



Basic nutritional investigation

Hypersensitivity linked to exposure of broad bean protein(s) in allergic patients and BALB/c mice



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ABSTRACT

Objective: Broad bean (*Vicia faba* L.), a common vegetable, belongs to the family Fabaceae and is consumed worldwide. Limited studies have been done on allergenicity of broad beans. The aim of this study was to determine if broad bean proteins have the ability to elicit allergic responses due to the presence of clinically relevant allergenic proteins.

Methods: Simulated gastric fluid (SGF) assay and immunoglobulin E (IgE) immunoblotting were carried out to identify pepsin-resistant and IgE-binding proteins. The allergenicity of broad beans was assessed in allergic patients, BALB/c mice, splenocytes, and RBL-2H3 cells.

Results: Eight broad bean proteins of approximate molecular weight 70, 60, 48, 32, 23, 19, 15, and 10 kDa that remained undigested in SGF, showed IgE-binding capacity as well. Of 127 allergic patients studied, broad bean allergy was evident in 16 (12%). Mice sensitized with broad bean showed increased levels of histamine, total and specific IgE, and severe signs of systemic anaphylaxis compared with controls. Enhanced levels of histamine, prostaglandin D₂, cysteinyl leukotriene, and β-hexosaminidase release were observed in the primed RBL-2H3 cells following broad bean exposure. The levels of interleukin IL-4, IL-5, IL-13 and regulated on activation, normal T-cell expressed and secreted were found enhanced in broad bean-treated splenocytes culture supernatant compared with controls.

Conclusion: This study inferred that broad bean proteins have the ability to elicit allergic responses due to the presence of clinically relevant allergenic proteins.

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Introduction

Food allergy is an immune provocation induced by certain foods in susceptible individuals. The prevalence of food allergy is a major health issue in several developed countries and reported to affect 4% of adults and 8% of children [1]. Approximately 3% to 4% of the U.S. population is reported to be affected by food-induced allergic reactions [2]. About 30,000 cases of food-induced anaphylaxis have been reported in the emergency

departments of U.S. hospitals including 200 deaths each year [3]. Legumes are the cheapest source of proteins in the majority of vegetarian foods and due to their high consumption, the prevalence of legume allergy is very high [4]. Broad bean (*Vicia faba* L.) is an annual herb that belongs to the family Fabaceae and tribe Viciaceae. It is commonly known as bakla, broad bean, fava bean, field bean, bell bean, faba bean, horse bean, windsor bean, tick beans, bakela (Ethiopia), boby kurmouvje (former USSR), faveira (Portugal), ful masri (Sudan), feve (French), yeshil bakla (Turkey), and baqla (in Persian language) in India. However, the term broad bean is the most common name and is produced in different parts of the world. Broad bean is the main ingredient of various dishes consumed worldwide, including vegetable, roasted seeds, falafel, bissara, nabet soup, habas saladas, habas con

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chile, broad bean puree with wild chicory, doubanjiang, ful medames, tlaoyos, and bolo-rei. It has been reported that broad bean seeds contain 20% to 41% protein [5]. Sustained consumption of any proteinaceous food, including legumes, may increase the probability of sensitization against the potential allergenic proteins in susceptible individuals. Allergenic manifestations to peanuts, soybeans, peas, chickpea, lentil, red gram, green gram, black gram, and red kidney bean have been well studied [6]. But, despite cosmopolitan consumption of broad beans, surprisingly, reports on its allergenicity are very few. In 2007, the first report of broad bean allergy appeared when a 25 year old Spanish woman showed itching in the tongue and pharynx 5 min after eating a sandwich containing broad bean flour [7]. A 49 year old Italian farmer showed adverse reactions to raw and boiled broad beans after ingesting or handling fresh broad bean vegetable. Following ingestion of fresh raw and boiled broad beans she demonstrated dyspnea, chest tightness, tachycardia, and malaise. Following the handling of fresh broad bean vegetable, she presented burning in the eyes and facial edema. In both cases, severity of the reactions reduced after 2 h [8]. Recently, 79.3% of children and 80.4% of adults of Moroccan population showed high levels of immunoglobulin E (IgE) against broad bean [9].

Because there are few studies on the allergenicity of broad beans and their sensitizing allergens; we conducted a thorough study of broad bean allergy in BALB/c mice and its splenocytes, RBL-2H3 cells, and humans.

Materials and methods

Preparation of broad bean crude protein extract

Broad bean seeds, popularly known as baqla seeds were purchased from a local certified seed vendor and the same lot was used throughout the study. The broad bean protein extraction technique employed was similar to a previously described method [10]. The concentration of broad bean crude protein extract (CPE) was determined by the Lowry method using bovine serum albumin (BSA) as the standard [11].

Digestibility of broad bean CPE by simulated gastric fluid assay

In vitro pepsin digestibility of broad bean proteins was performed by simulated gastric fluid (SGF) assay as previously described [12]. Densitometric analysis was carried out using gene tool software (Syngene Bio Imaging System, Cambridge, UK).

BALB/c mice

Healthy 6 to 8 week old female BALB/c mice (18 ± 3 g) were obtained from CSIR-Indian Institute of Toxicology Research, Lucknow, India, animal breeding colony. Mice were maintained under standard laboratory conditions and on broad bean and peanut-free diets.

Sensitization protocol of BALB/c mice

All BALB/c mice were sensitized with broad bean CPE according to a previously described method [13]. In brief, mice were randomly divided into three groups ($n=10$ mice/group). Mouse groups were treated intraperitoneally with 100 μ L phosphate-buffered saline (PBS; 20 mM Na₂HPO₄, 2 mM KH₂PO₄, 5.4 mM KCl, 0.5 M NaCl, pH 7.0), 100 μ g broad bean CPE in 100 μ L PBS and 100 μ g peanut CPE in 100 μ L PBS, respectively once a week up to 7 week. Blood was withdrawn from the retro-orbital sinus on days 15, 43, and 59 to measure different parameters like immunoglobulins, thymic stromal lymphopoietin (TSLP), and histamine. On day 60, five mice were challenged from each group with their respective proteins and anaphylactic symptoms were studied after 20 min. Tissue and blood samples were collected for histopathology and plasma, respectively.

IgE immunoblotting

We extended our study to find out the IgE-binding proteins of broad bean. For detection of IgE-binding proteins of broad bean, IgE immunoblotting was carried out as previously described [14]. Briefly, broad bean CPE was resolved on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and

electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon P 0.45 μ m, cat no- IPVH00010, Millipore, Bangalore) using a semidry blotting unit (Amersham Biosciences, St Francisco, USA). The IgE contents of the sera were concentrated by using Protein G-bead Agarose method. The nonspecific sites were blocked with 3% BSA in PBS plus 0.5 % of Tween 20 (PBS-T) and kept overnight at 4°C. Following incubation, the blot was washed in PBS-T five times. Pooled broad bean-sensitized mice sera were added as primary antibody (1:20), whereas peroxidase conjugated goat antimouse IgE (Southern Biotech, Birmingham, AL, USA, dilution 1:1000 in PBS-T + 1% BSA) was used as secondary antibody. The blot was developed by chemiluminescence method (Pierce, Thermo Scientific, Rockford, IL, USA) and image was acquired by a gel documentation system (Syngene Bio Imaging System, Cambridge, UK).

Serum total IgE level

Because of the pivotal role of IgE in the allergic reactions, the level of total IgE was estimated in different groups. Total IgE was estimated in the sera of control and broad bean-treated mice with the OptEIA mouse IgE kit (BD Biosciences, Germany) according to the manufacturers' instructions.

Estimation of specific IgE and IgG1 levels

Following total IgE estimation, we next sought to estimate the broad bean-specific IgE level in the sera of broad bean-treated mice. Specific IgE and IgG1 levels were estimated according to previously described methods with slight modifications [13]. In this study, BD OptEIA TMB substrate reagents set (BD eBioscience, San Jose, CA, USA) was used as a substrate and absorbance was taken at 450 nm (Power Wave XS2, BioTek Instrument, Winooski, VT, USA).

Splenocyte culture and cytokine levels

We further studied the levels of cytokines and chemokines at the cellular level following exposure to broad bean proteins. The splenocyte culture was carried out according to a previously described method [15]. In brief, the splenocytes obtained from broad bean-treated mice were divided into two groups; the spleen cells from control mice were used as naïve and challenged with PBS, whereas the second was treated with broad bean CPE. Supernatants were collected after 72 h of the challenge. The estimation of interleukin-IL-4, IL-5, and IL-13 levels in culture supernatant were carried by flow cytometry (BD FACS, Canto II, BD Biosciences, San Jose, CA, USA) using a CBA kit (BD Biosciences, San Jose, CA, USA) as per manufacturers' instructions. The level of regulated on activation, normal T-cell expressed and secreted (RANTES) was estimated by BD Flex set (BD Biosciences, San Jose, CA, USA).

Mouse mast cell protease-1 and eotaxin levels

Because mast cell mediators play a very important role in allergic manifestations, we have estimated the levels of mouse mast cell protease-1 (mMCPT-1) and eotaxin. Serum mMCPT-1 was quantified by enzyme-linked immunosorbent assay (ELISA) in the pooled sera from broad bean-treated and control mice (e Biosciences, San Diego, USA) according to manufacturers' instructions. The level of eotaxin was observed in mice sera using an ELISA kit (Ray Biotech, Norcross, GA, USA).

Systemic anaphylaxis score and rectal temperature

To observe the severity of allergic reactions induced after the challenge with a heavy amount of proteins in sensitized mice, we studied the clinical symptoms. The scoring system for systemic anaphylaxis symptoms was used as previously described [16]. In brief, mice were challenged by a heavy dose (5 mg) and anaphylactic symptoms were studied 20 min after challenge and given a score of 0 to 5. Score 0 indicated no symptoms; 1 indicated scratching and rubbing around the nose and head; 2 indicated puffiness around the eyes and snout, pilar erection, diarrhea, and reduced activity or standing still with an increased respiratory rate; 3 indicated wheezing, labored respiration, and cyanosis around the mouth; 4 indicated symptoms as in those scoring a 3, with loss of consciousness, tremors, and/or convulsion; 5 indicated death. Rectal temperature was measured before and after the challenge by a digital thermometer (Bioseb, France).

Histopathology

To visualize the pathologic changes in the tissue due to broad bean exposure, analysis of tissues were carried out. Histopathologic studies conducted in our experiment were semiquantitative. The lungs, spleen, and intestine were washed in cold normal saline solution, fixed in 10% formalin, and embedded in paraffin. Sections of 5- μ m thickness were cut and stained with hematoxylin and eosin for microscopic examination. The images were captured by microscope (Nikon Eclipse TE2000-S microscope).

Thymic stromal lymphopoietin

Our next intension was to observe the role of TSLP in the broad bean-induced allergic reactions. The TSLP level was quantified in the sera from broad bean and control mice by using the TSLP mouse ELISA kit (BD Biosciences, San Jose, CA, USA) according to manufacturers' instructions.

Mast cells staining

Mast cell plays a very important role in allergic onset via release of several allergic mediators, which is why mast cell count was carried out in the tissue. Detection of mast cells in the spleen, lungs, and intestine were performed using the mast cell staining method. In brief, the sections of organ tissues from control and broad bean-challenged mice (3–5 μ m) were rehydrated in xylene, xylene plus ethanol, and ethanol concentration (100%, 90%, 70%, and 50%). Furthermore, the sections were washed with PBS and stained with toluidine blue dye (Sigma Aldrich, St. Louis, MO, USA) for 30 min. Thereafter sections were washed in the running tap water gently and dehydrated using differentiating ethanol (50%, 70%, 90%, and 100%), xylene plus ethanol and xylene, respectively. Slides were mounted with DPX (BDH, India) and images were captured by microscope (Nikon Eclipse TE2000-S microscope).

Immunohistochemical analysis for eosinophil levels

We further studied another very important cell, eosinophils, which are frequently reported in allergic reactions to foods. Detection of eosinophils in the intestine and lungs of broad bean-challenged and control mice were performed using antibody against eosinophil major basic protein by immunohistochemical methods as previously described [17].

ELISA inhibition for cross-reactivity with other legumes

To find out the similarity of allergens among different legumes cross-reactivity was studied. The cross-reactivity of broad bean with peanut, soybeans, fenugreek, red gram, green gram, chickpea, and black gram was determined in vitro by ELISA inhibition following a previously described method [18]. The percentage inhibition of the specific IgE was expressed as:

Percentage inhibition = $1 - \text{OD of the sample with an inhibitor} / \text{OD of a sample without inhibitor} \times 100$.

Myeloperoxidase release assay

All the tissues were cleaned thoroughly with cold normal saline. The level of myeloperoxidase (MPO) was determined according to a previously described method [19]. Briefly, 100 mg of tissue from broad bean-treated and control mice was homogenized in two volumes of ice-cold buffer (0.1 M NaCl, 20 mM NaPO₄, 15 mM Na EDTA, pH 4.7) and centrifuged at 600 g for 15 min. Furthermore, the pellet was then subjected to a hypotonic lysis using a lysis buffer (900 μ L of 0.2% NaCl solution) and 30 sec later, 900 μ L of a solution containing 1.6% NaCl and 5% glucose was added. After centrifugation at 600 g for 15 min, the pellet was resuspended in 200 μ L of 50 mM NaPO₄ buffer, pH 5.4, containing 0.5% hexadecyl trimethylammonium bromide (Sigma Chemicals, St. Louis, MO, USA) and rehomogenized. The homogenate was then frozen, thawed three times and centrifuged at 3000 g for 15 min at 4°C. The MPO activity in the supernatant was assayed by measuring the change in absorbance at 450 nm using tetramethylbenzidine substrate (TMB reagent set, BD Biosciences, San Jose, CA, USA). The results were reported as change in absorbance at 450 nm/g tissue.

Mediators release from RBL-2H3 cells

The release of β -hexosaminidase from RBL-2H3 cells was studied according to a method previously described [20]. The RBL-2H3 cells were cultured and plated in 48-well plate. The cultured cells were incubated with sensitized mice sera (1:10 dilution) for 1 h as per a previously described method [21]. Plates were washed twice with Tyrode's buffer (135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 6 H₂O, 5.6 mM glucose, 20 mM HEPES, 0.1% BSA, pH 7.4) and added 150 μ L of serially diluted allergen (25 μ g, 50 μ g, 75 μ g, 100 μ g, 125 μ g) to the plate. The reaction was stopped by putting the plate on ice. The β -hexosaminidase activity was measured by incubating an equal volume of supernatant with substrate buffer (1.2 mM 4-nitrophenyl N-acetyl- β -D-galactoseaminide/0.05 M sodium acetate buffer brought to pH 4.5 with glacial acetic acid). The product of an enzymatic reaction, p-nitro phenol was measured at 405 nm. Histamine, cysteinyl leukotriene, and prostaglandin D₂ release from RBL-2H3 cells were measured using commercially available ELISA kits as per manufacturer's instructions (Cayman Chemical, Ann Arbor, Michigan, USA).

Skin prick test

Broad bean-allergic patients were selected on the basis of the clinical history of allergic rhinitis, bronchial asthma, urticaria, and food allergy that was further confirmed by skin prick test (SPT), total IgE, and specific IgE levels. The SPT was performed with pollens, insects, danders, dust, fungi, and food extracts on 127 (83 males and 44 females) allergic patients in the outpatient department of the Department of Pulmonary Medicine, King George Medical University, Lucknow, India. The SPT was carried out following a previously described method [22]. In brief, SPT was done with purified extracts of various allergens like pollens, fungi, insects, animal dander, and juicy and non-juicy foods obtained from Alcit India Pvt Ltd, New Delhi, India. Positive and negative controls were histamine acid phosphatase in glycerinated buffer saline 10 mg/mL and glycerinated buffer saline, respectively. The immediate response was read after 20 min. The grading was done as previously described [23]. Reactions of 2+ and above were labeled and scored. Blood was collected from SPT-positive broad bean patients (n = 16) and healthy volunteers (n = 10).

Total and specific IgE levels of allergic patients

The specific IgE levels were measured according to a previously described method [17]. Human total IgE ELISA kit (Immunology consultant's laboratory, Portland, OR, USA) was used to estimate the level of total IgE in humans.

Cross-reactivity with other legumes in allergic patients

The ELISA inhibition assay was performed to determine the cross-reactivity of broad bean proteins with chickpea, black gram, fenugreek, green gram, red gram, and red kidney bean proteins.

Statistical analysis

The statistical significance of the data obtained was determined using a software package from In Stat version 5 (Graph pad, San Diego, CA, USA; <http://www.graphpad.com>) using the Bonferroni analysis of variance test. Values for all measurements are expressed as mean $\pm$ SEM. Differences between groups were considered significant at $P < 0.05$.

Ethical permissions

The human study was performed at the Department of Pulmonary Medicine after informed consent from allergic patients and healthy volunteers. The study procedure was approved by the Human Ethical Committee (Reference no: 2938/R.Cell-11) of the King George Medical University, Lucknow, India. Animal study was carried out after approval of the Institutional Animal Ethics Committee (ITRC/IAEC/39/11) of CSIR-IITR, Lucknow, India.

Results

Pepsin digestibility assay

SGF assay of broad bean CPE showed that eight proteins of molecular weights 70, 60, 48, 32, 23, 19, 15, and 10 kDa remained undigested in SGF up to different incubation periods. Densitometry analysis revealed 38%, 30%, 29%, 28%, 28%, and 10% of 70 kDa; 55%, 52%, 45%, 24%, 22%, and 5% of 60 kDa; 100%, 78%, 76%, 52%, 54%, and 40% of 48 kDa; 79%, 79%, 73%, 66%, 55%, and 6% of 32 kDa; 88%, 86%, 72%, 62%, 47%, and 5% of 23 kDa; 76%, 45%, 34%, 27%, 27%, and 4% of 19 kDa; 87%, 80%, 79%, 65%, 60%, and 3% of 15 kDa; 80%, 76%, 62%, 40%, 10%, and 5% of 10 kDa remained undigested at the time period 0.25, 0.5, 1, 4, 15 and 60 min in SGF, respectively (Fig. 1A, B).

IgE binding proteins of broad bean

Immunoblotting with broad bean sensitized mice sera showed nine IgE-binding proteins of approximate molecular weight 95, 70, 60, 48, 32, 23, 19, 15, and 10 kDa. Interestingly, all these IgE-binding proteins, except protein of molecular weight 95 kDa, were found pepsin-resistant in SGF. No IgE-binding proteins were found by immunoblotting with pooled sera of the control mice (Fig. 2).

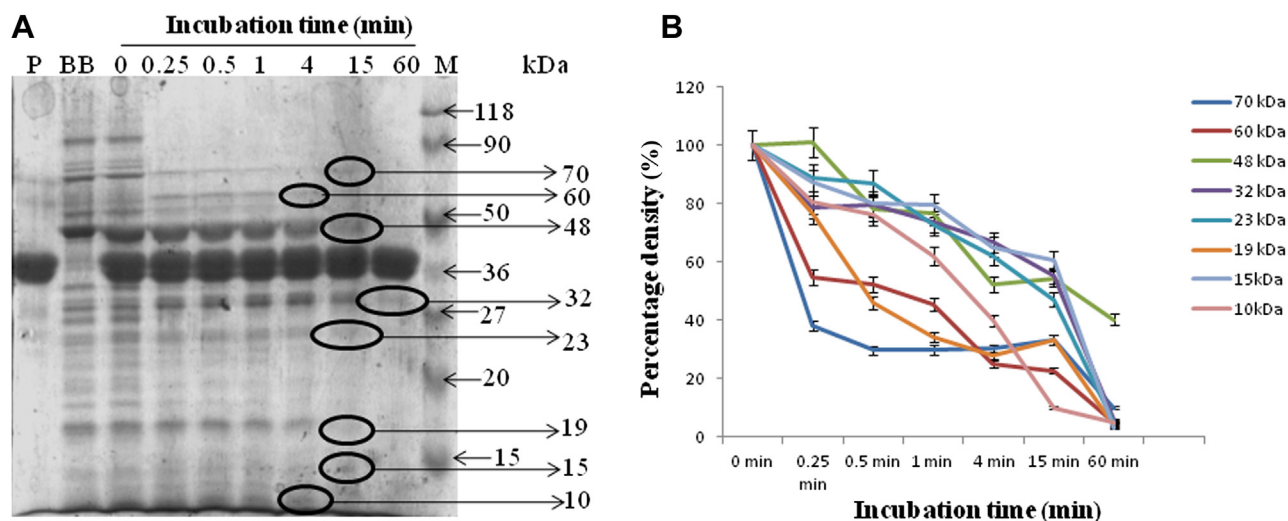


Fig. 1. (A) Simulated gastric fluid assay of broad bean crude protein extract (CPE). Where lane 1: Pepsin, lane 2: Broad bean CPE, lanes 3–9, incubation period of broad bean CPE in simulated gastric fluid at 0, 0.25, 0.5, 1, 4, 15, and 60 min. lane M, molecular weight markers. (B) Densitometry analysis of undigested proteins of broad bean at different time points.

Total IgE, specific IgE, and IgG1 antibodies

The mice group sensitized with broad bean CPE showed significantly higher levels of total and specific IgE ($P < 0.05$) compared with the respective control groups. Broad bean-

sensitized mice showed three, three, and four fold increases in the total IgE level on days 15, 43, and 59 days compared with respective control groups (Fig. 3A). Similarly, broad bean-sensitized mice showed 8, 12, and 13 fold increases in the specific IgE level on days 15, 43 and 59, respectively, when compared with controls (Fig. 3B). The specific IgG1 levels were also found enhanced in broad bean CPE-treated groups compared with controls. On days 15, 43, and 59, broad bean-sensitized mice showed 5, 11, and 13 fold increases in IgG1 compared with the control group (Fig. 3C).

Anaphylactic symptoms and rectal temperature

Clinical symptoms were observed 20 min after the broad bean CPE challenge. Of five mice from the broad bean-sensitized group, one showed puffiness around the eyes and snout, pilar erection, diarrhea, and reduced activity or standing still with an increased respiratory rate. Another mouse had symptoms such as wheezing, labored respiration, and cyanosis around the mouth. One mouse had wheezing, labored respiration, and cyanosis around the mouth, as well as loss of consciousness, tremors, and convulsion. The remaining two mice died. Control group animals showed no signs of anaphylaxis after broad bean CPE challenge, as depicted in Figure 3D. Twenty min after broad bean CPE challenge, sensitized mice showed marked reduction in body temperature in the range of 2°C to 4°C whereas the control group did not. One broad bean-sensitized mouse showed 4°C decrease, whereas three mice showed 3°C decrease in rectal temperature. The remaining mouse showed 2.5°C decrease in rectal temperature (Fig. 3E).

Histopathology

Abrupt histologic changes were observed in the organs, such as lungs, spleen and intestine of broad bean-treated mice. BALB/c mice exposure to broad bean CPE through intraperitoneal route led to inflammatory responses in airway tissue (lungs), lymphoid tissue (spleen), and gastrointestinal tract tissue (intestine). The lungs of sensitized mice showed infiltration of

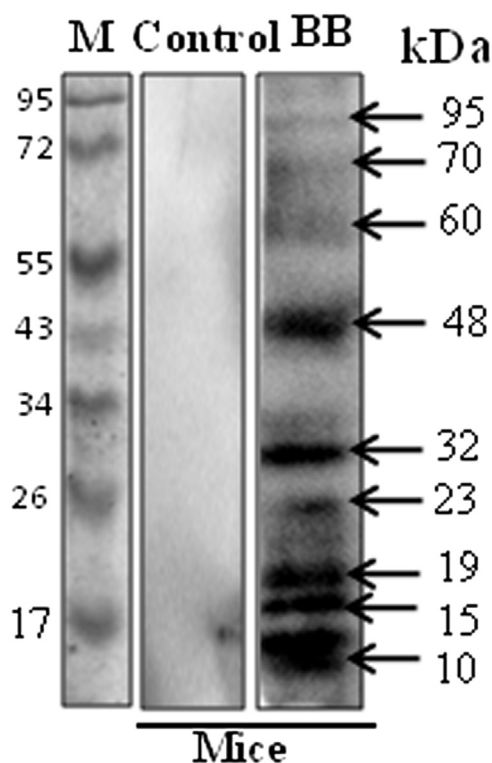


Fig. 2. IgE immunoblot of broad bean (BB) crude protein extract using control and broad bean-treated mice sera, where lane 1: Molecular weight markers (M), lane 2: Control—phosphate-buffered saline-treated mice, lane 3: BB—Broad bean-treated mice.

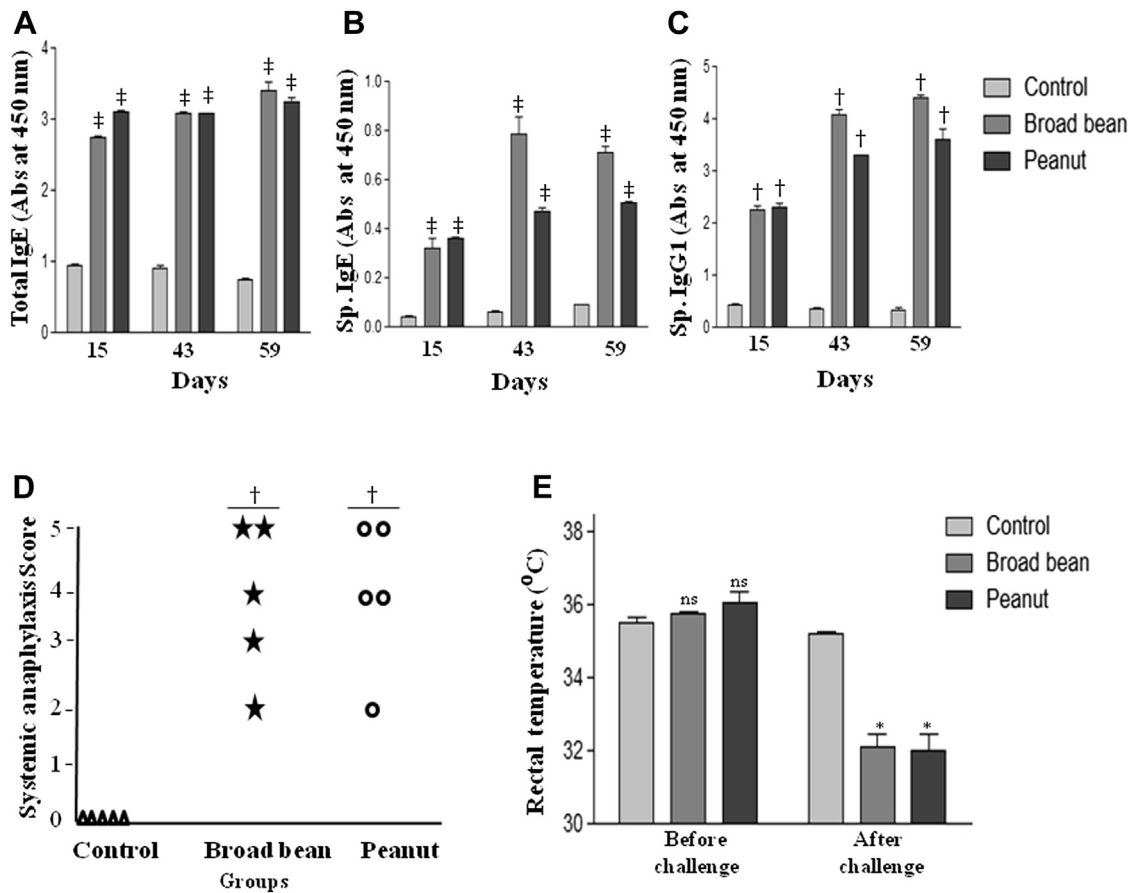


Fig. 3. Immunoglobulins (Ig) and anaphylactic symptoms. (A) serum total IgE level; (B) specific IgE level; (C) level of IgG1 on days 15, 43, and 59 in control, broad bean and peanut-treated mice; (D) anaphylactic symptoms after 20 min following challenge; (E) rectal temperature measured 20 min after challenge on day 60. Significant difference: * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$, $n = 3$ compared with control group.

mucus, hyperplasia of bronchial lining epithelium, and destruction as well as exfoliation of bronchiolar lining epithelium. Thickening in the alveolar wall among the treated group was moderately greater than in the control group. The lungs of control mice had no histologic changes as reported in the treated mice group. Broad bean intraperitoneally treated mice showed exfoliation of intestinal lumen, infiltration of leukocytes, destruction of the mucosal lining, and infiltration of the mucosa with inflammatory cells, whereas control mice had no such changes. Spleens from broad bean-treated mice showed megakaryocytic hyperplasia and lymphoid hyperplasia, whereas control mice had normal histology of spleen with red and white pulp (Fig. 4A).

Mast cells and eosinophils counts

The number of mast cells (Fig. 4B) and eosinophils (Fig. 4C) increased in broad bean-treated mice lungs and intestine compared the control group.

Cytokines and chemokines

Marked enhancement ($P < 0.05$) in the levels of IL-1, IL-4, IL-5, IL-12, and IL-13 was noticed in broad bean-exposed splenocytes culture supernatant compared with naïve controls (Fig. 5A–F). Furthermore, increased level of RANTES was observed in broad

bean-treated group compared with control (Fig. 5F). The level of eotaxin ($P < 0.05$) was found increased in the serum of sensitized mice compared with controls (Fig. 5G).

mMCPT-1 and TSLP

The level of mMCPT-1 increased by twofold in the serum of sensitized mice compared with controls (Fig. 6A). Furthermore, broad bean-sensitized mice sera showed a three fold increase in the level of TSLP compared with controls (Fig. 6B).

Myeloperoxidase assay

MPO activity was significantly ($P < 0.05$) increased in the spleen and skin of broad bean-treated mice compared with controls (Fig. 6C, D).

Cross-reactivity of broad bean with other legumes

Broad bean proteins exhibited a significant ($P < 0.05$) cross-reactivity with peanut, fenugreek, red kidney bean, red gram, green gram, chickpea, and black gram proteins in a dose-dependent manner (Fig. 7).

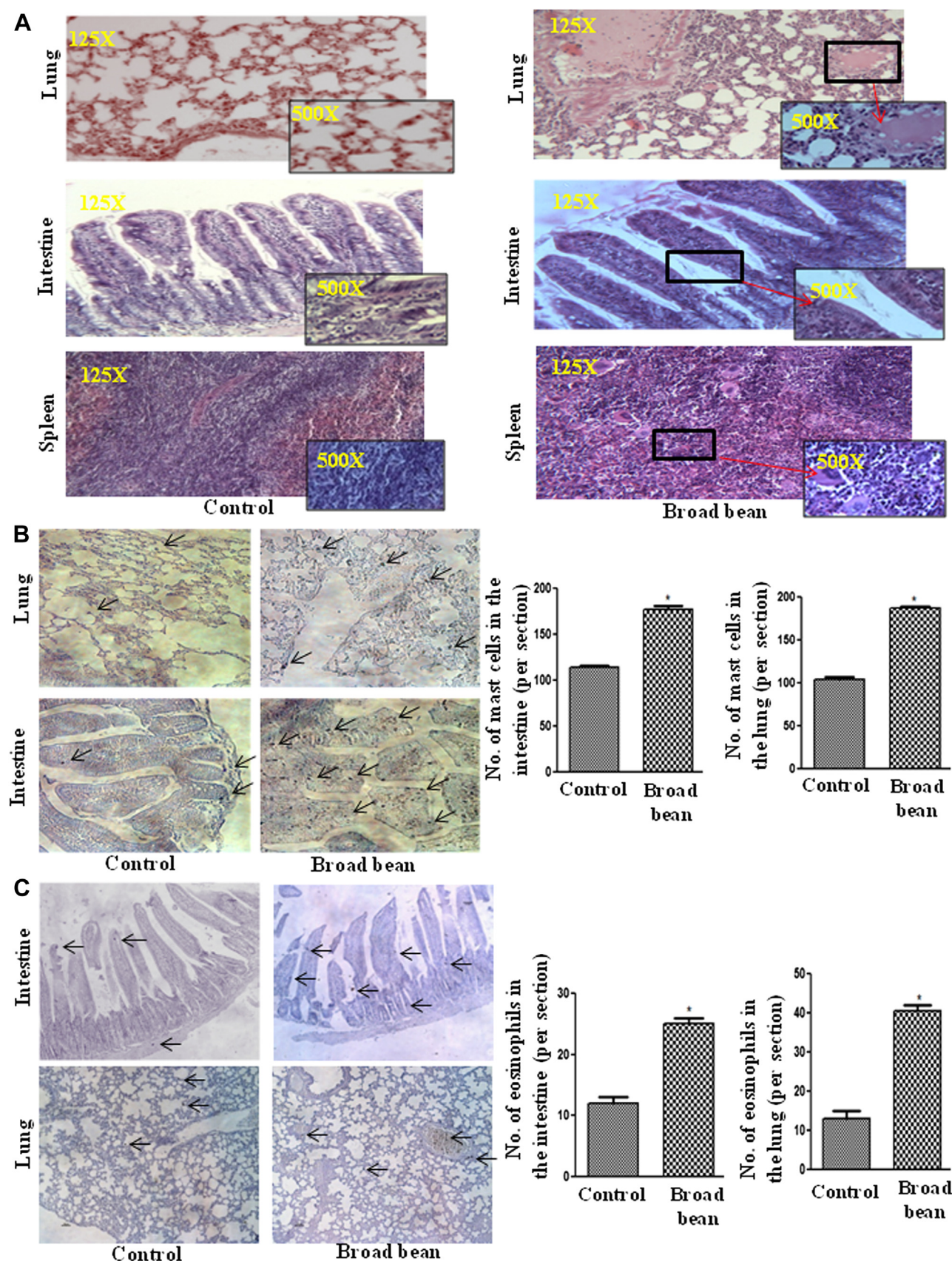


Fig. 4. Histopathology and immunohistochemistry. (A) Histopathologic analysis of the lung, intestine, and spleen of broad bean-treated mice. (B) Immunohistochemical staining of the lung and intestine for mast cells in broad bean-sensitized mice after challenge. Arrow indicates mast cells. (C) Immunohistochemical staining of lung and intestine for detection of the eosinophil in broad bean-sensitized mice after challenge. Arrow indicate eosinophils. * $P < 0.05$.

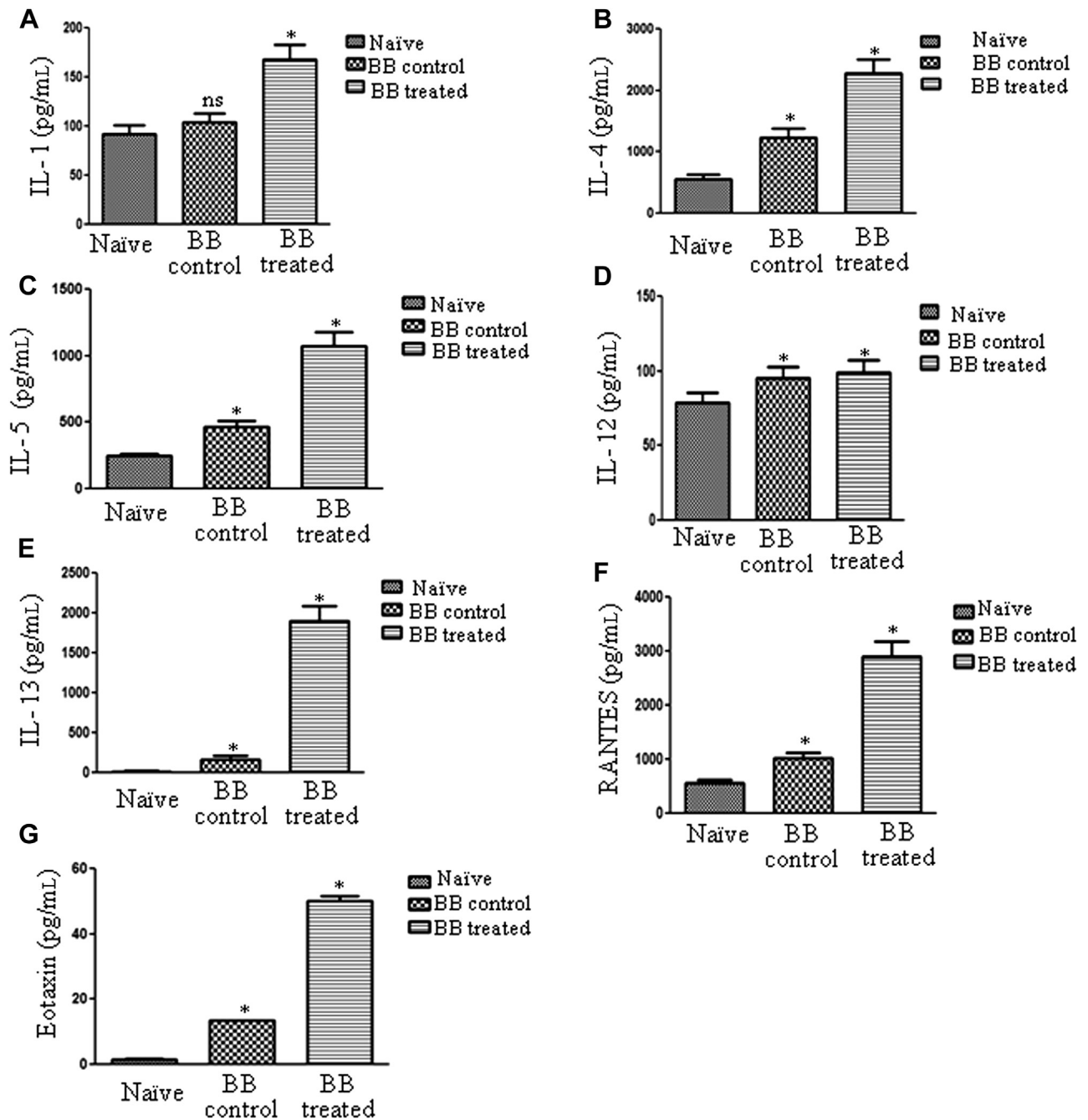


Fig. 5. Estimation of cytokine and chemokine levels in splenocytes culture supernatants collected at 72 h using flow cytometry. The levels of (A) IL-1, (B) IL-4, (C) IL-5, (D) IL-12, (E) IL-13, (F) RANTES, and (G) eotaxin. Results are expressed as mean $\pm$ SEM from three separate experiments (* $P < 0.05$, ns, nonsignificant; $n = 3$) compared with control. IL, interleukin; RANTES, regulated on activation, normal T-cell expressed and secreted.

Mediators release from RBL-2H3 cells

The release of β -hexosaminidase was found 59%, 62%, 64%, 66%, and 65% on the amount of 25, 50, 75, 100, and 125 μ g of broad bean CPE, respectively (Fig. 8A). Significantly enhanced ($P < 0.05$) release of histamine, prostaglandin D_2 , and cysteinyl leukotriene were observed in RBL-2H3 cells challenged with broad bean CPE compared with respective controls (Fig. 8B–D).

Human studies

One hundred twenty seven allergic patients were screened for this study and of them, sixteen (12%) showed positive SPT to broad beans. Results of SPT, total IgE, specific IgE, and allergic symptoms of broad bean-sensitive patients and healthy individuals are presented in Table 1. Total IgE levels in controls ranged between 123 and 326 kU/L, whereas in broad bean-sensitized patients, the range was 369 to 853 kU/L.

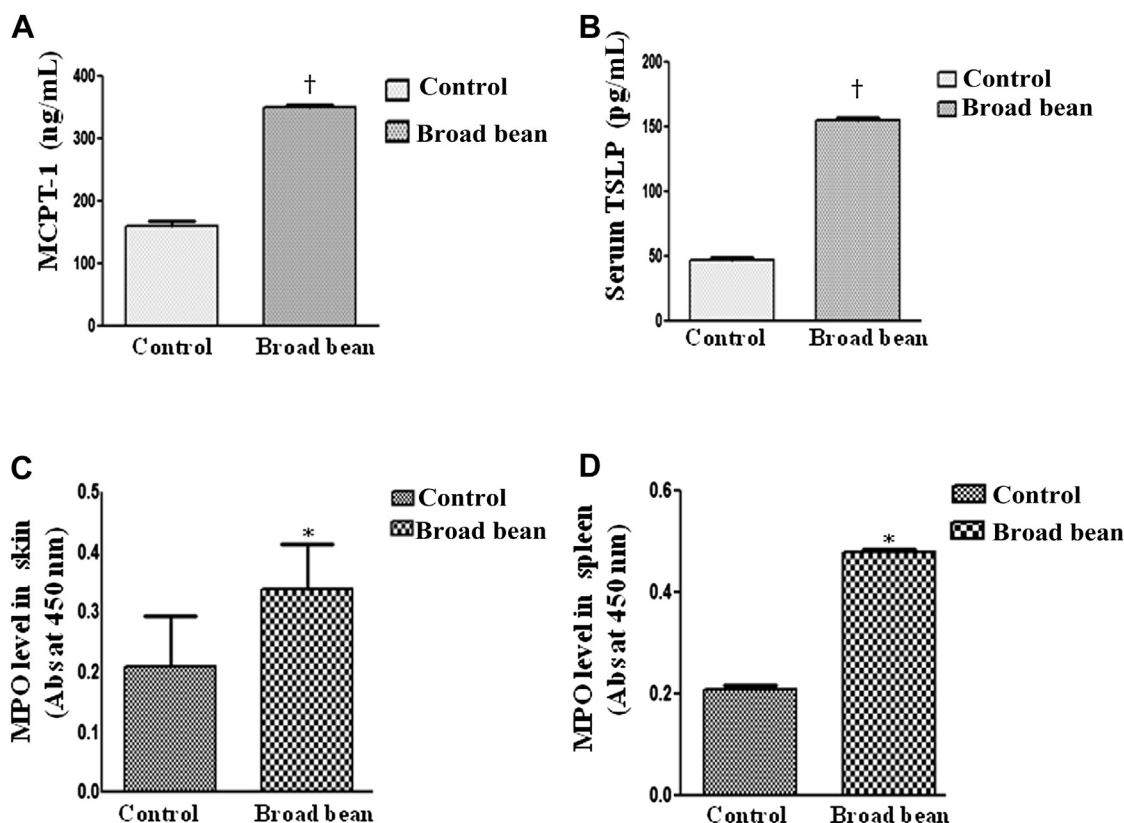


Fig. 6. Level of mMCPT-1 and TSLP. (A) Estimation of mMCPT-1 and (B) TSLP level was in broad bean-sensitized mice sera. (C) Level of myeloperoxidase was determined in the skin and (D) spleen lysate. Significant difference (* $P < 0.05$, † $P < 0.001$; $n, 3$) compared with control group. mMCPT-1, mouse mast cell protease-1; MPO, myeloperoxidase; TSLP, thymic stromal lymphopoietin.

Specific IgE levels ranged between 0.03 and 0.09 (absorbance at 450 nm) in the controls, whereas in broad bean-sensitive patients, it ranged between 0.1 and 1.4. Broad bean-sensitive

patients were also found to have concomitant sensitization to other allergens like pollens fungi, insects, and legumes (Table 2).

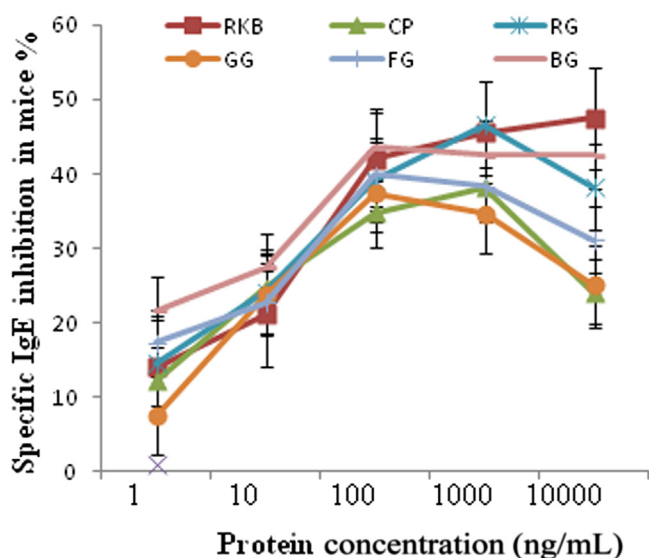


Fig. 7. Cross-reactivity of broad bean proteins with other legumes using ELISA inhibition with pooled sera of Broad bean-sensitized mice. BG, black gram; CP, chickpea; FG, fenugreek; GG, green gram; RG, red gram; RKB, red kidney bean.

IgE-binding proteins in human sera

Immunoblotting of electrophoresed broad bean CPE with pooled broad bean-allergic patient's sera showed nine IgE reactive proteins of approximate molecular weight of 95, 70, 60, 48, 32, 23, 19, 15 and 10 kDa. No IgE-binding proteins were found in IgE immunoblot with pooled sera of healthy individuals (Fig. 9A, B).

Cross-reactivity of sensitive patient's sera with other leguminous foods

The ELISA inhibition test showed a significant specific IgE inhibition with red kidney bean, chickpea, red gram, green gram, fenugreek, and black gram (Fig. 9C).

Discussion

Legumes, one of the main dietary sources of proteins, have been reported to cause life-threatening reactions in sensitized individuals [4]. Because reports on broad bean allergenicity are few, a thorough assessment of allergenic potential was undertaken in the present study.

Resistance to pepsin digestion and IgE-binding capacity are two important properties of allergenic proteins. The proteins

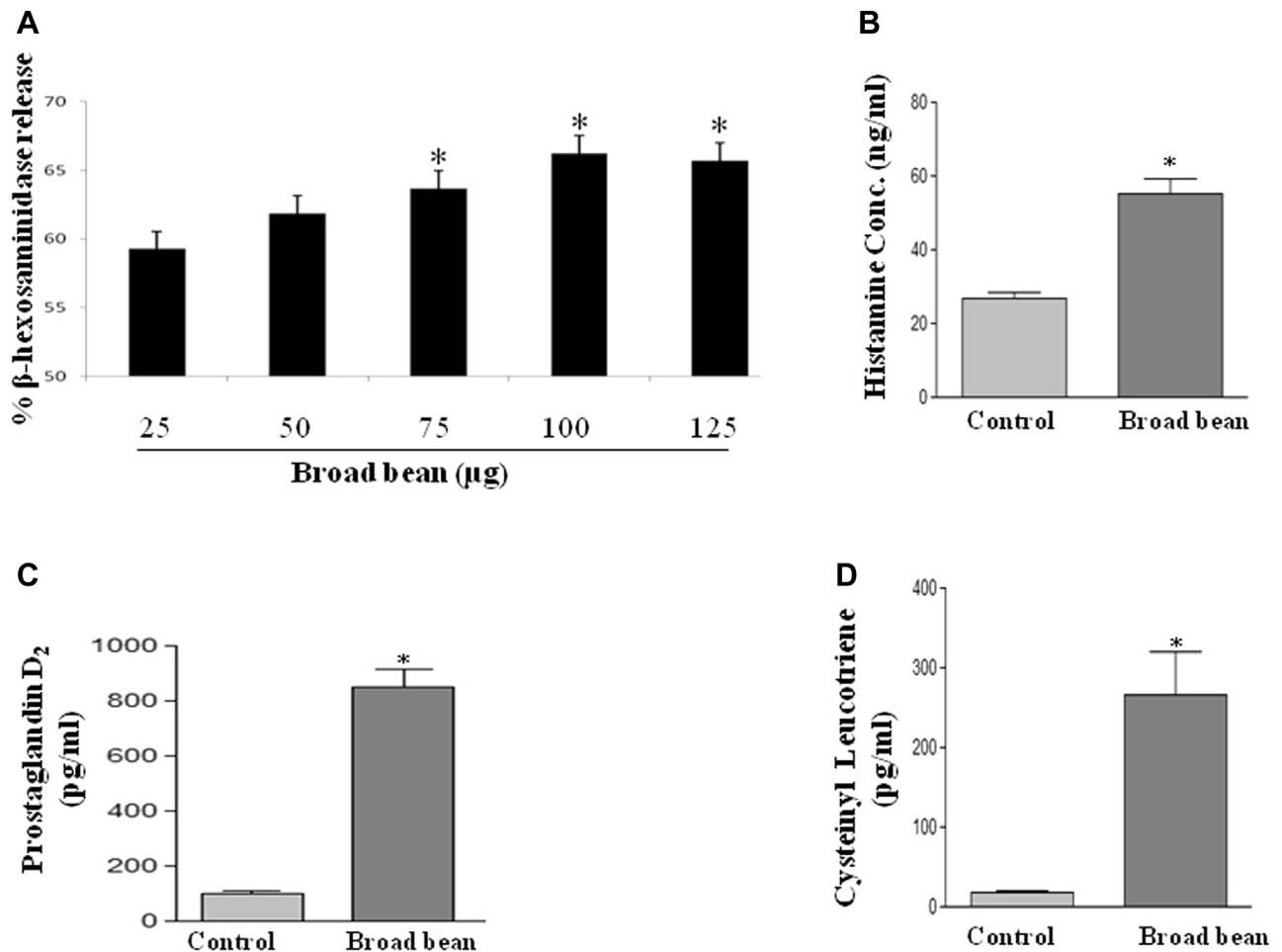


Fig. 8. Mediators released from RBL-2H3 cell lines primed with broad bean-treated mice sera. (A) Level of β -hexosaminidase determined at different doses of broad bean crude protein extract exposure. (B) Level of histamine, (C) prostaglandin D₂, and (D) cysteinyl leukotriene. Data are presented as means $\pm$ SEM; * P < 0.05; n = 3 versus control mice.

that are stable in SGF have potential to elicit allergic responses in the majority of cases [24]. The proteins that get digested in a relatively short time have little chance to pose adverse immune effects and therefore, are mostly non-allergenic. According to DBT guidelines [25], proteins with more than 10% stainable full-length protein band remaining at >30 to 60 min may be considered a stable protein. Proteins reduced to <10% stainable band at 5 to 30 min can be considered of intermediate stability. As per these guidelines, eight proteins of broad beans with molecular weight 70, 60, 48, 32, 23, 19, 15, and 10 kDa remained undigested for more than 2 min. Therefore, these proteins may have the capability to elicit allergic symptoms.

BALB/c mice have been used as a pivotal in vivo tool for the assessment of food allergic reactions due to its high IgE-responding properties [26]. Several studies regarding the food allergy assessment have been carried out in BALB/c mice following intraperitoneal exposure [10,17,26]. The intraperitoneal route has been found to surpass the oral tolerance that is frequently observed in orally treated mice.

Most of the food allergic reactions are IgE mediated and identification of IgE-binding components is very helpful in the

identification of allergic proteins [27]. IgE-immunoblotting with pooled sera of broad bean-sensitized mice as well as broad bean allergic humans showed nine IgE-binding proteins in broad bean CPE. Human and mice sera blotted proteins of similar molecular weight. The molecular weights of eight IgE-binding proteins were approximately similar to that of the pepsin-resistant proteins. Taken together, eight broad bean proteins had pepsin-resistant and IgE-binding properties, whereas one protein (95 kDa) had only IgE-binding ability. Pepsin-resistant proteins having IgE-binding properties have frequently been observed in the case of several food allergens like ovalbumin, Ara h2, and beta-lactalbumin [28,29].

Food allergens have a tendency to provoke the immune system via induction of immunoglobulins like IgE and IgG1 [30]. An increase in levels of total and specific IgE and IgG1 antibodies in broad bean-sensitized mice indicated the ability of broad bean proteins to induce allergic responses. Similar to murine model, the levels of total IgE as well as specific IgE were also found elevated in the sera of broad bean-allergic patients. Several molecules are involved in the food allergic reactions including immunoglobulins, cytokines and chemokines [31]. In the case of legume allergy, mixed T-cell helper Th1 and Th2 type cytokine

Table 1
Details of broad bean sensitive-patients

Serial number	Age/sex	SPT (weal)*	Total IgE [†] (kU/L)	Specific IgE [†] (Abs at 450)	Allergic symptoms
Control 1	57/M	– ve	326.0	0.0995	AR
Control 2	40/M	– ve	244.7	0.0345	BA
Control 3	10/M	– ve	320.3	0.064	AR
Control 4	32/F	– ve	247.5	0.0425	AR
Control 5	56/M	– ve	123.6	0.006	AR + BA
Control 6	20/F	– ve	212.0	0.010	AR + BA
Control 7	14/M	– ve	149.0	0.020	AR
Control 8	30/F	– ve	170.3	0.032	AR
Control 9	35/F	– ve	198.5	0.040	AR + BA
Control 10	20/F	– ve	247.5	0.380	AR + BA
Patient 1	42/M	3 +	737.2	1.3175	AR + BA
Patient 2	38/F	2 +	369.4	0.2095	AR, D
Patient 3	35/F	3 +	812.5	0.1995	AR
Patient 4	50/M	3 +	812.5	1.405	BA
Patient 5	20/F	2 +	853.3	0.693	AR, D
Patient 6	35/F	2 +	838.8	0.1255	AR
Patient 7	30/M	2 +	758.3	0.137	AR
Patient 8	14/F	2 +	401.1	0.113	AR + BA
Patient 9	21/F	3 +	649.1	0.79	AR
Patient 10	20/M	2 +	538.2	0.1235	AR
Patient 11	45/M	3 +	652.2	1.0015	BA
Patient 12	50/M	2 +	654.1	0.815	AR
Patient 13	15/F	3 +	678.4	0.289	AR + BA
Patient 14	18/M	2 +	518.0	0.650	BA
Patient 15	17/M	2 +	735.1	0.160	AR
Patient 16	25/F	2 +	692.0	0.561	AR, D

—Ve, SPT negative for broad bean; AR, allergic rhinitis; BA, bronchial asthma; D, dermatitis; Ig, immunoglobulin; SPT, skin prick test

* SPT measured according to weal size (in mm).

† Total and specific IgE presented in mean ± SEM.

responses have been reported frequently [32–34]. We also found a mixed cytokine (Th1/Th2) response in broad bean-sensitized splenocytes. Enhanced release of different allergic mediators like histamine, cysteinyl leukotriene, prostaglandin D₂ have been reported to play an important role in the food allergy reactions [35,36]. Eotaxin and RANTES levels have been reported to be enhanced in peanut allergy [36,37]. Similar response in cases of broad bean suggests its possible allergenic potential. TSLP, a protein of the cytokine family, is known to play an important role in the maturation of T-cell populations through activation of antigen presenting cells. TSLP triggers dendritic cell-mediated Th2 responses [38]. Furthermore, TSLP has been reported to be linked with allergic inflammatory diseases [39]. The level of TSLP increased in broad bean CPE-sensitized mice, which again indicates its allergenic potential. The mouse mMCPT-1 level has been reported to be enhanced in food allergies, including allergy to chickpeas [17]. The enhanced release of mMCPT-1 in broad bean-sensitized mice indicates the involvement of intestinal

Table 2
The concomitant sensitivity of broad bean-sensitive patients (N = 16) to other allergens

Groups	Allergens	Patients
1	Pollens	15
2	Fungi	6
3	Insects	8
4	Dust	9
5	Dander	2
6	Miscellaneous food items	10
7	Animal products	2
8	Other legumes	14

mast cells in broad bean allergy because mMCPT-1 is a known indicator of mucosal mast cell degranulation.

Broad bean-sensitized mice had marked anaphylactic symptoms and decreased rectal temperature on challenge. Similar to our results, mice intraperitoneally sensitized with peanut showed similar signs of anaphylaxis [39]. Drops in core body temperature (2°C–4°C) have been reported as in the case of red kidney bean and chickpea as well [17,26]. The inflammatory mediators released after challenge cause vasodilation resulting in decreased body temperature.

Hyperplasia and formation of megakaryocytes observed in the spleen of broad bean-treated mice are considered a sign of allergic responses. The probable reason behind this may be the massive proliferation and differentiation of T lymphocytes leading to histologic signs of chronic inflammation in the lymphoid organs [40]. Histology of the lungs showed that exposure to broad bean CPE recruits inflammatory cells resulting in airway blockage that is an indicator of immune inflammatory diseases like asthma. In this study, the levels of eosinophils and mast cell were found elevated in the lungs, intestine, and spleen of broad bean-treated mice. Eosinophilic infiltration is a frequently observed symptom of food allergy [26]. Mast cells are one of the most important cell types involved in the allergic reaction. The IgE binds to the mast cell receptor FcεR1 and following a crosslinking of the allergen, mast cells degranulate and release several allergic mediators [31]. The significant enhancement in the mast cell number in the broad bean-treated group indicated the severity of broad bean allergy.

RBL-2H3 cells have been used as a useful tool to demonstrate in vitro degranulation induced by certain proteins or chemicals [20]. Histamine, cysteinyl leukotrienes, prostaglandins D₂, and β-hexosaminidase are released in the acute allergic reaction [40]. In the case of broad bean-exposed RBL-2H3 cells, the levels of all these mediators were found elevated, which further validated the allergic potential of broad bean.

Neutrophils participate in asthma and the allergic processes and release MPO together with other granule enzymes upon activation [41]. The level of the MPO increased in the spleen and skin of broad bean CPE-sensitized mice, indicating the possible involvement of neutrophils in broad bean-induced allergy.

Cross-reactivity is a measure of the presence of same or similar epitopes among different proteins [26]. The ELISA inhibition study demonstrated the cross-reactivity of Broad bean proteins with fenugreek, red kidney bean, red gram, green gram, chickpea, and black gram proteins. Considering this outcome the possibility of broad bean allergy in these legume allergies cannot be ignored.

After observing allergenicity potential of broad beans in murine model, we extended our study to humans. The symptoms of allergic rhinitis, bronchial asthma, urticaria, and dermatitis have been frequently observed in food allergy [10,42]. These symptoms were observed in the broad bean-allergic patients as well. Due to cross-reactivity, broad beans may elicit allergic symptoms in sensitive individuals who may be allergic to red kidney bean, black gram, red gram, green gram, and chickpea, and so on.

The significantly elevated levels of total and specific IgE in broad bean-sensitive patients, along with IgE immunoblotting with broad bean-sensitized human pooled sera clearly demonstrated the allergenic potential of broad bean in humans.

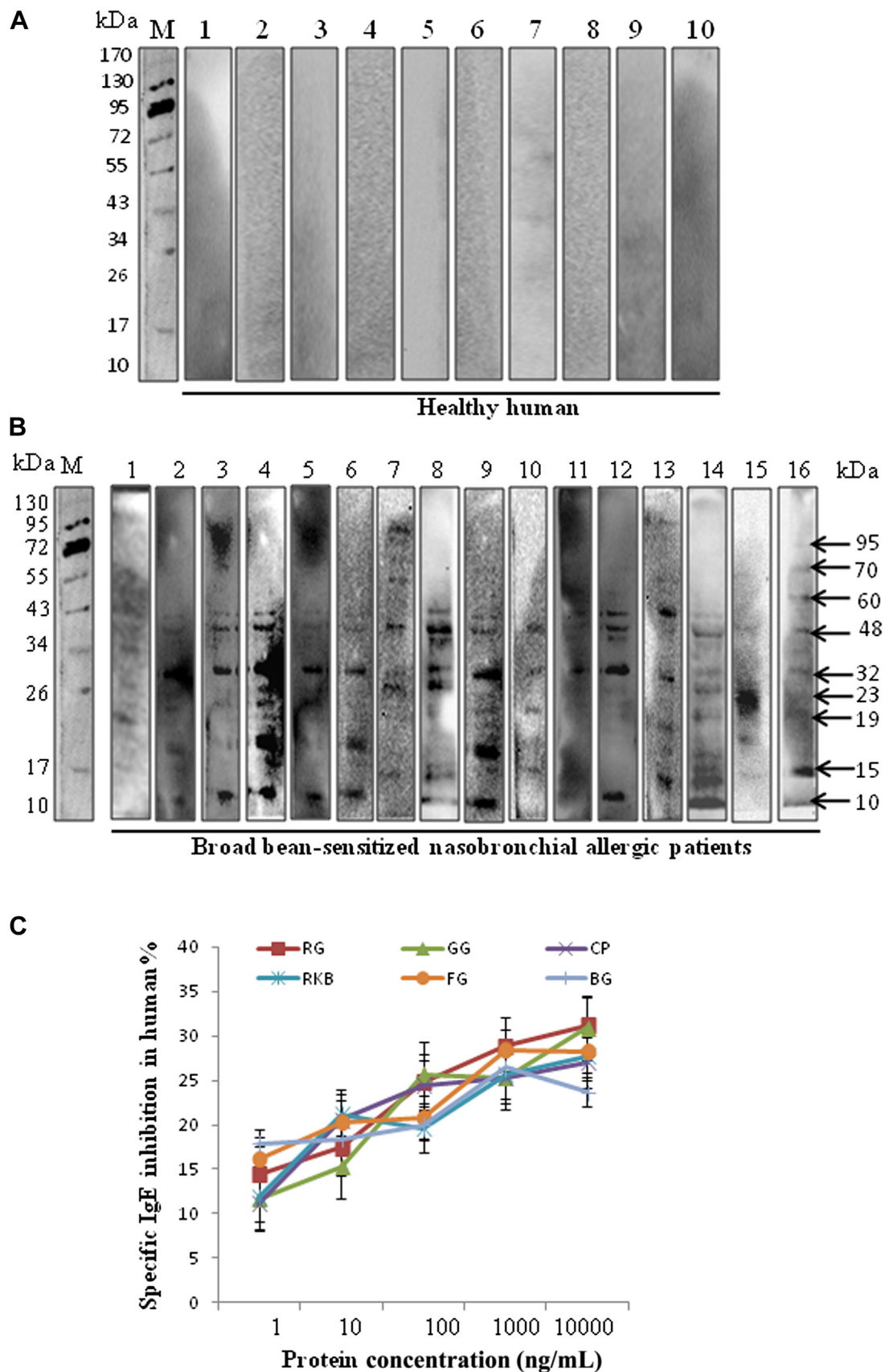


Fig. 9. Immunoglobulin (Ig)E immunoblots of (A) healthy human serum, (B) broad bean-allergic patients' serum, and (C) cross-reactivity of broad bean proteins with other legumes in the pooled sera of broad bean-sensitive patients. BG, black gram; CP, chickpea; FG, fenugreek; GG, green gram; RG, red gram; RKB, red kidney bean.

Conclusions

In summary, the allergic potential of broad bean was evidenced by the presence of eight pepsin-resistant proteins that also had IgE-binding ability. The increased levels of total IgE and specific IgE/IgG1, TSLP, eotaxin, mMCP-1, along with symptoms of systemic anaphylaxis, reduced body temperature and histopathological changes validated the sensitivity to broad bean proteins in BALB/c mice. Furthermore, the release of histamine, prostaglandin D₂, cysteinyl leukotriene, and β -hexosaminidase in the primed RBL-2H3 cells following broad bean CPE exposure confirmed the broad bean allergy in in vitro condition as well. Taken together, this study showed that broad bean proteins may have the ability to induce allergic responses in clinical, in vivo, in vitro, and ex vivo conditions.

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