

RESEARCH ARTICLE

Clinico-immunologic studies on allergy to pulses- a case report

Raj Kumar[#], Susheela Sridhara^{*}, Jyotsna Verma^{*}, Naveen Arora^{*} and B.P. Singh^{*}

[#]V.P. Chest Institute, Delhi and ^{*}Centre for Biochemical Technology, Delhi 110 007, India

Abstract

Worldwide about 6-8% children and 1-2% adults suffer from food allergy but no such data is available from India. A case with definite history of allergy (asthma) has been investigated using standard *in vivo* and *in vitro* tests. Double blind oral challenge test with dehusked horse gram (pulses) showed severe pulmonary obstruction (presenting reduced FEV1). Intradermal tests with seed extracts (pulses) revealed marked positive skin reactions. Further, ELISA with patient's serum showed significantly elevated IgE levels to various seed extracts compared to normal control. Immunoblotting with patient's serum showed 4-10 IgE binding proteins with MWs 45, 38, 35, 30, 28 and 14 kDa to be common in the food extracts, prepared from seeds of family leguminosae.

Key Words: Food allergens, Oral challenge test, Pulmonary function test, ELISA, Immunoblot.

INTRODUCTION

The term food allergy (hypersensitivity) is applied to a group of disorders characterized by abnormal or exaggerated response to various dietary proteins. This may result in breathlessness, rhinorrhoea, urticaria, dermatitis etc. in susceptible (atopic) individuals. IgE mediated food allergies were reported in 6-8% of children and 1-2% of adults¹⁻³. The prevalence of sensitivities to specific food is largely unknown except cow's milk allergy, which affects approximately 2% of young infants⁴. Severe food allergies have been poorly understood, however, the inadvertent ingestion of allergenic foods has resulted in death⁵. Fatal reactions were reported more commonly to peanuts, tree nuts and crustacea⁶ like egg, fish, etc.

Among the variety of foods available in India, the pulses viz. black gram, cajan pea, french bean, horse

gram, green gram and lentil from family leguminosae (subfamily papilionaceae) are used commonly in everyday diet. However, no systematic report is available about allergy to such foods of mass consumption. In the present study, a case with definite history of sensitivity to pulses has been investigated by skin tests, oral challenge tests and ELISA. Further, the IgE binding proteins of seed extracts were characterized by immunoblot using patient's serum.

MATERIALS AND METHODS

Clinical history: A 10 year's old girl presented with history of breathlessness, wheezing, nasal discharge, sneezing, conjunctivitis, nausea and vomiting after intake of cooked pulse or even water of raw pulse for last six years. Initially, she reported the development of black rashes and itching whenever she consumed pulses (local name-dal) or came in contact with them. She has a history of allergy with her mother suffering from urticarial rashes and rhinitis. The patient was normal on physical examination, hemoglobin 12 gm %. Total leuko counts = 10,000 mm³ and Differential

Corresponding author : Dr. B.P. Singh, Centre for Biochemical Technology Mall Road, Delhi University Campus, Delhi 110007, India.

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leuko counts-Polymorph 24, Leukocytes 61, Eosinophils 15. Urine, stool, X-ray chest Posterior/Anterior view, X-ray skull for Para nasal sinus was within normal limits. The absolute eosinophil count was $800/\text{mm}^3$ and total IgE was 375 IU/ml. She was diagnosed as asthmatic (bronchial asthma) after going through her history and spirometry *i.e.* forced expiratory volume (FEV1) reversibility > 20% with 2 puffs (200 μg) of inhaled salbutamol.

Skin tests and sera collection: The patient was advised to eliminate all suspected foods one week prior to skin test. The extracts were procured from Centre for Biochemical Technology, Delhi commercially supplying the allergens in India since the last 20 years. Intradermal tests (ID) were performed by injecting 0.02 ml (1:500 w/v) of each extract into the forearm of the patient at V. P. Chest Institute, Delhi. Intradermal tests were also carried out with serial dilutions (1:500- 1:500,000 w/v) of mixed food extracts. As a negative control, phosphate buffered saline was injected on another site. The skin tests were graded⁷ after 20 minutes in comparison with wheal size of negative control. Histamine diphosphate (100 $\mu\text{g}/\text{ml}$) was used as a positive control to assess the skin reactivity. Sera were collected from the ID positive patient (>12 mm wheal size) and few persons showing negative ID tests to the allergens tested. The consent of each subject was obtained prior to skin tests and sera collection.

Oral challenge test: Patient was administered an extract of cooked horse gram orally in the clinic. Pulmonary function test (PFT) was carried out^{7,8} prior and after the oral challenge. Double blind placebo controlled food challenge (DBPCFC) was also performed with two different foods. First challenge food contained placebo (wheat) to which the patient was not sensitive. The second challenge was performed with suspected food antigen (horse gram). Both the challenges were separated by 4 hrs. The PFT was carried out after both the oral challenges.

Pulmonary function tests: Spirometric evaluation of Forced vital capacity (FVC) and Forced expiratory volume at one second (FEV1) was made prior and after skin tests (ID) and oral food challenge. The bronchial reactions were graded after calculating the percentage reduction in FVC and FEV1 as given below:

$$\frac{\text{FEV1 prior to test} - \text{FEV1 after the test}}{\text{FEV1 prior to test}} \times 100$$

Reduction in FEV1 or increase in time taken to expel the total FVC were graded⁷ as mild (15% to 20%), moderate (21% to 50%) and severe (>50%).

Enzyme Linked Immunosorbant assay (ELISA): Specific IgE/IgG in patient serum against different food extract was quantitated using ELISA⁹. Allergens (2 $\mu\text{g}/\text{well}$ in 100 μl) were coated individually on a microtitre plate (Corning) using carbonate-bicarbonate buffer. After washing with Phosphate buffer saline (PBS)-Tween 20 (0.1%), the unbound sites were blocked with 1% bovine serum albumin for 3 hrs at room temperature. Plate was washed thoroughly and wells were incubated overnight with 1:10 v/v diluted patient sera in PBS-Tween 20. As a negative control, normal human serum was used. The plate was incubated overnight at 4°C. Washing was done with PBS-Tween 20 and 100 μl of 1:1000 v/v anti human IgE alkaline phosphatase conjugate was added in each well. The plate was incubated for 4 hrs at room temperature. The substrate p-nitro-phenyl-phosphate in glycine buffer was added and incubated at 37°C for 1 hr. The reaction was stopped by adding 3N NaOH and the absorbance read on a Nunc ELISA Reader. For specific IgG estimation, another set of ELISA was performed using anti IgG-horse radish peroxidase conjugate (Sigma).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot: Food extract proteins were separated on 10% gel using discontinuous buffer system as per Laemmli¹⁰. Resolved proteins were transferred on nitrocellulose paper in tris-glycine buffer with 20% methanol using dry blot apparatus¹¹. Non-specific sites were blocked by incubation with 1% bovine serum albumin for 2 hrs at room temperature. After washing with PBS-Tween 20, the strips were incubated overnight at 4°C with 1:10 v/v diluted patient's serum and/or normal human serum (NHS) as a negative control. The strips were washed with PBS-Tween20 and incubated with anti-human IgE (Sigma) conjugated to horse radish peroxidase for 4 hrs at 37°C. The unbound anti-IgE was washed off and specific IgE binding components were detected by addition of freshly prepared substrate *i.e.* diaminobenzidine with H_2O_2 in sodium

acetate buffer, pH 5.0. The blots were washed with distilled water to stop the reaction and the antigen-antibody binding was visualized as brown colour bands.

RESULTS

The patient reported with history of allergy to foods (seeds) when using them in different recipes. After routine clinical examination, intradermal tests (ID) were performed with various food and other allergen extracts (Table 1a). All six extracts of pulses demonstrated marked positive skin reactions. The extract of turmeric (haldi) a common ingredient used with foods also elicited positive skin reactivity in this patient. The other extracts such as pollen, fungi, insects and wheat showed negative ID reactions. A mixed allergen preparation from ID positive extracts was made and intradermal tests

Table 1a : Intradermal tests with seed (pulses), wheat, pollen and other allergen extracts

Seed Extracts (dilution w/v 1:500)	English name	Local name	I.D. test grading
<i>Cajanus cajan</i>	Cajan pea	Arhar	++
<i>Cicer arietinum</i>	Horse gram	Chana	++++
<i>Lens culinaris</i>	Lentil	Masur	+++
<i>Phaseolus aureus</i>	Green gram	Mung	++
<i>Phaseolus mungo</i>	Black gram	Urad	+++
<i>Phaseolus vulgaris</i>	French bean	Rajmah	+++
<i>Triticum aestivum</i>	Wheat	Genhu	—

Other extracts: = Pollen (16); Fungi (10); and Insect (5) elicited negative ID reactions. Turmeric (haldi) extract showed marked positive skin (2+) reaction.

Table 1b : Intradermal tests with mixed seed (pulses) extracts (ID +ve) in different dilutions

Extracts dilution (w/v)	Wheal size (mm)	ID test grading
1: 5000	20 × 18	>4+
1: 50,000	15 × 16	>4+
1: 100,000	13 × 14	>4+
1: 500,000	9 × 10	2+
control: PBS	3 × 3	-ve

PBS= phosphate buffer saline

were carried out with four concentrations (Table 1b). The skin tests were positive (2+) upto the extract dilution of 1:500,000 w/v.

Spirometric evaluation of patient was carried out before and after ID tests (Table 2). Mild reduction in FEV1 was recorded after the skin tests. Spirometry was also performed prior to and after oral challenge with cooked horse gram (Table 3). The patient showed severe reduction in FEV1 after the oral challenge test. Further DBPCFC was done using wheat recipe first (placebo) and later with horse gram recipe. Spirometric values are presented in Table 4. Severe broncho-constriction was recorded besides, nausea, vomiting, rhinorrhea and wheezing after oral challenge with horse gram.

ELISA with patient's serum showed highly raised specific IgE antibodies to different food extracts (Table 5). SDS-PAGE resolved 14-26 protein bands in the molecular weight (MW) range of 14-100 kDa in food extracts of pulses, turmeric and wheat (Fig. 1). The separated proteins were tested by immunoblot with food allergic patient's serum

Table 2 : Spirometric evaluation for FVC, FEV1, FEV1/FVC and FEF₂₅₋₇₅ of patient before and after intradermal tests

	Before (absolute value)	After	% change
FVC	1.70	1.80	5
FEV1	1.41	1.36	-3
FEV1/FVC%	83 %	76 %	-3
FEF ₂₅₋₇₅	1.36	1.01	-25

FVC Forced vital capacity; FEV Forced expiratory volume at one second.

Table 3 : Spirometric values before and after oral challenge with cooked horse gram

	Before (L)	After (L)	% change
FVC	1.94	0.99	-48
FEV1	1.81	0.64	-64
FEV1/FVC%	93 %	65 %	-34
FEF ₂₅₋₇₅	2.41	0.41	-82

FVC Forced vital capacity; FEV Forced expiratory volume at one second.

Table 4 : Spirometric values before and after challenge with wheat recipe and later horse gram recipe

	Pre (L)	Wheat recipe		Gram recipe	
		Post (L)	%change	Post (L)	%change
FVC	1.72	1.92	11	1.3	-30
FEV ₁	1.22	1.19	-2	0.56	-52
VEV ₁ /FVC	71%	62%	-12	42%	-32
FEF ₂₅₋₇₅	0.79	0.68	-13	0.24	-64

Table 5 : Specific IgE and IgG values estimated by ELISA in patient serum against different food allergens

Food extracts (english name)	IgE (O.D. 405 nm)	IgG (O.D. 490 nm)
Cajan pea	1.86	1.42
Lentil	2.32	1.56
Black gram	1.82	1.14
Green gram	1.97	1.18
Horse gram	2.83	1.18
French bean	2.41	1.19
Turmeric	2.86	1.27
Wheat	0.59	1.14

(Fig. 2). A number of allergenic proteins in the MW range 14-92 kDa were observed in food extracts. A 45 kDa protein was common in most of the extracts (Table 6). Wheat extract was used as a non-specific food extract. It showed 26 protein bands on SDS-PAGE but all the bands were undetectable on immunoblot using allergic patient's serum.

DISCUSSION

The proteins present in foodstuffs have been classified by the immune system as inert, non-pathogenic antigens and hence normally accepted by the body. But failure in oral tolerance mechanism

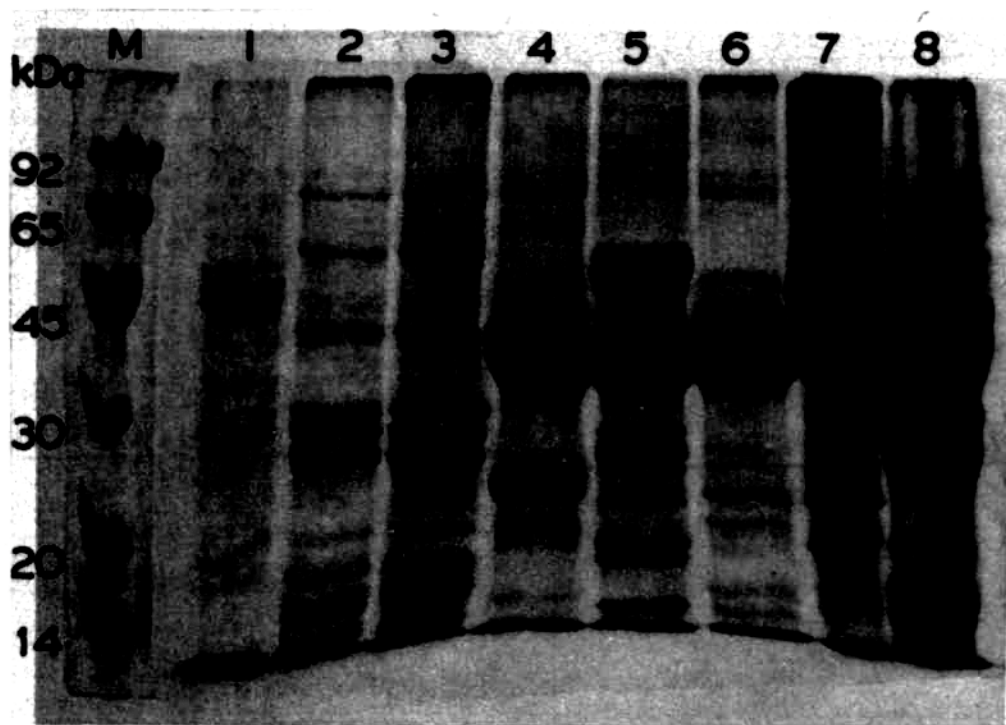


Fig. 1. SDS-PAGE profile of food extracts. M-Molecular weight markers, 1-Lentil, 2-Green gram, 3-Black gram, 4- Cajan pea, 5- French beans, 6-Turmeric, 7-Horse gram, 8-Wheat.

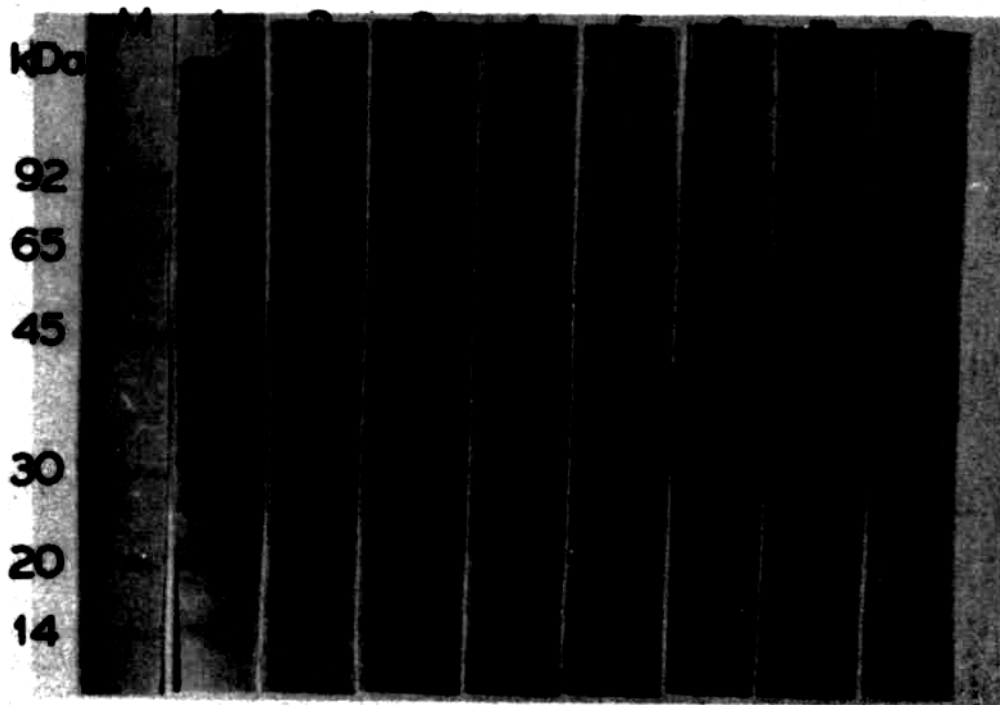


Fig. 2. Immunoblot showing IgE binding proteins in food extracts. M- Molecular weight markers, 1-Lentil, 2-Green gram, 3-Black gram, 4- Cajan pea, 5 French beans, 6-Turmeric, 7-Horse gram, 8-Wheat.

lead to a variety of enteropathies including classical food allergy^{12,13}. A range of environmental factors in particular inflammatory agents and gut microbial flora affect the process of oral tolerance^{14,15}.

The response to food antigens are driven by direct exposure (dietary components) during early post natal life that typically peak with respect to antibody titre (IgE) during first few years of life¹². In the present study, a case of food allergy has been investigated using standard *in vivo* and *in vitro* tests. The intradermal tests/ELISA revealed marked positive sensitivity to leguminous seed antigens (allergens) tested. Gradient (antigen/extracts) testing was also done and it showed marked positive skin reactivity, upto the dilution of 1:500,000 w/v *i.e.* the lowest concentration taken for immunotherapy prescriptions. To confirm the diagnosis further, the oral challenge and DBPCFC were performed with horse gram extract, placebo (wheat) and cooked horse gram, respectively. A significant pulmonary obstruction was

recorded after challenge with horse gram proteins (Table 4).

Among the large number of foods present in western diet, about 80% of allergic reactions in children occur due to hypersensitivity to legumes including peanuts and soybeans, cows milk and chicken eggs¹⁶. The overall prevalence of IgE mediated food allergies^{1,17} is presumed to be in the range of 1-2%^{17,18}. In our study, the patient showed significantly raised IgE levels by ELISA to all leguminous seed extracts tested indicating concomitant allergy or cross-reacting allergens present in them.

In general, the major food allergens identified are water soluble glycoproteins with MWs ranging from 10-70 kDa. They are stable to processing and cooking *i.e.* heating and freezing *etc.* and gastrointestinal digestion with acid and proteases¹⁸. It has been recently reported that boiled lentil extracts maintained strong allergenicity¹⁹. In the present study, immunoblot analysis of leguminous seed (pulses) and turmeric

Table 6 : A profile of IgE binding proteins in food extracts

Food extracts	SDS-PAGE Bands	Allergenic bands	Allergenic proteins (M.Wt)
Cajan pea	24	3	45, 35, 30 kDa
Lentil	14	3	42, 30, 14 kDa
Black gram	18	4	45, 38, 35, 28 kDa
Green gram	15	4	45, 38, 35, 28 kDa
Horse gram	25	10	92, 70, 45, 42, 30, 20, 18, 16, 15, 14, kDa
French bean	15	4	65, 45, 32, 30 kDa
Turmeric	25	7	92, 70, 45, 32, 20, 16, 14 kDa
Wheat	26	—	—

revealed 4 to 10 IgE binding bands in MW range of 14-92 kDa (Fig. 2). The proteins of MW 45, 38, 35, 30, 28 and 14 kDa present in these extracts may be common/cross-reacting allergens since they separated in the same electrophoretic zone (Table 6). Further, the purification of these IgE binding proteins and the structural studies will be helpful in understanding their IgE mediated allergic response.

In the present study, a case of food allergy to leguminous seeds (pulses) has been documented. The IgE binding proteins present in seeds were also characterized by immunoblotting with patient serum. Patient has been treated with allergen specific immunotherapy and showed significant clinical improvement. There is need to diagnose food allergy to pulses in children since they are common dietary ingredient in Indian families.

REFERENCES

- Niestijl Janson JJ, Kardinaal AFM, Huijbers GH, Vlieg Boestra BJ, Martens BPM, Ockhuizen T. Prevalence of food allergy and intolerance in the adult Dutch population. *J Allergy Clin Immunol* 1994; **93** : 446.
- Burks AW, Sampson HA. Food allergies in children. *Curr Prob Pediatrics* 1993; **23** : 230.
- Bock SA. Prospective appraisal of complaints of adverse reactions to foods in children during the first 3 years of life. *Pediatrics* 1987; **99** : 683.
- Host A, Halken S. A prospective study of cow milk allergy in Danish infants during the first three years of life. *Allergy* 1990; **45** : 587.
- Yunginger JW, Sweeny KY, Sturmer WQ. *et al.* Fatal food induced anaphylaxis. *J Am Med Assoc* 1988; **260** : 1450.
- Bock SA. The incidence of severe adverse reactions to foods in Colorado. *J. Allergy Clin Immunol* 1992; **83** : 900.
- Shivpuri DN, Agarwal MK. Studies on the allergenic fungal spores of Delhi, India, metropolitan area-clinical aspects. *J Allergy* 1969; **44** : 204.
- Sampson, H.A. Diagnosing food allergy. In: Provocation testing in clinical practice (Spector SL, editor), Marcel Dekker Inc, New York, **623** : 1995.
- Voller A, Bidwell D, Barlett A. Enzyme linked immunosorbent assay; In: Manual of Clinical Immunology, Rose NR and Feldman H (editors) American society for Microbiology, Washington, 1980.
- Laemmli UK. Cleavage of structural proteins during assembly of the head of the bacteriophage T₄. *Nature* 1970; **227** : 680.
- Towbin H, Gordon J. Immunoblotting and dot immunoblotting: Current status and outlook. *J Immunol Methods* 1984; **72** : 313.
- Holt PG. Mucosal immunity in relation to the development of oral tolerance/sensitization. *Allergy* 1988; **53** : 16.
- Mowat AM. The regulation of immune responses to dietary protein antigens. *Immunol Today* 1987; **8** : 93.
- Bruce MG, Strobel S, Hanson DG, Ferguson A. Irradiated mice lose the capacity to 'process' fed antigen for systemic tolerance of delayed-type hypersensitivity. *Clin Exp Immunol* 1987; **70** : 611.
- Sudo N, Sawamura SA, Tanaka K, Aiba Y, Kubo C, Koga Y. The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. *J Immunol* 1997; **159** : 1739.
- Sampson HA. Legumes, eggs and milk. *Allergy* 1998; **53** : 38.
- Young E, Stoneham MD, Petruckevitch A, Barton A, Rona R. A population study of food intolerance. *Lancet* 1994; **343** : 1127.
- Taylor SL, Lehrer SB. Principles and characteristics of food allergens. *Crit Rev Food Sci Nutr* 1996; **36** : 91.
- Sandin DI, San Ireneo MM, Lizana FM, Fernandez-Caldas, E, Lebrero EA, Borrego TL. Specific IgE determinations to crude and boiled lentil (*Lens culinaris*) extracts in lentil-sensitive children and controls. *Allergy* 1999; **54** : 1209.