Epitope analysis of peanut allergen Ara h1 with human monoclonal IgM antibody 92-2

Hiroshi Shinmoto · Yuji Matsuo ·
Yasunori Naganawa · Shinichi Tomita ·
Yuko Takano-Ishikawa

Received: 16 July 2009 / Accepted: 2 December 2009 / Published online: 19 December 2009
© Springer Science+Business Media B.V. 2009

Abstract A human-mouse hybridoma clone 92-2 secreting IgM-class human monoclonal antibody to peanut allergen protein Ara h1 was established. To detect antibody-binding sequences on Ara h1, we synthesized a series of peptides of the Ara h1 protein on a multi-pin apparatus for the pin-peptide ELISA. The 92-2 human monoclonal antibody was found to recognize a sequence of GREGEQEWGTPGSHVRE ETS. Further analysis with shorter pin-peptides with eight amino acid-long showed that the sequence of QEWGTPGS was an essential linear sequence of this epitope. When the QEW part of the sequence was replaced by alanine, the 92-2 monoclonal antibody did not bind to the substituted peptide, showing that those amino acids play an important role in the binding of the 92-2 monoclonal antibody.

Keywords Human monoclonal antibody · Allergen · Peanut · Epitope · IgM

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 (Imamura et al. 2008; Kanagawa et al. 2009). Several peanut proteins have been identified as allergens by using sera from patients allergic to peanut, and Ara h1 and Ara h2 have been reported as major peanut allergens (de Jong et al. 1998; Koppelman et al. 2004; Burks et al. 1991, Maleki et al. 2000). We have established five clones of mouse-human hybridomas secreting IgM-class human monoclonal antibodies to peanut allergens from peripheral blood lymphocytes transformed with Epstein-Barr virus followed by cell fusion with mouse myeloma cells (Naganawa et al. 2008; Shinmoto et al. 2004). In this paper, we present the results of a linear B-cell epitope analysis with synthesized Ara h1 oligo peptides recognized by IgM-class human monoclonal antibody 92-2.

Materials and methods

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

Peripheral blood lymphocytes (PBLs) prepared from peripheral blood from a healthy donor were transformed by Epstein-Barr virus infection as described in a previous report (Naganawa et al. 2005; Shinmoto

H. Shinmoto (✉) · Y. Matsuo · S. Tomita
Tamagawa University, Machida, Tokyo 194-8610, Japan
e-mail: shnmt920@agr.tamagawa.ac.jp

Y. Naganawa
Nagahama Institute of Bio-Science and Technology,
Nagahama, 526-0829 Japan

Y. Takano-Ishikawa
National Food Research Institute, Tsukuba 305-8642,
Japan

et al. 1992; Shinmoto et al. 1991). The resulting human B-lymphoblastoid cells (BLCs) secreting IgM-class antibody to peanut allergen Ara h1 were detected by Enzyme-Linked Immunosorbent Assay (ELISA). The B-lymphoblastoid cells and mouse myeloma SP2/O3 cells were cultured with ERDF medium (Kyokuto Co. Ltd., Japan) supplemented with 10% fetal calf serum (FCS, JRH Biosciences, KA, USA). The cell suspensions of BLCs (1×10^7) and SP2/O3 cells (1×10^7) were mixed and then centrifuged. After removing supernatant, 1 ml of 50% polyethyleneglycol (Hybrimax, average molecular weight of 1,450, Sigma Chemical Co., MO, USA) was added to the cell pellet. Then, the fusion mixture was diluted with 10 ml of ERDF medium and centrifuged again. The fused cells were suspended in 30 ml of ERDF medium supplemented with 15% FCS and the cells were cultured in two 96-well microculture plates for 24 h. The fused hybridoma cells were selected with O-HAT medium (ERDF medium supplemented with 10% FCS, 0.1 mmol/L hypoxanthine, 0.0003 mmol/L aminopterin, 0.016 mmol/L thymidine, and 0.002 mmol/ouabain) by replacing half of the culture medium three times a week (Shinmoto et al. 2004; Naganawa et al. 2005; Shinmoto et al. 1991). Antibody-producing hybridomas were analyzed by ELISA. Antibody-positive cells were cloned twice and a human-mouse hybridoma clone 92-2 secreting IgM-class anti Ara h1 antibody was established.

Enzyme-linked immunosorbent assay (ELISA)

Purified peanut allergen protein Ara h1 (kindly donated by Dr. S. J. Maleki, USDA, ARS, SRRC, New Orleans, LA, USA) was diluted with 0.05 mol/L NaHCO_3 (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, Dainippon Pharmaceutical 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 BLCs or 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 (1/2,000 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% H_2O_2 in 0.1 mol/L citrate buffer, pH 4.0).

Analysis of epitopes of peanut allergen Ara h1 by the multi-pin overlapping peptide method

First, we synthesized a series of overlapping peptides with 20-amino-acid length, based on an amino acid sequence of Ara h1 protein (Burks et al. 1995a; Burks et al. 1995b) as depicted in Figs. 1 and 2. The peptides were synthesized on a multi-pin apparatus (Mimotopes Pty Ltd., Clayton, Victoria, Australia) using a solid-phase method with immobilized C-terminus and acetylated N-terminus. Supernatants of hybridomas (0.1 mL) were 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 pins was measured by adding ABTS solution in a

Fig. 1 Amino acid sequence of peanut allergen protein Ara h1. IgE epitopes reported by by Burks et al. (1997) and Shin et al. (1998) are underlined and an IgM epitope found in this study was shown in bold letters (aa 156–163)

MRGRVSPLMLLLGILVLASV	SATHAKSSPYQKKTENPCAQ	RCLQSCQPEDDLKQKACES	60
RCTKLEYDPRCVYDPRGHTG	TTNQSRPPGERTRGRQPGDY	DDDRRQPRREEGGRWGAPG	120
<u>REEREEDWRQPREDWRRPS</u>	<u>HQQPRKIRPEGREGEQEWGT</u>	PGSHVREETS RNNPFYFPSR	180
RFSTRYGNQNGRIRVLQRFD	QRSRQFQNLQNHRIVQIEAK	PNTLVLPKHADADNILVIQQ	240
GQATVTVANGNNRKSFNLDE	GHALRIPSGFISYILNRHDN	QNLRVAKISMPVNTPGQFED	300
<u>FFPASSRDQSSYLQGFSRNT</u>	LEAAFNAEFNEIRRVLLEEN	AGGEQEERGQRRWSTRSSEN	360
NEGVIVKVSKEHVEELTKHA	KSVSKKGSEEGDITNPINL	REGEPDLSNNFGKLFYVKPD	420
KKNPQLQDLDMMLTCVEIKE	GALMLPHFNASKAMVIVVVK	GTGNLELVAVRKEQQQRGR	480
EEEEDEDEEEEGSNREVRRY	<u>TARLKEGDVFI</u> MPAAHPVAI	NASSELHLLGFGINAENNHR	540
<u>IFLAGDKDNVIDQIEKQAKD</u>	<u>LAFPGSGEQVEKLIK</u> NQKES	<u>HFVSARPQS</u> QSQSPSSPEKE	600
SPEKEDQEEENQGGKGPLLS	ILKAFN		626

Fig. 2 Pin peptide ELISA of Ara h1 20 amino-acid-overlapping peptides with antibody 92-2

1	MRGRVSPLMLLLGILVLASV	22	NHRIVQIEAKPNTLVLPKHA	43	KKNPQLQDLDMMMLTCVEIKE
2	LLGILVLASVSATHAKSSPY	23	PNTLVLPKHADADNIIQQ	44	MMLTCVEIKEGALMLPHFNS
3	SATHAKSSPYQKKTENPCAQ	24	DADNIIQQGQATVTVANG	45	GALMLPHFNSKAMVIVVNVK
4	QKKTENPCAQRCLQSCQQEP	25	GQATVTVANGNNRKSFNLDE	46	KAMVIVVNVKGTGNLELVAV
5	RCLQSCQQEPDDLKQKACES	26	NNRKSFNLDEGHALRIPSGF	47	GTGNLELVAVRKEQQQRGR
6	DDLKQKACESRCTKLEYDPR	27	GHALRIPSGFISYILNRHDN	48	RKEQQQRGRREEEDEDEEE
7	RCTKLEYDPRCVYDPRGHTG	28	ISYILNRHDNQNLRVAKISM	49	EEEEDEDEEEGSNREVRRY
8	CVYDPRGHTGTTNQRSPGGE	29	QNLRVAKISMPVNTPGQFED	50	EGSNREVRRYTARLKEGDFV
9	TTNQRSPGGERTRGRQPGDY	30	PVNTPGQFEDFFPASSRDQS	51	TARLKEGDFVIMPAHPVAI
10	RTRGRQPGDYDDDRQPPRE	31	FFPASSRDQSSYLQGFSRNT	52	IMPAHPVAINASSELHLLG
11	DDDRQPPREEGRWGPAGP	32	SYLQGFSRNTLEAAAFNAEFN	53	NASSELHLLGFGINAENNHR
12	EGGRWGPAGPREREREEDWR	33	LEAAAFNAEFNEIRRVLLEEN	54	FGINAENNHRIFLAGDKDNV
13	REREREEDWRQFREDWRRPS	34	EIRRVLLEENAGGEQEEERGQ	55	IFLAGDKDNVIDQIEKQAKD
14	QPRREDWRRPSHQPRKIRPE	35	AGGEQEEERGQRRWSTRSEN	56	IDQIEKQAKDLAFPSGSEQV
15	HQPRKIRPEGREGEQEWGT	36	RRWSTRSENNEGVIVKVS	57	LAFPSGSEQVEKLIKQKES
16	GREGEQEWGTPGSHVREETS	37	NEGVIVKVSKEHVEELTKHA	58	EKLIKQKESHFVSARPSQ
17	PGSHVREETSRRNPFYFPSR	38	EHVEELTKHAKSVSKKGSEE	59	HFVSARPSQSQSPSSPEKE
18	RNNPFYFPSRRFSTRYGNQN	39	KSVSKKGSEEGDITNPINL	60	SQSPSSPEKESPEKEDQEEE
19	RFSTRYGNQNGRIRVLQRF	40	EGDITNPINLREGEPDLSNN	61	SPEKEDQEEENQGGKGPLLS
20	GRIRVLQRFDRSRQFQNLQ	41	REGEPDLSNNFGKLFVEKPD	62	NQGGKGPLLSILKAFN
21	QRSRQFQNLQNHRIVQIEAK	42	FGKLFVEKPKDKKNPQLQDLD	63	ILKAFN

new 96-well plate. The absorbance of the reaction product was measured at 405 nm. Shorter peptides and alanine-substituted peptides were also synthesized to determine the fine epitope structure.

Results and discussion

In this study, we employed an IgM class human monoclonal antibody 92-2 to evaluate an amino acid sequence of antibody binding site (B-cell epitope). At first, we synthesized a series of peptides (overlapping peptides) with 20-amino-acid length (Fig. 2) with 10-amino-acid overlapping to detect 92-2 B-cell epitope on a peanut allergen Ara h1. Of 63 peptides analyzed, binding signals were observed at pins #16, #18, #28, and #54. Control analysis without 92-2 monoclonal antibody (peroxidase conjugated second antibody only) revealed that the signals at peptide #18, #28, and #54 were non-specific binding of the second antibody (data not shown). Then we concluded that the 92-2 human monoclonal antibody to Ara h1 bound to the pin #16 with the amino acid sequence of GREGEQEWGTPGSHVREETS (aa 151–170 of Ara h1).

From the amino acid sequence of the pin #16 of 20-amino-acid peptide, we then synthesized a series

GREGEQEWGTPGSHVREETS

- 1 GREGEQEW
- 2 REGEQEWG
- 3 EGEQEWGT
- 4 GEQEWGTP
- 5 EQEWGTPG
- 6 **QEWGTPGS**
- 7 EWGTPGSH
- 8 WTPGSHV
- 9 TPGSHVR
- 10 TPGSHVRE
- 11 PGSHVREE
- 12 GSHVREET
- 13 SHVREETS

Fig. 3 Pin peptide ELISA of Ara h1-8 amino-acid-overlapping peptides with antibody 92-2

of 8-amino-acid peptides (Fig. 3). The 92-2 antibody bound only to the pin #6 with the sequence of QEWGTPGS. This QEWGTPGS sequence (aa 156–163 of Ara h1) is thought to be a “core” amino acid sequence of an epitope recognized with the human monoclonal antibody 92-2.

Fig. 4 Pin peptide ELISA of Ara h1 epitope peptide with alanine substitution with antibody 92-2

Burks et al. (1997) and Shin et al. (1998) reported epitope sequences of Ara h1 detected with serum from patients allergic to peanut. Those sequences are shown in Fig. 1 (underlined). The epitope found with the IgM class human monoclonal antibody 92-2 in this study was not involved in the epitopes detected by serum IgE from patients allergic to peanut.

To evaluate which amino acid was essential for binding of 92-2 antibody, we synthesized a series of alanine substituted peptides with 8-amino-acid length (Fig. 4). When the first amino acids Q (glutamine) was replaced with alanine (peptide #2), the 92-2 antibody completely lost its binding activity to the peptides. Also the 92-2 antibody did not bind to the peptides when the second and the third amino acids of E (glutamic acid) and W (tryptophane) were replaced with alanine (peptides #3 and #4). This means that those Q (glutamine), E (glutamic acid), and W (tryptophane) amino acid residues played very important roles in antibody binding of the 92-2 antibody.

In a type I allergic reaction, IgE-class antibodies play a key role on the mast-cell surface (Ishizaka and Ishizaka 1978; Prussin and Metcalfe 2006; Miescher and Vogel 2002). When the allergens bind to IgE antibodies on the mast cells, the mast cells release chemical mediators such as histamine and other chemical mediators, which induce allergic symptoms. Contrary to this, the human monoclonal antibodies obtained in our previous study were all of the IgM class (Shinmoto et al. 2004). This seems to be due to

the fact that the population of cells transformed by the Epstein-Barr virus infection were B-cells expressing IgM antibodies on the cell surface (Crain et al. 1989; Zhu et al. 2008). IgM antibodies on the B-cell surface were thought to play the role of antigen receptors (O'Neill et al. 2005). B-cells also act as antigen-presenting cells (APC). 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 IL4 and IL13 to induce the B cell to an IgE-secreting plasma cell (Vries et al. 1993; Hajoui et al. 2004; Kajiwarra et al. 2004). The IgM antibody on the B cell may play a very important role in the Ig-class switch from IgM to IgE. Our finding of B-cell epitope with IgM-class human monoclonal antibody to Ara h1 in this study may contribute to further analysis of antigen presentation of Ara h1 by B-cells to helper T cells.

Conclusion

In this study, we demonstrated an antibody-binding amino-acid sequence (B-cell epitope) of the peanut allergen Ara h1 by using human IgM-class monoclonal antibody 92-2. The 92-2 antibody recognized a peptide sequence of QEWGTDGS, and the amino-acid sequence of Q, E, and W played an essential role in the binding of the 92-2 antibody. This human IgM-class monoclonal antibody seems to be useful for analyzing APC-T-cell interaction in vitro.

References

- Burks AW, Williams LW, Helm RM, Connaughton C, Cockrell G, O'Brien T (1991) Identification of a major peanut allergen, Ara h I, in patients with atopic dermatitis and positive peanut challenges. *J Allergy Clin Immunol* 88:172–179
- Burks AW, Cockrell G, Stanley JS, Helm RM, Bannon GA (1995a) Isolation, identification, and characterization of clones encoding antigens responsible for peanut hypersensitivity. *Int Arch Allergy Immunol* 107:248–250

- Burks AW, Cockrell G, Stanley JS, Helm RM, Bannon GA (1995b) Recombinant peanut allergen Ara h I expression and IgE binding in patients with peanut hypersensitivity. *J Clin Invest* 96:1715–1721. doi:[10.1172/JCI118216](https://doi.org/10.1172/JCI118216)
- Burks AW, Shin D, Cockrell G, Stanley JS, Helm RM, Bannon GA (1997) Mapping and mutational analysis of the IgE-binding epitopes on Ara h 1, a legume vicilin protein and a major allergen in peanut hypersensitivity. *Eur J Biochem* 245:334–339
- Crain MJ, Sanders SK, Butler JL, Cooper MD (1989) Epstein-Barr virus preferentially induces proliferation of primed B cells. *J Immunol* 143:1543–1548
- de Jong EC, Van Zijverden M, Spanhaak S, Koppelman SJ, Pellegrom H, Penninks AH (1998) Identification and partial characterization of multiple major allergens in peanut proteins. *Clin Exp Allergy* 28:743–751
- de Vries JE, Punnonen J, Cocks BG, de Waal Malefyt R, Aversa G (1993) Regulation of the human IgE response by IL4 and IL13. *Res Immunol* 144:597–601
- Hajoui O, Janani R, Tulic M, Joubert P, Ronis T, Hamid Q, Zheng H, Mazer BD (2004) Synthesis of IL-13 by human B lymphocytes: regulation and role in IgE production. *J Allergy Clin Immunol* 114:657–663
- Imamura T, Kanagawa Y, Ebisawa M (2008) A survey of patients with self-reported severe food allergies in Japan. *Pediatr Allergy Immunol* 19:270–274
- Ishizaka K, Ishizaka T (1978) Mechanisms of reaginic hypersensitivity and immunotherapy. *Lung* 155:3–22
- Kajiwara K, Shinazawa M, Morishima H, Yanagihara Y (2004) Differential effect of IL-4 and IL-13 on the expression of recombination-activating genes in mature B cells from human peripheral blood. *Cell Immunol* 227:121–128
- Kanagawa Y, Matsumoto S, Koike S, Imamura T (2009) Association analysis of food allergens. *Pediatr Allergy Immunol* 20:347–352
- Koppelman SJ, Wensing M, Ertmann M, Knulst AC, Knol EF (2004) Relevance of Ara h1, Ara h2 and Ara h3 in peanut-allergic patients, as determined by immunoglobulin E Western blotting, basophil-histamine release and intracutaneous testing: Ara h2 is the most important peanut allergen. *Clin Exp Allergy* 34:583–590
- Maleki SJ, Kopper RA, Shin DS, Park CW, Compadre CM, Sampson H, Burks AW, Bannon GA (2000) Structure of the major peanut allergen Ara h 1 may protect IgE-binding epitopes from degradation. *J Immunol* 164:5844–5849
- Miescher SM, Vogel M (2002) Molecular aspects of allergy. *Mol Aspects Med* 23:413–462
- Naganawa Y, Shinmoto H, Shimmoto M (2005) Generation of mouse-human hybridomas secreting human monoclonal antibodies to Japanese cedar pollen allergen Cry j1. *Hum Antibodies* 14:27–31
- Naganawa Y, Shimmoto M, Maleki SJ, Takase M, Shinmoto H (2008) Epitope analysis of peanut allergen Ara h1 with oligoclonal IgM antibody from human B-lymphoblastoid cells. *Cytotechnology* 57:177–180. doi:[10.1007/s10616-008-9142-3](https://doi.org/10.1007/s10616-008-9142-3)
- O'Neill SK, Shlomchik MJ, Glant TT, Cao Y, Doodles PD, Finnegan A (2005) Antigen-specific B cells are required as APCs and autoantibody-producing cells for induction of severe autoimmune arthritis. *J Immunol* 174:3781–3788
- Prussin C, Metcalfe DD (2006) IgE, mast cells, basophils, and eosinophils. *J Allergy Clin Immunol* 117(Suppl):S450–S456
- Shin DS, Compadre CM, Maleki SJ, Kopper RA, Sampson H, Huang SK, Burks AW, Bannon GA (1998) Biochemical and structural analysis of the IgE binding sites on ara h1, an abundant and highly allergenic peanut protein. *J Biol Chem* 273:13753–13759
- Shinmoto H, Dosako S, Tachibana H, Shirahata S, Murakami H (1991) Generation of human-mouse hybridoma secreting human IgM class anti-neocarzinostatin antibody and its application to hybrid hybridoma. *Agric Biol Chem* 55:2883–2885
- Shinmoto H, Dosako S, Tanaka S (1992) Transformation of human colostrum lymphocytes with Epstein-Barr virus. *Tokai J Exp Clin Med* 17:129–132
- Shinmoto H, Naganawa Y, Shimmoto M, Maleki SJ (2004) Generation of mouse-human hybridomas secreting antibodies against peanut allergen Ara h1. *Cytotechnology* 46:19–23. doi:[10.1007/s10616-005-1578-0](https://doi.org/10.1007/s10616-005-1578-0)
- Zhu F, Ramadan G, Davies B, Margolis DA, Keever-Taylor CA (2008) Stimulation by means of dendritic cells followed by Epstein-Barr virus-transformed B cells as antigen-presenting cells is more efficient than dendritic cells alone in inducing *Aspergillus* f16-specific cytotoxic T cell responses. *Clin Exp Immunol* 151:284–296