Development and Characterization of Neutralizing Monoclonal Antibodies Against the S1 Subunit Protein of QX-like Avian Infectious Bronchitis Virus Strain Sczy3

Nianli Zou,¹ Fuyan Wang,¹ Zhenzhen Duan,¹ Jing Xia,¹ Xintian Wen,^{1,2}
Qigui Yan,^{1,2} Ping Liu,¹ Sanjie Cao,^{1,2} and Yong Huang^{1,2}

Infectious bronchitis (IB) is a highly contagious disease in chickens caused by infectious bronchitis virus (IBV). The present study was carried out with the aim to develop anti-spike 1 (S1) subunit monoclonal antibodies (MAbs) that could react with IBV strains of different genotypes. The high antigenicity region of S1 gene of an QX-like IBV strain Sczy3 was amplified and ligated into the prokaryotic expression vector pET-32a(+), and the recombinant His-S1 fusion proteins were expressed and purified. The purified whole viral antigen of Sczy3 strain was used to immunize BALB/c mice to produce hybridoma-secreting anti-IBV MAbs. Eleven anti-IBV MAbs were generated, and two MAbs 1C8 and 2C10 were positive in indirect ELISA against both His-S1 protein and the purified whole viral antigen. These two MAbs showed positive reaction with IBV in Western blot, and the isotype were both IgM. These two MAbs react specifically with IBV but not with Newcastle disease virus (NDV) or avian influenza virus (AIV) subtype H9 or H5, and could cross-react with other 10 IBV strains in five different genotypes. End-point neutralizing assay performed in chicken embryo kidney (CEK) cells revealed that the neutralization titer of 1C8 and 2C10 against Sczy3 reached 1:2.82 and 1:4.70, respectively. The anti-S1 MAbs produced in the present work may be valuable in developing an antigen-capture ELISA test for antigen detection or a competitive ELISA test for antibody detection or therapeutic medicine for IB in poultry.

Introduction

AVIAN INFECTIOUS BRONCHITIS (IB) is a highly contagious respiratory infectious disease hazardous to the poultry industry. It can infect chickens at all ages and replicates in many tissues, causing respiratory symptoms, diarrhea, decline of egg production and quality, etc.^(1,2) Prevention of IB is of economic importance to the poultry industry due to the high morbidity and production losses associated with the disease.⁽³⁾ Although vaccines are now being used widely and extensively, outbreaks of IB still occur frequently, and epidemic IBV strains were mainly of QX-like strains.⁽⁴⁾ It is well known that little or no cross protection occurs between different serotypes of IBV, and new serotypes may appear in the future, complicating the prevention and control of IB.^(5–7)

The etiologic agent of IB is infectious bronchitis virus (IBV), a prototype of the Coronaviridae family, which is an enveloped, positive sense, single stranded RNA virus.⁽⁸⁾ The viral genome is around 27.6 Kb in length, and encodes four structural proteins, nucleocapsid protein (N), membrane

glycoprotein (M), spike glycoprotein (S), and small envelope protein (E).⁽⁹⁾ The S glycoprotein is post-translational cleaved at protease cleavage recognition motifs into the amino-terminal S1 and carboxyl-terminal S2 subunits by cellular protease.^(10,11) The S1 glycoprotein contains epitopes that induce virus-neutralizing, serotype-specific antibodies, hemagglutination inhibition antibodies, and cross-reactivity ELISA antibodies.^(12–16) It also plays an important role in tissue tropism and the degree of virulence of the virus.⁽¹⁷⁾

The development of monoclonal antibodies (MAbs) against coronavirus is critical for improvements in clinical diagnosis.^(18,19) Serological assays such as antigen capture enzyme-linked immune sorbent assay (AcELISA) can be used for antigen detection of clinical samples.⁽¹⁸⁾ Specific MAb against Taiwan IBV strain 2575/98 showed specificity to Taiwan IBV strains but had no cross-reactivity with the vaccine strain H120, and the MAb was used to establish a type-specific blocking ELISA to detect Taiwan IBV infection effectively.⁽¹⁹⁾ Moreover, MAbs are used widely as powerful tools for identifying linear epitopes, or for mimicking the epitopes of infectious agents.^(20,21) For example, two MAbs

¹College of Veterinary Medicine, ²Key Laboratory of Animal Disease and Human Health of Sichuan Province, Sichuan Agricultural University, Ya'an, Sichuan, People's Republic of China.

against nucleocapsid protein of the IBV were used to identify two linear B cell epitopes of N protein by phage display peptide library screening and peptide scanning.⁽²²⁾ In addition, MABs could be used as therapeutic material.⁽²³⁾ For example, a human-mouse chimeric antibody, engineered from MABs against the receptor-binding domain on spike protein of SARS-CoV, displayed high affinity and good neutralizing activity.⁽²⁴⁾

Although S1 subunit is a relatively variable protein of IB with antigenic variations occurring more quickly than that of other structural proteins, such as membrane glycoprotein (M) or nucleocapsid protein (N), there are still relatively conserved regions or epitopes in the S1 subunit, and the S1 subunit anchored to the external surface of viral particles, making it the antigen more easily recognized by IBV-specific antibody than other IBV antigens. It is also important to use the antigen from prevalent strains of IBV for the development of MABs for better sensitivity in subsequent MAB-based diagnostic methods. In the present work, development of MABs against the S1 subunit derived from China isolate of a QX-like IBV strain Sczy3 was targeted for possible use in antigen or antibody detection against different genotypes of IBV. The MABs against S1 would be useful in developing an antigen-capture ELISA test for antigen detection or a competitive ELISA test for antibody detection for IB in poultry.

Materials and Methods

Antigens, cells, and virus

IBV strain Sczy3 was isolated in 2009 from a broiler in Sichuan Province.⁽⁴⁾ The other ten IBV strains from five different genotypes were selected for cross-reaction analysis (Table 1). Newcastle disease virus (NDV) and subtype H9 avian influenza virus (AIV) and subtype H5 AIV antigen were selected for specificity analysis. All viruses were isolated by us before and stored at -70°C , except the H5 AIV antigen was purchased from Qingdao Yebio Bioengineering Co. (Qingdao, China). All viruses were propagated in 9- to 11-day-old SPF embryonated chicken eggs, and the presence of viral particles in the allantoic fluids of inoculated eggs was confirmed by RT-PCR.

Mouse myeloma SP20 cells were purchased from the ATCC (Rockville, MD) and cultured in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS) and maintained at 37°C and 5% CO_2 .

Preparation of whole viral antigen of IBV

IBV Sczy3 strain was propagated in 9- to 11-day-old specific pathogen-free (SPF) embryonated chicken eggs, and the allantoic fluids of inoculated eggs were harvested after 48 h incubation and the virus particles were further purified using differential velocity centrifugation. The allantoic fluids were first centrifuged at 12000 g for 30 min at 4°C ; then the supernatant fluid was centrifuged at 60000 g for 2 h at 4°C and the precipitate was resuspended with 0.01 M phosphate-buffered saline (PBS, pH 7.2).

Construction of recombinant prokaryotic expression vector with S1 gene

Viral RNA was extracted with Trizol Reagent (Tiangen Beijing Co., Beijing, China) and the cDNA was performed by RT-PCR with the PrimeScript™ One Step RT-PCR Kit (Takara Biotechnology, Dalian, China) as described by the manufacturer. The S1 sequence was amplified from Sczy3 genomic cDNA by PCR using forward primer S1F (5'-GGATCCATTGCACGTGATCATATTCG-3'; BamH1 site underlined) and reverse primer S1R (5'-CTCGAGTCATTAATACTAAGGGCTCCGT-3'; XhoI site underlined and termination codon italicized). The amplified S1 gene was ligated with the prokaryotic expression vector pET-32a (+) by the T4 DNA ligase (Takara Biotechnology) and transformed into *Escherichia coli* BL21 (DE3) competent cell. Confirmation of clones containing recombinant plasmid was achieved by PCR and restriction enzyme (RE) digestion. The recombinant plasmid was sequenced by Shanghai Sang-gong Biological Engineering Technology & Services (Shanghai, China).

Expression, purification, and identification of recombinant S1 subunit protein

The BL21 strains containing the recombinant prokaryotic plasmid were grown overnight with shaking at 37°C . The freshly 300 mL prepared LB broth was inoculated with 3 mL of overnight culture and incubated at 37°C with vigorous shaking for 3–4 h. The culture was induced by adding 1 mM IPTG and incubated for a further 4 h at 37°C . The cells were harvested and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). For extraction and purification of the inclusion body protein from

TABLE 1. BACKGROUND INFORMATION OF IBV STRAINS USED IN PRESENT STUDY

IBV strain	Country ^a	Year ^b	Type	Genbank accession no.
CK/CH/EM/09I	China	2009	LX4	GU384207
CK/CH/SCMS/10I	China	2010	LX4	HM106334
CK/CH/SCYA/10I	China	2010	Proventriculus	HM363027
CK/CH/SCMY/10I	China	2010	TW-I	HM363028
4/91	Vaccine	1992	4/91	AF093794
W93	Vaccine	–	Mass	AY842862
M41	Vaccine	1965	Mass	DQ834384
28/86	Vaccine	–	Mass	AY846750
Ma5	Vaccine	–	Mass	AY561713
H120	Vaccine	–	Mass	EU822341

^aCountry where the viruses were isolated.

^bYear of virus isolation.

the induced culture, the cells were harvested from liquid cultures by centrifugation and suspended in PBS (pH 7.2) containing 1 mg/mL lysozyme (1 mL/10 mL of the liquid cultures). After ice bathing for 30 min, the suspension was sonicated and centrifuged at 8000 rpm at 4°C for 10 min. The supernatant was then loaded into HisTrap FF crude Columns (GE Healthcare Life Sciences, Piscataway, NJ) with binding buffer (5 mM imidazole, 500 mM NaCl, 8 M urea, and 20 mM Tris-HCl [pH 7.9]). After washing, the recombinant proteins were eluted with imidazole buffer solution (250 mM imidazole, 500 mM NaCl, 8 M urea, and 20 mM Tris-HCl [pH 7.9]). The pooled fraction containing the recombinant proteins were successively dialyzed against PBS with 6 M urea, 4 M urea, 2 M urea, and 0 M urea. Each of the concentrations was dialyzed for 6 h. The purified recombinant S1 protein was analyzed by SDS-PAGE and characterized by Western blot with mice anti-serum against IBV Sczy3 (1:40) as the primary antibody and anti-mouse IgG horseradish peroxidase (HRP) conjugate (1:5000 diluted; Sigma, St. Louis, MO) as the secondary antibody.

Production of monoclonal antibodies to IBV whole viral antigen and recombinant S1 protein

Special pathogen-free 6- to 8-week-old female BALB/c mice were purchased from Chengdu Dossy Biological Technology (Chengdu, China). Each of five mice was immunized subcutaneously with the purified whole viral antigen of Sczy3 strain mixed with complete Freund's adjuvant (CFA), 0.1 g per mouse. At two-week intervals, two boosters of 0.15 g purified whole viral antigen virus in incomplete Freund's adjuvant (IFA) in a total volume of 200 μ L were administered. Seven days later, the mice received a final booster immunization with 0.05 g purified virus. Three days later, the splenocytes were collected from immunized mice and fused with SP2/0 myeloma cells (5:1) in the presence of polyethylene glycol (PEG 1500; Sigma Chemical). The cell fusion mix was then seeded in 96-well plates with peritoneal macrophages from non-immunized Kunming mice and cultured in DMEM medium contained with 10% FBS and 1 $\times$ hypoxanthine-aminopterin-thymidine (HAT; Sigma) for 7–12 days. The hybridoma culture supernatants of primary clones were screened by whole viral antigen-based indirect ELISA, and hybridoma cells from wells with a positive signal were further subcloned by limiting dilution for at least three rounds and screened by indirect ELISA.

The culture supernatants of hybridoma were checked for positive clones by whole viral antigen-based indirect ELISA. Briefly, 96-well microtiter plates (Nunc, Thermo Scientific, Roskilde, Denmark) were coated with 100 μ L/wells of purified whole viral antigen (1:40 dilution of stock antigen prepared as standardized in checkerboard titration) in coating buffer (0.05 M carbonate buffer, pH 9.6) and incubated overnight at 4°C. The antigen solution was discarded and the wells were washed three times with PBS containing 0.05% Tween-20 (PBST). The wells were then blocked with 100 μ L of 5% skim milk in PBS at 37°C for 2 h and washed three times with PBST. One hundred μ L of culture supernatant from each well of the hybridoma plate were transferred to a corresponding well in the ELISA plate. After incubation at 37°C for 1 h, the ELISA plate was washed with PBST three times and 100 μ L/well of anti-mouse horseradish peroxidase

(HRP) conjugate IgG (Sigma; diluted to 1:5000) were added. The plate was incubated at 37°C for 1 h. After washing three times with PBST, the color reaction was developed by adding 100 μ L/well of ready-to-use 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma). The plates were kept in the dark at room temperature for 15 min followed by the addition of 50 μ L/well of 2 M H₂SO₄ to stop the reaction. The plate was read at 450 nm in ELISA reader (Bio-Rad, Shanghai, China), and the wells with OD greater than the cut-off value (three times the mean OD of negative control wells) were considered positive for the presence of anti-IBV antibodies. The cultures that tested positive in whole antigen ELISA were used for further subcloning. After three rounds of subcloning, the positive hybridoma clones were further tested by recombinant S1-based ELISA with recombinant S1 protein as the coating antigen (2 μ g/wells as standardized in checkerboard titration).

Characterization of monoclonal antibodies against recombinant S1 protein

The MABs reacting positively with both whole viral antigen and recombinant S1 protein in indirect ELISA (hereinafter referred to as anti-S1 MABs) were used for further characterization. The hybridomas secreting S1 MABs were intraperitoneally injected into the 10-week-old BALB/c female mice with 1 $\times$ 10⁶ hybridoma cells per mouse. The ascites fluids were collected when the mice were on the verge of death, and the ascitic fluid containing the MABs was purified by Protein L Resin (Genscript, Nanjing, China) according to the manufacturer's instructions. The isotype of purified anti-S1 MABs were determined by antigen-mediated ELISA using a mouse monoclonal antibody isotyping reagents kit (Sigma) according to the manufacturer's instructions. The specificity and cross-reaction of anti-S1 MABs were analyzed by indirect ELISA using the method described above by using other avian viruses (NDV, AIV H5, and AIV H9) and other genotypes of IBVs (shown in Table 1) as coating antigen. It was noteworthy that the viruses were coated in 96-well plates with 1 μ g/well overnight at 4°C respectively, and whole viral antigen of Sczy3, uninfected allantoic fluid and BSA was coated in the same conditions as the positive, negative and blank control, respectively.

For Western blot analysis of purified anti-S1 MABs, the purified whole viral antigen or the His-S1 fusion protein were separated by 12% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) using a mini trans-blot system (Bio-Rad) respectively. The membranes were blocked with 5% skim milk in TBS at 37°C for 2 h. After washing three times with Tris-buffer saline (TBS) containing 0.05% Tween-20 (TBST), the membranes were incubated with anti-S1 MABs at 37°C for 1 h. Following three washes with TBST, the membranes were incubated with 1:5000 diluted goat anti-mouse IgG peroxidase conjugate (Sigma) for 1 h, followed by development of color reaction by DAB (3,3'-diaminobenzidine, Sigma).

Virus neutralization of anti-S1 MABs

IBV Sczy3 strain was passaged in 9- to 11-day-old SPF embryonated chicken eggs for six generations, and then 1:100 dilution of the allantoic fluid containing Sczy3 of the sixth generation was inoculated into CEK monolayer cells to

cultivate the cell-adapted strain. Monolayers of primary CEK cells were prepared from the kidneys of 18-day-old chicken embryos. The kidney cells were dispersed with 0.25% Trypsin, washed three times, and seeded in 24-well plates (Corning Inc., Corning, NY). Cell cultures were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO₂. The allantoic fluids containing IBV-Sczy3 strain was used to infect CEK cells at a confluency of 70–80%, and the culture medium was replaced by DMEM supplemented with 2% FBS. The supernatant was harvested 40 h post-inoculation and passaged in CEK cells for 20 generations until the characteristic cytopathic effect (CPE) such as syncytia was observed. The cell-adapted stock virus was stored at –70°C.

To determine the TCID₅₀ of the 20th generation of Sczy3 virus in CEK cells, the virus was serially diluted 10-fold with DMEM and was inoculated into 24-well plates (six wells for each dilutions) and maintained at 37°C with 5% CO₂ for 1 h. Then, 200 µL of DMEM containing 2% FBS were added to each well and the plates were incubated for 5 days. Three wells with no virus inoculation were taken in the same addition as blank control. The dilution of the cell-adapted stock virus that causes CPE in half of the cultures (TCID₅₀/mL) was calculated by the method of Reed and Muench.

For the end-point neutralizing assay of the anti-S1 MABs, equal volumes of 100 TCID₅₀ of the 20th generations of

Sczy3 and serial 2-fold dilutions of purified anti-S1 MABs were mixed and kept at 37°C for 1 h. 0.2 mL of the virus-antibody mix and 0.2 mL of DMEM with 2% FBS were then transferred into CEK cell cultures in 24-well plates (six wells for each dilutions). Sczy3 of 100 TCID₅₀ and the MABs of 1:2 dilution were also taken in the same addition solely as virus and antibody controls respectively. The plates were incubated until CPE developed in the virus control group, and the 50% end-point neutralizing titers were calculated by the method of Reed and Muench.

Results

The expression fragment about 882 bp long of the S1 gene was amplified by RT-PCR from the virus total RNA and cloned into pET-32a (+) in frame with the poly-His purification signal. PCR and RE analysis showed that prokaryotic expression plasmid pET-32a-S1 was successfully constructed (data not shown). The recombinant S1 subunit protein was expressed in BL21 strains, extracted, and purified and could react with anti-Sczy3 mouse polyclonal serum in Western blot. The size of the recombinant fusion protein was estimated to be approximately 46.0 kDa including the amino acids in the vector (Fig. 1).

After being immunized with purified whole viral antigen of Sczy3, the antiserum titer of immunized BALB/c mice before hybridoma fusion could reach 1:25,600 in whole viral

FIG. 1. Western blot (A) and SDS-PAGE (B) analysis of recombinant fusion protein pET-S1. M, protein standards (Bio-Rad); lane 1, Western blot analysis of purified recombinant protein with mice antisera against IBV; lane 2, Western blot analysis of His protein with mice antisera against IBV; lane 3, non-IPTG-induced cell lysate; lane 4, IPTG-induced cell lysate; lane 5, purified fusion protein pET-S1. In the Western blot analysis, the recombinant fusion protein pET-S1 and His protein were separated with 12% polyacrylamide. Western blot utilized mice anti-serum against IBV Sczy3 followed by anti-mouse IgG peroxidase conjugate (Sigma).

FIG. 2. Western blot analysis of MAbS (A, 2C10; B, 1C8). M, protein standards (Bio-Rad); lane 1, MAbS against purified Sczy3; lane 2, MAbS against purified fusion protein His-S1.

antigen-based indirect ELISA. The spleens were then used in hybridoma fusion to generate MAbS. After three rounds of subcloning of the primary positive clones, 11 MAbS positive in whole viral antigen-based indirect ELISA were generated, and only two, MAbS 1C8 and 2C10, were also positive in His-S1 protein-based indirect ELISA. The OD₄₅₀ of MAbS 1C8 and 2C10 could reach 0.689 and 0.710, respectively. MAbS 1C8 and 2C10 could react specifically with the recombinant His-S1 protein and whole viral antigen in Western blot (Fig. 2). Isotyping analysis showed that MAbS 1C8 and 2C10 were both of IgM isotype.

Specificity analysis showed that both MAbS 1C8 and 2C10 did not react with NDV or AIV subtypes H9 and H5 (data not shown). Cross-reaction analysis showed that both MAbS 1C8 and 2C10 could react with other 10 IBVs classified into five genotypes (Fig. 3).

After being passaged in 9- to 11-day-old SPF embryonated chicken eggs for six generations and then serially passaged in CEK cultures, specific CPE (syncytium) of IBV infection could be observed at 18 h post-infection of the fifth generation of CEK-adapted Sczy3 in the CEK monolayer cells (Fig. 4), suggesting that the CEK-adapted strain of Sczy3 was

FIG. 3. Cross-reaction analysis of MAbS with IBV strains of different genotypes.

FIG. 4. Specific cytopathic effect (CPE) of IBV (syncytia). (A) Normal CEK cells maintained at 37°C and 5% CO₂ for 14 h. (B) CEK cells were infected with 20th generation of Sczy3 at 37°C with 5% CO₂ for 14 h. Syncytia were observed (arrows).

generated successfully. As the passaging continues, the time when syncytia appeared became earlier, and more and more syncytia were observed. The syncytia could be observed about 12 h post-infection of the 20th generation of Sczy3 strain (data not shown). The TCID₅₀ of the 20th generation of Sczy3 strain was calculated as 10 – 5.64/0.2 mL (data not shown). End-point neutralizing assay of anti-S1 MAbs showed that the neutralization titers of 1C8 and 2C10 were 1:2.82 and 2C10, respectively (Table 2).

Discussion

S1 subunit protein is a main structural protein of IBV, although it is highly variable.⁽²⁵⁾ The previous studies demonstrated that it was an excellent candidate for developing a subunit vaccine against IB,⁽²⁶⁾ and S1 subunit is used as a marker for the identification, differentiation, and classification of IBV.^(27–29) The virus strain Sczy3 used in the present study was isolated in 2009 from a broiler in Sichuan

TABLE 2. RESULT OF END-POINT NEUTRALIZING ASSAY OF MAbs

MAbs	Dilutions	CPE wells	Non-CPE wells	Accumulated CPE wells	Accumulated non-CPE wells	Total wells	CPE wells (%)
1C8	1:2	1	5	1	9	10	10 (1/10)
	1:4	3	3	4	4	8	50 (4/8)
	1:8	5	1	9	1	10	90 (9/10)
	1:16	6	0	15	0	15	100 (15/15)
2C10	1:2	1	5	1	10	11	9 (1/11)
	1:4	2	4	3	5	8	37.5 (3/8)
	1:8	5	1	8	1	9	88.9 (8/9)
	1:16	6	0	14	0	14	100 (14/14)

1C8:

$$\text{PD} = [50\% - (\% \text{ next below } 50\%)] / [(\% \text{ next above } 50\%) - (\% \text{ next below } 50\%)] \\ = (50\% - 10\%) / (90\% - 10\%) = 0.5$$

$$\log (\text{dilution next above } 50\%) + \text{PD} \times \log (\text{dilution multiple}) = -0.3 + 0.5 \times (-0.3) = -0.45 \\ \text{Neutralization index} = 1:10^{-0.45} = 1:2.82.$$

2C10:

$$\text{PD} = [50\% - (\% \text{ next below } 50\%)] / [(\% \text{ next above } 50\%) - (\% \text{ next below } 50\%)] \\ = (50\% - 37.5\%) / (88.9\% - 37.5\%) = 0.24$$

$$\log (\text{dilution next above } 50\%) + \text{PD} \times \log (\text{dilution multiple}) = -0.6 + 0.24 \times (-0.3) = -0.672 \\ \text{Neutralization index} = 1:10^{-0.672} = 1:4.70.$$

Province, it was classified to QX genotype, and it was the prevalent genotype in Sichuan province and world-wide.^(4,7,30,31) Recombinant analysis of the Sczy3 genome demonstrated that Sczy3 is a chimeric strain derived from QX and H120.⁽³²⁾

Monoclonal antibodies are a powerful tool for virus detection and characterization. In recent years, a large number of MAbs have been developed for diagnosing diseases found in coronavirus. It is well known that it is very difficult to obtain the full-length S1 subunit from the purified IBV virions, and some expression systems are used to express it.^(33–35) Most mouse MAbs raised from the IBV virions were mainly directed against the N protein of IBV; only a few MAbs were specific for the S1 subunit.⁽³⁶⁾ In the present report, a highly antigenic and hydrophilic region of the S1 protein pET-S1 was expressed and used to screen the hybridoma clones specific for S1 protein. Out of 11 hybridoma clones positive in whole viral antigen-based indirect ELISA, only two MAbs (1C8 and 2C10) were positive in recombinant S1 protein-based indirect ELISA, which also had a high intensity positive reaction with IBV in Western blot. The cross-reaction test showed that these two MAbs were both group-specific antibodies as they could react with five different genotype IBVs, indicating that these MAbs could be used for the establishment of diagnostic assays such as IFA and AC-ELISA against IBVs. Moreover, these MAbs had the ability to neutralize the infection of Sczy3 strain in primary CEK cells, demonstrating that these MAbs had potential for immunotherapy. In recent years, studies also focused on mapping the antigenic regions in S1 subunit, and these MAbs could also be used for identifying the neutralization linear epitopes of S1 protein and characterize the role of neutralization linear epitopes in the IBV infection in further studies.

In conclusion, the present report describes the generation of two mouse MAbs specific to S1 protein of IBV. These MAbs were group-specific, and could be used for the establishment of diagnostic assays against IBVs. They also provide new material for the immunotherapy of IB and identification of linear neutralization epitopes on S1 protein due to their ability to neutralize the IBV infection *in vivo*.

Acknowledgment

This work was financially supported by the Program for Changjiang Scholars and Innovative Research Team in University “PCSIRT” (grant no. IRT0848).

Author Disclosure Statement

The authors have no financial interests to disclose.

References

1. Cavanagh D: Coronavirus avian infectious bronchitis virus. *Vet Res* 2007;38(2):281–297.
2. Abd ERS, El-Kenawy AA, Neumann U, Herrler G, and Winter C: Comparative analysis of the sialic acid binding activity and the tropism for the respiratory epithelium of four different strains of avian infectious bronchitis virus. *Avian Pathol* 2009;38(1):41–45.
3. Cavanagh D: Coronaviruses in poultry and other birds. *Avian Pathol* 2005;34(6):439–448.
4. Zou NL, Zhao FF, Wang YP, Liu P, Cao SJ, Wen XT, and Huang Y: Genetic analysis revealed LX4 genotype strains of avian infectious bronchitis virus became predominant in recent years in Sichuan area, China. *Virus Genes* 2010;41(2):202–209.
5. Li L, Xue C, Chen F, Qin J, Xie Q, Bi Y, and Cao Y: Isolation and genetic analysis revealed no predominant new strains of avian infectious bronchitis virus circulating in South China during 2004–2008. *Vet Microbiol* 2010;143(2–4):145–154.
6. Mahmood ZH, Sleman RR, and Uthman AU: Isolation and molecular characterization of Sul/01/09 avian infectious bronchitis virus, indicates the emergence of a new genotype in the Middle East. *Vet Microbiol* 2011;150(1–2):21–27.
7. Worthington KJ, Currie RJ, and Jones RC: A reverse transcriptase-polymerase chain reaction survey of infectious bronchitis virus genotypes in Western Europe from 2002 to 2006. *Avian Pathol* 2008;37(3):247–257.
8. Boursnell ME, Brown TD, Foulds IJ, Green PF, Tomley FM, and Binns MM: Completion of the sequence of the genome of the coronavirus avian infectious bronchitis virus. *J Gen Virol* 1987;68(Pt 1):57–77.
9. Lai MM, Brayton PR, Armen RC, Patton CD, Pugh C, and Stohlman SA: Mouse hepatitis virus A59: mRNA structure and genetic localization of the sequence divergence from hepatotropic strain MHV-3. *J Virol* 1981;39(3):823–834.
10. Jackwood MW, Hilt DA, Callison SA, Lee CW, Plaza H, and Wade E: Spike glycoprotein cleavage recognition site analysis of infectious bronchitis virus. *Avian Dis* 2001;45(2):366–372.
11. Cavanagh D, Davis PJ, Darbyshire JH, and Peters RW: Coronavirus IBV: virus retaining spike glycopolypeptide S2 but not S1 is unable to induce virus-neutralizing or haemagglutination-inhibiting antibody, or induce chicken tracheal protection. *J Gen Virol* 1986;67(Pt 7):1435–1442.
12. Niesters H G, Kusters JG, Lenstra JA, Spaan WJ, Horzined MC, and van der Zeijst BA: The neutralization epitopes on the spike protein of infectious bronchitis virus and their antigenic variation. *Adv Exp Med Biol* 1987;218:483–492.
13. Cavanagh D, Elus MM, and Cook JK: Relationship between sequence variation in the S1 spike protein of infectious bronchitis virus and the extent of cross-protection *in vivo*. *Avian Pathol* 1997;26(1):63–74.
14. Wang YF, Sun YK, Tian ZC, Shi XM, Tong GZ, Liu SW, Zhi HD, Kong XG, and Wang M: Protection of chickens against infectious bronchitis by a recombinant fowlpox virus co-expressing IBV-S1 and chicken IFN γ . *Vaccine* 2009;27(50):7046–7052.
15. Zhang J, Chen XW, Tong TZ, Ye Y, Liao M, and Fan HY: BacMam virus-based surface display of the infectious bronchitis virus (IBV) S1 glycoprotein confers strong protection against virulent IBV challenge in chickens. *Vaccine* 2014;32(6):664–670.
16. Promkuntod N, van Eijndhoven RE, de Vrieze G, Grone A, and Verheije MH: Mapping of the receptor-binding domain and amino acids critical for attachment in the spike protein of avian coronavirus infectious bronchitis virus. *Virology* 2014;448:26–32.
17. Casais R, Dove B, Cavanagh D, and Britton P: Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. *J Virol* 2003;77(16):9084–9089.
18. He Q, Du Q, Lau S, Manopo I, Lu L, Chan SW, Fenner BJ, and Kwang J: Characterization of monoclonal antibody against SARS coronavirus nucleocapsid antigen and

- development of an antigen capture ELISA. *J Virol Methods* 2005;127(1):46–53.
19. Chen HW, Wang CH, and Cheng IC: A type-specific blocking ELISA for the detection of infectious bronchitis virus antibody. *J Virol Methods* 2011;173(1):7–12.
 20. Yu D, Han Z, Xu J, Shao Y, Li H, Kong X, and Liu S: A novel B-cell epitope of avian infectious bronchitis virus N protein. *Viral Immunol* 2010;23(2):189–199.
 21. Hu JQ, Li YF, Guo JQ, Shen HG, Zhang DY, and Zhou JY: Production and characterization of monoclonal antibodies to (poly100) S1 protein of avian infectious bronchitis virus. *Zoonoses Public Health* 2007;54(2):69–77.
 22. Han Z, Zhao F, Shao Y, Liu X, Kong X, Song Y, and Liu S: Fine level epitope mapping and conservation analysis of two novel linear B-cell epitopes of the avian infectious bronchitis coronavirus nucleocapsid protein. *Virus Res* 2013; 171(1):54–64.
 23. Coughlin MM, Babcook J, and Prabhakar BS: Human monoclonal antibodies to SARS-coronavirus inhibit infection by different mechanisms. *Virology* 2009;394(1): 39–46.
 24. Bian C, Zhang X, Cai X, Zhang L, Chen Z, Zha Y, Xu Y, Xu K, Lu W, Yan L, Yuan J, Feng J, Hao P, Wang Q, Zhao G, Liu G, Zhu X, Shen H, Zheng B, Shen B, and Sun B: Conserved amino acids W423 and N424 in receptor-binding domain of SARS-CoV are potential targets for therapeutic monoclonal antibody. *Virology* 2009;383(1): 39–46.
 25. Cavanagh D, Davis PJ, Cook JK, Li D, Kant A, and Koch G: Location of the amino acid differences in the S1 spike glycoprotein subunit of closely related serotypes of infectious bronchitis virus. *Avian Pathol* 1992;21(1):33–43.
 26. Tan B, Wang H, Shang L, and Yang T: Coadministration of chicken GM-CSF with a DNA vaccine expressing infectious bronchitis virus (IBV) S1 glycoprotein enhances the specific immune response and protects against IBV infection. *Arch Virol* 2009;154(7):1117–1124.
 27. Jones RM, Ellis RJ, Cox WJ, Errington J, Fuller C, Irvine RM, and Wakeley PR: Development and validation of RT-PCR tests for the detection and S1 genotyping of infectious bronchitis virus and other closely related gammacoronaviruses within clinical samples. *Transbound Emerg Dis* 2011;58(5): 411–420.
 28. Meir R, Maharat O, Farnushi Y, and Simanov L: Development of a real-time TaqMan RT-PCR assay for the detection of infectious bronchitis virus in chickens, and comparison of RT-PCR and virus isolation. *J Virol Methods* 2010;163(2):190–194.
 29. Wang CH, Hong CC, and Seak JC: An ELISA for antibodies against infectious bronchitis virus using an S1 spike polypeptide. *Vet Microbiol* 2002;85(4):333–342.
 30. Gough RE, Cox WJ, de B WD, Worthington KJ, and Jones RC: Chinese QX strain of infectious bronchitis virus isolated in the UK. *Vet Rec* 2008;162(3):99–100.
 31. Sigrist B, Tobler K, Schybli M, Konrad L, Stockli R, Cattoli G, Luschow D, Hafez HM, Britton P, Hoop RK, and Vogtlin A: Detection of Avian coronavirus infectious bronchitis virus type QX infection in Switzerland. *J Vet Diagn Invest* 2012;24(6):1180–1183.
 32. Zhao F, Zou N, Wang F, Guo M, Liu P, Wen X, Cao S, and Huang Y: Analysis of a QX-like avian infectious bronchitis virus genome identified recombination in the region containing the ORF 5a, ORF 5b, and nucleocapsid protein gene sequences. *Virus Genes* 2013;46(3):454–464.
 33. Zhang X, Wu Y, Huang Y, and Liu X: Protection conferred by a recombinant Marek's disease virus that expresses the spike protein from infectious bronchitis virus in specific pathogen-free chicken. *Virol J* 2012;9:85.
 34. Armesto M, Evans S, Cavanagh D, Abu-Median AB, Keep S, and Britton P: A recombinant avian infectious bronchitis virus expressing a heterologous spike gene belonging to the 4/91 serotype. *PLoS One* 2011;6(8):e24352.
 35. Zeshan B, Mushtaq MH, Wang X, Li W, and Jiang P: Protective immune responses induced by in ovo immunization with recombinant adenoviruses expressing spike (S1) glycoprotein of infectious bronchitis virus fused/co-administered with granulocyte-macrophage colony stimulating factor. *Vet Microbiol* 2011;148(1):8–17.
 36. Ignjatovic J, and McWaters PG: Monoclonal antibodies to three structural proteins of avian infectious bronchitis virus: characterization of epitopes and antigenic differentiation of Australian strains. *J Gen Virol* 1991;72(Pt 12):2915–2922.

Address correspondence to:

Yong Huang
College of Veterinary Medicine
Sichuan Agricultural University
46# Xinkang Road
Ya'an, Sichuan 625014
P.R. China

E-mail: hyong601@163.com

Received: October 12, 2014

Accepted: December 8, 2014