Epitope analysis of human monoclonal antibody specific for rice allergenic protein generated by *in vitro* immunization

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Received 12 December 2000; accepted 8 June 2001

Key words: epitope, human monoclonal antibody, *in vitro* immunization, rice allergenic protein

Abstract

We previously established an *in vitro* immunization protocol for generating antigen specific human monoclonal antibodies (mAbs). *In vitro* immunization was performed against the soluble protein of rice allergenic protein (RA), resulting in the generation of three B cell clones, AC7-1/F9, CB7-1/E2 and CB7-8/F5, all of which produce a RA-specific human monoclonal IgM antibody. We attempted to map the epitope regions recognized by these mAbs to characterize their specificities. We performed two rounds of epitope mapping, rough mapping using 10-mer peptides covering the full-length RA with 5 amino acids overlapping, and fine mapping using 8-mer peptides covering the putative epitope regions from the rough mapping with 1 amino acid overlapping. As a result of the fine mapping, we identified the epitope regions of these three mAbs as ⁴⁵QVWQDCCRQ⁵⁴L, ⁵⁶AVDDGWCRCGA⁶⁷L and ⁹¹FPGCRRG⁹⁸D on the RA molecule and found to be identical. Furthermore, we determined the putative core epitope regions, which are critical for mAb binding to each region, ⁴⁷WQDCC⁵²R and ⁶⁰GWC⁶³R. The information about the epitope region on the RA molecule, which might trigger the allergenic response, would be useful to establish a specific immunotherapy against rice allergy.

Abbreviations: RA, rice allergenic protein; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; DMF, dimethylformamide; Fmoc, 9-fluorenylmethyloxycarbonyl; TFA, trifluoroacetic acid; FDNB, fluorodinitrobenzene; DIC, diisopropylcarbodiimide; BSA, bovine serum albumin; Leu-Leu-OMe, L-leucyl-L-leucine methyl ester; ABTS, p-2,2'-azino-di (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt.

Introduction

Allergic diseases triggered by aberrant immune responses cause urticaria, dermatitis, asthma, and anaphylaxis. The clinically important allergen sources in food are known to be eggs, milk, legumes, seafood, cereals, and meat. Rice is consumed and produced in large quantities around the world, and has been thought to be a source of severe atopic dermatitis in some adult patients, although the frequency of such rice allergy is lower than that of egg, milk or soybean allergies (Lezaun et al., 1994; Ikezawa et al.,

1992; Miyakawa et al., 1988). Rice seeds contain 5–10% alcohol soluble proteins (prolamines), 4–10% salt-soluble proteins (globulin and albumin), and 80–90% alkali soluble proteins (glutelin) (Cagampang et al., 1966). Rice sodium-salt soluble protein, especially the globulin and albumin fraction, has been reported to be the major allergenic components causing IgE-mediated rice allergy (Limas et al., 1990; Shibasaki et al., 1979). The allergenic proteins also show similarity to those of the wheat and cereal α -amylase/trypsin inhibitor family, which is a major allergen associated with asthma in wheat and barley

Figure 1. Amino acid sequence of RA and peptide design for rough mapping. A total of 26 peptides (10-mer) were synthesized to cover the full-length of RA sequence with 5 amino acids overlapping.

flour (Alvarez et al., 1995; Nakase et al., 1996; Barber et al., 1989; Gomez et al., 1990; Sanchez-Monge et al., 1992; Adachi et al., 1993; Izumi et al., 1992). The allergenic components have been identified and the molecular mass is between 14–16 kDa and the isoelectric point is between pI 6–8. Their stability against heat and proteolytic digestion have also been investigated (Matsuda et al., 1988, 1991). Tada et al. have reported the cDNA sequence encoding the rice allergenic protein, which has high homology to the cereal α -amylase/trypsin inhibitor (Tada et al., 1996). The allergenicity of rice allergenic protein has been shown to be reduced in the transgenic rice plant using antisense RNA, and various other approaches for producing hypoallergenic rice have been accessed (Tada et al., 1996; Nakamura et al., 1996). When producing hypoallergenic rice, it may be better to reduce the allergenicity of rice allergenic protein by genetic engineering, rather than removing the allergenic protein from the rice. To identify specific regions of allergenicity, we produced several rice allergenic protein specific human mAbs using *in vitro* immunization protocol.

We previously established an *in vitro* immunization protocol for generating antigen specific human mAbs (Kawahara et al., 1992; Shoji et al., 1994; Ichikawa et al., 1999). *In vitro* immunization was performed against human peripheral blood lymphocytes

by using 14–16 kDa RA, and several B cell lines (RA10B10, RA7H3, RA7C12, RA9G11, RA11C6 and RA10C3) were established by immortalizing with the *Epstein Barr Virus*. RA9G11, selected for further analysis because of its high proliferation potential, was fused with the human-mouse hetero myeloma cell line, RF-SI. After cloning, B cell clones (AC7-1/F9, CB7-1/E2 and CB7-8/F5) with the ability to produce as RA-specific human monoclonal IgM antibody were established. In this study, we attempted to analyze the epitope structure of these mAbs using the Multipin peptide synthesis kit.

RA-specific human mAbs could be useful for analysis of the allergenic structure of RA, which could provide valuable information for the creation of hypoallergenic rice, and further be applied to an *in vitro* allergy assay system, in which the allergenic structure and the interaction between allergen and antibody could be assessed by indexing the histamine release from a basophilic cell line.

Materials and methods

Reagents

The multi-pin peptide synthesis kit was purchased from Chiron Mimotope Tech. (Clayton, Australia). Dimethylformamide (DMF), piperidine, molecular

Figure 2. Rough epitope mapping of RA-specific human mAb. The epitope regions recognized by human mAb produced from B cell clones, AC7-1/F9 (A), CB7-1/E2 (B) and CB7-8/F5 (C) were determined by evaluating the binding ability of mAb to the peptides synthesized on the pin by ELISA. ELISA value of negative control at A405nm was 0.48 and the cut-off line indicated 3-fold greater value than negative control. Peptide #10 corresponds to ⁴⁶VWQDCCRQLA⁵⁵, #12 to ⁵⁶AVDDGWCRCG⁶⁵, #13 to ⁶¹WCRCGALDH⁷⁰, #15 to ⁷¹LSGIYRELGA⁸⁰, #19 to ⁹¹FPGCRRGDLE¹⁰⁰, #21 to ¹⁰¹RAAASLPAFC¹¹⁰ and #24 to ¹¹⁶NGPGGVCYWL¹²⁵.

Figure 3. Peptide design for fine epitope mapping. Total 74 peptides (8-mer) covering the reactive epitope region on RA with only 1 amino acid overlapping were synthesized.

Figure 4. Fine epitope mapping of RA-specific human mAb. Procedures are the same as Figure 2 except for the peptides used for epitope mapping. ELISA value of negative control at A405 nm was 0.66 and cut-off line indicated 3-fold greater value than negative control. The epitope regions recognized by human mAb producing B cell clones, AC7-1/F9 (A), CB7-1/E2 (B) and CB7-8/F5 (C) are shown.

sieves (1/16, 4A), trifluoroacetic acid (TFA), ethandithiol, thioanisole, methanol, fluorodinitrobenzene (FDNB), diisopropylcarbodiimide (DIC), acetic acid, sodium azide, ABTS (p-2,2'-azino-di (3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) were purchased from Wako (Kyoto, Japan). Glass fiber filter (GF/A) was purchased from Whatman Ltd. (Maidstone, U.K.). Bovine serum albumin (BSA) and L-leucyl-L-leucine methylester (Leu-Leu-OMe) were purchased from Boehringer GmbH (Mannheim, Germany). Goat anti-human IgM antibody and Goat anti-human IgM antibody-horseradish peroxidase (POD) conjugate were purchased from TAGO (CA, U.S.A.). RA was prepared according to the method reported by Matsuda et al. (1988, 1991).

Human monoclonal antibody

B cell lines producing human mAb specific for RA were generated by *in vitro* immunization established by Ichikawa et al. (1999). Briefly, Leu-Leu-OMe-treated human peripheral blood lymphocytes was im-

munized with RA in the presence of IL-2 and IL-4, immortalized with *Epstein Barr Virus* and fused with the human-mouse hetero myeloma cell line RF-S1. After cloning, B cell clones producing RA specific mAb were selected by ELISA, and used in this study.

Peptide synthesis

For the epitope mapping, two sets of peptides derived from RA were synthesized on activated polyethylene pins using the Multipin peptide synthesis system. For the rough epitope mapping, we synthesized twenty-six 10-mer peptides covering the full-length of RA, each of which overlap by 5 amino acids. For further fine epitope mapping, we synthesized seventy-four 8-mer peptides covering the epitope regions determined by the rough mapping, each of which overlap by 1 amino acid. The pins were deprotected in 20% piperidine/N, N-DMF for 20 min, washed twice in DMF for 2 min and four times in methanol for 2 min, allowed to air dry for 2 hr. For each coupling cycle, fresh 60 μ M stock solutions of the

Figure 5. Epitope regions recognized by anti-RA antibody and putative structure of RA. (A) Epitope regions recognized by anti-RA mAbs are shown in hatched bars, and the core epitope regions defined as indispensable regions for binding of anti-RA antibody to RA peptides are shown as black bars. (B) The locations of the epitope regions in RA molecule are shown. (C) The epitope regions in structure of RA are shown as broken lines. The open circles indicate cysteine residues with residue numbers and gray bars indicate disulfide bridges. The open squares represent the sheet. The gray and black arrows indicate the hydrophobic regions and helical structure regions, respectively.

Fmoc (9-fluorenylmethyloxycarbonyl)-amino acid esters were prepared in 72 μ M 1-hydroxybenzotriazole and 60 μ M DIC in DMF, and added to the appropriate wells in a polypropylene microtiter reaction tray (150 μ l/well). The pins were incubated in the reaction mixture for 24 hr at room temperature and washed for 2 min in methanol and DMF, respectively. The cycle of deprotection and coupling was repeated until all 10 or 8 amino acid residues were synthesized on the pins. The side-chain protecting groups were removed by incubating the pins in TFA/ethanedithiol/thioanisole (38:1:1) for 2.5 hr at room temperature. The pins were immediately fully immersed in methanol for 10 min, soaked in 0.5% acetic acid in methanol/water (1:1, v/v), and washed with methanol for 2 min. The blocks were allowed to air dry overnight and stored at 4 °C.

ELISA

For evaluating the binding of RA specific human mAb to the peptides synthesized on the pins, ELISA was performed according to the manufacturer's instructions. Briefly, pins bound to peptides were blocked in the blocking solution (2% BSA, 0.1% Tween 20 and 0.1% sodium azide in 0.01 M PBS) for 1 hr at room temperature and washed with PBS for 10 min. Pins were incubated in solution of primary antibody (anti-RA antibody) diluted 20 folds in blocking solution overnight at 4 °C. The pins were then washed with PBS and incubated with secondary antibody conjugate solution which was diluted 500 fold with buffer containing 1% sheep serum, 0.1% sodium caseinate and 0.1% Tween 20 in PBS. Pins were washed ten times with PBS and incubated in substrate buffer (0.1 M Na₂HPO₄, 0.8 M citric acid, pH 4.0) containing 0.05% ABTS and 0.01% hydrogen peroxide at room temperature for 45 min.

The reacted substrate solutions were monitored by measurement of optical density at 405 nm. After each epitope scan, bound antibody was removed from the pins by sonication for 10 min at 60 °C in buffer containing 1% SDS, 0.1% 2-mercaptoethanol in 10 mM PBS, pH 7.2. The pin block was then washed in distilled water at 60 °C for 30 min. Finally, the block was immersed in methanol at 60 °C for 15 sec, dried in air and stored at 4 °C.

Results and discussion

First, we performed rough epitope mapping using 10-mer peptides covering the full-length of RA, each

of which overlap by 5 amino acids (Figure 1). As shown in Figure 2, the AC7-1/F9 derived antibody reacted to the peptides #10, #13 and #24, CB7-1/E2 derived antibody to peptides #10, #12, #13, #15 and #19, and CB7-8/F5 to peptides #10, #12, #13, #15, #19 and #21. Peptides #10 and #13 were found to be reactive to all three antibodies, while peptides #12, #15 and #19 were demonstrated to be reactive to the two antibodies derived from CB7-1/E2 and CB7-8/F5. In addition, the peptide #24 and #21 were shown to be reactive to only one antibody derived from AC7-1/F9 and CB7-8/F5, respectively. However, because the peptides used in the rough epitope mapping study overlap by 5 amino acid with each other, we tried to finely map the epitope structure using newly synthesized peptides covering the amino acids sequence which were identified as epitope regions in the rough mapping study with only 1 amino acid overlapping. Amino acid sequences of RA corresponding to peptides #10 (⁴⁶VWQDCCRQLA⁵⁵), #12 (⁵⁶AVDDGWCRCG⁶⁵), #13 (⁶¹WCRGALDHM⁷⁰), #15 (⁷¹LSGIYRELGA⁸⁰), #19 (⁹¹FPGCRRGDLE¹⁰⁰), #21 (¹⁰¹RAAASLPAFC¹¹⁰) and #24 (¹¹⁶NGPGGVYWL¹²⁵) were included in the ⁴¹A to ⁸⁵H, ⁸⁶P to ¹¹⁵P and ¹¹¹N to ¹³⁰T. Then we newly synthesized seventy-four 8-mer peptides with 1 amino acid which covered the above epitope regions of RA (Figure 3), and performed fine epitope mapping by using these peptides. The three mAbs recognized almost the same regions, ⁴⁵QVWQDCCRQ⁵⁴L (peptides #5 to #7), ⁵⁶AVDDGWCRCGA⁶⁷L (peptides #16 to #20) and ⁹¹FPGCRRGD⁹⁸ (peptide #44) (Figure 4). The core epitope region is composed of the residues shared by all peptides, which are able to induce a positive response, and is considered to be essential for the binding of mAbs. We identified that the putative core epitope regions recognized by RA-specific human mAb are ⁴⁷WQDCC⁵²R and ⁶⁰GWC⁶³R (Figure 5).

The 14–16 kDa RA has been reported to be a member protein of the plant α -amylase/trypsin-inhibitor family where ten cysteine residues are well conserved (Nakamura and Matsuda, 1996; Izumi et al., 1999). Izumi et al. have reported five intramolecular disulfide bonds, ³⁴Cys-⁹⁴Cys, ²⁶Cys-⁵⁰Cys, ⁵¹Cys-¹¹⁰Cys, ¹²Cys-⁶²Cys and ⁶⁴Cys-¹²²Cys, contribute to the allergenic epitopes (Izumi et al., 1999). Four out of five disulfide bonds are included in the core epitope regions identified in this study, implicating that the core epitope regions of RA form a stable structure maintained by disulfide bonds. We suggest that analog peptides against the core epitope regions could

inhibit B cell responses and antagonize the antibody production against RA for immunotherapy of allergic diseases. Furthermore, we expect that these core regions can be a target for reducing the antigenicity and/or allergenicity of RA and used for the generation of hypoallergenic rice by genetic engineering.

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