

Original article

Sensitization to blackgram in patients with bronchial asthma and rhinitis: clinical evaluation and characterization of allergens

Background: Legumes are important causative agents of type I hypersensitivity in south Asia and Europe but such studies are lacking in Indian population. The present study investigates blackgram sensitization in asthma and rhinitis patients and identifies immunoglobulin E (IgE)-binding proteins.

Methods: Respiratory allergy patients were evaluated using standard questionnaire and skin prick tests (SPT) with common foods and aeroallergens. Blackgram-specific IgE level was estimated by enzyme-linked immunosorbent assay (ELISA) and sensitization was established by a double-blind, placebo-controlled food challenge (DBPCFC). The cross-reactivity of blackgram with other legumes was studied by immunobiochemical methods.

Result: Of 816 patients, 35 gave history of blackgram hypersensitivity. From these, 16 patients were SPT positive and 14 showed elevated specific IgE (three times of negative control) to blackgram. DBPCFC established blackgram sensitivity in four of 14 patients. Immunoblotting with individual patient's sera recognized eight most prevalent allergens of 78, 56, 47, 43, 40, 30, 28 and 16 kDa. Roasted blackgram showed six major allergens whereas 47, 43 and 28 kDa proteins retained IgE reactivity upon boiling. Blackgram extract required 14 ng of self protein for 50% ELISA inhibition whereas roasted and boiled blackgram required 16 and 120 ng protein. ELISA and immunoblot inhibition show partial inhibition to blackgram proteins by lentil, limabean and pea.

Conclusion: Blackgram induces IgE-mediated reactions in 1.7% of asthma and rhinitis patients and contains eight major IgE-binding components, of which six retained IgE reactivity after roasting. Blackgram shares allergenicity with lentil and limabean.

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Food hypersensitivity is estimated to be approximately 2% in adults and 8% in children (1). The incidence of perceived adult food hypersensitivity varies (1.4–19.1%) largely across different countries (2). Allergen-sensitization varying in different countries may be the result of interaction of genetic factors, cultural and dietary habits and/or exposure to new allergenic products early in life. Studies show higher prevalence of allergy to soy in Japan, peanut allergy in the UK, France, Switzerland and North America and sesame allergy in Israel (3). Legumes are an important source of immunoglobulin E (IgE)-mediated reactions in Mediterranean and Asian countries (4–6).

Allergic diseases have increased worldwide, including Asian countries (7, 8). Recent studies in India show a considerable increase in the prevalence of bronchial asthma (8–15%) (9, 10) when compared with the earlier report of <1% (11). Many asthma and rhinitis patients may be suffering from food allergy but our knowledge

about food hypersensitivity is limited to few studies carried out in the country (12–14). These studies indicate that foods such as egg, milk, cereals and legumes commonly induce IgE-mediated reactions in children and adult population.

Leguminosae is a large food family that includes variety of beans, peas and pulses. Globally legumes provide a major source of affordable protein. Allergens belonging to common botanical families are known to share antigens (15). Blackgram (*Phaseolus mungo*) from the family Leguminosae is extensively cultivated and consumed in India, Thailand, Sri Lanka and Indonesia. Moreover, neutral detergent fibre isolated from blackgram possesses significant hypolipidemic, hypoglycaemic and anticolon cancer activity (16). Previous reports show cases of bronchial asthma and/or anaphylaxis induced by inhaling vapours from cooking, eating lentil and/or chickpea belonging to the legume group (17). The present study was, therefore, undertaken to establish IgE-medi-

ated sensitization to blackgram in patients with asthma and allergic rhinitis. Blackgram extract was also characterized by immunobiochemical methods and its cross-reactivity with other common legumes was investigated.

Materials and methods

Preparation of extracts

Food and aeroallergens were defatted in diethyl ether at 4°C. The extraction was carried out by continuous stirring for 6 h at 4°C in 1 : 20 w/v ammonium bicarbonate buffer (50 mM, pH 8.0). The extracts were centrifuged at 10 000 g, the supernatant was filtered through a 0.22-µm filter, lyophilized and stored at -70°C. Protein content of extracts was estimated by Lowry's method (18).

Study subjects

A total of 816 (393 males and 423 females) cases aged 8–62 years reporting to the outpatient department, V.P. Chest Institute, Delhi, were screened for food hypersensitivity using a standard questionnaire (19). It is a referral chest hospital and receives patients from different states of the country for the diagnosis and treatment of allergy. The diagnosis of asthma was assigned to cases following the guidelines of the American Thoracic Society (20). The patients having any two of the symptoms, viz. sneezing, rhinorrhoea, nasal blockage, post nasal drip, etc., were diagnosed as having rhinitis (21).

From the total cases screened ($n = 816$), 35 patients with history suggestive of blackgram sensitivity were recruited for the present study. The control group consisted of 20 nonatopic subjects with no complaint of allergy. Consent was obtained from all the patients and nonallergic volunteers for participation in the study.

Skin prick tests

Lyophilized extracts were reconstituted in 50% glycerinated phosphate-buffered saline (PBS) for skin prick testing (SPT). A drop of the extract was placed on the volar aspect of the forearm and the skin was pricked by a 26 1/2" G sterile needle. SPT was also performed with a panel of common inhalant allergens such as pollen, fungi and insects. Glycerinated (50%) PBS was used as a negative control and histamine diphosphate (5 mg/ml) as a positive control. The skin tests were graded after 20 min in comparison with the wheal diameter of positive control. Wheal diameter equal to that of positive control or more (> 3 mm) were considered as marked positive skin reactions.

Patients' sera

Blood was collected from SPT-positive patients with a history of allergy to blackgram and from 20 normal individuals who did not show history or skin reactivity to any allergen extracts. Serum was separated and stored at -20°C.

Total IgE estimation

Serum total IgE level was determined by Bethyl Human IgE ELISA Quantitation Kit (Montgomery, TX) according to the manufacturer's instruction. In brief, anti-human IgE was coated (1 µg/100 µl) in carbonate buffer, pH 9.6 per well, and incubated for 60 min at room temperature. After washing with Tris buffer saline

(TBS)–Tween 20 (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0), the nonspecific sites were blocked with 1% bovine serum albumin in TBS (pH 8.0). The plate was washed again and incubated with human IgE calibrator antibody serially diluted to different concentrations of 15.625, 31.25, 62.5, 125, 500 and 1000 ng/ml along with 1 : 10 v/v sera of blackgram-hypersensitive patients and or normal human sera (NHS) for 60 min at room temperature. The plate was washed and incubated with 1 : 10 000 diluted goat anti-human IgE-peroxidase conjugate for 60 min at room temperature. Colour was developed with orthophenylene diamine (Sigma, St Louis, MO). The reaction was stopped after 20 min by adding 5 N H₂SO₄ and read on an ELISA reader at 492 nm. IgE concentration was defined using the standard curve and presented as IU/ml (1 IU/ml = 2.4 ng/ml).

Specific IgE estimation

Specific IgE in patient's sera was determined by the method of Voller et al. (22). Briefly, 1 µg protein of the blackgram extract was incubated in carbonate buffer (1 µg/100 µl per well) overnight at 4°C in a microtitre plate (Nunc MaxiSorp plates; Nalge Nunc International, Roskilde, Denmark). After washing with PBS–Tween 20 (0.05%), the nonspecific sites were blocked with 3% defatted milk for 3 h at 37°C. The plate was washed again and incubated with diluted (1 : 10 v/v) sera of blackgram-hypersensitive patients overnight at 4°C. Normal human sera were used as negative control. The plate was washed and incubated with antihuman IgE-horse radish peroxidase 1 : 1000 v/v (Sigma) for 3 h at 37°C. Colour was developed with orthophenylene diamine. The reaction was stopped after 20 min by adding 5 N H₂SO₄ and the absorbance was read at 492 nm.

ELISA inhibition

The allergenic potency of blackgram extracts was determined *in vitro* by ELISA inhibition. One microgram protein of raw blackgram extract was coated in carbonate buffer (1 µg/100 µl per well) overnight at 4°C in a microtitre plate (Nunc). Blackgram-hypersensitive pooled sera (1 : 10 v/v) were preincubated with 0.1 ng–10 µg of raw (self extract), roasted and boiled blackgram at 4°C and the mixture was then added to the raw blackgram-coated ELISA plate. Normal human sera pool was used as a negative control.

To study cross-reactivity, inhibition of IgE binding to blackgram was tested with sera of patients preincubated with 1 ng–10 µg of other legumes such as lentil, limabean, pea, soya bean and peanut as inhibitors. The mixture was then added to the solid phase raw blackgram extract in the ELISA plate. Blackgram antigen incubated with pooled patient's sera without inhibitors was taken as positive control. The rest of the procedure was similar to that of ELISA. Percentage inhibition was calculated using the formula given below:

$$1 - \frac{\text{OD of sample with inhibitor}}{\text{OD of sample without inhibitor}} \times 100.$$

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

To study protein profile, blackgram extract (80 µg protein per lane) was loaded on a 12% reducing gel (23) and stained with Coomassie brilliant blue. In addition, SDS-PAGE was performed for extracts prepared from boiled (100°C) and/or roasted blackgram for 15 and 30 min.

Immunoblot

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis-resolved proteins were transferred onto nitrocellulose membrane (NCM) as described by Towbin et al. (24). The unbound sites were blocked by 3% defatted milk for 3 h at 37°C. The NCM strips were washed and incubated with 1 : 10 v/v blackgram-hypersensitive patient's sera in PBS, overnight at 4°C. Normal human sera were taken as negative control. Blackgram-positive sera were also incubated with *Curvularia* fungus extract to observe the nonspecific binding. The strips were washed with PBS-Tween 20 and incubated with 1 : 1000 diluted antihuman IgE-peroxidase conjugate (Sigma). The IgE binding was detected by adding diaminobenzidine with hydrogen peroxide in sodium acetate buffer (pH 5.0).

Immunoblot inhibition

Immunoblot inhibition was performed to establish cross-reactivity of blackgram with five other common legumes. To this end, blackgram-hypersensitive pooled patient's sera were preincubated with 500 µg of blackgram extract (homologous inhibition) and other legume extracts such as lentil, limabean, pea, peanut and soy separately. Inhibition was also performed with *Curvularia* extract as inhibitor. The SDS-PAGE-resolved proteins were transferred onto NCM, strips were cut, blocked and incubated with the preincubated pooled sera. The rest of the procedure was similar to immunoblotting.

Double-blind, placebo-controlled food challenge

For food challenge, exact matching of the placebo test food and the active test food was chosen (25). We have used wheat flour as a vehicle for delivering the active test food. Blackgram was presoaked overnight and a paste was prepared in a grinder. The batter was prepared by mixing one part of the blackgram paste to three parts of wheat flour. The mixture was then steamed for 10 min. As placebo test food, wheat flour paste alone was used. The recipe was made in such a way that there was no perceivable sensory difference between the placebo and active test food. The patients were given about 10–30 g of active test food for food challenge test. Pulmonary function evaluation was made after 30 min and forced expiratory volume in 1 s (FEV₁) was recorded before and after placebo and active test food intake. Neither the patient nor the technician making the recordings was aware about the ingredients of the placebo or the active test food.

Results

Study subjects

Of 816 patients surveyed, 530 (64.9%) gave history of food allergy to one or more food(s). Most of these patients presented with symptoms of breathlessness (90%), cough (81%), wheezing (78.7%), sneezing (63%), rhinorrhoea (60.7%), nasal blockage (43%) and skin rashes (26%). A large number of the patients (75.9%) were suffering from both rhinitis and asthma whereas 19.1% and 3.9% were suffering from asthma and or rhinitis alone respectively (Table 1). Of the 816 patients, 35 (4.2%) patients with a mean age of 28.9 years (±11.3 s.d.) gave history of blackgram hypersensitivity. Of these 35 patients, 16 showed positive SPT to blackgram. In addition, seven

Table 1. Distribution of patients as per their symptoms and history of food allergy

Disease	Total patients (n = 816)		Patients with history of food allergy (n = 530)	
	n	%	n	%
Bronchial asthma	156	19.1	100	18.8
Allergic rhinitis	32	3.9	18	3.4
Asthma with rhinitis	620	75.9	409	77.2
Asthma with skin allergies	8	0.9	3	0.5

patients elicited marked positive skin reactions with few other food extract(s) such as lentil (3), two each with chickpea, frenchbean and rice and one each with cauliflower, limabean, maize, millet, mustard, orange, peanut and soy. Some of the blackgram-sensitive patients also demonstrated positive SPT to *Artemisia scoparia*, *Amaranthus spinosus* and *Prosopis juliflora* pollen extracts.

In vivo and in vitro IgE reactivity

Specific IgE were estimated in sera of 16 patients with marked positive SPT to blackgram. Fourteen patients showed elevated specific IgE ODs 0.40–0.92; more than three times of negative control (OD 0.13). The total IgE levels of blackgram-sensitive patients were higher, ranging from 675 to 3040 IU/ml in 14 cases than normal controls (3–212 IU/ml). However, patient 1 and patient 8 showed lower serum total IgE levels than normal controls (Table 2).

Fourteen asthma patients (SPT- and ELISA-positive) consented for DBPCFCs with blackgram. Four patients showed positive DBPCFCs with 15–20% reduction in FEV₁ after challenge with blackgram. All four patients experienced breathlessness, while one each with rhinorrhoea, itching in mouth, and skin rashes as additional symptoms.

Proteins in blackgram extract

Blackgram extract contained 208, 214, and 218 mg protein/g of powder in three batches of extracts. SDS-PAGE resolved raw blackgram extract into 31 protein bands of 12 to >97 kDa (Fig. 1A, lane 1) whereas 30 and 15 min roasted blackgram showed 23 and 26 bands respectively. Some new bands at 25 and 23 kDa appeared, whereas proteins below 20 kDa were not visible (Fig. 1A, lanes 2 and 3). Blackgram demonstrated 13 and 11 protein bands after 15 and 30 min of boiling respectively (Fig. 1A, lanes 4 and 5). Boiling substantially decreased the proteins of >70 kDa.

Allergenic proteins in blackgram extract

The IgE reactivity of extracts prepared from raw, roasted and boiled blackgram was analysed by Western blot with

pooled sera from blackgram-sensitive patients (Fig. 1B). Raw blackgram showed 14 IgE-binding proteins of 110, 104, 78, 70, 56, 47, 43, 40, 33, 30, 28, 20, 16 and 12 kDa (Fig. 1B, lane 1) whereas roasted blackgram (15 min) showed 17 IgE-binding proteins (Fig. 1B, lane 2). Some new allergenic proteins visualized were > 120, 38, 36, 25, 23 and 18 kDa whereas some allergenic proteins were absent (Fig. 1B, lane 2). The immunoblot profile of 30 min roasted blackgram was similar to the blackgram roasted

for 15 min except that, 40 and 38 kDa proteins were absent and two new allergenic proteins of 27 and 26 kDa appeared (Fig. 1B, lane 3). In the 15 min boiled blackgram, 11 allergenic proteins of 56, 47, 43, 30, 28, 25, 23, 20, 18, 12 and 7 kDa were detected (Fig. 1B, lane 4) whereas 30 min boiling showed nine allergenic proteins, in which 56 and 28 kDa proteins were absent (Fig. 1B, lane 5).

The IgE reactivity of raw blackgram extract was also analysed by individual patient's serum. The eight major allergens recognized by $\geq 80\%$ of the patient's sera were 78, 56, 47, 43, 40, 30, 28 and 16 kDa (Fig. 2, lanes 1–16) while proteins of 104 and 20 kDa were recognized by 69% and 44% of patients' sera respectively. The strips incubated with NHS did not show any band (Fig. 2, lane 17). Blackgram-positive sera incubated with *Curvularia* fungus extract recognized no protein (Fig. 2, lane 18). Blackgram extract was also incubated with PBS and showed no nonspecific binding (Fig. 2, lane 19).

Potency of blackgram extract and its cross-reactivity with other legumes

Allergenic potency of the raw, roasted and boiled blackgram was determined by ELISA inhibition. Raw blackgram required 14.0 ng of self protein for 50% inhibition whereas roasted and boiled blackgram required 16 and 120 ng respectively (Fig. 3A). To assess the cross-reactivity of blackgram with other legume allergens, ELISA inhibition was performed using legume extracts as inhibitors. Pea, limabean and lentil produced 50% inhibition of specific IgE binding to solid phase blackgram extract with 1.1 μ g, 200 ng and 70 ng protein(s) respectively (Fig. 3B). The maximum inhibition (88%) was observed with blackgram followed by 70–37% at 10 μ g protein by other legume extracts.

Pooled patients' sera reacted to 14 protein components in blackgram extract (Fig. 4, lane 1). Preabsorption of

Table 2. Details of blackgram-sensitive respiratory allergy patients

Patient no.	Age (years)/sex	Total IgE (IU/ml)	Sp. IgE (OD)	SPT	DBPCFC
1	11/M	150	0.716	2+	–
2	16/F	1142	0.404	2+	ND
3	13/M	1540	0.920	3+	+
4	26/M	3040	0.516	2+	+
5	18/F	750	0.667	3+	+
6	20/F	2028	0.323	2+	–
7	20/M	1245	0.230	2+	–
8	17/M	210	0.414	2+	–
9	35/F	725	0.688	3+	+
10	32/M	840	0.495	2+	–
11	32/M	725	0.66	2+	–
12	33/M	1110	0.585	2+	–
13	33/M	1466	0.399	2+	ND
14	29/F	720	0.429	2+	–
15	36/F	705	0.744	2+	–
16	46/M	675	0.479	2+	–

M, male; F, female; IgE, immunoglobulin E; SPT, skin prick test; DBPCFC, double-blind, placebo-controlled food challenge; ND, not done. Patient 2 and patient 13 did not agree to challenge test. Negative control for Sp. IgE: OD 0.13. Control for total IgE: 3–212 IU/ml.

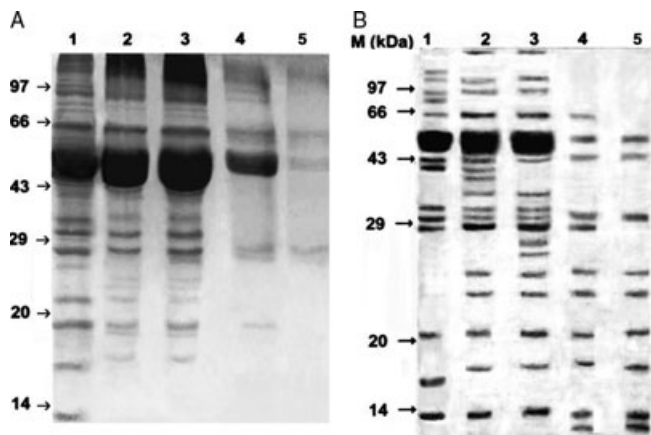


Figure 1. (A) SDS-PAGE – profile of raw blackgram extract (lane 1), blackgram roasted for 15 and 30 min (lanes 2 and 3 respectively) and boiled at 100°C for 15 and 30 min (lanes 4 and 5 respectively) stained with Coomassie brilliant blue. (B) Immunoblot of raw blackgram (lane 1), blackgram roasted for 15 and 30 min (lanes 2 and 3 respectively) and blackgram boiled at 100°C for 15 and 30 min (lanes 4 and 5 respectively) with pooled patient sera (1 : 10 v/v).

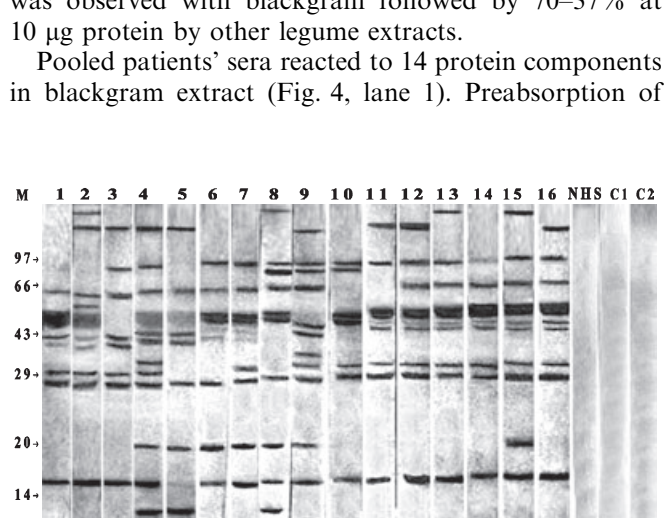


Figure 2. Immunoblot of individual blackgram-sensitive patient sera. Lanes 1–16: individual patient serum; NHS: pool of normal human sera; C1: *Curvularia* extract incubated with blackgram-sensitive patient sera; C2: blackgram extract incubated with PBS and antihuman IgE-HRP (1 : 1000).

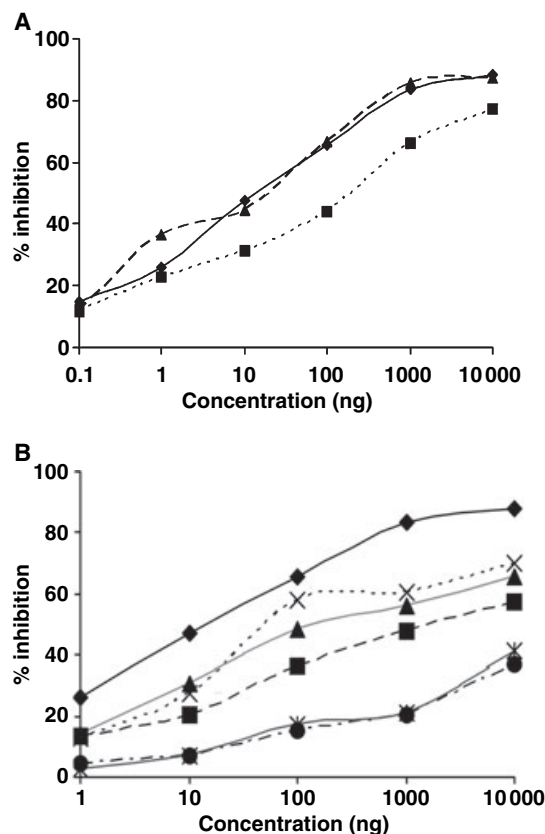


Figure 3. (A) ELISA inhibition with raw, roasted and boiled blackgram as inhibitors (0.1 ng to 10 µg protein) using raw blackgram on solid phase. Raw (—◆—), boiled (··■··) and roasted blackgram (---▲---). (B) ELISA inhibition of blackgram with legume extracts. Blackgram (—◆—), lentil (··×··), limabean (—▲—), pea (---■---), peanut (··●··) and soy (—*—).

pooled patients' sera with irrelevant fungal allergen *Curvularia* did not cause any change in IgE reactivity to blackgram (Fig. 4, lane 2). Preabsorption of pooled patients' sera with blackgram extract (self) totally abolished IgE reactivity to blackgram (Fig. 4, lane 3). Preabsorption of sera with other legume extracts resulted in a loss of IgE reactivity against high-molecular-weight blackgram proteins (> 70 kDa). But proteins of 47, 43, 30, 28, 20, 16 and 12 kDa could not be inhibited by any other legume (Fig. 4, lane 4–9). With soy as inhibitor, no cross-inhibition was observed except for > 70 kDa bands in blackgram (Fig. 4, lane 5).

Discussion

Studies of food allergy have primarily focused on the paediatric population (4, 5). The present study was undertaken to identify food sensitization in patients (aged 8–62 years) of bronchial asthma and allergic rhinitis reporting to a referral chest hospital at Delhi.

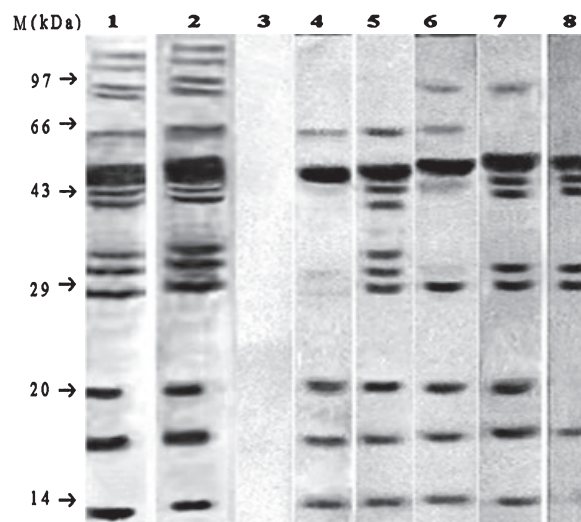


Figure 4. Immunoblot inhibition with raw blackgram extract. Inhibition of specific IgE binding to blackgram extract on immunoblot with 500 µg of legume proteins. Lane 1: without inhibitor (positive control); lane 2: *Curvularia* extract; lane 3: self-protein inhibitor; lane 4: lentil; lane 5: soy; lane 6: pea; lane 7: peanut; lane 8: limabean.

Many asthma and rhinitis patients presented history of allergy to different foods. Among them, 35 patients had a history of blackgram allergy whereas 16 patients showed positive SPT to blackgram extract. ELISA demonstrated significantly elevated IgE in 14 cases. Considering three parameters (history, SPT and ELISA), sensitization to blackgram was observed in 14 patients (1.7%) from a total of 816 cases. However, among the food allergic cases, blackgram figured within the top five food allergens including rice, banana, maize and frenchbean. In an earlier study of 1400 patients from Mumbai, 59 (4.2%) cases presented with a history of chickpea allergy. Of these, 41 patients were skin test positive to chickpea and 23 showed raised IgE levels (12).

The DBPCFC test has been suggested to obtain conclusive evidence of food allergy (25). Patil et al. (12) recorded 52.5% of DBPCFCs who tested positive for chickpea. Recently, oral food challenges showed 55% (milk) to 28% (soy) positive cases with respective food(s) (26). In the present study, four of 14 cases (28.5%) showed significant reduction in FEV₁ after challenge with blackgram, and were therefore graded as positive. Here, DBPCFCs were conducted with recipe prepared from steamed blackgram, which retained only three of the major allergens. Moreover, the boiled blackgram appeared to be approximately 10 times less potent than the raw blackgram. Probably this may be the reason for low positivity (28.5%) of DBPCFCs. However, all the four cases positive for DBPCFCs had remarkably high specific IgE to blackgram and SPT with ≥5 mm wheal diameter. In a study with buckwheat allergy, the use of 1.26 kU_A/l, which was three times higher than the

negative control (0.35 ± 0.15 kU_A/l), was considered useful in avoiding unnecessary risky food challenge in children with strong clinical history and skin test responses (27). In a recent study, 90% predicted probability was obtained by specific IgE levels of 6.3 kU/l for hen's egg and 88.8 kU/l for cow's milk (28).

Immunoblotting with individual patient's sera demonstrated eight major IgE-binding components of 78–16 kDa in blackgram extract. Patil et al. (12) reported five major allergens between 70 and 20 kDa as major allergens in chickpea extract. A study of lentil extract showed six allergenic proteins between 69 and 18 kDa by more than 50% of patients, and 53 kDa protein was recognized by 92% of the patients (4). Pascual et al. (5) showed 16 IgE-binding proteins in lentil, of which 54 and 38 kDa proteins were recognized by 77% and 82% of patients respectively.

Blackgram is consumed after boiling or roasting, therefore IgE reactivity of roasted and/or boiled forms was studied. Roasted blackgram showed 17 prominent IgE-binding proteins on immunoblot. Investigation with roasted peanut showed stable high-molecular-weight protein Ara h 1 (63 kDa) and its trimer forms (29). Boiling of lentils resulted in increase of low molecular bands in 12–16 kDa and decrease of bands in the 25- to 45 kDa range (6). Appearance of new allergenic determinants in roasted and boiled blackgram might be due to an increase in hydration, disulphide bond breakage, and loss of tertiary and secondary structures upon heating. ELISA inhibition with roasted blackgram showed comparable allergenic potency with raw blackgram whereas boiled blackgram was 10 times less potent.

Cross-reactivity of blackgram was studied by ELISA inhibition and immunoblot inhibition. Partial inhibition of blackgram was observed on immunoblot with pooled patients' sera preincubated with other legume extracts. To achieve 50% inhibition of IgE binding to blackgram proteins, 70–1100 ng of heterologous inhibitors were required. Peanut and soy extract could not produce 50% inhibition in blackgram, even with high dose of 10 µg protein. It appears that blackgram has more specific allergens than shared allergens with the legumes that were tested.

In conclusion, blackgram is a potent sensitizing agent in allergic asthma and rhinitis and contains eight most prevalent allergens of 16–78 kDa. Roasting of blackgram did not affect six major allergens whereas only 47, 43 and 28 kDa retained IgE reactivity after boiling. The patients with positive SPT (≥ 5 mm wheal diameter) and elevated specific IgE (four times or more of the normal control) should avoid blackgram in their diet without blinded food challenge.

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