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ORIGINAL ARTICLE

Characterization and utility of monoclonal antibodies against spike protein of transmissible gastroenteritis virus

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Abstract

Aims: This work aims to characterize the utility of four newly generated monoclonal antibodies (mAbs) against transmissible gastroenteritis virus (TGEV).
Methods and Results: Four monoclonal antibodies (mAbs) against the N-terminal half of spike protein (S1 protein) of TGEV were identified. Affinity constant of these mAbs was analysed. These mAbs were capable of reacting with the TGEV S1 protein analysed by ELISA and Western blot. A competition assay between the different mAbs was performed to determine whether the different antibodies mapped in the same or a different antigenic region of the protein. Investigation on the neutralizing ability of these mAbs indicated that two of these mAbs completely neutralized TGEV at an appropriate concentration. These mAbs were able to detect the TGEV-infected cells in immunofluorescence assays and Western blot. Moreover, they differentiated TGEV S protein from other control proteins.
Conclusions: The generated four mAbs are very specific, and the established immunofluorescence assays, Western blot and discrimination ELISA are useful approaches for detecting of TGEV.
Significance and Impact of the Study: It is a novel report regarding the use of the S1 protein of TGEV to generate specific mAbs. Their utility and the established immunoassays contribute to the surveillance of TGE coronavirus.

Introduction

Porcine transmissible gastroenteritis (TGE) caused by transmissible gastroenteritis virus (TGEV) is a highly contagious disease characterized by vomiting, diarrhoea and dehydration. The mortality rate of TGE in seronegative suckling piglets may reach 100%. TGE prevalence causes enormous economic losses to swine-breeding units.

TGEV is an enveloped virus with a positive-stranded RNA genome and belongs to the family *Coronaviridae*. The viral particle of TGEV is composed of four identified structural proteins: the spike (S), the integral membrane (M), the minor envelope (E) and the nucleocapsid (N) (Spaan *et al.* 1988; Ren *et al.* 2008). TGEV S protein is a major viral antigen and can elicit the neutralizing antibodies in hosts (Jiménez *et al.* 1986). The interaction between the S protein and porcine aminopeptidase N (pAPN), the cellular receptor of TGEV, mediates the virus entry and subsequent membrane fusion (Delmas *et al.* 1992; Liu *et al.* 2009). Consequently, the S protein of TGEV can be selected as a candidate for antigen detection and vaccine design. Four major antigenic sites (A, B, C and D) located on the amino-terminal half of protein S have been identified (Delmas *et al.* 1990). In this study, using the bacterially expressed TGEV S1 protein and hybridoma technique, four monoclonal antibodies (mAbs) against the S1 protein were generated and characterized. The availability and utility of these mAbs are helpful for detecting and analysing TGEV infection.

Materials and methods

Virus and cells

Swine testis (ST) cells were grown in Eagle's minimum essential medium (EMEM) supplemented with 10%

newborn bovine serum (NBS; Excell Bio, Shanghai, China). TGEV strain PUR46-MAD was provided by Dr L. Enjuanes of CSIC-UAM Canto Blanco, Madrid, Spain. The virus was propagated in ST cells and passaged twice a week. SP2/0 myeloma cells were stored in our laboratory.

Generation of anti-TGEV S1 protein monoclonal antibodies

Recombinant plasmid bearing full-length TGEV S gene (GenBank accession number: No. M94101) was used as PCR template (Schwegmann-wessels *et al.* 2009). Sense primer 5′-GGGGGGATCCATTGAAACCTTCCTTCTA and antisense primer 5′-CCCCGAATTCGTTAGTTTGTCTA-ATAATA were used to amplify a truncated S gene encoding the 5′ end half of the TGEV S gene designated S1 (*c.* 2·0 kb in length) by conventional PCR. The PCR product purified with a DNA purification kit (KeyGen, Nanjing, China) was cloned into BamH I and EcoR I sites of a prokaryotic expression vector, pGEX-6P-1. The resulting plasmid, pGEX-S1, was transformed into *Escherichia coli* BL21(DE3) pLysS, and protein expression was induced with isopropyl *β*-D-thiogalactoside (IPTG) at a final concentration of 1 mmol l^{-1} at 37°C followed by gel purification. The purified protein plus equal volume of Freund's complete adjuvant were used to immunize 6-week-old BALB/c mice (50 mg per mouse) via intraperitoneally. The immunization was boosted four times with the same antigen plus Freund's incomplete adjuvant at 2-week intervals. The anti-S1 protein serum titre of immunized mice was detected using indirect ELISA using purified TGEV S1 protein as coating antigen. Spleen cells from the best immunized mice were fused with SP2/0 myeloma cells. Hybridomas were generated as previously described (Li *et al.* 2010; Meng *et al.* 2010). Positive hybridomas were cloned three times to harvest monoclonal hybridomas. These mAbs harvested from hybridoma grown in 1640 medium without NBS were isotyped by a Mouse MAb Isotyping kit (Sigma, USA) according to the manufacturer's instructions.

Indirect immunofluorescence assays

For indirect immunofluorescence assay, ST cells cultured on glass coverslips in 24-well plates were infected with TGEV (10^5 PFU ml^{-1}) for 24 h followed by fixation with 4% (w/v) paraformaldehyde in PBS for 20 min. The cells were incubated with undiluted anti-S1 mAbs followed by incubation with fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG (1 : 100 dilution in 1% bovine serum albumin, BSA) at room temperature for 1 h. The nuclei of the cells were stained with propidium iodide at 37°C for 15 min prior to fluorescence microscopy (Ren *et al.* 2006; Meng *et al.* 2010; Sui *et al.* 2010).

Western blot

TGEV S1 protein was isolated in 12% SDS-PAGE and then transferred to nitrocellulose (NC) membranes. The NC membranes were blocked overnight at 4°C using 5% nonfat dry milk in PBS – 0·05% Tween 20 (PBST), sliced into strips and incubated with either the supernatant of the hybridomas or SP2/0 myeloma cell culture (1 : 500 dilution in PBS) at room temperature for 1 h. After washing three times with PBST, these membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1 : 2000 dilution in PBS) at 37°C for 1 h. The protein bands were visualized using 3,3′-diaminobenzidine (DAB) substrate.

Analysis of affinity constant of the mAbs

The affinity constant of the mAbs was determined with ELISA as previously described (Li *et al.* 2010). Briefly, purified S1 proteins at concentrations of 2000, 1000 or 500 ng ml^{-1} were coated in ELISA plates (100 μl per well) at 4°C overnight followed by incubation with the serially diluted mAbs. After complete washing, the HRP-conjugated goat anti-mouse IgG was added into the wells followed by the addition of *o*-phenylenediamine dihydrochloride (OPD) substrate. The equation for calculating affinity constant $Kaff = (n - 1)/2(n[Ab']t - [Ab]t)$, derived from law of mass action, where $n = [Ag]t/[Ag']t$. $[Ag]t$ and $[Ag']t$ are the total antigen concentrations measured in the wells, while $[Ab']t$ and $[Ab]t$ are the total antibody concentrations in the wells at OD-50 (50% of OD-100, the upper plateau) and OD-50′ of plates coated with $[Ag]t$ and $[Ag']t$.

Specificity of the mAbs

To analyse the specificity of the mAbs, a discrimination ELISA was performed. Briefly, the plate was coated with the S1 protein of TGEV, S1 protein of porcine epidemic diarrhoea virus (PEDV), GP5 protein of porcine reproductive and respiratory syndrome virus (PRRSV), BSA diluted in PBS buffer (pH 7·2) at 4°C overnight. The concentration of the proteins was 10 μg ml^{-1} (100 μl per well). After blocking with PBS containing 1% BSA (PBSB) for 2 h at room temperature and washing with PBST, they were consecutively incubated with hybridoma supernatants (100 μl per well) and HRP-conjugated goat anti-mouse IgG antibody (1 : 5000 dilution) followed by addition of OPD substrate. The reaction was stopped with 2 mol l^{-1} H_2SO_4. OD_{490} value was measured using an ELISA plate reader.

Analysis of antigenic epitopes

To analyse the recognizing antigenic epitopes in the S protein by the mAbs, a modified double-antibody-binding ELISA was performed to calculate the additivity index (AI). Briefly, ELISA plates were coated with 100 μl purified S1 protein (10 μg ml^{-1}) diluted in 0·1 mol l^{-1} bicarbonate carbonate buffer (pH 9·6) at 4°C overnight. After blocking with PBSB for 1 h at room temperature and washing with PBST, the wells were incubated with undiluted supernatant from either individual hybridoma (100 μl per well) or two hybridomas. Then the wells were incubated with HRP-conjugated secondary antibody and OPD substrate as described earlier. The amounts of the bound antibodies were quantified by measuring the OD_{450} value. The AI of two hybridomas was calculated according to equation: $AI = [2A_{1+2}/(A_1 + A_2) - 1]100\%$, where A_1, A_2, and A_{1+2} represent the OD_{450} values of MAb1, MAb2, and the mixture of the two mAbs, respectively. If the AI is above 50%, it shows that the two mAbs recognize different antigenic epitope; if below 50%, it demonstrates that the two MAbs recognize same antigenic epitope.

Viral neutralizing activity of the mAbs

TGEV (10^7 or 10^5 PFU ml^{-1}) was incubated with the mAbs serially diluted in medium at 37°C for 1 h. Then, the treated viruses were used to infect ST cells in 24-well

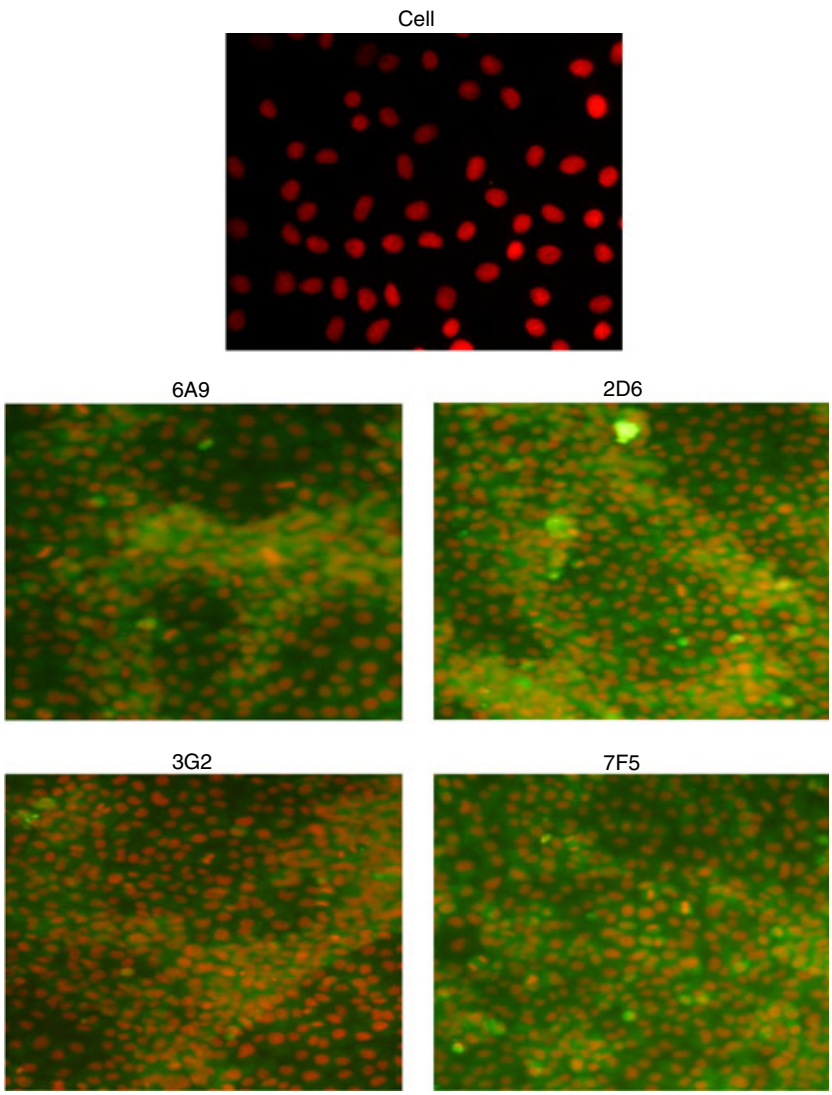

Figure 1 Detection of cell infection by indirect immunofluorescence assay using these mAbs. ST cells were infected with transmissible gastroenteritis virus followed by conventional immunofluorescence using the mAbs as primary antibody. The mock-infected ST cells were used as control. The nuclei were stained with propidium iodide. A representative comparison is shown.

plates at 37°C for 1 h. After PBS washing, the cells were overlaid with 1% methylcellulose in medium and cultured for 48 h and subjected to plaque assays (Ren *et al.* 2008; Li *et al.* 2009).

Results

Generation of anti-S1 protein mAbs

The bacterially expressed TGEV S1 protein was purified and used to immunize BALB/c mice. The mouse splenic cells were fused with SP2/0 myeloma cells to generate mAbs. In this study, four positive mAbs against the S1 protein of TGEV were generated. These mAbs were designated 7F5, 2D6, 3G2 and 6A9, respectively. Their isotypes identified using a Mouse MAb Isotyping kit were IgG. The isotype of 3G2 is IgG3 and the isotypes of other mAbs identified in this study are IgG1.

Recognizing of the mAbs to the S1 protein

The recognizing of the mAbs to TGEV S1 protein was examined first by immunofluorescence assays after TGEV-infected cells were fixed. As shown in Fig. 1, these mAbs recognized the virus-infected cells rather than mock-infected cells. The reactivity between supernatants from these hybridomas and the S1 protein was further analysed by Western blot (Fig. 2). Our results showed that these mAbs recognized the TGEV S1 protein specifically; in contrast, no positive reaction band on the NC membrane was detected, when the culture supernatant from SP2/0 myeloma cells was used as control.

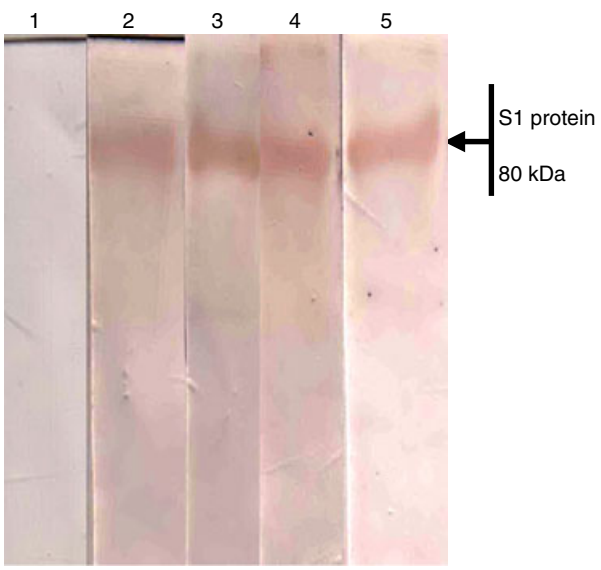

Figure 2 Western blot analysis on transmissible gastroenteritis virus (TGEV) S1 protein. After the S1 protein of TGEV was transferred onto nitrocellulose membranes, the membranes were incubated with the supernatant from either SP2/0 cell culture or the mAbs followed by incubation of horseradish peroxidase-conjugated secondary antibody. Lanes 1–5 are the blot results using the supernatant from SP2/0, 6A9, 2D6, 3G2 and 7F5 as primary antibody, respectively.

Determination of affinity constant of these mAbs

The affinity constant of these mAbs was determined with noncompetitive ELISA method described using serial dilutions of coated antigen and mAbs (Table 1). The results demonstrated that the mean Kaff of mAbs 6A9, 2D6, 3G2 and 7F5 were $3{\cdot}638 \times 10^{10}$, $1{\cdot}125 \times 10^{11}$, $2{\cdot}024 \times 10^{11}$ and $7{\cdot}278 \times 10^{11}$, respectively.

Antigenic epitopes recognized by the mAbs

To analyse epitopes of the S1 protein, which were recognized by each of these mAbs, the AI assays were

Table 1 Affinity constant of the mAbs

mAbs	[Ag] (ng ml^{-1})	OD-50 (OD_{450})	[Ab]at OD-50 (ng ml^{-1})	Kaff (M^{-1})	Average Kaff (M^{-1})
6A9	2000	0·9812	1·162	$4{\cdot}950 \times 10^{10}$	$3{\cdot}638 \times 10^{10}$
	1000	0·87145	1·338	$2{\cdot}326 \times 10^{10}$	
	500	0·79495	2·279	NA	
2D6	2000	0·9385	0·293	$1{\cdot}190 \times 10^{11}$	$1{\cdot}125 \times 10^{11}$
	1000	0·90795	0·462	$1{\cdot}059 \times 10^{11}$	
	500	0·7149	0·585	NA	
3G2	2000	0·8924	0·325	$2{\cdot}309 \times 10^{11}$	$2{\cdot}024 \times 10^{11}$
	1000	0·70925	0·325	$1{\cdot}739 \times 10^{11}$	
	500	0·65115	0·378	NA	
7F5	2000	0·95925	0·334	$7{\cdot}092 \times 10^{10}$	$7{\cdot}278 \times 10^{11}$
	1000	0·88105	0·697	$7{\cdot}463 \times 10^{10}$	
	500	0·7181	0·851	NA	

NA, not applicable.

Table 2 Additivity index (AI) of the mAbs

Monoclonal antibody	6A9	2D6	7F5	3G2
6A9	NA	NA	NA	15·65%
2D6		NA	NA	7·96%
7F5			NA	7·47%
3G2				NA

NA, not applicable.

Competition for each antibody was determined by calculating the AI using the formula $AI = [2(A_{1+2})/(A_1 + A_2)-1]100$, where A_1 and A_2 represent the absorbance values for each of two mAbs tested and A_{1+2} represents the absorbance value when the two were combined.

performed. As shown in Table 2, the AI resulting from the combination of the mAbs generated in this study was lower than 50%.

Specificity of the anti-TGEV-S1 mAbs

The specificity of these mAbs was examined using ELISA. As shown in Fig. 3, these mAbs exclusively reacted with TGEV S1 protein. In contrast, they did not recognize unrelated proteins, such as PRRSV GP5 protein or PEDV S1 protein.

Virus neutralizing effect of the mAbs

The neutralizing effect of the mAbs on TGEV was evaluated. The incubation between the high dose of viruses (1×10^7 PFU ml^{-1}) and the serially diluted mAbs led to a decreased infectivity of TGEV in a dose-dependent manner. The virus titre was reduced to *c.* 60–70% by mAbs 2D6 and 3G2; other two mAbs had poor neutralizing ability to TGEV (data not shown). Based on the results, we analysed the neutralizing activity of mAbs 2D6 and 3G2 to lower virus titre (1×10^5 PFU ml^{-1}). The result showed that both mAbs neutralized TGEV completely (Fig. 4).

Discussion

TGEV S protein is often used as immunogen or diagnostic target. The aim of this study is to identify and characterize mAbs against the S protein for virus surveillance and function analysis. TGEV S gene encoding the S protein is *c.* 4300 bp in length, and four major antigenic sites are located in the N-terminal half of the S protein (Delmas *et al.* 1990). Therefore, the N-terminal half of the S gene (S1 gene) was expressed in the *E. coli* system because this system has advantages consisting of low cost, convenience and high fermentation potential (Yin *et al.* 2007). Many heterologous proteins have been expressed in this system (Ren *et al.* 2010a,b,c). In this study, the S1 protein of TGEV was expressed in the same system and used to immunize BALB/c mice. Using conventional hybridoma techniques, four positive mAbs against the S1 protein of TGEV were generated, and the isotype of 3G2 is IgG3. And the isotypes of other mAbs identified in this study are IgG1. The data indicate that this protein can induce the generation IgG antibody in the immunized mice.

To analyse the recognition of these mAbs to the S1 protein, we tested the reaction between these mAbs and the TGEV by immunofluorescence assays. Our result indicates that the mAbs can react with the native S protein of TGEV. The reactivity between supernatants from these hybridomas and the S1 protein was confirmed by Western blot. Previously, we have used a known monoclonal antibody, (6A.C3) against the S protein of TGEV (Schwegmann-wessels *et al.* 2009). The mAbs generated in the study have a similar recognizing ability with the 6A.C3.

Determination of affinity constant of these mAbs indicated that the affinity ability between the S1 protein and the mAbs is increased with the increasing concentrations of these mAbs. To further characterize these antibodies, antigenic epitopes recognized by the mAbs were further evaluated, which indicates that these antibodies reacted with the same antigenic epitope in the S1 protein of TGEV.

To verify the specificity of the developed mAbs, reactivity of the antibodies with the S protein of a closely related

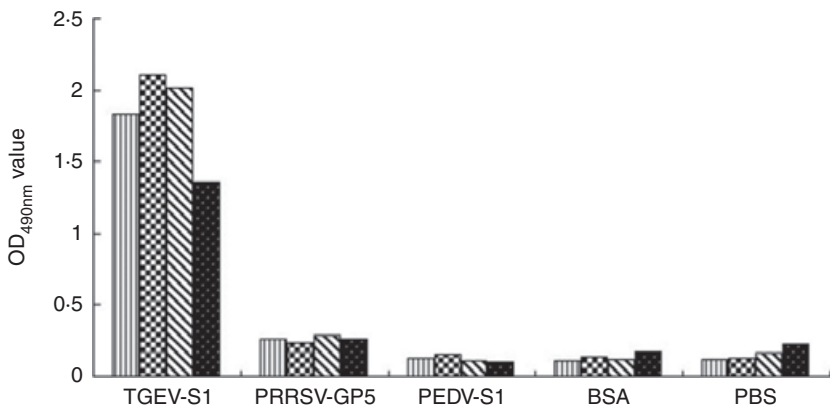

Figure 3 Analysis of specificity of the mAbs. The S1 protein of transmissible gastroenteritis virus, GP5 protein of porcine reproductive and respiratory syndrome virus, S1 protein of porcine epidemic diarrhoea virus, BSA and PBS were used as coating antigen. The mAbs generated in this study were used as primary antibody for indirect ELISA. The OD_{490} value is the mean value from three independent assays. (▥) 7F5; (▩) 6A9; (▧) 2D6; (■) 3G2.

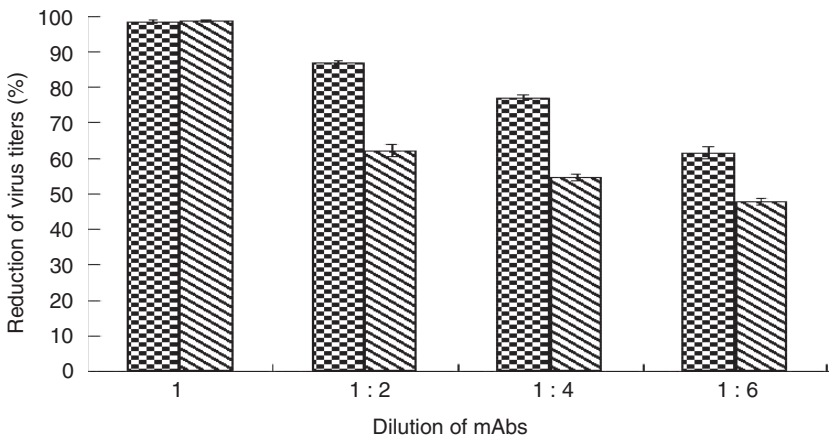

Figure 4 Neutralizing effect of the mAbs to transmissible gastroenteritis virus (TGEV). TGEV (1×10^5 PFU ml^{-1}) was incubated with undiluted or serially diluted mAbs (2D6 and 3G2) for 1 h. The treated viruses were used to infect ST cells. The supernatant from SP2/0 cells was used as control in conventional plaque assays. The reduction in virus titres are shown. (▩) 2D6; (▨) 3G2.

coronavirus, PEDV or GP5 protein of PRRSV was analysed. The specific reactivity of these mAbs to TGEV S protein as shown in Western blot and to TGE virions as shown in immunofluorescence assays suggests that they may assist in the laboratory diagnosis of TGE. In the future, we will test the cross-reaction between these mAbs and the S proteins of other group 1a coronaviruses or the S protein of some group II coronavirus that have been reported to be highly related to TGEV in the amino-terminal domain of the S protein (Decaro *et al.* 2009). More importantly, the virus-neutralizing assays indicated that two mAbs 2D6 and 3G2 were shown neutralizing TGEV completely. They are better experiment materials that can be used for investigating the biological function of TGEV.

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