

## Peanut Allergenicity and Cross-Reactivity with Pea Proteins in the *In Vivo* Model

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The aim of the study was to analyse the potential pea-peanut cross-reactivity using the mice BALB/c as a biological *in vivo* model in the research on immune response to peanut proteins (PnE). BALB/c mice were three-fold sensitised (on days 1, 7, and 21) by oral or intraperitoneal (IP) administration of PnE in 0.5 mg or 1 mg dose, with or without adjuvant – aluminum hydroxide gel (Alum). Serum immunoglobulins (IgE, IgG, IgG1 and IgG2a) and level of cytokines (IL-4, IL-10, IFN- $\gamma$ ), secreted by the isolated lymphocytes were examined. The highest increase in total IgE and peanut-specific IgG1 was noted in the group sensitised by IP administration of PnE in the presence of Alum. Lymphocytes from peanut-sensitised (with and without Alum) mice showed a significantly high level of IL-4 and this cytokine was secreted to a much higher extent as compared to IFN- $\gamma$ . Stimulation of a culture of lymphocytes with pea proteins resulted in high IFN- $\gamma$  secretion. A weak reaction of peanut-specific IgG1 present in mice serum with pea globulins (vicilin – PV and legumin – PL) can suggest that the cross-reactivity between peanut and pea proteins results from the presence of proteins other than 7S and 11S globulins. Due to the demonstrated low cross-reactivity between peanut proteins and pea globulins, the possibility of applying pea proteins in peanut-allergy immunotherapy may be suggested.

### INTRODUCTION

Improper immune system response to food occurs in almost 5% of small children and 3–4% of adults and has a growing tendency [Sicherer & Sampson, 2010]. Peanut allergy affects almost 1% of children and 0.6% of adults in the United States [Burks; 2008; Sicherer *et al.*, 2003]. Thyagarajan *et al.* [2010] reported that the problem concerns over 1% of the population of the Western countries. Peanut causes the highest number of cases of severe anaphylaxis and deaths [Finkelman, 2010]. The mechanism of peanut allergy still remains unclear. A significant role in anaphylaxis evocation is assigned to the capability of peanut proteins to activate the complement system [Khodoun *et al.*, 2009]. This probably differentiates such allergy from hypersensitivity to milk or eggs. The effect of the glycation process during roasting on the peanut allergenicity should also be considered [Kopper *et al.*, 2005; Bielikowicz *et al.*, 2010]. There is plenty of information about cross-reactivity between leguminous plants (including peanuts), but cases confirming their clinical reactivity are rare [Fæste & Namork, 2010]. Majority of studies in this area are based on the determination of the level of specific antibodies in blood serum using *in vitro* methods [de Leon *et al.*, 2007; Maloney *et al.*, 2008; Martinez San Ireño *et al.*, 2008]. The possibility of cross reactivity can be assessed on the basis

of the identity of local amino acid sequence in the examined protein with those in epitopes in known allergenic proteins [Minkiewicz *et al.*, 2011]. Implementation of a biological model with the use of laboratory animals appears to be another, maybe more proper solution. *In vivo* studies on the animal model are supported by FAO and WHO [FAO/WHO, 2001] and numerous national organizations engaged in studying food and health [Bowman & Selgrade, 2008]. Interest in the use of the model based mainly on rodents (mice) in food allergy research in humans has increased considerably during the last few years [Knippels *et al.*, 2004; Lehrer & McClain, 2009; McClain & Bannon, 2006]. Food allergy usually proceeds according to IgE-mediated and non-IgE-mediated (cellular) mechanism [Sicherer & Sampson, 2010]. IgE occurs in trace amount under physiological conditions, and its synthesis is regulated by various factors. The most important role in the activation of these antibodies' production is played by Th2, which produces interleukin 4 (IL-4) and signal B cells after an interaction between TCR/CD3 and class-II MHC. IgE synthesis can also be stimulated by other cytokines produced by Th2, like IL-13, IL-5, and IL-6. On the other hand, some cytokines, produced by Th1, especially gamma interferon (IFN- $\gamma$ ) and IL-12, inhibit IgE synthesis [Kaplan, 2001; Male *et al.*, 2008].

The utility of the model based on mice depends on whether in a given species a genetic tendency to food allergy is connected with Th2-Th1 response change [Morafo *et al.*, 2003]. It was clearly shown that C3H/HeJ mice can be used in studies on induced anaphylaxis (IgE-mediated) and that

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its clinical course (T- and B-cells responses) was similar to that occurring in individuals with hypersensitivity to peanuts [Li *et al.*, 2000]. Also other species of mice: BALB/c, DBA/2, A/J, BDF-1, C57B/6, are used in studying the mechanisms of food allergy [Ladics *et al.*, 2010; Ganeshan *et al.*, 2009; Lifrani *et al.*, 2005; Sumiyoshi *et al.*, 2010; Yang & Mine, 2009]. The majority of investigations conducted with the use of the murine model determine the influence of isolated antigens on the immune system [Dearman & Kimber, 2007; Helm, 2002; Hilton *et al.*, 1997]. However, this model has an error because purified proteins (allergens) stimulate murine immune system with less efficacy than crude extracts [Strid *et al.*, 2004]. Moreover, the efficiency of stimulation depends not only on the purity of protein, but also on the presence of contamination, like lipopolysaccharides (LPS) [Eisenbarth *et al.*, 2002], endotoxins [Delayre-Orthez *et al.*, 2004], or lectins [Prasad *et al.*, 2007; van Wijk *et al.* 2005]. In our studies, BALB/c mice were used to determine their utility in studies related to food reaction caused by peanut protein administration (crude extract).

The aim of this study was to analyse the potential peanut cross-reactivity with using the BALB/c mice as a biological *in vivo* model in the research on immune response to peanut proteins.

## MATERIALS AND METHODS

### Mice and reagents

Five-week-old female BALB/c mice purchased from Medical Research Centre, PAS, Warsaw, Poland, were maintained on legume-free diet. Standard guidelines for care and use of animals were followed. Aluminum hydroxide gel (Alum) was used as an adjuvant (A8222, Sigma).

### Preparation of peanut extract

Crude peanut extract (PnE) and pea extract (PE) was prepared from defatted raw flour. The flour (1:10 w/v) was extracted for 2 h with PBS (pH 7.4) at room temperature. After centrifugation at 20,000  $\times g$  for 60 min, the supernatant was filter-sterilised, frozen and stored at  $-20^{\circ}\text{C}$  until further use. Protein concentrations were determined using the Bradford analysis with BSA as the standard. 2-D analysis from the extract showed spots between 6–100 kDa and pH 5–7 (not shown). The major peanut and pea globulins were extracted as described before [Freitas *et al.*, 2000] and then fractionated by ammonium sulfate precipitation [Maleki *et al.*, 2000]. Isolated 11S and 7S fractions were purified by ion exchange chromatography on a column with DEAE-Sepharose (DFF100, Sigma). Protein sample (250 mg) was applied to the column (HR 16  $\times$  10) and eluted in the gradient of two buffers: A – 50 mmol/L phosphate buffer (pH 7.2) and B – 50 mmol/L phosphate buffer (pH 7.2), containing 0.5 mol/L NaCl. As a result of chromatographic separations, the pea and peanut legumin (PL and PnL) fractions were obtained, which on SDS-PAGE gel yielded bands corresponding to the molecular masses of 40 kD and 20 kD. Pea vicilin (PV) purified on DEAE-Sepharose column yielded bands corresponding to the molecular masses of 50 kD, 33 kD and three 19–16 kD bands, and peanut vicilin (PnV) – 63 kD (not shown).

### Mouse treatment protocol

Peanut sensitization and challenge was adapted from the previously described protocol [Pons *et al.*, 2004]. Sensitization was performed by intraperitoneal (IP) administration of 0.5 or 1.0 mg PnE in PBS only or together with Alum (0.3 mL injected/mouse) on days 1, 7 and 21. The control group received only PBS (0.3 mL). Two weeks after last sensitization, mice were challenged with PnE. Next day all mice were killed by  $\text{CO}_2$  inhalation.

The Local Care Use of Animal Committee (authorization 11/2007) approved animal handling and experimental procedures.

### Measurement of serum peanut-specific IgG1, IgG2a, IgE and total IgE levels

Tail vein blood was obtained during sensitization on days 14, 28 and after completion of the experiment (day 35). The blood was incubated for 1 h at room temperature, centrifuged at 14,000  $\times g$  for 10 min. Then, the sera were collected and stored at  $-20^{\circ}\text{C}$  [Dearman & Kimber, 2007].

Levels of peanut-specific IgG1 and IgG2a were measured by ELISA as described previously [Lee *et al.*, 2001] with minor modifications. Plates were coated with PnE, PE at 20  $\mu\text{g}/\text{mL}$  and globulin fractions at 10  $\mu\text{g}/\text{mL}$ , incubated overnight at  $4^{\circ}\text{C}$  and blocked with 3% BSA (A7906, Sigma) in PBS-T (PBS with 0.05% Tween 20). Sera were diluted at 1:50,000, 1:1,000 and 1:5 for detection of IgG1, IgG2a and IgE, respectively. After incubation for 2 h at  $37^{\circ}\text{C}$ , plates were washed (PBS-T) and biotinylated rat anti-mouse IgG1 (550331, Becton Dickinson), IgG2a (553388, Becton Dickinson) or IgE (553414, Becton Dickinson) antibodies were added. After washing, streptavidin peroxidase conjugate (E2886, Sigma) was added (30 min). The enzymatic reaction was developed with TMB (T5525, Sigma) for 15 min. Absorbance was measured at 450 nm using Sunrise-Tecan automatic reader.

Total IgE was determined by employing ELISA according to the manufacturer's instructions (OptEIA™ Mouse IgE Set – 555248, Becton Dickinson). Serum was diluted at 1:100. For each mouse, concentrations of immunoglobulins were the mean of triplicates.

### Spleen lymphocyte isolation, cytokine analysis

Lymphocytes were isolated from pooled spleens removed from mice ( $n=5$ ) of each group according to a standard method [Maddaloni *et al.*, 2006]. A total of  $2 \times 10^6$  cells per 1 mL were cultured in the complete medium at  $37^{\circ}\text{C}$  in the atmosphere of 5%  $\text{CO}_2$ . Complete cell culture medium containing RPMI 1640 medium (R8758, Sigma) was supplemented with 10% heat-inactivated newborn calf serum (N4762, Sigma), 1% non-essential amino acids (M7145, Sigma) and 1% penicillin-streptomycin solution (P4333, Sigma). Cells were cultured in the medium only (negative control) or stimulated with PnE (100  $\mu\text{g}/\text{mL}$ ), peanut globulin fractions (50  $\mu\text{g}/\text{mL}$ ) or ConA (5  $\mu\text{g}/\text{mL}$ ) (positive control).

Levels of IFN- $\gamma$ , IL-4 and IL-10 in the supernatants of 48-h cultures were determined in triplicate runs using commercial enzyme-linked immunosorbent assay (ELISA) kit reagents according to the manufacturer's instructions (BD Pharmingen, Mississauga, Canada: Mouse IFN- $\gamma$  ELISA set – BD

555138, Mouse IL-4 ELISA set – BD 555232 and Mouse IL-10 ELISA set – BD 555252).

### B cell Ab ELISPOT

For antigen-formatting cells (AFC), IgG antigen specific ELISPOT was done according to a standard method [Maddaloni *et al.*, 2006]. MultiScreen-IP plate (Millipore, MSIPS4510) were then coated with 20  $\mu\text{g}/\text{mL}$  of antigen in sterile PBS overnight at 4°C. The plates were blocked with cRPMI at 37°C for 1 h, and then washed five times with PBS. A total of 100  $\mu\text{L}$  of cells from spleen at  $1 \times 10^5$  concentrations was added to the well. The plates were incubated overnight at 37°C in 5%  $\text{CO}_2$  atmosphere. To detect AFC response, anti-mouse IgG-HRP conjugates (A4416, Sigma) were added, and the plates were incubated overnight at 4°C. After washing, the plates were developed with AEC substrate set (551951, Becton Dickinson) for 15 min at room temperature, and the reaction was stopped with water. Spot quantitation was performed by counting under stereo zoom microscope (OLYMPUS, SZX 9) the following day.

### Statistical analysis

All values are expressed as means  $\pm$  SEM for 5 animal/group. Data were analysed by one-way ANOVA, and then differences among means were analysed using Fisher-Protected Least Significant difference for multiple – comparison procedure. The level of significance was set at  $p < 0.05$ .

## RESULTS AND DISCUSSION

The BALB/c mice were tested for their use in studies on immunomodulation of food reaction induced by peanut proteins. To sensitize BALB/c mice, peanut proteins (PnE) were administered in 0.5 to 1 mg dose to mice 6–8 weeks of age on the 1<sup>st</sup>, 7<sup>th</sup>, and 21<sup>st</sup> day of the study. A dose higher than recommended induced weak production of IgE [Li *et al.*, 2000] and could promote production of IgG2a [Arps *et al.*, 1998]. The antigen was administered orally and intraperitoneally in a native form or in the presence of Alum. The concentration of antibodies in serum was monitored during immunization. The highest level of total IgE (Figure 1) and PnE-specific IgG (Figure 2) was observed in the sensitized group after intraperitoneal administration of antigen in the presence of adjuvant. However, it was observed that the administration of the third dose of adjuvant significantly increased the studied antibodies (day 28). The presence of adjuvant affected mainly the level of total IgE (Figure 1). The concentration of specific-IgG in groups vaccinated without adjuvant was lower as compared to groups administered with PnE with Alum; although, this difference was not so significant as in the case of IgE, which indicated huge immunogenicity of peanut proteins that were administered intraperitoneally (Figure 2). It is interesting to observe that the presence of Alum stimulated mainly the production of PnL-specific IgG (Figure 3). Oral administration of peanut proteins induced food tolerance in BALB/c mice (Figures 1, 2, 3).

It is commonly considered that the level of specific IgE in serum determines protein allergenicity [Fæste & Namork, 2010]. In our studies, peanut-specific IgE was assayed

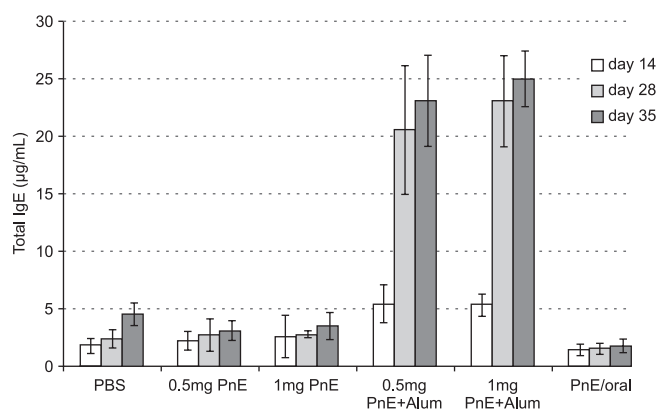


FIGURE 1. Total IgE levels in the serum of mice during sensitization with peanut proteins (PnE); PBS – control group; 0.5 mg PnE and 1 mg PnE – IP sensitised groups without Alum; 0.5 mg PnE + Alum and 1 mg PnE + Alum – IP sensitised groups with Alum; PnE/oral – orally-sensitised group.

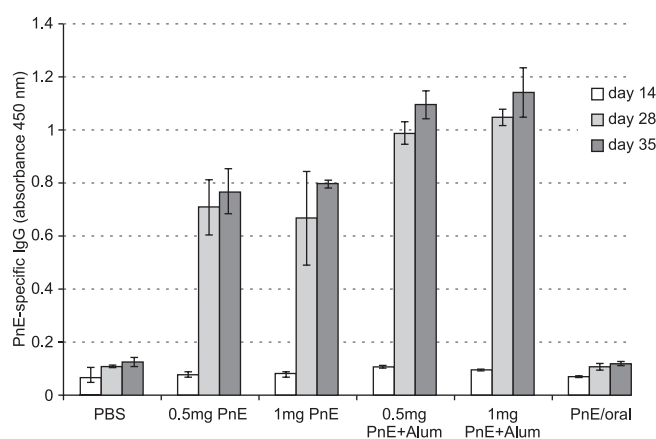


FIGURE 2. PnE-specific IgG levels in the serum of mice during sensitization with peanut proteins (PnE); serum dilution 1:50,000; PBS – control group; 0.5 mg PnE and 1 mg PnE – IP sensitised groups without Alum; 0.5 mg PnE + Alum and 1 mg PnE + Alum – IP sensitised groups with Alum; PnE/oral – orally-sensitised group.

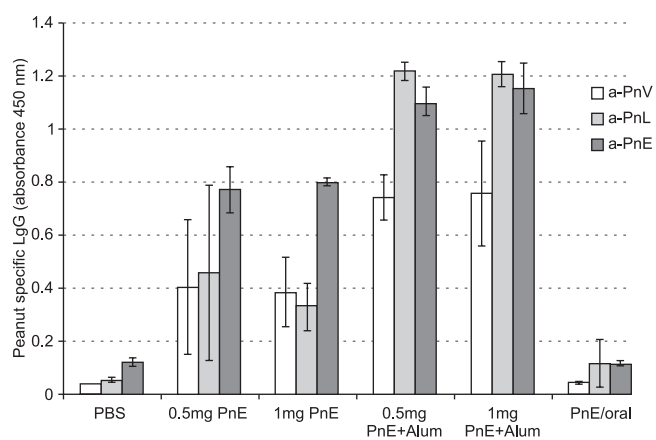


FIGURE 3. Peanut specific IgG levels in the serum of mice after sensitization with peanut proteins (PnE); serum dilution 1:50,000; PBS – control group, 0.5 mg PnE and 1 mg PnE – IP sensitised groups without Alum; 0.5 mg PnE + Alum and 1 mg PnE + Alum – IP sensitised groups with Alum; PnE/oral – orally-sensitised group; a-PnV – peanut vicilin – specific IgG; a-PnL – peanut legumin – specific IgG; a-PnE – peanut protein extract – specific IgG.

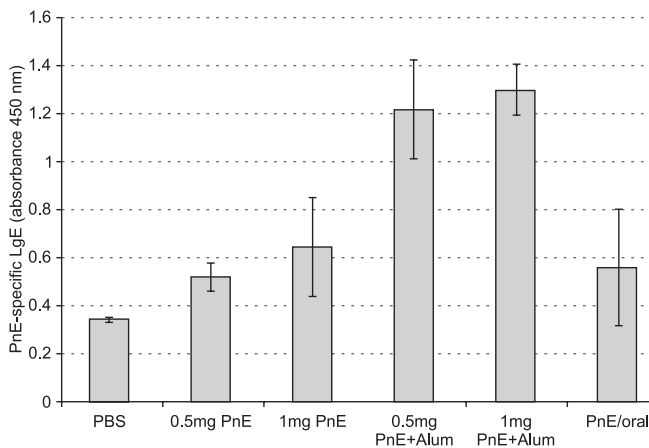


FIGURE 4. PnE-specific IgE levels in the serum of mice after sensitization with peanut proteins (PnE); serum dilution 1:5; PBS – control group; 0.5 mg PnE and 1 mg PnE – IP sensitised groups without Alum; 0.5 mg PnE + Alum and 1 mg PnE + Alum – IP sensitised groups with Alum; PnE/oral – orally-sensitised group.

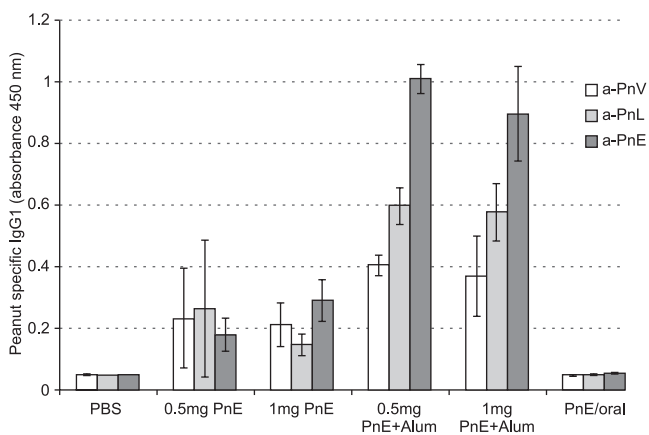


FIGURE 5. Peanut specific IgG1 levels in the serum of mice after sensitization with peanut proteins (PnE); serum dilution 1:50,000; PBS – control group, 0.5 mg PnE and 1 mg PnE – IP sensitised groups without Alum; 0.5 mg PnE + Alum and 1 mg PnE + Alum – IP sensitised groups with Alum; PnE/oral – orally-sensitised group; a-PnV – peanut vicilin – specific IgG1; a-PnL – peanut legumin – specific IgG1; a-PnE – peanut protein extract – specific IgG1.

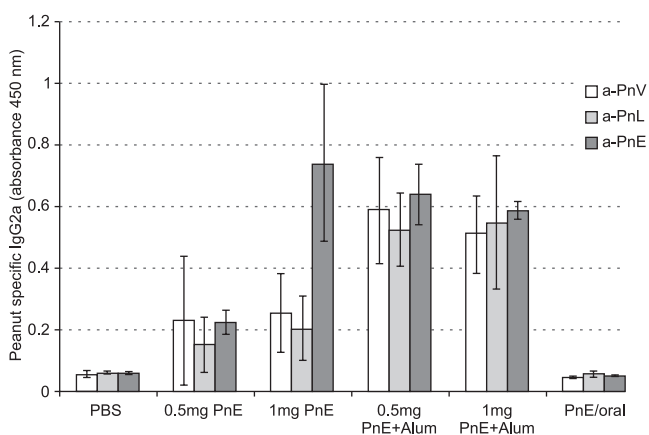


FIGURE 6. Peanut specific IgG2a levels in the serum of mice after sensitization with peanut proteins (PnE); serum dilution 1:1,000; PBS – control group; 0.5 mg PnE and 1 mg PnE – IP sensitised groups without Alum; 0.5 mg PnE + Alum and 1 mg PnE + Alum – IP sensitised groups with Alum; PnE/oral – orally-sensitised group; a-PnV – peanut vicilin – specific IgG2a; a-PnL – peanut legumin – specific IgG2a; a-PnE – peanut protein extract – specific IgG2a.

by ELISA method. The results obtained indicated an increase in the secretion of specific IgE in mouse serum after intra-peritoneal administration with and without Alum; however, the determined concentration of given antibodies was quite low (Figure 4). We assume that this could result from the method which was used to determine specific IgE. It is possible that antigen-specific IgG, which occurs at a very high concentration, will inhibit the detection of binding of antigen-specific IgE in ELISA tests. Goodman *et al.* [2008] demonstrated that *in vitro* serum IgE measurements did not always correlate with biological reactivity, as measured by PCA (passive cutaneous anaphylaxis assay), ACA (active cutaneous anaphylaxis assay) or ASA (active systemic anaphylaxis assay). Additionally, it was considered that the *development of peanut allergy* was accompanied not only by the increase in specific IgE but also through the elevated level of other biomarkers, such as specific IgG1 or cytokines profile, which play a significant role in this process [Baeza & Zubeldia, 2007; Miyajima *et al.*, 1997; Van Wijk & Knippels, 2007].

In our study, 3-fold administration of PnE resulted in a significant increase in the level of peanut-specific IgG1 as compared to PBS-immunised group (Figure 5). The presence of adjuvant induced significantly a higher level of these antibodies. The concentration of specific IgG2a was also higher than in the control group (Figure 6). It is interesting that Alum stimulated much better the production of specific IgG2a than IgG1. Similar results were cited by Ladics *et al.* [2010] based on unpublished TNO data. However, a high ratio of IgG1/IgG2a indicated the immunological response according to Th2 type, a characteristic trait of food allergy [Aldemir *et al.*, 2009; Blumchen *et al.*, 2010; Jones *et al.*, 2009]. Gizzarelli *et al.* [2006] reported that a high level of both IgE and IgG1 as well as a lower level of IgG2a, together with the production of IL-4 and IL-5, were observed in the case of Th2 type responses to soybean proteins. A relatively high concentration of PnL-specific IgG1 in BALB/c mouse serum indicated a stronger allergenicity to peanut arachin (Ara h 3) than to conarachin (Aha h 1). Van Wijk *et al.* [2005] demonstrated that oral exposure of C3H/HeJ mice to peanut proteins resulted in the production of specific IgE antibodies against the peanut allergens Ara h 3 and Ara h 6, and to a lesser degree against Ara h 1 and Ara h 2. However, it is known that Ara h 1 and Ara h 2, being allergens of vicilin (7S type) and albumin (2S), respectively, are the most potent peanut allergens [Burks *et al.*, 1998]. Similar studies, which were performed on animal models, demonstrated that oral exposure [Van Wijk *et al.*, 2005] and IP vaccination (our results) did not clearly confirm this finding.

High homology of amino acid sequences was observed among globulin proteins of leguminous plants [Barre *et al.*, 2005a,b; 2007; 2008; Shutov *et al.*, 1995], which could result in cross-reactivity [Beardslee *et al.*, 2000; Fæste & Namork, 2010; Ibañez *et al.*, 2003]. Mice presented a peanut-specific IgG1 response that cross-reacted with pea proteins (Figure 7). The strongest reactions were observed in relation to the extract from pea proteins (PE). Weak reaction with PV and PL may prove that cross-reactivity between peanut and pea proteins results from the presence of proteins other than globulin proteins of 11S and 7S type (Figure 7).



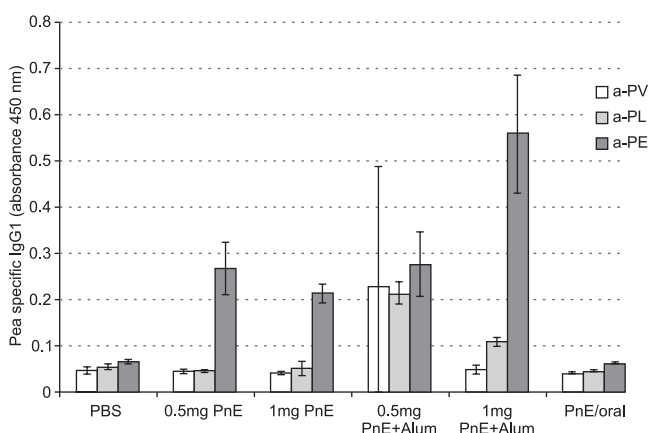


FIGURE 7. Pea specific IgG1 levels in the serum of mice after sensitization with peanut proteins (PnE); serum dilution 1:1,000; PBS – control group, 0.5 mg PnE and 1 mg PnE – IP sensitised groups without Alum; 0.5 mg PnE + Alum and 1 mg PnE + Alum – IP sensitised groups with Alum; PnE/oral – orally-sensitised group; a-PV – pea vicilin – specific IgG1; a-PL – pea legumin – specific IgG1; a-PE – pea protein extract – specific IgG1.

The administered amount of peanut protein (0.5 or 1 mg) had no significant effect on the level of the studied peanut- and pea-specific antibodies. The results obtained by ELISA on the cellular level were confirmed by ELISPOT test. A statistically significant 2-fold increase in AFC was observed in groups vaccinated with adjuvant addition as compared to the control groups; however, no effect of the amount of the administered peanut protein on the lymphocyte reaction was observed (Table 1).

Not only change in the level of specific antibodies, but also cytokine profile, which promotes Th2 response, determines the development of food allergy. It is known that development of helper lymphocytes of Th2 type is related to initiation and maintenance of IgE-mediated immunological response. Interleukin-4 (IL-4), which is produced by Th2 cells, is essential for the production of IgE. Cytokine (IFN- $\gamma$ ) secreted by Th1 type cells represents the antagonist of IgE response [Male *et al.*, 2008, Akdis & Akdis, 2009].

In PnE-sensitised groups without Alum, a high level of IL-4 (Figure 8) and this cytokine was secreted to a much higher extent as compared to IFN- $\gamma$  (Figure 9). In addition, the isolated peanut fractions (PnL and PnV) activated lymphocytes towards Th2 response. A slight increase in the PnL-specific IFN- $\gamma$  was detected during stimulation of lymphocyte culture derived from mice vaccinated with PnE in the presence of Alum. This peanut fraction also induced a significant increase in the secretion of IL-10 (Figure 10). Contrary to the role of IL-4, it is difficult to explain the function of the elevated level of IL-10. IL-10 is described as a cytokine of Th2 profile [Fiorentino *et al.*, 1989], which causes suppression of IFN- $\gamma$  and IL-12 secretion [Hsu *et al.*, 1992] and induces inflammatory responses in autoimmune diseases [Strobel, 2002]. However, it may also be related to induction of oral tolerance [Strobel, 2002] as well as suppression of allergenic inflammatory responses induced by an increased activity of Th2 lymphocytes [Barnes *et al.*, 2001; Romagnani, 2000]. Based on our studies, it may be suggested that the IL-10 level is related to the development of oral tol-

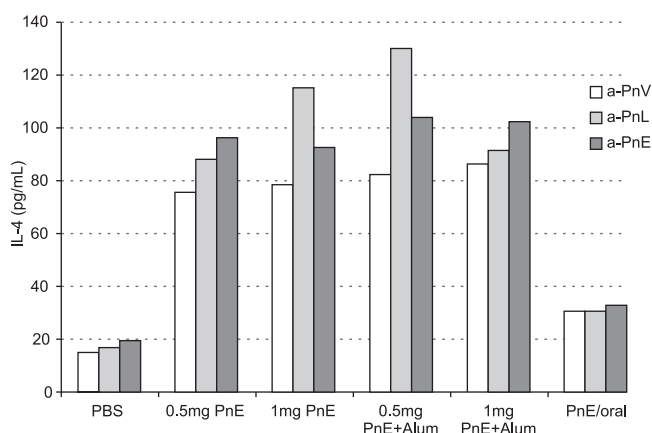


FIGURE 8. IL-4 levels in lymphocytes culture supernatants. Cells cultured in medium with presence of peanut vicilin (a-PnV), peanut legumin (a-PnL) or peanut proteins extract (a-PnE). Medium was the negative control and medium containing ConA the positive control (data not shown). PBS – control group; 0.5 mg PnE and 1 mg PnE – IP sensitised groups without Alum; 0.5 mg PnE + Alum and 1 mg PnE + Alum – IP sensitised groups with Alum; PnE/oral – orally-sensitised group.

TABLE 1. The B-cells ELISPOT for PnE-specific IgG; PBS – control group; 0.5 mgPnE and 1 mgPnE – IP sensitised groups without Alum; 0.5 mgPnE+Alum and 1 mgPnE+Alum – IP sensitised groups with Alum; PnE/oral – orally-sensitised group.

Groups	PnE-specific AFC/4x10 <sup>5</sup> lymphocytes
Control (PBS)	31 ± 1.69
0.5 mg PnE	38 ± 1.63
1 mg PnE	56 ± 2.35
0.5 mg PnE + Alum	87 ± 9.97
1 mg PnE + Alum	81 ± 3.29
PnE – orally-immunised	23 ± 1.63

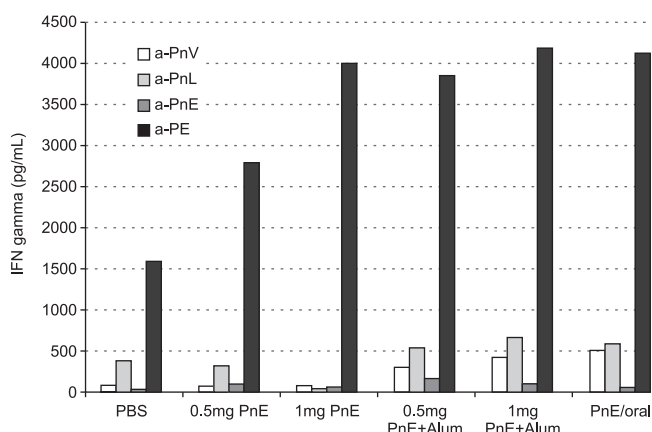


FIGURE 9. IFN- $\gamma$  levels in lymphocytes culture supernatants. Cells cultured in medium with presence of peanut vicilin (a-PnV), peanut legumin (a-PnL), peanut proteins extract (a-PnE) or pea protein extract (a-PE). Medium was the negative control and medium containing ConA the positive control (data not shown). PBS – control group; 0.5 mg PnE and 1 mg PnE – IP sensitised groups without Alum; 0.5 mg PnE + Alum and 1 mg PnE + Alum – IP sensitised groups with Alum; PnE/oral – orally sensitised group.

erance to peanut proteins (orally administered) (Figure 10). Morafo *et al.* [2003] observed that an increase in IL-10 concentration by splenocytes stimulated with milk proteins was related to an increase in IL-4 level and a decrease of IFN- $\gamma$  to the zero value. This clearly demonstrates the reaction according to Th2 type in C3H mice. In BALB/c mice, milk proteins stimulated huge secretion of IFN- $\gamma$  and at the same time maintained a low level of IL-4 and IL-10 (allergic response of Th1 type). According to researches, likewise in humans, the allergic process to milk proteins occurs in C3H mice based on the obtained information. In the case of administration of peanut proteins, these trends are not so explicit [Morafo *et al.*, 2003]. Opposite to milk proteins, peanut proteins induced production of specific IgE antibodies in BALB/c mice (not very high as in C3H mice). Milk proteins did not induce synthesis of IgE antibodies. It is worth noticing that the mice were sensitised orally with proteins in the presence of cholera toxins – adjuvant, which enhances the secretion of sIgA in some mice species, which then activates Th2 cells and promotes the production of IL-4 and IgE [Marinaro *et al.*, 1995]. In our studies, sensitization of BALB/c mice with IP method also resulted in the secretion of IL-4 in groups where no adjuvant was administered. When BALB/c mice were vaccinated intraperitoneally with peanut proteins in the presence of Alum, a slight increase in the IFN- $\gamma$  level was observed as compared to the control groups; although, this increase concerned only the trials that were stimulated *in vitro* by purified fraction of PnL. The PnL and PnV fractions also stimulated higher secretion of IFN- $\gamma$  by splenocytes isolated from mice, to which the antigen was administered orally. In this group, the level of IL-4 was only slightly higher than in the control group. In the remaining groups, there was a significant shift of balance towards reaction according to Th2 type. Intense secretion of IFN- $\gamma$  was observed in cultures of peanut-sensitised mice lymphocytes cultured in the presence of pea proteins (Figure 9), thus it may be concluded that pea proteins can activate Th1 cells.

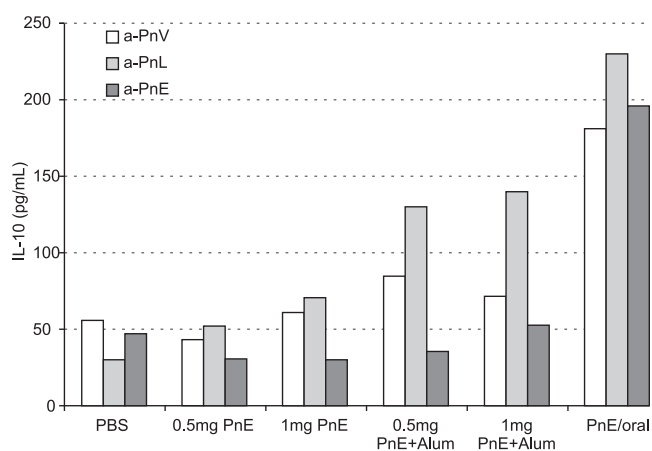


FIGURE 10. IL-10 levels in lymphocytes culture supernatants. Cells cultured in medium with presence of peanut vicilin (a-PnV), peanut legumin (a-PnL) or peanut proteins extract (a-PnE). Medium was the negative control and medium containing ConA the positive control (data not shown). PBS – control group; 0.5 mg PnE and 1 mg PnE – IP sensitised groups without Alum; 0.5 mg PnE + Alum and 1 mg PnE + Alum – IP sensitised groups with Alum; PnE/oral – orally-sensitised group.

## SUMMARY

The low cross-reactivity between peanut proteins and pea globulins was found with using the mice BALB/c as a biological *in vivo* model in the research. Based on results achieved, it may be concluded that the pea globulins can be applied in immunotherapy of peanut allergy with a potential success.

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## REFERENCES

1. Akdis C.A., Akdis M., Mechanisms and treatment of allergic disease in the big picture of regulatory T cells. *J. Allergy Clin. Immunol.*, 2009, 123, 735–746.
2. Aldemir H., Bars R., Herouet-Guicheney C., Murine model for evaluating the allergenicity of novel proteins and foods. *Reg. Toxicol. Pharmacol.*, 2009, 54, S52–S57.
3. Arps V., Sudowe S., Kölsch E., Antigen dose-dependent differences in IgE antibody production are not due to polarization towards Th1 and Th2 cell subsets. *Eur. J. Immunol.*, 1998, 28, 681–686.
4. Baeza M.L., Zubeldia J.M., Immunology of anaphylaxis: lessons from murine models. *Curr. Allergy Astma Rep.*, 2007, 7, 49–55.
5. Barnes P.J., IL-10: a key regulator of allergic disease. *Clin. Exp. Allergy*, 2001, 31, 667–669.
6. Barre A., Borges J.P., Culerrier R., Rouge P., Homology modelling of the major peanut allergen Ara h 2 and surface mapping of IgE-binding epitopes. *Immunol. Lett.*, 2005a, 100, 153–158.
7. Barre A., Borges J.F., Rougé P., Molecular modelling of the major allergen Ara h1 and other homotrimeric allergens of the cupin superfamily: a structural basis for their IgE-binding cross-reactivity. *Biochimie*, 2005b, 87, 499–506.
8. Barre A., Sordet C., Culerrier R., Rance F., Didier A., Rouge P., Vicilin allergens of peanut and tree nuts (walnut, hazelnut and cashew nut) share structurally related IgE-binding epitopes. *Mol. Immunol.*, 2008, 45, 1231–1240.
9. Barre A., Jacquet G., Sordet C., Culerrier R., Rougé P., Homology modelling and conformational analysis of IgE-binding epitopes of Ara h 3 and other legumin allergens with a cupin fold from tree nuts. *Mol. Immunol.*, 2007, 44, 3243–3255.
10. Beardslee T.A., Zeece M.G., Sarath G., Markwell J.P., Soybean glycinin G1 acidic chain shares IgE epitopes with peanut allergen Ara h 3. *Int. Arch. Allergy Immunol.*, 2000, 123, 299–307.
11. Bielikowicz K., Wojtacha P., Kostyra E., Iwan M., Jarmolowska B., Kostyra H., Influence of glycation of wheat albumins and globulins on their immunoreactivity and physicochemical properties. *Pol. J. Food Nutr. Sci.*, 2010, 60, 335–340.
12. Blumchen K., Ulbricht H., Staden U., Dobberstein K., Beschoner J., de Oliveira L.C., Shreffler W.G., Sampson H.A., Niggemann B., Wahn U., Beyer K., Oral peanut immunotherapy in children with peanut anaphylaxis. *J. Allergy Clin. Immunol.*, 2010, 126, 83–91.
13. Bowman Ch.C., Selgrade M.K., Differences in allergenic potential of food extracts following oral exposure in mice reflect differ-

- ences in digestibility: potential approaches to safety assessment. *Toxicol. Sci.*, 2008, 102, 100–109.
14. Burks A.W., Peanut allergy. *Lancet*, 2008, 371, 1538–1546.
  15. Burks A.W., Sampson H.A., Bannon G.A., Peanut allergens. *Allergy*, 1998, 53, 725–730.
  16. De Leon M.P., Drew A.C., Glaspole I.N., Suphioglu C., O’Hehir R.E., Rolland J.M., IgE cross-reactivity between the major peanut allergen Ara h 2 and tree nut allergens. *Mol. Immunol.*, 2007, 44, 463–471.
  17. Dearman R.J., Kimber I., A mouse model for food allergy using intraperitoneal sensitization. *Methods*, 2007, 41, 91–98.
  18. Delayre-Orthez C., de Blay F., Frossard N., Pons F., Dose-dependent effects of endotoxins on allergen sensitization and challenge in the mouse. *Clin. Exp. Allergy*, 2004, 34, 1789–1795.
  19. Eisenbarth S.C., Piggott D.A., Huleatt J.W., Visintin I., Herrick C.A., Bottomly K., Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J. Exp. Med.*, 2002, 196, 1645–1651.
  20. Fæste C.K., Namork E., Differentiated patterns of legume sensitisation in peanut-allergic patients. *Food Anal. Methods*, 2010, 3, SI, 357–362.
  21. FAO/WHO 2001. Evaluation of allergenicity of genetically modified foods. Report of a joint FAO/WHO expert consultation on allergenicity of foods derived from biotechnology. Rome: Food and Agriculture Organization of the United Nations.
  22. Finkelman F.D., Peanut allergy and anaphylaxis. *Curr. Opin. Immunol.*, 2010, 22, 783–788.
  23. Fiorentino D.F., Bond M.W., Mosmann T.R., Two types of mouse T-helper cell. IV.Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J. Exp. Med.*, 1989, 170, 2081–2095.
  24. Freitas R.L., Ferreira R.B., Teixeira A.R., Use of a single method in the extraction of the seed storage globulins from several legume species. Application to analyse structural comparisons within the major classes of globulins. *Int. J. Food Sci. Nutr.*, 2000, 51, 341–352.
  25. Ganeshan K., Neilsen C.V., Hadsaitong A., Schleimer R.P., Luo X., Bryce P.J., Impairing oral tolerance promotes allergy and anaphylaxis: a new murine food allergy model. *J. Allergy Clin. Immunol.*, 2009, 123, 231–238.
  26. Gizzarelli F., Corinti S., Barletta B., Iacovacci P., Brunetto B., Butteroni C., Afferni C., Onori R., Miraglia M., Panzini G., Di Felice G., Tinghino R., Evaluation of allergenicity of genetically modified soybean protein extract in a murine model of oral allergen-specific sensitization. *Clin. Exp. Allergy*, 2006, 36, 238–248.
  27. Goodman R.E., Vieths S., Sampson H.A., Hill D., Ebisawa M., Taylor S.L., van Ree R., Allergenicity assessment of genetically modified crops – what makes sense? *Nat. Biotech.*, 2008, 26, 73–81.
  28. Helm R.M., Food allergy animal models – an overview. *Ann. N. Y. Acad. Sci.*, 2002, 964, 139–150.
  29. Hilton J., Dearman R.J., Sattar N., Basketter D.A., Kimber I., Characteristics of antibody responses induced in mice by protein allergens. *Food Chem. Toxicol.*, 1997, 35, 1209–1218.
  30. Hsu D.H., Moore K.W., Spits H., Differential effects of IL-4 and IL-10 on IL-2-induced IFN-gamma synthesis and lymphokine-activated killer activity. *Int. Immunol.*, 1992, 4, 563–569.
  31. Ibañez D., Martínez M., Sánchez J.J., Fernández-Caldas E., Legume: cross-reactivity. *Allergol. Immunopathol.*, 2003, 31, 151–161.
  32. Jones S.M., Pons L., Roberts J.L., Scurlock A.M., Perry T.T., Kulis M., Shreffler W.G., Steele P., Henry K.A., Adair M., Francis J.M., Durham S., Vickery B.P., Zhong X., Burks A.W., Clinical efficacy and immune regulation with peanut oral immunotherapy. *J. Allergy Clin. Immunol.*, 2009, 124, 292–300.
  33. Kaplan A.P., Chemokines, chemokine receptors and allergy. *Int. Arch. Allergy Immunol.*, 2001, 124, 423–431.
  34. Khodoun M., Strait R., Orekov T., Hogan S., Karasuyama H., Herbert D.R., Kohl J., Finkelman F.D., Peanuts can contribute to anaphylactic shock by activating complement. *J. Allergy Clin. Immunol.*, 2009, 123, 342–351.
  35. Knippels L.M., van Wijk J., Penninks A.H., Food allergy: what do we learn from animal models? *Curr. Opin. Allergy Clin. Immunol.*, 2004, 4, 205–209.
  36. Kopper R.A., Odum N.J., Sen M., Helm R.M., Stanley J.S., Burks A.W., Peanut protein allergens: the effect of roasting on solubility and allergenicity. *Int. Arch. Allergy Immunol.*, 2005, 136, 16–22.
  37. Ladics G.S., Knippels L.M.J., Penninks A.H., Bannon G.A., Goodman R.E., Herouet-Guicheney C., Review of animal models designed to predict the potential allergenicity of novel proteins in genetically modified crops. *Reg. Toxicol. Pharmacol.*, 2010, 56, 212–224.
  38. Lee S.Y., Huang C.K., Zhang T.F., Schofield B.H., Burks A.W., Bannon G.A., Sampson H.A., Oral administration of IL-12 suppresses anaphylactic reactions in a murine model of peanut hypersensitivity. *Clin. Immunol.*, 2001, 101, 220–228.
  39. Lehrer S.B., McClain S., Utility of animal models for predicting human allergenicity. *Reg. Toxicol. Pharmacol.*, 2009, 54, S46–S51.
  40. Li X.M., Serebrisky D., Lee S.Y., Huang C.K., Bardina L., Schofield B.H., Stanley J.S., Burks A.W., Bannon G.A., Sampson H.A., A murine model of peanut anaphylaxis: T- and B-cell responses to a major peanut allergen mimic human responses. *J. Allergy Clin. Immunol.*, 2000, 106, 150–158.
  41. Lifrani A., Dubarry M., Rautureau M., Aattouri N., Boyaka P.N., Tome D., Peanut-lupine antibody cross-reactivity is not associated to cross-allergenicity in peanut-sensitized mouse strains. *Inter. Immunopharmacol.*, 2005, 5, 1427–1435.
  42. Maddaloni M., Staats H.F., Mierzejewska D., Hoyt M., Robinson A., Callis G., Kozaki S., Kiyono H., McGhee J.R., Fujihashi K., Pascual D.W., Mucosal vaccine targeting improves onset of mucosal and systemic immunity to botulinum neurotoxin A. *J. Immunol.*, 2006, 177, 5524–5532.
  43. Male D., Brostoff J., Roth D.B., Roitt I., *Immunologia*. 2008, Elsevier Urban & Partner, Wrocław, Poland, pp. 217–226 (in Polish).
  44. Maleki S.J., Kopper R.A., Shin D.S., Park C.W., Compadare C.M., Sampson H., Burks W., Bannon G.A., Structure of the major peanut allergen Ara h 1 may protect IgE-binding epitopes from degradation. *J. Immunol.*, 2000, 164, 5844–5849.
  45. Maloney J.M., Rudengren M., Ahlstedt S., Bock S.A., Sampson H.A., The use of serum-specific IgE measurements for the diagnosis of peanut, tree nut, and seed allergy. *J. Allergy Clin. Immunol.*, 2008, 122, 145–151.
  46. Marinaro M., Staats H.F., Hiroi T., Jackson R.J., Coste M., Boyaka P.N., Okahashi N., Yamamoto M., Kiyono H., Bluethmann H., Mucosal adjuvant effect of cholera toxin in mice results from induction of T helper 2 (Th2) cells and IL-4. *J. Immunol.*, 1995, 155, 4621–4629.

47. Martínez San Ireneo M., Ibáñez M.D., Sánchez J.J., Carnés J., Fernández-Caldas E., Clinical features of legume allergy in children from a Mediterranean area. *Ann. Allergy Asthma Immunol.*, 2008, 101, 179–84.
48. McClain S., Bannon G.A., Animal models of food allergy: opportunities and barriers. *Curr. Allergy Asthma Rep.*, 2006, 6, 141–144.
49. Minkiewicz P., Dziuba J., Gładkowska I., Update of the list of allergenic proteins from milk, based on local amino acid sequence identity with known epitopes from bovine milk proteins – a short report. *Pol. J. Food Nutr. Sci.*, 2011, 61, 153–158.
50. Miyajima I., Dombrowicz D., Martin T.R., Ravetch J.V., Kinet J.P., Galli S.J., Systemic anaphylaxis in the mouse can be mediated largely through IgG1 and Fc-gamma-RIII: assessment of the cardiopulmonary changes, mast cell degranulation, and death associated with active or IgE- or IgG1-dependent passive anaphylaxis. *J. Clin. Invest.*, 1997, 99, 901–914.
51. Morafo V., Srivastava K., Huang C.K., Kleiner G., Lee S.Y., Sampson H., Li X., Genetic susceptibility of food allergy is linked to differential TH2-TH1 responses in C3H/HeJ and BALB/c mice. *J. Allergy Clin. Immunol.*, 2003, 111, 1122–1128.
52. Pons L., Ponnappan U., Hall R.A., Simpson P., Cockrell G., West M., Sampson H.A., Helm R.M., Burks A.W., Soy immunotherapy for peanut-allergic mice: Modulation of the peanut-allergic response. *J. Allergy Clin. Immunol.*, 2004, 114, 915–921.
53. Pramod S.N., Venkatesh Y.P., Mahesh P.A., Potato lectin activates basophils and mast cells of atopic subjects by its interaction with core chitobiose of cell-bound non-specific immunoglobulin E. *Clin. Exp. Immunol.*, 2007, 148, 391–401.
54. Romagnani S., The role of lymphocytes in allergic disease. *J. Allergy Clin. Immunol.*, 2000, 105, 399–408.
55. Shutov A.D., Kakhovskaya I.A., Braun H., Baumlein H., Muntz K., Legumin-like and vicilin-like seed storage proteins: evidence for a common single-domain ancestral gene. *J. Mol. Evol.*, 1995, 41, 1057–1069.
56. Sicherer S.H., Muñoz-Furlong A., Burks A.W., Sampson H.A., Prevalence of peanut and tree nut allergy in the United States determined by means of random digit dial telephone survey: a 5-year follow-up study. *J. Allergy Clin. Immunol.*, 2003, 112, 1203–1207.
57. Sicherer S.H., Sampson H.A., Food allergy. *J. Allergy Clin. Immunol.*, 2010, 125, S116–S125.
58. Strid J., Thomson M., Hourihane J., Kimber I., Strobel S., A novel model of sensitization and oral tolerance to peanut protein. *Immunology*, 2004, 113, 293–303.
59. Strobel S., Oral tolerance, systemic immunoregulation and autoimmunity. *Ann. N. Y. Acad. Sci.*, 2002, 958, 47–58.
60. Sumiyoshi M., Sakanaka M., Kimura Y., Effects of Red Ginseng extract on allergic reactions to food in Balb/c mice. *J. Ethnopharm.*, 2010, 132, 206–212.
61. Thyagarajan A., Varshney P., Jones S.M., Sicherer S., Wood R., Vickery B.P., Sampson H., Burks A.W., Peanut oral immunotherapy is not ready for clinical use. *J. Allergy Clin. Immunol.*, 2010, 126, 31–32.
62. Van Wijk F., Knippels L., Initiating mechanisms of food allergy: Oral tolerance versus allergic sensitization. *Biomed. Pharmacother.*, 2007, 61, 8–20.
63. Van Wijk F., Nierkens S., Hassing I., Feijen M., Koppelman S.J., de Jong G.A.H., Pieters R., Knippels L.M.J., The effect of the food matrix on *in vivo* immune responses to purified peanut allergens. *Toxicol. Sci.*, 2005, 86, 333–341.
64. Yang M., Mine Y., Novel T-cell epitopes of ovalbumin in BALB/c mouse: potential for peptide-immunotherapy. *Biochem. Biophys. Res. Commun.*, 2009, 378, 203–208.

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