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THE EFFECT OF *TYPHA DOMINGENSIS* PERS. POLLEN ON BALB/C MICE MIMICKING LOCAL ALLERGIC RHINITIS LIKE SYMPTOMS

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Abstract

Airborne pollen grains found commonly in the air usually cause hay fever or pollinosis. *Typha* sp. pollen grain has been frequently reported in various aerobiological surveys from Pakistan. This study was designed to investigate the allergenic potential of *Typha domingensis* Pers. Animal model was developed for *In vivo* testing of pollen allergy. Freshly collected pollen powder was used for intranasal sensitization of Balb/c mice. *In vitro* testing of the pollen was also done quantitatively (Bradford's method) and qualitatively (SDS-PAGE analysis). The results of *In vivo* study showed that the group of mice exposed to the dried pollen powder, experienced allergy symptoms including itching and rubbing of eyes, feet, and tail along with frequent episodes of sneezing. The results were confirmed by performing differential blood count of mice. Obtained data was statistically analyzed by using Sigma plot that revealed significant 4-fold increase in eosinophil count in sensitized mice as compared to control (Two tailed t-test with p-value <0.001). SDS-PAGE analysis of extracted proteins showed that the proteins resolved into six bands of molecular weight ranging from 20 KDa- 55 KDa. In current study, data strongly depicts that *T. domingensis* could be possible allergenic particle. This study would also aid in allergy suffering patient therapy.

Key words: Allergic rhinitis, Laboratory animal models, Pollen allergy, Respiratory hypersensitivity.

Introduction

Air current carries various biological particles including pollen grains, fungal spores as well as various pathogens, which can harm humans, plants, and animals (Waqar *et al*., 2010). Some organisms are obligatory airborne while others are carried by the wind. Pollen from floral resources are usually present in air. A difference in the pattern of their count and type is observed depending upon the time of day, climatic conditions and local flora (Perveen *et al*., 2014).

Pollen grains contain proteins and glycoproteins that cause the allergic reaction (Knox & Suphioglu, 1996). Bradford's method is an accurate and fast method for the protein estimation. This method is usually preferred for the quantification of protein in laboratories. This technique is not only simple and quick but also more sensitive than other methods. Moreover, when this method is compared with the other methods, its results are not disturbed by non-protein constituents of the biological samples (Walker, 2008).

SDS-PAGE is the most frequently used technique for qualitative analysis protein mixtures. It is useful for purification and separation of protein in accordance to their molecular weight and shape etc. In this technique, protein is reacted with sodium dodecyl sulphate (SDS), an ionic detergent. After reaction, the protein converts to a negatively charged complex. The amount of charge and SDS bounded by the protein is equivalent to the protein size. The cross-linked polyacrylamide matrix acts as a molecular sieve. Large molecular weight proteins travel slowly while low molecular weight proteins travel faster in the matrix. Negatively charged proteins then separate and resolve as distinct bands (Osborne & Brooks, 2006; Walker, 2008).

The allergenic particles cause various types of allergies, including skin or atopic dermatitis / contact dermatitis, ocular or allergic conjunctivitis, respiratory allergy (hay fever and asthma), gastrointestinal allergies (food allergy). Mice, rats, and guinea pigs are considered suitable for the research on gastrointestinal and respiratory allergies. Aerosolized biological particles are capable of inducing allergic reactions in the body either directly as the allergen particles source or indirectly by exacerbation of allergic rhinitis symptoms. For the detection of allergy symptoms the mice model is considered most efficient *in vivo* animal model. In various other experiments, Balb/c mice have been utilized to analyze the allergic effects of pollen grains (Hau & Hoosier, 2004).

Introduction to the study area: The province Sindh is located at Southeast of Pakistan (Fig. 1). It is bordered by Balochistan province to the west, and Punjab province to the north. Sindh also borders the Indian states of Gujarat and Rajasthan to the east, and Arabian Sea to the south. Sindh is the second largest province (in terms of Population) of Pakistan. This province has a total area of 140,914 km^2. Karachi is the largest and most populous city of Sindh (Perveen *et. al.,* 2015). Hyderabad is second largest city of Sindh province and Tando-Jam is a town of Hyderabad District. Khairpur is also a city of Sindh province. Aerobiological investigations were performed previously in those areas (Perveen *et al*., 2012; Perveen *et al*., 2014; Perveen *et al*., 2015). *Typha* sp. pollen was detected in various aerobiological surveys from Sindh, Pakistan. Therefore, this plant pollen was used in allergy testing in murine model. *T. domingensis* pollen type was analyzed for its allergenic property. Balb/c mice model was developed to test its allergenic property. Pollen grains have low molecular weight proteins or glycoproteins (<70 KDa) that surprisingly are the major cause of allergic reaction in hypersensitive individuals. The proteins present in pollen are stable under high temperature (up to 100°C) (Puc, 2003).Further confirmation of the allergenic potential of *T. domingensis* pollen and its protein analysis has also been performed along with the *In vivo* animal model testing.

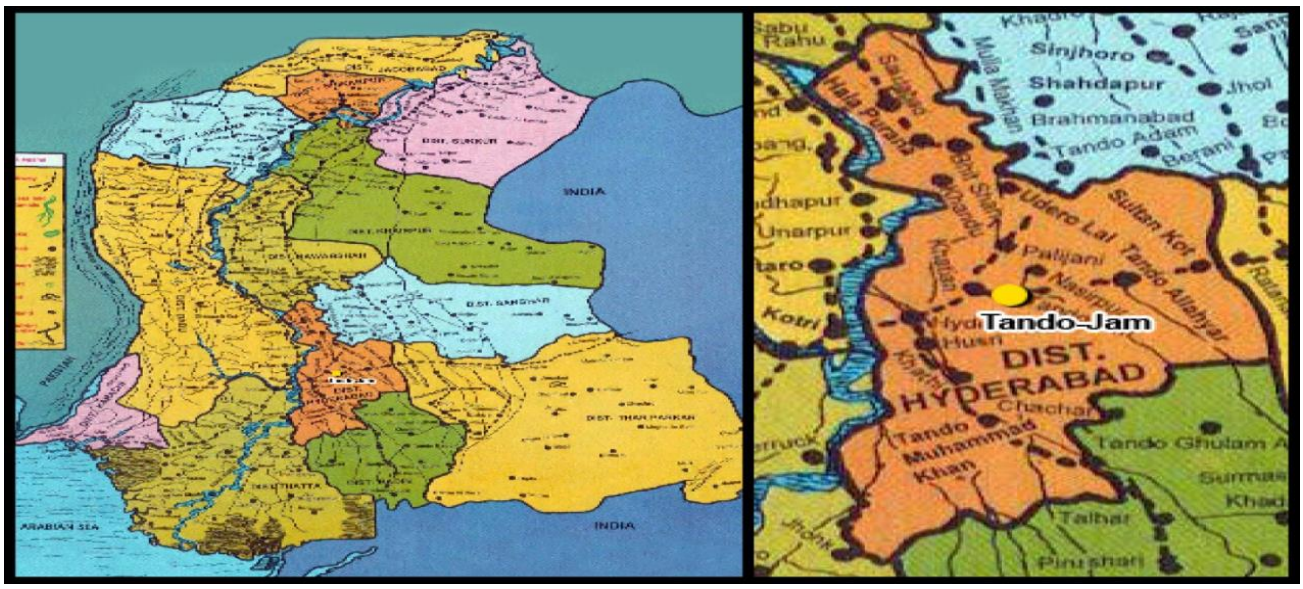

Fig. 1. Map of Sindh, Pakistan.

Materials and Methods

Pollen grains collection and protein extraction: *Typha domingensis* Pers. Pollen were collected from University of Karachi. Voucher specimen (G.H.No. 89538) was submitted in Karachi University Herbarium (KUH), Centre for Plant Conservation, University of Karachi. Pure pollen powder was obtained by directly dusting male inflorescence on the sieve set (300 μm mesh size and 100μm mesh size). Defatting of the pollen was done by adding diethyl ether on pollen grains and allowed to air dry. The procedure was repeated three times. Extraction of proteins was done in phosphate buffer saline solution (PBS). Protein quantification was done by Bradford assay 1976, A quick and rapid method for protein estimation in micrograms was used (He, 2011). BSA (Bovine Serum Albumin) was used as protein standard.

SDS-PAGE analysis: SDS-PAGE analysis was done to identify the protein bands present in the pollen extract. Label 1.5mL microfuge tubes for protein samples. Take 20 μL (1.5 μg/μL) mix with 20μL sample lysis buffer (with 2-mercaptoethanol). Vortex the mixture and incubate in 95˚C at water bath for 05 minutes. Cool to room temperature. Resolving gel concentration was 15% and stacking gel concentration was 4.5%. About 20 μL of prepared protein sample was loaded.

Animal model: A total of 42 male Balb/c mice were purchased from the animal house of International Centre for Chemical and Biological sciences (ICCBS), University of Karachi. The mice were about six to seven weeks old weighing about 25-30g. Those were kept in normal husbandry conditions. Animal study was endorsed by the 'Institutional Animal Care and Use Committee' of International Centre for Chemical and Biological sciences (ICCBS), University of Karachi. The animals were housed in labeled cages in a room maintained at 21 ± 1˚C temperature 57% humidity with 12 hour light and 12 hour dark period. Standard feed was given to the mice containing 14% protein, 82.5% carbohydrates, and 3.5% fat. International guidelines were used for the care and use of laboratory experimental animals. The mice were divided in 7 groups each containing six mice (n= 6). One group was untreated and used as control. Other 6 groups were exposed to pure pollen powder of *T. domingensis*.

Test mice groups were sensitized through nasal exposure to 0.1g dry pollen powder on 1st week of experiment. The mice were again exposed for 2nd and 3rd week of experiment for the confirmation of allergy symptoms due to pollen. All the clinically important allergy symptoms were recorded. Differential leukocyte count was also done for further validation of allergic sensitization caused by the allergens.

Statistical Analysis: Statistical analysis of differential blood cell count was performed by using Systat Software, Inc. Sigma Plot for Windows ver. 13.0. Correlation of leucocytes count of control and test group was done by using two tailed *t-test*.

Results

Pollen grain powder of *Typha domingensis* were subjected to various biochemical analysis *In vitro* and allergy testing *in vivo* models. Allergenic property of the pollen was determined by using SDS-PAGE analysis and animal model testing. Allergy to pollen of *T. domingensis* has been reported for the first time from Pakistan.

Typha domingensis Pers. belongs to the family Typhaceae. It is a Unigeneric family having 10 species; represented in Pakistan by 5 species (Omer & Hashmi, 1987). *Typha* pollen has been reported in various aerobiological surveys performed in Sindh, Pakistan and in other countries as well (Kaplan, 2004; Waqar *et al*., 2010; Perveen *et al*., 2012). In Pakistan, this pollen has been detected from Sindh province especially airborne pollen studies conducted in the cities of Karachi, Hyderabad; Tando-Jam and Khairpur. The city of Karachi is the biggest metropolitan city of Pakistan while Hyderabad and Khiarpur are also important cities of Sindh. This *Typha* pollen has been repeatedly reported in those regions (Table 1).

Table 1: Occurrence of *Typha* pollen in various cities of Pakistan*.

City	Highest pollen count/m^3	Total yearly pollen count /m^3
Tando-Jam	106	257
Karachi	20	61
Khairpur	47	223

*Note: Data obtained from previously published reports (Perveen *et al*., 2012; Perveen *et al*., 2014; Perveen *et al*., 2015)

Protein analysis of *Typha domingensis*: Pollen proteins in PBS extract were determined by Bradford method. SDS-PAGE analysis of extracted protein was done. Results showed that *T. domingensis* pollen grain extract contained 0.196μg/μL of proteins. Pollen protein concentration was 3.934 mg/g of pollen.

20μL (3.92 μg proteins) of the extract was taken in SDS-PAGE. The SDS-PAGE analysis of pollen grain extract showed 06 protein bands of molecular weight < 55 KDa (Fig. 2).

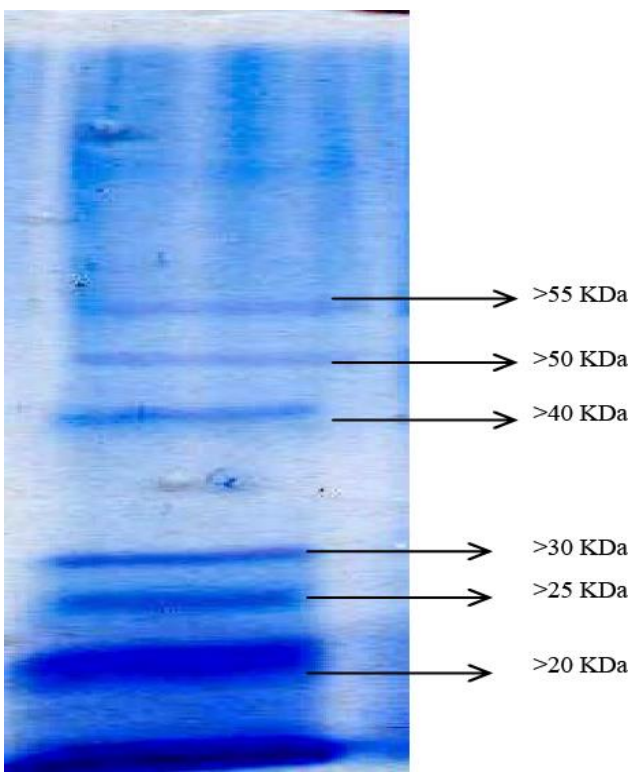

Fig. 2. SDS-PAGE gel of *Typha domingensis* pollen proteins.

Intranasal sensitization of mice model: Dry pollen grains of *T. domingensis* were exposed to the mice through nasal exposure. On the 1st week of exposure, the mice were sensitized to the pollen and only 50% of the mice showed allergy symptoms including itching and sneezing few mice showed puffiness in eyes. On the 2nd week, the mice showed sharp allergy symptoms. After 05 minutes of pollen exposure, all of 36 mice started rubbing eyes, itching on the nose, face, limbs, and tail. After 10 minutes of exposure, 30 out of 36 mice started sneezing frequently with 10-15 numbers of sneezes per minute. Remaining 06 mice also sneezed but the rate of sneezing was below 9 sneezes per minute showing mild allergy symptoms. On the 3rd week of experiment, the mice were again sensitized and all of the 36 mice showed clear and high allergy symptoms including puffiness of eyes, itching of face, eyes, nose, limbs, tail and sneezing (Table 2). Decrease of physical activity was observed for almost 1 hour after the pollen exposure. Blood samples were collected on 3rd week of study after 12 hours of pollen exposure. The blood test showed positive allergy test. Microscopy of blood samples revealed that there was a sharp increase in the eosinophils count in sensitized mice while the control mice showed normal counts. In total 5000 cells were counted for confirmation of allergy symptoms. In the control mice, the white blood cells (WBC) counts were as eosinophils 4%, neutrophil 60%, lymphocytes 34%, monocytes 2%, and basophil 0%. However, in the sensitized mice the average cell count (presented in % values of total count) i.e. eosinophils, neutrophils, lymphocytes, monocytes, and basophils were 16%, 55%, 25%, 2%, and 2%, respectively (Fig. 3-7). Statistically significant results was obtained by comparing the eosinophils count of control and test mice of animal model with the two tailed P-value= <0.001 (Table 3)

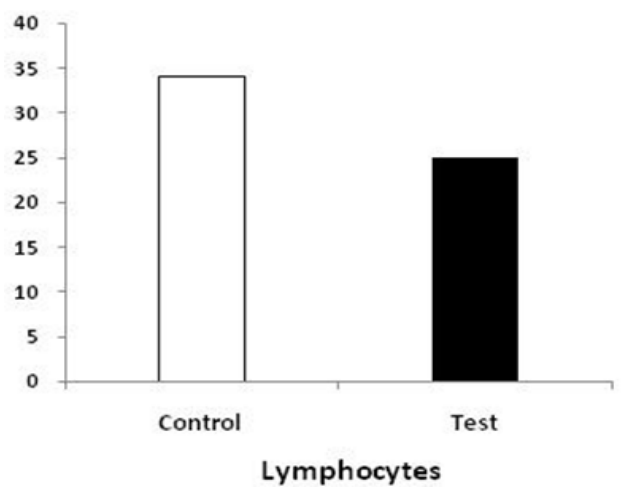

Fig. 3. Comparison of lymphocytes cell count.

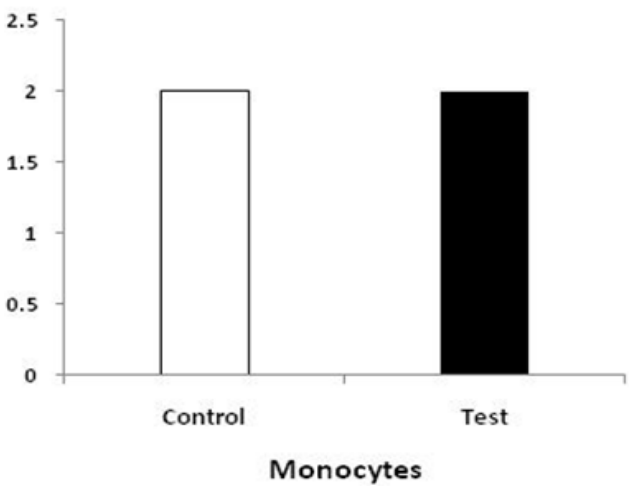

Fig. 4. Comparison of monocytes cell count.

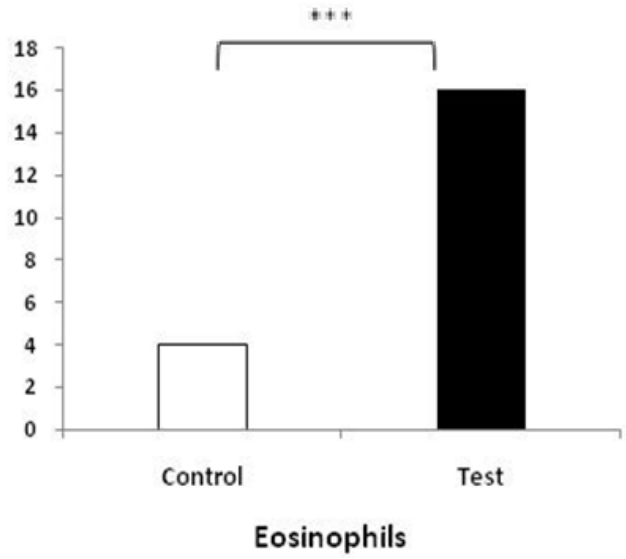

Fig. 5. Comparison of eosinophils cell count count.

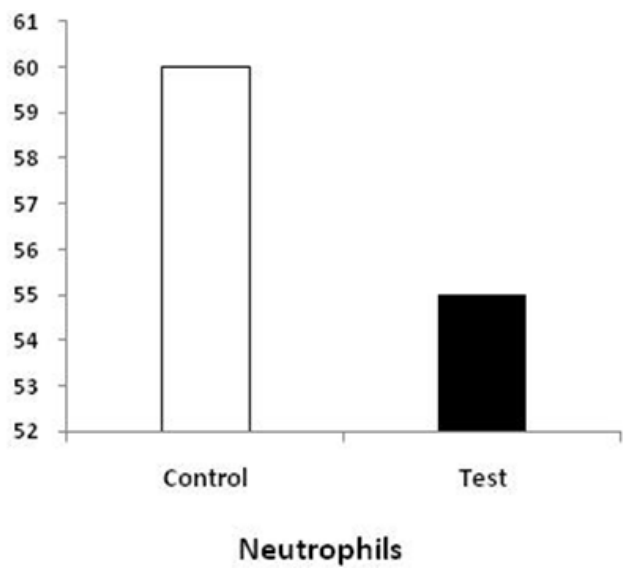

Fig. 6. Comparison of neutrophils cell
(***Level of significance P-value: <0.001)

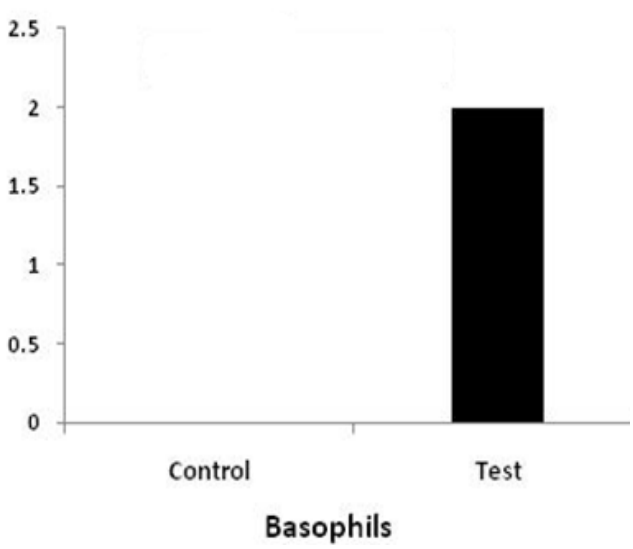

Fig. 7. Comparison of basophils cell count.

Table 3. Statistical analysis of differential leukocytes count of control and test mice (exposed to pollen).

Cell type	Control	Test	P-value
Eosinophils	4%	16%	<0.001
Neurtrophils	60%	55%	>0.01
Lymphocytes	34%	25%	>0.01
Monocytes	2%	2%	>0.01
Basophils	0%	2%	>0.01

Discussion

According to a report 54% of the total people in the world live in cosmopolitan cities (United Nations report, 2014).People living in cities spend major part of their life while working in offices, eating, and sleeping. Various transportation means, children schools, offices, restaurants, hospitals, libraries, and community centers can all have pollen encounter that can elicit allergic reaction (Rantio-Lehtimaki, 1991; Chanda, 1994; Reponen, 1994; Verhoeff, 1994; Nikkels *et al*., 1996; Garrett, 1997; Flannigan *et al*., 2001).

Pollen grains are the specialized structures produced by the plants that contain male sperms or male gametes of the flowering angiosperms. Pollen allergy has been reported to play a key role in development of allergy symptoms in sensitive patients. Proteins, glycoproteins and sometime an amino acid in pollen can be the cause of bronchial anaphylaxis (Cresti & Tiezzi, 1992; Hrabina *et al*., 2008).

Further confirmation of *T. domingensis* pollen allergy was done by developing of animal model. Purified pollen grains from this species were tested on mice Balb/c animal model to check the allergenic property. Observations were recorded after the exposure of pollen to the individual animal for 2 hours. Mice were sensitized through intranasal pathway to pollen allergens for 10 minutes once a week for consecutive three weeks. Various research workers have done intranasal sensitization of mice for allergy testing (Yang *et al*., 2013; Kato *et al*., 2014). In the first week of experiment mild allergy symptoms were observed in the test mice. The allergy symptoms appeared more clearly in the 2nd and 3rd week of experiment. Assessment of allergy responses were made according to the guidelines (Li *et al*., 1999). Signs of anaphylaxis attack appeared in the Balb/c mice after 5 to 10 minutes of exposure to pollen. After exposure to allergen, the mice stared scratching and rubbing around the nose, head, tail, and limbs. Decreased activity, wheezing, and sneezing were observed in all of the mice in the last week of exposure. Similar signs of allergy were observed in a study of allergen testing (Li *et al*., 1999). The symptoms of allergy were further confirmed by the differential counting of white blood cells (WBC) after 24 hours of pollen exposure. Increased levels of eiosinophils were observed in the blood smear of mice confirming allergy conditions. Increased eosinophil count is typical during anaphylactic attack. Various studies have reported high eosinophil count in the blood and nasal mucosal membrane (Stampfli *et al*., 1992; Yamamoto *et al*., 2000; Hansen, 2007.

Table 2. Appearance of local Allergic rhinitis symptoms in mice exposed to 0.1g of *T. domingensis* pollen.

Weeks	% of Mice showing		
	Sneezing (>10 sneeze/min)	Puffiness of eyes	Atopic dermatitis
1st week of pollen exposure	50%	25%	50%
2nd week of pollen exposure	83.33%	100%	100%
3rd week of pollen exposure	100%	100%	100%

Conclusion

The aim of the current study was to identify the allergenicity of *T. domingensis* pollen grains. *T. domingensis* pollen grain protein extract was subjected to SDS-PAGE analysis for the protein test. SDS-PAGE analysis revealed that the pollen proteins of *T. domingensis* were resolved in six bands (Less than 55KDa). Low molecular weight allergenic proteins have also been detected in earlier protein extracts of allergenic pollen types (Corden *et al*., 2003; Kato *et al*., 2014). The pollen grains were able to induce allergic reaction in mice also. Thus, present study suggests that *T. domingensis* is important pollen allergen, which may become the cause of allergy in humans. Further investigations are required to identify the allergenic proteins that are responsible for eliciting allergic reactions.

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