



Chickpea (*Cicer arietinum*) proteins induce allergic responses in nasobronchial allergic patients and BALB/c mice

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ABSTRACT

Allergy to chickpea or Garbanzo bean (*Cicer arietinum*) has been reported in the Indian population. Little information is found regarding allergenic events involved in the chickpea allergy; therefore, chickpea allergenicity assessment was undertaken. *In vivo* and *ex vivo* studies were carried out using BALB/c mice. Chickpea skin prick test positive patients have been used to extend this study in humans. Identification of allergens was carried out by simulated gastric fluids assay for pepsin resistant polypeptides and validated by IgE western blotting using chickpea sensitive humans and sensitized mice sera. Our data have shown the occurrence of a systemic anaphylactic reaction resulting in reduced body temperature after challenge along with significantly increased levels of IgE, IgG1, MMCP-1, CCL-2 as well as histamine. Further, increased Th1/Th2 (mixed) cytokine response was observed in spleen cell culture supernatants. Jejunum, lungs and spleen showed prominent histopathological changes specific for allergic inflammation. Immunoblotting with pooled sera of either sensitized mice or human sera recognized seven similar IgE binding polypeptides that may be responsible for chickpea induced hypersensitivity reactions. This study has addressed the allergenic manifestations associated with chickpea consumption and identifies the proteins responsible for allergenicity which may prove useful in diagnosis and management of allergenicity of legumes especially chickpea.

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1. Introduction

The past few decades have witnessed a remarkable increase in the prevalence of allergic diseases and food allergies constitute a major part of this increase (Yocum et al., 1999; Sorensen et al., 1989). The incidence of food allergy varies among different countries. The overall prevalence of food allergy is 3–4% in adults and 5% in children (Sicherer and Sampson, 2010). The major cause of anaphylactic reactions treated in emergency rooms in western countries is due to consumption of food. Sampson (2003) has reported that about 29,000 patients are treated in the emergency department per year due to food anaphylaxis in the USA (Sampson, 2003).

The incidence of food allergy may vary according to the consumption habit of a particular country and increased consumption of a particular food may lead to increased sensitization among susceptible consumers (Dalal et al., 2002). Leguminous crops (family Fabaceae) are consumed worldwide due to their high protein

content. Because of this, the chances of being allergic to these proteins are high and can play a major role in the food allergy (Dalal et al., 2002). Several reports have been published regarding allergenicity of legumes worldwide including India (Misra et al., 2009). Legumes are the fifth most common cause of food allergy in Spanish children (Martinez et al., 2008). Allergenicity due to some leguminous crops like peanuts, soy beans, lentils, red gram, green gram, red kidney bean and peas are well known (Koppelman et al., 2003; Helm et al., 2000; Sanchez Monge et al., 2000, 2004; Misra et al., 2010, 2011; Kumar et al., 2011). However, a detailed study on allergenic responses of humans to chickpea (CP) or Garbanzo bean (*Cicer arietinum*) is still lacking, in spite of the crop being one of the most commonly consumed legumes used in different ways, mostly as dal, whole seeds, and several types of traditional, fermented, deep fried, sweetened and puffed products especially in India, Spain and other Mediterranean countries. Multiple IgE-binding protein bands were detected in western blot of chickpea extracts in the molecular weight range of 10–106 kDa. Majority of these peptides were heat-stable (Niphadkar et al., 1997; Martínez et al., 2000, 2008; Patil et al., 2001; Dibek et al., 2011). The existence of cross-reactivity between allergens from latex and chickpea, has been reported (Branco et al., 2004). Food-dependent exercise-induced anaphylaxis to chickpea has been reported in a 17-year-old boy (Orhan and Karakas, 2008).

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However, detailed studies for better understanding of chickpea induced immunological reactions are required. Since, a systematic study has not been carried out on chickpea allergenicity therefore, the present investigation was undertaken to assess the allergenic potential of chickpea in BALB/c mice and the allergenic responses of naso-bronchial patients sensitive to chickpea were correlated.

2. Materials and methods

2.1. Test materials and reagents

All the chemicals were of highest grade purity available. RPMI-1640, antibiotic–antimycotic solution (containing penicillin, streptomycin and amphotericin-B), Fetal Bovine Serum (FBS), pepsin from porcine gastric mucosa (Cat No. P-6887), O-phenylenediamine (OPD), bovine serum albumin (BSA), reagents for sodium dodecyl sulphate 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. Chickpea crude protein extraction

Chickpea seeds (Pusa-1003 variety) were purchased from a local certified seed vendor and the same lot was used throughout the study. Crude chickpea protein extract (CP-CPE) was prepared as previously described (Misra et al., 2009; Astwood et al., 1996). The soluble protein content was measured by the Lowry method (Lowry et al., 1951).

2.3. Pepsin digestibility assay

To identify pepsin-stable fragments in chickpea, simulated gastric fluid (SGF) digestibility assay as described by Thomas et al. (2004) with slight modifications was performed (Thomas et al., 2004). 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. Boiled samples (20 µL/well) were subjected to 12% SDS–PAGE at 22 mA for 4 h 30 min. 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).

2.4. Animal and experimental protocol

Healthy 6–8 week old female BALB/c mice (22 ± 3 g) were obtained from the CSIR-IITR, Lucknow, India animal breeding colony. Mice were maintained under standard laboratory conditions in specific pathogen free environment and fed chickpea and peanut free diet. Mice were sensitized according to the previously described protocol by intraperitoneal route because it is one of the accepted routes of allergic response development in animals (Misra et al., 2011). Blood samples from treated groups were collected from retro-orbital sinus to measure serum IgE and IgG1 antibodies, Chemokine (C–C motif) ligand 2 (CCL2) and Mouse Mast Cell Protease-1 (MMCP-1) on 15, 43 and 59 days. On day 60, mice were challenged intraperitoneally with 10 mg CP-CPE (Fig. 1a). Peanut protein was chosen as positive control as peanuts are considered to be one of the most potent food allergens causing severe allergic reactions (Burks et al., 1998).

2.5. Measurement of specific IgE, IgG1 and IgG2a antibody

Specific IgE (1:20 dilution), IgG1 (1:1000 dilution) and IgG2a (1:1000 dilution) levels against chickpea were estimated using enzyme-linked immunosorbent assay (ELISA), plates coated with soluble chickpea protein 1 µg/100 µL per well overnight at 4 °C, according to the method described earlier (Misra et al., 2010). Absorbance was measured at 492 nm using an ELISA Plate Reader (Biotek, Power Wave XS2).

2.6. Measurement of total IgE

Level of total IgE in mice serum was determined by the OptEIA™ mouse IgE Kit (BD Biosciences Pharmingen, Heidelberg, Germany) according to the manufacturers' instructions. The reaction was stopped after 30 min by adding 2 N H₂SO₄ and absorbance was taken using ELISA reader (Biotek, Power wave XS2).

2.7. Measurement of mouse mast cell protease-1 level and CCL2 level

Serum MMCP-1 level was also quantified using ELISA (Cat No. 887503, eBioscience, Inc., CA, USA) according to the manufacturers' instructions. Level of CCL-2 (also known as monocyte chemoattractant protein-1) in serum sample was estimated by ELISA method using BD optia kit (BD Biosciences, Germany) according to manufacturers' instructions.

2.8. Immunoblot analysis

To detect IgE binding proteins of chickpea, IgE immunoblotting was performed according to the earlier described procedure (Misra et al., 2011; Towbin et al., 1979).

2.9. Systemic anaphylaxis score and rectal temperature measurement

Anaphylactic reactions were scored using a previously described scoring system (Li et al., 2000). 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; and 5 – death.

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

2.10. Measurement of histamine levels

Histamine levels in plasma, lungs, spleen and jejunum were determined 30 min after challenge using enzyme immunoassay kit (SPI-BIO, Montigny le Bretonneux, France) following manufacturers' protocol. Quantification of histamine concentration was done by standard histamine curve which was run together with the assay.

2.11. Th1/Th2 cytokine

Single spleen cell suspensions were prepared aseptically in RPMI-1640 (Sigma, St. Louis, MO) according to previously described method with slight modifications (O'Donnell and Openshaw, 1998). For stimulation, spleen cells from control mice were used as naive and treated with 50 µg chickpea crude protein extracts. CP-CPE treated mice splenocytes were divided in two groups; the first one was treated with PBS (CP Control) while second was treated with 50 µg CP-CPE (CP Treated). 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 TNFα) were estimated using CBA cytokines kit (BD Biosciences, Catalog No# 560485) and analyzed by flowcytometry (BD FACS Canto) as per manufacturers' instructions. Standard cytokines were run together with the samples to quantify the levels of the individual cytokines present.

2.12. Histopathology

The mice were sacrificed after challenge by cervical dislocation. Spleen, lungs and jejunum were taken for histological analysis. Tissues were fixed in 10% formalin in PBS, embedded in paraffin, and cut into 3–5-µm thick sections.

2.13. Eosinophil levels in jejunum and lungs

Immunohistochemical detection of eosinophils in jejunum and lungs was performed as previously described (Li et al., 1995). In brief, rabbit anti mouse eosinophils major basic protein (EMBP (S-20), sc-18241, Santa Cruz biotechnology, Inc.) was used as primary antibody and HRP conjugated anti-rabbit serum 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).

2.14. Cross reactivity determination with other legumes

The cross reactivity of chickpea with other legumes like peanut, soybeans, red kidney bean and blackgram was determined *in vitro* by ELISA inhibition following the method as described by Kumari et al. (2006) with slight modifications. The inhibition percent of the specific IgE binding was expressed as

$$= 1 - \frac{\text{Absorbance of samples preincubated with allergens}}{\text{Absorbance of samples preincubated with PBS}} \times 100$$

2.15. Screening of chickpea sensitive patients

Human study protocol was followed as depicted in Fig. 1(b). Skin prick tests (SPT) were performed with allergen kit (Alicit India Private Limited) on allergic patients (n=200) and normal healthy control (n=10) at the Department of Pulmonary Medicine, Chhatrapati Shahuji Maharaj Medical University (CSMMU), Lucknow. Weal diameter equal to positive control or more were considered as marked positive skin reactions (Aggarwal et al., 2000). If smaller weal as compared

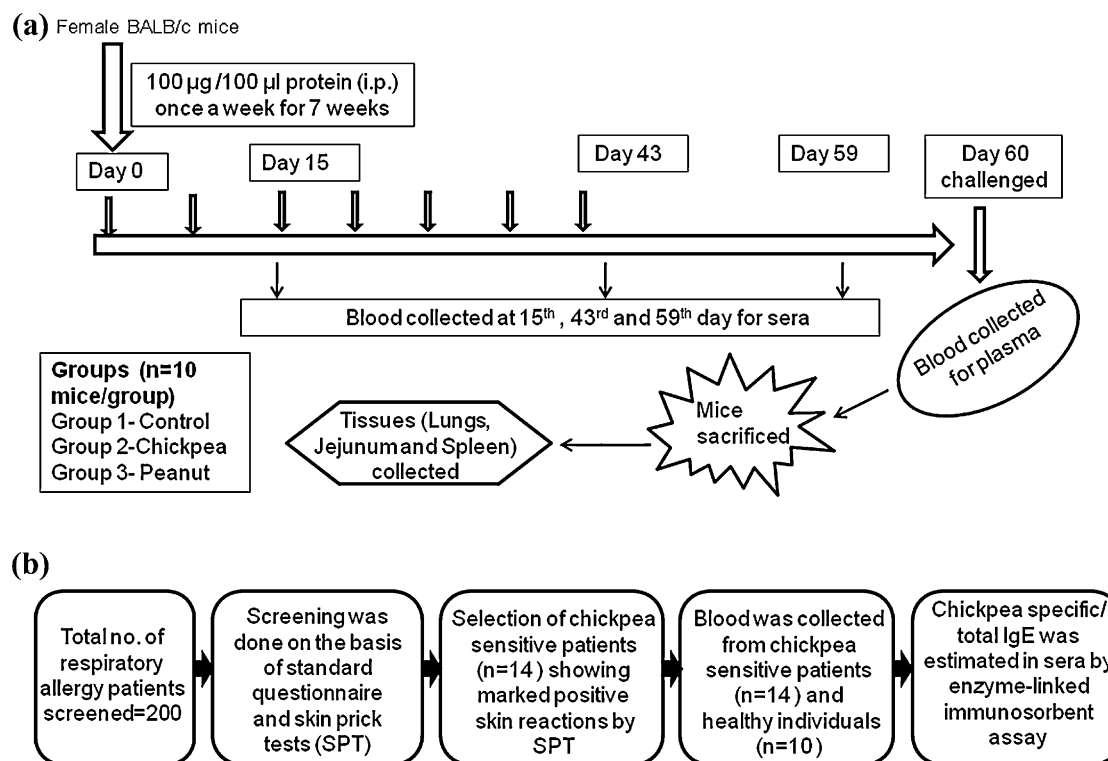


Fig. 1. (a) Experimental design of animal sensitization. (b) Protocol followed for the screening of chickpea hypersensitivity patients.

to positive control then +1, equal +2, larger 3 and for bigger with pseudopodia then +4.

2.16. Total and chickpea specific IgE determination

Serum total IgE in patients and healthy individuals was determined using ELISA (Immunology Consultants Laboratory, Inc., Newberg, OR) according to the manufacturers' instructions. Specific IgE level was determined as described earlier (Misra et al., 2011).

2.17. Statistical analysis

The data are expressed as mean $\pm$ SEM. All the data were analysed using software package from Instat version 3.0 (Graph pad, San Diego, CA, USA; <http://www.graphpad.com>). Differences between values were compared by using one-way analyses of variance (ANOVA) with Bonferroni as a *post hoc* test. A value of $p < 0.05$ was considered statistically significant.

2.18. Ethical permissions

Skin Prick Test and blood collection were carried-out with patient's consent and the study protocol was approved by the human ethics committee of the CSMMU, Lucknow (IEC No. 2938/R. Cell-11). Animal study protocol was approved by the institute's Ethics Committee (ITRC/IAEC/07/2009).

3. Results

3.1. Effect of *in vitro* pepsin digestion on chickpea crude protein extracts (CP-CPE)

Fig. 2(a and b) shows the digestibility profile and densitometry analysis of the chickpea CPE. It was found that seven proteins of molecular weights 95, 70, 55, 45, 35, 26 and 20 kDa approximately were resistant to digestion in the simulated gastric fluid. Densitometry analysis revealed that the percentages of 20, 26, 35, 45 and 55 kDa protein components that remained undigested were 48%, 39%, 51%, 29% and 26% respectively, up to 60 min in SGF. Density of 70 and 95 kDa protein components were found 18% and 20% respectively, at time point 8 min and degraded within 15 min.

3.2. IgE-binding polypeptides in chickpea extract

Multiple immunoreactive proteins were identified in CP-CPE in the range of 15–95 kDa. Immunoblotting analysis performed with pooled sera of sensitized mice and hypersensitive patients showed the presence of seven IgE-specific binding proteins in CP-CPE (Fig. 2c). Prominent IgE-binding bands could be observed at 20, 26, 35, 45, 55, 70 and 95 kDa. Fig. 2c(i), (ii) and (iii) depict clearly that molecular weight of pepsin resistant proteins and mice and human sera IgE bindings were almost similar. No IgE binding proteins were detected by Western blot analysis with sera of healthy individuals, and PBS treated mice [Fig. 2c(iv)].

3.3. Levels of total and specific IgE antibody in sensitized mice

The group sensitized with CP-CPE showed significantly higher levels of total IgE and specific IgE ($p < 0.05$) as compared to control group as shown in Fig. 3(a and b).

3.4. Levels of IgG1 and IgG2a antibody in sensitized mice

In order to evaluate the effect of CP-CPE on IgG subclass antibody involved in allergic reactions, we measured the levels of chickpea-specific IgG1 and IgG2a levels in sensitized mice. Significant differences were found in the levels of sera of control and sensitized mice collected at different time points. Sensitized mice showed significantly increased levels of IgG1 ($p < 0.001$) and IgG2a antibody ($p < 0.001$) (Fig. 3c and d), the increase being more pronounced in the former.

3.5. Measurement of MMCP-1 and CCL2 level in serum

There was a two to three-fold increase in MMCP-1 at all time points in chickpea sensitized mice (Fig. 3e). In chickpea sensitized mice higher induction of CCL2 was observed as compared to PBS

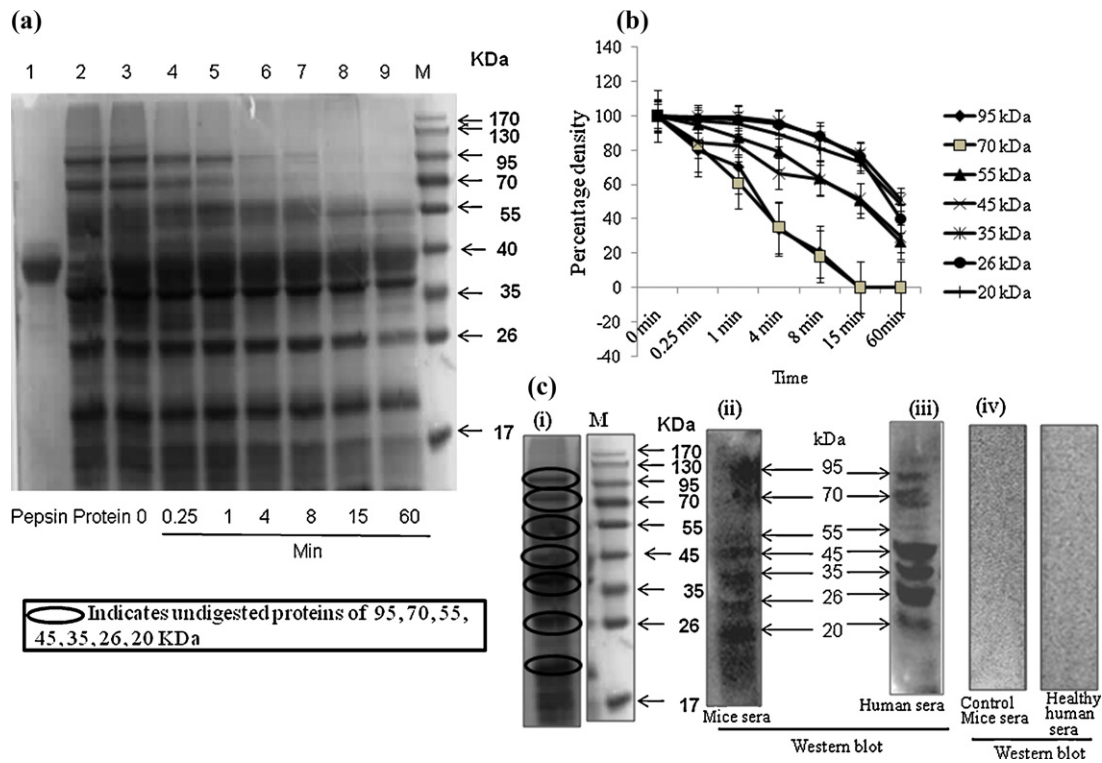


Fig. 2. (a) Pepsin digestibility profile of chickpea CPE. Lane 1, Pepsin, lane 2, chickpea CPE, lanes 3–10, SGF digestion pattern of chickpea CPE at 0, 0.25, 1, 4, 8, 15 and 60 min, lane M, molecular weight markers. (b) Densitometry plot of undigested proteins at different time points. (c) (i) Pepsin resistant proteins along with marker (M); (ii) IgE immunoblot of chickpea treated mice; and (iii) chickpea sensitive humans; (iv) IgE immunoblot with PBS treated mice and healthy human's sera.

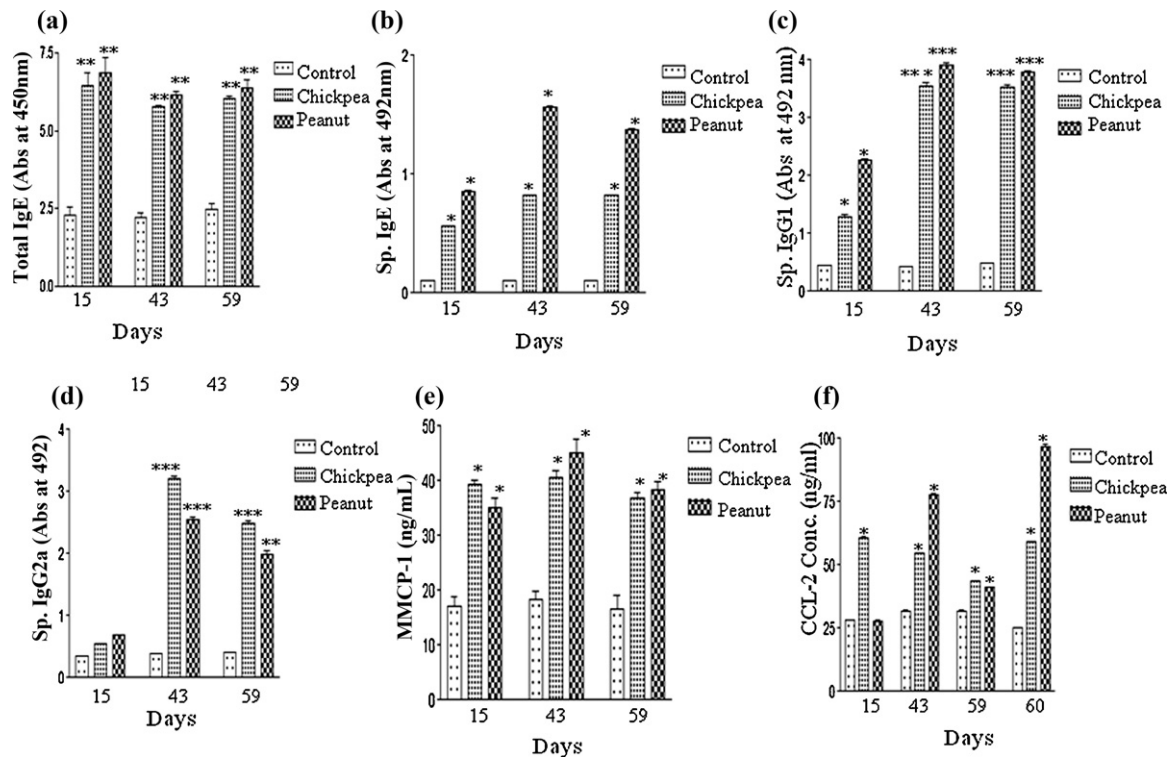


Fig. 3. Serum levels of total/specific IgE (a, b), IgG1, IgG2a response (c, d), mouse mast cell protease-1 (MMCP-1) and CCL2 Chemokine (e, f) was determined in chickpea sensitized mice by ELISA. The level of CCL2 was also estimated in serum after challenge. Significant difference (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) as compared to control group.

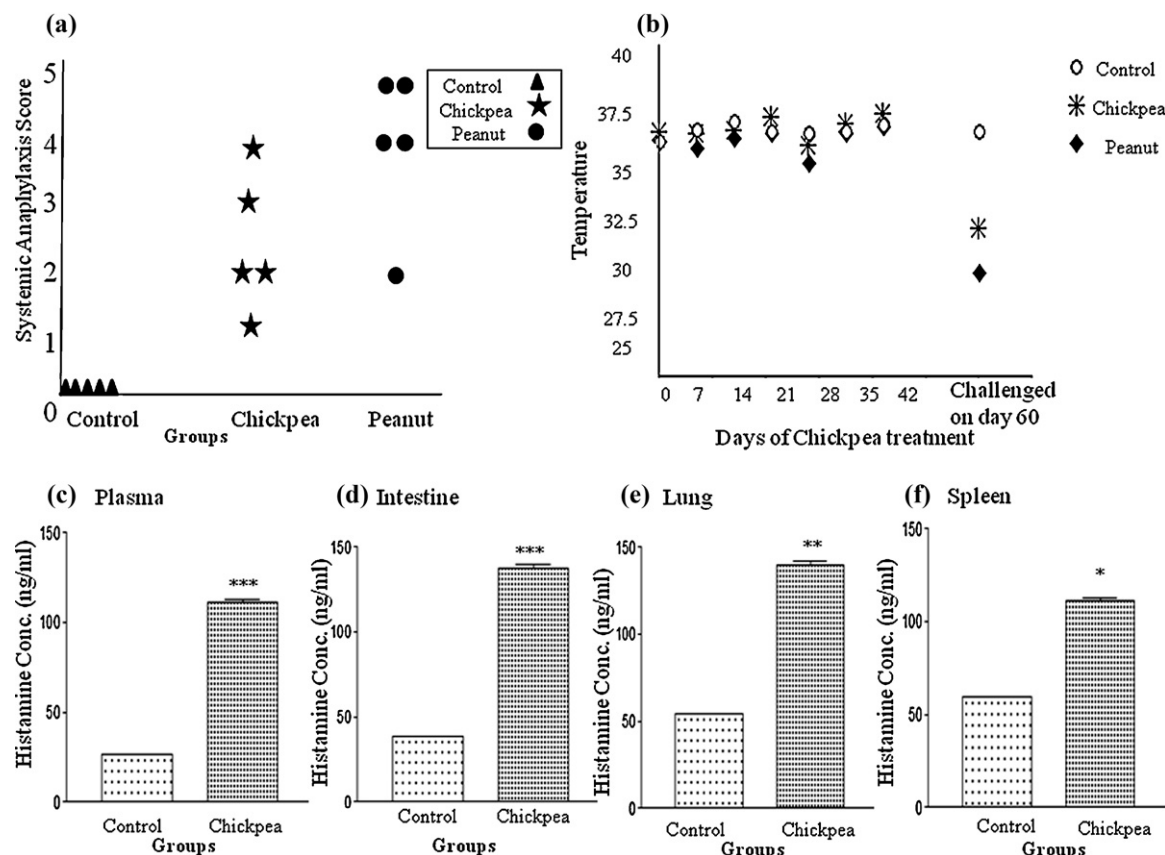


Fig. 4. (a) Anaphylactic symptoms scored after 20–30 min following challenge, presented as median value for control (0), chickpea treated (2.5) and peanut (4) treated mice respectively. (b) Rectal temperature was measured on day 0th, 7th, 14th, 21st, 28th, 35th, 42nd and 20 min after challenge on day 60. Histamine levels were estimated in (c) plasma (d) intestine (e) lungs and (f) spleen. Plasma and organs were obtained 30 min after Chickpea challenge. Histamine levels were determined using histamine release assay. Data are presented as means $\pm$ SEM (* p < 0.05, ** p < 0.01, *** p < 0.001) versus control mice.

treated mice (Fig. 3f). Increased concentration of CCL2 was noted after challenging with CP CPE on day 60th with CP-CPE as compared to 59th day.

3.6. Higher anaphylaxis score and reduced body temperature in sensitized mice after challenge

Five mice from each group were challenged intraperitoneally with CP-CPE (10 mg dose/mice). Clinical symptoms were observed 20–30 min after challenge. Out of 5 mice from chickpea sensitized group, one mouse showed scratching and rubbing around the nose and head (score 1/5). In two mice, the symptoms were diarrhea and increased respiratory rate (score 2/5). Remaining two mice were affected the most with no response to external stimuli with labored respiration (score 3, 4/5). Control group animals showed no signs of anaphylaxis upon CP-CPE challenge as depicted in Fig. 4(a).

As shown in Fig. 4(b), 20 min following challenge, sensitized mice, showed a marked drop in body temperature in the range of 4–7°C but not in control group. As anaphylaxis symptoms progressed after challenging the mice with CP-CPE, core body temperature dropped sharply.

3.7. Measurement of histamine levels in plasma and different organs

CP-CPE sensitized mice showed increased histamine level in plasma as compared to control mice. As shown in Fig. 4(c), the histamine level in the control group was 26.6 ng/mL; however, in sensitized mice histamine level was increased up to 111.6 ng/mL. So, there was ~4 fold increase in concentration of histamine in

sensitized mice as compared to control mice. Intestine, lungs and spleen of CP-CPE sensitized mice also demonstrated a significant histamine release as compared to the control group mice (p < 0.05) as depicted in Fig. 4(d–f).

3.8. Th1 and Th2 cytokine production in sensitized mice

We determined the levels of the Th2-related cytokines (IL-2, IL-4, IL-6, and IL-17), Th1-related cytokine (IFN- γ , TNF α) as well as the T regulatory-associated cytokine IL-10 in CP-CPE exposed spleen cell culture. No significant difference was observed in the case of IL-2 level on chickpea exposure (Fig. 5a). There was a considerable increase in the production of IL-4, IL-6, IL-17, IL-10, IFN- γ and TNF α from splenocytes obtained from sensitized mice compared to naive controls (Fig. 5b–g).

There was a significant increase in IL-4, IL-6, IL-17, IL-10, IFN- γ and TNF α compared to non-stimulated spleen cells. Conclusively, mixed cytokine responses were observed as CP exposure resulted in increased levels of Th1/Th2/Treg cytokine production in the spleen.

3.9. Histopathological analysis of lungs, jejunum and spleen

Prominent histopathological changes in lungs, spleen and jejunum were found in CP-CPE treated mice. Lungs from intraperitoneally sensitized mice with CP-CPE had hyperplasia of bronchiolar lining epithelium, destruction as well as exfoliation of bronchiolar lining epithelium. Exfoliated mucosal mass within the bronchiole was seen. Thickened alveolar septa were another prominent change that was observed. PBS treated mice had normal histological structure of alveoli and bronchioles. Analysis of

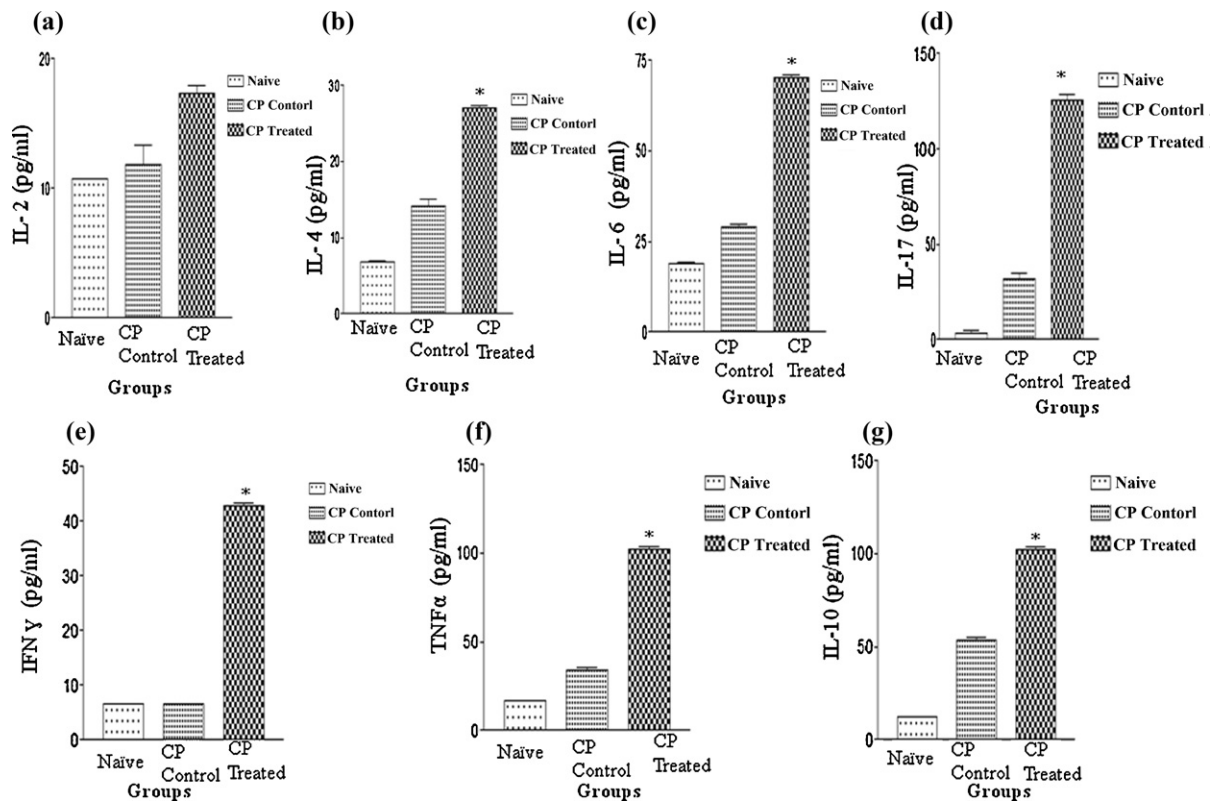


Fig. 5. Cytokine levels in splenocytes culture supernatants. Cell suspensions were cultured in complete medium in the presence of Chickpea CPE or medium alone. Th2 cytokines (a) IL-2, (b) IL-4, (c) IL-6, (d) IL-17 Th1 cytokine, (e) IFN- γ , (f) TNF α and T regulatory cytokine, and (g) IL-10 levels were determined using flow cytometry. Results are expressed as mean of values $\pm$ SEM. Statistically significant difference (* $p < 0.05$) from control group.

jejunum of CP-CPE treated mice revealed destruction of mucosal lining and infiltration of mucosa with inflammatory cells while control mice had normal structure with no distortion in mucosa. The effect of CP-CPE treatment was also observed on spleen that had starry sky appearance and lymphoid hyperplasia. In contrast, normal histology of red pulp and white pulp was observed in control mice (Fig. 6). Histopathological analysis of peanut sensitized mice which was used as positive control showed changes similar to CP-CPE with more severity in all three organs i.e. lungs, spleen and jejunum.

3.10. Eosinophil levels in lungs and jejunum

Eosinophil numbers were increased in CP-CPE treated mice in comparison to control group. The elevated level of eosinophils in CP-CPE sensitized mice similar to peanut was found when compared to control group (Fig. 7).

3.11. Cross reactivity determination with other legumes with mice sera

The sera samples of CP-CPE sensitized mice preincubated with peanut, red kidney bean, soybean and black gram exhibited a significantly reduced antibody binding to CP-CPE in a dose-dependent manner as compared to control serum samples preincubated with PBS alone. ELISA inhibition test showed specific IgE inhibition with peanut, red kidney bean, soybean and black gram (Fig. 8).

3.12. Study on chickpea sensitive respiratory allergy patients

We screened 200 patients for this study and out of which fourteen (7%) showed positive SPT for CP allergen. Clinical data like

SPT, total IgE, specific IgE and allergic symptoms, related to CP sensitive patients and healthy individuals are presented in Table 1. In control individuals, total IgE levels ranged between 55.5–232 kU/L, while in CP patients it ranged between 96 and 664 kU/L. Specific IgE levels ranged between 0.14 and 0.54 kAU/L in control individual and between 0.08 and 11.9 kAU/L in CP sensitive patients. CP sensitive patients also had concomitant sensitization to other food allergens as shown by skin prick test. In our study, CP allergic patients were found to be mainly sensitive to pollens, fungi, insects and other leguminous crops (Table 2) (Supplementary Table S1).

4. Discussion

Legume allergy is an emerging health concern throughout the globe. Our understanding of allergenicity potential of commonly consumed legumes like peanut, soybean, peas, lentils, green gram, red gram and red kidney beans has increased manifold in recent years (Koppelman et al., 2003; Helm et al., 2000; Sanchez Monge et al., 2000, 2004; Misra et al., 2010, 2011; Kumar et al., 2011). Some preliminary reports on allergenicity of CP suggest the probable allergenicity potential of CP, but to better understand its allergenic mechanism, further studies are required. Therefore, in this work we have tried to unravel the detailed mechanism of allergenic potential of CP.

The main criterion of selecting allergenic proteins was on the basis of their resistance to pepsin. It is known that proteins stable to SGF digestion have the potential to elicit allergic response in majority of cases (Misra et al., 2010, 2011; Kumar et al., 2011). The food proteins that get digested relatively in less time have little opportunity to exert adverse effects and mostly non-allergenic. According to DBT, India guidelines (2008), proteins with more than

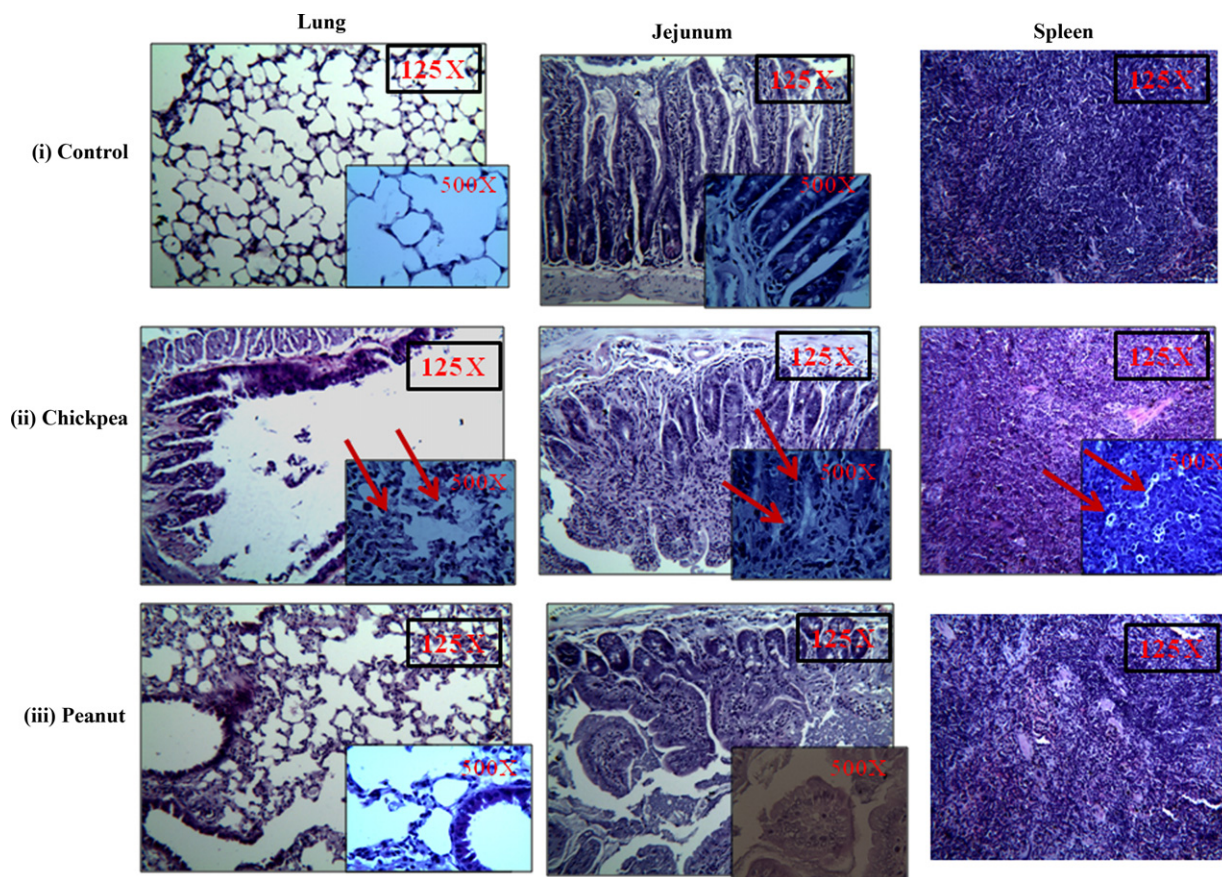


Fig. 6. Histopathological analysis of lungs, jejunum and spleen of chickpea treated mice: (i) lungs, jejunum and spleen of control group, (ii) lungs (arrow indicating exfoliation of epithelial lining at the bronchioles), jejunum (arrow indicating exfoliation of intestinal villi with inflammatory cells) and spleen (arrow indicate starry sky appearance) of chickpea sensitized mice, and (iii) lungs, jejunum and spleen of peanut sensitized mice.

10% stainable full-length protein band remaining at >30–60 min may be considered stable. Proteins reduced to <10% stainable band at 5–30 min can be considered of intermediate stability (DBT, 2008). With above said criteria we could get 7 proteins (mol. wt. 95, 70, 55, 45, 35, 26 and 20 kDa) survived digestibility upto 0.25, 1, 4 and 8 min. Therefore, it was hypothesized that these proteins were

probably capable of elicitation of allergenic symptoms in gastrointestinal tract. This could be explained with specific IgE-binding bands against CP-CPE observed at 20, 26, 35, 45, 55, 70 and 95 kDa with sensitive humans as well as CP-CPE exposed mice sera. Further experiments were conducted to confirm the allergenic potential of CP proteins/peptides.

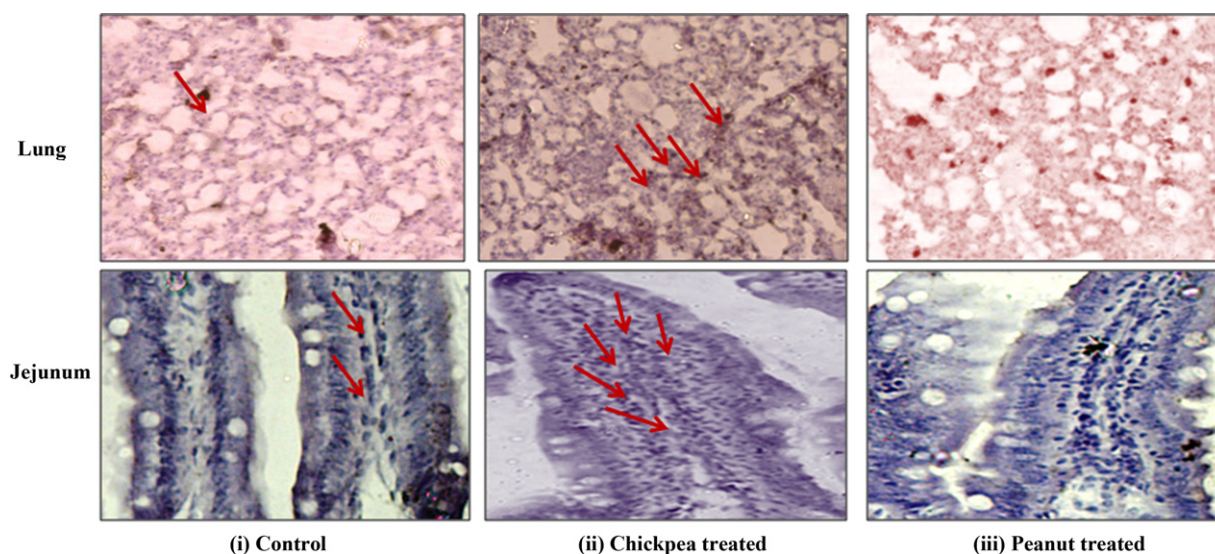


Fig. 7. Immunohistochemical staining of lungs and jejunum of chickpea sensitized mice after challenge. Detection of eosinophil in lungs (i) control, (ii) chickpea and (iii) peanut. Detection of eosinophil in jejunum (i) control, (ii) chickpea and (iii) peanut. Arrow indicating eosinophils.

Table 1
Details of chickpea-sensitive respiratory allergy patients.

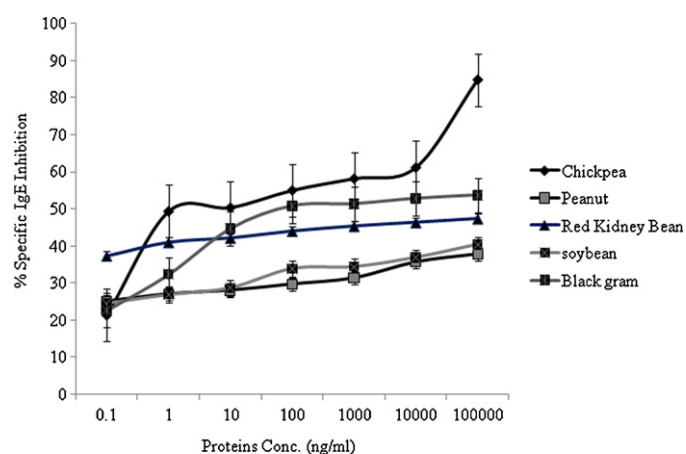
S.N.	Age (years)/sex	SPT (weal)	Total IgE (kU/L)	Specific IgE (kAU/L)	Allergic symptoms
Control-1	30/F	–Ve	96 ± 6.5	0.47 ± 0.15	None
Control-2	42/F	–Ve	77 ± 1.7	0.32 ± 0.05	None
Control-3	20/M	–Ve	59.5 ± 4.0	0.27 ± 0.16	None
Control-4	34/M	–Ve	143 ± 0.7	0.35 ± 0.11	None
Control-5	45/F	–Ve	232 ± 35	0.547 ± 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	46/M	+2	521 ± 17.8 [*]	2.7 ± 0.26 [*]	AR
Patient-2	32/M	+3	433 ± 2 [*]	7.6 ± 0.5 [*]	BA
Patient-3	51/M	+2	482.5 ± 4.4 [*]	8.7 ± 0.25 [*]	BA, AR, D
Patient-4	48/M	+3	531 ± 8 [*]	9.0 ± 0.4 [*]	AR, BA
Patient-5	32/M	+2	306 ± 1.7 [*]	2.75 ± 0.15 [*]	AR, BA
Patient-6	23/M	+1	310 ± 58.3 [*]	4.5 ± 0.15 [*]	BA
Patient-7	45/F	+3	96 ± 3	0.08 ± 0.02	AR, BA, D
Patient-8	21/F	+2	232.5 ± 15	1.4 ± 0.1	AR
Patient-9	37/M	+2	134.5 ± 0.3	0.35 ± 0.04	AR, BA
Patient-10	55/M	+3	664 ± 8 [*]	11.9 ± 0.5 [*]	BA, D
Patient-11	24/F	+1	98 ± 2	2.0 ± 0.4 [*]	AR, BA
Patient-12	36/M	+3	269 ± 3	7.8 ± 0.7 [*]	BA
Patient-13	48/M	+3	636 ± 1 [*]	11 ± 0.3 [*]	AR, BA
Patient-14	52/M	+2	409 ± 5 [*]	9.9 ± 1.0 [*]	D, AR

M, male; F, female; IgE, immunoglobulin E; SPT, skin prick test; –Ve, SPT negative for Chickpea; AR, allergic rhinitis; BA, bronchial asthma; D, dermatitis; U, urticaria. SPT (skin prick test) measured according to wheel size (in mm). Total and specific IgE presented in mean ± SEM.

^{*} Significant, $p < 0.05$.

BALB/c mice has been widely used to evaluate the sensitizing potential of novel proteins as this strain is known to favour the development of Th2-type immune responses and the production of IgE antibody (Dearman and Kimber, 2001).

Allergenic proteins have the ability to induce production of IgG1 antibody along with specific IgE. An increase in level of specific IgE and IgG1 antibody in sensitized mice compared to control mice indicate the ability of CP-CPE to induce allergenic response that needs further confirmation.



Legumes	Inhibition Percentage	
	Minimum	Maximum
Peanut (PN)	25	37
Soybean	24	40
Red kidney bean	37	48
Black gram	22	53

Fig. 8. Cross reactivity of chickpea with other legumes like peanut, red kidney bean, soybean and blackgram using ELISA inhibition with pooled chickpea exposed mice sera.

CP induced anaphylaxis reaction was studied in mice after injecting 10 mg of CP-CPE on day 60. It has been earlier reported by Li et al. (2000) that orally sensitized mice with peanut after challenge have shown visible signs of anaphylaxis (Li et al., 2000).

It is well known that a drop in body temperature (4–7 °C) is inversely proportional to anaphylaxis symptom scores (Sato et al., 2010) and the drop in temperature observed in our study signifies the development of systemic anaphylaxis. This was evident by a marked increase in histamine and specific IgE levels in CP-CPE treated mice compared to control mice. Earlier reports have shown that antigen induces cross-linking of IgE bound to mast cells/basophils, and results in release of mediators such as histamine that may cause the symptoms of allergy in a sensitized individual (Metzger, 1991). Marked increase in histamine level in the plasma, intestine, lungs and spleen is a clear indicator of CP induced anaphylaxis in sensitized mice. This demonstrates that CP-CPE has the ability to crosslink IgE antibody present on mast cell and thus results in subsequent release of histamine following challenge in blood as well as different tissues.

MMCP-1 is stored and secreted by intestinal mucosal mast cells (IMMC) (Caughey, 2007). Secretion of MMCP-1 is up-regulated during hypersensitivity reaction, suggesting the role of this protease in a sensitized individual. Raised concentrations of MMCP-1 (~2–3 fold) in serum at different time intervals in sensitized mice indicate increased epithelial permeability leading to enhanced absorption

Table 2
Prevalence of concomitant sensitization of chickpea sensitive patients ($n = 14$) to other food allergens.

Groups	Food allergens	Patients
1	Pollens	8
2	Fungi	8
3	Insects	10
4	Dust	6
5	Dander	4
6	Miscellaneous food items	4
7	Animal products	4
8	Other legumes	13

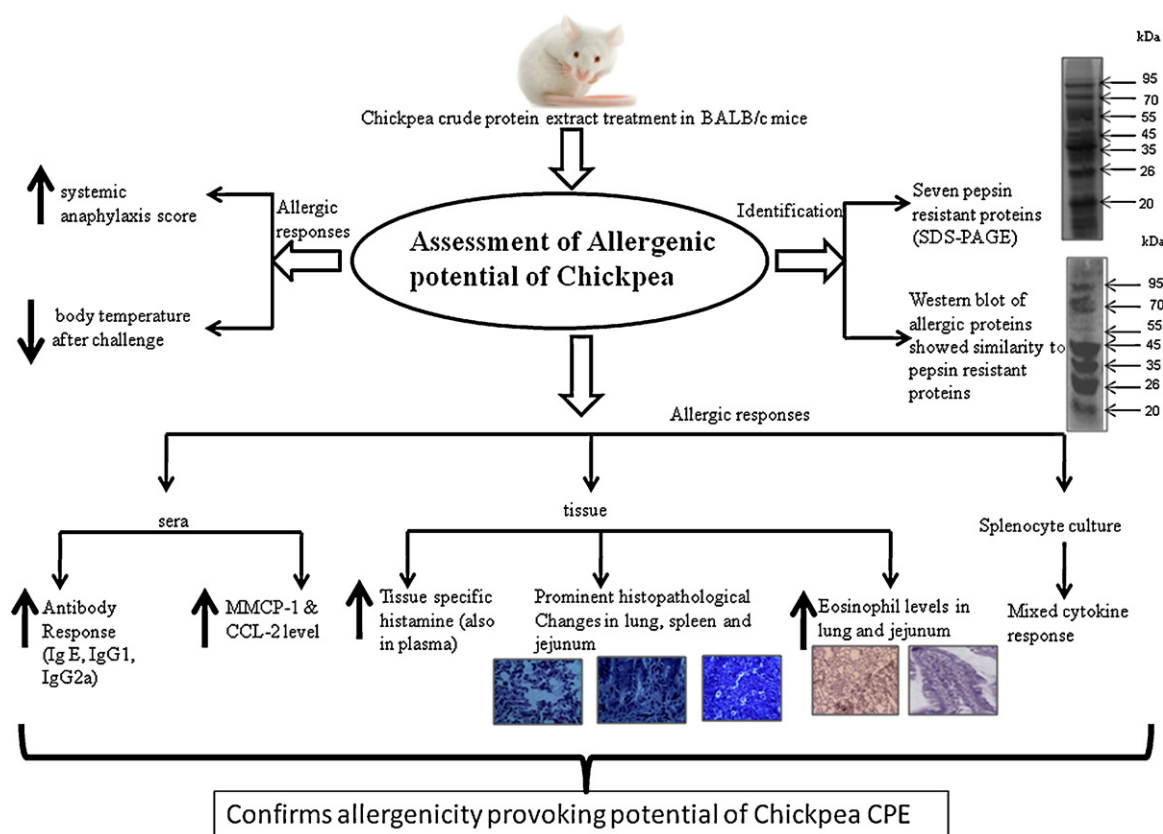


Fig. 9. Immuno-allergic action of pepsin resistant chickpea proteins.

of undigested proteins and peptides that may cause subsequent development of allergic response.

CCL2, a chemokine recruits monocytes, memory T cells and dendritic cells to sites of tissue injury, infection, and inflammation and its secretion increases in the case of allergenic condition (Carr et al., 1994). In the light of above, we investigated the level of CCL2 in CP-CPE treated mice and found significant increase in its concentration. The increase in the level of CCL2 was more in sera collected after challenging the sensitized mice and is an indicator of allergenic responses induced by CP.

Shifting of the Th1/Th2 balance in favour of Th2 cytokine is considered to be a key factor for development of allergy (Maggi, 1998). However, in case of food allergy antigen-specific mixed Th1 and Th2-type cytokine response had occurred (Holen et al., 2001; Smart and Kemp, 2002; Eigenmann and Frossard, 2003).

Our data demonstrated mixed cytokine (Th1/Th2) response in splenocyte culture of CP sensitized mice. Increased amounts of Th2 cytokines (IL-4, IL-6, and IL-10) as well as IL-17 observed by us may trigger and aggravate the allergic and inflammatory responses (Ozdemir et al., 2010). Higher levels of IFN- γ and TNF α activate mast cells and its activation may lead to production of higher IgE and enhanced release of different mediators like histamine, leukotriene responsible for allergic reaction (Metcalfe et al., 1997).

Jejunum and lungs histology revealed that exposure to CP-CPE recruits inflammatory cells and caused airway blockage which is an indicator of immune-inflammatory diseases like asthma. The lymphoid hyperplasia of spleen observed in present study is again a sign of allergenic response. The probable reason behind this may be massive proliferation and differentiation of T lymphocytes which are the main histological signs of chronic inflammation in the lymphoid organ (Ryan and Majno, 1977). These histological changes

support our findings and explain the modulation in cytokines, CCL-2, IgE and IgG1 levels following exposure to CP-CPE. CP probably has allergenic proteins that may cause problems in sensitized consumer group gets support from elevated levels of eosinophils in CP-CPE sensitized mice as compared to 0 control.

Significantly elevated levels of specific and total IgE in CP patients were found as compared to normal healthy individuals. The patients with CP allergy were suffering from allergic rhinitis, bronchial asthma, dermatitis and urticaria. These allergic manifestations in CP SPT positive patients may be an outcome of direct CP induced allergy or cross-reactivity of CP with other allergens. CP has the ability to induce allergic symptoms in sensitive individuals who may be allergic to red kidney bean and black gram as these have shown higher cross reactivity with CP-CPE as compared to peanut and soybean. Therefore, CP may induce allergic symptoms in sensitive individuals who may be allergic to any of the above legumes.

Taken together, our study unravel the facts that CP-CPE is responsible for allergenic manifestations in more than 7% individuals who had allergic rhinitis and bronchial asthma. This study demonstrated conclusively that CP-CPE could induce specific IgE and IgG1, CCL-2, MMCP-1, histamine, eosinophils in an animal model that was facilitated by heightened mixed cytokines responses. In addition, seven novel putative clinically relevant allergens were identified (Fig. 9). Identification of allergenic proteins can be the first step for allergen specific immunotherapy (SIT) and sublingual immunotherapy (SLIT) and can also be helpful in the production of hypoallergenic transgenic crops using plant breeding and GM methods.

Conflict of interest statement

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.toxlet.2012.01.011.

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