

Full Length Research Paper

A study of an ideal animal model for evaluating the potential allergenicity of novel foods proteins

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Accepted 09 April, 2017

Food allergy is now an important health issue, and there is urgent need for a developmental approach to identify allergenic potential of food. We present an approach that shows some promise for assessment of shrimp allergenicity using BALB/c strain mice. The mice were immunized by intraperitoneal injection of shrimp allergen. Mast cell degranulation in combination with serologic methods was used to monitor protein allergy. The results showed the method could continuously reflect the variation of allergic symptom and actualize dynamic determination of shrimp allergenicity. Furthermore, it is feasible, sensitive and repeatable. The approach will provide some valuable reference for identifying allergenicity of novel food proteins by using animal model in the future.

Key words: Shrimp allergen, allergenicity, mast cell, serology.

INTRODUCTION

The prevalence of food allergy has been estimated recently at 3 - 4% for adults and approximately 6 - 8% for young children and infants in the past decade (Dearman and Kimber, 2001; Hanson and Telemo, 1997). With increased interest in the development of novel foods including some food products derived from transgenic plants, the problem is receiving more attention. There is need to establish safety assessment strategies for food allergy (FAO/WHO, 2001; Kimber et al., 2003a; Ladics et al., 2003). Currently, the most important issue is to find ideal model and method for the characterization of allergenic potential of food. Animal models have been introduced which is expected to provide the basis for a direct evaluation of food inherent sensitization potential. But now there are no still valid and widely accepted methods or animal models that are available for the evaluation of allergenic potential of food (Kimber et al., 2003b; Knippels and Penninks, 2005).

In this paper, shrimp allergenic potential was evaluated. The major food items responsible for inducing allergic symptoms are milk, eggs, citrus fruits, peanuts, cereals and particularly seafood. Seafood allergies are frequently reported (Daul et al., 1990; 1993). Shrimp that is very

widely eaten as delicacy is one of the most important allergen (Hoffmann, 2000). BALB/c strain mice was chosen as animal model in this research. Mouse pleural mast cell degranulation in combination with serologic method is introduced in evaluating shrimp allergenicity. Previous studies have demonstrated that BALB/c mouse strain is favorable for the development of Th2 type immune responses and the production of IgE antibody. Using the BALB/c mouse, it is possible to measure the quality and vigor of immune responses after systemic exposure to proteins and to define these proteins as having inherent sensitizing potential if they provoke clear IgE antibody responses (Kimber et al, 2003a). In this study, BALB/c mouse pleural mast cell degranulation assays and serum antibody (IgE) assays were performed. The aim of this work is to examine the possible effectiveness of the new method using BALB/c mouse pleural mast cell degranulation combining with serologic methods to evaluate allergic potential of shrimp. Furthermore, it is hoped to provide some useful reference for developing ideal animal model to evaluate the potential allergenicity of novel foods proteins.

MATERIALS AND METHODS

Materials and reagents

Shrimp allergen (36 kDa) was supplied by Food Safety Laboratory of Ocean University of China (purity \geq 99.8%). Phosphatase (no

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allergenicity), phthaldialdehyde and bovine serum albumin (purity \geq 99.8%) were obtained from Sigma Chemical Co. USA. Goat Anti-Mouse IgE-Biot, (SBA Company, Germany), HRP-Streptavidin, (KPL company, USA), calf serum and RPMI 1640 medium were obtained from GBICO Company, USA. All other chemicals were at analytical grade and obtained from Qingdao Alp Science and Technology Co., Ltd, China.

Animals and treatments

BALB/c strain mice (8 weeks old) from Beijing Experimental Animal Center were used. They were maintained under hygienic conditions with free access to food and water. The composition of the diet was monitored and where possible proteins from the same source as the test protein was avoided. They were allowed to acclimate to the environment for a week prior to experiment. Mice were divided into experimental and control groups. The route of exposure was the same as described by Dearman and Kimber (2007). Initial Sensitization: experimental groups of mice ($n = 120$) were systemically sensitized by intraperitoneal injection (IP) of 5 mg/ml of protein in a volume of 0.25 ml. Booster sensitization was given seven days later. Experimental groups of mice were challenged by IP of high dose protein solution (10 mg/ml protein), with strict monitoring for anaphylactic responses following the second intraperitoneal injection and choosing the mice that showed allergic behavior and symptom characteristics. Control groups: Negative control mice was exposed to phosphatase (no allergenicity) by the same way; blank control of mice was only injected with 0.25 ml Hanks' balanced salt solution (HBSS) every time.

Blood samples collection

Mice were exsanguinated on day 4 and day 7 after the initiation of exposure and on 15, 30 min; 2, 6, 12, 24 h; day 4, day 7, day 14, day 21, and day 28 after booster challenge. Every five mice were decapitated and blood samples were collected on each time endpoint. Every five serum samples of each endpoint were pooled respectively, equal volumes of serum from each individual animal contributed to the pool. Samples were stored at -80°C until analysis.

Isolation, purification and culture of mast cell from mouse peritoneum

Mice were executed and disinfected in alcohol and immediately given a 5 ml IP injection of HBSS (without Ca^{2+} or Mg^{2+}). This was followed by kneading the abdominal region of mice for two minutes and then opening the abdominal cavity, collecting lavage fluid with a haustorial tube. The lavage fluid was centrifuged ($500 \times g$, 10 min) to separate the cells from the fluid portion at 4°C . The supernatant was discarded and the cells were collected and suspended in 1 ml HBSS. 4 ml 90% Percoll (9 ml Percoll and 1 ml 10-fold concentrations HBSS) was added in (Enerbäck and Svensson, 1980). After the mixture was agitated and swirled completely, 1 ml HBSS was dropped in slowly. The mixture was centrifuged ($1000 \times g$) for 5 min; the cells were collected and then were washed by HBSS three times. The cell count was approximately $0.5 \times 10^6 \sim 1 \times 10^6$ from each mouse. Cells purity was identified by neutral red staining and cell vigor was identified by trepan blue staining. The cells from each individual animal were adjusted to an equal concentration and those cells that come from five mice of each endpoint at equal volumes were mixed. The cell samples were cultured in RPMI 1640 culture medium supplemented with 10% heat-inactivated FCS at a cell density of 1×10^6 cells per ml at 37°C in a humidified atmosphere with 5% CO_2 for 4 h before experiment.

Mouse pleural mast cells (MPMC) provocation and degranulation *IN VITRO*

Prior to all experiments, MPMC of experimental groups were challenged by incubating with 100 $\mu\text{g}/\text{ml}$ of shrimp allergen for 1 h at 37°C . MPMC of blank control groups: con 1 was incubated with HBSS, con 2 was challenged by shrimp allergen at the same dose as experimental groups; MPMC of negative control group: con 3 was challenged by shrimp allergen as above. The cell density was all $5 \times 10^5/\text{ml}$. The incubation was stopped by placing the cell on ice. The supernatant and cells were collected respectively for assays.

MPMC degranulation assay

0.5 ml of caustic soda solution (0.4 m/l) was added to 1 ml of the MPMC supernatant and phthaldialdehyde (0.1 ml, 0.05%) was added. The solution was mixed and placed for 10 min at 37°C and then the reaction was stopped by addition of 0.5 ml hydrochloric acid (0.1 M). Correspondingly, the remaining cell pellets were suspended by 1 ml HBSS and the rupture of membrane by addition of Triton X-100. The following procedures were the same as the treatments of cell supernatants. Histamine of the supernatant and cell pellet fractions were assayed by an spectrofluorometer auto-analyzer (Bran+Luebbe GmbH, Norderstedt, Germany) (Wang and Lau, 2007). Experiments were independently repeated at least thrice and comparable results between the experiments were obtained. Data are presented as percentage of histamine released into the supernatant relative to total cellular histamine.

Anti-protein IgE antibody analysis

ELISA-techniques were used to measure sera antibodies specific for protein according to Knippels and Penninks, (2003). Positive and negative controls were incorporated for each 96-wells plate. The average extinction in negative control wells, to which three times the standard deviation was added, provided the reference value taken to determine the titer in the test sera. Each test serum was titrated starting at a 1:10 dilution and the reciprocal of the furthest serum dilution giving extinction higher than the reference value was read as the titer. All analyses were performed in triplicates.

Assay for serum histamine

The assay of histamine in serum was performed using a 96-wells kit of rat anti-mouse histamine determination (ADL, Catalogue No.QRCT-301301EIA/UTL, USA) following the manufacturer's procedures.

Statistics

The data were analyzed using SPSS 11.0 software. One way analysis of variance followed by Duncan's Multiple Range test was adopted, and $*P < 0.05$ was considered for significant difference compared to the control.

RESULTS

MPMC degranulation

Histamine release assay was performed on day 4 and 7 after the initiation of exposure and on day 1, 4, 7, 14, 21 and 28 after booster challenge. The histamine release on

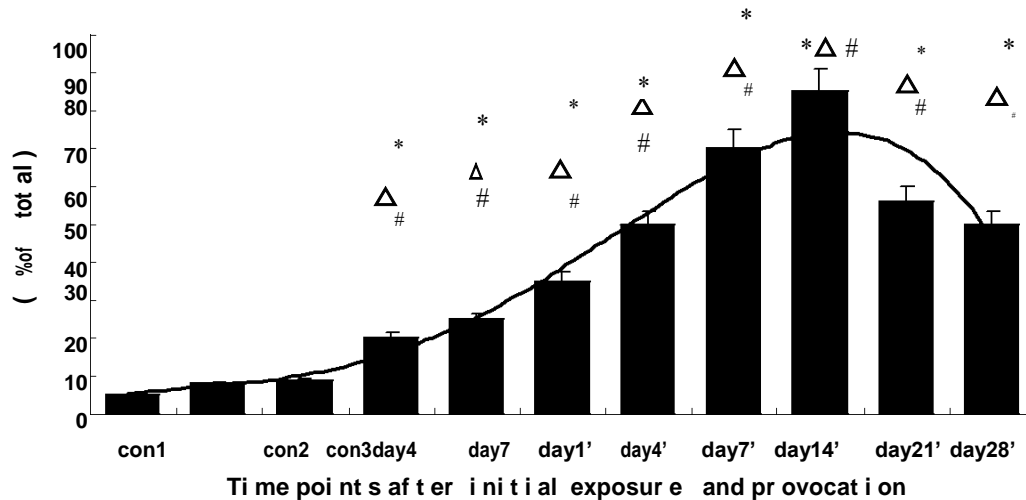


Figure 1. MPMC degranulation: Histamine release on every time point was found to be regular with time alteration in mouse pleural mast cells.

every time point was recorded completely and it was found to be regular with time alteration (Figure 1). Blank control group: con1, the spontaneous release of MPMC in HBSS buffer alone was in general less than 5%; con 2, the release of MPMC incubated with shrimp allergen was in general less than 10%. Negative group: con 3, the release of MPMC incubated with shrimp allergen was in general less than 10%. Experimental group: after initial intraperitoneal exposure, the release rate of MPMC presented rising tendency until it reached the peak value $79 \pm 5.3\%$ after booster challenge and then it took on decreased tendency.

Experimental group: mast cell degranulation assays were performed on day 4 and 7 after the initiation of exposure and on day 1, 4, 7, 14, 21 and 28 after booster challenge (Booster challenge was performed on day 7 since initial exposure). Control groups: Con 1, normal mice mast cell spontaneous release; Con 2, normal mice mast cell that was challenged by shrimp allergen *in vitro* release; Con 3, negative control mice mast cell release after suffering the challenge of shrimp allergen *in vitro*. Data were means \pm S. D. of 5 mice of each endpoint; * represents $p < 0.05$ when compared with the con 1, # represents $p < 0.05$ when compared with the con 2, # represents $p < 0.05$ when compared with the con 3.

IgE titer determination

The blood samples of multiple time points associated with sensitizing exposure were detected. The assays demonstrated that there was a regular alteration in IgE level of mice. It was observed to increase in a time dependent manner after initial exposure, especially after booster challenge IgE level rose very quickly until reached a vertex and then decreased slowly. The variation is just as the exhibit of Figure 2. But IgE antibody titre can not be

detected in negative control group of mice.

IgE titres in time following IP exposure to sensitizing mice ($n=5$ for each endpoint). Data were displayed as serumal IgE titres (Mean \pm SD) in time. On day 7 since initial exposure the animals were booster challenged. Day 1 represents after being booster challenged for 24 h and the rest may be deduced by analogy. Con represents the normal mice, *represents the significant differences compared to the control group with $p < 0.05$.

Histamine variation in sera

The histamine assay of sequential and multiple blood samples on the all time points showed that the histamine levels in those sera of sensitized mice are generally higher than control group within 24 h. However beyond 24 h, no significant difference between experimental and control group. The nearest time point presents the most significant difference compared with control group. The histamine level of control mice almost kept constancy in the term (Figure 3).

The histamine content in mice sera of multitude endpoints: Control group was normal mice. The endpoints were 15, 30 min; 6, 12, 24 h; day 4, 7, 14, 21 and 28 after the booster challenge. * represents the significant differences compared to control group with $p < 0.05$.

DISCUSSION

Food allergy belongs to hypersensitivity mediated by IgE. Mast cell is important producer of inflammatory responses (Robbie-Ryan and Brown, 2002). Immediate-type allergic reaction is mediated by histamine release in response to the antigen cross-linking of immunoglobulin E (IgE) bound to Fc ϵ R1 on the mast cells. After activation

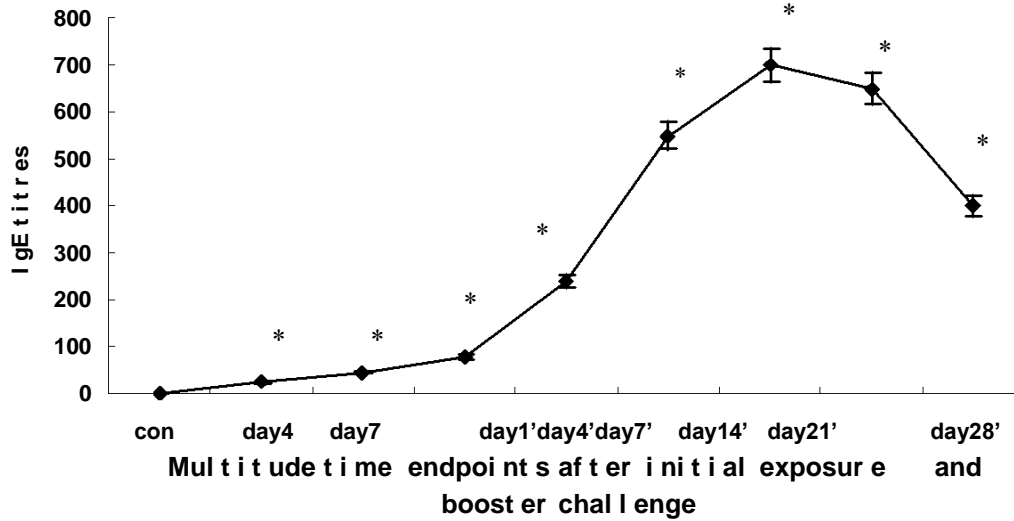


Figure 2. IgE titer determination: IgE was observed to increase in a time dependent manner after initial exposure. After booster challenge IgE level rose very quickly until reached a vertex and then decreased slowly.

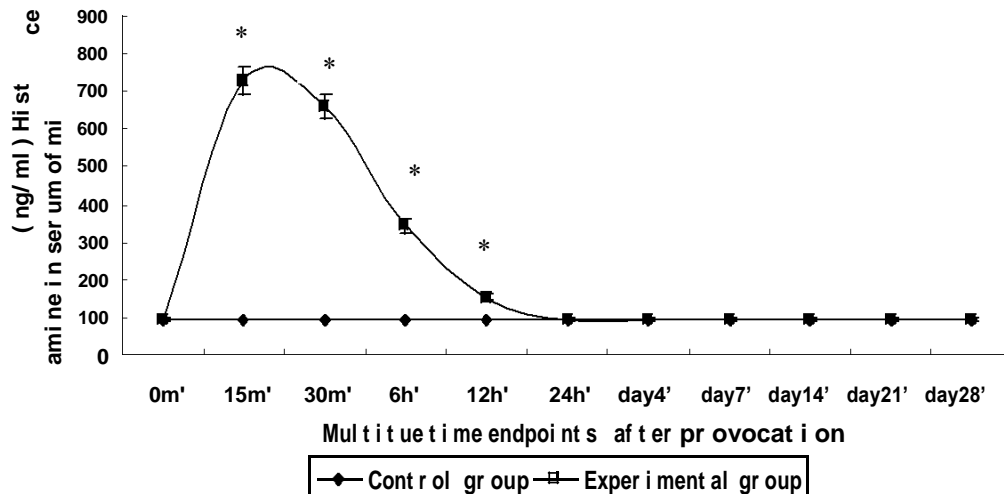


Figure 3. Histamine variation in sera after provocation.

via the FcεRI, the mast cells start the process of degranulation which results in the release of mediators, such as products of arachidonic acid metabolism and an array of inflammatory autacoids (Hogan and Schwartz, 1997). Degranulation of mast cell makes important contribution for hypersensitivity, considered as general phenomenon for sensitization happening (Untersmayr and Jensen, 2006). So we try to investigate sensitizing state via mast cell degranulation. We detected BALB/c MPMC degranulation on multiple time points. The experimental results disclosed the release of mast cell in sensitized mice could take on regularity in different time stages. One important point was found that there was favorable concordance between mast cell degranulation

and serum antibody titers (Figures 1, 2). This will provide us one possibility to accomplish dynamic monitoring protein allergy by detecting mast cell degranulation on sequential time points. The fact proved that it is feasible. In the study, mast cell release and IgE levels both rose after initial IP exposure in a short term and then presented a swift increase after provocation treatment. They reached a vertex after two weeks and then decreased slowly. Correspondingly, the badly symptom of mice allergy appeared within two or three weeks after being challenged. They could take on good anastomosis in the process of allergy.

The serumal histamine could not well reflect mice allergy in a long time. The results only showed that it was

higher than control group in short term. Furthermore, it varies among individual animals. The main reason is possible that the half-life period of histamine *in vivo* is transient (Schwartz et al., 1987). We can find from Figure 3 that histamine peak value appeared at about 20 min after provocation and then decreased quickly and it came back to baseline level within 24 h. So the blood samples should be collected quickly after allergic response happening to determine histamine. Otherwise the result will not be reliable. In conclusion, histamine in animal serum is not a good parameter for evaluating allergenicity of food protein.

By now, there is no single method to fully assess the potential allergenicity of food proteins; especially it is very difficult for estimating some new proteins introduced from transgenic products since no amino acid sequence homology is recorded and no allergic serum can be provided. Animal model may become an effective way to evaluate the safety of novel food in future (Kimber et al., 2003b). In this research, mast cell degranulation in combination with serologic methods using BALB/c strain mice proved ideal for evaluating shrimp allergenicity. The study will provide some useful reference to develop animal model for evaluating food allergenicity in the future.

ACKNOWLEDGMENTS

This work was supported by National High Technology Research and Development Program of China (863 Program) (NO.2006AA09Z427) and Nature Science Foundation of China. No. 30800859 and No. 30871948.

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