A Novel Monoclonal Antibody Against the Constant Region of Goose Immunoglobulin Light Chain

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A monoclonal antibody (MAb) against the antigenic determinant of the constant region of goose immunoglobulin light chain (GoIgCL) was produced and characterized for the first time here. Goose immunoglobulin (Ig) in serum was purified by immunoaffinity chromatography and the resulting protein was used as immunogen to immunize BALB/c mice. At the same time, the GoIgCL gene was expressed and purified as the screening antigen for selecting MAb against GoIgCL. One hybridoma that produces antibodies against GoIgCL was selected by indirect ELISA. Then the characterization of the MAb was analyzed by ELISA, Western blot, and flow cytometry. It was found to be IgG1 with κ light chain; the MAb has high specificity to Ig in goose serum, bile, and B lymphocytes from peripheral blood, reacts only with the light chain of goose Ig, and can distinguish Ig from other birds. Therefore, the MAb generated in this study can be used as a specific reagent for detection of goose disease-specific antibodies and as a powerful tool for basic immunology research on geese.

Introduction

IMMUNOGLOBULIN (Ig) IS AN IMPORTANT effector molecule of humoral immunity that is composed of two identical heavy chain polypeptides and two identical light chain polypeptides. A light chain has two successive domains: one variable (VL) domain and one constant (CL) domain.⁽¹⁾ Very little genetic variability is found in the CL domain, which made the C region of L chain important for the preparation of specific antibody used for immunoassay. The same type of Ig light chain (IgL) has the same antigenicity,^(1,2) which made the C region of L chain important for the preparation of specific antibody used for immunoassay. In addition, one type of light chain is only present in a typical antibody, and the mammals have two types of light chain, κ and λ , but only the λ chain is expressed in the avian species including goose.^(3,4,6,7) Therefore, the level of CL can represent that of Ig in goose.

Thus far, there has been little research on the goose immune system due to a lack of well-characterized immunological reagents with specificity for immune system components, including Ig isotypes and subclasses. On the basis of our previous studies, having first described the gene sequences encoding goose Ig alpha chain and light chain,^(5,7) the major objective of this study was to generate monoclonal antibodies (MAb) with specificity for goose Ig light chain constant region (GoIgCL). This has been achieved by immunizing BALB/c mice with purified goose Ig, fusing the immunized spleen cells with myeloma, and selecting for cloned cell hybrids that recognize GoIgCL determinants by

recombinant protein containing GoIgCL gene (rGoCL). The MAb against GoIgCL was then characterized by Western blot, ELISA, and flow cytometry. Our data showed that the MAb is a useful reagent for goose basic immunological research and infectious disease.⁽⁸⁾

Materials and Methods

Animals

BALB/c mice (5~6 weeks old) were purchased from the veterinary institute in Harbin and maintained under standard conditions with free access to laboratory food and water.

Purification of goose Ig

Ig was purified roughly by anhydrous sodium sulfate from goose serum and further purified using protein A affinity chromatography.⁽⁹⁾ The purified Ig mixed with sodium dodecyl sulfate (SDS)-loading buffer was subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Protein expression and purification

A pair of specific primers F/ λ IgCL (5'-TAGGATCCGTC CTGGGCCAGCCCAAGG-3', *Bam*H I site underlined) and R/ λ IgCL (5'-CCGAAGCTTTTACGAGCACTCGGATTTG TTCA-3', *Hind* III site underlined) that were used to amplify the GoIgCL gene (309bp) were designed according to the sequence of GoIgL (GenBank ID: HQ852946).⁽⁷⁾ The PCR products were cloned into pET30a (+) and the positive recombinant plasmid was transformed into *Escherichia coli*

strain *Rosetta(DE3) Lys* cells; then rGoCL was induced with isopropyl β -D-thiogalactoside (IPTG) (Sigma-Aldrich, St. Louis, MO) and the expression product was analyzed by SDS-PAGE.⁽¹⁰⁾ The supernatant was purified by a nickel-charged column (GenScript, Nanjing, China), according to the manufacturer's protocol, and rGoCL was dialyzed as screening antigen, which was used to detect the MAb against GoIgCL.

Cell fusion

BALB/c (6~8 weeks old) mice were immunized with subcutaneous (s.c.) injections of 50 μ g/mouse purified goose Ig emulsions in Freund's complete adjuvant (Sigma-Aldrich) and boosted with an additional 50 mg/mouse of goose Ig intraperitoneally (i.p.) without adjuvant on day 21. After 3 days, the serum was separated to detect the indirect enzyme-linked immunosorbent assay (I-ELISA) method for selecting the positive hybridoma cells, and the spleen was removed for the fusion process. The spleen cells were separated from the spleen, removed according to conventional methods, and then fused with SP2/0 cells at a ratio of 9:1 in serum-free medium using PEG3500 (Sigma-Aldrich). The resulting hybridoma cells were plated onto 96-well plates (Costar, Corning, NY) and cultured in selection medium 1640 (Gibco, Carlsbad, CA) with hypoxanthine, aminopterin, and thymidine (HAT, Sigma-Aldrich). Five days post-fusion, half medium was changed into selection medium 1640 with hypoxanthine and thymidine (HT, Sigma-Aldrich). After 10–14 days, the hybridoma supernatants were screened using the I-ELISA detected to select hybridomas secreting specific antibodies against GoIgCL. After 14 days post-fusion, the selected hybridoma clones were subcloned by limiting dilution and rescreened by I-ELISA three to four times until all the subclones secreting antibodies reacted with rGoCL. The selected hybridomas were expanded for storage in liquid nitrogen after being checked for sterility.⁽¹¹⁾

Establishment of screening method

An I-ELISA method used for selection of hybridoma-secreting antibodies against GoIgCL was developed. 96-well polystyrene plates (Jet, Toronto, Canada) were coated with 0.5 μ g/mL rGoCL diluted in a binding solution (0.01 M NaOH buffer [pH 12.0]) with a 100 μ L volume and incubated

at 4°C overnight. The plate was then washed with PBST (0.01 M PBS [pH 7.4], 0.05% (v/v) Tween-20) four times. The coated wells were blocked by 300 μ L blocking buffer (PBST containing 5% skimmed milk) at 37°C for 2 h. After four washes with PBST, the serum separated above was diluted by multiproportion with PBST in a 100 μ L volume per well at 37°C for 1 h. Unbound serum was removed by washing four times with PBST, and volumes (100 μ L) of HRP-conjugated goat anti-mouse (ZSGB, Beijing, China) were diluted at 1:10,000 and dispensed into all wells. After 1 h incubation at 37°C and subsequent washing, the color was developed using the chromogen/substrate mixture TMB/H₂O₂ (TMB, 3,3',5,5'-tetramethyl-benzidine; Sigma-Aldrich). The reaction was stopped after 15 min by adding 1 M H₂SO₄ and the OD450 nm of each well was read using a microplate reader (Molecular Devices, Sunnyvale, CA).⁽¹²⁾

Basic characterization of MAb

The isotype of the selected MAb was identified using a mouse monoclonal antibody isotyping kit (Sigma-Aldrich) according to the manufacturer's instructions. The chromosome number of hybridoma cells was analyzed by the conventional method using colchicines (Sigma-Aldrich) with the control of SP2/0 cells.^(13,14) The stability of passage and cryopreservation was also measured by conventional techniques.^(13,15) The ascites of MAb against GoIgCL was prepared using standard procedures as described previously⁽¹⁶⁾ and purified by affinity chromatography using Protein G (Genscript). The titer of ascites produced here was tested by I-ELISA.

Analysis of MAb by Western blot, ELISA, and flow cytometry

Western blot was performed to analyze the immunoreactivity of MAb with goose Ig under standard conditions. The goose serum, goose bile (our lab conserved), and fusion protein rGoCL were separated on 12% gels and then electrotransferred onto a nitrocellulose membrane (NC membrane) in transfer buffer. After blocking with 5% skimmed milk in PBST overnight at 4°C, the NC membrane was incubated with hybridoma supernatant for 2 h at 37°C. Then the membrane was washed with PBST four times and incubated with HRP-conjugated goat anti-mouse (1:1000) for 1 h at 37°C. The HRP substrate, 4-chloro-I-naphthol (4CN, Sigma-Aldrich)

FIG. 1. SDS-PAGE analysis of protein for MAb preparation and Western blot analysis of the MAb 1B11. (A) SDS-PAGE analysis of protein purified from serum. Lane M, protein molecular weight marker; lane 1, purified goose serum Ig. (B) SDS-PAGE analysis of recombinant protein rGoCL. Lane M, protein molecular weight marker; lane 1, negative control of empty vector pET-30a (+); lane 2, thalline of rGoCL; lane 3, sedimentation of rGoCL; lane 4, supernatants of rGoCL; lane 5, purified rGoCL. (C) Western blot analysis of MAb 1B11 against GoIgCL. Lane M, EasySee Western marker; lane 1, goose serum; lane 2, thalline of rGoCL; lane 3, goose bile.

TABLE 1. TITER OF ASCITES PRODUCED BY MAB 1B11

Dilution (1000)	1	2	4	8	16	32	64	128	256	512
1B11	1.154	1.078	1.048	1.027	1.016	1.002	0.958	0.935	0.912	0.854
SP2/0	0.091	0.085	0.086	0.083	0.080	0.078	0.078	0.075	0.073	0.070

was used for color development and visualization of reacted bands.

The reactivity of MAb with other animal Ig was tested by the I-ELISA as described above. The plates were coated with serum (four animals per species) diluted 1:100 in 0.01 M NaOH buffer (pH 12.0), and each serum was repeated three times. At the same time, the purified rGoCL and serum dilution were used as control. Finally, the results were analyzed by conventional statistical methods. The sera that were examined for immunoreactivity analysis were from *Gallus gallus*, *Gallus domesticus*, turkey, duck, goose, cow, sheep, pig, rabbit, *Anas platyrhynchos*, and *Grus japonensis*.

The reactivity of MAb with native Ig expressed on the surface of B lymphocytes was analyzed by flow cytometry. First, the lymphocytes were isolated from goose peripheral blood by lymphocyte separation medium (TBD, Tianjin, China) according to the manufacturer's instructions. Two $\times 10^6$ cells were added to the centrifuge tube respectively; then the MAb and SP2/0 diluted with PBA (0.01 M PBS [pH 7.4], 0.1% BSA) at the concentration of 0.2 mg/mL in a 200 μ L volume were mixed with the cell for 2 h at 4°C. Then the cell was washed with PBS twice and incubated with FITC-conjugated goat anti-mouse (diluted with PBA at 1:100, ZSGB) for 30 min at 4°C. After washing twice with PBS, the cells treated with MAb and SP2/0 were analyzed by flow cytometry.⁽¹⁶⁾

Results

Purification of goose serum immunoglobulin as immunogen

SDS-PAGE was used to analyze the purified protein. The bottom of the band was the light chain, which is noted by the

arrow at the location of 26 kDa in Figure 1A. The protein concentration of purified Ig was about 1.5 mg/mL.

Expression and purification of rGoCL

rGoCL was mainly expressed as a soluble form in the bacterial cytosol after induction with 0.8 mM IPTG at 16°C for 24 h; the expressed rGoCL was 20 kDa, as expected. Then rGoCL was purified using a nickel-charged column and the result revealed a single band (Fig. 1B), whose concentration was tested as 0.3 mg/mL.

Production and basic characterization of MAb

After the fusion, approximately 20% (75 hybridoma cultures) of the wells yielded hybridomas. One hybridoma, designated 1B11, was selected following subcloning by limiting dilution until the secreting antibody was stable. Isotyping results classified the MAb as the subclass IgG1 with κ light chain. The number of chromosomes of hybridomas 1B11 ranged from 85 to 95, which is consistent with the chromosome analysis of hybridoma. The ascites produced by hybridoma 1B11 has a high titer (Table 1), which can be used for further study.

Analysis of MAb by Western blot, ELISA, and flow cytometry

Western blot analysis showed that MAb 1B11 has a clear reaction with goose serum and bile at a molecular weight of 26 kDa (Fig. 1C), which is the location of the light chain of goose Ig. It also shows a specific reaction with the thalline of rGoCL at a molecular weight of 20 kDa, which corresponds with the expression analysis of rGoCL. The result of

FIG. 2. Reactivity of MAb 1B11 with serum from different species. The mean of the OD450 nm for each serum sample was used to analyze the histogram. Error bars were generated by the function of STDEV.

FIG. 3. Flow cytometry analysis of the reactivity of MAb 1B11 with B lymphocytes from goose peripheral blood. (A) Flow cytometry analysis of SP2/0 control. (B) Flow cytometry analysis of MAb 1B11 against GoIgCL.

I-ELISA showed that the MAb 1B11 only has reactivity with goose serum, and not with the serum of other animals tested (Fig. 2). Flow cytometry analysis demonstrated that MAb 1B11 can recognize the majority of peripheral blood B lymphocytes; the B lymphocytes that reacted with MAb 1B11 took up about 20.5% in the total lymphocytes isolated from peripheral blood, while SP2/0 had no reactivity with the peripheral blood B lymphocytes (Fig. 3). They both demonstrated that the produced MAb has a high specificity with the light chain of goose Ig.

Discussion

With the development of poultry aquaculture, waterfowl including duck and goose, are important food sources for humans. At the same time, they are the source of infection for pathogenic microorganisms and parasites that can threaten the consumer. Avian influenza, for example, is regarded as a zoonosis that is a threat to human life, and whose host is waterfowl.⁽¹⁷⁾ The natural host of egg drop syndrome, a viral disease that can make hens drop their eggs, is also waterfowl.⁽¹⁸⁾ Therefore, there is an urgent need to develop a reagent with high specificity that can be used for the surveillance of the antibody generated in response to pathogens or vaccines. Moreover, the well developed and robust mouse MAb technology can generally supply the means necessary for investigating goose immune response.

In this study, goose Ig from serum, which was first purified by affinity chromatography with protein A, was used as immunogen, with the character of protein A probably binding goose Ig through unique histidine residues occurring predominantly in the CH1 domain.⁽⁹⁾ Moreover, the recombinant protein containing GoIgCL gene⁽⁷⁾ was expressed for screening the hybridoma-secreted antibody against GoIgCL, which avoids the development of false positives. Then the characterization of the selected hybridoma was analyzed according to the conventional method, and the MAb in ascites were easily purified by affinity chromatography using protein G with the characterization that IgG1 antibody can bind strongly to the bacterial cell wall protein isolated from group G streptococci.⁽¹⁶⁾ The purified antibody was further analyzed by Western blot, ELISA and flow cytometry. In goose serum, there is an abundance of Ig, including IgY, IgY (Δ Fc),

and IgM, while IgA is mainly expressed in goose bile,⁽²⁾ so Western blot and ELISA analyses suggested that the MAb can react with goose Ig from serum and bile at the location of the light chain. The specificity of B cells is a function of the Ig genes that encode antibody molecules, which can bind with anti-Ig antibodies,⁽¹⁹⁾ and the flow cytometry result showed that the MAb can react with goose B lymphocytes expressing Ig on their surface. In other words, the MAb 1B11 can specifically react with the light chain of native goose Ig. As a single Ig molecule uses only one L light chain isotype and the goose Ig has only one type of L chain,^(2,3,7) the MAb prepared here can combine all types of goose Ig. In addition, it has good specificity that can be used as an excellent reagent for diagnosing goose diseases.

In summary, one hybridoma-secreted antibody against GoIgCL was produced and characterized in this study. To our knowledge, it is the first time that the goose species-specific Ig MAb has been obtained, and this specific antibody provides valuable tools for the study of basic goose immunization, as well as the research and development of immunodiagnostics and immunotherapy for goose diseases.

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Author Disclosure Statement

The authors have no financial interests to disclose.

References

- Burton DR: Structure and function of antibodies. *Molecular Genetics of Immunoglobulin*. Elsevier, New York, 1987, pp. 1–55.
- Davison F, Magor K, and Kaspers B: Structure and evolution of avian immunoglobulins. *Avian Immunology*. Elsevier, New York, 2008, pp. 107–128.
- Edholm ES, Wilson M, and Bengten E: Immunoglobulin light (IgL) chains in ectothermic vertebrates. *Dev Comp Immunol* 2011;35:906–915.
- Magor KE, Higgins DA, Middleton DL, and Warr GW: cDNA sequence and organization of the immunoglobulin

- light chain gene of the duck, *Anas platyrhynchos*. *Dev Comp Immunol* 1994;18:523–531.
5. Guo Y, Gao M, Luo X, Sheng Q, Ma B, and Wang J: Molecular cloning and characterization of the α -chain gene of goose immunoglobulin heavy chain. DOI: 10.1007/s10529-013-1415-z, 2013.
 6. Reynaud CA, Dahan A, and Weill JC: Complete sequence of a chicken lambda light chain immunoglobulin derived from the nucleotide sequence of its mRNA. *Proc Natl Acad Sci USA* 1983;80:4099–4103.
 7. Guo Y, Gao M, and Wang J: A novel gene encoding goose immunoglobulin lambda light chain. *Acta Biochim Biophys Sin* 2012;44:805–806.
 8. Chen Y, Niu Q, Su J, Zhao J, and Zhang G: Production and characterization of monoclonal antibodies against chicken secretory IgA. *Hybridoma* 2008;27:375–379.
 9. Higgins DA, Cromie RL, Liu SS, Magor KE, and Warr GW: Purification of duck immunoglobulins: an evaluation of protein A and protein G affinity chromatography. *Vet Immunol Immunopathol* 1995;44:169–180.
 10. Wang J, Guo Y, and Gao M: Preparation of polyclonal antibody specific for light chain of goose immunoglobulin using recombinant protein. *J Northeast Agric Uni* 2012;43:60–63.
 11. Balu S, Rothwell L, and Kaiser P: Production and characterisation of monoclonal antibodies specific for chicken interleukin-12. *Vet Immunol Immunopathol* 2011;140:140–146.
 12. Gao M, Zhang R, Li M, Li S, Cao Y, Ma B, and Wang J: An ELISA based on the repeated foot-and-mouth disease virus 3B epitope peptide can distinguish infected and vaccinated cattle. *Appl Microbiol Biotechnol* 2012;93:1271–1279.
 13. Liu D: Preparation and application of monoclonal antibodies against nonstructural protein 3A and 3B of foot-and-mouth disease virus. *Harbin Northeast Agric Uni* 2011;53.
 14. Meng F, Yin J, Li X, Yang W, Li G, and Ren X: Production and characterization of a monoclonal antibody against spike protein of transmissible gastroenteritis virus. *Hybridoma* 2010;29:345–350.
 15. Chen CH, Abi-Ghanem D, Njongmeta L, Bray J, Mwangi W, Waghela SD, McReynolds JL, Ing NH, and Berghman LR: Production and characterization of agonistic monoclonal antibodies against chicken CD40. *Dev Comp Immunol* 2010;34:1139–1143.
 16. Coligan J, Bierer B, and Margulies D: *Short Protocols in Immunology*. John Wiley & Sons, Hoboken, NJ, 2005.
 17. Garamszegi LZ, and Moller AP: Prevalence of avian influenza and host ecology. *Proc Biol Sci* 2007;274:2003–2012.
 18. Cha SY, Kang M, Moon OK, Park CK, and Jang HK: Respiratory disease due to current egg drop syndrome virus in Pekin ducks. *Vet Microbiol* 2013;165:305–311.
 19. Ratcliffe MJH: *B cells, the bursa of fabricius and the generation of antibody repertoires*. *Avian Immunology*. Elsevier, New York, 2008, pp. 67–89.

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