Allergenic responses of red kidney bean
(Phaseolus vulgaris L.) crude protein
extract in BALB/c mice
1. Introduction

Most of the food allergic reactions are caused by IgE mediated immediate type hypersensitivity reaction, but non IgE mediated reactions have also been reported (Noh et al., 2012). Recently, mixed type of allergic reactions has been reported in several legumes including peanut and chickpea (van Wijk et al., 2004; Verma et al., 2012b). Th1/Th2 balance is a very crucial aspect for determination of fate of allergic reactions whether immediate, late or both. The transcription factors like signal transducer and activator of transcription 6 (STAT-6), trans-acting T-cell-specific transcription factor GATA-3, T-bet, nuclear factor of activated T cells (NFATc), transcription factor Maf or (c-maf), mast/stem cell growth factor receptor (SCFR) or proto-oncogene (c-Kit), and nuclear factor κB (NF-κB) which actively involved in Th1/Th2 reactions (Kumar et al., 2012b). Food allergic reactions are generally induced by an impaired oral tolerance and controlled by several immune molecules including Foxp3 and other immune molecules (Noh et al., 2010; Noh et al., 2012). But, there is rarely any study in this regard in the other legumes except, the peanut. Therefore, we have studied several immunological aspects behind the RKB induced allergic manifestations.

A large fraction of RKB seed protein is represented by its storage protein phaseolin and lectin-related protein family (Yin et al., 2011; Fitches et al., 2001; Yamaguchi, 1993). The lectin-related protein family consists of three major components arcelin, α-amylase inhibitor, and the true lectin, phytohemagglutinins (PHA, mainly PHA-P). Both PHA-P and α-amylase inhibitor are responsible for the lowering of the nutritional value of beans (Pusztai & Palmer, 1977). Several
studies regarding PHAs have been carried out to reveal blood grouping, erythrocyte polyagglutination activity, mitogenic stimulation of lymphocytes, lymphocyte subpopulation studies, fractionation of cells and other particles, histochemical studies of normal and pathological conditions (Pusztai & Palmer, 1977; Venter & Thiel, 1995). Initially, it was reported that low-dose intragastric administration of PHA does not induce IgE production in Sprague–Dawley rats (Haas et al., 2001). But, still the role of PHAs in RKB allergy has not been resolved, completely.

After establishing prevalence of RKB allergy and also finding pepsin resistant and IgE binding proteins in crude protein extract of RKB in chapter 2, it was an obvious step to study the mechanism of action in high IgE responder mice, BALB/c. The aim of this study was to explore the allergenicity of RKB proteins following oral exposure in BALB/c mice in the absence and presence of an adjuvant, splenocytes, bone marrow mast cells (BMMC), peritoneal cells derived mast cells (PCMC) and rat basophilic leukaemia (RBL-2H3) cells. This study was further extended to understand the role of PHA in RKB induced allergenicity.

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), 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.

2.2. Extraction of Red kidney bean crude protein extract

Red kidney bean crude protein extract (RKB-CPE) was prepared as earlier method described in chapter 2 (Astwood et al., 1996).

2.3. Animals

Healthy 6–8 week old female BALB/c mice (22±3 g) were obtained from the animal breeding colony of Gehru campus, CSIR-IITR, Lucknow, India. Animals were maintained under standard laboratory conditions in pathogen free environment and on red kidney bean free diet. Animal studies were carried out after approval of the animal ethics committee of CSIR-IITR, Lucknow.

2.4. Animal treatment

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=15/group). Groups of mice were gavaged daily with PBS (100 μL), RKB-CPE (100μg in 100μl PBS) and peanut-CPE (100μg in 100μl PBS),
respectively. Blood samples were withdrawn from the retro-orbital sinus to estimate serum allergic parameters on day 15, 43 and 59. On day 60, all groups of mice were challenged with 1 mL of 5 mg/mL RKB-CPE.

2.5. Serum immunoglobulins

Total IgE level in the sera of control and RKB-CPE treated mice were estimated by Optia mouse IgE kit (BD Biosciences, Germany) as per the manufacturer's instructions. Specific IgE levels were estimated with the earlier method described in chapter 2 with slight modifications (Misra et al., 2011). Specific IgE level was estimated by ELISA using method of Voller et al. (1980). Briefly, the wells of microtiter plate (Nunc, Denmark) were coated with 1 lg of protein in carbonate buffer (pH 9.6). After blocking, the plates were incubated with diluted sera (1:10 v/v) of RKB-CPE treated mice overnight at 4 °C. The plate was washed and incubated with HRP-conjugated goat-anti mouse IgE (1:1000 v/v, Sigma Chemical Co., St. Louis, USA) for 3 h at 37 °C. Colour was developed with orthophenylenediamine. The reaction was stopped after 20 min by adding 5 N H₂SO₄ and the absorbance was read at 492 nm. Specific IgE levels were measured in triplicate and average value is depicted. Measurement of specific IgG1 in sera samples was also performed by ELISA. In brief, 100 μL of RKB-CPE 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 was 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 antimouse IgG1 (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).

2.6. IgE immunoblotting

To detect IgE binding proteins in RKB-CPE, IgE immunoblotting was carried out using pooled sera of RKB-CPE sensitized mice sera according to the methods described in the chapter 2.

2.7. Anaphylactic symptoms and mediators release

The clinical symptoms induced after challenge in the control and RKB-CPE sensitized mice. Anaphylactic reactions were scored using as earlier described scoring system (Li et al., 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). Plasma histamine, serum mouse MCPT-1 and TSLP levels were determined 40 min after the challenge using ELISA kits (SPI-BIO, Montigny Le
Bretonneux, France and bioscience, Inc. CA, USA, respectively) following manufacturers' instructions.

2.8. Vascular permeability assay

Vascular permeability or vascular leakage was studied 40 min after the challenge as per the earlier described method (Sun et al., 2007). In brief, 3 ml of PBS, 10 mM EDTA injected into the peritoneal cavity of control as well as RKB-CPE treated mice. Following, a 1-min abdominal massage, peritoneal lavage fluid was aspirated gently. Further, peritoneal lavage fluids were centrifuged at 600 rpm for 6 min at 4°C and the supernatant was collected. The albumin content in the supernatants was estimated by BCA kit (Thermo Scientific, USA). Albumin levels of control mice challenged with the same amount of RKB-CPE was used as background levels. BSA was used as standard to calculate the concentrations of albumin.

2.9. Histopathology

The challenged and control mice were sacrificed by cervical dislocation after 40 min and intestine were taken for histopathology and fluorescence histochemistry. For histopathology the tissues were fixed in 10% formalin in PBS, embedded in paraffin, and cut into 3– 5-μm thick sections.

2.10. RBL-2H3 cells

Rat basophilic leukemia cells (RBL-2H3 cells) were obtained from American type cell culture (ATCC, Manassas, VA) and maintain as per manufacturer’s instruction.
2.11. Priming and Sensitization of RBL-2H3 cells

The priming of RBL-2H3 cells with the sera of RKB-CPE sensitized mice was carried out according to the earlier described method (Diesner et al., 2008). In brief, RBL-2H3 cells were seeded in a 24 well flat bottom plate and after 24 h incubation at 37°C and 5% CO₂ cells were incubated with either sera of control or red kidney bean sensitized mice (dilution 1:10) for 1 h at 37°C and 5% CO₂. Tyrode buffer (135mM NaCl, 5mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂.6H₂O, 5.6 mM Glucose, 20 mM HEPES buffer, 1% BSA, pH 7.4) was used to wash the cells. Furthermore, the cells were challenged with different concentrations (20, 40, 60, 80, 100 and 120 μg) of RKB-CPE up to 90 min. The control group was challenged either with PBS or lysis buffer (0.1% Triton-X 100). The plate was put on the ice to terminate the ongoing reactions. The supernatant were kept for the estimation of β-hexosaminidase release assays.

2.12. β-hexosaminidase release assay

The release of β-hexosaminidase was estimated according to the earlier described protocol (Blanc et al., 2009). In brief, 75 μl from aforesaid supernatants and substrate buffer for β-hexosaminidase (1.2 mm 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide in 0.05 M CH3COONa, pH 4.4) were mixed in a 96 well plate, and incubated at 37°C for 90 min. The quenching buffer (0.1 M Glycine, 0.1M Na₂CO₃, pH 10.0) was used to stop the reaction. The absorbance was taken at 405 nM (ELISA plate reader, Biotek, Power Wave XS2). The percentage of β-hexosaminidase release was calculated as per the formula given below:
2.13. Immunofluorescence for transcription factors

The expressions of Th1/Th2 transcription factors GATA-3, STAT-6, T-bet, c-MAF and NFATc1 were observed by immunofluorescence. In brief, RBL-2H3 cells (1X10^6 cells) were primed and challenged with a dose of 100 μg RKB-CPE as per described in above section. Immunofluorescence was carried out according to the protocol described online (Source: Immunofluorescence General Protocol). Formaldehyde was used to fix the cells while 5% goat serum was used for blocking the nonspecific sites. The antibodies for GATA-3, STAT-6, c-MAF, NFATc1 and T-bet were obtained from the BD Biosciences, San Jose, California and Santa Cruz biotechnology, inc. Santa Cruz, CA, respectively. These antibodies were used as primary antibodies (dilution 1:200) while Alexa Fluor® 488 Goat anti-Mouse IgG (Invitrogen, Carlsbad, CA, USA) was used as secondary antibody. Further, Prolong gold antifade reagent with 4’, 6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI; Invitrogen, Carlsbad, CA, USA) was used as mounting the mounting media. The Images were captured by confocal microscope (Leica Microsystems, Germany).


Animals were sensitized according to the earlier described protocol (Sun et al., 2007). Briefly, mice (n= 20/group) were randomly divided into control, RKB and
peanut groups and treated orally with 100 μl PBS, 1 mg of RKB-CPE and 1mg of peanut-CPE, respectively along with 10 μg of cholera toxin (Sigma-Aldrich, St Louis, MO) weekly up to 4 weeks. Two weeks after the last sensitization, mice (n=10/group) were challenged with 10 mg of RKB-CPE and peanut CPE, respectively. Anaphylactic reactions, rectal temperature, histamine, mMCP1 (SPI-bio, Massy Cedex, France and eBiosciences, San Diego, CA) and histopathology were carried out in control, peanut and RKB-CPE fed mice according the earlier described protocols as in the case of oral exposure without any adjuvant. In ELISA, 3,3′,5,5′-Tetramethylbenzidine or TMB (BD Biosciences, San Jose, USA) was used as substrate with a serum dilution 1:20 and reading was taken at 450 nm. The mast cell levels were studied in the lungs, intestinal mucosa and spleen using toluidine blue (Sigma-Aldrich, St Louis, Mo) staining.

2.15. Type 1 skin test

To evaluate the in vivo relevance of RKB CPE induced specific antibodies, type I skin tests were carried out according to the earlier method (Diesner et al., 2008) Compound 48/80 (Sigma-Aldrich, St Louis, Mo), which is known to induce mast cell degranulation was given as a positive control and the result was evaluated after 20 min.

2.16. Immunohistochemistry for eosinophil levels

Immunohistochemical detection of eosinophils in the organs were performed as previously described (Li et al., 1995). In brief, anti mouse eosinophils major basic protein (Santa Cruz biotechnology, Inc.) was used as primary antibody and anti
mouse HRP conjugated antibody was used as secondary antibody. DAB staining followed by counter stain with hematoxylin was done and the picture was taken using microscope (Nikon Eclipse TE2000-S).

2.17. GATA-3 and T-bet levels in the intestine

The levels of transcription factors GATA-3 and T-bet were observed in the intestinal sections of RKB challenged and control mice using immunofluorescence histochemistry (Kirwan et al., 2009). Briefly, goat antimouse GATA-3 (BD Biosciences, San Jose, California) and T-bet (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) with a dilution of 1:200, were used as primary antibodies, while Alexa Fluor® 488 goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) was used as secondary antibody (dilution of 1:1000). Slides were mounted by prolonged gold antifade reagent with 4’, 6-diamidino-2-phenylindole, dihydrochloride or DAPI (Invitrogen, Carlsbad, CA, USA) and images were captured by a confocal microscope (Leica microsystem, Germany).

2.18. Cytokine and chemokine levels

Single cell suspensions of the spleen from control and RKB challenged mice (n=5) were prepared aseptically using the earlier method (Misra et al., 2011). The splenocytes obtained from the spleen of RKB-CPE group was divided into two subgroups, RKB-U (challenged with PBS) and RKB-T group (challenged with 75 μg RKB-CPE), while the splenocytes obtained from the spleen of control group mice were treated with PBS. Cytokines were estimated in the splenocytes culture supernatant by CBA kits (BD Biosciences, San Jose, California) using flowcytometry (BD FACS Canto II).
2.19. Western blots for Th1/Th2 transcription factors

The expression of Th1/Th2 transcription factors GATA-3, STAT-6, T-bet and c-MAF in the splenocytes were detected by immunoblot (Towbin et al., 1979). In brief, proteins from RKB-U, RKB-T and control group were isolated using RIPA lysis buffer (1X RIPA buffer containing 10 µl PMSF, 10 µl Sodium Orthovanadate and 10-20 µl protease inhibitor cocktail solution per ml). Proteins from each group were resolved in 12% SDS-PAGE and electrophoretically transferred to PVDF membranes (Millipore, Billerica, Massachusetts) using a semi-dry blotting unit (Amersham Biosciences, St Francisco, USA). Blots were blocked with 3% BSA in PBS-T buffer (PBS containing 0.5% Tween-20, pH 7.4) and kept overnight at 4 °C. The goat antimouse GATA-3 and STAT-6 (BD Biosciences, San Jose, California), T-bet, c-MAF and β-tubulin (Santa Cruz biotechnology, inc. Santa Cruz, CA) were used as primary antibodies (1:200) while goat anti-mouse IgE peroxidase conjugated (Sigma Chemical Company, USA) with a dilution 1:1000 was used as secondary antibody. The β-tubulin was used as an experimental control.

2.20. Immunophenotyping for CD4+ and CD8+T and B-cells

The level of T-cells (CD4+ and CD8+) and B-cells population in the spleen was estimated using flowcytometry according to the previously described method (Yadav et al., 2012). Briefly, spleen from challenged RKB and control groups were taken and single cell suspensions were prepared. Following 24 h incubation, 1X106 cells from each group were suspended in staining buffer (2% FBS, 1% sodium azide in PBS) and stained with APC-Cy7-conjugated anti-CD3e antibodies, FITC-conjugated anti-CD4, APC-conjugated CD8 and Alexafluor 700-conjugated anti-CD19 (BD Biosciences, San Jose, California).
for 20 min on ice. The samples were analyzed within 1 h by flow cytometry (BD Biosciences, FACS Canto II).

2.21. Mediator release from BMMC, PCMC and RBL-2H3 cells

BMMC and PCMC cultures were carried out according to the previously described method (Noguchi et al., 2005; Yeatman et al., 2000). The β-hexosaminidase release was carried out in BMMC, PCMC and RBL-2H3 cells (ATCC, USA) according to the earlier described method (Diesner et al., 2008). Further, levels of histamine and CysL were estimated in RBL-2H3 cells by EIA kits (Cayman, Ann Arbor, Michigan; SPI-bio, Massy Cedex, France and eBiosciences, San Diego, CA, respectively).

2.22. Assay of hemagglutinating activity

Hemagglutinating activities of RKB, autoclaved RKB (RKB-A) and PHA-P (Sigma Aldrich, USA) were studied according to the earlier described protocol (Wang et al., 2000).

2.23. Role of PHA in RKB induced allergy

Female BALB/c mice were sensitized intraperitoneally according to the earlier described protocol (Misra et al., 2011). In brief, mice were randomly divided into four groups (n=15/group). A first group of mice (designated as Control group) were injected 100 µL PBS. A second group of mice (designated as PHA-P group) were treated with 100µg PHA-P in 100µl PBS. A third group of mice (designated as RKB-A group) were treated with 100µg RKB–A in 100µl PBS. A fourth group of mice (designated as RKB group) was treated with 100µg RKB in 100µl PBS. Mice from each group received aforesaid doses once a week up to 7 weeks. Blood
was withdrawn from the retro-orbital sinus on days 15, 43 and 59 and serum was collected. Mice (n=10/group) from each group were challenged intraperitoneally with 10 mg proteins of their respective group on day 60. Tissue samples from organs like lung, spleen and intestine were collected for histopathological studies. Specific IgE and IgG1 level estimation, systemic anaphylaxis score and core body temperature, mMCP -1 and TSLP levels and mediators release assays in RBL-2H3 cells were carried out as described in the earlier sections.

2.2.4. Statistical analyses

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 have been expressed as mean± SEM. Differences between groups were considered significant when p<0.05.

3. Results

3.1. Allergic symptoms following oral exposure of RKB-CPE in BALB/C mice

The level of total IgE was found significantly increased on 15 (p < 0.01), 43 (p < 0.001) and 59 days (p < 0.01) in the sera of RKB-CPE treated mice over control mice (Fig. 3.1A). Further, the levels of specific IgE were also found significantly enhanced in 15 (p < 0.05), 43 (p < 0.001) and 59 (p < 0.01) days in the sera of RKB-CPE treated mice over control (Fig. 3.1B). The level of IgG1 were significantly (p<0.001) increased in on day 15, 43 and 59 in the serum of RKB-CPE treated mice over control mice sera (Fig. 3.1C). The IgE binding potential of
RKB-CPE was also confirmed by the pooled sera of RKB treated mice which indicated five IgE binding proteins with approx molecular weight 170, 100, 43-50, 34 and 20-25 kDa (Fig. 3.1D).

The mice sensitized and challenged with RKB-CPE showed scratching around the nose and mouth (anaphylactic score 2; 30%), forced respiration (score 3, 30%); and 30% showed symptoms of score 4, like, severe diarrhea including unconsciousness or negligible response despite the gentle touch and score 5 in 10% (Fig. 3.2A). A decrease of 2–3 °C in the rectal temperature of red kidney bean sensitized and challenged mice was noticed after 40 min of challenge while control mice failed to show any decrease in the rectal temperature (Fig. 3.2B). The severity of anaphylaxis induced post red kidney proteins challenge was found to be augmented in form of plasma histamine (p<0.001) and serum mMCP-1 (p<0.001) in treated and challenged mice as compared to control as shown (Fig. 3.3A and 3.3B). An enhanced level (p<0.05) of the peritoneal albumin level was observed in the serum of RKB treated and challenged mice, as compared to control as shown (Fig. 3.3C). Further, significant (p<0.001) increase in the serum TSLP level was observed in RKB treated mice, as compared to that of the control as shown (Fig. 3.3D).

3.2. Abnormal histopathology

Histopathology of the lungs of RKB-CPE treated mice showed thickening in the alveolar septa and mucus infiltrations. Exfoliations in the intestinal walls and
Fig. 3.1. Total IgE, specific IgE and IgG1 levels and IgE-Immunoblot. (A) Total IgE (B) Specific IgE and (C) Specific IgG1 levels in the serum of control red kidney bean crude protein extract (RKB-CPE) and peanut crude protein extract (Peanut) treated mice groups on day 15, 43 and 59. (D) IgE-immunoblotting using pooled sera of Control and RKB-CPE treated mice. (Values for all measurements are expressed as means± SEM and **p<0.01; ***p<0.001).
Fig. 3.2: Anaphylactic symptoms in mice. (A) Following 43 days oral treatment mice were challenged on day 60 and systemic anaphylaxis score and (B) Core body or rectal temperatures were measured in control, red kidney bean crude protein extract (RKB-CPE), and peanut crude protein extract (Peanut) treated mice. (Values for all measurements are expressed as means SEM, *p<0.05).
Fig. 3.3. **Estimation of allergic mediators.** The levels of allergic mediators like (A) plasma Histamine, (B) Serum mouse mast cell protease-1 (mMCPT-1), (C) Peritoneal albumin and (D) Serum thymic stromal lymphopoietin (TSLP) were estimated in control, red kidney bean crude protein extract (RKB-CPE) and peanut crude protein extract (Peanut) treated mice. (Values for all measurements are expressed as means± SEM and *p<0.05; ***p<0.001).
Fig. 3.4. **Histopathological studies.** Mice were exposed orally either by phosphate buffer saline (PBS) of red kidney bean crude protein extract (RKB-CPE) up to 42 days and challenged on day 60. Following sacrifice, the lungs, intestine and spleen were collected from RKB-CPE treated and control mice. The inset images were taken at 500X resolution while the main images were at 125X.
leukocytes infiltrations were evident in the intestine of RKB-CPE treated mice. The spleen of RKB-CPE treated mice showed the presence of megakaryocytic structures. No such changes were evident in control groups (Fig. 3.4).

3.3. Enhanced mediator release and transcription factors in RBL-2H3 cells

A significant (p<0.05) release of β-hexosaminidase was observed following exposure of different doses of RKB-CPE in RBL-2H3 cells. The percentage release was 58.96, 64.22, 68.24, 72.16, 75.36, and 74.57% in 20, 40, 60, 80, 100 and 120 μg RKB-CPE (Fig. 3.5A). Further, the significantly enhanced mean intensity of GATA-3 (p<0.001), STAT-6 (p<0.001), c-MAF (p<0.01), T-bet (p<0.001) and NFATc1 (p<0.05) were found in RKB-CPE treated group when compared to that of control (Fig. 3.5B-F).

3.4. RKB-CPE along with adjuvant induces hypersensitivity manifestations in BALB/c mice

The allergenicity of RKB was evident by significantly enhanced levels of total IgE and specific IgE, prominent anaphylaxis symptoms, reduced core body temperature, higher plasma histamine and mMCP-1 levels (Fig. 3.6), eosinophil counts and MPO level (Fig. 3.7) in RKB-CPE treated mice over control. Increased mast cell counts were evident in the lungs; intestine and spleen of RKB challenged mice when compared to control (Fig. 3.8). RKB-CPE treated mice showed a positive reaction in the type 1 skin test in sensitized mice when compared to that of control (Fig. 3.9). Significantly enhanced mean intensity of
**Fig. 3.5. Mediator release and Th1/Th2 transcription factors in rat basophilic leukemia-2H3 (RBL-2H3) cells.** (A) Release of β-hexosaminidase from RBL-2H3 cells at 20, 40, 60, 80, 100 and 120 μg red kidney bean crude protein extract (RKB-CPE). (B) Expression of GATA-3 (C) STAT-6 (D) c-MAF (E) T-bet and (F) NFATc1 in control and RKB-CPE treated cells, respectively. Goat antimouse IgG Alexa fluor-488 antibody (AF-488) was used as secondary antibody while DAPI was used for nuclear staining. Merged image is indicating both AF-488 and DAPI staining. (Values for all measurements are expressed as means± SEM and *p<0.05; **p<0.01; ***p<0.001).
Fig. 3.6. Allergic reactions in following oral exposure of red kidney bean crude protein extract (RKB-CPE) along with cholera toxin sensitized BALB/c mice. (A) Serum total IgE (B) Specific IgE using sera of RKB-CPE treated and the control mice on day 15 and 30. (C) Systemic anaphylactic score or clinical symptoms and (D) Core body or rectal temperatures of RKB-CPE treated and the control mice (E) Serum mMCP-T-1 (F) Plasma histamine level of control and RKB treated mice. (Values for all measurements are expressed as means±SEM and *p<0.05; **p<0.01; ***p<0.001).
Fig. 3.7. Eosinophil levels. Immunohistochemical studies of eosinophils in the lungs, intestine and spleen of control and red kidney bean crude protein extract (RKB-CPE) treated mice. (Values for all measurements are expressed as means± SEM and ***p<0.001).
Fig. 3.8. *Mast cells level in the lungs, intestinal mucosa and spleen.* Toluidine blue staining of mast cells in the lungs, intestinal mucosa (IM) and spleen in red kidney bean crude protein extract (RKB-CPE) treated and control groups (Values for all measurements are expressed as means± SEM and *p<0.05).
Fig. 3.9. Type 1 skin test in red kidney bean crude protein extract (RKB-CPE) treated mice. To evaluate the in vivo relevance of RKB-CPE induced specific antibodies, type I skin tests were carried out. Compound 48/80 was taken as a positive control.
Fig. 3.10: Fluorescence histochemistry of GATA-3 and T-bet in the intestine. The levels of Th2 transcription factor GATA-3 and Th1 transcription factor T-bet were studied in the intestine of control and red kidney bean crude protein extract (RKB-CPE) treated mice. Goat anti mouse IgG- Alexa Fluor 488 was used as secondary antibody while DAPI was used for nuclear staining (Values for all measurements are expressed as means± SEM and *p<0.05).
3 and T-bet was obtained in the RKB CPE group when compared to control (Fig. 3.10).

3.5. Elevated cytokines secretion in splenocytes following RKB-CPE exposure

We next sought to determine the ex vivo responses of RKB-CPE on splenocytes of RKB sensitized vs. untreated groups. The RKB-U and RKB-T splenocytes secreted enhanced levels of IL-1β, IL-2, IL-4, IL-5, IL-12 and IL-13 when compared to the respective control (Fig. 3.11).

3.6. Up regulated Th1/Th2 transcription factors in the splenocytes

After analyzing cytokines, we tried to explore the role of transcription factors involved in the regulation of allergic manifestations induced by RKB-CPE in splenocytes. Expression of GATA-3 was found to enhance in RKB-T group as well as RKB-U group. The STAT-6 level was elevated in both RKB-U and RKB-T group over the control. The T-bet and c-MAF expressions were found elevated in the RKB-U group but not in RKB-T group (Fig. 3.12). An increase of 8% in CD4+T cells and 1% decrease in CD8+T cell population was observed in RKB group when compared to control (Fig. 3.13A). The B-cells population in RKB exposed group was increased by 11.6% over the control (Fig. 3.13B).

3.7. Enhanced allergic mediators in BMMC, PCMC and RBL-2H3 cells

We next sought to reveal the release of allergic mediators in the in vitro and in vivo conditions following RKB CPE exposure. The BMMC, PCMC and RBL-2H3 cells showed a significant enhancement in the release of β-hexosaminidase
Fig. 3.11: Cytokines level in the splenocytes culture supernatants. The levels of (A) IL-1β (B) IL-2 (C) IL-4 (D) IL-5 (E) IL-12 and (F) IL-13 were measured after 72 hours in the splenocytes culture supernatants of control and red kidney bean crude protein extract (RKB-CPE) treated groups using flowcytometer. The splenocytes obtained from the spleen of RKB-CPE treated mice were divided into two subgroups, RKB U (challenged with PBS) and RKB T group (challenged with RKB-CPE); while the splenocytes obtained from the spleen of control group mice were treated with PBS. (Values for all measurements are expressed as means± SEM and *p<0.05; **p<0.01; ***p<0.001).
**Fig. 3.12: Th1/Th2 transcription factors in the splenocytes.** The splenocytes obtained from the spleen of RKB-CPE treated mice were divided into two subgroups, RKB U (challenged with PBS) and RKB T group (challenged with RKB-CPE); while the splenocytes obtained from the spleen of control group mice were treated with PBS. The levels of GATA-3, STAT-6, T-bet, c-MAF and β-Tubulin was studied using western blot and densitometry analysis in control, RKB U and RKB-T groups (Values for all measurements are expressed as means± SEM and *p<0.05; **p<0.01; ***p<0.001).
Fig. 3.13: Immunophenotyping analysis of T and B cells in splenocytes of red kidney bean crude protein extract (RKB-CPE) treated mice. (A) Immunophenotyping for CD4+ and CD8+ T-cells and (B) B-cells population in the spleen of control and RKB-CPE treated groups (Values for all measurements are expressed as means± SEM and *p<0.05; **p<0.01; ***p<0.001).
Fig. 3.14: Mediators release assay in bone marrow mast cell (BMMC), peritoneal cells derived mast cells (PCMC) and RBL-2H3 cells following exposure of red kidney bean crude protein extract (RKB-CPE) exposure. (A) β-hexosaminidase release in BMMC. (B) PCMC and (C) RBL-2H3 cells using different doses of RKB-CPE. (D) Histamine and (E) Cysteinyl leukotriene (CysL) levels in RKB-CPE challenged and control RBL-2H3 cells. (Values for all measurements are expressed as means± SEM and *p<0.05; **p<0.01; ***p<0.001).
following RKB-CPE (at 100 and 125 µg in all groups) treatment over control (Fig. 3.14A-C). The increased levels of histamine and CysL got elevated by 5 and 6 fold, respectively in RKB-CPE treated group when compared to control in RBL-2H3 cells (Fig. 3.14D-E).

3.8. Specific hemagglutination activity of RKB, RKB-A and PHA-P

The specific hemagglutination activity of RKB, RKB-A and PHA-P was 204, 0.2 and 204 titer/mg (Fig. 3.15A and 3.15B).

3.9. Immunoglobulin levels in PHA-P sensitized animals

We further extended our study in female BALB/c mice to quantify specific IgE and IgG1 levels induced by PHA-P treatment. The level of specific IgE was found significantly (p<0.001) enhanced in RKB, RKB-A and PHA-P, respectively when compared to that of control on day 15th, 43rd and 59th (Fig. 3.15C). The level of specific IgG1 was found significantly (p<0.001) enhanced in RKB, RKB-A and PHA-P treated groups in comparison to control (Fig. 3.15D).

3.10. Anaphylaxis symptoms in PHA-P sensitized animals

The mice from different PHA-P treated group exhibited symptoms of systemic anaphylaxis 40 min after challenge. Mice in the PHA-P treated and challenged group exhibited scratching and rubbing around the head and snout (score 1) in 10% mice; pilar erection, puffiness around the eyes and snout, reduced activity or standing still, increased respiratory rate and diarrhea (score 2) in 20% mice; symptoms of score 1 and score 2 along with labored respiration (score 3) was
shown in 30%; near fatal reactions such as loss of consciousness or little activity despite gentle prodding (score 4) was evident by 30% mice. Mortality (score 5) was noted in 10% mice (Fig. 3.1A). In this study, 8 out of 10, PHA-P treated and challenged mice showed 3 to 4°C decrease in core body temperature (Fig. 3.1B).

3.11. Histamine, TSLP and mMCPT-1 level in PHA-P sensitized animals

A 2 fold increase in the plasma histamine level was observed in PHA-P treated mice when compared to control (Fig. 3.1C). In our result, PHA-P sensitized mice showed 2.5 fold increases in the concentration of TSLP in comparison to control (Fig. 3.1D). More than 2 fold enhancement in mMCPT-1 was observed in the serum of mice treated with PHA-P when compared to control (Fig. 3.1E).

3.12. Mediators release in RBL-2H3 cells following PHA-P exposure

We further extended this study using RBL-2H3 as in vitro model and assessed mediator release after exposure of RKB, RKB-A and PHA-P. The β-hexosaminidase levels were found elevated (p<0.001) at all concentration of RKB, RKB-A and PHA-P (Fig. 3.17A). Further, PHA-P treated group indicated up to 3 fold enhanced level of β-hexosaminidase release at 25, 50, 75, 100 and 125 µg concentrations when compared to control. The level of histamine, PGD₂, and CysL were found to be enhanced in RKB, RKB-A and PHA-P treated RBL-2H3 cells (Fig. 3.17B-D).
Fig. 3.15: Hemagglutination activity and specific immunoglobulins levels. (A) Hemaglutinin titer and (B) hemagglutination activity of phytohemagglutinins (PHA-P), autoclaved red kidney bean crude protein extract (RKB-A) and autoclaved red kidney bean crude protein extract (RKB) using rabbit blood (C) Specific IgE and (D) Specific IgG1 levels on day 15th, 43rd and 59th in control, PHA-P, RKB-A and RKB treated mice. Data represented in means ±SE; n=3; (***p<0.001) when compared with control.
Fig. 3.16: Allergic manifestations in phytohemagglutinins (PHA-P) sensitized BALB/c mice (A) Systemic anaphylactic score (B) Core body temperatures (°C) before and after 40 min of challenge (C) Plasma histamine (D) Serum TSLP and (E) Serum mMCP-1 levels in challenged PHA-P, autoclaved red kidney bean crude protein extract (RKB-A) and red kidney bean crude protein extract (RKB) and control mice (Data represented in means ±SE and *p<0.05; **p<0.01; ***p<0.001).
Fig. 3.17: Mediators release in RBL-2H3 cells (A) β-hexosaminidase release in RBL-2H3 cells using different doses of control, PHA-P, RKB-A and RKB (B) Cysteinyl leukotriene (CysL) levels (C) Histamine (D) Prostaglandin D$_2$ (PGD$_2$) levels in control, phytohemagglutinins (PHA-P), autoclaved red kidney bean crude protein extract (RKB-A) and autoclaved red kidney bean crude protein extract (RKB) challenged RBL-2H3 cells. Data represented in means ± SE and *p<0.05; **p<0.01; ***p<0.001).
4. Discussion

There is a growing need for the development of an animal model to better understand the allergenicity of food proteins and the immunological and aspects underlying the development of food allergy. In the present study, along with total and specific IgE, clinical symptoms, histamine and mouse MCPT-1 release, peritoneal albumin and TSLP were also found enhanced. PCA is used to observe the immediate type allergic reactions in several foods (Herouet-Guicheney et al., 2009). In this study, a significant increase was observed in the skin of naïve mice indicating the possibility of IgE mediated reactions in RKB-CPE treated and sensitized mice.

Release of β-hexosaminidase is an indicator of cell degranulation. When allergen comes in contact with IgE primed cells it cross-linked to the two IgE that ultimately go to the cascade and finally degranulate the cells. This degranulation is resulted in the release of several mediators including beta-hexosaminidase (King et al., 2005). Transcription factors are key molecules involved in the determination of Th1/Th2 balance. Transcription factors like STAT6, GATA-3, T-bet, NFATc, c-MAF, and T-bet are actively involved in Th1/Th2 balance. Higher expression of transcription factors in RKB-CPE treated RBL-2H3 cells indicated the prevalence of the mixed type of allergic reaction during RKB allergy. Similar to our study, expressions of Th2 inducing genes including GATA-3 were up regulated in mucosal samples of cow’s milk allergy (CMA) animals (Smaldini et al., 2012). STAT-6 plays an important role in signal transduction pathway used by interleukin-4 (IL-4) and interleukin-13 (IL-13) as
well as in class switch to IgE and Th2 cytokine production (Kaplan et al., 1996). It has been reported that mice deficient in STAT-6 show reduced IL-4-mediated functions (Davey et al., 2000). GATA-3 promotes the secretion of IL-4, IL-5, and IL-13 cytokines from activated Th2 cells (Yamashita et al., 2004). It has been reported that GATA-3 along with T-bet plays an important role in the shift of Th2 or Th1 reactions (Kiwamoto et al., 2006). It has been reported that IL-4 gene transcription is critically regulated by c-Maf (Yang et al., 2005). It has been shown that in c-maf−/− mice, CD4+ T cells had significantly reduced capacity of IL-4 production, while the levels of IL-13 and IgE were normal (Dent et al., 1998). NFATc transcription factors may play an important role in allergic events especially in the Ca++ signaling during the degranulation of mast cells. Its role in protein kinase C (PKC) activation has been revealed but complete exploration regarding its involvement in food allergy is yet to be done (Hermann-Kleiter et al., 2010). Regulation of expression of cytokines and other genes in eosinophils by subtypes of NFAT, like NFATp and NFATc has been reported but such studies regarding mast cells are still elusive (Seminario et al., 2001). The other sub types of NFAT i.e., NFATc1 and NFATc2 get accumulated in memory CD4+T cells, indicating their roles in early activation following antigen attachment (Dienz et al., 2007). The dysfunction of T-bet may be involved in the pathogenesis of severe asthma that may be responsible for accumulation of neutrophils as well as eosinophils in the airways and is considered a hallmark of disease (Fujiwara et al., 2007).
Animal models of food allergy have been used to identify mechanisms involved in the development of sensitization to food proteins as well as immunological mechanisms of adverse reactions to allergen exposure. Considering this fact, we have extended the study in BALB/c mice following oral exposure of RKB-CPE in the presence of cholera toxin as an adjuvant. Enhanced total and specific IgE levels along with prominent clinical manifestations in RKB-CPE treated mice advocate the severity of RKB induced anaphylactic reactions. This study shows similar outcomes reported in several foods induced anaphylactic reactions (Cianferoni & Muraro, 2012). Higher concentrations of plasma histamine and serum mMCP-T-1 level, have been reported to be encountered during food allergy, were also evident in RKB-CPE treated mice (Neurath et al., 2002; Srivastava et al., 2009; Perrier & Corthésy, 2011). Mast cells having high affinity IgE binding receptor, FcεRI play a pivotal role in the food allergy (Stone et al., 2010). Prominent infiltrations of mast cells in the lung, intestine and spleen indicate its role in RKB allergy. Enhanced count of eosinophils in the lungs and intestine suggests the probability of RKB induced eosinophilic esophagitis and eosinophilic gastroenteritis. The prevalence of eosinophilic esophagitis and eosinophilic gastroenteritis has been reported in food allergy (Hong & Vogel, 2010; Temiz et al., 2012). The positive type 1 skin test in the RKB CPE treated mice indicated the possibility of immediate type hypersensitivity. Similar to our study, positive type 1 skin test has also been reported in the OVA sensitive mice (Diesner et al., 2008). In this present study, the GATA-3 and T-bet level were
found enhanced, in the intestine of RKB treated groups indicating a mixed type of allergic manifestations. Similar to our study, expressions of GATA-3 were upregulated in mucosal samples of animals suffering from cow’s milk allergy (Smaldini et al., 2012). Further, T-bet has been reported to play an important role in non IgE mediated reactions (Cornejo-Garcia et al., 2007).

It has been reported that enhanced levels of IL-1β and IL-2 can enhance the possibility of AD in allergic individuals (Zeyrek et al., 2008; Chen et al., 2011). In the RKB sensitized group the enhanced level of IL-1β and IL-2 suggests that RKB consumption may lead to AD in susceptible animals. Eosinophils, mast cells and T lymphocytes are important cells in the allergic inflammatory reactions, which are regulated by cytokines like IL-3, and IL-5 (Asquith et al., 2008). In this study, eosinophils, mast cells and CD4+ T cells were found elevated post RKB administration indicating the role of enhanced IL-3, and IL-5 in RKB allergy. During Th2 cytokine responses, IL-4 and IL-13 induce B-cell class switching to IgE production, whereas IL-5 is the principal eosinophil-activating cytokine (Brandt et al., 2009). Further, it has been reported that peanut induced intestinal allergy is mediated through a mast cell–IgE–FcεRI–IL-13 pathway (Wang et al., 2010). Elevated IL-3, IL-4, IL-13 and IL-12 levels in RKB treated group indicates a Th2-type environment induced by RKB, which perhaps triggers the shift from a Th1 to Th2 environment (Pochard et al., 2010). Similar to our results, peanut has been reported to demonstrate such responses, which include increased production of Th2 and inflammatory cytokines induced inflammation evident by the presence
and activation of eosinophils, basophils, mast cells and, to a variable extent, neutrophils (Finkelman, 2010; Berin & Mayer, 2009).

Enhanced GATA-3 and STAT-6, and reduced T-bet and c-MAF expression in RKB-T group indicates a shift towards the immediate type I reaction because of the presence of allergens, while enhanced levels of GATA-3, STAT-6, c-MAF and T-bet in RKB-U group advocated possibility of both immediate as well as a late phase allergic reaction (Kumar et al., 2012b). Further, enhanced CD4+T cell population indicates potential involvement of CD4+T cells during RKB induced allergic reactions. The CD4 blockade has been found to play an important role in protecting from peanut-induced anaphylaxis (Duarte et al., 2011). In this study, B-cells population got enhanced in RKB treated group that suggests positive regulation of immune responses by cells producing antigen-specific antibodies and further supports the possibility of RKB induced atopic dermatitis (AD). Allergen-specific B cell subset responses in cow’s milk allergy of late eczematous reactions in AD have been well established (Lee et al., 2010).

The β-hexosaminidase release assay from BMMC, PCMC and RBL-2H3 cells has been reported as one of the important tools for in vitro elucidation of mast cell degranulation (Kim et al., 2012). The allergic mediators like histamine and CysL level have been reported to elevate in the case of food allergy (Pynaert et al., 1999). In our study, these mediators were found to increase in the treated group which suggests the allergenicity potential of RKB-CPE.

The presence of lectins in foodstuffs is an important health concern which may induce acute toxicity and its chronic exposure lead to harmful consequences to
human health (Peumans & Van Damme, 1996). These dietary lectins are protein that bind to surface glycoproteins (or glycolipids) on erythrocytes or lymphocyte membranes and may function as allergens as well as hemagglutinating agents (Nachbar et al., 1980). Considering the above facts, this study was initiated to explore the allergenic potential of PHA using in vivo, ex vivo and in vitro approaches.

Hemagglutination activity is defined as the lowest sample dilution that showed hemagglutination (Correia & Coelho, 1995). The outcome of this study reveals the fact that PHAs are present in a significant amount in RKB while negligible in the RKB-A. Therefore, while assessing the allergenic potential of RKB, role of PHAs cannot be ignored.

We further extended our study in female BALB/c mice to quantified specific IgE and IgG1 levels induced by PHA-P treatment. The enhanced specific IgE and IgG1 levels in PHA-P treated group suggested the possibility of PHAs induced allergic reactions. The mice from different PHA-P treated group exhibited symptoms of systemic anaphylaxis 40 min after challenge. The systemic anaphylaxis score has been reported as one of the most vital parameters during assessment of food allergy (Li et al., 1999). Enhanced allergic symptoms with decreased core body temperature reflect the severity of food induced systemic anaphylactic reactions (Sato et al., 2010). Most of the food allergic manifestations have been reported to be mediated via release of allergic mediators like histamine, PGD₂ and leukotrienes (Bloemen et al., 2007). The enhanced level of histamine, TSLP, PGD₂ and mMCPT-1 further validated the possibility of PHA-P induced
allergic manifestations. We further extended this study using RBL-2H3 as in vitro model and assessed mediator release after exposure of RKB, RKB-A and PHA-P. The β-hexosaminidase levels were found elevated. The allergic mediators like histamine, PGD$_2$ and CysL level have been reported to get elevated in case of food allergy manifestations (Bloemen et al., 2007; Boden & Burks, 2011; Kraneveld et al., 2012).

5. Conclusions

In summary, RKB-CPE exposure showed enhanced levels of total and specific IgE, anaphylactic symptoms, histamine, mouse MCPT-1 and TSLP over control. An enhanced release of β-hexosaminidase along with an up regulated level of transcription factors GATA-3, STAT-6, T-bet, c-MAF and NFATc1 in the RBL-2H3 cells indicating the mixed fate of allergic reactions. Further, RKB-CPE exposure in the presence of cholera toxin exposure may provoke the immune system of the susceptible subjects via a cascade of immune reactions, mediated by several components including IgE, mast cells, cytokine, chemokines, transcription factors, eosinophils and neutrophils. Furthermore, the presence of PHA may augment the allergenic potential of RKB, evident by enhanced levels of immunoglobulins, prominent anaphylactic manifestations, release of β-hexosaminidase and other allergic mediators, pathological symptoms of allergy in the intestine, lungs and spleen in the BALB/c mice.