



Epitope analysis of a rice allergen with a monoclonal antibody secreted by a human-mouse hybridoma

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Abstract

Five human human-mouse hybridomas secreting human monoclonal antibodies to rice allergens were established. Antibodies secreted from the hybridomas reacted with 14–16 kDa rice major allergen proteins. Analysis with overlapping peptides synthesized on a multi-pin apparatus revealed a binding sequence of the major rice allergen protein RA17. The antigenic determinant was located in the C-terminus region of the RA17 protein.

Introduction

To analyse structures of food allergens, we plan to obtain human monoclonal antibodies to food allergens. As it appeared difficult to prepare a standard sera library for allergenicity testing (Taylor 1995), we have developed a new system to evaluate the allergenicity of foods employing the B-cell transforming technique. Our method employed the transformation of human B cells with Epstein–Barr virus (EBV) followed by cell fusion with mouse myeloma cells to establish stable human-mouse hybridomas (Shinmoto *et al.* 1990, 1991a,b, 1992). We have already obtained over 2200 multi-clone, transformed human B cell library stocks from seven healthy donors by transforming peripheral blood B cells with EBV (Shinmoto *et al.* 1998). The library contained transformed human B cells secreting antibodies against rice, soybeans, peanuts, wheat, milk proteins, and egg-white proteins.

Whereas we tried to clone the transformed B lymphoblastoid cells (BLCs) by limiting dilution and soft agar methods, 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 small population of BLCs having telomerase activity could grow continuously. That

seemed to be the reason 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 human-mouse hetero hybridomas. In this study, we report the preparation of human-mouse hybridomas secreting antibodies to rice allergens and the result of B cell epitope analysis.

Materials and methods

Transformation of human B cells

As described in the previous paper (Shinmoto *et al.* 1988), we obtained an Epstein–Barr virus (EBV) transformed human B cell library from peripheral blood lymphocytes (PBLs) from seven healthy donors. Our transformed B lymphoblastoid cell (BLC) library consisted of 2202 stocks of transformed cell ampoules and frozen supernatants. Analysis of antibodies to food allergens showed that this library contained antibodies to rice, soybeans, milk proteins, and egg-white proteins (Shinmoto *et al.* 1998). We chose five BLCs secreting antibodies to rice allergens for cell fusion with mouse myeloma cells.

Cell fusion of BLCs with mouse myeloma cells

8-Azaguanine-resistant (HAT sensitive) mouse myeloma cell line SP2/O was cultured in an ERDF medium (Kyokuto Co. Ltd., Tokyo, Japan) supplemented with 10% (v/v) fetal calf serum (FCS, JRH Biosciences, Lenexa, KS, USA) and 0.05 mM ouabain to obtain an ouabain-resistant cell line SP2/O3. B lymphoblastoid cells (1×10^7) and SP2/O3 cells (1×10^7) were fused in 50% (v/v) polyethyleneglycol. The fused cells were then plated into three 96-well microculture plates. The cells were cultured in HAT medium (the ERDF medium supplemented with 10% FCS, 0.1 mM hypoxanthine, 0.4 μ M aminopterin and 0.016 mM thymidine) containing 0.002 mM ouabain (O-HAT). In this medium, the BLCs were killed with ouabain, and the myeloma cells with aminopterin. After 2 to 4 weeks of culture, the number of wells with cell growth was scored; antibodies in the supernatant were assayed by enzyme-linked immunosorbent assay (ELISA). Antibody positive hybridomas were cloned twice by the limiting dilution method. Briefly, 100 hybridoma cells were plated into three 96 well plates and cultured for three weeks. Supernatants from wells with cell growth were assayed by ELISA and antibody positive hybridoma clones were established.

Enzyme-linked immunosorbent assay of culture supernatants to rice allergens

Antibody-producing cells were analysed by an enzyme-linked immunosorbent assay (ELISA). Crude rice allergens were extracted by stirring rice powder with 0.5 M NaCl at room temperature for 6 h. ELISA plates (Sumitomo Bakelite Co., Tokyo, Japan) were coated with antigen solutions (protein contents of 0.001 mg ml⁻¹, dissolved with 0.05 M NaHCO₃) and then blocked with Block Ace (Dainippon Pharmaceuticals, Osaka, Japan). Supernatants of hybridomas were placed on the plates, and the plates were incubated for 1 h at room temperature. The plates were then washed with phosphate-buffered saline (PBS) containing 0.05% Tween20 (PBS-Tween) and then reacted with anti-human IgA, IgG, IgM or IgE antibody conjugated with horse radish peroxidase (Biosource International, Camarillo, CA, USA) diluted with Block Ace. Peroxidase activities on the wells were determined with substrate solution (0.3 mg ml⁻¹ 2,2'-azino-di-(3-ethyl benzthiazolin sulfonic acid) (ABTS), 0.03% (v/v) H₂O₂ in 0.1 M citrate buffer pH 4.0). Absorbancy of the reaction product

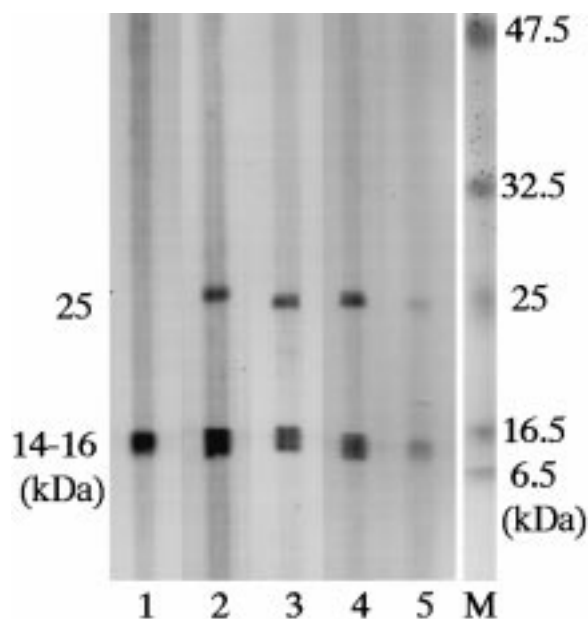


Fig. 1. Western blot analysis of rice allergens with human monoclonal antibodies. 1: 3-207-2-6, 2: 5-39-147-932, 3: 5-133-64, 4: 5-175-7-2, 5: 5-215-24, M: Markers.

was measured at 405 nm by a MPRA4 multi-plate reader (Tosoh Co., Tokyo, Japan).

Western blot analysis of rice allergens with human monoclonal antibodies

Crude rice allergens were boiled for 5 min with SDS-sample buffer containing 2.5% (v/v) 2-mercaptoethanol. The crude allergens were separated by an 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 (NEN Life Science Products, Boston, MA, USA), and the membrane was blocked with Block Ace. The membrane was placed on a multi-slot reacting apparatus (ATTO Co., Tokyo, Japan), and the culture supernatants of the hybridomas were reacted with the membrane. Anti-human IgM antibodies conjugated with horseradish peroxidase were then reacted with the membrane. Finally, the membrane was developed with a Konika immunostaining kit (Konika, Tokyo, Japan). Pre-stained molecular marker proteins (New England Bio Labs, Beverly, MA, USA) were used as standard marker proteins.

10	20	30	40	50	60	70
DHHQVSPGE	QCRPGISYPT	YSLPQCRTL	RRQCVGRGAS	AADEQVWQDC	CRQLAAVDG	WCRGALDHM
80	90	100	110	120	130	135
LSGIYRELGA	TEAGHPMAEV	FPGCRRGDLE	RAAASLPAFC	NVDIPNGPGG	VCYWLGYPR	PRTGH

Sequence	Pin number
DHHQVSPGE	1
HQVSPGEQC	2
VYSPGEQCRP	3
SPGEQCRPGI	4
GEQCRPGISY	5
QCRPGISYPT	6
RPGISYPTSY	7
GISYPTSYSLP	8
SYPTSYSLPQC	9
PTSYSLPQCRT	10
YSLPQCRTL	11
LPQCRTLVR	12
QRTLVRRCG	13
RTLVRRQCVG	14
LVRRQCVGRG	15
RRQCVGRGAS	16
QCVGRGASAA	17
VGRGASAADE	18
RGASAADEQV	19
ASAADEQVWQ	20
AADEQVWQDC	21
DEQVWQDCCR	22
QVWQDCCRQL	23
WQDCCRQLAA	24
DCRQLAAVD	25
CRQLAAVDG	26
QLAAVDGWC	27
AAVDGWCRC	28
VDDGWCRCGA	29
DCWCRCGALD	30
WCRGALDHM	31
RCGALDHMLS	32
GALDHMLSGI	33
LDHMLSGIYR	34
HMLSGIYREL	35
LSGIYRELGA	36
GIIYRELGA	37
YRELGA	38
ELGATEAGHP	39
GAEGHPMA	40
TEAGHPMAEV	41
AGHPMAEVFP	42
HPMAEVFPGC	43
MAEVFPGCR	44
EVFPGCRGD	45
FPGCRGDLE	46
GCRGDLE	47
RRGDLE	48
GDLE	49
LERAAASLPA	50
RAAASLPAFC	51
AASLPAFCNV	52
SLPAFCNVDI	53
PAFCNVDIPN	54
FCNVDIPNGP	55
NVDIPNGPGG	56
DIPNGPGGVC	57
PNGPGGVCYW	58
GGPGGVCYWL	59
GGVYWLGYPR	60
VCYWLGYPR	61
YWLGYPRPR	62
LGYPRTG	63
YPRTPRTGH	64

Fig. 2. Amino acid sequence of rice allergen RA17 and sequences of pin-peptides.

Analysis of epitopes of rice allergen RA17 by multi-pin overlapping peptide method

We synthesized the series of overlapping deca-peptides shown in Figure 2 from an amino acid sequence of a rice RA17 allergen (Adachi 1995, Alvarez *et al.* 1999). The peptides were synthesized on a multi-pin apparatus (Chiron Technologies Pty. Ltd., Clayton, Victoria, Australia) by the solid-phase synthesis method with immobilized C-terminus and free NH₂. Pin-peptides were washed with PBS-Tween and then blocked with Block Ace. Supernatants of a hybridoma (0.1 ml) diluted with Block Ace 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 horse radish peroxidase. After washing, enzyme activity was measured by the addition of ABTS solution and absorbancy of the reaction product was measured at 405 nm.

Results and discussion

Cell fusion of BLCs with mouse myeloma SP2/O3

We have obtained more than 150 BLC-stocks containing cells secreting antibodies to rice allergens (Shinmoto *et al.* 1998). These cells secreted IgM class antibodies because of preferential infection of EBV to B cells expressing IgM molecules on the cell surface (Brown & Miller 1982). BLCs secreting antibodies to rice allergens were fused with 8-azaguanine and ouabain resistant mouse myeloma SP2/O3 cells and the fused cells were cultured in a HAT medium containing ouabain. Hybridomas were observed in all the

Table 1. Establishment of human-mouse hybridomas secreting antibodies against rice allergens.

BLCs ^a	Hybridoma clones established ^a
3-207	3-207-2-6
5-39	5-39-147-932
5-133	5-133-64
5-175	5-175-7-2
5-215	5-215-24

^a) B-lymphoblastoid cells (BLCs) were fused with mouse myeloma SP2/O3 cells and hybridomas secreting antibodies to rice allergens were cloned.

culture wells. The clones listed in Table 1 were established after repeated cloning by limiting dilution. We obtained five stable human-mouse hybridomas secreting human monoclonal IgM class antibodies to rice allergens.

Western blot analysis of rice allergens with five human-mouse hybridomas

Figure 1 shows the result of Western blot analysis. Four human monoclonal antibodies reacted with rice proteins having molecular weights of 14–16 kDa and 25 kDa. One antibody, 3-207-2-6, reacted only with 14–16 kDa allergens. Proteins of 14–16 kDa are thought to be rice major allergens reported by Nakamura & Matsuda (1996). These proteins were reported to include several proteins with similar molecular weight and primary structures. The allergen proteins were reported to have alpha-amylase inhibitor activity. The cDNAs of the rice allergens were classified to the alpha-amylase/trypsin inhibitor gene family (Nakamura & Matsuda 1996, Alvarez *et al.* 1995). The binding of four monoclonal antibodies to 25 kDa protein remains to be solved.

Epitope analysis of rice allergen RA17 with human monoclonal antibody 5-175-7-2

Molecular cloning of rice allergens revealed that there were several allergen molecules having similar primary structures (Adachi 1995, Alvarez *et al.* 1999). We chose one major allergen, RA17, for the epitope analysis. To detect the binding sequence of the RA17 allergen, we synthesized series of peptide (overlapping peptides) as shown in Figure 2 on a multi-pin apparatus. Technique of overlapping synthetic peptides has been used to map the antibody-binding epitope of al-

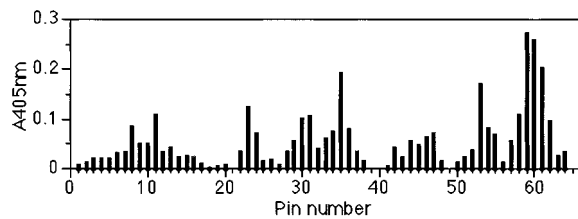


Fig. 3. Binding analysis of rice allergen RA17 with the human monoclonal antibody 5-175-7-2. Pin-peptides were reacted with supernatants of a hybridoma 5-175-7-2 and IgM molecules adsorbed on the pins were detected with anti-human IgM antibody conjugated with horse radish peroxidase. Peroxidase activity was measured by the addition of ABTS solution and absorbancy of the reaction product was measured at 405 nm.

lergens such as a major soybean allergen and a major latex allergen (Beezhold *et al.* 1997, Helm *et al.* 1998). We prepared 63 deca- and one nona-peptides by repeating the shift of every two peptides in the direction from *N* to *C* terminus of the sequence of the RA17 allergen. Monoclonal antibody from human-mouse hybridoma 5-175-7-2 was reacted with the pins. Figure 3 shows the result of the binding of the monoclonal antibody 5-175-7-2. The strongest binding of the monoclonal antibody was on the pins 59-61; near the *C*-terminus region of the RA17 allergen. The result suggested that the epitope would exist in the sequences of those peptides 59, 60 and 61.

Conclusion

A B-cell library consisting of more than 2000 stocks of BLCs could be an excellent source of antibodies to a wide spectrum of allergens, as it appears to contain antibodies to various food allergens. The disadvantages of this method lie in the instability of the BLC clones secreting antibodies of interest because of the absence of telomerase activity (Counter *et al.* 1994). In contrast, cell fusion of human lymphocytes (not transformed) with mouse myeloma cells yields high fusion efficiency and enables cloning by limiting dilution. However, it is difficult to establish human-mouse hybridomas secreting antibodies with a certain specificity. The method described in this paper combined the advantages of the EBV transformed cell library and cell fusion with mouse myeloma, and eliminates the disadvantages. This would be the first report about the binding sequence of a rice allergen with a human monoclonal antibody. Similar studies will be possible to analyze milk, egg, or peanut allergens with

EBV transformation of human B cells followed by cell fusion with mouse myeloma cells.

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