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Decreased neutralizing antigenicity in IBV S1 protein expressed from mammalian cells



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ABSTRACT

We evaluated the antigenicity of recombinant infectious bronchitis virus (IBV) S1 protein expressed in mammalian cells. Recombinant S1 was expressed as a secreted protein fused with a trimerization motif peptide, then purified using Ni Sepharose. The purified protein was analyzed by Western blotting, mixed with oil adjuvant, and administered to 29-day-old specific-pathogen-free chickens. Six weeks after immunization, anti-IBV neutralizing titer and anti-S1 ELISA titer were determined; immunized chickens then were inoculated with IBV via the trachea and ciliary activity was observed. Results showed that the recombinant S1 protein was highly glycosylated, and the neutralizing antigenicity of recombinant S1 protein was lower than that of inactivated virus. However, anti-S1 ELISA indicated that the recombinant S1 protein induced antibodies against S1. These results suggest that the recombinant S1 may retain non-neutralizing epitopes but have unnatural glycosylation pattern and conformation, resulting in lacking neutralizing conformational epitopes. In conclusion, the neutralizing antigenicity of recombinant S1 protein expressed from mammalian cells was decreased, and was not sufficient to induce neutralizing antibodies.

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1. Introduction

Infectious bronchitis virus (IBV) belongs to the order *Nidovirales*, family *Coronaviridae*, genus *Gammacoronavirus*, and causes respiratory disease and pathology in the kidney and gonads of chickens and other birds (Boltz et al., 2004; Cavanagh, 2007). IBV is an economically important disease in the poultry industry, and several vaccines have been used to prevent the spread of IBV. IBV shows extensive antigenic variation, reflecting mutation of the spike protein gene (Cavanagh et al., 1988, 1997; Wang and Huang, 2000). Vaccines targeting individual IBV serotypes yield poor cross-protection; therefore, various attenuated and inactivated multivalent vaccines (derived from several different serotypes) are used (Deguchi et al., 1998; Sjaak de Wit et al., 2011).

The spike protein (S) is an envelope glycoprotein that forms a dimer or trimer, and has been shown to play an important role in viral infection (Cavanagh et al., 1986; Ignjatovic and Galli, 1994;

Wickramasinghe et al., 2011). S is highly glycosylated, and based on its amino acid sequence, the spike protein is predicted to contain 21 to 35 N-glycosylation sites. S has two main functions: to attach the virus to the host cell receptor, and to activate fusion of the virion membrane with the host cell membrane (Casais et al., 2003; Wickramasinghe et al., 2011). The S protein is the most important antigen in inducing neutralizing antibodies against IBV, and the N-terminal S1 region is especially important (Cavanagh et al., 1986; Ignjatovic and Galli, 1994; Kant et al., 1992; Koch et al., 1990; Promkuntod et al., 2014). The S1 domain forms the bulbous head of the spike protein, and several virus neutralization (VN) epitopes have been reported to reside within the first and third quarter of the S1 sequence (Cavanagh et al., 1988; Kant et al., 1992; Koch et al., 1990; Sjaak de Wit et al., 2011). Thus, analysis of the antigenicity of the S1 domain is expected to be critical to the development of effective anti-IBV vaccines.

To prevent IBV infection, several recombinant subunit vaccine developments have been attempted using the recombinant S1 protein or other proteins. Immunization with recombinant S1 protein expressed from baculovirus has been shown to provide in chicken effective protection against IBV infection (Song et al., 1998). Other groups also have reported that immunization with recombinant S1

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epitope peptide expressed from *Escherichia coli* (*E. coli*) protected chickens against IBV infection (Yang et al., 2009a, 2009b). Furthermore, viral vectored vaccines co-expressing the S1 protein and host cytokines have been reported to induce anti-S1 antibodies (Chen et al., 2010; Shi et al., 2011; Tomley et al., 1987; Wang et al., 2009; Zeshan et al., 2011; Zhang et al., 2012). However, in these reports of protection, recombinant antigen did not provide perfect protection and antibody titers were evaluated only by hemagglutination inhibition (HI) or enzyme-linked immunosorbent assay (ELISA); the respective authors did not indicate whether these antigens retained their native neutralizing antigenicity (Song et al., 1998; Yang et al., 2009a, 2009b). Therefore, it remains unknown whether recombinant S1 protein, without the S2 domain, completely retains its conformational epitopes and neutralizing antigenicity.

To address these issues, we analyzed the recombinant S1 expressed from mammalian and avian cells by Western blotting and analyzed the neutralizing antigenicity of recombinant S1 protein and compared titers against those of inactivated virus antigen. For the immunization experiment, recombinant S1 protein was expressed as the secreted protein with a trimerization motif because some researchers have reported that secreted recombinant S1 protein, when expressed fused to the trimerization motif peptide, retains the ability to bind the cell receptors (Promkuntod et al., 2013; Wickramasinghe et al., 2011) and it seemed to be suited to the vaccine antigen.

2. Materials and methods

2.1. Viruses and cells

The present work employed the TM86 IBV strain, an isolate of genotype JP-II that originally was recovered from a field chicken; this strain subsequently has been used as a vaccine strain (Ariyoshi et al., 2010; Mase et al., 2004). TM86 was propagated in specific-pathogen-free (SPF) chicken embryonated eggs. IBV TM86 adapted to chicken kidney (CK) cells was used for the VN test and ELISA of the antigen. CK cells and chicken embryo fibroblast (CEF) cells were incubated in Eagle's medium (EMEM) supplemented with 10% tryptose phosphate broth and 5% heat-inactivated fetal bovine serum (FBS; Hyclone), along with 100 units of penicillin and 100 µg of streptomycin per mL, and cells were grown at 37 °C in 5% CO₂ incubators. 293T cells and 293 cells lacking *N*-acetylglucosaminyltransferase I (293 GnT1⁻, Reeves et al., 2002) were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS along with 100 units penicillin and 100 µg of streptomycin per mL, and cells were grown at 37 °C in 5% CO₂ incubators.

2.2. Construction of the expression plasmid

A segment of the S1-encoding gene, coding for the protein from the N-terminus to the cleavage site between S1 and S2, was amplified from the *spike* gene of IBV strain TM86 (Accession No. AB120655) by reverse transcriptase polymerase chain reaction (RT-PCR). The primer set used for RT-PCR was 5'-CAAATTATTGGTCAGAGATGTTGG-3' (S1.1) and 5'-GAATCATTAACAGACTTTTGGTCT-3' (S2R1). After amplification, the DNA encoding the signal sequence of S1 (MLVKSFLVTLFALCS) was replaced with a DNA sequence encoding the signal sequence of Marek's disease virus (MDV)-glycoprotein A (gA) (MLTPRVLRALGWTLGFLFLLSPSNVL). Furthermore, DNA sequences encoding a 6× His-tag peptide sequence, with or without those encoding a T4 phage fibrin coiled-coil trimerization motif (GSGYIPEAPRDGQAYVRKDGWVLLSTFLG), were inserted in-phase and downstream of the recombinant S1-encoding gene. The resulting

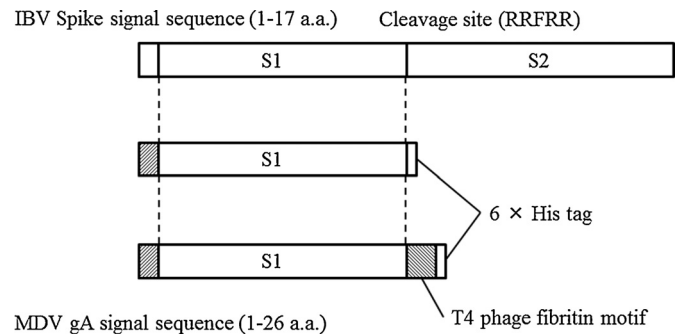


Fig. 1. Schematics of the IBV S1 segment expressed as a secreted protein. To construct the secreted S1 expression plasmid, the nucleotide sequence encoding the S1 domain lacking the cleavage sequence (RRFRR) was amplified from the IBV TM86 genome. To increase expression levels of the recombinant S1 protein, the nucleotide sequence encoding the signal sequence was replaced with a nucleotide sequence encoding MDV gA. To facilitate purification and multimerization, the resulting sequence was cloned upstream and in-phase with nucleotide sequences permitting expression as C-terminal fusions to a 6× His-tag peptide sequence with or without a T4 phage fibrin coiled-coil trimerization motif.

loci encoded the recombinant S1 protein with C-terminal fusions to the indicated domains (Fig. 1). The constructed sequence was cloned into the *Sal*I site of the expression plasmid pCAGGS, which contains a CAG promoter (modified chicken β -actin promoter with cytomegalovirus immediate-early (CMV-IE) enhancer), rabbit β -globin gene sequence including polyadenylation signal, and simian virus 40 (SV40) *ori* (Niwa et al., 1991). The constructed plasmids were named pCAGGS-S1-T4-His and pCAGGS-S1-His, respectively. For transfection, the plasmid was purified using a QIAprep Spin Miniprep Kit (QIAGEN).

2.3. Mouse monoclonal antibody (mAb)

The mouse mAbs against IBV S1 or S2 region were established in our institute. It was confirmed that these mAbs recognize non-conformational epitopes by Western blotting under denaturing condition. It was also confirmed that these mAbs do not show neutralizing activity in VN test. Before examination, the mAbs were purified using protein G column.

2.4. Expression of the recombinant protein

293T, 293 GnT1⁻, and CEF cells were transfected with either of the constructed plasmids using polyethylenimine (PEI). Transfection using PEI was performed according to the protocol established by Boussif et al. (1995). Briefly, 88 µg of the plasmid was mixed with 440 µL of PEI (2 mg/mL) and then transfected into approximately 2×10^7 of 293T cells. Transfected cells were incubated in OPTI-MEM (Life Technologies) and the supernatant of transfected cells was harvested at 72–96 h post-transfection. The recombinant protein was purified using Ni sepharose (GE healthcare) according to the manufacturer's protocol.

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

SDS-PAGE was carried out under denaturing and non-denaturing conditions. Under denaturing conditions, the purified protein was mixed with 2× sample buffer (100 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.2% bromophenol blue) containing 200 mM dithiothreitol (DTT) and boiled for 5 min at 95 °C. In contrast, non-denaturing SDS-PAGE was performed according to the protocol reported by Bender et al. (2005). Briefly, the purified protein was mixed with 2× sample buffer containing 0.4% SDS

(in the absence of a reducing agent) and loaded directly onto a gel (without boiling of the sample). The protein was separated by 5–20% polyacrylamide gradient gel (e-PAGEL, ATTO) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was incubated in 5% skim milk (Wako) in T-PBS buffer (phosphate-buffered saline, pH 7.2 (PBS) containing 0.05% Tween 20) for 60 min at 37 °C, and then incubated with a mAb against S1 protein in 5% skim milk in T-PBS buffer. Next, the membrane was incubated with a peroxidase-conjugated second antibody, goat anti-mouse IgG (H + L) (Jackson). The reacted protein was visualized using a TMB substrate kit (Invitrogen).

2.6. Immunization and virus challenge in chickens

The immunization experiment consisted of 4 groups of 5 or 10 chickens. The SPF chickens (layer-type) used for this study were maintained in our institute and 10 chickens were immunized with the purified recombinant S1 protein, which was His-tagged with trimerization motif, or inactivated virus, respectively. Five chickens were immunized with PBS-mock vaccine (mock group) and 10 chickens were left untreated to serve as an unvaccinated control group. Light liquid paraffin, sorbitan monooleate, and polysorbate 80 in a volume ratio of 9:36:4:1 was used as oil adjuvant. Recombinant S1 protein was mixed with oil adjuvant to make an S1 suspension at a concentration of 20 µg/dose. The IBV TM86 strain was inactivated using formaldehyde and mixed with oil adjuvant to generate a suspension harboring inactivated virus at a virus concentration of $10^{7.0}$ EID₅₀/dose. Chickens were inoculated intramuscularly. At 6 weeks post-inoculation, blood was collected from each animal, and the resulting sera were used to determine antibody titers. Each chicken then was challenged with $10^{3.5}$ EID₅₀ IBV TM strain administered via the trachea. Animals were sacrificed at 4 days post-challenge and ciliostasis was assessed by a slightly modified version of the previously reported protocol (Cook et al., 1999). Briefly, tracheas were removed aseptically from euthanized chickens. Cilia were observed microscopically and the cessation of ciliary movement was considered to be a sign of symptoms.

2.7. VN test

Sera were serially diluted two-fold with EMEM in a microplate and mixed with 200 TCID₅₀ of CK-adapted IBV TM strain. After incubation for 1 h at 37 °C, CK cells were inoculated with IBV and incubated at 37 °C in 5% CO₂ incubators. VN titer was defined as the reciprocal of the highest dilution showing no cytopathic effect (CPE).

2.8. Indirect sandwich ELISA using S1 protein

The mAb against IBV S1 protein was diluted to 2 µg/mL with PBS and 50 µL was added to each well of a 96-well microplate (Maxisorp; Nunc, Denmark). After incubation overnight at 4 °C, plates were washed three times with T-PBS. Next, 300 µL of T-PBS containing 5% skim milk was added and incubated at room temperature (RT) for 1 h. After washing three times with T-PBS, 50 µL of S1 antigen was added to each well and incubated at RT for 1 h. For the recombinant S1 ELISA, supernatant of 293T cells transfected with the expression plasmid pCAGGS-S1-His was used. For the native spike ELISA, lysate of IBV-infected CK cells was used as the antigen. CK cells were infected with IBV at a MOI of 0.2 and extracted 72 h post-infection by treatment with RIPA buffer (containing 1% TritonX-100, 1% sodium deoxycholate, 0.05 M Tris-HCl (pH 8.0), 0.1 M NaCl, and 1 mM EDTA). Antigens were diluted with dilution buffer (T-PBS containing 5% skim milk) and added to wells. The levels of recombinant and native S1 protein used for the ELISA test were selected as the respective concentrations that yielded

absorbances (following reaction with the positive serum) of 1.0 to 1.5. The contents of the wells were washed with T-PBS; an aliquot (50 µL) of sera (diluted 1:100 with dilution buffer) was dispensed to each well, and plates were incubated at RT for 1 h. In the recombinant S1 ELISA, primary sera were pre-treated with 1 µg/mL of purified His-tagged IBV E protein at 4 °C overnight to remove antibodies against the His-tag peptide. The contents of the wells were washed with T-PBS; an aliquot (50 µL) of peroxidase-conjugated anti-chicken immunoglobulin (donkey anti-chicken IgY (H + L) (Jackson)) was dispensed to each well, and plates were incubated at RT for 30 min. The contents of the wells were washed with T-PBS; an aliquot (100 µL) of TMB substrate kit (Dako) was dispensed to each well, and plates were incubated at RT for 15 min. After incubation, the enzymatic reaction was stopped by addition of 100 µL/well of 1 M sulfuric acid. The absorbance was measured using a spectrophotometer (VersaMax), with a 450-nm and 650-nm filter. A hyperimmune serum against IBV, which was prepared by immunization with attenuated and inactivated vaccines, was used as the positive serum. Separate work (data not shown) using chicken antisera against IBV E, M and N proteins and anti-S2 mouse mAb (described above) confirmed that native S1 ELISA does not react with antibodies raised against IBV component proteins S2, E, M, or N. Antisera against IBV E, M and N were prepared from chickens immunized with purified recombinant proteins expressed from *E. coli* and it was confirmed that these antisera reacted to IBV component by ELISA (data not shown).

2.9. Statistical analysis

All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University), which is a graphical user interface for R (The R Foundation for Statistical Computing) (Kanda, 2013).

3. Results

3.1. Recombinant S1 protein has a different glycosylation character from native spike protein

The constructed expression plasmids, pCAGGS-S1-T4-His and pCAGGS-S1-His, were transfected into cells and the recombinant protein was expressed as secreted protein. The secreted S1 protein was purified using Ni sepharose and analyzed by Western blotting. The result of Western blotting under denaturing condition showed that recombinant S1 protein expressed from 293T cells ran as a broad band of nominal molecular weight ranging from 100 to 150 kDa. The size range of this band was increased compared to that of native S1 protein (derived from IBV propagated in chicken embryonated eggs), with the native protein running on the denaturing gel as a single sharp band at a nominal size of approximately 100 kDa (Fig. 2). Recombinant S1 protein derived from 293 GnTI⁻ cells, which lack the ability to synthesize complex N-glycans, ran on

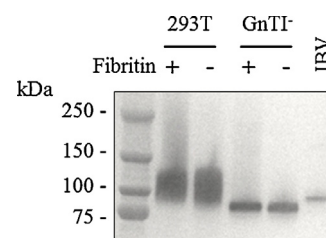


Fig. 2. Western blotting analysis of the recombinant S1 protein with (+) and without (-) fibrinin motif expressed in 293T and 293 GnTI⁻ cells. IBV derived from chicken embryonated eggs was included as a positive control. SDS-PAGE was carried out under denaturing conditions.

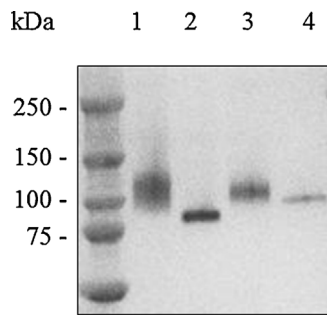


Fig. 3. Western blotting analysis of the recombinant S1 protein with fibritin motif expressed in 293T (lane 1), 293 GnTI⁻ (lane 2), and CEF cells (lane 3). IBV derived from chicken embryonated eggs was included as a positive control (lane 4). SDS-PAGE was carried out under denaturing conditions.

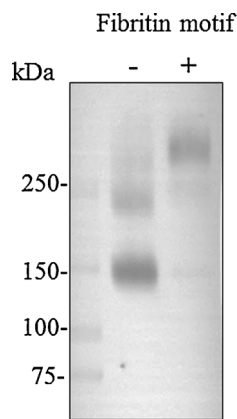


Fig. 4. Western blotting analysis of the recombinant S1 protein derived from 293T cells with (+) and without (-) fibritin motif. Recombinant proteins were purified using Ni Sepharose and SDS-PAGE was carried out under non-denaturing conditions.

the denaturing gel as a sharp band at a nominal molecular weight of 75 to 100 kDa, with a size smaller than that observed for recombinant protein expressed from 293T cells (Fig. 2). Recombinant S1 protein expressed in CEF cells (cells that are derived from the natural host for IBV) also ran under denaturing conditions as a slightly broader and larger band than native S1 obtained from IBV virions; notably, the protein produced in CEF cells ran as a sharper band than protein produced in 293T cells (Fig. 3). These results indicated that recombinant S1 protein might have an unnatural glycosylation pattern.

Using Western blotting under non-denaturing conditions, we examined the conformation of recombinant S1 protein produced and secreted from 293T cells. We observed that recombinant S1 protein lacking the fibritin motif existed in monomeric, dimeric, and trimeric conformations. On the other hand, recombinant S1 protein with the fibritin motif existed primarily in the trimeric conformation (Fig. 4).

3.2. Recombinant S1 protein induces neutralizing antibodies against IBV, but the titer was lower than that induced by the inactivated virus antigen

For the immunization experiment, the purified recombinant S1-fibritin protein expressed from 293T cells was mixed with adjuvant to generate a S1 inoculum at a concentration of 20 µg/dose. VN titer induced by the recombinant S1 protein was lower ($P < 0.05$) than that induced by the inactivated virus antigen (Fig. 5). To compare the efficacy against IBV infection, ciliostasis was evaluated via IBV challenge in animals previously immunized with S1 or with inactivated IBV virus; as a control, unvaccinated and mock-vaccinated

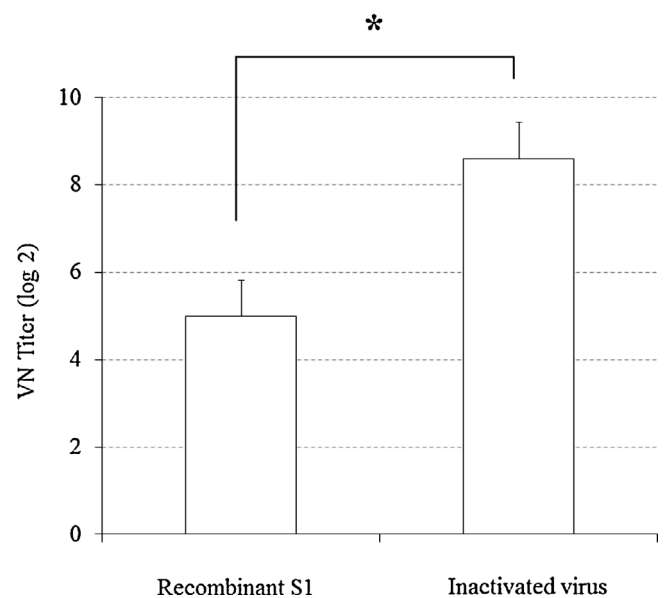


Fig. 5. Anti-IBV viral neutralization (VN) titer of immunized chickens. Sera were collected at 6 weeks post-immunization. Sera were serially diluted two-fold with EMEM in microplates, then mixed with 200 TCID₅₀ of IBV. VN titer was defined as the reciprocal of the highest dilution showing no cytopathic effect (CPE). The asterisk indicates statistical significance ($P < 0.05$) and the error bar indicates standard deviation. Comparison between two groups was performed using *t* test.

Table 1

Protection defined by ciliostasis in the trachea.

Group	Protection ^a
Recombinant S1	4/10
Inactivated virus	10/10
Unvaccinated control	3/10
PBS-mock	0/5

^a Birds showing active ciliary movement/total.

chickens also were subjected to IBV challenge. Following challenge, all (10/10) chickens immunized with inactivated virus antigen retained ciliary movement. In contrast, post-challenge ciliostasis was observed in 60% (6/10) of animals immunized with recombinant S1, 70% (7/10) of unvaccinated animals, and all (5/5) mock-vaccinated chickens (Table 1). These results indicated that immunization with recombinant S1 protein was not sufficient to provide protection from subsequent IBV infection.

3.3. Recombinant S1 protein induces non-neutralizing antibodies against S1 protein

To analyze the antigenicity of the recombinant S1 protein, sera obtained from the immunization experiment were analyzed by ELISA, using recombinant or native S1 protein as antigen. Recombinant S1 ELISA showed that sera from chickens immunized with recombinant S1 protein had a slightly high, but not significant ($P = 0.14$), titer than sera from chickens immunized with inactivated virus (Fig. 6a). Native S1 ELISA detected similar titers in the sera of animals immunized with recombinant S1 protein compared to those in sera of chickens immunized with inactivated virus (Fig. 6b) (Control experiments (data not shown) demonstrated that mock and unvaccinated control groups did not differ from each other in VN and ELISA tests of anti-S1 activity.) These results indicated that while recombinant S1 protein retained antigenicity (the ability to induce antibodies against S1 protein), the resulting antibodies was decreased its neutralizing activity, in contrast to those induced by inactivated virus.

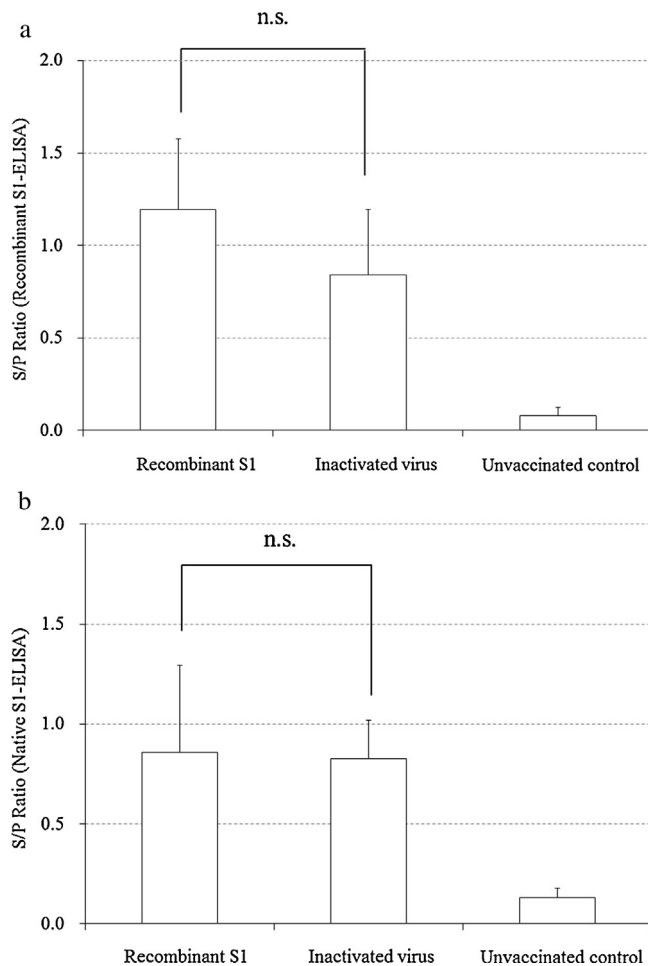


Fig. 6. Indirect sandwich ELISA using recombinant S1 protein (a) and the lysate from IBV-infected cells (b). Primary sera were collected from immunized chickens and diluted 1:100. In recombinant S1 ELISA, primary sera were pre-adsorbed with purified recombinant His-tagged protein to remove the antibodies against the His-tag peptide sequence. Reactivities are shown as the S/P ratio of absorbance at 450 nm and 650 nm. Comparisons between three groups were performed using steel-Dwass test and n.s. indicated not significant.

4. Discussion

This study showed that recombinant S1 protein exhibited decreased ability to induce a neutralizing antibody response, although the recombinant S1 induced non-neutralizing antibodies. The ELISA using recombinant S1 used sera that already had been adsorbed with His-tag peptides, precluding a role for the purification (His-tag) domain in the induced antibody response. In the ELISA using native S1, the native protein may have contained non-cleaved spike protein, thus including both S1 and S2 domains as part of the antigen. However, we found (data not shown) that inclusion (“spiking”) of S2 protein did not affect the results of the native S1 ELISA. This observation is consistent with other reports suggesting that the S2 region contains fewer neutralizing epitopes than does the S1 region; the literature indicates that most epitopes are located in the S1 region (Cavanagh et al., 1986; Ignjatovic and Galli, 1994; Kant et al., 1992; Koch et al., 1990; Promkuntod et al., 2014). These results excluded the possibility that the other viral components affect the titer in ELISA. Thus, although recombinant S1 protein induced antibodies, this recombinant protein was decreased the ability to induce neutralizing antibodies, suggesting the recombinant S1 expressed in mammalian cells may change its character. In a separate test, we observed that antigen from inactivated virus that was denatured by boiling (5 min, 95 °C) did not induce neutralizing

antibodies (data not shown). This result suggested that the correct conformation is important for S1 protein to induce neutralizing antibodies.

In the present study, the recombinant S1 protein used for immunization was expressed in mammalian cells, rather than in avian cells; the expression efficiency of recombinant proteins in avian cells was decreased compared to that in mammalian cells, and expression in avian cells was insufficient to obtain recombinant proteins in the quantities needed for the experiments. The present work also used recombinant S1 expressed as secreted protein because secretion permitted accumulation of recombinant protein to higher levels and made it easy to purify recombinant protein. These modifications, using mammalian cells and expressing recombinant protein as the secreting form, may affect the conformation of the S1.

Western blotting using denaturing conditions indicated that recombinant S1 protein expressed in 293T cells exhibited increased (and less uniform) sizes compared to the native protein. In contrast, recombinant S1 protein expressed in 293 GnT1⁻ cells (which lack N-acetyl-glucosaminyltransferase I activity and so do not generate complex N-glycans) ran as a sharper band of a size similar to that of native S1 protein. Together, these data indicated that the glycosylation pattern of recombinant S1 protein might differ from that of native S1, and that this difference reflects the level and nature of N-glycosyl modifications. We hypothesize that the native spike protein is typically decorated with high-mannose or hybrid-type N-glycans, and/or that the complex-type glycans decorating the native spike proteins are few in number and are homogeneous. It is known that glycosylation characteristics differ between species, and glycosylation affects the conformation of glycoproteins (Helenius, 1994). Therefore, glycosylation pattern differences may affect the conformation and antigenicity of the S1 protein. Furthermore, glycans attached on the surface of the envelope protein are known to mask antigenic sites, thereby permitting evasion of the host immune system (Sun et al., 2011; Zhang et al., 2015). We infer that recombinant S1 protein antigenic sites also may be masked by glycans. From these hypotheses, there is the possibility that S1 expressed in other expression systems, which have different glycosylation character, also changes its antigenicity.

Other reports suggest that the S2 region of the IBV spike protein is important for the correct conformation of S (Callison et al., 1999; Promkuntod et al., 2013). Therefore, the recombinant S1 protein secreted in this study might assume a non-native conformation. We hypothesize that expression of the S1 domain without the S2 region results in the assumption of an unnatural conformation, resulting in unnatural glycosylation patterns, or that unnatural glycosylation of S1 protein causes the recombinant peptide to assume an unnatural conformation. Recombinant S1 protein may expose hidden epitope(s) that are normally latent in the protein. However, some researchers have reported that secreted recombinant S1 protein, when expressed fused to the trimerization motif peptide, binds the cell receptors (Promkuntod et al., 2013; Wickramasinghe et al., 2011). These reports suggested that the secreted S1 protein retains receptor binding ability. In addition, expression of other viral envelope proteins (specifically, human immunodeficiency virus (HIV) gp120 and influenza virus hemagglutinin (HA)) as secreted proteins have been reported to induce neutralizing antibodies (Pancera et al., 2005; Wei et al., 2008). These reports indicated that secreting envelope proteins with a trimerization motif is an effective method for creating vaccine antigens. Additionally, Cavanagh (2007) reported that IBV S1 protein was sufficient for the induction of neutralizing antibodies (though S1 was not sufficient for protection against IBV infection). Furthermore, cytotoxic T-lymphocyte (CTL) activity induced by immunization of nucleocapsid protein (NP) has been shown to be important for virus clearance (Collisson et al., 2000; Seo and

Collisson, 1997). However, in the present study, while immunization with recombinant IBV S1 (expressed as a secreted protein fused to the fibrin motif) induced antibodies, the response mainly consisted of non-neutralizing antibodies. Therefore, recombinant S1 protein (expressed as a secreted protein in mammalian cells) may be insufficient for use as a vaccine antigen. Recently, virus-like particle (VLP)-based antigens have been tested as new candidates for subunit vaccines against IBV (Cavanagh, 2003; Lv et al., 2014). Receptor binding domain (RBD) peptides also have been tested as vaccine candidates against severe acute respiratory syndrome (SARS) coronavirus (Jiang et al., 2012). These alternative approaches to antigen production may overcome the challenges observed in the present work.

In conclusion, we demonstrated that recombinant S1 protein (expressed as a secreted protein in mammalian cells) was decreased the ability to induce neutralizing antibodies in chicken. We infer that the recombinant protein lacked conformational epitopes as a result of changes in glycosylation and changes in conformation compared to the native S1 protein. Further research will be required to identify factors necessary to establish a recombinant S1 antigen that retains native neutralizing antigenicity.

Conflict of interest

The authors declare that they have no conflict of interest.

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