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Allergen-specific immunoglobulin, histamine and T-cell responses induced by soybean glycinin and β-conglycinin in BALB/c mice of oral sensitisation

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# Allergen-specific immunoglobulin, histamine and T-cell responses induced by soybean glycinin and $\beta$ -conglycinin in BALB/c mice of oral sensitisation

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Glycinin and  $\beta$ -conglycinin are major soybean allergens involved in food hypersensitivity. However, the mechanism of immune responses induced by glycinin and  $\beta$ -conglycinin has not been fully understood. Balb/c mice were oral sensitised with different doses (0.1, 1.0 and 10 mg/day) of soybean glycinin and  $\beta$ -conglycinin for five weeks. Allergen-specific immunoglobulin (Ig), serum histamine and T-cell responses were tested to assess the allergenic activity of glycinin and  $\beta$ -conglycinin. Mice sensitised with 0.1 or 1.0 mg/day allergens induced high levels of specific IgE, IgG1 and serum histamine compared with mice treated with saline. Furthermore, specific T-cell proliferation and significant up-regulation of interleukin (IL)-4, IL-5 and interferon (IFN)- $\gamma$  were observed in splenocytes from mice gavaged with 0.1 or 1.0 mg/day soybean proteins. Low doses of glycinin or  $\beta$ -conglycinin can induce allergic reactions in BALB/c mice, which might be associated with increased IgE and cytokine production.

Keywords: glycinin; β-conglycinin; Balb/c mice; anaphylaxis; oral sensitisation

#### Introduction

Soybeans contain approximately 36% to 38% protein with a wide range of applications both in the feed and food industries (Friedman & Brandon, 2001). The two major proteins in soybeans are glycinin and  $\beta$ -conglycinin, which stored in vacuoles of seed cells, and account for about 40% of the total seed proteins (Golubovic, van Hateren, Ottens, Witkamp, & van der Wielen, 2005). Glycinin and  $\beta$ -conglycinin are of interest, in addition to their anti-nutritional properties, because most of them have been observed to be allergenic (i.e. via IgE responses) in human or laboratory animals (Hou & Chang, 2004; Maruyama et al., 2001; Petruccelli, Chirdo, & Anon, 2005). Surveys have found the prevalence of soy allergy to be nearly 0.5% in the infants of western countries, although most of the allergic infants become tolerant of soy by the age of 3 years (Bock, 1987; Bruno et al., 1997; Sicherer, Sampson, & Burks, 2000). However, with the increasing consumption of soy-containing products, the incidence of soycaused allergies is expected to increase (FAO, 1995).

Soy foods have a long history of safe usage in Asian populations and are generally recognised as safe (US Food and Drug Administration, 1999). Merritt and Jenks (2004) suggested that dietary soy protein with isoflavones in soy infant formulas had no adverse effect on human health. However, food allergy caused by

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the ingestion of soybean products has been documented in many reports about clinical symptoms, which ranged from mild gastrointestinal distress to suspected anaphylaxis (Adachi et al., 2003; Helm et al., 2000). Nevertheless, unlike peanut allergy, it is uncommon to induce severe and fatal reactions in the soy allergy (Sicherer, Sampson, & Burks, 2000).

Much of our understanding of the responses induced by allergens has been obtained from the study of animal models. As a result, pigs (Hao, Zhan, Guo, Piao, & Li, 2009; Huang, Xu, Yu, Gao, & Liu, 2010; Sun, Li, Dong, Qiao, & Ma, 2008; Wang, Qin, Sun, Zhao, & Zhang, 2010; Zhao, Qin, Sun, Zhang, & Wang, 2010), rats (Guo, Piao, Ou, Li, & Hao, 2007; Han, Ma, & Yin, 2010) and mice (Gizzarelli et al., 2006; Oakes, Piller, & Bost, 2009) have been used for study of allergic reactions to soybean allergens. However, to our knowledge, there are few reports available on allergenic effects of purified soybean glycinin and  $\beta$ -conglycinin in mice. In addition, several studies have demonstrated that immune responses after oral sensitisation are influenced by the dosage of allergens (Adel-Patient, Bernard, Ah-Leung, Creminon, & Wal, 2005; Capobianco et al., 2008), but this has not been reported in soybean-induced anaphylaxis.

Our previous study has demonstrated anaphylactic reactions in BALB/c mice caused by soybean glycinin and  $\beta$ -conglycinin, including increased specific serum antibodies and histamine release (Liu, Feng, Xu, Wang, & Liu, 2008). In the present study, we aim to elucidate the effects of various doses of soybean allergens (glycinin or  $\beta$ -conglycinin) on the specific antibody and T-cell responses induced in BALB/c female mice using the established model. Features obtained in our study may provide valuable information for the development of a suitable *in vivo* model of soybean allergens and for exploring the underlying mechanisms related to soy-induced allergic reactions.

#### Material and methods

#### Mice and reagents

Second-generation female BALB/c mice (six-week-old, body weight,  $20.0\pm2.1$  g) purchased from the Zhejiang Chinese Medical University (Hangzhou, China) were kept in stainless steel wire cages (4 mice/cage) with soy-free feed and water *ad libitum*. To avoid oral tolerance, pregnant mice and mice after birth were both fed soy-free chow. Cages were put in a specific pathogen-free room maintained at  $22\pm3^{\circ}$ C, with a relative humidity of 55% to 65% and controlled lighting (10 h to 12 h/day) according to the guidelines of the Animal Care and Use Ethics Committee of the Zhejiang University (Hangzhou, China). Purified glycinin and  $\beta$ -conglycinin were offered by Professor Shuntang Guo of the Food Institute of China Agricultural University (Patent No. 200410029589.4, China). The protein samples contained over 95% glycinin and  $\beta$ -conglycinin. The purity was validated by sulfate polyacrylamide gel electrophoresis followed by Quantity One<sup>TM</sup> Software (Bio-Rad, Hercules, USA) analysis.

#### Intragastric antigen sensitisation

Mice were sensitised with glycinin or  $\beta$ -conglycinin as previously described (Liu et al., 2008). Briefly, fifty-six mice were divided into seven groups (n = 8/group). Mice

were sensitised by an intragastric administration of 0.1 mg, 1 mg or 10 mg of soybean glycinin or  $\beta$ -conglycinin/1 ml/mouse daily for 5 weeks without an adjuvant using an 18-gauge stainless steel gavage needle (Popper & Sons, Inc., NY, USA), while control animals received an equal volume of saline. Mice were bled from the retro-orbital plexus on days 0, 7, 14, 21, 28 and 35 using a 2-ml Clot Activator tube (Kremsmunster, Austria) 1 hour after the administration of soybean allergens. Samples were centrifuged for 20 minutes at 2000 × g and kept at 4°C to obtain sera. All samples were stored at 20°C until further analysis.

#### Measurement of soybean allergen-specific IgE, IgG1 and IgG2a levels

Serum allergen-specific IgE, IgG1 and IgG2a were detected by an indirect sandwichtype enzyme-linked immunosorbent assay (ELISA). In brief, 96-well microtiter plates (Costar, Corning Inc., NY, USA) were coated with 100 µl glycinin or  $\beta$ -conglycinin solution (50 µg/ml soybean allergens in 50 mM carbonate buffer (NaHCO<sub>3</sub>/Na<sub>2</sub>CO<sub>3</sub>, pH 9.6)) for 24 hours at 4°C. Plates were washed and blocked with 5% skim milk powder/phosphate buffered saline (PBS) at 37°C for 1 hour. Serum samples diluted in PBS containing 0.05% (s/v) Tween 20 (PBS/Tween) or standards (Sigma Chemical Co., Milan, Italy) were added and incubated for 1 hour at 4°C in 3 duplicates (diluted 1:20 for IgE measurement, 1:40 for IgG1 or IgG2a). Plates were again washed, and the bound antibodies were detected by adding a peroxidase-labelled rat monoclonal anti-mouse IgE (Bethyl, Inc., TX, USA) for 5 hours at room temperature or peroxidase-labelled rat monoclonal anti-mouse IgG1 and IgG2a (Bethyl, Inc., TX, USA) for 1 hour. Secondary antibodies were diluted 1:500. The peroxidase substrate (ortho-phenylenediamine chloride; Sigma-Aldrich, Milan, Italy) was then added, and absorbance at 490 nm was measured by a microtiter reader (Bio-Rad 550, Hercules, USA) after incubated at 37°C for 10 minutes. The data were expressed in optical density units. The ELISA assays were standardised by adding an irrelevant protein (e.g. ovalbumin) as a positive control to test specificity.

#### Measurement of serum histamine level

Concentrations of serum histamine on days 0, 7, 14, 21, 28 and 35 were determined using an enzyme immunoassay kit (Beckman Coulter) according to the manufacturer's instructions.

#### Spleen cell culture

After the last blood sampling at week 5, mice from each group were killed by vertebral dislocation, and individual spleens were aseptically removed and minced. After erythrocyte lysis, spleen cells were resuspended in complete medium (RPMI 1640 containing 10% fetal bovine serum, 2 mM L-glutamine, 25 mM Hepes, 100 IU/ ml penicillin, 100 mg/ml streptomycin). Spleen lymphocytes from individual mice were used in further analysis of proliferation assays and cytokine measurements.

#### Spleen cell proliferation

Lymphocyte proliferation assays were performed in triplicate according to 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method (Mosmann, 1983). Aliquots (100  $\mu$ l) of splenocyte suspensions at a concentration of  $5 \times 10^6$  cells/well were daily stimulated with 50 µl of 0.1, 1, 10 mg/ml glycinin or β-conglycinin, respectively (spleen cells that separated from mice received different daily dosages of glycinin, or  $\beta$ -conglycinin were stimulated *in vitro* with 50  $\mu$ l of the same concentration), whereas splenocytes from the mice treated with saline were added with 50 µl of saline as controls (un-stimulated). All cell cultures were incubated in 96-well flat bottom plates (Corning Costar Corp., Cambridge, USA). The plates were covered and incubated for 48 hours at 37°C in a 5% CO<sub>2</sub> incubator with 95% humidity. Then, 20 µl of freshly prepared MTT in serum-free RPMI1640 (5 mg/day) was added to each well after 44 hours. Cultures were incubated for a further 4 hours and then solubilisation reagent [100 µl of acidified isopropanol: distilled water (2:1, v/v)] was added. The absorbance was measured at 570 nm, based on the optical wavelengths for differentiating MTT using a microtiter reader (Bio-Rad 550, Hercules, USA) (Kong, Hu, Rui, Wang, & Li, 2004; Zhou et al., 2005). The stimulation index was calculated as the absorbance ratio of stimulated cells to that of un-stimulated cells.

#### Quantification of specific cytokines in splenocytes

Spleen lymphocytes prepared as noted above were cultured in 24-well plates ( $5 \times 10^6$  cells/ml/well), and treated with 50 µl of glycinin or  $\beta$ -conglycinin (0.1, 1 or 10 mg/ml), or un-stimulated (saline treated), respectively, as described above. The cell-free supernatants were collected after 3 days of culture at 37°C with 5% CO<sub>2</sub>, and aliquots were stored at  $-80^{\circ}$ C until analyzed. Levels of cytokines IFN- $\gamma$ , IL-4 and IL-5 were determined using commercially available ELISA kits according to the manufacturer's instructions (Biosource, Camarillo, CA, USA). Assays were performed in triplicate.

#### Statistical analysis

All data in experiment were expressed as the mean  $\pm$  standard deviation (SD) and analyzed by using SPSS 16.0 for Windows (SPSS Inc., Chicago, USA). The differences between the groups were analyzed by one-way ANOVA followed by Turkey's post hoc test. Values of p < 0.05 were considered as significant.

#### Results

#### Induction of soybean allergen-specific antibody response by oral sensitisation

To evaluate soybean protein–induced anaphylactic reactions in sensitised mice, doseresponses of antigen-specific IgE, IgG1 and IgG2a were compared kinetically by ELISA with three different amounts of glycinin or  $\beta$ -conglycinin (0.1, 1.0 and 10 mg/ mouse/day) on days 0, 7, 21 and 35 (Figure 1). The administration of glycinin or  $\beta$ -conglycinin induced soybean-specific IgE antibody after 2 weeks of sensitisation. A clear different IgE level was detected among the three dosages of allergen



Figure 1. Serum levels of specific IgE (a), IgG1 (b) and IgG2 (c) in BALB/c mice. Sera were collected on days 0, 7, 14, 21, 28 and 35 from groups of mice (n = 8) sensitised with different doses of glycinin or  $\beta$ -conglycinin. Glycinin 0.1, 1.0, 10 and  $\beta$ -conglycinin 0.1, 1.0, 10 represent 0.1, 1, 10 mg/day glycinin and 0.1, 1, 10 mg/day  $\beta$ -conglycinin, respectively. Levels of IgE, IgG1 and IgG2 were measured by ELISA. The data are presented as mean (OD<sub>490 nm</sub>)±SD from three separate experiments. \*, p < 0.05; \*\*, p < 0.01 versus saline-treated group.

(p < 0.05), with the maximum level in mice administered with 1.0 mg/day of glycinin or  $\beta$ -conglycinin on day 28 (Figure 1(a)).  $\beta$ -conglycinin,  $0.8 \pm 0.09$ ; glycinin,  $0.72 \pm$ 0.1; p < 0.01 vs. control). Then, IgE levels induced by gavage with 0.1 or 1.0 mg/day of soybean proteins were somewhat decreased on day 35. Control mice, immunised with saline, did not produce any detectable level of allergen-specific IgE (Figure 1(a)). Similarly, levels of specific IgG1 started to increase after week 2, reaching its plateau level in mice treated with 1.0 mg/day of glycinin or  $\beta$ -conglycinin (Figure 1(b)). glycinin,  $0.52 \pm 0.04$ ;  $\beta$ -conglycinin,  $0.66 \pm 0.02$ ; p < 0.01 vs. control). Specific IgG2a also increased from day 7 to day 28, but had a low production level (Figure 1(c)). No allergen-specific IgG responses were induced in control group of mice gavaged with saline. Additionally, no signals could be detected when testing ELISA specificity by irrelevant proteins (e.g., ovalbumin) (data not shown).

#### Induction of serum histamine release

We also measured the serum histamine levels 1 hour after soybean allergens sensitisation on days 0, 7, 14, 21, 28 and 35, because histamine is closely associated with the anaphylactic reactions. We found that serum histamine level was rapidly increased (p < 0.05) 2 weeks after oral sensitisation with the maximal release at day 28 in mice gavage with 1.0 mg/day glycinin or  $\beta$ -conglycinin (glycinin,  $1100 \pm 90$  nM;  $\beta$ -conglycinin,  $1220 \pm 80$  nM; p < 0.01 vs. control). As observed for Ig responses, histamine release was less intense after 28 days, but still showed a significant increase in the 1.0 mg/day-sensitised mice than in the control group gavaged with saline (Figure 2).



Figure 2. Response of serum histamine levels. Sera were collected on days 0, 7, 14, 21, 28 and 35 from groups of mice (n = 8) sensitised with different doses of glycinin or  $\beta$ -conglycinin. Glycinin 0.1, 1.0, 10 and  $\beta$ -conglycinin 0.1, 1.0, 10 represent 0.1, 1, 10 mg/day glycinin and 0.1, 1, 10 mg/day  $\beta$ -conglycinin, respectively. Histamine levels were determined using an enzyme immunoassay kit. The data are presented as mean  $\pm$  SD. \*, p < 0.05; \*\*, p < 0.01 versus saline-treated group.

#### Induction of spleen cell proliferative responses by oral sensitisation

To determine whether oral sensitisation with soybean proteins affected systemic T-cell responses, we assessed spleen cell proliferative responses to glycinin or  $\beta$ -conglycinin stimulation *in vitro*. As shown in Figure 3, splenocyte from groups of mice gavaged with 0.1 and 1.0 mg/day glycinin or  $\beta$ -conglycinin depicted significant proliferation activity in response to antigen stimulation (Figure 3). No differences were observed among splenocytes from the 0.1, 10 mg/day and saline-treated groups (p > 0.05).

#### T-cell cytokine profile induced by soybean protein stimulation

To determine the primed pathway of systemic T help (Th)1/Th2 responses, the cytokines produced by splenocyte were measured in culture supernatants. *In vitro* stimulation with 50 µl of 0.1 and 1 mg/ml glycinin or  $\beta$ -conglycinin induced significant levels of IL-4, IL-5 and IFN- $\gamma$  compared with control (Figure 4). Among the three different doses of allergens, splenocytes from mice gavaged with 1 mg/day glycinin or  $\beta$ -conglycinin exhibited the strongest proliferation responses with levels of IL-4 (613.5 pg/ml and 651.5 pg/ml), IL-5 (1384.0 pg/ml and 1530.3 pg/ml) and IFN- $\gamma$  (1372.0 pg/ml and 1432.1 pg/ml), respectively. Cytokine production after allergen stimulation was not changed in mice administered with 10 mg/day glycinin or  $\beta$ -conglycinin compared with the control mice (p > 0.05). Additionally, almost undetectable levels of cytokines were found in culture supernatants of saline-treated cells (un-stimulated cells).

#### Discussion

The development of *in vivo* murine models against soybean proteins provides an optimal tool for revealing the possible mechanism of anaphylactic symptoms induced by allergen ingestion. Recently, we have established a suitable BALB/c mice model by



Figure 3. Spleen cell proliferative response. Spleen cells from individual mice dosed with 0.1, 1 or 10 mg/day glycinin or  $\beta$ -conglycinin, were cultured with 50 µl of 0.1, 1.0 or 10 mg/ml soybean glycinin or  $\beta$ -conglycinin for 48 hours *in vitro*, respectively (spleen cells from mice dosed with 0.1 mg/day glycinin or  $\beta$ -conglycinin stimulated with 0.1 mg/ml allergens *in vitro*, and so on). The data are presented as mean ±SD of 8 spleens per group. \*, p < 0.05; \*\*, p < 0.01 versus saline-treated group.



Figure 4. Cytokine levels in splenocyte culture supernatants. Spleen cells from individual mice dosed with 0.1, 1 or 10 mg/day glycinin or  $\beta$ -conglycinin, were cultured with 50 µl of 0.1, 1.0 or 10 mg/ml soybean glycinin or  $\beta$ -conglycinin for 72 hours *in vitro*, respectively (spleen cells from mice dosed with 0.1 mg/day glycinin or  $\beta$ -conglycinin stimulated with 0.1 mg/ml allergens *in vitro*, and so on). IL-4 (a), IL-5 (b) and IFN- $\gamma$  (c) were determined by ELISA. Results are expressed as mean  $\pm$ SD of 8 spleens per group. \*\*, *p* < 0.01 versus saline-treated group.

oral administration of soybean allergens (Liu et al., 2008). In the present study, we demonstrated in the same model that systemic anaphylactic symptoms were induced in sensitised BALB/c mice, and reported the characterisation of the antigen-specific cellular responses *in vitro*. In addition, we also examined the effect of oral feeding dose on sensitisation in BALB/c mice.

IgE plays an important role in type I hypersensitivity reactions by inducing mast cell degranulation and release of histamine after antigen sensitisation or challenge. IgG1 is considered to be a Th2-associated antibody, whereas IgG2a is recognised as a Th1-associated antibody (Snapper & Paul, 1987). Our results showed that mice sensitised with 0.1 and 1 mg/day glycinin or  $\beta$ -conglycinin induced high levels of allergen-specific IgE and IgG1, which are in accordance with Gizzarelli et al. (2006), who sensitised BALB/c mice with soybean extracts and cholera toxin as an adjuvant by oral administration. Christensen, Bruun, and Frokiaer (2003) also reported that soybean protein induced strong levels of specific IgE and IgG1 vs. low levels of IgG2a. This pattern indicates the skewing of the immune responses towards a prevalent Th2-type. On the other hand, Liu et al. (2008) demonstrated an increased level of IgE and IgG1 in mice sensitised with 1.0 mg/day soybean allergens. We further found that low dose given orally induced much stronger antigen-specific IgE. IgG1 and IgG2 responses than 10 mg/day administration of soybean proteins. In fact, the increase in the allergen doses may affect specific IgE levels and plasma histamine concentrations, suggesting an optimisation procedure for allergen administration (Capobianco et al., 2008). Our result confirms the statement that oral immunisation requires a low antigen dose to prevent the induction of oral tolerance (Akiyama et al., 2001; van Wijk & Knippels, 2007). Compared with the data of Liu et al. (2008), we interestingly found that the kinetics of antibody production demonstrated a falling from the fourth week while the antigen intake was done for a week further (five weeks). This is consistent with the serum histamine release observed by Liu et al. (2008), suggesting that mice might develop oral tolerance after the continuous administration of antigens (Stokes et al., 1983). However, there is no published evidence that feeding normal laboratory chow (containing soybean proteins) is detrimental to adult mice. This discrepancy may be due to the prelimitation of soybean proteins in the diets of the pregnant mice and their newborn mice. Additionally, more general aspects, including the origin of mouse strain and the maintenance conditions, also exert an influence on optimal in vivo sensitisation. The variability of these factors may underline the difficulty to develop animal models of food allergy.

Histamine regulates body temperature by function on histaminergic neurons in the central nervous system (Makabe-Kobayashi et al., 2002). The increased histamine level could reflect mast cell degranulation and is considered to be one of the major indicators of rapid allergic reactions. Interestingly, BALB/c mice displayed a maximal increase of serum histamine level at day 28 when induced by 1.0 mg/day soybean allergens compared with those gavaged with 0.1 or 10 mg/day. This appears to correlate mainly with the increase of specific IgE and IgG1 in BALB/c mice, suggesting a dose-dependent difference in histamine release in our model. Our result also consists with the serum histamine level responding to ovalbumin in B10A mice, which also occurred in a dose-dependent manner (Akiyama et al., 2001).

Besides the effect on B cells, oral administration of glycinin or  $\beta$ -conglycinin influenced T-cell proliferative responses. We found that splenocytes from mice treated with 0.1 and 1.0 mg/day soybean proteins (glycinin or  $\beta$ -conglycinin) exhibited significant proliferative responses compared with those from other groups. Previous study has reported that splenocytes may develop resistance to spontaneous apoptosis by specific antigen or cytokines induced by allergens (Budd, 2002), which might be consistent with our results.

To investigate the role of Th1/Th2 cytokines in systemic immune responses in BALB/c mice orally induced by soybean glycinin and  $\beta$ -conglycinin, IL-4, IL-5 and IFN- $\gamma$  in splenocytes were measured. It is well known that IFN- $\gamma$ , IL-4 and IL-5 play pivotal roles in the development, differentiation and maintenance of the allergic response. Therefore, the identification of the cytokine profiles from spleen cells in vitro may help to determine the pathway whereby allergen-specific antibodies were induced. In the current study, a significant production of IL-4, IL-5 and IFN- $\gamma$  were observed *in vitro* cell culture when stimulated by 50 µl of 0.1 mg/ml or 1 mg/ml soybean proteins. IFN-y belongs to Th1 cytokines, which favors IgG2a production, while IL-4 and IL-5 are strong promoters of switching on IgE and IgG1 production, which belongs to Th2 cytokines (Howard et al., 1983). Thus, the result seems to suggest that a mixed Th1/Th2 response was stimulated. It is uncommon, to our knowledge, to find a high IFN- $\gamma$  level combined with a low content of IgG2a after sensitisation. Although similar results were reported by Hofman (1995), who demonstrated that serum IL-4 and IFN- $\gamma$  levels were twice and four times as high in children with food allergy as those in the control group, the results may still not be fully explained by the T lymphocytes differentiation paradigm (Mosmann & Coffman, 1989). Stevens et al. (1988) suggested that other factors may be involved for inducing the levels of IgG2a production in vivo. Therefore, further studies into other splenocytes development pathway will be necessary. Additionally, our study focused on systemic immune responses induced by soybean allergens. Cytokine expression in other tissues, such as mesenteric lymph nodes, which provides valuable information of local immune responses, may also help to explain the pattern in cytokine response induced by allergen-specific stimulation of spleen cells.

The model exhibited that lower doses of soybean allergens induced more severe immune responses than the higher sensitising dose (10 mg/day). Especially, 10 mg/ day administration of soybean allergens was inefficient in priming the BALB/c mice for subsequent systemic immune responses. This is consistent with other studies showing that high allergen doses induce tolerance (Capobianco et al., 2008; Hao et al., 2009). These data provide evidence that the administration of the higher dose of glycinin or  $\beta$ -conglycinin through the oral route is capable of eliciting prevalent type 2-dependent antibody responses in BALB/c mice, which prevents persistent tissue injury (Cardoso et al., 2008). Similarly, Akiyama et al. (2001) suggested that a low antigen dose and rapid antigen intake are required for oral immunisation. But the mechanism of this effect is still not fully understood due to the shortage of literatures. The animal diet and the maintenance conditions also exert an influence on optimal in vivo sensitisation (Capobianco et al., 2008). On the other hand, low and high doses are relative and may be changed in the different animals. Therefore, we can not guarantee that an increase of soybean meal in feed composition will reduce the allergic diarrhoea in animals. Further studies will be required to clarify the effects of different allergen doses on gut immune responses in our model.

Additionally, several mechanisms whereby oral tolerance occurs have been described, including T-cell active suppression and deletion (Faria & Weiner, 2005). There is solid evidence that a high dose of antigen induces clonal deletion and active suppression (Whitacre, Gienapp, Orosz, & Bitar, 1991). However, the underlying cellular mechanisms of oral tolerance and possible sites of oral tolerance induction remain to be answered. Our result suggests that a long-lasting administration of

high-dose soybean proteins would be used to develop a tolerance model. In future studies, optimisation of oral administration techniques will be needed for building such a model.

We acknowledged that the results are somewhat in conflict with Gizzarelli et al. (2006) and Oakes et al. (2009), who detected no allergen-specific response in mice immunised with soybean protein alone. The discrepancy is probably due to the differences in animal sensitisation procedures, as mice used in these experiments received allergens on separated days. Other main contributing factors could also affect the outcome of the anaphylactic response, including different source of allergens, composition of food, and conditions of maintenance of the mice (Chatel, Song, Bhogal, Orson, 2003). Moreover, minipig models have already been established to investigate the anaphylactic reactions induced by soybean glycinin or  $\beta$ -conglycinin without any adjuvant (Sun et al., 2008).

Taken together, our study demonstrated that low doses (0.1 or 1.0 g/day) of glycinin or  $\beta$ -conglycinin for BALB/c mice could induce allergic reactions, including high levels of allergen-specific IgE and IgG1 associated with a concomitant increase of IL-4, IL-5 and IFN- $\gamma$  production in spleen cells stimulated *in vitro*. Our results also suggest that BALB/c mice are suitable *in vivo* models for investigating the allergic responses caused by soybean protein.

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