



Allergenic responses of red kidney bean (*Phaseolus vulgaris* cv *chitra*) polypeptides in BALB/c mice recognized by bronchial asthma and allergic rhinitis patients

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ABSTRACT

Allergenicity potential of red kidney beans (*Phaseolus vulgaris* cv *chitra*) was assessed and attempts were made to identify the responsible proteins by pepsin digestibility assay and IgE immunoblotting. To evaluate allergenic potential, BALB/c mice were sensitized with red kidney bean proteins and levels of specific immunoglobulin, histamine, mast cell protease-1, cytokines and CCL-2 were measured. To confirm our findings in BALB/c, the studies were also extended to human subjects. Human sera collected from control subjects and allergic patients after skin prick test were used for IgE immunoblotting, measuring the levels of total and specific IgE and determining cross reactivity of red kidney bean with other legumes. Red kidney bean allergenic potential was evident by significant increase in specific IgE, IgG1, histamine, mast cell protease-1 and Th2 cytokine levels in comparison to control. Enhanced level of eosinophils in jejunum, prominent anaphylactic symptoms, and eruptive histopathological changes give indication towards red kidney beans allergenicity. IgE immunoblotting detected five protein components with molecular weights of approximately 170, 100, 43, 34 and 20 kDa. Red kidney bean proteins showed cross reactivity with peanut, soybean, chickpea and black gram. Finally, this work demonstrated that red kidney beans may induce allergic response in mice similar to human subjects, with identification of five clinically relevant allergenic protein components.

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1. Introduction

Food allergy is an important health issue of growing interest. It is estimated that 29,000 cases of anaphylactic reactions to food are treated in the emergency departments of US hospitals and 150–200 deaths because of food anaphylaxis occur each year (Sampson, 2003). Legumes may play a major role in allergenicity due to their high protein contents (Dalal et al., 2002). Red kidney bean (*Phaseolus vulgaris* cv *chitra*), a herbaceous annual plant and commonly known as rajmash, belongs to the family Fabaceae. The commercial production of red kidney bean (RKB) is well-distributed worldwide with countries in Asia, Africa, Europe, Oceania, South and North America. Its popularity can be

Abbreviations: RKB, (red kidney bean); SGF, (simulated gastric fluid); SPT, (skin prick test).

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attributed to its full-bodied flavor. RKB is the main ingredient of several dishes in raw and cooked form like red kidney beans and rice, macaroni with red kidney beans, red kidney bean casserole, red kidney bean salad, red kidney bean dip, red kidney bean vinaigrette and red kidney bean burgers etc. United States Department of Agriculture (USDA) reported that the consumption of red kidney beans in USA is 0.58 lb per capita (Lucier, Lin, Allshouse, & Kantor, 2000). India is one of the major markets of red kidney beans (CICILS/IPTIC Convention, 2009). Phaseolin is a major protein of RKB and its diversity has been reported as a possible strategy to improve the nutritional value (Montoya, Lallès, Beebe, & Leterme, 2010). Chemoprotective function of RKB polysaccharides against colon cancer by modulation of gene expression in human colon cancer cells (HT-29) has been reported recently (Campos-Vega, Guevara-Gonzalez, Guevara-Olvera, Oomah, & Loarca-Piña, 2010).

High consumption of any proteinaceous food including some commonly consumed legumes may increase the probability of sensitization against the potential allergenic proteins in susceptible individuals (Dalal et al., 2002). Pulses containing oligosaccharides were well tolerated with negligible perceived changes in flatulence and overall gastrointestinal function when incorporated into the diet of healthy adult males (Veenstra et al., 2010). Allergens of peanuts and soybeans have been isolated, identified, cloned and well defined

(Helm et al., 2000; Koppelman et al., 2003). Some data also exist on pea (Sanchez-Monge et al., 2004), chickpea (Mercedes et al., 2000), lentil (Sanchez-Monge et al., 2000) and red gram (Misra et al., 2010) as well. There are a few reports on RKB allergenicity. IgE antibodies were detected against RKB, white bean, pinto bean, chickpea, pea and black-eyed in human (Zacharisen & Kurup, 1998). Pepsin resistant proteins have also been reported in RKB by some groups using simulated gastric fluid (SGF) assay (Misra, Prasad, Das, & Dwivedi, 2009; Moma, 2006) but their purification and characterization is not well established.

Since, a systematic study has not been carried out on RKB allergenicity and its sensitizing allergens therefore, the present investigation was undertaken to assess the allergenic potential of RKB in BALB/c mice and the findings were then confirmed in RKB sensitive human population. Therefore, in this study, we performed SGF assay, IgE immunoblotting, IgG antibody responses (IgG1 and IgG2a), IgE antibody response, mMCPT-1, histamine levels, systemic anaphylaxis score, rectal temperature and histopathologic studies to characterize the allergenic potential of RKB proteins. Moreover, to understand the mechanism of RKB allergy, we also tried to analyze the cytokines and chemokine profile stimulated by RKB.

It was also attempted to identify the allergenic protein components, though a thorough characterization of the proteins needs to be done further. The assessment of allergenic potential of leguminous food is important from the purview of susceptible individuals who may be forewarned not to consume the crop and related legumes showing cross reactivity. Moreover this study paves the way for taking and advancing research for remedial immunotherapeutic measurements. The identification of allergens may also be helpful for plant biotechnologists for the development of transgenic non allergenic varieties of RKB.

2. Materials and methods

2.1. Test materials and reagents

All the chemicals were of highest grade purity available. RPMI-1640, antibiotic–antimycotic solution, Fetal Bovine Serum (FBS), pepsin from porcine gastric mucosa (Cat No. P-6887), O-phenylenediamine (OPD), bovine serum albumin (BSA), reagents for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and goat anti-human IgE peroxidase conjugate (Cat No. A9667) were purchased from Sigma Chemical Company, St. Louis, USA. The anti-human IgE antibody preparation is specific for human IgE when tested against purified human IgG, by manufacturer using Ouchterlony Double Diffusion (ODD) and immunoelectrophoresis (IEP). Goat anti-mouse IgG1 and IgE-HRP linked were obtained from Southern Biotech, Birmingham, USA. All the other chemicals used were of the highest purity available from other commercial sources. Red kidney bean seeds, popularly known as “Rajmash (cv chitra)” were purchased from a local certified seed vendor and the same lot was used throughout the study. The dry seeds were stored at 5–8 °C and 25–30% relative humidity. Seeds were put in a water proof container with a thin layer of colored silica gel desiccant as indicator of dryness.

2.2. Preparation of crude protein extract (CPE)

Crude red kidney bean protein extract was prepared as earlier described (Astwood, Leach, & Fuchs, 1996; Misra et al., 2009). Briefly, powdered seeds were defatted with n-hexane. Defatted flour was macerated in the phosphate buffer (20 mM Na₂HPO₄, 2 mM KH₂PO₄, 5.4 mM KCl, 0.5 M NaCl, pH 7.0). The mixture was agitated overnight at 4 °C, centrifuged for 30 min at the 10,000 × g and supernatant was recovered, filtered through 0.45 µm syringe filter and stored as aliquots at –80 °C until used.

2.3. Digestibility of RKB crude protein extracts in SGF

In vitro SGF assay was performed as described earlier with slight modifications (Thomas et al., 2004). The goal was to determine whether RKB protein components are resistant to digestion in SGF. SDS–PAGE analysis was used to measure the rate of protein degradation. The amount of pepsin used in the SGF assays was about 13 times more than that of test proteins (by weight) to ensure sufficient degradation. In brief, 3.2-mg pepsin was dissolved in 1 mL of 0.03 M NaCl, pH 2.0 for preparation of SGF. SGF (200 µL) was incubated at 37 °C prior to addition of 10 µL of test protein. After addition of test protein, the contents of the tube were mixed by mild vortexing and the tube was immediately placed in a water bath at 37 °C. Reaction was stopped at different time points (0.25, 0.5, 1, 2, 4, 8, 15 and 60 min) by 70 µL of the 1N NaHCO₃ followed by addition of 70 µL of 5X Laemmli sample buffer (pH 6.8). SGF and RKB CPE were added directly to the stopping solution and mixed by vortexing prior to the incubation for zero min control. Each sample was boiled for 5 min at 100 °C. Boiled samples (20 µL/well) were subjected to 12% SDS–PAGE according to the method of Laemmli (1970) at 22 mA for 4:30 h. Standard molecular weight (mol. wt.) markers were included to estimate the mol. wt. of the proteins. Coomassie brilliant blue (G-250) stain was employed for staining the gel. Gel image was captured using Syngene Bio Imaging System (Syngene, Cambridge, UK). Densitometry analysis was performed for validation of SGF results using VERSA DOC Imaging System model 1000 (Bio Rad, USA). Densitometry of each protein band was performed thrice and the average values have been used.

2.4. BALB/c mice

Healthy 6–8 week old female BALB/c mice (22 ± 3 g) were obtained from the IITR, Lucknow, India animal breeding colony. Mice were maintained under standard laboratory conditions in specific pathogen free environment and on RKB and peanut free diet.

2.5. Sensitization protocol

Mice were sensitized according to the earlier described protocol with slight modifications (Singh et al., 2006). In brief, mice were randomly divided into three groups (n = 10/group). Groups of mice were injected intraperitoneally with 100 µL Phosphate Buffer saline (PBS), 100 µg RKB protein in 100 µL PBS and 100 µg peanut protein in 100 µL PBS respectively once in a week for 7 weeks. Blood samples were collected from retro-orbital sinus to measure serum IgE and IgG1 antibodies, CCL-2 and mMCPT-1 on 15, 43 and 59 days. On day 60, groups of mice (n = 10/group) were challenged intraperitoneally with 1 mL of 10 mg/mL CPE. Tissue and blood samples were collected as per schedule depicted in Fig. 1. Peanut seeds were chosen as positive control as peanuts are considered to be one of the most potent food allergens causing severe diseases (Burks, Sampson, & Bannon, 1998; Fries, 1982; Van Wijk et al., 2005).

2.6. IgE immunoblotting

To detect IgE binding proteins of RKB, IgE immunoblotting was performed (Towbin, Staehelin, & Gordon, 1979). RKB crude protein extracts were resolved on 12% SDS–PAGE and electrophoretically transferred on PVDF membrane using a semidry blotting unit (Amersham Biosciences, St Francisco, USA). Blot was blocked with 3% BSA in TBS-T buffer (Tris–HCl 20 mM, 500 mM NaCl containing 0.1% Tween-20, pH 7.4) and kept overnight at 4 °C. Pooled RKB sensitized mice and SPT positive human sera were used as primary antibodies while goat anti-mice IgE peroxidase conjugated and goat anti-human IgE peroxidase conjugated (Sigma Chemical Company, dilution 1:1000 in TBST + 1% BSA) were secondary antibody.

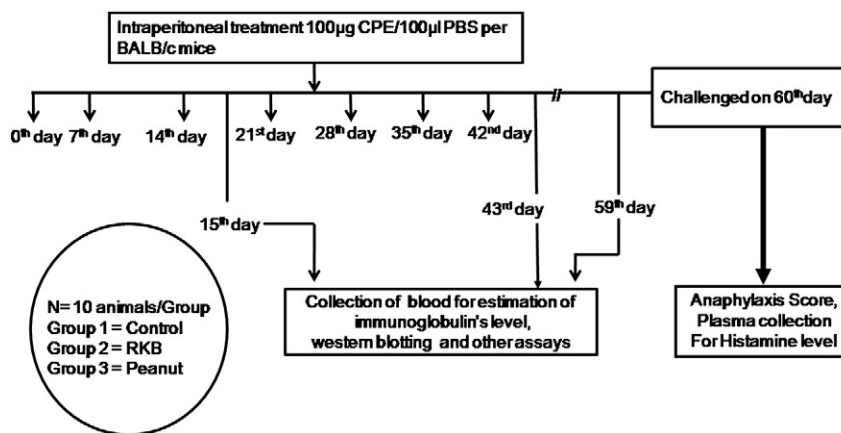


Fig. 1. Diagrammatic representation of animal treatment protocol. BALB/c mice were treated intraperitoneally for 7 weeks and blood was exsanguinated at day 15th, 43rd and 59th. Mice were challenged on day 60th for study of systemic effects caused by RKB proteins.

2.7. Total serum IgE assay

Total IgE was estimated with the Optia mouse IgE kit (BD Biosciences, Germany) according to the manufacturer's instructions. Briefly, 96 well Nunc maxisorp microtiter plates (Nunc, Denmark) were coated with 100 µL of antimouse IgE antibody at 1:250 dilution in 0.1 M carbonate buffer (pH 9.5) and kept overnight at 4 °C. The plate was washed with PBS washing buffer having 0.05% Tween 20 and blocked with PBS with 3% Fetal Bovine Serum (Assay Diluent, BD Biosciences, Germany). Serum samples containing 3% Fetal Bovine Serum were added to the wells in triplicate. After 2 h at room temperature, the plate was washed and incubated with biotinylated antimouse IgE antibody and avidin horseradish peroxidase reagent for 1 h at room temperature. The plate was read at 450 nm (Biotek, Power Wave XS2).

2.8. Specific IgE, IgG1 and IgG2a level estimation

Specific IgE, IgG1 and IgG2a levels were estimated with the earlier described methods with slight modifications (Misra et al., 2010; Voller, Bidwel, & Barlett, 1980). Briefly, microtiter plates (Maxisorp; NuncTM Immunomodule, Roskilde, Denmark) were coated with 1 µg protein in 100 µL per well in carbonate buffer (pH 9.6). Non specific sites were blocked with 3% BSA, washed and incubated with the sera of the RKB treated mice (1:10 v/v). The sera from PBS treated mice were treated as control. After washing, the plates were incubated for 2 h with anti-mice IgE-HRP (1:1000 v/v; Sigma Chemical Co., St Louis, MO, USA) and the absorbance was read at 492 nm (Biotek, Power Wave XS2).

Measurement of specific IgG1 and IgG2a in sera samples was also performed by ELISA. In brief, 100 µL of RKB crude protein extracts (5 µg/mL) was coated onto 96-well micro plates and kept overnight at 4 °C and then blocked with 200 µL of 3% BSA. Diluted serum samples (1:1000 dilutions for specific IgG1 levels and 1:500 for specific IgG2a levels) were added to each well and incubated. The plates were incubated for 2 h at 37 °C and then washed 3 times with washing buffer (PBS-T). To each well, 100 µL of HRP conjugated goat anti-mouse IgG1 and IgG2a antibodies (1:1000; Southern Biotech, Birmingham, USA) were added and incubated for 1 h at 37 °C. The plates were washed with washing buffer. A 50 µL substrate solution (5 mg ortho-phenylenediamine in 10 mL substrate buffer and 10 µL H₂O₂) was added to each well and the plate was incubated for 30 min at 37 °C in the dark. The reaction was stopped by the addition of 50 µL of stopping solution (5N H₂SO₄) and the absorbance was taken at 492 nm in ELISA plate reader (Biotek, Power Wave XS2).

2.9. Splenocyte culture and cytokine levels

Single spleen cell suspensions were prepared aseptically in RPMI-1640 (Sigma, St. Louis, Mo) according to earlier described method with slight modifications (O'Donnell & Openshaw, 1998). In brief, spleen cell suspensions were prepared immediately by cutting, mincing, and filtering through 70 µm nylon meshes. Cells were resuspended in RPMI medium, supplemented with 10% fetal calf serum, 1% L glutamine and 1% penicillin/streptomycin. Cells were plated (4 × 10⁶ cells/mL) in sterile round-bottom 24-well tissue culture plates (Costar, NY, USA). For stimulation, spleen cells from control mice were used as naïve and treated with 75 µg RKB crude protein extracts. RKB treated mice splenocytes were divided in two groups; the first one was treated with PBS while the second was treated with 75 µg RKB extracts. The cells were cultured for 72 h at 37 °C and 5% CO₂. Supernatants were harvested and stored at −80 °C until further use for cytokine measurements.

Cytokine levels (IL-4, IL-6, IL-10, IL-17, IFN-γ and TNF-α) were estimated using CBA cytokines kit (BD Biosciences) and analyzed by flow cytometry (BD FACS Canto) as per manufacturers' instructions. Standard cytokines were run together with the samples to quantify the levels of the individual cytokines present.

2.10. Mouse chemotactic protein-1 (MCP-1 or CCL-2) and Mouse Mast Cell Protease-1 (mMCPT-1) level

Serum mouse chemotactic protein-1 (MCP-1 or CCL-2) was quantified on day 15th, 43rd and 59th by ELISA using BD Optia kit (BD Biosciences, Germany) according to manufacturers' instructions. Serum mMCPT-1 levels were also quantified using ELISA (Cat No. 887503, eBioscience, Inc. CA, USA) according to the manufacturers' instructions. In brief, coating antibody was incubated overnight and FBS (Fetal Bovine Serum) was used for blocking, followed by incubation of 100 µL mice sera and standards followed by addition of detection antibody and HRP. Absorbance was taken at 450 and 570 nm (Biotek, Power Wave XS2).

2.11. Histamine level, systemic anaphylaxis score and rectal temperature

Plasma, lung, spleen and jejunum histamine levels were determined 20 min after challenge using ELISA (SPI-BIO, Montigny le Bretonneux, France) following manufacturers' instructions. Anaphylactic reactions were scored using as earlier described scoring system (Li, Schofield, Huang, Kleiner, & Sampson, 1999) 0 – no symptoms; 1 – scratching and rubbing around the snout and head; 2 – puffiness around the eyes and

snout, pilar erection, diarrhea, and reduced activity or standing still with an increased respiratory rate; 3 – wheezing, labored respiration, and cyanosis around the mouth; 4 – symptoms as in no-3 with loss of consciousness, tremors, and/or convulsion; 5 – death.

Rectal temperature was monitored once a week during treatment and 20 min after challenge using digital rectal thermometer (Bioseb, France).

2.12. Immunohistochemical analysis of eosinophil levels in jejunum and lung

Detection of eosinophils in jejunum and lung was performed using immunohistochemical methods as described earlier (Li, Sun, Satoh, Fisher, & Spry, 1995). In brief, rabbit anti mouse eosinophil major basic protein (EMBP (S-20): sc-18241, Santa Cruz biotechnology, Inc.) was used as primary antibody and anti rabbit serum was used as secondary antibody. DAB (3, 3' Diamino Benzidine Tetrahydrochloride) staining followed by counter stain with hematoxylin was carried out and the picture was taken using Nikon Eclipse TE2000-S microscope.

2.13. Histopathology

The mice were challenged and later sacrificed by cervical dislocation and spleen, lung and jejunum were taken for histopathology. Tissues were fixed in 10% formalin in PBS, embedded in paraffin, and cut into 3–5-µm thick sections.

2.14. ELISA inhibition test for cross reactivity with other legumes

The cross reactivity of red kidney bean extracts with other legumes like peanut, soybeans, chickpea and black gram was determined in vitro by ELISA inhibition following the earlier described method with slight modifications (Kumari et al., 2006). Sera of mice preincubated with 0.1 ng–100 µg of other legumes such as peanut, soybean, chickpea and black gram as inhibitors. The mixture was then added to the solid phase raw RKB extract in the ELISA plate (Nunc, Denmark). RKB antigen incubated with pooled mice sera without inhibitors was taken as positive control. The rest of the procedure was similar to that of ELISA. The inhibition percent of the specific IgE was expressed as

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

2.15. Allergic patients, SPT and blood collection

Allergic patients were selected on the basis of clinical history which was further confirmed by skin prick test (SPT), total IgE and specific IgE levels. The symptoms were from the description of the subjects and perhaps from multiple experiences of consuming a food. It cannot be said that the symptoms were clearly associated with RKB consumption as patients were not challenged with RKB alone. However, SPT showed 2+ to 3+ score and their ELISA and immunoblot demonstrated specific IgE binding as well. SPT scores were mostly positive for RKB. The age of the patients included in the study ranged from 16 to 75 years. SPT was performed with pollens, insects, danders, dust, fungi and food extracts on 200 (120 males and 80 females) bronchial asthma and allergic rhinitis patients at the outpatient department (OPD) of the Department of Pulmonary Medicine, Chhatrapati Shahuji Maharaj Medical University (CSMMU), Lucknow (Fig. 2). Glycerinated buffer saline and glycerol histamine acid phosphate (1:100) were used as negative and positive control respectively. Comparison was performed with histamine wheel, and scoring was done as: smaller wheel then +1, equal +2, larger +3 and for bigger with pseudopodia then +4. The results were read 20 min after allergens prick. Blood was collected from RKB SPT positive (RKB SPT +Ve)

patients (n=17) and normal healthy volunteers (n=10). Patients showing no wheels against RKB were designated as RKB SPT negative (RKB SPT –Ve) patients (n=183) and they were not included in this study. Serum was separated from whole blood (Fergusson, 1996) and stored at –80 °C.

2.16. Total and specific IgE in allergic patients

Serum total IgE was determined using ELISA (Immunology Consultants Laboratory, Inc, Newberg, OR) according to the manufacturers' instructions and specific IgE level was determined as described earlier (Misra et al., 2010). Results for both serum total IgE and specific IgE measurements were expressed in kilounits per liter and equilibrated against the World Health Organization (WHO) International Reference Preparation for Human IgE, standard for 1 kU for total IgE and 1 kAU for specific IgE were equal to 2.4 ng/mL, respectively.

2.17. Cross reactivity with other legumes in allergic patients

ELISA inhibition was performed to determine the red kidney bean cross-allergenicity with other legumes like peanut, soybeans, chickpea and black gram as described in mice (Section 2.14) with slight modifications. Sera of RKB SPT positive patients preincubated with 0.1 ng–100 µg of other legumes such as peanut, soybean, chickpea and black gram were used as inhibitor. Healthy human patient's sera were used as control. The mixture was then added to the solid phase raw RKB extract in the ELISA plate (Nunc, Denmark). RKB antigen incubated with RKB SPT positive patient's pooled sera without inhibitors was taken as positive control and anti-human IgE (Sigma Chemical Company, St. Louis, USA) was use as secondary antibody. The rest of the procedure was similar to that of ELISA. The percentage inhibition was calculated according to the formula given in Section 2.14.

2.18. Statistical analysis

The statistical significance of the data obtained was determined using a software package from InStat version 3.0 (Graph pad, San Diego, CA, USA; <http://www.graphpad.com>) using the Benferroni analysis of variance (ANOVAs) test. Values for all measurements are expressed as mean ± SEM. Differences between groups were considered significant at p<0.05.

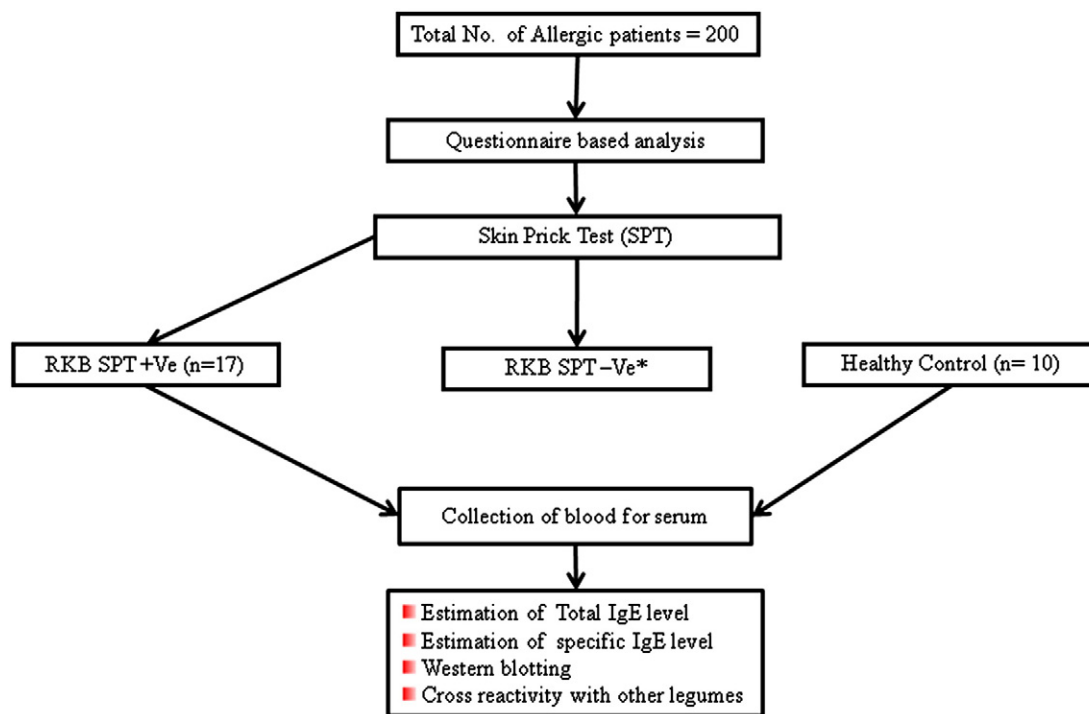
2.19. Ethical permissions

SPT and sera collection were carried-out with patient's consent and the study protocol was approved by the human ethics committee of the CSMMU, Lucknow. Animal study was performed after approval of animal ethics committee of CSIR-IITR, Lucknow.

3. Results and discussion

Different legumes are used for supplementing the protein requirement of vegetarians in the Indian subcontinent. Studies on peanut and soybean are well established but many other legumes like RKB is not well studied.

Five protein components of molecular weight approximately 170, 100, 43, 34 and 20 kDa were found to be pepsin resistant. Densitometry analysis revealed that percentage of 170 kDa protein components that remained undigested was 95, 88, 87, 85, 68 and 61% at time period 0, 0.25, 0.5, 1, 2, 15 and 60 min in SGF respectively. Similarly, 100 kDa protein components remaining undigested after SGF digestion was 87, 72, 63, 63, 62, 54, and 17% at time period mentioned above. Also, 43 kDa protein component the undigested protein was 99, 96, 93, 92, 86, 85 and 68%. Densitometry analysis of 34 kDa protein component after SGF digestion showed 97, 97, 92, 90, 86, 54 and 50%. Analysis of 20 kDa protein component density of



*RKB SPT -Ve patients were not included in this study.

Fig. 2. Allergenicity studies in human subjects and their serum.

remaining protein was 98, 93, 87, 83, 77, 53 and 19% (Fig. 3A). We compared the digestibility of food allergens in SGF with taking bovine serum albumin as an easily digestible protein (Fig. 3B) and densitometry analysis showed 96, 0, 0 and 0% density at time period 0, 0.25, 0.5, 1, 2, 15 and 60 min in SGF respectively. β -Lactoglobulin B was taken as an example of nondigestible protein (Fig. 3C) and its densitometry analysis showed 91, 89.5, 89 and 78% density at 0, 5, 15 and 60 min in SGF respectively as shown in Fig. 3b–c. On the basis of densitometry analysis, the order of pepsin resistibility of different protein components was observed to be as follows $43 > 170 > 34 > 20 > 100$ kDa.

Resistance to enzymatic digestion with pepsin is a feature common to many, but not all, allergenic food proteins. Guidelines for pepsin digestibility have been prescribed by FAO/WHO (2001, 2003) and others to assess the allergenicity (Astwood et al., 1996; Fu et al., 2002; Herman et al., 2005). In this study pepsin digestion was performed in a standard simulated gastric fluid assay. The stability of proteins in the SGF assay may be important for both sensitization of the immune system after reaching mucosa and for the elicitation of gastrointestinal symptoms of food allergy. Proteins with more than 10% stainable full-length protein band remaining at >30 to 60 min are considered stable. Proteins reduced to <10% stainable band at 5 to 30 min are considered of intermediate stability. Proteins reduced to <10% stainable band by 2 min are considered labile (DBT, 2008). The densitometry analysis clearly revealed that all the five proteins found resistant in SGF assay viz. 170, 100, 43, 34 and 20 kDa, were having more than 16% density even after 1 h indicating that all the five allergenic protein components were found pepsin resistant and as per DBT guideline can be considered stable.

Immunoblotting with pooled sera of mice sensitized with RKB (Fig. 3D) and hypersensitive patients (Fig. 3E) showed five IgE reactive protein components of molecular weight approximately 170, 100, 43, 34 and 20 kDa. In our study SGF stable and IgE binding protein of RKB were found to have similar molecular weights and may probably be the same proteins. The strip incubated with normal human and control mice sera did not show any band.

Murine models of sensitization against food allergens provide an optimal tool for allergic risk assessment and studying the mechanism of food allergen sensitization including allergies and asthma (Bashir et al., 2002; Bashir et al., 2004; Helm, 2002).

Total and specific IgE production was significantly increased ($p < 0.05$) in the RKB treated mice when compared to control mice (Fig. 4A–B). A significant increase in specific IgG1 ($p < 0.05$) while moderately significant enhancement in specific IgG2a (Fig. 4C–D) was observed in RKB treated mice sera as compared to vehicle treated controls. The total IgE level in food allergy is generally enhanced while increased specific IgE level demonstrates specific allergen participation. Moreover, there was significant enhancement in the levels of IgG1 as compared to IgG2a. The allergic reactions favor a marked shift in the balance of IgG1 and IgG2a and it has been reported that enhanced level of IgG1 supports Th2 reactions (Katayama & Mine, 2006).

RKB stimulated splenocytes secreted significantly enhanced levels of IL-4, IL-6, IL-10, IL-17, IFN- γ and TNF- α ($p < 0.05$) (Fig. 5A–F). B cells proliferate and differentiate into immunoglobulin-secreting plasma cells under the influence of a combination of cytokines derived from T cells. In particular, a subset of T helper lymphocytes (Th2 cells) produces a variety of Th2 cytokines, including IL-3, IL-4, IL-5, IL-6, IL-10 and IL-13 (Ozdemir et al., 2010). In our study, substantially higher amounts of Th2 cytokines IL-4, IL-6, IL-10 and IL-17 were found, which may trigger and aggravate the allergic and inflammatory responses. Antigen presentation in a Th2 dominated microenvironment leads to the formation of IgE antibodies and enhances the migration of eosinophils and mast cells into regions of inflammation. Higher levels of IFN- γ and IFN- α activate mast cells (Metcalfe et al., 1997) and their activation leads to higher IgE and enhanced release of different mediators responsible for allergic reactions. Higher level of CCL-2, a chemokine was also observed in RKB treated mice (Fig. 6A) when compared to control (p value < 0.05). Regulation of leukocyte migration and activation by chemokines are recognized as potentially important functions in the induction of acute and chronic inflammatory reactions. The Th2 cytokines and other

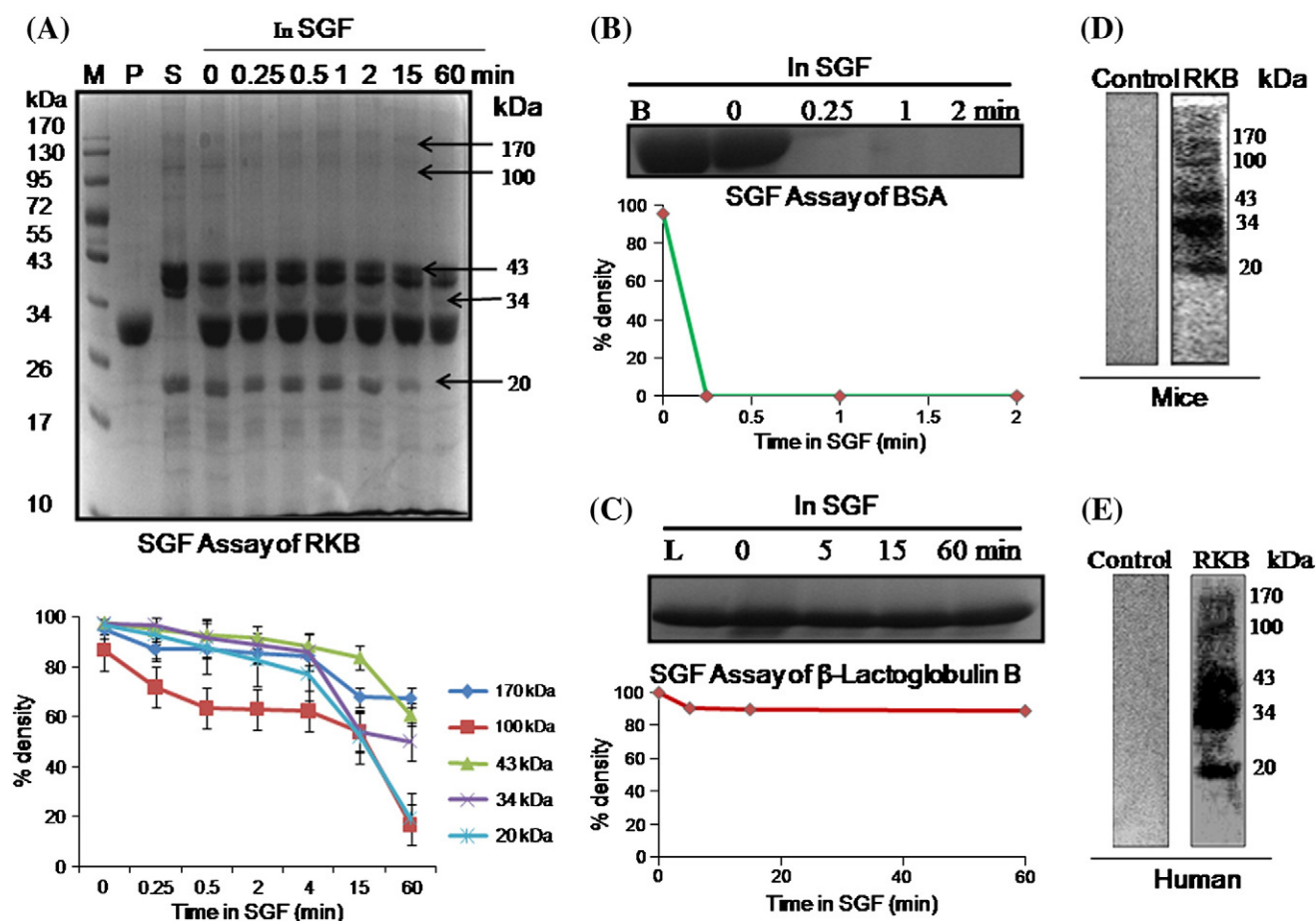


Fig. 3. Pepsin digestibility assays and IgE immunoblot of RKB CPE (A) Pepsin digestibility assay and its densitometry (pH 2.0). Where M = Marker; P = Pepsin; S = RKB proteins; PS = RKB proteins in SGF (at 0 min). 0.25 min, 0.5 min, 1 min, 2 min, 15 min and 60 min are indicating incubation period of RKB proteins in SGF (B) Pepsin digestibility assay and densitometry of BSA as an easily digestible protein and (C) Pepsin digestibility assay and densitometry of β Lactoglobulin B as non digestible protein. IgE immunoblot of were performed using sera of (D) Control and RKB treated mice and (E) healthy control and RKB sensitive humans.

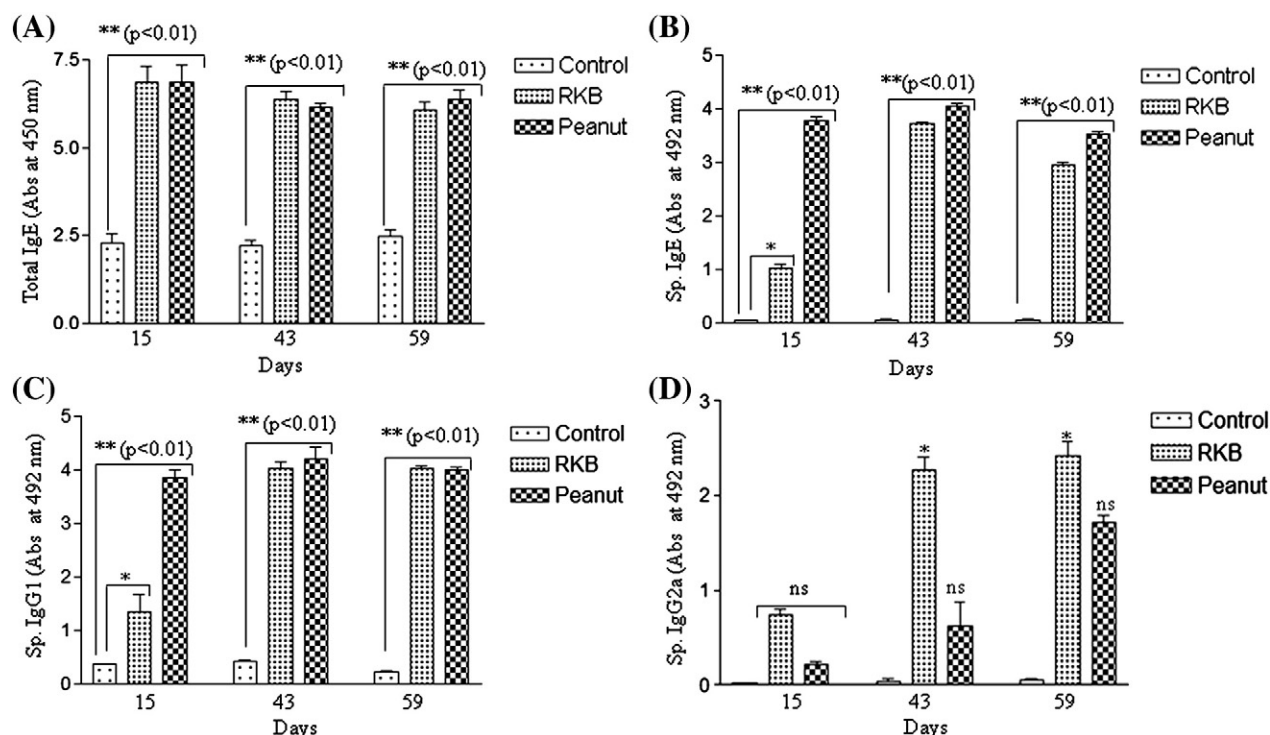


Fig. 4. (A) Total serum IgE (B) specific IgE (C) specific IgG1 and (D) specific IgG2a level on day 15th, 43rd and 59th, in control, RKB and peanut treated mice. Data represents in mean $\pm$ SE; n = 3; (*p < 0.05, **p < 0.01) when compared with control.

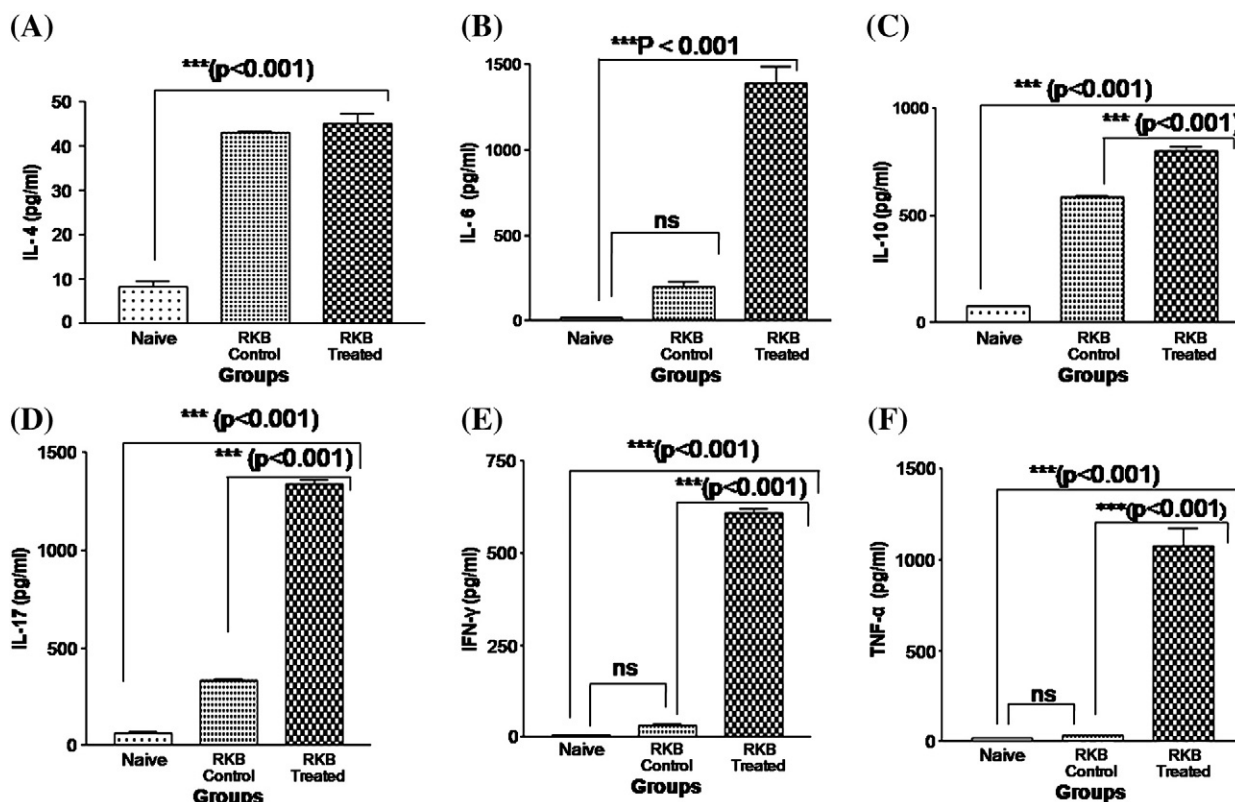


Fig. 5. Cytokine levels in splenocytes culture supernatants using Flowcytometry. Supernatants were collected at 72 hrs and levels of different cytokines were estimated. (A) IL-4, (B) IL-6, (C) IL-10, (D) IL-17A (E) IFN and (F) TNF levels. Results are expressed as mean $\pm$ SEM of from three separate experiments (***) ($p < 0.001$) when compared with control.

chemokines like CCL-2 are crucially involved in the local infiltration of eosinophils (Soehnlein et al., 2008). Immunohistochemical studies also show that there was marked increase in eosinophils of lung and jejunum of RKB treated mice in comparison to control mice (Fig. 9A–C).

There was a significant increase in the levels of mMCP-1 in the serum of RKB treated animals on day 15th, 43rd and 59th as compared to control animals. The level was highest on the 43rd day with a mean value of 43 ng/mL, representing a 2-fold increase over control values ($p < 0.05$) (Fig. 6B). Mouse Mast Cell Protease-I (MCPT-1, mMCP-I) is a β -chymase, a type of serine protease stored and secreted in a tissue-specific manner by mucosal mast cells. MCPT-1 is the only chymase expressed by intestinal mucosal mast cells, which are found in the intestinal epithelium. Although it is expressed constitutively and is detectable in the sera of normal mice an elevated MCPT-1 level

indicates intestinal allergic hypersensitivity reactions. Serum MCPT-1, apart from being a marker of mucosal mastocytosis and increased gut permeability is a new systemic serum marker of intestinal mast cell activation in murine food allergy models (Caughey, 2007; Knight et al., 2000).

Mice in the RKB-treated and challenged group exhibited symptoms of labored respiration (score 3, 40%); and near fatal reactions such as loss of consciousness or little activity despite gentle prodding (score 4, 40%) and death (Score 4, 10%). The median score was 3.5 (range: 3–5). Naive mice showed no reaction to challenge (Fig. 7A).

Rectal temperatures were obtained 20 min after the RKB challenge to measure the severity of systemic anaphylaxis. Temperatures in the naive group ranged between 37 °C and 38 °C after RKB challenge, whereas rectal temperatures in 8 out of 10 mice in the RKB treated

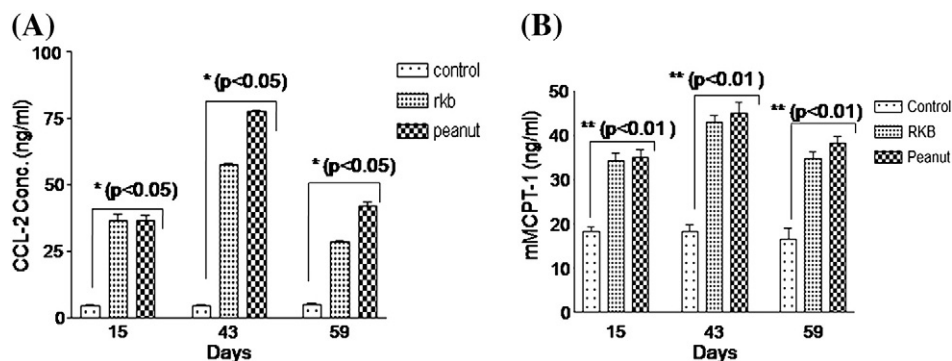


Fig. 6. CCL-2 level and mMCP-1 levels determination in mice serum using ELISA. (A) CCL-2 level was estimated in serum at day 15th, 43rd and 59th and (B) mMCP-1 levels on day 15th, 43rd and 59th. Data are presented in mean $\pm$ SEM. * ($p < 0.05$) and ** ($p < 0.01$).

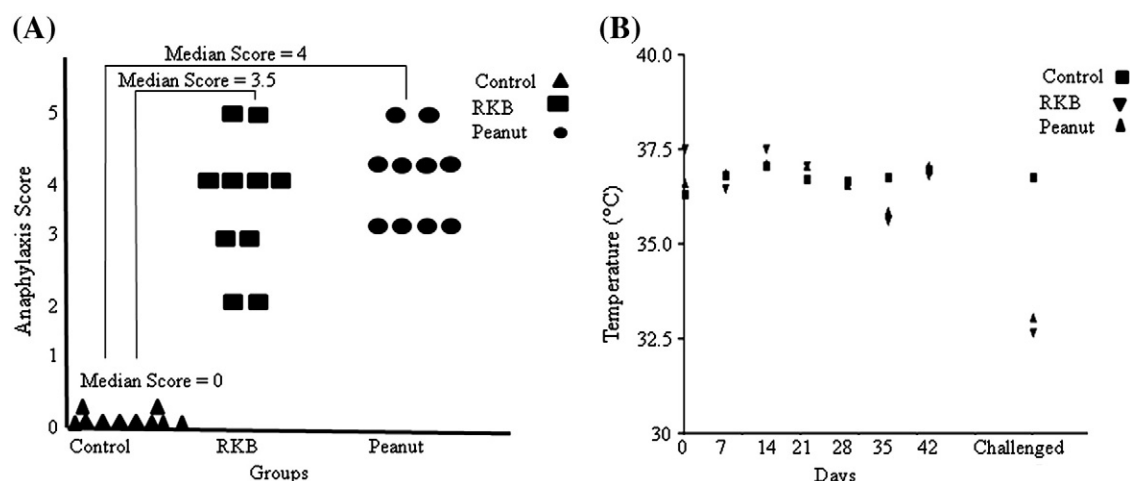


Fig. 7. (A) Anaphylactic symptoms scored after 20 min following challenge, presented as median value for control (0), RKB treated (3.5) and peanut (4) treated mice respectively (B) Rectal temperature was measured on day 0th, 7th, 14th, 21st, 28th, 35th, 42nd and 20 min after challenge, 2–4 °C decrease was found in RKB as well as in peanut.

group were 2° to 4 °C below normal range of the control group (Fig. 7B). Decreased body temperatures reflect the severity of systemic anaphylaxis in mice (Sato et al., 2010).

Most allergies involve the release of histamine and other pro-inflammatory substances. A ten fold increase in the histamine level was observed in plasma of RKB treated mice, whereas it's level was 4 fold in intestine. Lung and spleen of RKB treated mice also demonstrated significant histamine release as compared to control ($p < 0.05$) as shown (Fig. 8A–D). Histamine is an important mediator involved in allergic reactions (Ivan et al., 2009).

In RKB treated mice, mixed inflammatory cells infiltration throughout the lung parenchyma, edema to some extent and moderate congestion were also observed. Prominent exfoliations of mucosa in lumen, infiltration of inflammatory cells, and denudation of mucosa were observed in jejunum. The spleen was observed to have severe lymphoid hyperplasia (Fig. 10B). In peanut treated group lung showed moderate congestion, at some places edema and moderate infiltration of mixed inflammatory cells were observed. The spleen exhibited megakaryocytic hyperplasia, intestine showed hyperplasia of enterocytes, exfoliation of mucosa in lumen (Fig. 10C). The control group showed normal histology

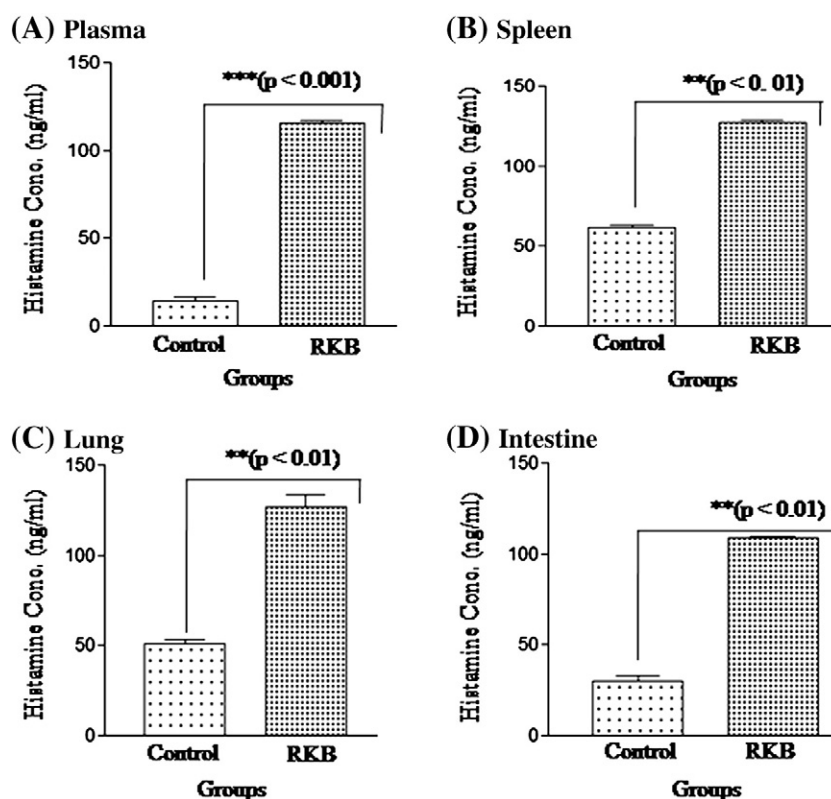


Fig. 8. Histamine levels were estimated in (A) plasma (B) spleen (C) lung and (D) intestine. Plasma and organs were obtained 20 min after RKB challenge. Histamine levels were determined using histamine release assay. Data are presented as means $\pm$ SEM (** $p < 0.01$, *** $p < 0.001$) versus control mice.

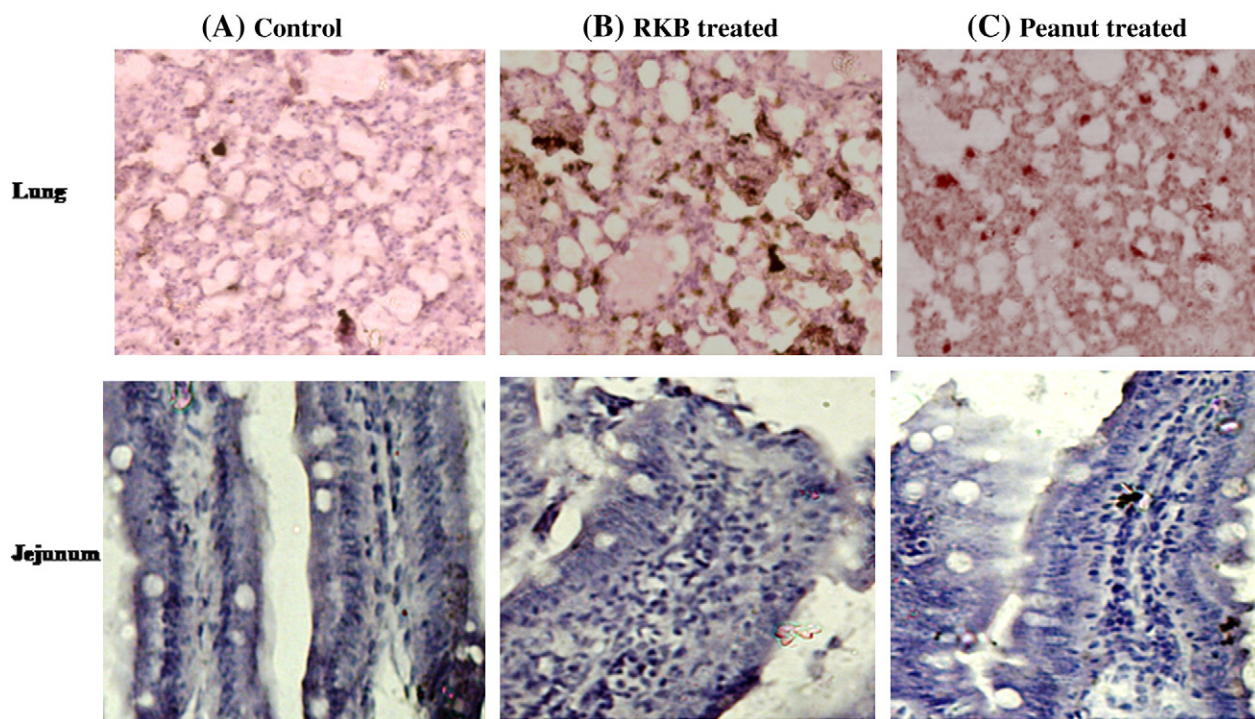


Fig. 9. Detection of eosinophils by immunohistochemistry in (C) control (D) RKB treated and (E) peanut treated lung and jejunum.

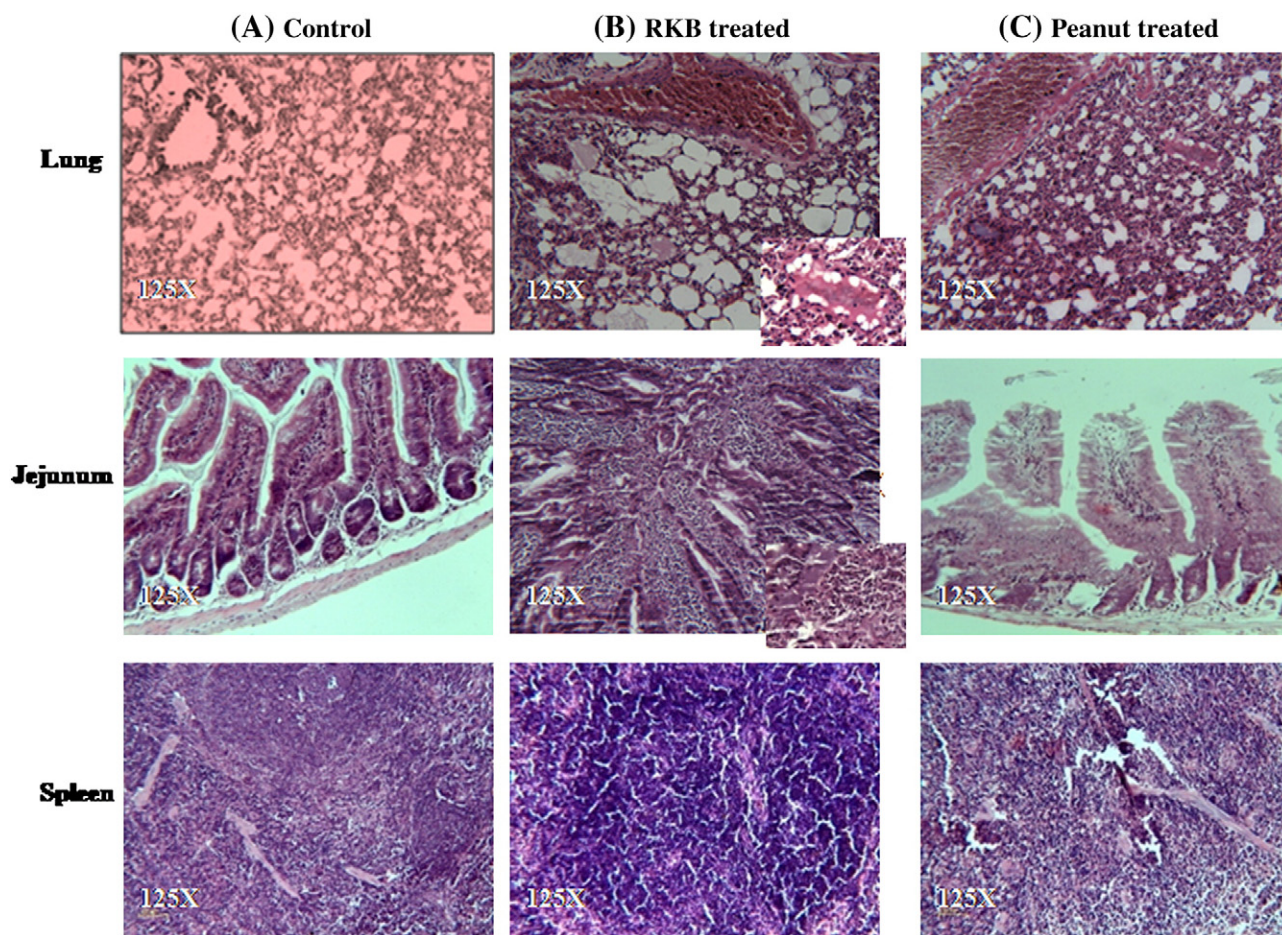


Fig. 10. Histopathology of lung, jejunum and spleen. Tissues were collected 20 min after challenge (A) control group lung, jejunum and spleen (B) RKB treated mice, lung, jejunum and spleen (C) peanut treated group lung, jejunum and spleen.

of intestine and spleen (Fig. 10A). Histopathological studies of RKB treated mice lung, intestine and spleen explore the allergenic reactions as described in red gram (Misra et al., 2010).

A significant increase in histamine in the plasma, spleen, lung and intestine, prominent systemic score, decreased rectal temperatures in 80% challenged animals and eruptive changes in histopathology indicate that RKB has the potential to induce anaphylaxis.

Skin tests were performed in 200 patients with allergy symptoms and from these, 150 patients (75%) showed sensitivity to various common allergens such as pollens, fungi, house dust and mites. SPT, total IgE, specific IgE and symptoms in patients and normal healthy controls are presented in Table 1. In RKB sensitized individuals total IgE level ranged between 196 and 663 kU/mL while in control it ranged between 39.5 and 232 kU/L. Specific IgE level in RKB sensitized individuals ranged between 1.74 and 11.9 kAU/L, while in normal healthy individuals it was in the range of 0.27–0.55 kAU/L.

Over 20% of the world population suffers from immunoglobulin E (IgE) mediated allergic diseases such as asthma, food allergy, rhino conjunctivitis, eczema and anaphylaxis. In India alone, approximately 20% of the population suffers from allergic rhinitis and 15% from bronchial asthma. The prevalence of nasobronchial allergy has increased in the last two to three decades possibly due to change in indoor and outdoor environment. Besides a genetic predisposition, environmental and other surrounding factors may contribute to the development of the allergic phenotype. Even local and systemic inflammatory processes also seem to be involved (Braunstahl & Hellings, 2003). Allergens are one out of the many factors which can cause and trigger nasobronchial allergy. Skin prick may be very useful

to identify the offending allergen but, it is not fully reliable (Prasad et al., 2009). It has been documented for food allergy cases that serum IgE measurement gave more positive results than did skin prick testing (Elizabeth et al., 2010). In our study, 17 (8.5%) allergic patients, showed positive SPT and enhanced specific IgE level to RKB which show involvement of RKB in allergy. The serum total and specific IgE levels against RKB were found to be elevated significantly in these patients when compared with normal healthy persons. These RKB sensitized individuals were suffering from allergic rhinitis, bronchial asthma, dermatitis and urticaria. Interestingly, RKB was found to be cross-reactive to peanut, soybean, chickpea and bengal gram. The serum samples preincubated with peanut, chickpea, soybean and black gram exhibited a significantly reduced antibody binding to RKB extracts in a dose dependent manner as compared to control serum samples preincubated with PBS alone. ELISA inhibition test with peanut has showed inhibition up to 57% in mice and up to 74% in humans at 100 µg/mL. Chickpea showed inhibition up to 64% in mice and up to 74% in humans at 100 µg/mL. Soybean showed inhibition up to 60% in mice and up to 55% in humans at 100 µg/mL. Black gram showed inhibition up to 54% in mice and up to 71% in human at 100 µg/mL (Fig. 11A–B). Therefore, RKB may induce allergic symptoms in individuals who may be allergic to any of the above legumes.

We observed IgE immunoblotting and cross reactivity results with human sera which were similar to those obtained with RKB sensitized mice sera, demonstrating the usefulness of BALB/c mice model replicating humans at least for specific IgE responses. Although, murine models are not exactly identical to humans' but it holds good promise for screening and risk assessment.

4. Conclusions

The allergenic potential of RKB was evident by significant increase in specific IgE, IgG1, histamine, mast cell protease-1 and Th2 cytokines levels, enhanced level of eosinophils in jejunum, prominent anaphylactic symptoms and eruptive histopathological changes. Five protein components with molecular weights of approximately 170, 100, 43, 34 and 20 kDa were found to be pepsin resistant and IgE reactive. RKB proteins showed cross reactivity with peanut, soybean, chickpea and black gram. Finally, this work demonstrated that red kidney beans may induce allergic response in mice that could be probably similar in human subjects, with identification of five clinically relevant allergenic protein components.

Overall, this study demonstrated that RKB proteins may be a contributing factor in development of allergy in susceptible individuals and further studies are required to thoroughly characterize the individual proteins and understand the underlying mechanisms of allergy.

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The authors have declared that there is no conflict of interest.

Table 1
Total and specific IgE levels in RKB sensitive patients.

S.N.	Age ^a /Sex (M ^b , F ^c)	SPT ^h (wheel)	Total IgE (kU/L)	Specific IgE (kAU/L)	Allergic symptoms
Control-1	30/F	–Ve	96 ± 6.5	0.47 ± 0.15	None
Control-2	42/F	–Ve	77 ± 1.7	0.32 ± 0.05	None
Control-3	20/M	–Ve	59.5 ± 4.0	0.27 ± 0.16	None
Control-4	34/M	–Ve	143 ± 0.7	0.35 ± 0.11	None
Control-5	45/F	–Ve	232 ± 35	0.547 ± 0.32	None
Control-6	25/M	–Ve	60 ± 21	0.23 ± 0.14	None
Control-7	39/M	–Ve	123 ± 18	0.14 ± 0.22	None
Control-8	49/M	–Ve	78.5 ± 5	0.33 ± 0.18	None
Control-9	41/F	–Ve	191 ± 23	0.29 ± 0.13	None
Control-10	28/F	–Ve	55.5 ± 2	0.46 ± 0.39	None
Patient-1	23/M	+2	521 ± 17.8*	2.7 ± 0.26*	AR ^d
Patient-2	45/M	+3	651 ± 3.7*	7.7 ± 0.35*	AR, D ^e , U ^f
Patient-3	33/M	+3	647 ± 9.87*	6.5 ± 0.13*	BA ^g , AR
Patient-4	50/M	+2	572 ± 16.8*	11.2 ± 0.18*	AR, D, U
Patient-5	29/M	+2	306 ± 1.7*	2.75 ± 0.15*	AR, BA
Patient-6	25/F	+2	19 ± 4.5	1.74 ± 0.30	AR, U
Patient-7	18/M	+1	435 ± 27.1*	2.73 ± 0.23*	AR, BA
Patient-8	26/M	+3	471 ± 2.5*	11.5 ± 1.37*	AR, D, BA
Patient-9	54/M	+2	663 ± 19.2*	11.9 ± 1.33*	AR, U
Patient-10	23/M	+1	310 ± 58.3*	4.5 ± 0.15*	BA
Patient-11	66/M	+3	552 ± 43.5*	10.5 ± 0.5*	D, AR
Patient-12	51/F	+3	660 ± 13*	7 ± 0.35*	AR, BA
Patient-13	48/M	+3	427 ± 19.5*	8.5 ± 0.2*	U, BA
Patient-14	32/M	+2	508 ± 31*	3 ± 0.45*	U, D, AR
Patient-15	57/F	+3	278 ± 23*	6 ± 0.3*	AR, BA
Patient-16	21/M	+1	304 ± 41.5*	1 ± 0.6	BA
Patient-17	37/F	+2	467 ± 28*	11.5 ± 0.4*	AR, BA, D

^a Age in years.

^b M = male.

^c F = female.

^d AR = allergic rhinitis.

^e D = dermatitis.

^f U = urticaria.

^g BA = bronchial asthma.

^h SPT (Skin Prick Test) measured according to wheel size (in mm).

* (significant, $p < 0.05$). Total and specific IgE presented in mean ± SEM.

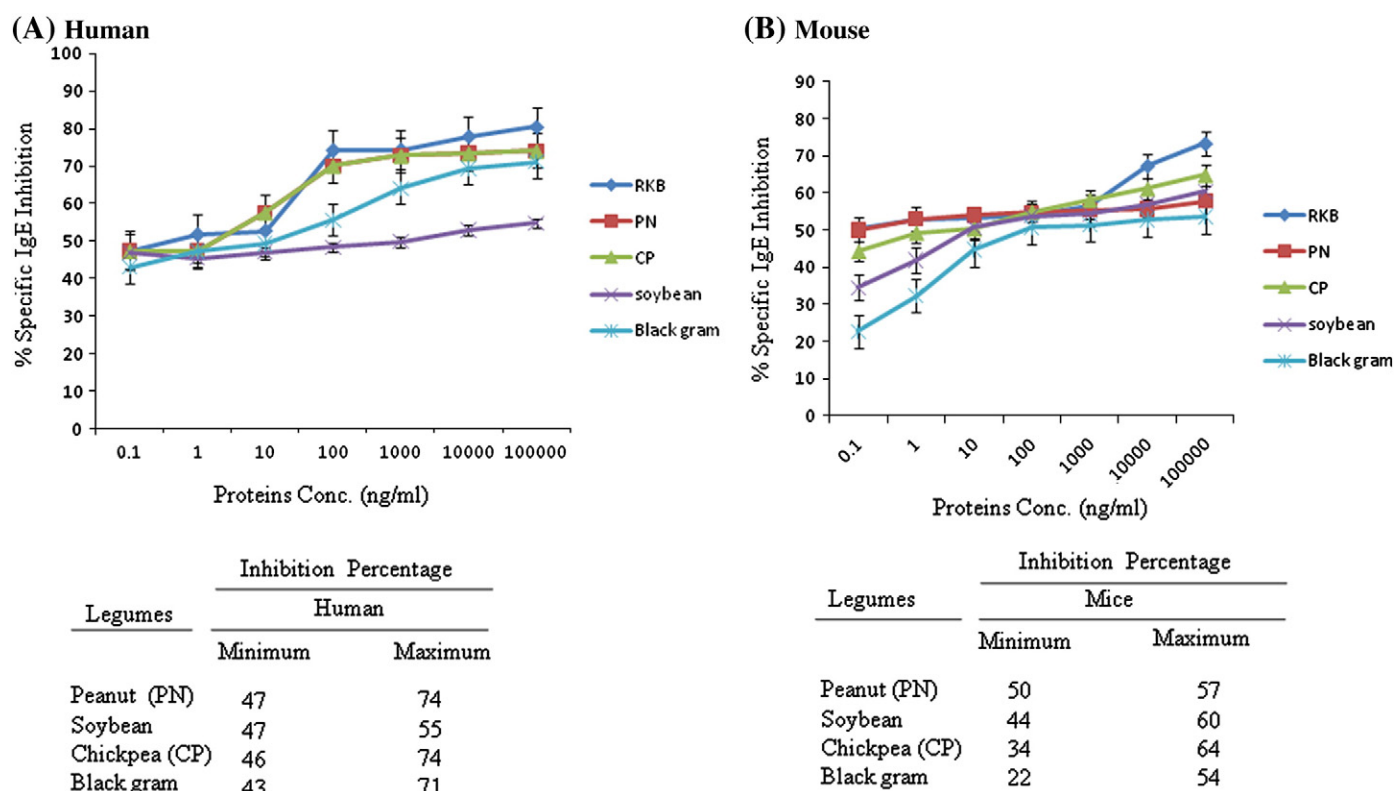


Fig. 11. Cross reactivity of RKB was observed using ELISA inhibition with peanut, chickpea, soybean and black gram in (A) human and (B) mice pooled sera.

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