

Generation of mouse-human hybridomas secreting human monoclonal antibodies to Japanese cedar pollen allergen Cry j1

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Abstract. Peripheral blood lymphocytes from 13 donors were transformed with Epstein-Barr virus (EBV) to establish immortalized human B-cells secreting antibodies to Japanese cedar (*Cryptomeria japonica*) pollen allergens. EBV-transformed B-lymphocytes were then fused with mouse myeloma SP2/O3, and three clones of mouse-human hybridomas secreting human IgM class monoclonal antibodies to a cedar pollen allergen Cry j1 were established.

Keywords: Human-mouse hybridoma, cedar pollen allergen, human monoclonal antibody, epitope, IgM, Epstein-Barr virus

1. Introduction

Japanese cedar (*Cryptomeria japonica*) trees spread pollen over most areas of Japan from late January to April and cause severe pollen allergy [5,6]. Major Japanese cedar pollen allergens Cry j1 and Cry j2 were identified with sera from patients, and cDNAs of the allergens were cloned [2,4,13].

To analyze the B-cell epitope structures of cedar pollen allergens, we plan to obtain human monoclonal antibodies to Cry j1. We transformed human peripheral blood B cells with Epstein-Barr virus (EBV) and then performed cell fusion with mouse myeloma SP/2O3 cells to establish stable mouse-human hybridomas. In this study, we established three hybridomas secreting human monoclonal antibodies reacting with the Cry j1 allergen and present the results of B-cell epitope (antibody binding site) analysis with synthetic Cry j1 peptides.

2. Materials and methods

2.1. 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 donors by discontinuous density centrifugation on lymphocyte separation medium (Organon Teknika Co., USA). The PBLs were washed twice with ERDF medium (Kyokuto Seiyaku, Co. Ltd., 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 ($2 \mu\text{g/ml}$, Sigma-Aldrich Japan) and 1/10 volume of culture supernatants of marmoset B95-8 cells containing Epstein-Barr virus (EBV) [1,9]. 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 culturing, transformed cells (B-lymphoblastoid cells, BLCs) and the culture supernatants were frozen and stored.

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[Amino acid sequence of Cry j1 precursor]

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MDSPCLVALL VLSFVIGSCF SDNPIDSCWR GDSNWAQNRM KLADCAVGFG 50
SSTMGGKGGD LYTVTNSDDD PVNPAPGTLR YGATRDRLW IIFSGNMNIK 100
LKMPMYIAGY KTFDGRGAQV YIGNGGPCVF IKRVSNVHH GLHLYGCSTS 150
VLGNVLINES FGVEPVHPQD GDALTLRTAT NIWIDHNSFS NSSDGLVDVT 200
LSSTGVTISN NLFFNHHKVM LLGHDDAYS DKSMTKTVAF NQFGPNCQQR 250
MPRARYGLVH VANNNYDPWT IYAIGGSSNP TILSEGSFT APNESYKKQV 300
TIRIGCKTSS SCSNWWQST QDVFYNGAYF VSSGKYEGGN IYTKKEAFNV 350
ENGNATPQLT KNAGVLTCSL SKRC

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[Synthesized Cry j1 overlapping peptides]

1 MDSPCLVALL VLSFVIGSCF	21 LSSTGVTISN NLFFNHHKVM
2 <u>VLSFVIGSCF</u> SDNPIDSCWR	22 NLFFNHHKVM LLGHDDAYS
3 <u>SDNPIDSCWR</u> GDSNWAQNRM*	23 LLGHDDAYS DKSMTKTVAF
4 GDSNWAQNRM KLADCAVGFG	24 DKSMTKTVAF NQFGPNCQQR
5 KLADCAVGFG SSTMGGKGGD	25 NQFGPNCQQR MPRARYGLVH
6 SSTMGGKGGD LYTVTNSDDD**	26 MPRARYGLVH VANNNYDPWT**
7 LYTVTNSDDD PVNPAPGTLR**	27 VANNNYDPWT IYAIGGSSNP**
8 PVNPAPGTLR YGATRDRLW	28 IYAIGGSSNP TILSEGSFT
9 YGATRDRLW IIFSGNMNIK	29 TILSEGSFT APNESYKKQV
10 IIFSGNMNIK LKMPMYIAGY	30 APNESYKKQV TIRIGCKTSS
11 LKMPMYIAGY KTFDGRGAQV	31 TIRIGCKTSS SCSNWWQST
12 KTFDGRGAQV YIGNGGPCVF	32 SCSNWWQST QDVFYNGAYF
13 YIGNGGPCVF IKRVSNVHH	33 QDVFYNGAYF VSSGKYEGGN
14 IKRVSNVHH GLHLYGCSTS	34 VSSGKYEGGN IYTKKEAFNV
15 GLHLYGCSTS VLGNVLINES	35 IYTKKEAFNV ENGNATPQLT
16 VLGNVLINES FGVEPVHPQD	36 ENGNATPQLT KNAGVLTCSL
17 FGVEPVHPQD GDALTLRTAT	37 KNAGVLTCSL SKRC
18 GDALTLRTAT NIWIDHNSFS**	
19 NIWIDHNSFS NSSDGLVDVT**	
20 NSSDGLVDVT LSSTGVTISN	

Fig. 1. Sequences of synthesized Cry j1 overlapping peptides. Sequence with underline (1–21) was a signal sequence of Cry j1.

2.2. Enzyme-linked immunosorbent assay (ELISA)

Crude Japanese cedar pollen allergen was purchased from LSL Co. Ltd. (Japan), and affinity-purified Japanese cedar pollen allergens Cry j1 and Cry j2 were purchased from Seikagaku Kogyo Co., Ltd. (Japan). The allergens were diluted with 0.05 mol/L NaHCO₃ (protein concentration of 0.1–1 µg/ml), 0.05 ml of the solution was plated onto 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 (0.05 ml) were pipetted onto

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, 1/2,000 dilution, Biosource International, USA) conjugated with horseradish peroxidase (HRPO) 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) (ABTS), 0.03 % H₂O₂ in 0.1 mol/L citrate buffer pH 4.0).

2.3. 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

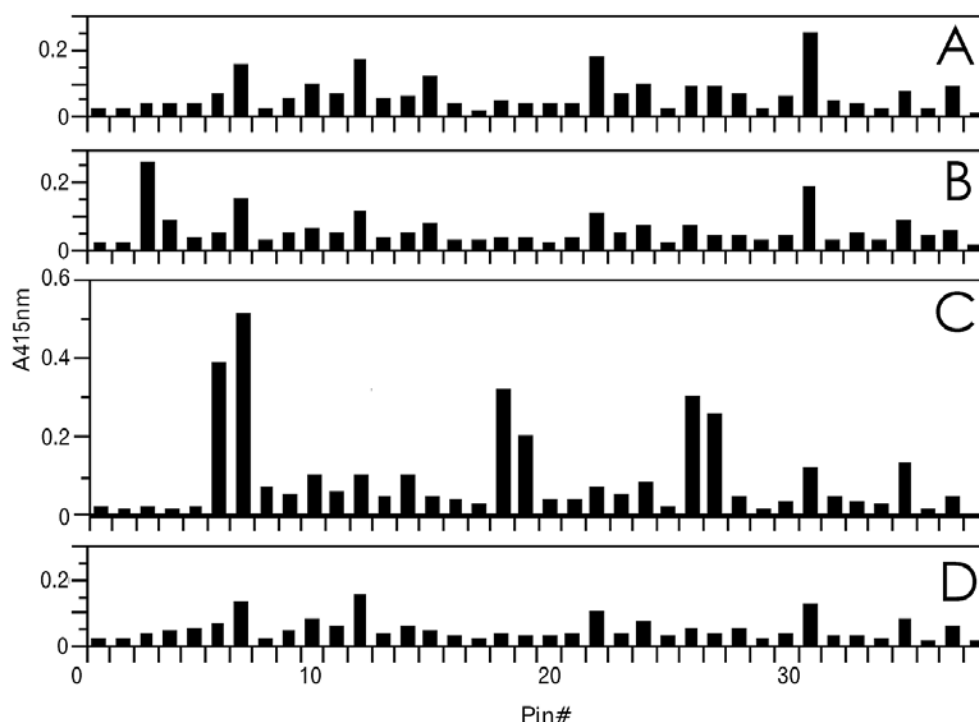


Fig. 2. Epitope analysis of Cry j1 with the human monoclonal antibodies

Synthesized overlapping peptides based on an amino acid sequence of Cry j1 protein reacted with human monoclonal antibodies (A: control (no monoclonal antibody), B: 9-464-H1-2, C: 2-103H6-26, and D: 3-52H1-21). 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.

medium supplemented with 10% FCS and 0.05 mmol/L ouabain to obtain an ouabain-resistant cell line, SP2/O3. BLCs (2×10^7) and SP2/O3 cells (2×10^7) were fused in 50% (v/v) polyethyleneglycol (Sigma-Aldrich Japan) [10–12]. 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 hybridomas were cloned twice by limiting dilution method.

2.4. Analysis of epitopes of a cedar pollen allergen Cry j1 by the multi-pin overlapping peptide method

A series of overlapping peptides (20 amino acids long) based on an amino-acid sequence of Cry j1 precursor protein [2] including signal peptide (amino acids: #1–21) was synthesized. 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 NH_2 . Diluted supernatants of hybridomas (0.1 ml) were placed on 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 HRPO. After washing, enzyme activity was measured by adding ABTS solution, and absorbancy of the reaction product was measured at 415 nm.

3. Results and discussion

3.1. Transformation of peripheral blood lymphocytes with EBV and establishment of mouse-human hybridomas

We obtained more than 5,000 transformed human B-lymphoblastoid cells (BLCs) from peripheral blood lymphocytes from 13 donors. The supernatants of the BLCs were analyzed by ELISA for antibodies against crude cedar pollen allergen and purified allergens, Cry

Table 1

Transformation of human peripheral B cells and detection of the cells secreting antibodies specific to Japanese cedar pollen allergens

Ig-class	number of BLCs with antibody reacted with		
	crude allergen	Cry j1	Cry j2
IgA	1*	0	1
IgG	0	2	1
IgM	68	11	8
IgE	0	0	0
Total	69	13	10

*Transformed cells analyzed = 5,142.

Table 2

Specificities of human monoclonal antibodies against Japanese cedar pollen allergens

Hybridoma clones	crude allergen	Cry j1	Cry j2
9-464-H1-2	+	++	—
1-254-H8-10	+	—	—
2-103H6-26	+	++	—
3-52H1-21	+	++	—
10-404H10-8	±	—	+

++: Strongly positive, +: positive, ±: slightly positive, and —: negative.

j1 and Cry j2. Table 1 shows the results. Many BLCs secreted antibodies to cedar pollen allergens. 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 [1]. Of those cells, five BLCs secreting antibodies to crude allergen were fused with mouse myeloma SP2/O3 cells. Three stable human-mouse hybridoma clones secreting human monoclonal IgM-class antibodies reacting with Cry j1 were established (Table 2).

3.2. B-cell Epitope analysis of Cry j1 with the human monoclonal antibodies

Cry j1 is the major allergen isolated from Japanese cedar pollen, and Cry j1 precursor was reported to have 374 amino acids by cDNA analysis (mature protein had 353 amino acids) [2]. To detect the antibody-binding sequences (B-cell epitopes) in Cry j1 allergen, a series of peptides (overlapping peptides) was synthesized. We prepared a series of overlapping peptides of 20 amino acids long by repeating the shift of every ten amino acids in the direction from N to C terminus of the sequence of Cry j1 precursor protein. Monoclonal antibodies from human-mouse hybridomas 9-464H1-2, 2-103H6-26, and 3-52H1-21 were reacted with the pins shown in Fig. 1.

Figure 2 presents results of B-cell epitope analysis. A control run without antibody to Cry j1 (only with secondary polyclonal antibody against human IgM conjugated with HRPO) exhibited several non-specific reactions with pins # 7, 12, 15, 22 and 31. Monoclonal antibody 9-464H1-2 reacted with pin # 3 with an amino acid sequence of ²¹SDNPIDSCWRGDSNWAQNRM⁴⁰ (N-terminus region of mature Cry j1 protein). While the monoclonal antibody 2-103H6-26 was cloned twice, the antibody reacted with pins #6–7, 18–19 and 26–27. Monoclonal antibody 3-52H1-21 produced a very similar pattern to “non-specific” reaction of the secondary antibody to human IgM, suggesting that the antibody 3-52H1-21 did not recognize the primary structure of Cry j1.

Kawashima et al. [3] and Sakaguchi et al. [7,8] reported B-cell epitope analysis using mouse monoclonal antibodies to Cry j1, but they did not present any epitope amino acid sequences of a primary structure of Cry j1. Tania et al. [14] reported an enzyme-digested fragment of Cry j1 with a sequence near C-terminus of ³⁵²NGNATPQLTKNAGVLTCSLSKR³⁷³ was an IgE binding epitope with serum of a patient and a mouse monoclonal antibody to Cry j1. Tamura et al. [15] also reported an epitope analysis of the cedar pollen allergen Cry j with mouse serum antibodies to Cry j1. They demonstrated that there was only one B-cell epitope peptide with a sequence of ¹⁶⁶VHPQDGDGA¹⁷³.

In this study, several amino acid sequences were recognized by human monoclonal antibodies but were not identical to the previously reported B-cell epitope sequences of Cry j1. Human monoclonal antibodies to Japanese cedar pollen allergens would be very useful for evaluation of B-cell epitope of the allergens. Epitope analysis with shorter peptides (8–10 amino acids) will be required for detecting fine epitope structures of the allergens with 9-464H1-2 and 2-103H6-26 monoclonal antibodies.

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