

Occupational asthma caused by soybean flour in bakers—differences with soybean-induced epidemic asthma

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Summary

Background Soybean dust has been identified as the causative agent of occupational asthma and asthma epidemics. Two main soybean hull allergens responsible for asthma outbreaks, Gly m 1 and Gly m 2, have been identified and purified.

Objective The soybean allergens causing occupational asthma in exposed bakers were investigated and compared with those involved in epidemic asthma.

Methods We report four bakers or confectioners with work-related respiratory symptoms who were exposed to soybean flour used as a baking additive. The causative role of soybean flour was investigated by immunological tests and specific inhalation challenge tests. Soybean flour allergens causing occupational asthma were characterized by immunoblotting. Immunoglobulin (Ig) E-reactivity to Gly m 1 and Gly m 2 was assessed using enzyme-linked immunosorbent assay.

Results Sensitization to soybean flour was demonstrated by skin and serological tests and was confirmed by positive inhalation tests. Bronchial challenge test to soybean flour extract elicited immediate or dual asthmatic responses. Immunoblotting with soybean flour and soybean hull extracts showed IgE-binding mainly to high molecular weight (MW) allergens. There was an important individually different allergic response to inhalant soybean components. None of the patients showed IgE-reactivity against Gly m 1 and only one patient showed IgE-reactivity to the soybean hull allergen Gly m 2.

Conclusion These bakery workers had developed IgE-mediated occupational asthma to soybean flour. The allergens involved in occupational asthma caused by soybean flour are predominantly high MW proteins that are present both in soybean hull and flour, and they are different from the allergens causing asthma outbreaks, which are mainly low MW proteins concentrated in the hull.

Keywords: allergens, baker's asthma, bronchial challenge test, epidemic asthma, occupational asthma, soybean.

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Introduction

Exposure to inhalant allergens from soybean (*Glycine max*) dust and flour can give rise to allergic manifestations, such as rhinitis [1], occupational asthma [2,3], epidemic asthma [4–6] and hypersensitivity pneumonitis [7]. Although cereal flour proteins are known to be the main allergens causing respiratory sensitization among bakers and confectioners,

flours from different sources, such as soybean [8] and buckwheat [9], and α -amylase from *A. oryzae* [10] can also cause occupational asthma. Soybean flour is a common baking additive, which is routinely added to dough in order to improve the baking products and to bleach dough carotinoids. Baur *et al* [8] reported that the frequency of sensitization of symptomatic bakers to soybean flour was 21%, indicating that soybean flour is an important allergen in the baking industry.

In the last years several asthma outbreaks occurred in the Spanish cities of Barcelona [4] and Cartagena [5].

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Table 1. Clinical characteristics of the study patients

Subject no.	Age (years)	Job	Duration of exposure (months)	Duration of symptoms (months)	FVC (L) (% pred.)	FEV ₁ (L) (% pred.)	FEV ₁ /FVC	Eos./mm ³
1	29	Confectioner	144	130	4.28 (114%)	3.61 (103%)	77%	280
2	35	Confectioner	240	72	4.76 (112%)	3.04 (84%)	64%	1030
3	39	Baker and confectioner	240	125	4.94 (99%)	3.62 (88%)	73%	106
4	36	Baker	96	80	5.05 (94%)	3.91 (88%)	77%	310

FVC: forced vital capacity; FEV₁: forced expiratory volume in the first second; Eos. absolute count of eosinophils in peripheral blood/mm³.

Subsequent studies linked the asthma epidemics to soybean dust released during the unloading of soybean into harbour silos. Soybean hull allergens have been partially characterized where two main allergens, Gly m 1 [11] and Gly m 2 [12], have been identified. The similarities between soybean epidemic asthma and occupational asthma have been described [13]. However, the soybean allergens involved in occupational and epidemic asthma have not been compared previously.

In this article we present the clinical and immunological studies carried out in four bakers or confectioners in whom allergic respiratory symptoms developed upon exposure to soybean flour and other baking ingredients. Since bakers and confectioners are usually exposed to soybean flour we investigated whether sensitization to the main soybean hull allergens (Gly m 1 and Gly m 2) may be relevant in these bakery workers.

Materials and methods

Subjects

Four bakers and/or confectioners who developed symptoms of cough, chest tightness, shortness of breath and wheeze were evaluated. They were all male and their ages ranged from 29 to 39 years. They had been working in the baking industry from between 96 and 240 months. The mean duration of asthma symptoms in these patients was 101 months (range 72–130 months). Patients' characteristics and demographic data are presented in Table 1. They were all ex-smokers and they were still working at the time of evaluation. Sneezing, itching and watery eyes and nose preceded the development of chest symptoms in all patients. All of them improved when on holiday and during sick leave. Besides cereal flour, these patients also routinely handled flour additives containing soybean flour and fungal

amylase. All the patients could eat bread and baking products without any symptoms.

They were taking inhaled corticosteroids on a regular basis and short-acting beta-agonists as needed at the time of the study. The subjects were told to refrain from taking inhaled bronchodilators for at least 6 h before inhalation testing and the dose of inhaled corticosteroids was taken the night before bronchial inhalation test.

Allergenic extracts

Additive-free intact soybeans with hulls (El Granero Integral SL, Madrid, Spain) were ground with a coffee grinder and extracted with 0.9% NaCl at a 10% (w/v) ratio, by stirring for 1 h at room temperature. The extract was separated from the solid material by centrifugation at 22000 g for 20 min, and dialysed against distilled water for 24 h. Afterwards, the extract was centrifuged again as before, filtered through a 0.22 µm pore size membrane (Millipore, Bedford, MA, USA) and aliquoted. The soybean flour extract was stored at less than 0 °C until use for both *in vivo* (skin and bronchial challenge) and *in vitro* tests. Trypsin inhibitor from soybean (T-9003) supplied by Sigma Chemical Co. (St Louis, MO, USA) was prepared at a concentration of 10 mg/mL in saline solution 0.9% and passed through a 0.22 µm pore size membrane for sterilization. Moreover, an extract from soybean hulls 5% w/v was obtained as previously described [14] and used in immunoblotting.

Skin tests

Skin-prick tests were performed with the soybean flour extract 10% w/v and trypsin inhibitor from soybean 10 mg/mL, as well as with commercially available extracts of soybean flour 10 mg/mL (CBF Leti, Madrid, Spain),

wheat flour 5% w/v (ALK-Abelló, Madrid, Spain) α -amylase from *Aspergillus oryzae* 20 mg/mL (Stallergènes SA, Antony, France) and with a panel of common inhalant allergens (ALK-Abelló, Madrid, Spain), including pollens (grass, trees and weeds), mites (*Dermatophagoides pteronyssinus* and *D. farinae*), storage mites (*Acarus siro*, *Lepidoglyphus destructor*, and *Tyrophagus putrescentiae*), animal dander (cat and dog), and moulds (*Alternaria*, *Aspergillus* and *Penicillium*).

Histamine phosphate at 10 mg/mL and normal saline were used as positive and negative controls, respectively. A weal with a mean diameter 3 mm or greater with erythema, occurring 15 min after testing, was defined as a positive reaction, the skin-prick test with the saline was defined as a negative reaction or control.

Total and specific IgE determinations

Total serum IgE was measured by Pharmacia CAP System IgE fluoroenzyme immunoassay (FEIA) (Pharmacia Diagnostics, Uppsala, Sweden). The serum levels of specific IgE-antibodies against soybean and wheat flour and α -amylase were measured in every patient by Pharmacia CAP System RAST FEIA (Pharmacia Diagnostics, Uppsala, Sweden). CAP scores higher than 0.35 kU/L were regarded as positive, as recommended by the manufacturer. Moreover, the serum concentration of specific IgE-antibodies against purified Gly m 1 [11] and Gly m 2 [12] was measured by enzyme-linked immunosorbent assay (ELISA) technique as described elsewhere. Serum from an atopic patient unexposed to soybean flour was used as a control. A result was considered positive when serum bound four times more specific IgE than the titre of the control.

Quantification of Gly m 1 in soybean extracts and in baking additives

The content of the major soybean hull allergen Gly m 1 was measured in the soybean flour and soybean hull extracts and in different baking additives containing soybean flour by using a monoclonal antibody-based sandwich ELISA (detection limit <0.2 ng/mL) as previously described [15]. The baking additives handled by the patients: Lecibrem complet (Lecibrem, Madrid, Spain) and Tolerant (Ireks-Arcady, Kulmbach, Germany) contain a mixture of ingredients, including soybean flour, fungal α -amylase, malt flour and several preservatives. These baking additives, as well as roasted soybean flour (Nido, Barcelona, Spain), were extracted with phosphate-buffered saline (PBS) at a 10% (w/v) ratio, by stirring for 16 h at 4 °C, and then the Gly m 1 content in these extracts was quantified.

SDS-PAGE and immunoblotting

Polyacrylamide gel electrophoresis in the presence of dodecyl sulphate (SDS-PAGE) was performed with a 8–25% acrylamide gradient in a high molarity Tris buffer system under nonreducing conditions [16]. SeeBlue prestained standards (Novex, San Diego, CA, USA) were used as molecular weight (MW) markers. An amount of 100 μ g of protein of soybean flour (1.22 ng Gly m 1) and soybean hull (12.5 μ g Gly m 1) extracts and 10 μ g of purified Gly m 1 (ALK-Abelló, Madrid, Spain) were applied per lane. Protein bands were visualized with Coomassie brilliant blue.

After running, the separated proteins were electrophoretically transferred to Hybond ECL membranes (Amersham, Buckinghamshire, UK) following the method of Towbin *et al* [17]. Unreacted membrane sites were blocked with PBS containing 1% BSA and 0.05% polysorbate 20 (Tween 20) for 1 h at room temperature. After four washes with PBS-Tween 20 0.05%, blots were incubated with 2.5 mL of human serum diluted 1 : 5 in PBS containing 1% BSA and 0.05% Tween 20. After washing, 2.5 mL of a solution of antihuman IgE HE2 mouse monoclonal antibody was added and incubated for 1 h at room temperature. The blots were washed four times, as previously, and were incubated with peroxidase-conjugated rabbit antimouse immunoglobulin (DAKO, Glostrup, Denmark) diluted 1 : 5000 for 1 h at room temperature. Thereafter, they were incubated with Supersignal substrate (Pierce, Rockford, IL, USA) for 5 min at room temperature. IgE-binding proteins were visualized by autoradiography with appropriate exposure times. Individual serum samples from the four bakers, a serum pool from 20 individuals affected by the asthma outbreaks that occurred in Cartagena [11] and from an atopic patient unexposed to soybean flour (control) were used in immunoblotting experiments.

Bronchial provocation tests

Methacholine inhalation test was performed according to Cockcroft [18] with some modifications. The aerosolized particles were generated by a continuous pressurized nebulizer model DeVilbiss 646 (DeVilbiss Co., Somerset, PA, USA) with an output of 0.28 mL/min. The result of this test was expressed as the provocative concentration of methacholine causing a 20% fall in forced expiratory volume in 1 s (PC₂₀) and it was determined by interpolation of the last two concentrations.

Specific bronchial challenge was carried out with the water-soluble soybean flour extract, following the method previously described [19]. The patient inhaled the aerosolized allergen using the nebulizer method mentioned above in progressive concentrations at tidal breathing for 2 min. A control challenge with PBS was carried out before antigen

Table 2. Results of skin-prick tests and bronchial challenge tests

Subject no.	SPT					Inhalation tests	
	Soybean 10%	Tryp inh. 1%	Wheat 5%	α -amylase 2%	Other	PC ₂₀ met. mg/mL	BPT fall in FEV ₁
1	6	0	7	8	pollen, dog	2	22%
2	5	0	5.5	0	Pollen	0.29	26%
3	8	3	5.5	14	pollen, dust mites	0.25	23%*
4	8	5.5	4	0	—	0.31	40%

SPT: skin-prick tests (mean of the largest weal orthogonal diameters in mm); BPT: bronchial provocation test with soybean flour extract (maximum fall in FEV₁ observed during the EAR). *Patient 3 showed a dual asthmatic response.

provocation. Increasing concentrations of soybean flour extract were given by inhalation starting with a concentration that induced a 2–3 mm weal on skin-prick testing. The dose was increased in twofold increments at intervals of 10 min and FEV₁ and FVC were measured at 5 and 10 min after inhalation of each concentration. Bronchial challenge test was discontinued when there was a fall in FEV₁ of 20% or greater from the lowest postsaline value or when the highest concentration had been given. At the end of the inhalation challenge test, spirometry was performed at 20, 30, 40 and 60 min after challenge, and again the following day. Peak expiratory flow rate (PEF) was measured before and every hour after bronchial challenge until bedtime, and again the day after. A fall in FEV₁ of 20% or more from the lowest postsaline within 60 min of challenge was considered a positive immediate reaction, whereas a fall in PEF greater than 25% between 2 and 24 h after challenge was considered a positive late reaction if no change was observed during the control day.

Results

Skin tests

After skin testing all patients showed a positive response to both the aqueous and commercial soybean flour extracts and also to wheat flour extract. Moreover, a positive response to soybean trypsin inhibitor was observed in patients 3 and 4. A positive response to fungal α -amylase was also observed in two of the patients. Skin tests results are shown in Table 2. Three out of the four patients were atopic as determined by positive skin tests to common inhalant allergens.

Specific IgE determinations

Measurement of soybean-specific IgE-antibodies was positive

in three out of the four patients. Moreover, specific IgE-antibodies against wheat flour were present in the sera from all the patients, and IgE against α -amylase was detected in two of them. Determinations of specific IgE-antibodies by ELISA technique against the purified soybean hull allergen Gly m 1 were negative in all the patients, as were to Gly m 2, with the exception of patient 1 who showed a positive result to the latter allergen. Measurement of specific IgE against Gly m 1 with the serum pool from patients with soybean epidemic asthma was strongly positive. Results of specific IgE determinations are shown in Table 3.

Quantification of Gly m 1 in soybean extracts and baking additives

The Gly m 1 content of the soybean hull and the soybean flour extracts was 125 μ g/mL and 12.2 ng/mL, respectively. The concentration of Gly m 1 in the baking additives was as follows: Lecibrem complet 187.89 μ g/g, Tolerant 24.66 μ g/g, and roasted soybean flour 72.1 μ g/g.

SDS-PAGE immunoblotting

Figure 1 shows the protein composition of soybean flour, soybean hull and Gly m 1. Molecular weight analysis showed that soybean flour contains a wide range of protein sizes, being relevant especially between those of 18 and 51 kDa, whereas soybean hull mainly contains low MW polypeptides. A strong band with an apparent MW of 7.5 kDa was present in the soybean hull extract, corresponding to the same MW as Gly m 1.

The IgE-binding bands of the soybean flour and soybean hull extracts studied by immunoblotting are shown in Fig. 2. In the soybean flour extract, serum from patient 1 reacted to several allergens with MWs between 39.8 and 55.3 kDa, whereas serum from patient 2 reacted to three IgE-binding

Table 3. Results of total and specific IgE determinations

Subject No.	Soybean kU/l	Wheat kU/l	α -amylase kU/l	Gly m 1 OD	Gly m 2 OD	Total IgE kU/l
1	6.1	18	1.5	0.058	0.634	271
2	1.9	74.2	0	0.042	0.071	1637
3	1.36	25.8	4.63	0.037	0.059	1387
4	0	1.66	0	0.049	0.033	107
Control	<0.35	<0.35	<0.35	0.036	0.032	229
Epidemic patients*	ND	ND	ND	1.120	ND	ND

OD, absorbance in optical densities at 450 nm; ND: not done. *Serum pool from 20 patients with soybean-induced epidemic asthma.

bands with estimated MWs of 43.6, 37.1 and 35.5 kDa, and serum from patient 3 showed IgE-binding with bands of 35, 33.6 and 25 kDa. However, serum from patient 4, which presented very low levels of circulating specific IgE against soybean (negative CAP), did not show IgE-reactivity to any soybean flour allergen (lane 4). When immunoblotting was performed with the soybean hull extract, with serum from patient 1 several bands with ability to bind IgE with MWs of 43.4, 37.2, 33.6, 9.3 and 8 kDa were visualized. Serum from patient 2 showed strong IgE-binding bands at 56, 44.7, 42.7 and 33.9 kDa and additional faint bands at 28.2, 25.9 and 11.5 kDa. Serum from patient 3 reacted to a very faint band at 43.2 kDa. None of the patients showed IgE-binding to a low MW allergen such as Gly m 1 (7–7.5 kDa), and immunoblotting with purified Gly m 1 showed no reactivity (not shown). Only patient 1 showed IgE-binding to an

8-kDa allergen in soybean hull, that very likely corresponds to Gly m 2, in agreement with ELISA results in this patient. With the serum pool of individuals with epidemic asthma, up to 10 bands with ability to bind IgE could be visualized in the soybean hull extract, especially those with MWs of 7–8 kDa, which correspond to Gly m 1 and Gly m 2 (lane 6).

Bronchial challenge tests

All patients had nonspecific bronchial hyper-responsiveness as measured by methacholine inhalation test. Specific bronchial challenge with soybean flour extract resulted in early asthmatic responses in all patients; this was followed by a late asthmatic response only in patient 3. The concentration of soybean flour extract causing a fall in FEV₁ of 20% or

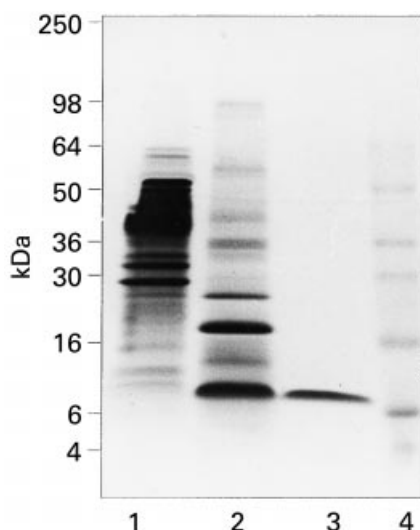


Fig. 1. SDS-PAGE of the soybean flour extract (lane 1), soybean hull extract (lane 2), purified Gly m 1 (lane 3), and molecular weight markers (lane 4)

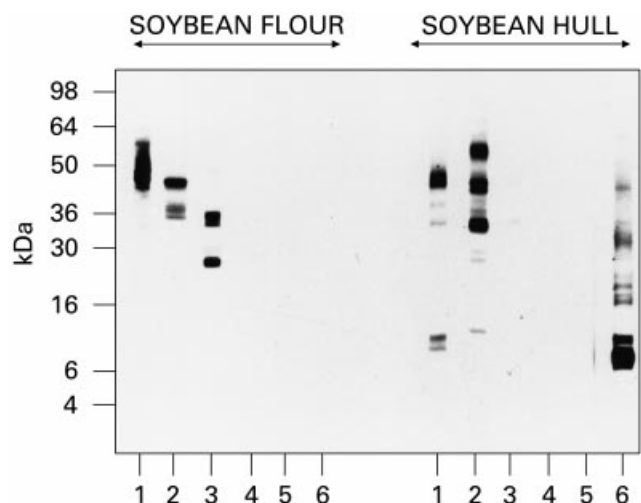


Fig. 2. IgE-immunoblots of the soybean flour and soybean hull extracts incubated with individual serum from four bakers (lanes 1–4), control serum (lane 5) and a serum pool from 20 patients with soybean-induced epidemic asthma (lane 6)

greater was 1/160 w/v in three patients and 1/20 w/v in the remaining subject (patient 1). The results of bronchial provocation tests are shown in Table 2.

Discussion

Soybean is considered a very potent source of both food [20] and inhalant allergens [2–8]. However, there are few studies demonstrating the causative role of soybean allergens in asthma as confirmed by specific inhalation tests. The immunological studies performed in the bakers reported herein showed that they had developed IgE-mediated hypersensitivity to soybean flour allergens as well as to cereal flour allergens or fungal α -amylase. Moreover, bronchial provocation test with soybean flour demonstrated the clinical relevance of exposure and sensitization to soybean allergens as a cause of asthma. Polysensitization to occupational agents seems to be common in bakers [8,10,21] and it may result from an atopic background or perhaps it is due to the fact that the ongoing immune-inflammatory reaction in the airways may facilitate sensitization to several allergens found in the work environment.

Studies performed by Baur *et al.* [22] revealed that 20% and 32% of bakers who suffered from asthma or rhinitis had elevated soybean flour specific IgE-antibodies, and in a previous study we found that nine out of 21 (43%) cereal workers were sensitized to soybean flour [21]. However, soybean flour allergens causing occupational asthma have rarely been investigated. In a worker in a food-processing plant in whom soybean flour-induced occupational asthma developed, nine allergens with MWs ranging from 14.8 to 54.5 kDa were identified [2]. In a recent study of 14 bakers suffering from work-related respiratory symptoms who were sensitized to soybean flour, it was found that the trypsin inhibitor (MW 20 kDa) was an important soybean inhalant allergen [22], as we corroborated in two of our patients who showed a positive skin test to this allergen. Nevertheless, there appears to be an important individually different allergic response to inhalant soybean components.

The asthma outbreaks that took place a few years ago in the Spanish cities of Barcelona [4], Cartagena [5] and Tarragona [23], as well as in New Orleans [6], were associated with the unloading of soybeans in the harbour. There is a good correlation between the soybean-induced asthma and the presence of specific IgE to soybean hull allergens [24]. Atopy as well as cigarette smoking are risk factors for this disease [25], and the risk of epidemic asthma is particularly associated with the HLA-DRB1*13 gene [26]. Asthma outbreaks in Barcelona showed some characteristics features such as the sudden onset, high severity and rapid recovery. However, some intriguing findings have been reported: there was no evidence of late phase allergic response, severe attacks occurred most often in

men, children were not affected and no cases of epidemic asthma were detected among workers in the harbour [27]. Moreover, asthma attacks could not be reproduced in the laboratory with the standard methods of antigen challenge [27]. This fact has been related to differences in the size of allergen particles, and it has been hypothesized that during unloading of soybean in the harbour particles of the allergenic agent were emitted into the atmosphere in a way that is difficult to reproduce with the methods currently used in antigen challenge [27]. This finding is quite surprising since the major soybean hull allergens are hydrophilic glycoproteins, which are easily extracted from soybean hulls [14] and IgE-mediated asthmatic responses caused by water-soluble allergens can be consistently reproduced by specific inhalation challenge [19]. In fact, bronchial provocation tests with soybean hull extract carried out in three patients with epidemic asthma from Cartagena elicited positive asthmatic responses [28]. It has been postulated that stored soybeans may be contaminated with moulds, mites and bacteria that may be partially responsible as causative agents for soybean-induced asthma outbreaks [29]. Likewise, it has been recently shown that soybean hulls are contaminated by *Aspergillus* and *Penicillium* species and that the sera of asthma-induced epidemic patients contain higher levels of specific IgE to several *Aspergillus* and *Penicillium* species compared with that of asthmatic-nonepidemic patients [29].

An initial and partial characterization study of the allergens involved in soybean-epidemic asthma demonstrated two glycoproteins concentrated mainly in the hull with a MW less than 14.4 kDa [30]. Thereafter, a soybean hull allergen, Gly m 1, with two isoallergens, Gly m 1A (7.5 kDa) and Gly m 1B (7 kDa), was purified and identified as the main allergen responsible for the asthma outbreaks in Cartagena [11]. A second soybean hull allergen, Gly m 2, with a MW of 8 kDa was further purified and associated with the Barcelona asthma outbreaks [12]. Nevertheless, additional higher MW allergens have also been incriminated in soybean-induced epidemic asthma [14,30,31].

In our patients sensitized to soybean flour, the pattern of IgE-binding is quite individualistic. However, despite the fact that they reacted to several soybean proteins and that they were exposed to baking additives containing Gly m 1, this major allergen involved in soybean epidemic asthma does not seem to be relevant in patients with occupational asthma caused by soybean flour. This finding could be due to differences in the type and level of exposure, particle size, allergen load and possibly to the different populations affected by epidemic and occupational asthma. Actually, the concentration of Gly m 1 of the soybean flour was much less than that of the soybean hull extract, suggesting that exposure to soybean hull allergens responsible for epidemic asthma is significantly less among bakers. Nonetheless, the

possibility of sensitization to soybean hull allergens among bakers should not be ignored, as we were able to confirm sensitization to Gly m 2 in one of our patients.

Taken together, these data suggest that soybean inhalant allergy involves several major allergens and that different soybean-allergic individuals may react to different proteins. Whereas soybean allergens causing asthma outbreaks are mainly low MW proteins concentrated in the hull, the allergens involved in occupational asthma caused by soybean flour are predominantly high MW proteins that are present both in soybean hull and flour. Whether the different pattern of allergen recognition is associated with differential clinical features, such as severity of the disease, remains to be elucidated.

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