

Research Article

Research on Preparation of Human Immune Cell *in vitro* with Response to Shrimp Allergen

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Abstract: Shrimp is one of the most important food allergens. Tropomyosin is its major allergen. Wherein Pen a 1, contains five antibody binding regions, has been identified as the only major shrimp allergen. However, the study on IgE with response to shrimp allergen is still a serious lack, compared with the allergenic proteins. Particularly in the aspects of the preparation of IgE *in vitro*, it is restricted and can only obtain the complete IgE molecules by polyclonal or monoclonal technology. As for the preparation of small molecule IgE to the shrimp allergen has not yet been reported. This study attempts to carry out research on obtaining of cell materials that are used to clone. It sets up a convenient and efficient immune system *in vitro* which combines dendritic cell differentiation, allergens immune, mixed lymphocyte culture and so on. Finally the system successfully activates the proliferation of specific B cells and the secretion of a large number of specific IgE antibodies to shrimp allergen.

Keywords: B lymphocytes, epitope, IgE, *in vitro* immune, shrimp-allergen

INTRODUCTION

Aquatic crustaceans represented by shrimp is one of the eight food allergens identified by the FAO (Metcalf *et al.*, 1997), it's also the main food allergen of adults. Especially for the Asian region, shrimp is the most important source of food allergies in dietary structure (Li *et al.*, 2011). In China, consumption of shrimp in 2006 reached 2.5 million tons, ranking first in the world (Liao and Ning, 2009). There are a huge number of shrimp allergy populations in china and showed an increasing trend in the incidence. In addition, implementation of food allergen ingredient labeling method and HACCP management of European and American countries has become the biggest obstacle of shrimp exports (Taylor and Hefle, 2006; Hiroshi *et al.*, 2011; Hu *et al.*, 2009). So researches of shrimp allergens have important practical significance on China's food safety and breaking trade technical barrier.

Currently, shrimp antigen has been relatively clear; the molecular weight of 36 kD tropomyosin has been identified as the major allergenic protein in shrimp (Jeoung *et al.*, 1997). In which Pen a 1, was identified five major antibody binding regions (Ayuso *et al.*, 2002), is the most representative allergenic proteins, can response to more than 80% shrimp allergy serum (Lehrer *et al.*, 2003). Relatively, research of shrimp allergen antibodies was seriously insufficient. Especially in preparation of antibody *in vitro* for complete shrimp allergen IgE molecules is limited by

the polyclonal (Zhao *et al.*, 2013) or monoclonal (Huang *et al.*, 2012) techniques; genetic engineering small-molecule antibody has not been involved in.

The single-chain antibody scFv is built based on the antibody heavy and light chain variable regions. Because it's easy to penetrate the cells and low hepatic clearance, it's the focus direction of small molecules preparation *in vitro* (Qin *et al.*, 2011). There are two main sources of cloned material: the first is taken directly from allergy cells, such as peripheral blood mononuclear cells and spleen cells, peripheral blood is mainly used limited by the acquisition conditionality, but it's seriously lack of specific B cells (Ji, 1990); the second is hybridoma cell strain taken from immunized animals, but this method is complex, lengthy and difficult to control. Therefore, the establishment of a short preparation period, abundance of specific B cells *in vitro* and single epitope immune stimulation, will be an effective source of shrimp allergen small-molecule antibody clone materials.

The production of IgE is dependent on antigen stimulation and complex synergies of antigen-presenting cells (such as dendritic cells), T cells and B cells. But the peripheral blood is lack of adequate antigen-presenting cells and B cell-specific. This study attempted to use shrimp allergen Pen a 1 single epitope peptide to stimulate human peripheral blood monocyte-derived dendritic cells and mix with mononuclear cells to co-culture; thus activate the proliferation of B lymphocytes which secrete specific IgE.

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MATERIALS AND METHODS

Materials: Shrimp allergy sufferer diagnosed by the Ningbo Second Hospital, female, 20 years old; non-allergic healthy controls, male, 25 years old. Both are school student volunteers. Fresh peripheral blood was collected from the vein inside of the elbow and anticoagulated with heparin.

Shrimp allergen single epitope peptide synthesis: Shrimp allergen Pen a 1 is a polypeptide chain contains 284 amino acid residues, which was reported by Ayuso *et al.* (2002), mainly contains five antigen binding region. According to the amino acid sequences of the five binding regions, 16-39 amino acids length epitope peptide were synthesized by Shanghai Ziyu Company, the purities were above 98%. To enhance its immunogenicity and consider the different roles of subsequent immune stimulation and screening, the five epitope peptides were coupled to the carriers BSA and KLH as being the immunogen and the coating antigen. In addition, to facilitate the epitope peptide to couple to carrier, (Table 1).

Optimum dilution of serum and high response epitope screening: Using five epitope peptides coupled with KLH carrier to coat 96-well plates, coat concentration was determined as 1 ug/mL referring to previous studies, 4°C overnight. The next day added shrimp allergic and non-allergic serum respectively, serum diluted with 0.1 M PBS to 1:1, 1:10, 1:100, 1:500, 1:1000, 1:5000, 1:10000 times, 37°C incubated for 1h. After that adding enzyme-labeled goat anti-human IgG, (1:8000), 37°C incubated 1 h. Added TMB substrate solution, 37°C dark incubated 15 min, the reaction was stopped with 2 M H₂SO₄. OD value is then determined at 450 nm, which the OD value is about 1.0 and there were significant differences was determined as the best serum dilution multiple and referring to this screening the highest response immunostimulatory epitope.

Isolation of mononuclear cells: Human peripheral blood anticoagulated with heparin was diluted with the same volume of prewarmed RPMI-1640 culture medium. Then slowly added to the same volume of lymphocyte separation medium along the test tube wall and ensure a clear demarcation. Four hundred grams horizontal centrifuge 20 min. Carefully transfer the intermediate layer of white mist mononuclear cells PBMCs to a Pasteur tube, this layer mainly comprises mononuclear cells (i.e., precursor cells of dendritic cells), T cells and B cells. Then washed twice with 5 volumes of RPMI-1640 medium 350 g, 10 min, the supernatant was discarded.

Dendritic cell differentiation culture: Re-suspended cells With RPMI-1640 medium and counted with cell counter, adjusted the concentration not less than 1 million cells per mL. Inoculated into 12-well plates, each well 2 mL, 37°C, 5% CO₂ culture. After 4 h, suspension cells were aspirated and washed twice with RPMI-1640 medium. Add 2 mL RPMI-1640 medium containing 10% fetal bovine serum, cytokines rhGM-CSF (100 ng/mL) and rhIL-4 (100 ng/mL), continued culturing. On the 3rd and 5th day, half volume of the medium was changed and supplemented with cytokines.

Immunostimulatory: On the 5th day, added high response single-epitope peptide screened by serum to immune stimulation, the positive control well was added rhTNF-α (100 ng/mL) and set up a blank control well. Added epitope peptide and freshly isolated PBMCs to co-culture in another well and set up a non-allergic parallel control well, cells were harvested on the seventh day.

ELISA was used in the screening of allergy serum high response epitope peptide and the measurement of IgE secretion in cell culture process, flow cytometry was used in analysis and identification of changes of cell surface-associated protein markers in dendritic cells during differentiation.

Table 1: Preparation of single-epitope peptides

Amino acid position in pen a 1	Amino acid sequence
43-57	KLH-C-VHNLQKRMQQLENDL BSA-C-VHNLQKRMQQLENDL
85-105	KLH-C-VAALNRRRIQLLEEDLERSEER BSA-C-VAALNRRRIQLLEEDLERSEER
133-153	RSLSDDEERMDALENQLKEARF-C-KLH RSLSDDEERMDALENQLKEARF-C-BSA
187-202	KLH-C-ESKIVELEEEELRVVER BSA-C-ESKIVELEEEELRVVER
247-284	KLH-C-ARLQKEVDRLEDELVNEKEKYKSITDELDTQTFSELSGY BSA-C-ARLQKEVDRLEDELVNEKEKYKSITDELDTQTFSELSGY

Table 2: Screening of serum and single-epitope peptides

Amino acid position	Allergy serum								Non-allergy serum 1:1000
	1:1	1:10	1:100	1:500	1:1000	1:5000	1:10000	PBS	
43-57	2.844	2.959	2.787	2.688	0.744	0.271	0.181	0.065	0.298
85-105	2.789	2.902	2.868	2.562	0.705	0.272	0.175	0.072	0.265
133-153	2.666	2.838	2.807	2.568	0.684	0.266	0.171	0.067	0.322
187-202	2.719	2.852	2.870	2.560	0.696	0.261	0.175	0.073	0.139
247-284	2.924	3.114	3.044	2.703	1.059	0.345	0.207	0.070	0.249

RESULTS AND DISCUSSION

Screening of optimum dilution of serum and high response to a single-epitope peptide: ELISA results (Table 2) showed that the optimal dilution of serum was 1:1000, in this dilution (P-C) / (N-C) values were greater than 2.1 and positive. Five shrimp allergy epitope peptides corresponding OD values presented the same regularity under each dilution multiple, all have highest OD value at 247-284 amino acid epitope peptide. And there were significantly different OD values compared with the other four peptide epitopes at a dilution of 1:1000, indicating it's the highest level response to the epitope and also the major allergenic epitope of this allergy (Fig. 1). Non-allergy serum OD value had no regularity. It's predicted that the epitope peptide sequence (247-284 sites) of C-A R L Q K E V D R L E D E L V N E K E K Y K S I T D E L D Q T F S E L S G Y, would be able to get a better immune stimulating effect.

Dendritic cell morphology: Observed under an inverted microscope (Fig. 2), after the 1st day culture removed suspension cells by adherent method, the remaining monocytes were homogeneous size and round adherent cells, smooth and bright surface. On the third day a part of the cell volumes increased about two to three times and occasionally had two or three cell clusters, the cells remained adherent state. On the fifth day cell clusters increased significantly and increased to eight to ten cell clusters, most still on adherent state, clusters cell surface occasionally had fine dendritic cell structures. On the 7th day cells in epitope peptide

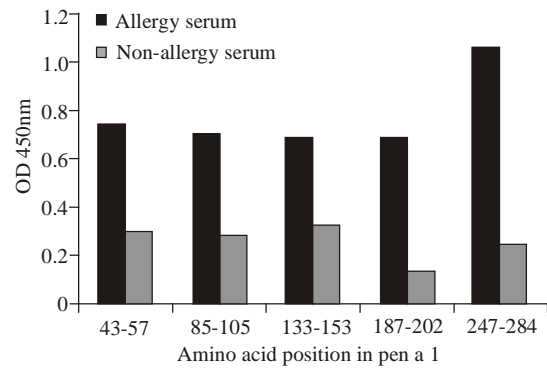


Fig. 1: Epitope screening results at 1:1000 dilution of serum

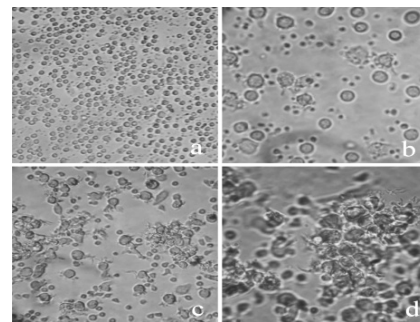


Fig. 2: The morphology of dendritic cells in different stages, (a) day 1, mononuclear cells attached to grow, (b) day 3, part of mononuclear cell volume increase, (c) day 5, part of the dendritic cells gathered a small colony, (d) day 7, most of the dendritic cells gathered for colonies and apparent dendritic structure on the cell surface

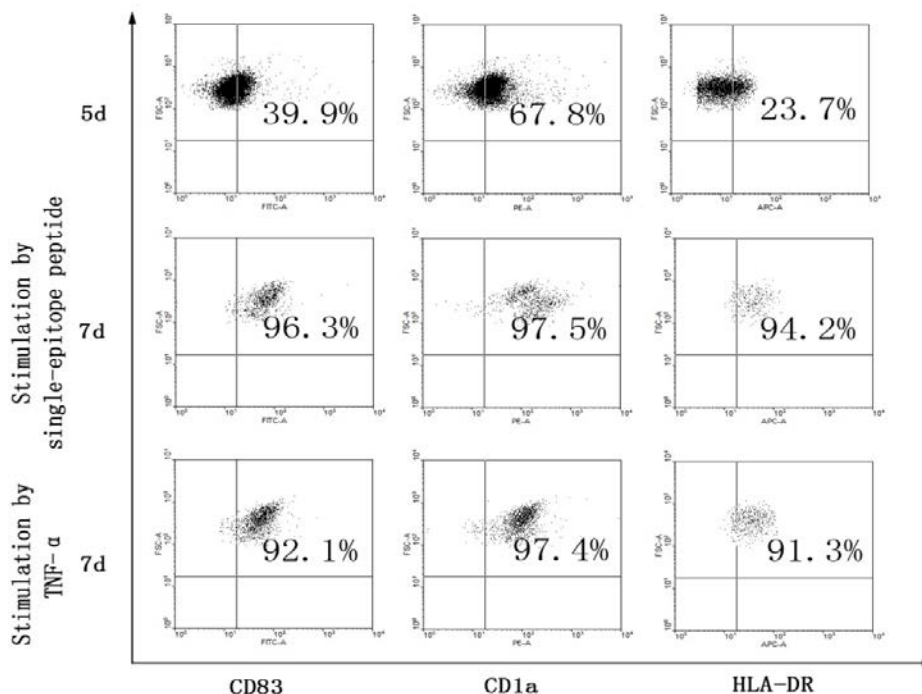


Fig. 3: The detection results of dendritic cell surface marker protein

Table 3: The test results of specific IgE in the cell culture supernatant

	3 day	5 day	5 day PBMCs	7 day single-epitope peptide	7 day TNF- α	7 day PBMCs and single-epitope peptide	PBS	Medium
Allergy	0.088	0.079	0.127	0.098	0.097	0.571	0.069	0.070
Non-allergy	0.082	0.075	0.105	0.068	0.079	0.185	0.070	0.071

stimulation well and TNF- α well continued to grow more and bigger, suspension or semi-suspension; more clearly dendritic structure can be seen on the clusters cell surface. Observations showed that the peripheral blood source mononuclear cells induced by GM-SCF and IL-4 and immunogenic stimulation, differentiate into mature dendritic cells. In addition, stimulation with peptide epitopes, can obtain the same positive control TNF- α stimulation effect. Morphological change dendritic cells were obtained consistent with the report.

Determination of dendritic cell phenotype: Flow measurement results (Fig. 3) indicated that, dendritic cells cultured 5 days, cell surface markers CD83, CD1a, HLA-DR expressed in different degrees, were 39.9, 67.8 and 23.7%, respectively. After epitope peptide stimulated and became mature, three cell surface marker expression rates had significantly increased, 96.3, 97.5 and 94.2%, respectively. The expression of TNF- α positive control well rose analogously. The results meet with the phenotypic changes of dendritic cells in the literature, can be identified as mature and having the function of antigen presenting dendritic cells.

Immune cell function test-measurement of IgE in the culture supernatant: ELISA results (Table 3) indicated that, supernatant IgE concentration of cells before stimulation did not change obviously. After epitope peptide and TNF- α stimulation for 2 days, allergy OD value slightly elevated, non-allergy OD value irregularly varied. After epitope peptides and PBMCs added for 2 days, allergy OD values increased significantly, non-allergy OD value slightly varied. Considered the mixed PBMCs of the 5th day may affect the OD values, its OD value was subtracted, then $(P-C) / (N-C) = 37.4$, much higher than the positive judgment indicator: $(P-C) / (N-C) > 2.1$; while this value can also be used as indicator of stimulus rate. The measurement results showed that when the mature dendritic cells, B cells, T cells simultaneously exists, the epitope peptide stimulation in vitro can activate specific B cell proliferation and secreted a large number of specific IgE. While the one lacking of new mixed PBMCs (mainly T cells and B cells accounted for more than 90%), had no significant change after stimulation.

CONCLUSION

In this experiment, peripheral blood from shrimp allergy patients was used as materials. Firstly, we stimulated mononuclear cells to differentiate into

dendritic cells by the cell factor GM-SCF and IL-4. And put it as antigen presenting cells. Then the high response single epitope peptide screened by sera was used to stimulate immunization, mixed with freshly isolated mononuclear cells (approximately 90% T cells and B cells, 10% monocytes) to co-culture. It's identified by ELISA and flow cytometry, the function, morphology and phenotype were meeting with reports of mature dendritic cells, in the while specific IgE secretion stimulation rate was identified up to 37.4. Finally the system was proved that it an successfully activate the proliferation of specific B cells and the secretion of a large number of specific IgE antibodies to shrimp allergen. And it ensured the effectiveness of the immune materials used as cloning experiments.

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