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Production of a monoclonal antibody against chicken immunoglobulin G: A valuable molecule with research and diagnostic applications

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Abstract

Monoclonal antibodies (MAbs) are invaluable molecules which have several advantages over polyclonal immunoglobulins (Igs) including consistency and higher specificity and hence can be used in biological researches, diagnosis and treatment of diseases. The present study was conducted to produce monoclonal antibody against chicken IgG. The IgG molecules were purified from chicken serum and used as antigens to immunize several mice. Thereafter, a well-immunized mouse was chosen and used for fusion process. After production of hybridoma cells, several rounds of cloning were carried out and produced MAbs were examined by various immunological assays including enzyme-linked immunosorbent assay (ELISA) and western and dot blotting. Assessment of grown hybridomas indicated that only one clone (5B8) has produced desired MAb against chicken IgG. Meanwhile, using an indirect ELISA, it was shown that this MAb successfully recognizes chicken IgG molecules attached to influenza virus nucleoprotein. Evaluation of cross reactivity of MAb 5B8 with several avian serum samples revealed that this molecule specifically identifies chicken antibody molecules. However, it also recognized turkey antibodies with less affinity. In addition to research applications like isolation and purification of chicken and turkey IgG molecules, such a MAb can be applied to design and development of various immunoassays (e.g. ELISA) in these avian species.

تولید آنتی بادی منوکلونال علیه ایمونوگلوبولین G مرغ: مولکولی ارزشمند با کاربردهای پژوهشی و تشخیصی

چکیده

آنتی‌بادی‌های منوکلونال مولکول‌های ارزشمندی هستند که نسبت به ایمونوگلوبولین‌های (Igs) پلی‌کلونال از مزایای بسیاری از جمله همسانی و اختصاصیت بالاتر برخودار می‌باشند و بنابراین می‌توانند در پژوهش‌های زیست‌شناختی، تشخیص و درمان بیماری‌ها مورد استفاده قرار گیرند. مطالعه حاضر با هدف تولید آنتی‌بادی منوکلونال علیه IgG مرغ انجام شد. مولکول‌های IgG از سرم مرغ خالص شد و به عنوان آنتی‌ژن برای ایمن‌سازی موش‌های متعدد استفاده گردید. سپس یکی از موش‌های به خوبی ایمن شده، انتخاب شد و جهت انجام فرآیند همجوشی مورد استفاده قرار گرفت. پس از تولید سلول‌های هیبریدوما، مراحل کلونینگ متعددی انجام پذیرفت و آنتی‌بادی‌های منوکلونال تولید شده با استفاده از انواع آزمون‌های ایمنی‌شناختی از جمله الایزا، وسترن و دات بلات ارزیابی شدند. بررسی هیبریدوماهای رشد یافته نشان داد که تنها یک کلون (5B8)، آنتی‌بادی منوکلونال مورد نظر علیه IgG مرغ را تولید کرده است. به علاوه، با استفاده از الایزای غیرمستقیم نشان داده شد که این آنتی‌بادی منوکلونال، مولکول-های IgG مرغی متصل به نوکلئوپروتئین ویروس آنفلوانزا را به خوبی شناسایی می‌کند. ارزیابی واکنش متقاطع آنتی‌بادی منوکلونال 5B8 با چندین نمونه سرمی پرندگان، آشکار ساخت که این مولکول به طور اختصاصی مولکول‌های آنتی بادی مرغ را شناسایی می‌کند. با این حال، این مولکول، آنتی بادی‌های بوقلمون را نیز با تمایل کمتری تشخیص داد. علاوه بر کاربردهای پژوهشی مانند جداسازی و خالص‌سازی مولکول‌های IgG مرغ و بوقلمون، چنین آنتی‌بادی منوکلونالی می‌تواند جهت طراحی و توسعه آزمایشات ایمنی‌شناختی مختلف (مانند الایزا) در این گونه‌های پرنده، به کار رود.

واژه های کلیدی: آنتی بادی منوکلونال، الایزا، ایمونوگلوبولین G، مرغ

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Introduction

Poultry industry is one of the most important chicken meat and egg industries throughout the world. However, infectious diseases of chickens have a huge impact on this industry and can lead to major economic losses.

There are various diseases affecting chicken flocks including those caused by devastating bacterial and viral agents such as mycoplasmas, influenza and Newcastle disease viruses. Several methods have been used in the preventive and surveillance programs to battle against such conditions, most of which are based on the diagnosis of infections through detection of antibodies against infectious agents in chicken sera. Currently, immuno-enzyme assays, in the form of commercial enzyme-linked immunosorbent assay (ELISA) kits, are the most common serological tests for the diagnosis of poultry diseases. In all these kits, detector secondary antibodies labeled with an enzyme used to recognize chicken immunoglobulin (Ig) G molecules. Chicken IgG, also known as IgY, is the major antibody isotype in the chicken serum and is composed of two heavy and two light chains, each of them with molecular mass of 67 to 70 and 25 kDa, respectively and as it has one additional constant region in heavy chains, its molecular mass (~ 180 kDa) is higher than mammalian IgG.[1] In many cases, anti-chicken IgG antibodies consist of polyclonal antibodies produced in rabbits or goats. Although polyclonal antibodies can detect several epitopes on the whole molecules of chicken IgG, they often lack specificity. However, the story is different for monoclonal antibodies (MAbs) which can distinguish their antigens more specifically. It has been shown that mouse MAbs could be applied as secondary antibodies to identify chicken IgG molecules in ELISA.[2] As MAbs recognize a single epitope; they have major advantages over polyclonal antibodies including their homogeneity, consistency and higher specificity. Because of these benefits, enzyme conjugated MAbs could be more suitable to develop diagnostic tests.[3-5] Using such indicative MAbs against chicken IgG molecule, it is possible to detect chicken antibodies produced in response to different infectious agents in various indirect ELISA tests. Meanwhile, chicken antibodies can also be used as alternatives for mammalian antibodies.[6,7] Nowadays, chickens have become the preferred species to provide antibodies against mammalian antigens, as they are phylogenetically distant from their mammalian counterparts and enzyme conjugated MAbs produced against chicken IgG could also be a valuable tool in those researches.[6-10]

The present study, for the first time in Iran, was conducted to produce a functional MAb against chicken IgG (IgY) molecule and evaluate its possible application as a detector antibody in ELISA compared to a commercial enzyme conjugated anti-chicken antibody.

Materials and Methods

Collection of serum samples. Chicken serum was used as a source of chicken IgG molecules. On the other hand, sera from several avian species including turkey, duck, geese, ostrich, pigeon and quail were also collected and used to investigate any possible cross reaction.

Purification of chicken IgG. Purified chicken IgG was used as antigen to immunize mice for MAb production. Purification process was carried out based on a previously described method.[11] Briefly, 10 mL of chicken serum was mixed with acetate buffer and pH was adjusted to 4.50. Caprylic acid was then added to the mixture (25 µL mL^{-1} serum) followed by centrifugation at 10000 *g* for 30 min. The supernatant was filtered through a filter paper and mixed 10:1 with phosphate-buffered saline (PBS) 10X. Once again, pH was adjusted to 7.40 and ammonium sulfate was gradually added (0.27 g mL^{-1}) followed by another centrifugation step. The precipitate was dissolved in 1 mL of PBS 1X and the solution was dialyzed against PBS 1X for an overnight period with at least two changes of the buffer.

Immunization. The BALB/c mice were used for immunization. Five to 8-week-old female BALB/c mice were intraperitoneally immunized by injection of 50-100 µg purified chicken IgG on days 0, 15 and 30. To induce an appropriate immune response, the first injection was carried out using complete Freund's adjuvant (Sigma, St. Louis, USA), while the other two injections were performed using incomplete Freund's adjuvant. Ten days after the last injection, mice were tail-bled and the sera were assayed for antibody molecules against chicken IgG in an indirect ELISA. Mice with the highest titer of anti-chicken IgG antibodies were selected for further steps.[12,13]

Evaluation of mice immune response by ELISA. The mice sera were examined to determine antibody titer against injected chicken IgG molecules. Briefly, 100 ng of purified chicken IgG was added into each well of a 96-well polystyrene microtiter plate as ELISA antigen and incubated for 16 hr at 4 ˚C. All subsequent incubation steps were performed at room temperature and the plate was washed four times with PBS containing 0.05% Tween 20 (PBST) after each step. Unreacted sites blocking was carried out using PBST containing 5% skim milk for 2 hr. After washing the plate, three dilutions of each of the mice sera (1/200, 1/400 and 1/800 in PBST containing 5% skim milk) were added into the wells and the plate was incubated for 1 hr. After a washing step, a 1/2000 dilution of goat anti-mouse IgG-horseradish peroxidase (Sigma) in PBST containing 0.50% skim milk (Merck, Darmstadt, Germany) was added into the wells and followed by another incubation step for 1 hr. The ELISA plate was washed again with PBST and tetramethylbenzidine substrate solution (Sigma) was added into the wells. After 10 min, the reactions were stopped by addition of 0.10 M

HCl and optical density (OD) values were measured at 450 nm with a plate reader.

Preparation of myeloma and mouse feeder cells. The Sp2/0 murine myeloma cells were prepared and cultured with 8-azaguanine (Sigma) to check their sensitivity to hypoxanthine aminopterin thymidine (HAT; Sigma) selection medium used after cell fusion. About 1×10^7 Sp2/0 cells in the logarithmic phase with viability of more than 95% were used for fusion. Meanwhile, mouse peritoneal cells (feeder cells), mostly macrophages, used to provide effective source of soluble growth factors for hybridoma cells. To prepare feeder cells, adult Balb/c mice were sacrificed and 8 mL of 0.34 M chilled sucrose solution was peritoneally injected and after a gentle massage of the abdomen, the fluid was withdrawn. The viable cells were then counted and diluted with HAT medium to 1×10^5 feeder cells per mL. This cell suspension was dispensed into 60 inner wells of 96-well plates, 24 hr prior to fusion.[13]

Production of hybridoma cells. The Sp2/0 myeloma cells (1×10^7) in the logarithmic phase were fused with 1×10^8 spleen cells from an immunized mouse using polyethylene glycol (Roche, Mannheim, Germany) as a fusing agent. The cells in the fusion mixture were suspended in 35 mL of HAT medium and incubated for at least 30 min in a CO_2 incubator. Thereafter, 100 µL of the fusion mixture was added into each of 60 wells of the plates containing feeder cells and incubated for five days in a CO_2 incubator. After incubation, 100 µL of HAT medium was added into each well and the medium was replaced every day.[13]

Screening of grown hybridomas. The supernatants of grown hybridomas were assessed for antibodies against chicken IgG using indirect ELISA and western blotting. The procedure of indirect ELISA was the same as the previous one used to examine immunized mice sera except that this time the supernatants of cultured hybridomas, instead of serum samples, were added into the wells. Reactivity of produced MAb (by an ELISA positive hybridoma) against whole chicken IgG molecule and each of the light and heavy chains were checked using western blotting. Thus, reduced and non-reduced purified chicken IgG molecules were electrophoresed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and protein bands were transferred onto the nitrocellulose membranes. The membranes were blocked using PBST containing 5.00% skim milk for 2 hr followed by three times of washing with PBST. Thereafter, the supernatant of an ELISA positive hybridoma was incubated with the membranes for 1 hr followed by three times washing with PBST. Finally, the membranes were incubated for 1 hr with diluted (1:2000 in PBST) peroxidase-conjugated goat anti-mouse IgG (Sigma). After washing, the membranes were developed using 4-chloro 1-naphtol (Sigma) and H_2O_2 (Merck) as substrates.[13]

Isolation and cloning of hybridoma cells. Several cloning rounds were carried out until more than 90% of the wells containing single clones were positive for antibody production which indicated that the cells were identical and from the same origin. For this purpose, positive clones were expanded in 24-well plates containing feeder cells and cultured in a CO_2 incubator. Afterward, positive hybridomas were cloned by limiting dilution (8 cell per mL^{-1}) in hypoxanthine thymidine medium and dispensed into 96-well plates. The single clones with the highest anti-chicken IgG antibody titers were sub-cloned at least three times. After three rounds of cloning, positive clones were grown to larger volumes and stored frozen in liquid nitrogen in fetal bovine serum (Gibco, Waltham, USA) containing 10.00% dimethyl sulfoxide (Gibco).[13]

Evaluation of produced MAb in an indirect ELISA. A microplate was coated by a recombinant nucleoprotein (NP) of type A influenza virus (1 µg per well) at 4 ˚C for 16 hr.[14] The plate was washed three times with PBST and blocked with PBST containing 5.00% skim milk at room temperature for 2 hr. After washing the plate, 40 chicken sera (diluted 1/400 in PBST) were duplicately added into the wells. These samples were included 20 sera collected from broiler chickens infected with H9N2 serotype of influenza virus and 20 sera obtained from non-vaccinated and uninfected broiler chickens as negative controls. The plate was then incubated at room temperature for 1 hr and washed three times with PBST. Thereafter, each of the produced MAb and cell culture medium were added into half of the wells and the plate was incubated at room temperature for another hour. Following washing, peroxidase-conjugated anti-mouse IgG (diluted 1/2000 in PBST) and peroxidase-conjugated anti-chicken IgG (diluted 1/1000 in PBST) were added into those wells incubated with the MAb and cell culture medium, respectively. After 1 hr incubation at room temperature, the plate was washed again and tetramethylbenzidine substrate solution was added into the wells. The reactions were stopped after 10 min by addition of 0.1 M HCl and finally, OD values were measured at 450 nm with a plate reader.

Evaluation of cross-reactivity of MAb with other sera. Serum samples of aforementioned species along with the chicken serum as the main target were diluted 1:10 in PBS and examined for any cross reaction. Briefly, 5 µL of each of the diluted serum samples were dotted onto the nitrocellulose strips and unreacted sites were blocked with PBST containing 5% skim milk for 1 hr. The strip was then immersed in 5 mL of prepared anti-chicken MAb (diluted 1/5 in PBST containing 5% skim milk) for 1 hr followed by a washing step. Finally, the strip was incubated with diluted (1:2000 in PBST) goat anti-mouse IgG peroxidase for 1 hr. After washing, the strip was developed using 4-chloro 1-naphtol (Sigma) and H_2O_2. Two

same strips were also allocated to commercial peroxidase-conjugated anti-chicken and anti-mouse IgG as positive and negative controls, respectively.

Results

Purification of chicken IgG. The purity of chicken IgG was examined by SDS-PAGE. As shown in Figure 1A, reduced purified IgG constituted two protein bands of about 20-25 and 65-70 kDa respectively representing light and heavy chains, while non-reduced purified IgG (without using 2-mercaptoethanol) formed a major single protein band with a molecular weight of about 180 kDa.

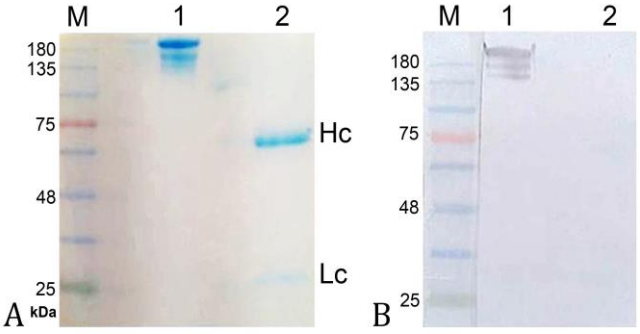

Fig. 1. A) Polyacrylamide gel electrophoresis of non-reduced (lane 1) and reduced (lane 2) purified chicken IgG. M: Pre-stained protein marker; Hc and Lc: Heavy and light chains, respectively. **B)** Reactivity of monoclonal antibody 5B8 with non-reduced (lane 1) and reduced (lane 2) chicken IgG in western blotting. M: Pre-stained protein marker (kDa).

Evaluation of mice immune response by ELISA. The ELISA results revealed that all of the injected mice had been properly immunized against chicken IgG in comparison with an uninjected mouse (control). Three dilutions of sera from these animals were assayed. The obtained OD values are given in Table 1.

Production of hybridoma cells. One of the well-immunized mice was chosen and used for fusion process. After cultivation of the cells in HAT cell culture medium (only hybridoma cells can grow in this medium), the plates were observed for growth of hybridomas. The fusion process resulted in the production of at least 140 hybridoma cells.

Screening and cloning of grown hybridomas. Examination of supernatants of hybridoma cells by ELISA depicted that among 140 hybridomas only one clone (5B8) permanently produced antibody against chicken IgG. This hybridoma clone was cultured in 24-well plates and frozen. On the other hand, western blotting was performed to check the reactivity of produced MAb with whole and each of the light and heavy chains of chicken IgG. The results indicated that MAb 5B8 reacted only with whole molecule of chicken IgG (non-reduced IgG). This is presented in Figure 1B. This clone which was found to be positive by ELISA and western blotting was cloned three times and its subclones were examined in each step by ELISA. After the third cloning step, one subclone was stored frozen in liquid nitrogen for long preservation.

Evaluation of MAb 5B8 cross-reactivity with other avian sera. The reactivity of produced MAb with serum samples of various avian species was assessed by dot blotting. As shown in Figure 2, the results indicated that MAb 5B8 only recognized sera from chicken and turkey (with less affinity, almost as the same as the commercial peroxidase-conjugated anti-chicken IgG) and did not show any reaction with other tested serum samples.

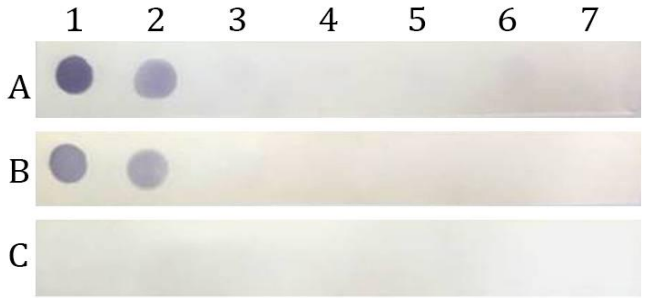

Fig. 2. Reactivity of prepared monoclonal antibody against sera of various avian species. **A)** Commercial peroxidase-conjugated anti-chicken IgG; **B)** Produced monoclonal antibody; **C)** Commercial peroxidase-conjugated anti-mouse IgG (negative control); Lanes 1 to 7: Sera from chicken, turkey, quail, duck, geese, ostrich and pigeon, respectively.

Evaluation of MAb 5B8 by an influenza indirect ELISA. The OD values obtained from this ELISA showed that produced MAb 5B8 was functional in the experiment and could truly recognize chicken IgG molecules while they had been attached to NP antigens. The results of this MAb were comparable to those obtained from a commercial peroxidase-conjugated goat anti-chicken IgG. The data are given in Figure 3.

Discussion

Nowadays, use of MAbs has been dramatically increased in various fields including biomedical researches, diagnosis and treatment purposes. This referred to their advantages over polyclonal antibodies such as homogeneity, consistency and higher specificity.

Table 1. Optical density values of mice sera 10 days after injection.

Serum dilution	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Not-injected mouse
1/200	2.36	2.56	2.61	2.46	2.52	0.15
1/400	2.50	2.64	2.67	2.66	2.53	0.10
1/800	2.46	2.64	2.55	2.64	2.70	0.09

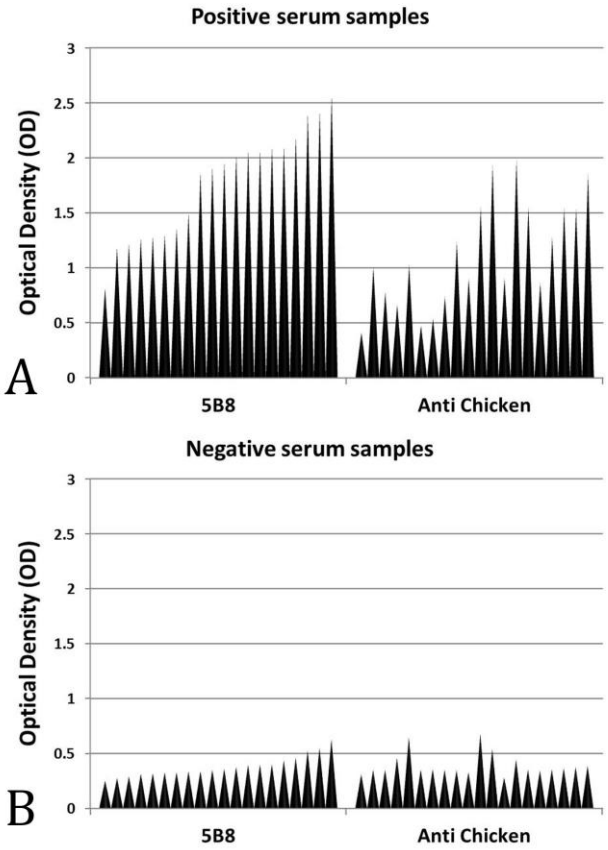

Fig. 3. Reactivity of monoclonal antibody 5B8 in comparison with commercial anti-chicken IgG in an indirect ELISA for detection of chicken antibodies against influenza virus nucleoprotein in positive serum samples (A). No false positive reaction was observed for negative serum samples (B).

Consequently, production of MAbs has many benefits regarding to their applications. In this case, MAbs against chicken IgG are invaluable molecules as chicken IgG is the major and most important isotype of Igs in this species. Moreover, chicken IgG is not only an indicator of infections but it is also becoming an alternative for Igs of mammalian origin.[6,7]

Few studies have been done to produce MAbs against chicken IgG. Erhard *et al.* reported development of a specific ELISA for quantitation and qualification of chicken Ig isotypes G, M, and A using MAbs.[15] In 2004, Narat *et al.* produced several MAbs against chicken IgG. Application of these antibodies in blotting immunological assays has showed that one of produced antibodies was very potent as a secondary antibody and was able to detect chicken antibodies against different pathogens.[10] Raj *et al.* produced several MAbs against chicken IgG and used one of them as a capture antibody in ELISA for estimation of purified IgY from egg yolk.[9]

Regarding the development of poultry industry in our country and the necessity of having reagents to detect infectious diseases of chickens; here, we produced a Mab 5B8 against chicken IgG. The results of ELISA and western blotting showed that this MAb reacted with whole molecule of IgG under non-reduced condition suggesting that this molecule may react with a structural epitope on the IgG molecule. Meanwhile, based on the results of indirect ELISA for the detection of antibodies against influenza virus in sera of infected chickens, it was determined that this MAb is able to detect chicken antibodies, indicating that attachment of these molecules to the influenza antigen does not interfere in their reaction with produced MAb. Although another step for addition of a commercial goat anti-mouse IgG was done in the influenza indirect ELISA, the results obtained from these two antibodies indicated that the reactivity of produced MAb with the commercial one was comparable. As this MAb detected antigen-bound and unbound chicken IgG molecules, it can be used as an anti-chicken antibody in several immunological diagnostic tests. Moreover, this molecule can also be applied for laboratory experiments such as purification and quantification of chicken IgG molecules. On the other hand, evaluation of cross-reactivity of MAb 5B8 with polyclonal sera of various avian species showed that this molecule specifically identifies chicken IgG and this is an advantage in development of specific ELISAs. Interestingly, MAb 5B8 also recognized turkey polyclonal serum implying its possible application in immunoassays development in this species.

It should be noted that beside their applications in biological researches, production of such applicable MAbs may address needs for designing new diagnostic and/or treatment methods for various infectious diseases.

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Conflict of Interest

The authors declare no conflict of interest.

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