

23473

Production of Monoclonal Antibodies Specific to Major Allergens of *Cryptomeria japonica* Pollen

KAZUYUKI HONDA,¹ HIDEYUKI SHIRAE,² KIYOSHI MIWA,²
SHO MATSUSHITA,³ KAZUMI MAKISHIMA,¹ YASU HARU NISHIMURA,³
and TAKEHIKO SASAZUKI³

¹Department of Otorhinolaryngology, School of Medicine, University of Occupational and Environmental Health, Kitakyushu 807, Fukuoka, Japan

²Ajinomoto Research Institute, Kawasaki 210, Fukuoka Japan

³Department of Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812, Fukuoka, Japan

ABSTRACT

Monoclonal antibodies (MAbs) directed against *Cryptomeria japonica* pollen antigen (CPAg) were prepared. CPAg was precipitated with these MAbs and both MAbs CPA7 (IgG_{2a}) and CPA9 (IgG₁) recognized two major glycoproteins (m=41kDa and 46 kDa) of CPAg. MAb CPA7 or CPA9 was coupled to CNBr activated sepharose and affinity purification of the major allergen of CPAg from the crude extract was performed. The affinity purified CPAg bound IgE antibody present in patients with Cedar pollinosis.

INTRODUCTION

Cryptomeria pollinosis is type I allergy caused by *Cryptomeria japonica* (Japanese cedar) pollen and is one of the most common pollinosis in Japan. *Cryptomeria* pollinosis prevails every year from February to April, except in the northern and southern areas of Japan. We reported that *Cryptomeria* pollinosis was inherited as an HLA-linked single recessive trait and that the resistance to *Cryptomeria* pollinosis was an HLA-linked dominant phenotype (1,2). Analyses of the cellular mechanism of low responsiveness, using an in vitro *Cryptomeria japonica* pollen antigen (CPAg) specific IgE production system, showed the resistance to be mediated by CPAg-specific CD8⁺ suppressor T cells (3). We thus concluded that the low responsiveness of IgE to CPAg was not due to the absence of an immune response gene to CPAg, but rather was mediated by the immune suppression gene for CPAg controlling the induction of CPAg-specific suppressor T cells (1-3). To investigate the immunochemical properties of CPAg, we prepared monoclonal antibodies (MAbs) specific to CPAg and purified two major allergens of CPAg, by affinity chromatography using MAbs.

MATERIALS AND METHODS

Preparation of antigens

CPAg was prepared as described(3,4). Briefly, dry pollen of *Cryptomeria japonica* was treated with ether, placed in 0.125M ammonium bicarbonate, and stirred for 48 hours at 4°C. Solid ammonium sulfate was added to the extract, to 100% saturation. The precipitate was dissolved in 0.05M Tris-HCl pH 7.8, dialyzed against the same buffer (fraction A), applied to a DEAE-cellulose (DE-52; Whatman, Springfield Mill, Kent, England) column, eluted by 0.05M Tris-HCl pH 7.8 and dialyzed against 0.01M acetate buffer pH 5.0 (fraction B). This was followed by chromatography using a CM-cellulose (CM-52; Whatmann) column. The column was eluted first with 0.01M acetate buffer pH 5.0 then with 0.1M phosphate buffer, pH 7.0 and the second elute was concentrated (fraction C). The antigenic purity of CPAg fraction C was much the same as Cry j I described by Yasueda et al(4).

Preparation of MAbs to CPAg

Balb/c mice were immunized intraperitoneally with 30µg of CPAg fraction B in 0.3 ml of phosphate buffered saline (PBS) and then were given the same intraperitoneal injection as a booster three times at two week intervals. The last booster was given intraperitoneally 3 days before cell fusion.

Spleen cells from mice were fused with P3-X63-Ag8 myeloma cells by the method described by Kennett et al(5). Briefly, ten million spleen cells were mixed with an equal number of P3-X63-Ag8 in a 50ml plastic tube. The mixture was centrifuged and the pellet in the tube was gently tapped and kept at 37°C in a water bath. Using a 1ml pipette, 1ml of warm 45%(w/v) polyethylene glycol (Sigma Mr 3350, St Louis, MO) was added dropwise over a 1-min period. The cell pellet was then gently stirred. The cell suspension was left for 5 min at room temperature and then 2ml of MEM (Gibco, Grand Island, NY) medium were added during a 4 min period. Finally, 10ml of MEM without serum were added to the suspension and the preparation was centrifuged at 1500rpm for 10 min at room temperature. After centrifugation, 40ml of RPMI-1640 medium (GIBCO) supplemented with 100u/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine, 15% heat-inactivated fetal bovine serum and hypoxanthine (136.1mg/ml) aminopterin (176mg/ml) and thymidine (33.8mg/ml) (HAT) were added to the cell pellet and the preparation stirred to suspension form. This suspension was seeded into each well of a 96-well flat bottom tissue culture plate (200µl/well) and cultured at 37°C in a 5% CO₂ incubator. Half of the HAT selection medium in each well of the plate were exchanged with fresh HAT selection medium every week. Initial screening of IgG producing hybridomas and clones was performed by radioimmunoassay (RIA) detecting a CPAg specific antibody. The 96-well plates were

coated overnight with 50µg/ml CPAg fraction B in PBS. After three washes with PBS, the wells were filled with 10% fetal bovine serum/PBS for 2.5 hr at 4°C to block unsaturated protein-binding sites. The plates were then washed 8 times with PBS, and 100µl of culture supernatant were added to each well followed by incubation for 5hr at 37°C. After 10 washes with PBS, 100µl of ¹²⁵I-labelled goat anti-mouse IgG (Cappel Laboratories, West Chester, PA) were placed to each well and the preparation was allowed to react for 3 hr at room temperature. The wells were washed extensively with PBS, and then with tap water and radioactivities were counted in an automatic gamma counter. For cloning, 100 cells from anti-CPAg antibody positive wells were suspended in 20ml of HAT selection medium and 100µl of cell suspension were seeded in each well (0.5 cell/well). The positive clones were selected and the specificities were confirmed. Balb/c mice were given approximately one million viable, cloned hybridoma cells i.p. after being pretreated with 0.5ml pristane(6) to obtain ascites form of MABs. The immunoglobulin isotypes of these MABs were determined using mouse MAB isotyping kits (Amersham).

Immunoprecipitation and electrophoretic analysis

Varying amounts of ascites fluids containing anti-CPAg MABs were incubated with CPAg fraction C (1,465,570 cpm/5λ) radiolabelled with ¹²⁵I, using the solid-phase lactoperoxidase method(7) for 4 hours at room temperature. The mixture was incubated with rabbit anti-mouse IgG (Cappel) for 2 hours, followed by incubation with 5mg pre-swollen protein A bound Sac cells for 2 hours. Immunoprecipitates were eluted from Sac cells by 0.1M SDS sample buffer and were analyzed by 10% homogeneous sodium dodecyl sulfate polyacrylamide gel electrophoresis, as described(8), followed by autoradiography. In some experiments, electrophoresed CPAg was visualized with a silver stain kit (Daiichi Co., Tokyo).

Affinity purification of CPAg

Ascitic fluid recovered from 5 Balb/c mice injected intraperitoneally with hybridoma producing anti-CPAg MAB CPA7 was collected, and ammonium sulfate was added to 40% saturation. The precipitate was dissolved in Ca²⁺ and Mg²⁺ free PBS, and the IgG fraction was isolated with protein A-sepharose Cl-4B (Pharmacia) and coupled to CNBr activated-sepharose 4B(Pharmacia), as suggested by the manufacturer. The IgG-coupled sepharose 4B was packed in a column and washed with Ca²⁺ and Mg²⁺ free PBS. The ammonium sulfate precipitate of the pollen crude extract fraction (fraction A), dissolved and dialysed in Ca²⁺ and Mg²⁺ free PBS was placed on a column. After washing with a sufficient volume of Ca²⁺ and Mg²⁺ free PBS, CPAg was eluted with 0.2M Gly-HCl (pH2.0) and neutralized with Tris base (fraction D).

Radioimmunoassay for anti-CPAg IgE antibody

CPAg-specific IgE antibody were quantified using solid-phase RIA as described elsewhere(3,7). The lower limit of specific IgE concentration measured in this system was 10pg/ml. The standard deviation of the triplicate assay was less than 20%. The count of 10% fetal bovine serum/PBS, as negative control, was 314 ± 21 cpm.

RESULTS AND DISCUSSION

Of the 863 growing hybridomas obtained, 74 produced IgG bound to CPAg. Four of the latter were selected for further cloning and 2 clones (CPA7 and 9) were characterized in detail. CPA7 and CPA9 are of mouse IgG_{2a}, k and IgG₁, k isotype respectively. To identify the specificity of MAbs CPA7 and CPA9, immunoprecipitates of CPAg with MAbs were electrophoretically analyzed. As shown in Figure 1B, lanes 7 and 8, two MAbs precipitated two glycoproteins of CPAg (m=41kDa and 46kDa). Using MAbs CPA7 and CPA9, we attempted affinity purification of the major bands of CPAg. The fraction D of CPAg eluted from the affinity column of CAP7 by 0.2M Gly-HCl pH 2.0 is depicted in Figure 1A, lane 4. This fraction consisted of two major glycoproteins of CPAg (m=41kDa and 46kDa), thereby indicating that the affinity purification of major glycoproteins of CPAg was successful.

Using three preparations of CPAgs (fraction B, C and affinity purified fraction D), we identified IgE specific to CPAg by RIA in sera from two patients with Cryptomeria pollinosis. As shown in Table I, all CPAgs especially fractions C and D, bound IgE antibody, thereby indicating that the affinity purified major allergens conserved epitopes for IgE antibody specific to CPAg.

The allergen of Cryptomeria pollinosis is *Cryptomeria japonica* pollen and CPAg is a complex of at least 13 glycoproteins with various molecular weights. Yasueda et al found that Cry j I was the major allergen of CPAg, determined by Radioallergosorbent test inhibition analysis(4). Matsushita et al obtained the same results using Western blot analysis(3). They also noted that other glycoproteins of CPAg possessed minor allergenic activity.

Table I Binding of IgE antibody derived from patients with cedar pollinosis to the affinity purified CPAg.

Serum donor	IgE bound to CPAg. (cpm) ^a		
	fraction B	fraction C	fraction D
KU	2,004 $\pm$ 271	3,275 $\pm$ 224	4,029 $\pm$ 49
KH	4,902 $\pm$ 20	6,164 $\pm$ 71	5,932 $\pm$ 178

^a IgE antibody specific to CPAg was quantitated in triplicate by RIA and the mean cpm $\pm$ S.D. is given. Background cpm (10% fetal bovine serum/PBS) was 314 ± 21 cpm.

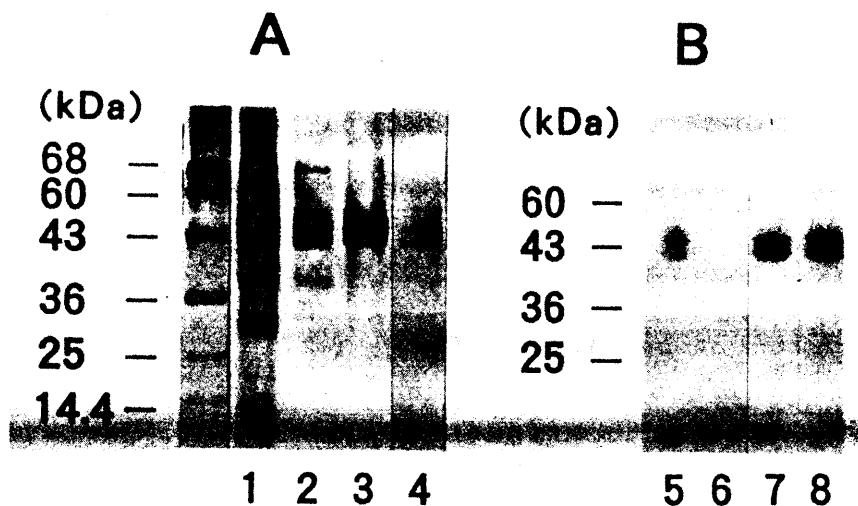


Figure 1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis profiles of CPAg purified by various methods:

The left lane of the panel A indicates size markers as follows, Bovine serum albumin (m=68kDa), Catalase (m=60kDa), Ovalbumin (m=43kDa), Lactate dehydrogenase (m=36kDa), α -chymotrypsinogen (m=25kDa) and Lysozyme (m=14.4kDa).

Panel A lanes 1-4 are profiles of CPAg, followed by silver-staining. Lane 1 : CPAg fraction A, Lane 2 : CPAg fraction B, Lane 3 : CPAg fraction C, Lane 4 : second elute of affinity column, CPAg fraction D. Panel B lanes 5-8 indicate the profiles of immunoprecipitates from the ^{125}I labelled CPAg fraction with the MAb indicated below, followed by autoradiography. Lane 5 : CPAg fraction B, Lane 6 : P3-X63-Ag8 as control, Lane 7 : CPA9 MAb, Lane 8 : CPA7 MAb.

For a better understanding of the allergic immune response to CPAg, CPAg has to be characterized. In the present immuno-logical analysis of CPAg using MAbs, we obtained evidence that two MAbs bound to the molecular mass 41kDa and 46kDa glycoproteins of CPAg, based on the electrophoretical profiles of the immunoprecipitates. Purification of CPAg was feasible using affinity chromatography and MAbs. The antigen specific IgE titers in sera obtained from patients with Cedar pollinosis measured using the purified CPAg fraction D were higher than those measured by using CPAg fraction B. Earlier studies showed that peripheral blood lymphocytes from both patients KU and KH responded to CPAg in vitro, as the CPAg-driven IgE response. The molecular mass of major allergen of CPAg which binds to IgE antibody derived from these two patients with cedar pollinosis was determined to be 41kDa and 46kDa. As each preparation of CPAg was adjusted at 0.5mg/ml for RIA, the proportion of major allergen in CPAg fraction D must be relatively larger than that in CPAg fraction

B to bind to a larger amount of IgE antibody to CPAg fraction D, as compared with fraction B.

ACKNOWLEDGMENTS

We thank Dr.T.Furukoshi (Kanto Forest Tree Breeding Institute, Japan) for providing the *Cryptomeria japonica* pollen and M. Ohara for helpful comments. This work was supported in part by Grants-in-Aid 59870019, 60480177, 03857220 from the Ministry of Education, Science and Culture, Japan and by a research grant for intractable diseases from the Ministry of Health and Welfare, Japan.

REFERENCES

- 1) SASAZUKI,T.,NISHIMURA,Y.,KIKUCHI,I.,HIRAYAMA, K., TSUKAMOTO,K., YASUNAMI,M.,MATSUSHITA,S.,MUTO,M.,SANO,T., and HIROSE,T.(1986) HLA-linked immune suppression gene maps within HLA-DQ subregion. Regulation of Immune Gene Expression.197-206. Humana Press: New Jersey
- 2) SASAZUKI,T.,NISHIMURA,Y.,MUTO,M., and OHTA,N. (1983) HLA-linked genes controlling the immune response and disease susceptibility. Immunol.Rev. 70,51-75.
- 3) MATSUSHITA,S., MUTO,M., Suemura,M., SAITO,Y., and SASAZUKI,T. (1983) HLA-linked nonresponsiveness to *cryptomeria japonica* pollen antigen. I. Nonresponsiveness is mediated by antigen-specific suppressor T cell. J.Immunol. 138,109-115.
- 4) YASUEDA,H.,YUI,U.,SHIMIZU,T., and SHIDA,T. (1983) Isolation and partial characterization of the major allergen from Japanese cedar (*Cryptomeria japonica*) pollen. J. Allergy Clin. Immunol. 71,77-86.
- 5) KENNETT,R.H., MCKEARN,T.J., and BCHTOL,K.B. (eds.). (1980) Monoclonal antibodies. Plenum Press:New York.
- 6) POTTER,M.(1972). Immunoglobulin-producing tumors and myeloma proteins of mice. Physiol.Rev. 52,631-719.
- 7) NONAKA,M.,ZURAW,B.,O'HAIR,C., and KARTZ,D. (1981) Stimulation of primary in vitro IgE antibody responses in cultures of human peripheral mononuclear cells. J.Exp.Med. 153,1574-1580.
- 8) WEBER,K., and OSBORN,M. (1969) The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. J.Biol.Chem. 244,4406-4412.

Address for correspondence and reprints:

Dr. Takehiko Sasazuki, Department of Genetics, Medical Institute of Bioregulation, Kyushu University 69, Maidashi 3-1-1, Higashi-ku, Fukuoka 812, JAPAN.
Phone:92-641-1151 Ext.3771 Facsimile:92-632-0150

Received for publication: 1/7/92

Accepted after revision: 3/10/92