

Production and Characterization of IgA Monoclonal Antibody Against Ovalbumin

TAKASHI TANIKAWA, KOHTA KUROHANE, and YASUYUKI IMAI

ABSTRACT

We established a hybridoma clone secreting an immunoglobulin A (IgA) monoclonal antibody (MAb) against ovalbumin (OVA). The MAb was produced using nasal-associated lymphoid tissues (NALT) of BALB/c mice that had been intranasally immunized with OVA together with cholera toxin. The isotype of the MAb was determined to be IgA, κ . The established IgA MAb exhibited saturable and dose-dependent binding to immobilized OVA on ELISA. The majority of the antibodies formed a dimer on immunoblot analyses. To determine the affinity of each binding site, we performed surface plasmon resonance analysis, in which the binding of soluble OVA to immobilized IgA was measured. The results revealed a slow association rate and relatively low affinity of each binding site. Despite this, the stable binding of the MAb to the immobilized OVA suggests that IgA may gain high avidity through formation of the dimer. This hybridoma will provide a unique source of genuine IgA MAb, not an IgG-IgA chimeric one, against food allergens.

INTRODUCTION

SECRETORY IMMUNOGLOBULIN A (S-IgA) is the most prominent immunoglobulin on mucosal surfaces.⁽¹⁾ S-IgA is believed to provide host defense against pathogens without host tissue damage.⁽²⁾ Monoclonal antibodies (MAb) of the IgA class directed against cholera toxin (CT) have been shown to prevent the toxin from binding to intestinal epithelial cells.⁽³⁾ Moreover, the ability of IgA antibodies to neutralize intracellular viruses suggests that IgA is important for host defense.⁽⁴⁾ The targets of IgA are not restricted to pathogens because it has also been suggested that IgA prevents food antigens from entering the blood circulation.⁽⁵⁾

Airborne allergens such as house dust and pollens can trigger the development of allergic asthma. On repeated exposure to airborne allergens, inflammation may cause changes in mucosal tissues. When ragweed allergen-specific recombinant chimeric IgG-IgA was administered to the respiratory tract, an increase in airway responsiveness was prevented in allergen-sensitized mice.⁽⁶⁾ Clinical studies have also suggested that IgA deficiency and allergy are associated.⁽⁵⁾ However, there has been a report that IgA-deficient mice are less prone to ovalbumin (OVA)-induced allergic pulmonary inflammation.⁽⁷⁾ Because many complications were observed

in this mouse model, such as a decrease in OVA-specific IgG1 production and reduced levels of IL-4 and IL-5, it is difficult to conclude that secretory IgA contributes to the development of lung inflammation. In humans, the concentrations of allergen-specific and bacterial antigen-specific IgA were found to be increased in sputum from patients with asthma.⁽⁸⁾ Furthermore, it has been shown that IgA is capable of inducing the degranulation of human eosinophils.⁽⁹⁾ Recent studies also indicated that IgA is a potent inducer that activates human eosinophils.⁽¹⁰⁾ With this background, IgA MAb against allergens will be useful for addressing questions concerning the therapeutic or pathogenic roles of IgA in allergic conditions.

It is more difficult to produce IgA MAb than IgG MAb. There are various problems, such as how to optimize the route of immunization, how to prepare IgA-switched B cells, and how to obtain sufficient numbers of cells from mucosal-associated lymphoid tissues. Successful studies have included mouse mammary tumor virus (MMTV) and reovirus type 1,⁽¹¹⁾ respiratory syncytial virus,⁽¹²⁾ poliovirus,⁽¹³⁾ *Vibrio cholerae* LPS,⁽¹⁴⁾ *Shigella flexneri* LPS,⁽¹⁵⁾ *Porphyromonas gingivalis* fimbriae,⁽¹⁶⁾ *Salmonella enteritidis* LPS,⁽¹⁷⁾ and cholera toxin.⁽³⁾ We have also succeeded in the production of IgA MAb against Shiga toxin B subunits.⁽¹⁸⁾

Laboratory of Microbiology and Immunology, COE Program in the Twenty-first Century, University of Shizuoka School of Pharmaceutical Sciences, Shizuoka City, Shizuoka, Japan.

OVA is a major protein of chicken eggs and is one of the major allergens. OVA has been used as a model allergen for analyzing allergic responses to food proteins.⁽¹⁹⁾ It is not known whether OVA-specific IgA is capable of modifying allergic responses. There has been no report on OVA-specific IgA MAb. Here we report the production and characterization of an OVA-specific mouse IgA MAb.

MATERIALS AND METHODS

Mice

Specific pathogen-free, female BALB/c mice (8 weeks of age) were purchased from Japan SLC (Shizuoka, Japan). Animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals of the University of Shizuoka.

Reagents

Purified rabbit anti-mouse IgA (α chain-specific), goat anti-mouse IgA (α chain-specific), horseradish peroxidase (HRP)-goat anti-rabbit IgG (H+L), HRP-goat anti-mouse IgA (α chain-specific), and a Mouse MonoAb ID kit were purchased from Zymed (South San Francisco, CA). Goat anti-rabbit IgG microbeads and MS columns were purchased from Miltenyi Biotec Gladbach (Bergisch Gladbach, Germany). Cholera toxin (CT) (*Vibrio cholerae*, type Inaba 569B, azide-free) was purchased from Calbiochem (San Diego, CA). Streptomycin sulfate, Tween-20, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and 4-chloro-1-naphthol were purchased from Wako Pure Chemicals (Osaka, Japan). HAT media supplement, HT media supplement, OPI media supplement, polyethylene glycol (waxy solid, Hybri-Max, P-7777), purified mouse myeloma protein (TEPC 15, IgA, κ), BSA (fraction V), albumin from chicken egg white (OVA, Grade V), and 2-mercaptoethanol were purchased from Sigma (St. Louis, MO); HEPES from Nacalai Tesque (Kyoto, Japan); and penicillin G from ICN (Aurora, OH). CD Hybridoma Medium was purchased from Gibco Invitrogen (Carlsbad, CA), DMEM from Nissui Pharmaceuticals (Tokyo, Japan), and fetal bovine serum (FBS) from Hyclone (South Logan, UT). Precision Plus Protein Standards Dual Color was purchased from Bio-Rad (Hercules, CA), polyvinylidene fluoride (PVDF) membranes (Immobilon-P) from Millipore (Bedford, MA), and skimmed milk from Yukijirushi (Sapporo, Japan).

Cells

Non-secreting mouse myeloma P3X63/Ag8.653 cells were purchased from the American Type Culture Collection (Rockville, MD) and cultured in DMEM containing 10 mM HEPES (pH 7.4), 2 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 50 μ M 2-mercaptoethanol (complete DMEM) containing 10% FBS under a humidified atmosphere of 5% CO₂/95% air.

Immunization

As an immunogen, 100 μ g of OVA was mixed with 1 μ g of CT in 15 μ L of PBS. BALB/c mice were anesthetized by in-

traperitoneal injection of ketamine/xylazine, and then the mice were intranasally immunized with 7.5 μ L of the antigen and CT mixture per nostril on days 0, 7, 14, and 21. On day 52, the mice were boosted intranasally with the same dose of antigen and CT.

Production of hybridoma cells

Mouse hybridoma cells producing MAb against OVA were generated using the protocol described previously with modifications.⁽¹⁸⁾ Briefly, BALB/c mice were killed 4 days after the intranasal booster by cervical dislocation, and lymphocytes from nasal-associated lymphoid tissues (NALT) were collected. To obtain surface IgA (sIgA)-positive cells, the cells were incubated with rabbit anti-mouse IgA followed by goat anti-rabbit IgG microbeads, and then sIgA-positive cells were obtained using a magnetic cell sorter (Vario MACS, Miltenyi). Surface IgA-positive NALT cells and P3X63/Ag8.653 myeloma cells were packed in a ratio of 3:1, and then 60% polyethylene glycol diluted with the complete DMEM (0.1 mL per 10⁷ total cells) was added for cell fusion. The fused cells were cultured at 1 $\times$ 10⁵ cells/well in the complete DMEM containing OPI media supplement, HAT media supplement, and 20% FBS (HAT medium) for 9 days. The culture medium was gradually replaced by HT medium (DMEM containing OPI media supplement, HT media supplement, and 20% FBS) and then by the complete DMEM containing OPI media supplement and 20% FBS. Hybridoma cloning was carried out by means of two consecutive limiting dilution cultures. To obtain MAb samples under serum-free conditions, cloned hybridoma cells were cultured in protein-free CD Hybridoma Medium.

ELISA

For hybridoma screening, OVA-specific IgA antibodies in the culture supernatant were detected by a modified ELISA, as described previously.^(18,20,21) Briefly, samples containing IgA were allowed to bind to immobilized OVA (500 ng/well) for 1 h at room temperature. The samples had been diluted with PBS containing 0.1% BSA and 0.1% Tween-20. The antibody binding was detected with rabbit anti-mouse IgA (1:1000) followed by HRP-goat anti-rabbit IgG (H+L) (1:1000). As a substrate, 100 μ L of 1 mM ABTS dissolved in 0.1 M citrate buffer (pH 4.2) containing 0.03% H₂O₂ was added. After 5 to 10 min of incubation at room temperature, the absorbance was measured with a microplate reader (Tecan Spectra Readers, Tecan, Salzburg, Austria) at 405 nm. Antibody subclasses and light chain types were determined with the Mouse MonoAb ID kit. To determine the IgA antibody contents of samples, a sandwich ELISA was performed as described previously.⁽¹⁸⁾ Rabbit anti-mouse IgA was immobilized on an ELISA plate (Costar 9018, Corning, NY), and then hybridoma culture supernatants were added. The captured mouse IgA was detected with HRP-goat anti-mouse IgA. As standards, purified mouse IgA myeloma proteins (TEPC 15) were used. The antibody concentration in the culture supernatant was determined based on a dose-response curve generated with the standard TEPC 15 proteins.

Immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out under Laemmli's non-reducing

(5.6% gel) and reducing (10% gel) conditions as described previously.⁽¹⁸⁾ Proteins were transferred to a piece of PVDF membrane using a semi-dry blotting apparatus (ATTO, Tokyo, Japan). To block non-specific binding sites, the membrane was incubated in 5% skimmed milk in PBS for 1 h at room temperature. IgA was detected with a 1:1000 dilution of rabbit anti-mouse IgA followed by incubation with a 1:500 dilution of HRP-goat anti-rabbit IgG (H+L), or directly with a 1:500 dilution of HRP-goat anti-mouse IgA, for 1 h each at room temperature. The antibodies were diluted with 0.05% Tween-20–0.1% skimmed milk. The signals representing α heavy chains were enzymatically detected using 4-chloro-1-naphthol (0.5 mg/mL) and H₂O₂ (0.0125%) as a substrate dissolved in a buffer comprising 20 mM triethanolamine (pH 7.5), 130 mM NaCl, and 17% methanol.

Surface plasmon resonance analysis

Kinetic analyses of the antigen-antibody interaction were performed with a Biacore 2000 (Biacore K.K., Tokyo, Japan). HBS-EP (Biacore) was used as the running buffer. To remove sodium azide, the IgG fraction of goat anti-mouse IgA was dialyzed against PBS using Slide-A-Lyzer dialysis cassettes K10 (P7430, Pierce, Rockford, IL). A CM5 sensor chip (Biacore) was activated with 50 μ L of a 1:1 (v/v) mixture of NHS and EDC reagents (Biacore). The goat anti-mouse IgA (20 μ g proteins/mL diluted with 70 μ L of 10 mM sodium acetate, pH 5.0) was immobilized on the activated sensor chip, and then excess reaction groups were inactivated with 70 μ L of ethanolamine-HCl (pH 8.5). The sensor chip was washed with 10 mM glycine-HCl (pH 1.8). These procedures were carried out at a flow rate of 10 μ L/min at 25°C. The measurements were performed by repeated cycles of capture of MAb followed by injection of OVA at a flow rate of 20 μ L/min at 25°C: that is, 60 μ L of the MAb preparation (5 μ g/mL in HBS-EP) was injected to be captured. In the reference lane, no MAb was injected to evaluate non-specific binding of OVA. To record the binding of OVA to the captured MAb, samples of various concentrations (60 μ L) were injected in the Kinject mode. Between each measurement cycle, the sensor chip was regenerated by injection of 20 μ L of 10 mM glycine-HCl (pH 1.8) before injection of MAb. Data were analyzed with BIAevaluation 4.1 software (Biacore), and curve fitting was performed assuming a one-to-one interaction (Langmuir).

RESULTS

Production of IgA MAb to OVA

BALB/c mice were intranasally immunized with the OVA and CT mixture four times, and then intranasally boosted with the same antigen 4 days before cell fusion. Surface IgA-positive cells from NALT were enriched and then fused with mouse myeloma cells. Hybridoma cells producing OVA-specific IgA were screened and located by means of ELISA. The positive wells were subjected to cloning twice by means of limiting dilution cultures, and the clone exhibiting a positive signal was designated as A11F4. The isotype of A11F4 was determined to be IgA, κ .

Dose-dependent binding of IgA MAb A11F4 to immobilized OVA was measured by using a serum-free culture supernatant. As a control, purified IgA myeloma protein TEPC 15 was used. The concentration of IgA in the serum-free culture supernatant

of A11F4 was determined by sandwich ELISA. Concentration-dependent and saturable binding of A11F4 to the immobilized OVA was observed (Fig. 1). TEPC 15 did not bind to the immobilized OVA. The IgA concentration of A11F4 that gave half-maximal binding was 0.625 μ g/mL.

Biochemical characterization of the OVA specific-IgA antibody

To determine whether dimeric IgA was produced, immunoblotting was carried out using anti-mouse IgA. A11F4 and TEPC 15 gave similar bands corresponding to molecular masses of more than 250 kDa under non-reducing conditions. The major bands presumably represent a dimeric IgA form (Fig. 2A). The band corresponding to much higher molecular mass may represent a polymeric form of IgA. Under reducing conditions, a single band corresponding to the IgA heavy chain (65 kDa) was observed for the A11F4 and TEPC 15 samples (Fig. 2B).

Kinetic analysis of the binding of the OVA-specific IgA antibody

Surface plasmon resonance analysis was performed to determine the rates of binding and dissociation of antigens. Because dimeric IgA has four binding sites, the binding of IgA in solution to immobilized antigens is influenced by complex effects based on avidity. To determine the affinity of a single binding site, we chose a method in which the antigen in solution was allowed to bind to immobilized MAb. We first immobilized anti-IgA antibodies on a sensor chip to capture the MAb. The OVA-specific IgA MAb was captured, and then the surface plasmon resonance was recorded after the injection of various concentrations of OVA. Typical sensorgrams with various OVA concentrations were obtained (Fig. 3A). The resonance units obtained with each concentration of OVA at the report point (190 s) demonstrated dose-dependent binding (Fig. 3B). Based on curve fitting of the

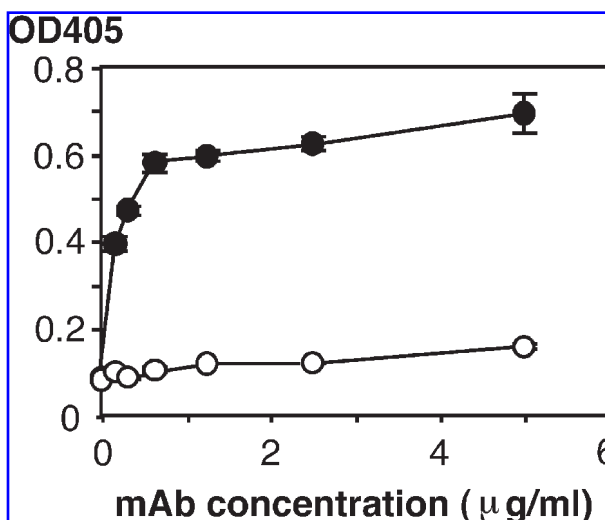


FIG. 1. Binding of IgA MAb to OVA. Binding of the IgA (ordinate) to immobilized OVA in relation to the antibody concentration (abscissa) was determined by ELISA. OVA-specific IgA MAb A11F4 (solid circles) and IgA myeloma protein TEPC 15 (open circles) are compared. Data represent means $\pm$ standard deviations of triplicate determinations.

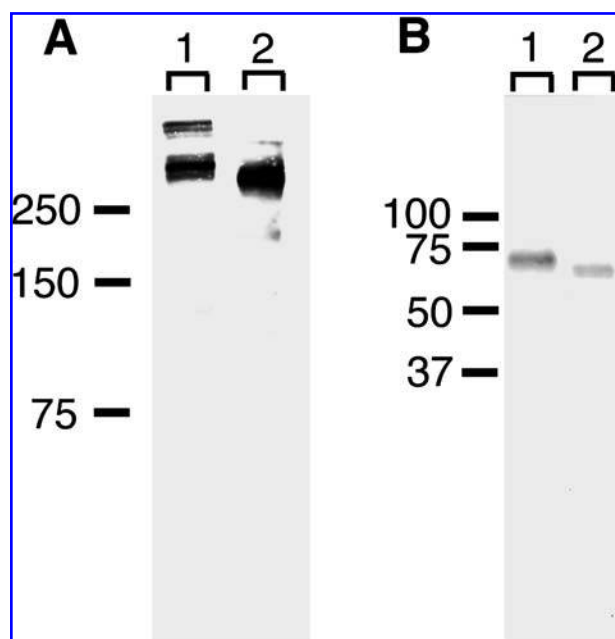


FIG. 2. Immunoblot analyses of IgA MAb. Proteins were separated by SDS-PAGE on a 5.6% gel under Laemmli's non-reducing conditions (A) or on a 10% gel under reducing conditions (B). OVA-specific IgA MAb A11F4 (lane 1) and IgA myeloma protein TEPC 15 (lane 2) are compared. Each lane contained 0.15 μ g of IgA. Rabbit anti-mouse IgA plus HRP-goat anti-rabbit IgG (A) or HRP-goat anti-mouse IgA (B) revealed the IgA heavy chain. The positions of molecular weight standards are shown on the left in kDa (molecular mass).

sensorgrams, the association rate constant (k_{ass}) was calculated to be 164 ($\text{M}^{-1} \text{s}^{-1}$) and the dissociation rate constant (k_{diss}) to be $7.59 \times 10^{-4} (\text{s}^{-1})$. The dissociation constant at steady state (K_D) was calculated to be $4.63 \times 10^{-6} (\text{M})$.

DISCUSSION

IgA production is usually induced in mucosal tissues by immunization via the oral or nasal route. Monoclonal IgA antibodies have been produced by means of oral^(3,13-15,17) or nasal^(12,16) immunization. The collection of IgA-switched B cells is necessary to obtain IgA-producing hybridomas, which have been typically generated through fusion between myeloma cells and Peyer's patch cells^(3,11,14,15) until now. B cells from spleen,⁽¹⁷⁾ lung,⁽¹²⁾ and submandibular gland⁽¹⁶⁾ have also been examined as alternative sources. In this study, mice were intranasally immunized with OVA, and NALT were used as inductive sites for a mucosal lymphoid system such as Peyer's patches. Because there have been reports that IgA class-switched plasmablasts express cell surface IgA,^(22,23) we enriched surface IgA-positive (sIgA⁺) B cells by means of positive selection. The surface IgA⁺ B cells were fused with myeloma cells, and we established a clone, A11F4, that produces OVA-specific IgA MAb.

The binding of IgA MAb A11F4 to immobilized OVA appears to exhibit sufficient avidity, as judged on ELISA. The lack of binding of an IgA myeloma, TEPC 15, to OVA indicates the specificity of A11F4. The saturated binding of A11F4

also suggests specific binding. Immunoblot analyses revealed that the majority of the IgA was dimeric. That is, there are four binding sites per molecule. The presence of multiple binding sites per molecule makes measurement of the affinity of individual binding sites difficult. When the binding of MAb to immobilized OVA is measured directly by means of surface plasmon resonance, the results do not represent the affinity of an individual binding site.

We attempted to avoid this problem by using the antibody capture method, in which the IgA MAb was first captured by means of immobilized antibodies directed to the α heavy chain. Various concentrations of OVA were then injected to determine surface plasmon resonance. With this method, global fitting based on multiple curves became possible. Interestingly, the association rate appears to be slow for this particular MAb. The net dissociation constant was calculated to be of the order of 10^{-6} M , suggesting relatively low affinity of the binding site. Despite the low affinity

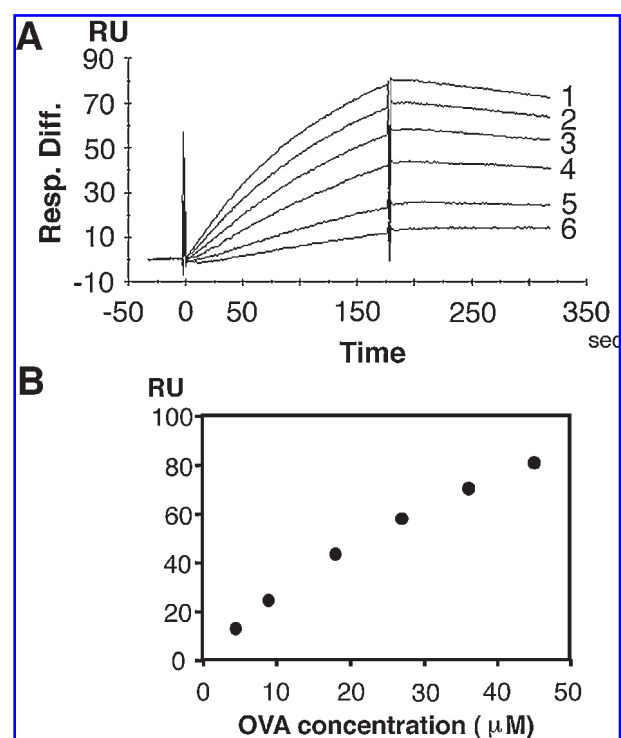


FIG. 3. Kinetic analyses of the antigen-antibody interaction involving the capture method. (A) Surface plasmon resonance of OVA binding to the captured IgA MAb A11F4. The MAb (5 μ g/mL) was captured with goat anti-mouse IgA immobilized on a CM5 sensor chip. OVA samples of various concentrations (4.5–45.2 μ M) were injected for 180 s and then allowed to dissociate for 3 min. Then the sensor chip was regenerated, followed by the capture of the same concentration of MAb. The ordinate represents net resonance units (RU) after subtraction of RU for the reference lane (no MAb captured). Each sensorgram corresponding to a given concentration of OVA was overlaid. The number to the right of each sensorgram indicates the concentration of the OVA sample as follows: 45.2 μ M (1), 36.2 μ M (2), 27.1 μ M (3), 18.1 μ M (4), 9.0 μ M (5), and 4.5 μ M (6). Curve fitting of the sensorgrams was carried out assuming a 1:1 binding model (Langmuir). (B) Net RU at the report point (at 190 s) reflecting specific binding of OVA (ordinate) was plotted in relation to the OVA concentration (abscissa).

ity of each binding site, the binding of the MAb to the immobilized OVA was stable, suggesting that the dimeric organization of IgA contributed to the high avidity binding.

In summary, we were able to obtain an IgA MAb against OVA by using NALT cells from BALB/c mice that had been intranasally immunized with OVA and CT. Surface plasmon resonance analyses revealed the relatively low affinity of each binding site, but the dimeric organization of IgA molecules contributes to the high avidity. It remains to be determined whether this MAb will be useful as a tool for studies on allergic disorders.

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Address reprint requests to:

Yasuyuki Imai, Ph.D.

Laboratory of Microbiology and Immunology

University of Shizuoka School of Pharmaceutical Sciences

52-1 Yada, Suruga-ku, Shizuoka-shi

Shizuoka 422-8526

Japan

E-mail: imai@u-shizuoka-ken.ac.jp

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