

Epitope analysis of peanut allergen Ara h1 with human monoclonal IgM antibody clone #86

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Abstract. A human-mouse hybridoma clone #86 secreting IgM-class human monoclonal antibody to peanut allergen protein Ara h1 was newly established. To detect an antibody-binding sequence (epitope) on Ara h1, the monoclonal antibody #86 was reacted with multi-pin apparatus with a series of peptides synthesized from the amino acid sequence of Ara h1. The antibody #86 was found to bind to a peptide with amino acid sequence of 481EEEEDEDEEEEGSNREVERRY500. Further analysis with shorter pin-peptides with ten amino acid-long showed that the peptides reacted with the antibody #86 contained a sequence of 485DEDEEE491. This might be an essential linear sequence of this epitope. When the 485DED487 part of the peptide was replaced by alanine, decreased binding of antibody #86 was observed.

Keywords: Human monoclonal antibody, allergen, peanut, epitope, IgM

1. Introduction

The number of patients suffering from peanut allergy has been increasing, and the peanut is thought to be the sixth most common food allergen in Japan [1,2]. Several peanut proteins have been identified as allergens by using sera from patients allergic to peanut, and Ara h1, Ara h2, Ara h3, and Ara h4 have been reported as major peanut allergens [3–6]. In the previous report, we established a mouse-human hybridoma clone #92-2 secreting IgM-class human monoclonal antibodies to Ara h1 from peripheral blood lymphocytes [7–9], and showed novel linear B-cell epitope of Ara h1. In this study, an another clone of human-mouse hybridoma (clone #86) secreting human IgM class monoclonal antibody to Ara h1 was established for further epitope analysis of Ara h1.

2. Materials and methods

2.1. Preparation of a human-mouse hybridoma secreting anti-Ara h1 human monoclonal antibody

Peripheral blood lymphocytes from a healthy donor were transformed by Epstein-Barr virus infection [8,9,12]. The resulted human B-lymphoblastoid cells secreting IgM-class antibody to Ara h1 were fused with mouse myeloma SP2/O3 cells with 50% polyethylenglycol. Hybridoma cells were cultured in O-HAT medium (ERDF medium (Kyokuto Co. Ltd., Japan) supplemented with 10% fetal calf serum (FCS, SAFC Biosciens, MO, USA), 0.1 mmol/L hypoxanthine, 0.0004 mmol/L aminopterin, 0.016 mmol/L thymidine, and 0.002 mmol/L ouabain) [9,10,12]. Antibody-positive hybridomas were cloned twice and a human-mouse hybridoma clone #86 secreting IgM-class anti Ara h1 antibody was established.

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MRGRVSPLMLLLGILVLASV	SATHAKSSPYQKKTENPCAQ	RCLQSCQQEPDDLKQKACES	60
RCTKLEYDPRCVYDPRGHTG	TTNQSRSPGERTGRQPGDY	DDDRRQPRREEGGRWGPAGP	120
REREREEDWRQPREDWRRPS	HQQPRKIRPEGREGGEQEWGT	PG SHV REETSRNNPFYFPSR	180
RFSTRYGNQNGRIRVLQRFD	QRSRQFQNLQNHRIQIEAK	PNTLVLPKHADADNILVIQQ	240
GQATVTVANGNNRKSENLDE	GHALRIPSGFISYILNRHDN	QNLRVAKISMPVNTPGQFED	300
FFPASSRDQSSYLQGFSRNT	LEAAFNAEFNEIRRVLLEEN	AGGEQEERGQRRWSTRSSEN	360
NEGVIVKVSKEHVEELTKHA	KSVSKKGSEEGDITNPINL	REGEPDLSNNFGKLFVEVKPD	420
KKNPQLQDLDMMLTTCVEIKE	GALMLPHFNSKAMVIVVVK	GTGNLELVAVRKEQQQRGR	480
EEEE DEDEEEE GSNREVRRY	TARLKEGDVFIMPAAHPVAI	NASSELHLLGFGINAENNHR	540
IFLAGDKDNVIDQIEKQAKD	LAFPGSGEQVEKLIKQKES	HFVSARPSQSQSPSSPEKE	600
SPEKEDQEEENQGGKGPLLS	ILKAFN	626	

Fig. 1. Amino acid sequence of peanut allergen protein Ara h1. IgE epitopes reported by Burks et al. [15] and Shin et al. [16] were underlined and an IgM epitope found in this study was shown in bold letters with double underline (485DEDEEEE491).

2.2. Enzyme-Linked Immunosorbent Assay (ELISA)

Purified Ara h1 (kindly donated by Dr. S. J. Maleki, USDA, ARS, SRRC, New Orleans, LA, USA) was diluted with 0.05mol/L NaHCO₃ (0.2–1 micro g protein/mL), 0.06 mL of the solution was plated into ELISA plates, and the plates were kept at 4°C for 16 h. The allergen solution was discarded and the plates were then blocked with a blocking reagent (Block Ace, DS Pharma Biomedical Co. Ltd., Japan) for 1 h. The plates were then washed three times with phosphate-buffered saline containing 0.05% Tween-20 (PBS-Tween). The culture supernatants of hybridomas (0.05 mL) were pipetted into the wells of the plates and incubated for 1 h at room temperature. The plates were washed three times with PBS-Tween, and 0.05 mL of anti-human IgM antibody conjugated with horseradish peroxidase (1/2000 dilution, Biosource International, CA, USA) was added to the plates. After 1 h of incubation at room temperature, the plates were washed six times and peroxidase activity immobilized on the wells was measured by adding 0.1 mL of substrate (ABTS) solution (0.3 mg/ml 2,2'-azino-di-(3-ethyl benzthiazolin sulfonic acid), 0.03% H₂O₂ in 0.1mol/L citrate buffer, pH 4.0).

2.3. Epitope analysis of Ara h1 by the multi-pin overlapping peptide method

As described in the previous paper [7], a series of overlapping peptides with 20-amino-acid length synthesized on a multi-pin apparatus (PepSets, Mimotopes Pty Ltd., Victoria, Australia), based on an amino acid sequence of Ara h1 [13,14] was used for epitope analysis. Supernatant of hybridoma clone #86 (0.1 ml) was pipetted into the wells of a 96-well micro titer plate, and multi-pin peptides were placed on the wells of the plate and reacted for 1 h at room temperature. The

multi-pin peptides were washed with PBS-Tween and reacted with anti-human IgM antibody conjugated with horseradish peroxidase. After washing, enzyme activity bound to the pin-peptides was measured by adding ABTS solution in a new 96-well plate [7,9]. Shorter peptides and alanine-substituted peptides were also synthesized to determine the fine epitope structure.

3. Results and discussion

In this study, we established a hybridoma clone #86 secreting IgM class human monoclonal antibody to Ara h1 and evaluated an amino acid sequence of antibody binding site (B-cell epitope). Of 63 pin-peptides of 20-amino acid-long analyzed, binding signals were observed at pins 12-13, 27, 34, and 49 (Fig. 2). Control analysis without monoclonal antibody #86 (peroxidase conjugated secondly antibody only), revealed that the signals at peptide 12-13, and 27 were non-specific binding of secondly antibody.

From the amino acid sequence of 20-amino-acid peptides, 34 and 49, we then synthesized two sets of 10-amino acid peptides. While the antibody #86 did not give enough signals with 10-amino acid peptides from 20-aa peptide number 34 (Fig. 3), relatively stronger signals with 10-amino-acid peptides from 20-aa peptide number 49 was observed (10-amino acid peptides number 1-5, Fig. 4). Because the common sequence of those 10-aa peptides was 485DEDEEEE491, this sequence might play the important role for antibody binding of antibody #86.

Burks et al. [15] and Shin et al. [16] reported IgE-binding epitope sequences of Ara h1 detected with serum from patients allergic to peanut. Those sequences were shown in Fig. 1 (underlined). The epitope found with the IgM class human monoclonal antibody #86 in this study (shown in bold letters with

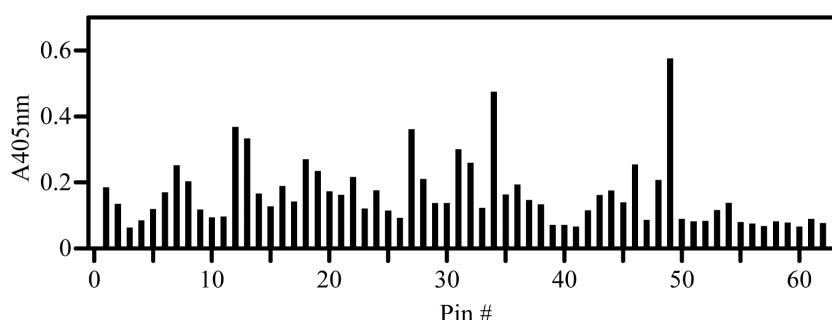


Fig. 2. Epitope analysis of 20 amino acid-peptides synthesized from amino acid sequence of Ara h with human monoclonal antibody #86.

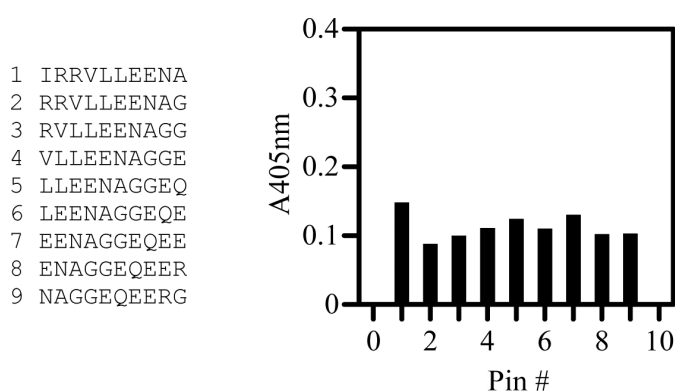


Fig. 3. Binding analysis of 10 amino acid-peptides from 20 amino acid-peptide #34 with antibody #86.

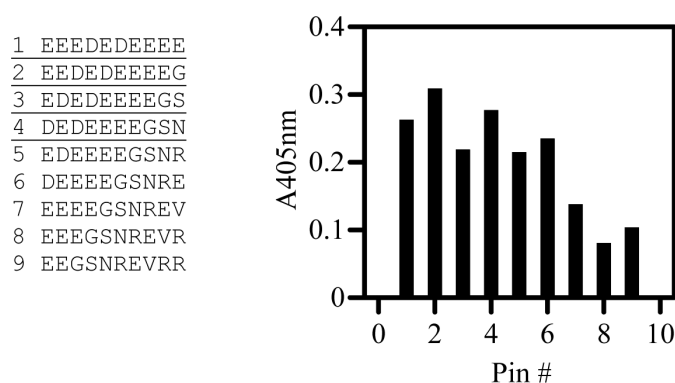


Fig. 4. Binding analysis of 10 amino acid-peptides from 20 amino acid-peptide #49 with antibody #86.

doubly underlined) was not involved in those epitopes detected by serum IgE from patients allergic to peanut.

To evaluate which amino acid was essential for binding of antibody #86, we synthesized a series of alanine substituted peptides of 14-amino-acid 484ED-EDEEEEGSNREV497. When the second, third and fourth amino acids D, E and D of the peptide were replaced with alanine (peptide #3, 4 and 5), the binding of antibody #86 was significantly decreased as shown in Fig. 5.

IgM antibodies on the B-cell surface were thought to play the role of antigen receptors [17]. When certain antigens bind to IgM antibody on B-cells, the B-cells incorporate the antigen molecule and process the antigen by proteolytic digestion. The processed antigen peptides are then presented on a class II major histocompatible complex (MHC) molecule on the cell surface. The complex of the antigen peptide and class II MHC is recognized by a T-cell receptor on T-cell surface, and the T-cell secretes specific cytokines such as

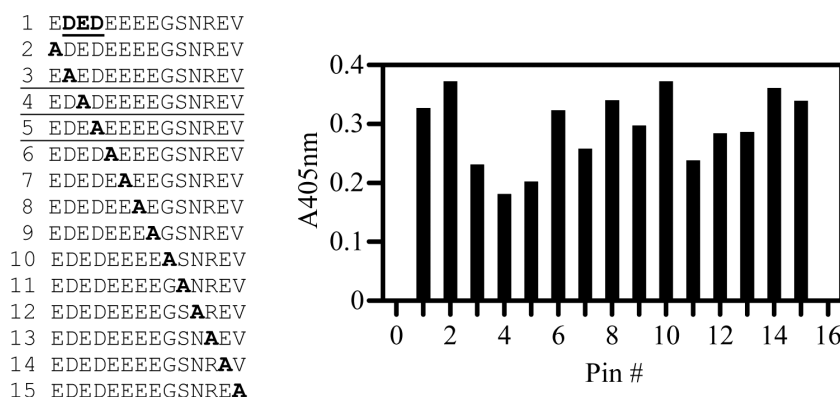


Fig. 5. Binding analysis of alanine-substituted peptides with antibody #86.

IL4 and IL13 to induce the B cells to an IgE-secreting plasma cells [18–20]. The IgM antibodies on the B cells may play a very important role in the Ig-class switch from IgM to IgE.

We have shown two IgM-epitopes with IgM class human monoclonal antibodies, clone #92-2 [7] and clone #86 (this study). Those B-cell epitope sequences will become a strong tool for analyzing B-cell-T-cell interaction of Ara h1 recognition.

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