational exposition. The symptoms of the previous case report, described by Spanish authors (13), were associated to the inhalation of powders from dried flowers and both in vitro and in vivo tests were performed using the powder obtained by the grinding of the dried flower of Lt. In this paper we described another worker of the floriculture industry that had asthma attacks when handling fresh flowers but only during fluorescence period. For this reason, a fresh allergen extract derived from of Lt was obtained and used for the above described in vivo and in vitro tests.
The patient was certainly asthmatic, as suggested by the clinical history and confirmed by the results of methacoline test. The specific sensitization to the Limonium tataricum was also demonstrated by the positivity of SPT, while specific IgE detection, performed using a non-commercial method, were only slightly positive. In vivo, the nasal provocation test and in ex-vivo, the activation of basophils, were both positive. Of note ELISA-inhibition demonstrated clearly the absence of any cross-reactivity with other pollen extracts against which the patient was sensitized, suggesting that the case report considered is a genuine allergy. To these functional and laboratory results, it should be added that, in this patient, asthma occurred only during the blooming period. Along this line, a sensitization was also observed by detecting specific IgE against Artemisia, whose florescence period is clearly different from that of Lt. The novel finding of this paper is that, in the studied patient, symptoms were related to the presence of fresh allergen, while the previous paper described a patient who had allergic symptoms in the presence of dried flowers. This is interesting not only for the worldwide diffusion of Lt as decorative plant, but also for the large number of employers of the flower industry who could, at least potentially, sensitize to the pollen during the period from June to September, when the plant is in bloom, and risk an asthmatic attack.
Some other observations can derive from this study. First, despite the large number of allergens available commercially for highly sensitive tests, such as specific IgE detection, not all allergens are sold. In particular, novel or extremely rare allergens are not available. A similar consideration can be done on highly purified (or recombinant) allergens, in particular those spotted on the matrix of allergen microarray. Thus, in these situations, "classic" laboratory tests based on sIgE binding to a given allergen are irrelevant for the diagnosis. On the contrary, other "functional" tests, such as SPT, provocation tests and BAT, may be more useful. This must be kept in mind in the presence of a clinical picture suggesting a role of an allergen in which every other know allergen is negative (or the rare positive allergens observed cannot explain the signs and symptoms observed). In this context, it should be noted that "functional" tests, such as SPT and provocation test (two approaches belonging to the classic armamentarium of allergy diagnosis) require the preparation of a specific extract to be employed. These "homemade" extracts have a great potential for the diagnosis but the results obtained by their use should be carefully taken into consideration. Similarly, the use of BAT, recently introduced in the diagnosis of allergy, has a great potential not only for its intrinsic capacities (namely, the high specificity of the method and the very good sensitivity of the assay) but also for the large number of controls (negative subjects, mock allergens etc.) that should be carried out in order to improve the clinical significance and reliability of the laboratory result.
Thus, it should be considered that, despite the poor number of specific IgE against Lt, the patient's history reports a massive exposition to the allergen, related to his occupational activity. This finding suggests that a serious clinical event may occur even in the absence of a high score of sIgE, specific for the suspect allergen. In addition, functional tests, that were always significantly high and specific in this patient, clearly overcome any laboratory evidence, based on classic assays. These two considerations should be always kept in mind when the allergologist is facing complex cases, in particular those sustained by non conventional allergens.

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