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Inbred Chinese Wuzhishan (WZS) Minipig Model for Soybean Glycinin and β-Conglycinin Allergy

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Validated murine models have been built to assess the potential allergenicity of novel proteins. Large animals, such as pigs, share more similarities to humans in physiology and immunology than murine. Among Chinese minipigs, Wuzhishan (WZS) minipigs have the highest inbreeding coefficient, more stable heredity, and less variability, which were gastrically sensitized and excitated with diets containing 4% glycinin or 4% β-conglycinin or neither to induce anaphylactic reactions in the present study. In glycinin- and β-conglycinin-sensitized animals, diarrhea symptoms and skin wheal and flare responses were observed. In comparison to the control, after oral excitation with glycinin or β-conglycinin, the serum IgE was increased by 34.4 or 38.4% and the serum histamine was increased by 42.1 or 46.9%, respectively. In addition, the serum IFN-γ were reduced by 12.4 or 30.0%, respectively. The jejunum histamine level of β-conglycinin-sensitized animals was increased by 196.6%, while the number of mast cells in the submucosa of jejunum and ileum of the glycinin-sensitized animals declined by 48.1 and 45.0%. In conclusion, the WZS minipig allergy models induced by soybean glycinin and β-conglycinin represent type-I hypersensitivity reactions mediated by IgE, which could potentially be useful in determining the potential allergenicity of novel proteins.

KEYWORDS: Chinese WZS minipig; food allergy; glycinin; β-conglycinin; soybean protein

INTRODUCTION

IgE-mediated food allergies are characterized by a variety of cutaneous, gastrointestinal, and systemic symptoms induced by food proteins that are associated with the production of an allergen-specific IgE antibody. The prevalence of food allergies has been increasing for many years, especially in the Western world (*1*). In the 1970s, scientists began to research and plant genetically modified organisms (GMOs). In 2001, GMO varieties accounted for about 26% of the corn, 68% of the soybeans, and 69% of the cotton planted in the United States (*2*). Many people have expressed concern about the potential association between GMOs and allergenicity (*3*, *4*). The Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) decision tree is widely used and is continuously being improved. One new recommendation is the use of animal models (*5*). Because of ethical and other reasons, sensitization in humans is not possible. Validated animal models that mimic the allergic responses seen in humans would make it possible to assess the potential allergenicity of proteins. These models could also be used to derive sensitization and elicitation thresholds and to define the conditions under which tolerance (the failure to develop an allergic response to potential food allergens) is induced (*6*). According to Selgrade et al. (*7*), serum from clinically well-defined food allergic individuals needs to be immediately banked for use in screening proteins of unknown allergenicity. Maybe an animal food allergy model is another good serum bank source.

Inbred strains of mice have been characterized as either high or low IgE responders for both inhalant and food allergens (*8*). Pigs share many physiological and immunologic similarities with humans, which have made the pig an important large-animal model for biomedical research (*9*). Earlier at 1978, investigations performed on pigs have showed similar hypersensitivity reactions that are more closely aligned with human responses following ingestion of soy protein (*10*). Piglets in development have a sensitization to soy proteins that are similar to what is seen in young children (*11*, *12*). However, disadvantages include cost and the limited experimental facilities, and immunologic reagents for large animals have made the pig model on food allergy investigation still limited. Large-animal food allergy models have not been effectively used to evaluate the potential allergenicity of GMO proteins.

Owing to the small size, simple operation, and striking similarities to man with respect to anatomy and physiology, minipigs are commonly used as experimental animals in research on the cardiac and vascular system, digestive system, reproductive system, dermatology, and nutriology (*13*). Currently, there are at least five kinds of minipig strains in China, including the Chinese Banna minipig, Guizhou minipig, Guangxi Bama minipig, Wuzhishan (WZS) minipig, and Tibet minipig, all of which have been successfully cultivated and used in life sciences research.

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Among them, the WZS minipig was the first to be cultivated and inbred after being introduced from Wuzhishan, Hainan, China in 1987 (*14*). After 20 generations of inbreeding, the WZS minipig now has the highest inbreeding coefficient (more than 0.965) (*15*), stable heredity, and little variability between individual animals. According to a study by Yang et al., the WZS minipig is most similar to humans regarding hematology, as compared with other Chinese minipigs, the Göttingen minipig and the Landrace pig (*16*). The histologic structures of immune organs in the minipigs are generally similar to those of humans and other mammals (*17*). Two aims of this study are to determine whether inbred WZS minipigs demonstrate more advantages regarding repeatability and stability compared to outbred pigs as food allergy animal models and whether this highly inbred minipigs strain is a good IgE responder or more consistent with the human allergic response. In this study, we chose to use the WZS minipig as an animal model to achieve more convincing evidence.

Soybeans are widely used by the food industry as a cheap source of protein or as an additive for improving the functional properties of foods. At the same time, soybeans represent one of the major foods causing severe allergic reactions in humans, which have received great attention worldwide (*18*−*20*). Glycinin and β-conglycinin are the two major soybean allergen proteins (*12*, *21*). To investigate whether glycinin and β-conglycinin are also suitable as positive controls for the inbred minipig food allergy test, inbred Chinese WZS minipigs were administered glycinin or β-conglycinin and were subjected to skin prick tests. Additionally, levels of serum IgG, IgE, and histamine, concentrations of histamine, the number of mast cells in the small intestine, and cytokine expression levels were also measured for evaluation.

MATERIALS AND METHODS

Animals. WZS minipigs obtained from the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (Beijing, China), were housed and treated according to the guidelines for minipig care from the Chinese Academy of Agricultural Sciences Animal Care and Use Committee.

Diet Formulation and Soybean Protein. Soy-free diets according to the formulation of the National Research Council (NRC) (*22*) were provided by the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, and contained 1.55% lysine and 21.42% crude protein. To avoid oral tolerance, a pregnant sow at day 108 of gestation and piglets from 30 days after birth were both fed soy-free diets.

Soybean-extracted glycinin (11*S*) and β-conglycinin (7*S*) were kindly provided by Prof. Shuntang Guo of the Food Institute of China Agricultural University (Patent 200410029589.4; purity, 80−85%).

Pig Experimental Design. In brief, piglets were used to eat diets alone and weaned at 45 days after birth. After 4 days of adaptation (no adverse reactions observed), 12 WZS minipigs were randomly allotted to three groups on day 0 of the experiment. Each treatment had four replicates. All experimental minipigs were orally fed soy-free diets with a quantity of 3% of weight throughout the whole experiment period. For the 11*S*- and 7*S*-sensitized animals, during the sensitization phase (days 0−10 of the experiment) and excitation phase (days 16−18 and day 32 of the experiment), 4% of daily diet 11*S* or 7*S* protein were orally given to pigs before feeding. At 3 h after the last time of excitation (day 32 of the experiment), all the piglets were slaughtered with an ear injection of anesthetic (Zoletil 100, 5 mg/kg body weight) and jugular exsanguination.

Activity and Diarrhea. All pigs were weighed on days 0, 7, 14, 21, 28, and 31 of the experiment to calculate weight gain. Physical activity and incidence of diarrhea were recorded daily throughout the entire experiment. Pasty, semi-liquid, and liquid pig feces were considered to be diarrhea.

Cutaneous Skin Testing. Cutaneous skin testing was conducted on day 25 of the experiment. After animals were anesthetized with Zoletil 100, six kinds of solutions (0.1 mL per site) were respectively injected intradermally under previously shaved flank regions. Protein concentrations were determined by Coomassie Brilliant Blue (CBB) R-250 staining and adjusted to 100 μg of protein per injection site. Soybean-extracted 11*S* and 7*S* were used to assess skin reactivity to soybean proteins. Physiological saline solution and soybean protein carrier solution (0.05 M Na_2CO_3 and $NaHCO_3$) served as the negative and carrier controls, respectively. Potato acid phosphatase solution (0.01 g/mL, Sigma-Aldrich) was used as a non-allergen control, and histamine HCl solution (1 mg/mL, Sigma-Aldrich) was used as a positive control. At 15 min after the injection, wheal and flare diameters were measured and recorded.

Figure 1. Cutaneous skin testing was conducted at day 25. Representative photographs of skin prick test results from (A) glycinin-sensitized animals, (B) β-conglycinin-sensitized animals, and (C) control animals. 1, physiological saline solution (PBS); 2, potato acid phosphatase (PAP); 3, histamine HCl (HIS); 4, carrier solution (CA); 5, glycinin (11*S*); and 6, β-conglycinin (7*S*).

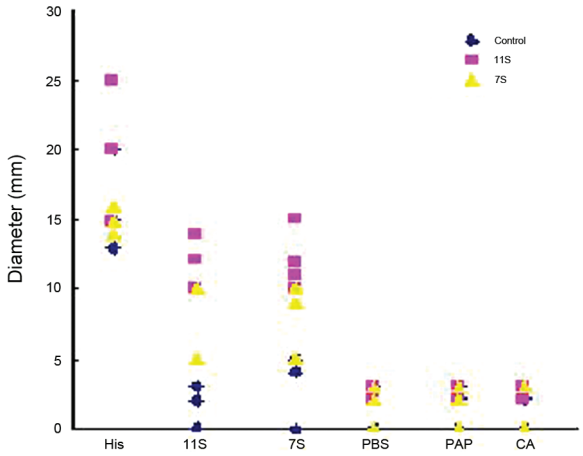

Figure 2. Intradermal skin challenge in glycinin- and β-conglycinin-sensitized and control pigs at day 25. The diameters at different injection sites for glycinin (11*S*)-sensitized (red), β-conglycinin (7*S*)-sensitized (yellow), and control (blue) animals are shown.

Analysis of Antibodies, Histamine, and Cytokines by an Enzyme-Linked Immunosorbent Assay (ELISA). Blood was collected from the precaval vein on days 11, 19, and 32. The serum was separated and stored at −80 °C for serum antibody, histamine, and cytokine analyses. On day 32, segments were removed from the duodenum, the jejunum, and the ileum, stored at −80 °C, and prepared as tissue homogenate samples for intestinal histamine analysis according to the kit protocol. Swine serum IgG, IgE, histamine, TNF-α, IL-2, IFN-γ, IL-4, IL-10, and intestinal histamine ELISA kits (RapidBio Lab, Calabasas, CA) were used for quantification.

Histological Analysis of Intestinal Mast Cells (*23*, *24*). Small-intestine samples removed from the duodenum, jejunum, and ileum were fixed in Carnoy's fluid. Following routine paraffin embedding and sectioning, mast cells were specifically stained with toluidine blue (Amresco). The mast cells in the mucosa and submucosa were quantified by randomly numbering mast cells in 10 areas of one segment histological slide with a Microcheck Grid (Shanghai ShanYi Instrument, Ltd., Shanghai, China), containing 100 microchecks (0.04 mm^2), under 200× amplification.

Statistical Analysis. One-way analysis of variation (ANOVA) tests, Mann−Whitney tests, and bivariate correlation tests were performed using SPSS 11.0 software (SPSS, Inc., Chicago, IL). $p < 0.05$ or $p < 0.01$ was considered significant.

Table 1. Serum IgG, IgE, and Histamine Levels in Glycinin- and β-Conglycinin-Sensitized and Control Pigs at Various Times[a]

	IgG (logarithmic transforming)			IgE (logarithmic transforming)			histamine (logarithmic transforming)		
group	11 days	19 days	32 days	11 days	19 days	32 days	11 days	19 days	32 days
control	2.19 ± 0.16	2.29 ± 0.25	2.17 ± 0.12	1.65 ± 0.14	1.77 ± 0.23	1.64 ± 0.07	1.33 ± 0.14	1.45 ± 0.23	1.31 ± 0.07
glycinin	2.43 ± 0.54	2.91 ± 0.21^b	2.61 ± 0.12^b	1.89 ± 0.51	2.38 ± 0.18^b	2.13 ± 0.17^b	1.57 ± 0.51	2.06 ± 0.18^b	1.81 ± 0.18^b
β-conglycinin	2.14 ± 0.36	2.95 ± 0.31^b	2.49 ± 0.14^b	1.65 ± 0.30	2.45 ± 0.30^b	2.01 ± 0.13^b	1.33 ± 0.30	2.13 ± 0.30^b	1.68 ± 0.12^b

[a] The data are presented as mean ± standard deviation (SD) of four animals per group. [b] In comparison to the control group, $p < 0.01$.

Table 2. Pearson Correlation Analysis between Serum IgG, IgE, and Histamine Levels in 12 Pigs at Various Times[a]

		serum IgE			serum histamine		
factor		11 days	19 days	32 days	11 days	19 days	32 days
serum IgG	11 days	0.994^b	0.304	−0.047	0.994^b	0.303	−0.057
	19 days	0.375	0.899^b	0.712^b	0.375	0.902^c	0.705^c
	32 days	0.091	0.731^b	0.949^b	0.091	0.735^b	0.948^b
serum IgE	11 days	1	0.294	−0.041	1.000^b	0.294	−0.052
	19 days	0.294	1	0.788^b	0.294	1.000^b	0.781^b
	32 days	−0.041	0.788^b	1	−0.041	0.791^b	1.000^b

[a] The Pearson correlation coefficients are presented. [b] Correlation test, $p < 0.01$. [c] Correlation test, $p < 0.05$.

RESULTS

Performance and Diarrhea Symptoms Throughout the Trial. Pigs fed 11*S* or 7*S* had lower average daily weight gain, but statistic differences were not significant. At 2 days after the pigs were orally sensitized with soybean protein, two of the 11*S*-sensitized animals and two of the 7*S*-sensitized animals showed diarrhea symptoms (pasty or semi-liquid feces), which lasted for 2−4 days. Control animals fed soybean-free diets failed to show any symptoms.

Cutaneous Sensitization. Positive histamine wheal and flare responses ranged from 13 to 25 mm in diameter. Control animals did not react to phosphate-buffered saline (PBS), potato acid phosphatase (PAP), glycinin, and β-conglycinin (diameter < 5 mm). Skin prick tests were positive in both 11*S*-sensitized and 7*S*-sensitized animals (flare diameters ranging from 5 to 15 mm). A typical skin response is shown in **Figure 1**, and the data from the different treatment groups are shown in **Figure 2**. As shown in **Figure 2**, in comparison to the control, the responses of 11*S*-sensitized ($p = 0.000$) or 7*S*-sensitized ($p = 0.014$) animals are evident.

Serum IgG, IgE, and Histamine Levels. The logarithmic transforming values of serum antibody and histamine in pigs are summarized in **Table 1**. In contrast to the control, serum IgG, IgE, and histamine of 11*S*- and 7*S*-sensitized animals were all increased. After 3 days of oral challenge, on day 19, in the 11*S* and 7*S* groups, the serum IgE increased by 34.4% (2.38 versus 1.77) and 38.4% (2.45 versus 1.77) and the serum histamine increased by 42.1% (2.06 versus 1.45) and 46.9% (2.13 versus 1.45), respectively. As presented in **Table 2**, serum IgG, IgE, and histamine of the 12 pigs at days 11, 19, and 32 were positively correlated ($p < 0.05$ or 0.01).

Histamine and Mast Cell Numbers in the Small Intestine. The number of intact mast cells in the mucosa of the jejunum and ileum was higher than that of the duodenum, while the histamine release in the jejunum and ileum on day 32 was lower than that in the duodenum. In comparison to the control, the jejunum histamine level of 11*S*- and 7*S*-sensitized animals was increased by 95.2% (145.66 versus 74.61) and 196.6% (221.32 versus 74.61) and the ileum histamine of 11*S* and 7*S*-sensitized animals was increased by 22.2% (101.58 versus 83.16) and 75.9% (146.32 versus 83.16), respectively, while the number of mast cells in the submucosa of the jejunum and ileum of 11*S*-sensitized animals declined by 48.1% (6.8 versus 13.1) and 45.4% (7.1 versus 13.0) and the number of mast cells in the submucosa of the jejunum and ileum of 7*S*-sensitized animals declined by 16.0% (11.0 versus 13.1) and 10.0% (11.7 versus 13.0) (**Table 3**). Representative photographs of mast cells in the submucosa of the small intestine of 11*S*- and 7*S*-sensitized animals are shown in **Figures 3** and **4**.

Cytokine Concentrations in the Serum. To examine changes in Th1/Th2 cytokines in 11*S* and 7*S* orally sensitized and challenged pigs, IL-2, TNF-α, IFN-γ, IL-4, and IL-10 levels were measured on days 11, 19, and 32. Except for IFN-γ, the levels of these serum Th1/Th2 cytokines were too low to measure and analyze. In comparison to the control animals, the serum IFN-γ (Th1 cytokines) levels on day 19 of 11*S*- and 7*S*-sensitized pigs were reduced by 12.4% (44.77 versus 51.1) and 30.0% (35.78 versus 51.1), respectively (**Figure 5**).

DISCUSSION

In comparison to mice food allergy models, the minipig model is advantageous because of similarities of the appearance between pigs and humans regarding systemic anaphylaxis, such as diarrhea and typical skin wheal and flare responses, observed in the present study. Another advantage is that the multiple sampling on minipigs makes the kinetic observation on serum antibody, histamine, and cytokines more feasible and convenient.

IgE and histamine are both known to play important roles in type-I hypersensitivity (*25*). High levels of IgE and IgG responses in BALB/C mice sensitized with the soybean proteins glycinin and β-conglycinin have been shown (*26*). A recent study by Sun et al. showed similar reactions on serum antibody in crossbred piglets orally induced by glycinin (*23*). In our study, inbred Chinese WZS minipigs were orally sensitized with glycinin or β-conglycinin without any adjuvant. The increases in serum IgE, IgG, and histamine in animals sensitized with soybean proteins, as compared to control animals, suggest that soybean hypersensitivity reactions have been induced.

Similar to other studies (*27*, *28*), a decreased number of intact mast cells and an increased histamine release, which is usually initiated by mast cell activation and degranulation after cross-linking of IgE bound to FcεRI (8−34) were also found in the present study. Liu et al. suggested that glycinin or β-conglycinin could induce oral challenge reactions in mice by triggering the release of mediators, such as histamine (*26*). Similar results regarding histamine and mast cells in the small intestine seen in this animal study provide more evidence supporting this idea (*29*). However, the hypersensitive physiologic reaction may be stronger if day 19 is chosen instead of day 32 as the investigation end date.

To investigate the role of Th1/Th2 cytokines in anaphylactic reactions in WZS minipigs orally induced by soybean glycinin and β-conglycinin, serum IL-2, TNF-α, IFN-γ, IL-4, and IL-10 were measured. However, except for IFN-γ, levels of other Th1/Th2 cytokines were undetectable. In future studies, examination of intestinal tissues or more sensitive methods, such as measuring cytokines mRNA levels, will be required.

The physical symptoms following glycinin and β-conglycinin ingestion in our study included decreased body weights and

Table 3. Histamine Content (ng/g) and Mast Cell Numbers (Numbers/0.04 mm^2) in the Small Intestine of Glycinin- and β-Conglycinin-Sensitized and Control Pigs[a]

	intestinal histamine (ng/g)			mast cell numbers in the mucosa (numbers/0.04 mm^2)			mast cell numbers in the submucosa (numbers/0.04 mm^2)		
group	duodenum	jejunum	ileum	duodenum	jejunum	ileum	duodenum	jejunum	ileum
control	116.05 ± 38.97	74.61 ± 19.9	83.16 ± 29.24	9.0 ± 1.8	13.3 ± 1.5	13.8 ± 2.5	12.1 ± 1.7	13.1 ± 1.0	13.0 ± 1.5
glycinin	129.87 ± 23.20	145.66 ± 19.11	101.58 ± 25.28	5.5 ± 0.8	9.0 ± 3.3	8.4 ± 1.7	8.8 ± 1.5	6.8 ± 1.0[b]	7.1 ± 0.6[b]
β-conglycinin	206.18 ± 74.44	221.32 ± 55.15[b]	146.32 ± 65.43	8.5 ± 2.5	8.6 ± 2.5	12.4 ± 2.1	12.8 ± 1.7	11.0 ± 3.1	11.7 ± 3.6

[a] The data are presented as mean ± standard error of the mean (SEM) of four animals per group. [b] In comparison to the control group, $p < 0.05$.

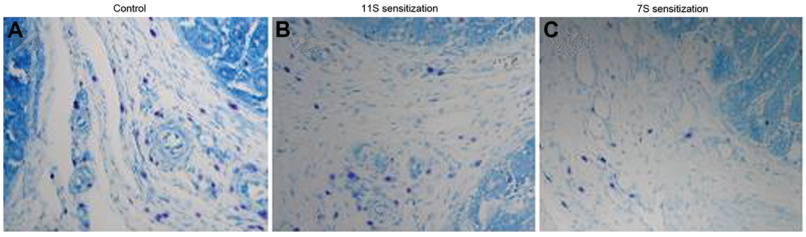

Figure 3. Effects of the soybean proteins glycinin or β-conglycinin on mast cell numbers in the submucosa of the jejunum in WZS minipigs. (A) Control, (B) glycinin sensitization, and (C) β-conglycinin sensitization. Samples were stained with toluidine blue and photographed at 200× magnification using a light microscope.

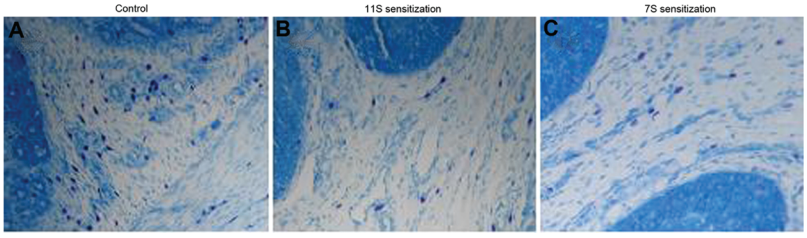

Figure 4. Effects of the soybean proteins glycinin or β-conglycinin on mast cell numbers in the submucosa of the ileum in WZS minipigs. (A) Control, (B) glycinin sensitization, and (C) β-conglycinin sensitization. Samples were stained with toluidine blue and photographed at 200× magnification using a light microscope.

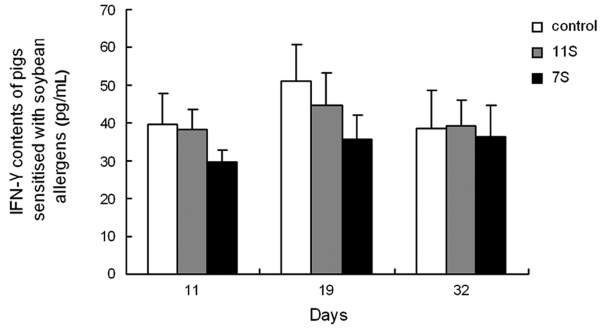

Figure 5. Serum IFN-γ levels (pg/mL) at different days in glycinin (11*S*)- or β-conglycinin (7*S*)-sensitized and control pigs. The data are presented as mean ± SD of four animals per group.

average daily gain and increased diarrhea, which are less severe than the respiratory distress and anaphylaxis seen in newborn piglets following oral challenge with peanuts by Rciki et al. (*29*). First, just as in humans, diarrhea is the frequently allergic symptom induced by soybean, which is not so severe as that by peanuts. In addition, the piglets in the present study were inbred animals, which could not be weaned before 45 days after birth. To avoid stress resulting from weaning, minipigs were fed with soybean-free diets 30 days after birth and weaned on 45 days. On the contrary, outbred pigs can be weaned at 21 days after birth or even earlier. Younger animals often show an higher allergenic sensitivity or response.

Sun et al. studied the differences between anaphylactic reactions in piglets induced by including 2, 4, and 8% glycinin in soybean-free diets (*23*). It seems that higher doses may cause more severe anaphylactic symptoms. However, considering the risk of oral tolerance induced by an 8% high dosage of antigen protein, we chose a 4% dosage for this study. Furthermore, in comparison to murine animal models, the large-animal model will require a larger amount of pure antigen proteins for sensitization, especially in the context of oral exposure.

In conclusion, this report suggests that glycinin and β-conglycinin can orally induce soybean allergic hypersensitivity physician reactions on inbred WZS minipigs that mimic symptoms of human soybean allergy. More evidence on immune reactivity and possible mechanisms involved in allergic responses in these animals should be investigated.

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