Brief Report

Studies on the Specific Degranulation of Mast Cell Sensitized by Several Allergens *in vitro*

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Food allergy is a major health issue worldwide. Mast cells play a very important role in the immediate hypersensitivity for which mast cell degranulation needs to be studied extensively. In this study, an approach was taken to study the characteristics of sensitized mast cell degranulation *in vitro,* which associated with the study of mast cells and animal models. BALB/c mice were immunized respectively by several food allergens, then blood and peritoneal mast cells were collected at different time points. A dynamic determination was carried out between mast cells and serumal IgE. Comparative analysis on sequential time points showed that there was a close coincidence between mast cell degranulation and IgE antibody titers in sensitized BALB/c mice. Furthermore, it is interesting that sensitized mast cells could implement specific degranulation against the challenges *in vitro*, but the closely tropomyosins induced mast cell degranulation displayed cross reactions. This is very similar to IgE resisting the allergens *in vivo*. The study disclosed some characteristics on mast cells, coming from sensitized BALB/c mice, degranulation *in vitro*. *Cellular & Molecular Immunology*. 2009;6(2):149-153.

Key Words: food allergy, allergen, mast cell degranulation

Introduction

The prevalence of food allergy has been estimated as 2-3% of adults and approximately 6-8% of young children and infants in the past decade (1, 2). With increased interest in the development of novel foods, the problem is becoming serious. It is well known that mast cells are important producer of inflammatory responses (3, 4). Immediate-type allergic reaction is mediated by histamine release in response to the antigen cross-linking of IgE bounding to FcεRI on mast cells. Therefore mast cell degranulation makes important contribution in food allergy and is considered as a general phenomenon for sensitization (5). The studies on mast cells to unfold their functions and mechanism are being explored and researched. To study mast cells *in vitro* is still a problem due to the lack of convenient preparation source. Previous studies were focused on RBL-2H3 cell strain that is used to simulate real mast cells *in vitro* (6, 7). In fact RBL-2H3 cells are rat basophilic leukemia cell line, a kind of tumor cell analog of mast cells for which it can not replace the real mast cells completely. Furthermore, the mast cells coming from mouse resource have not been studied extensively. In our article, mast cells coming from BALB/c mice were studied *in vitro*. The cells were obtained from the abdominal cavity of mice. The characteristics on mast cell degranulation *in vitro* were thought to be the preliminary investigation in our study.

The aim of this work is to explore whether the mast cells which were sensitized *in vivo* can keep specific degranulation *in vitro* and whether some correlation can be proved to exist between IgE antibody titer and mast cell degranulation in BALB/c mice. Thereby some inspiration is expected to obtain for finding any useful models to evaluate food allergenicity *in vitro*.

Materials and Methods

Materials and reagents

Shrimp allergen (36 kDa), and clam allergen (35 kDa) were obtained from food safety lab of Ocean University of China (purity ≥ 99.8%); ovalbumin (OVA) from eggs, β-lactoglobulin B from bovine milk and phosphatase acid were obtained from Sigma-Aldrich (purity ≥ 99.8%). Goat anti-mouse IgE-Biot (SBA), HRP-streptavidin (KPL). Calf serum and RPMI 1640 medium were obtained from GBICO Company. All other chemicals were analytical grade and

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Animals and treatments

Female BALB/c mice (8 weeks old) were procured from Beijing Experimental Animal Center. They were maintained under hygienic conditions with free access to food and water. The composition of the diet was monitored and possible proteins from the same sources with allergens were avoided in the experiments. The ambient temperature was maintained between 20°C and 24°C as well as the relative humidity was also maintained between 40% and 70% with a 12 h light/dark cycle. The mice were allowed to acclimatize the environment for a week prior to experiment. Mice were divided into multi-experimental groups and control groups randomly. Experimental groups included several sensitized groups: the mice were sensitized by OVA, β-lactoglobulin B and shrimp allergen, respectively. Control groups included the negative group and the blank group. Negative group: the mice were exposed to phosphatase acid which has no allergenicity. Blank group: the mice were exposed to HBSS. The route of exposure was performed as described by Rebecca et al. (8). Strict monitoring was done for anaphylactic responses following the provocation. The mice which appeared allergic behavioral and symptom were chosen. PCA experiments were performed as the criterion to identify the anaphylaxis of mice (9). No sensitized mice were found in control groups.

Blood sample and mast cell collection

Each group was exsanguinated on day 0, day 7, day 14, day 28 and day 56 after booster challenge. Blood and peritoneal mast cells of every five mice were collected at each time point. Every five sera samples on each time point were pooled respectively. Equal volumes of serum from each individual animal contributed to the pool (8). Cell sample pools were obtained in the same way as serum samples. Cell density was 5×10^5/ml.

Anti-protein IgE antibody titer determination

Serum titers of specific IgE were determined according to a previously reported method (10) with some modifications. Ninty-six-well microtiter plates were coated overnight at 4°C with 100 μl/well of 10 μg/ml protein solution in carbonate buffer, pH 9.6. The plates were washed with ultrapure water containing 0.4% Tween 20. This was followed by the addition of 100 μl/well PBS containing 1% BSA and 0.02% Tween 20. After 2 h incubation at 37°C, the plates were washed and serial dilutions of mouse sera in PBS-Tween 20 were added to the wells and incubated for 2 h at 37°C. After washing, 100 μl/well goat anti-mouse IgE-Biot (diluted 1:10,000) in PBS-Tween 20 was added. After incubation for 2 h at 37°C, the plates were washed and 100 μl/well HRP-Streptavidin (diluted 1:2,000) was added. After incubation for another 2 h, an enzyme substrate solution of 3, 3',5,5'-tetramethylbenzidine (TMB; Sigma, 100 μl/well; 6 mg/ml DMSO) was added. The plates were developed at 37°C for 15 min. Finally, 50 μl/well of 2 N H_2SO_4 were added. Optical density was read spectrophotometrically at 450 nm with an ELISA plate reader (Microplate Reader, Bio-Rad Laboratories, Richmond, USA). Blank group of serum pools were used as negative control. 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 1:4 dilution, and the reciprocal of the furthest serum dilution giving extinction higher than the reference value was read as the titer. Positive and negative control samples were incorporated for each 96-well plate. All analyses were performed thrice.

Isolation, purification and culture of mast cells from mice peritoneum (MPMC)

Mice were executed and disinfected in alcohol following 5 ml *i.p.* injection of HBSS (without Ca^{2+} or Mg^{2+}). Kneading the abdominal region for two minutes and then lavage fluid was collected from the abdominal cavity (11, 12). The lavage fluid was centrifuged (500 × g, 10 min) to separate the cells from the fluid portion at 4°C. The supernatant was discarded; the cells were collected and suspended in 1 ml HBSS. Four milliliter of 90% Percoll was added in as previous report (13). After the mixture was agitated and swirled completely, 1 ml HBSS was dropped in slowly. The mixture was centrifuged (1,000 × g) for 5 min. The cells were collected and washed with HBSS for three times. The cell numbers were approximately 1-5 × 10^5 of each mouse. Cell purity was identified by neutral red staining and cell vigor was identified by trypan blue staining. Those cells coming from each time point composed the corresponding cell samples. The cell samples were cultured in RPMI 1640 culture medium supplemented with 10% heat-inactivated FCS at cell density of 5 × 10^5/ml at 37°C in a humidified atmosphere with 5% CO_2 for 4 h before experiment.

MPMC provocation and degranulation in vitro

Mast cell degranulation was performed *in vitro*. MPMC of every group were challenged by incubating with 100 ng/ml of food proteins that included OVA, β-lactoglobulin B, shrimp allergen, phosphatase, and clam allergen for 1 h at 37°C. The cell density was 5 × 10^5 in all the reaction. The incubation was stopped by placing the cells on ice. Cell suspension was centrifuged as above. The supernatant and cells were collected respectively for assay.

MPMC degranulation assay

Spectrofluorimetric assays were performed on cell supernatants and lysates after exocytic stimulation. Caustic soda solution (0.5 ml) was dropped in 1 ml MPMC supernatants following phthaldialdehyde (0.1 ml, 0.05%) (14). The solution was mixed and placed for 10 min at 37°C. The reaction was stopped by addition of 0.5 ml hydrochloric acid. The corresponding cell pellets were suspended by 1 ml HBSS. The cell membranes were ruptured by addingTriton X-100. The following procedures were the same as the treatments of cell supernatants as above. Histamine in the supernatant and cell pellet fractions were assayed using a Bran + Luebbe Auto Analyzer (Bran + Luebbe GmbH,

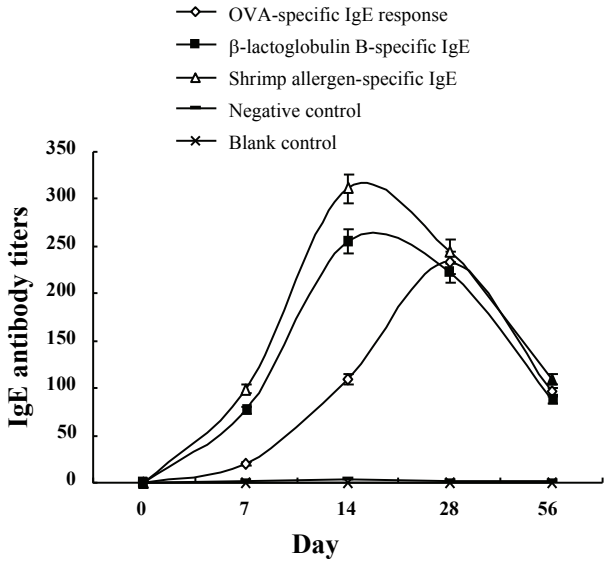

Figure 1. IgE antibody titer. The mice were sensitized by OVA, β-lactoglobulin B, shrimp allergen, phosphatase acid or HBSS respectively. IgE titers were determined in blood samples obtained at each time point. The data are presented as mean ± SD of five mice/group.

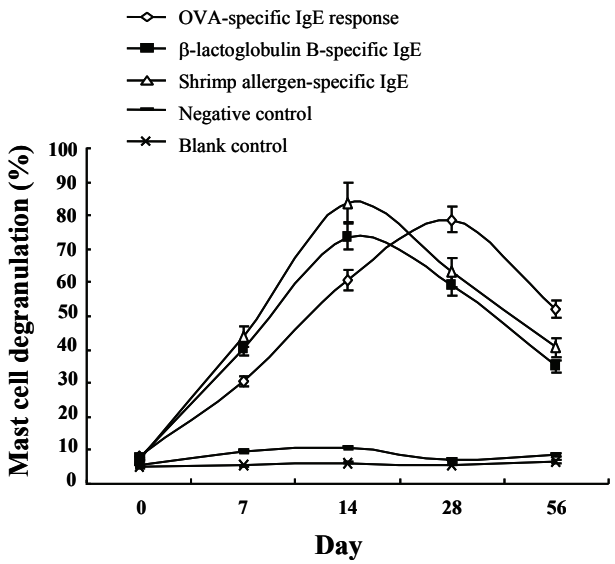

Figure 2. Specific degranulation of MPMC. MPMC of different allergens sensitized mice were stimulated by corresponding antigens *in vitro* and the histamine releases were assay. Experimental group, mast cells from BALB/c mice that were sensitized by OVA, β-lactoglobulin B and shrimp allergen; negative group, mast cells coming from the mice that were exposed to phosphatase acid; blank group, mast cells coming from normal mice.

Norderstedt, Germany) (15, 16). Experiments were independently repeated thrice and comparable results among the experiments were obtained. Data were shown as percentage of histamine released into the supernatant relative to total cellular histamine. Ratio of histamine release (%) = (OD sample - OD spontaneous)/(OD total - OD spontaneous).

Data analysis

Data were expressed as mean ± SD, and analyzed by SPSS 11.0 software. One way analysis of variance followed by Duncan's Multiple Range test, $p < 0.05$ was considered significant with the control.

Results

Serumal IgE titer determination

The IgE titers of each group were detected and the data were shown in Figure 1. The result showed IgE antibody titer took on fluctuating tendency after the sensitized mice received antigen provocation. But for negative group and blank group, IgE could not be detected.

MPMC degranulation assay

Histamine assay was performed on the same time points as IgE detection. The variation on MPMC degranulation at each time point was recorded completely and it was found to be regular (Figure 2). The spontaneous degranulation of blank group was less than 10% in general, and that of negative group was less than 15% in general. MPMC of sensitized experimental group mice were incubated with corresponding allergen, which presented high releasing rate and accompanied some variation. The negative group and blank group almost kept constancy all the time. From above results, some similarity was found between IgE titer and mast cell degranulation rate. The tendency changed since time has kept the elemental concordance.

The orthogonal experiment showed mast cells from the sensitized mice could implement specific degranulation against the corresponding allergen (Table 1). The results showed that the degranulation rates of OVA group, β-lactoglobulin B group and shrimp allergen group were all higher than 50% when incubated with corresponding allergen, and showed significant differences with blank group. However, for other proteins, the degranulation rates were very low (< 20%) and had no statistical difference with negative group. For seafood allergens, obvious cross reactions were found between shrimp allergen and clam allergen and the cross reaction rate was 33.3 ± 4.3%.

Discussion

Protein specific IgE-mediated allergic reactions are known to be major in food allergy (17). In recent years, new proteins have been artificially produced in special foods, such as genetically modified foods (18). Some of the new proteins might be strongly allergenic in humans (19, 20). Nevertheless, at present there are only a few methods to predict the allergenicity of food proteins (21, 22). Although several attempts have been made to develop animal models for investigating the allergenicity of food proteins, mainly in the

Table 1. Orthogonal experiment on mast cell degranulation

MPMC Allergens	OVA	β-lactoglobulin B	Shrimp allergen	Phosphatase	HBSS	Clam allergen
OVA	60.7 ± 9.8*	11.1 ± 2.1	12.7 ± 2.7	12.8 ± 2.3*	8.5 ± 1.7	13.3 ± 2.8*
β-lactoglobulin B	13.1 ± 2.9	73.7 ± 10.7*	13.6 ± 3.1*	14.7 ± 3.0*	8.9 ± 2.2	13.3 ± 2.7*
Shrimp allergen	11.0 ± 3.3	10.4 ± 3.4	83.8 ± 12.3*	10.7 ± 3.1	6.7 ± 2.7	33.3 ± 4.3*
Negative control	8.4 ± 2.5	8.8 ± 2.1	9.1 ± 2.8	9.5 ± 2.3	6.5 ± 1.8	9.3 ± 2.2
Blank control	7.3 ± 3.3	7.5 ± 3.1	7.8 ± 3.5	7.0 ± 2.5	7.2 ± 2.8	7.9 ± 2.0

The data were expressed as mean ± SD of five mice/endpoint; *$p < 0.05$, compared with blank group.

mouse, guinea-pig and rat (23), it is expected that some simple and sensitive models can be developed (24). To date, cell model has not be adopted for application. In our study, the preliminary investigation on mast cell (BALB/c mice resource) degranulation *in vitro* was performed.

The study revealed that there is some close concordance between mast cell degranulation and IgE titers in sensitized BALB/c mice with the passage of time. Mast cell degranulation took on corresponding tendency with the variation of IgE titers in BALB/c mice, which can be found from comparing the result in Figure 1 with that in Figure 2. The point is interesting and requires consideration. It is inferred that more IgE can cohere on the surface of mast cells when IgE titer becomes higher *in vivo*. So once encountering antigen challenge, more cross linking of IgE bounding can be created which leads to more degranulation in mast cells. This phenomenon is confirmed to be happened in BALB/c mice.

It is well known that allergic response happens *in vivo* associated with antigen-antibody specific combination on mast cells. In this study, we found that sensitized mast cells could implement directional degranulation against the antigen stimulus *in vitro*. No cross reactivity was found among OVA, β-lactoglobulin B, phosphatase acid, and shrimp allergen. However, cross reactivity was found to occur among shrimp allergens and clam allergens. The phenomenon is very obvious and crossing degranulation rates have exceeded 30%, which is similar to that IgE resists seafood allergens *in vivo* (25, 26). It is inferred that protein-specific IgE cohering on mast cells should be the fundamental reason to accomplish specific degranulation on mast cells *in vitro*. When sensitized mast cells encountered the non-corresponding antigens, the degranulation rates were less than 20% in general, which was much lower than specific degranulation. Some of them could present significant difference comparing with the blank control. But no significant difference was found between negative group and blank group. This disclosed the fact that sensitized mast cells are easier to release than normal mast cells when encountering extrinsic stimulus. It is probably due to the fact that mast cells coming from negative group were not sensitized for which they can possess more similarity to normal cells.

The experiment has simulated mast cell degranulation occurring in BALB/c mice. The results demonstrated that BALB/c mice is favorable for development of Th2 type immune responses and production of IgE antibody. By using BALB/c mice, 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. The mast cells coming from BALB/c mice can keep favorable specificity against antigens *in vitro*, so it is also hoped that this approach will provide a helpful tool and evolve new idea to study allergenicity of food proteins *in vitro*.

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