

Generation of mouse–human hybridomas secreting antibodies against peanut allergen Ara h1

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Abstract

Two clones of mouse–human hybridomas, secreting human monoclonal antibodies to a peanut allergen Ara h1, were generated from human peripheral blood lymphocytes transformed with Epstein–Barr virus, followed by cell fusion with mouse myeloma cells. Epitope analysis with overlapping peptides synthesized on a multi-pin apparatus revealed antibody-binding sequences of Ara h1 protein.

Introduction

To analyze the structures of food allergens, we plan to obtain human monoclonal antibodies to food allergens. To begin with, we transformed human peripheral B cells with Epstein–Barr virus (EBV). We then tried to clone the EBV-transformed B-lymphocytes (B-lymphoblastoid cells, BLCs) by limiting dilution and soft agar methods. However, we could not obtain any stable BLC-clone secreting antibodies to food allergens. The BLCs were reported to grow for a limited period because of the absence of telomerase activity (Counter et al. 1994) and only a small population of BLCs having telomerase activity grew continuously. That seemed to be why the preparation of human monoclonal antibodies by EBV-transformation of human lymphocytes was not successful. To solve this problem, we employed a method to prepare hu-

man–mouse hetero hybridomas (Shinmoto et al. 1990).

In this study, we established two hybridomas secreting human monoclonal antibodies reacting with a major peanut allergen (Ara h1) and show the results of epitope analysis with synthetic Ara h1 peptides.

Materials and methods

Preparation of peripheral blood lymphocytes and transformation of the lymphocytes with Epstein–Barr virus

Peripheral blood lymphocytes (PBLs) were prepared from peripheral blood from 13 healthy donors by discontinuous density centrifugation on lymphocyte separation medium (Organon Teknika,

USA). The PBLs were washed twice with ERDF medium (Kyokuto Seiyaku, Japan), and were then suspended into ERDF medium (10^6 cells/ml) supplemented with 10% fetal calf serum (FCS, JRH Biosciences, Lenexa, KS, USA), cyclosporin A ($2 \mu\text{g/ml}$) and 1/10 volume of culture supernatants of marmoset B95-8 cells, containing Epstein–Barr virus (EBV). The cell mixture was then plated into 96-well microculture plates (10^5 cells/well). The medium (ERDF medium supplemented with 10% FCS) was changed every two or three days. After three weeks of the culture, transformed cells (B-lymphoblastoid cells, BLCs) and the culture supernatants were frozen and stored.

Enzyme-linked immunosorbent assay (ELISA)

Powdered peanut (1.0 g) was used for extraction with 10 ml of 0.5 mol/l NaCl for 2 h at room temperature. The mixture was centrifuged at 15,000 rpm for 10 min. The supernatant (protein concentration of 12.5 mg/ml) was used as a crude peanut allergen. The crude peanut allergen was diluted with 0.05 mol/l NaHCO_3 ($1 \mu\text{g/ml}$), 0.05 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, Dai-nippon Pharmaceutical, 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 (0.05 ml) were pipetted into the plates and incubated for 1 h at room temperature. The plates were then washed three times, and 0.05 ml of antibodies (anti-human IgA, IgG, IgM, and IgE, 1/2000 dilution) conjugated with horseradish peroxidase (Bio-source International, 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 plates was measured by adding 0.1 ml of substrate 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).

Cell fusion of BLCs with mouse myeloma cells

An 8-azaguanine-resistant (HAT sensitive) mouse myeloma cell line, SP2/O, was cultured in an

ERDF medium supplemented with 10% (v/v) FCS and 0.05 mM ouabain to obtain an ouabain-resistant cell line, SP2/O3. BLCs (1×10^7) and SP2/O3 cells (1×10^7) were fused in 50% (v/v) poly-ethyleneglycol. The fused cells were then plated into three 96-well microculture plates. The cells were cultured in O-HAT medium (the ERDF medium supplemented with 10% FCS, 0.1 mmol/l hypoxanthine, 0.0004 mmol/l aminopterin, 0.016 mmol/l thymidine, and 0.002 mmol/l ouabain). Antibody-producing cells were analyzed by an enzyme-linked immunosorbent assay (ELISA).

Western blot analysis of peanut allergens with human monoclonal antibodies

Crude peanut allergens or peanut allergen Ara h1 protein were boiled for 5 min with an SDS-sample buffer containing 2.5% (v/v) 2-mercaptoethanol. The allergens were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10 to 20% (w/v) gradient gel (Daiichi-Kagaku, Tokyo, Japan). The proteins on the gel were transferred to a PolyScreen transfer membrane. The membrane was placed on a multi-slot reacting apparatus (ATTO, Tokyo, Japan), and the culture supernatants of the hybridomas were reacted with the membrane. The culture supernatants were diluted to produce absorbancy of 0.2–0.5 at 415 nm at ELISA. Anti-human IgM antibody conjugated with horseradish peroxidase (1/2000 dilution) was then reacted with the membrane. Finally, the membrane was developed with a Konika immunostaining kit (Konika, Tokyo, Japan).

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

We synthesized the series of 20 overlapping peptides based on an amino acid sequence of Ara h1 protein (Burks et al. 1995). The peptides were synthesized on a multi-pin apparatus (Chiron Technologies, Clayton, Victoria, Australia) by the solid-phase synthesis method with immobilized C-terminus and free NH_2 . Supernatants of hybridomas (0.1 ml) were placed in a 96-well microtiter plate, and multi-pin peptides were placed on 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 was measured by adding ABTS solution and absorbance of the reaction product was measured at 415 nm.

Results and discussion

Transformation of peripheral blood lymphocytes with EBV and establishment of mouse–human hybridomas

We obtained more than 5000 transformed human B-lymphoblastoid cells (BLCs) from peripheral blood lymphocytes from 13 healthy donors. The supernatants of the BLCs were analyzed by ELISA for antibodies against crude peanut allergen. Table 1 shows the result. Many BLCs secreted antibodies to crude peanut allergen. These cells mostly secreted IgM-class antibodies. Preferential infection of EBV to B cells expressing IgM molecules on the cell surface could account for the high occurrence of IgM antibodies (Brown and Miller 1982). Of those cells, we chose seven BLCs with a high titer of antibodies and good cell growth for cell fusion with mouse myeloma.

Seven BLCs secreting antibodies to peanut allergens were fused with mouse myeloma SP2/O3 cells. Two stable human–mouse hybridoma clones 4 and 5, secreting human monoclonal IgM-class antibodies reacting with a peanut allergen Ara h1, were established.

Western blot analysis of peanut allergen Ara h1 with human monoclonal antibodies

Figure 1 shows the results of the Western blot analysis. Human monoclonal antibodies 4 and 5 exhibited stained bands of 60–67 kDa (Figure 1:

Table 1. Transformation of human peripheral B cells and detection of the cells secreting peanut specific antibodies.

BLCs established	5142
Anti-peanut antibody	80
IgA	0
IgG	5 ^a
IgM	79 ^a
IgE	0

^aFour B-lymphoblastoid cells secreting IgG-class antibody also secreted IgM-class antibodies.

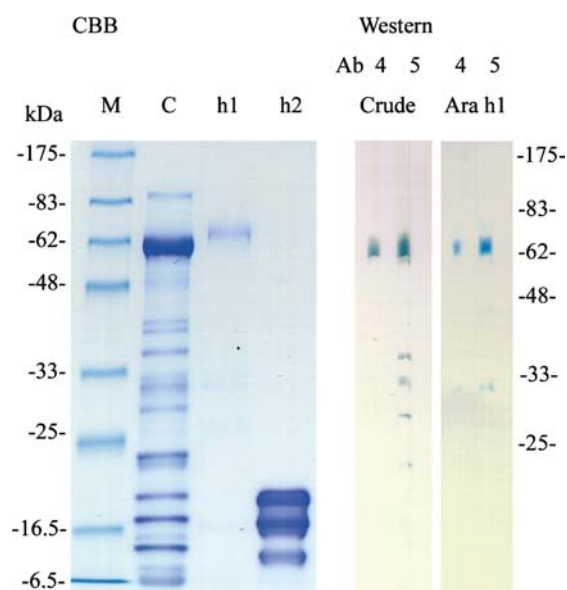


Figure 1. Western blot analysis of peanut allergens. The proteins on the PAGE-gel were transferred to a PolyScreen transfer membrane. The culture supernatants of the hybridomas were reacted with the membrane. The culture supernatants were diluted to produce absorbance of 0.2–0.5 at 415 nm at ELISA. Anti-human IgM antibody conjugated with horseradish peroxidase was then reacted with the membrane and the membrane was developed with a Konika immunostaining kit. CBB: Coomassie brilliant blue staining, M: Marker, C: crude extract, h1: Ara h1, h2: Ara h2. Western: Crude extract (Crude) and Ara h1 were reacted with monoclonal antibodies (Ab) 4 and 5.

crude); molecular weights were similar to that of Ara h1. Results of Western blot analysis of purified Ara h1 with those two human monoclonal antibodies (Figure 1: Ara h1) demonstrated that antibodies 4 and 5 reacted with Ara h1.

Epitope analysis of Ara h1 with the human monoclonal antibodies 4 and 5

To detect the binding sequences in Ara h1 allergen, we synthesized a series of peptides (overlapping peptides). We prepared 20 peptides by repeating the shift of every 10 peptides in the direction from N to C terminus of the sequence of Ara h1 protein (Figure 2). Monoclonal antibodies from human–mouse hybridomas 4 and 5 were reacted with the pins (Figure 3). Antibody 4 reacted with Pin #5 with an amino acid sequence of RCLQSCQQEPDDLKQKACES, and antibody 5 reacted with Pin #46 with a sequence of

[Amino acid sequence of Ara h1]

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MRGRVSPLMLLLGILVLASV SATHAKSSPYQKKTENPCAQ RCLQSCQQEPDDLKQKACES 60
RCTKLEYDPRCVYDPRGHTG TTNQRSPPGERTRGRQPGDY DDDRRQPRREEGGRWGPAGP 120
REREREEDWRQPREDWRRPS HQQPRKIRPEGREGEQEWGT PGSHVREETSRNNPFYFPSR 180
RFSTRYGNQNGRIRVLQRFD QRSRQFQNLQNHRIQIEAK PNTLVLPKHADADNILVIQ 240
QATVTVANGNNRKSFNLDE GHALRIPSGFISYILNRHDN QNLRVAKISMPVNTPGQFED 300
FFPASSRDQSSYLQGFSSNT LEAAFNAEFNEIRRVLLEEN AGGEQEERGQRRWSTRSEN 360
NEGVIVKVSKEHVEELTKHA KSVSKKGSEEGDITNPINL REGEPLSNNFGKLFVVKPD 420
KKNPQLQDLDMMLTCVEIKE GALMLPHFNSKAMVIVVVK GTGNLELVAVRKEQQQRGR 480
EEEEDEDEEEEGSNREVRRY TARLKEGDVFIMPAHPVAI NASSELHLLGFGINAENNR 540
IFLAGDKDNVIDQIEKQAKD LAFPGSGEQVEKLIKQKES HFVSARFQSQSQSPSSPEKE 600
SPEKEDQEEENQGGKGPLLS ILKAFN 626
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[Synthesized Ara h1 overlapping peptides]

1	22	43
MRGRVSPLMLLLGILVLASV	NHRIVQIEAKPNTLVLPKHA	KKNPQLQDLDMMLTCVEIKE
2	23	44
LLGILVLASVSATHAKSSPY	PNTLVLPKHADADNILVIQ	MLLTCVEIKEGALMLPHFNS
3	24	45
SATHAKSSPYQKKTENPCAQ	DADNILVIQQGQATVTVANG	GALMLPHFNSKAMVIVVVK
4	25	46
QKKTENPCAQRCLQSCQQEP	GQATVTVANGNNRKSFNLDE	KAMVIVVVKGTGNLELVAV
5	26	47
RCLQSCQQEPDDLKQKACES	NNRKSFNLDEGHALRIPSGF	GTGNLELVAVRKEQQQRGR
6	27	48
DDLKQKACESRCTKLEYDPR	GHALRIPSGFISYILNRHDN	RKEQQQRGRREEEDEDEEE
7	28	49
RCTKLEYDPRCVYDPRGHTG	ISYILNRHDNQNLRVAKISM	EEEEDEDEEEEGSNREVRRY
8	29	50
CVYDPRGHTGTTNQRSPPGE	QNLRVAKISMPVNTPGQFED	EGSNREVRRYTARLKEGDVF
9	30	51
TTNQRSPPGERTRGRQPGDY	PVNTPGQFEDFFPASSRDQS	TARLKEGDVFIMPAHPVAI
10	31	52
RTRGRQPGDYDDRRQPRRE	FFPASSRDQSSYLQGFSSNT	IMPAHPVAINASSELHLLG
11	32	53
DDRRQPRREEGGRWGPAGP	SYLQGFSSNTLEAAFNAEFN	NASSELHLLGFGINAENNR
12	33	54
EGGRWGPAGPREREREEDWR	LEAAFNAEFNEIRRVLLEEN	FGINAENNRIFLAGDKDNV
13	34	55
REREREEDWRQPREDWRRPS	EIRRVLLEENAGGEQEERGQ	IFLAGDKDNVIDQIEKQAKD
14	35	56
QPREDWRRPSHQQPRKIRPE	AGGEQEERGQRRWSTRSEN	IDQIEKQAKDLAFPGSGEQV
15	36	57
HQQPRKIRPEGREGEQEWGT	RRWSTRSENNEGVIVKVS	LAFPGSGEQVEKLIKQKES
16	37	58
GREGEQEWGTPGSHVREET	NEGVIVKVSKEHVEELTKHA	EKLIKQKESHFVSARFQSQ
17	38	59
PGSHVREETSRNNPFYFPSR	EHVEELTKHAKSVSKGSEE	HFVSARFQSQSQSPSSPEKE
18	39	60
RNNPFYFPSRRFSTRYGNQN	KSVSKKGSEEGDITNPINL	SQSPSSPEKESPEKEDQEEE
19	40	61
RFSTRYGNQNGRIRVLQRFD	EGDITNPINLREGEPLSNN	SPEKEDQEEENQGGKGPLLS
20	41	62
GRIRVLQRFQSRQFQNLQ	REGEPLSNNFGKLFVVKPD	NQGGKGPLLSILKAFN
21	42	63
QRSRQFQNLQNHRIQIEAK	FGKLFVVKPDKKNPQLQDL	ILKAFN

Figure 2. Sequences of synthesized overlapping Ara h1 peptides.

KAMVIVVVKGTGNLELVAV. The sequence of Pin #5 included a 48–57 sequence QEPDDLKQKA that was reported to be one of the Ara h1 epitopes determined by using sera from patients allergic to peanuts (Burks et al. 1997; Shin

et al. 1998). Pin #46 did not contain any known epitope sequence of Ara h1 protein.

Swiss model plot (Westbrook et al. 2003) for Ara h1 partial peptide sequence (P43237, <http://swissmodel.expasy.org/repository/sms.php?spt>

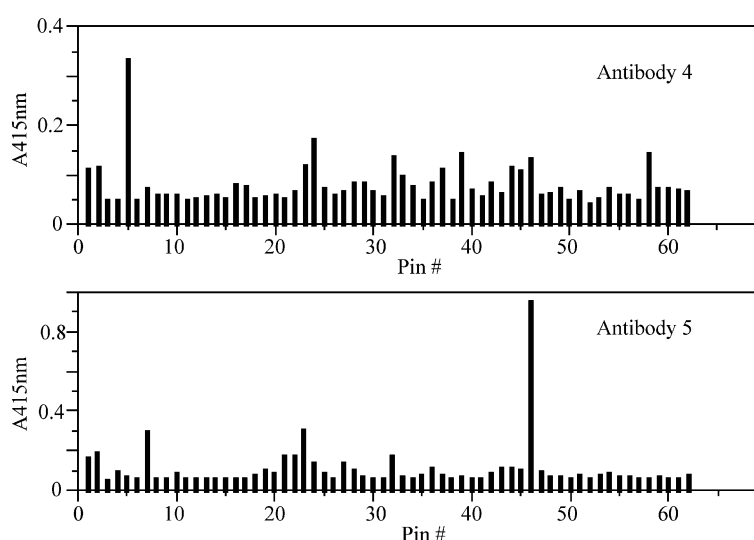


Figure 3. Epitope analysis of Ara h1 with the human monoclonal antibodies 4 and 5. Synthesized overlapping peptides based on an amino acid sequence of Ara h1 protein reacted with antibody 4 (upper) and antibody 5 (lower). Supernatants of hybridomas were placed in a 96-well microtiter plate, and multi-pin peptides were placed on the plate and reacted for 1 h at room temperature. Multi-pin peptides were then reacted with anti-human IgM antibody conjugated with horseradish peroxidase. An enzyme activity was measured by adding ABTS solution.

r_{ac}= P43237) showed the peptide #46 was calculated to have two strand structures to make arch structure and the middle part of the oligo peptide located on the surface of the molecule of Ara h1 protein.

Additionally, we performed BLAST analysis (Altschul et al. 1997). The result showed amino acid sequence of Pin #46 was homologous to peanut conarachin (*Arachis hypogaea*, 100%), French vetch convicilin (*Vicia narbonensis*, 70%), lentil convicilin (*Lens culinaris*, 75%), soybean beta-conglycinin alpha subunit (*Glycine max*, 61%). Further study for cross reactivity of the antibody 5 to tree nuts allergens should be needed.

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