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Monoclonal and Polyclonal Antibodies Specific for Foot and Mouth Disease Virus Type A and Type O VP1

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The foot and mouth disease virus (FMDV) is an RNA virus composed of single stranded positive sense RNA. FMDV has been known to infect cloven-hoofed animals, including pigs, cattle, and sheep. FMDV is rapidly spreading outward to neighboring regions, often leading to a high mortality rate. Thus, early diagnosis of FMDV is critical to suppress propagation of FMDV and minimize economic losses. In this study, we report the generation and characterization of polyclonal and six monoclonal antibodies against VP1 through immunoblotting and immunofluorescence microscopy analyses. These VP1 antibodies will be useful as tools to detect serotypes A and O of FMDVs for diagnostic usage.

Introduction

Foot and mouth disease virus (FMDV) is an RNA virus composed of single-stranded positive sense RNA of around 8.4 kb, which encodes FMD virus particle (structural protein) or non-structural proteins (3ABC, 2C, or 3D).[1] FMDV is classified in the Aphthovirus genus as a member of the Picornaviridae family. There are seven different serotypes of FMDV, including A, O, C, SAT1, SAT2, SAT3, and Asia1, with numerous subtypes within each serotype. FMDVs are virions forming icosahedral shells composed of 60 copies each of four structural proteins VP1–VP4. The FMDV structural protein VP1 elicits neutralizing and protective antibody and has the Arg-Gly-Asp (RGD) ligand for integrin receptors of host cells for virus entry.[2–5] Amino acid sequence at residues about 141–160 of VP1 in the O serotype, which corresponds to the G-H loop, contain the most immunogenic site and RGD ligand. Therefore the determination of serotype and vaccine protection of an FMDV must refer considerably to this region due to its critical role in the immunogenicity and virus infection.

FMD is generally regarded as the most contagious of all diseases of farm animals. FMDV has been known to infect cloven-hoofed animals, including pigs, cattle, and sheep, often leading to a high mortality rate (i.e., above 90%) in young livestock species. In addition, the FMDV rapidly spreads to neighboring regions through birds, human beings, and various transportation systems. The prevalence of FMDV severely affects the welfare and productivity of high-value farm animals and leads to enormous economic losses. In addition, the disease status of FMD in each country has a great influence on the conditions in trade of animals and animal products between countries. Thus many countries invest a great amount of their budget to control or eradicate FMD by means of vaccination, movement restriction, quarantine, or sampling-out depending on their situation.[6]

FMD outbreaks of types O and A occurred in Korea during the last 2 years, and induced a huge amount of economical loss, totaling about three billion USD. Thus, to determine an early stage of whether a suspected animal is infected with FMDV or not is critical for effective disease control because, in some cases, with clinical symptoms alone, FMD cannot be differentiated from other diseases like swine vesicular disease (SVDV).[7] Laboratory diagnosis using ELISA, PCR, and sequencing should be performed following clinical examination for suspicious cases. In the diagnosis using FMDV protein, there are two kinds of ELISA; one is for FMDV antibody-detecting ELISA, the other for FMDV antigen-detecting ELISA. The application of FMDV antibody-detecting ELISA should be carefully considered because FMDV antibody could be detectable at least 7–10 days following infection of FMDV, which implies that FMDV could be actively spreading out in the meantime without seroconversion. Thus an FMDV antigen-detecting ELISA or PCR is more preferable than an FMDV antibody-detecting ELISA to confirm FMD in the very early stage of infection accompanying clinical symptoms. On the other hand, after recovery of the disease or disappearance

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of clinical symptoms, an antibody-detecting ELISA becomes a more suitable diagnostic method to find evidence of the infection. Serotype-specific liquid-phase blocking ELISAs (LPB-ELISAs) have been developed and used for detecting antibodies against structural protein, even though there is cross-reactivity among different serotypes to some extent.[8,9] Recently, competitive ELISAs using monoclonal or polyclonal antibodies to replace theses LPB-ELISA have been developed and used effectively.[10–12]

The essential components of these ELISAs are mono- or polyclonal antibodies specific to the FMD virus particle (structural protein) or non-structural proteins (3ABC, 2C, or 3D).[13,14] Thus, in this study, our goal was to generate polyclonal and monoclonal antibodies against VP1 to detect serotypes A and O of FMDVs for diagnostic use.

Materials and Methods

Generation of recombinant VP1 protein

VP1 genes encoding 212 amino acids of VP1 of serotype A strain, A/HuBWH/CHA/2009 (accession no. JF792355), and 213 amino acids of type VP1 of a serotype O strain, O/IRN/2/2010 (accession no. JF916985) were synthesized from Bioneer (Daejon, Korea), and inserted into EcoRI/SalI site of pET28a (Novagen, Darmstadt, Germany). The resulting clones were transformed into the *Escherichia coli* strain BL21-DE3. The expression of the VP1 proteins was induced by 0.1 mM IPTG at 25°C for 6 h. The cells expressing the recombinant proteins were harvested, resuspended in lysis buffer (6 M guanidine-HCl, 20 mM KH_2PO_4, 500 mM NaCl [pH 7.8]), and then lysed by ultrasonication. After centrifugation of the lysate at 25,000 *g*, the supernatants were recovered and loaded to nickel affinity chromatography. The column was washed with washing buffer (20 mM KH_2PO_4, 500 mM NaCl, 2 mM β-mercaptoethanol [pH 7.8]) and eluted with elution buffer (20 mM KH_2PO_4, 500 mM NaCl, 300 mM imidazole, 2 mM β-mercaptoethanol [pH 8.0]). Proteins were quantified with Bradford solution (Bio-Rad, Hercules, CA) and stored at −70°C until use.

Generation of monoclonal and polyclonal antibodies

The CHA Animal Care and Use Committee approved all animal studies, and the investigation conformed to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD). To generate mouse monoclonal antibody against type A VP1 and type O VP1, purified protein (1.5 mg) was used as an immunogen, 1 mg for ELISA assays. Female BALB/c mice (8 weeks old) were immunized intraperitoneally. The primary injection was performed on week 0 and consisted of 100 μg/mouse of immunogen in CFA (Sigma-Aldrich, St. Louis, MO). Booster injections were conducted during week 4. The mouse serum antibody titers were assessed by an indirect ELISA kit (Abfrontier, Seoul, Korea) using a protein antigen. The mouse showing positive immune response activity was subjected to a final boost injection during week 8. The mouse harboring the highest reactivity against protein antigen was sacrificed and splenocytes were isolated from the spleen. The splenocytes were fused to SP2/0 cells and the resulting hybridomas were screened by culturing in HAT medium as described previously.[15] Hybridomas showing positive reactivity in ELISA were sub-cloned by standard limiting dilution method. The hybridomas producing monoclonal antibody were grown in a 175T flask, and the supernatant was harvested and stored at −80°C. The isotyping was performed using a Beadlyte-Mouse Immunoglobulin Isotyping Kit (Upstate, Lake Placid, NY). In addition, to generate polyclonal antibody against type A VP1 and type O VP1 in rabbit, New Zealand White rabbits (1 kg, male) were purchased from Orient Bio (Seoul, Korea) and maintained for 1 week for adaptation. Pre-immune blood was harvested, and Western blot analysis was performed using 30 μg of HEK293 human cell lysates to rule out the rabbit harboring cryptogenic antibodies. The purified recombinant VP1 (500 μg) was mixed with complete Freund's adjuvant at 1:1 volume ratio and then subcutaneously injected into the NZ white rabbits. Booster injections were conducted three times at 1-week intervals using the same amount of the protein mixed with the incomplete adjuvant at a 1:1 ratio. The rabbits were euthanized with isoflurane (Hana Pharm, Korea), and then blood was harvested from heart puncture. To harvest the anti-VP1 serum, the blood was incubated at 4°C for 1 h, and centrifuged with 8000 *g* at 4°C for 30 min. The antibody was purified by protein A column chromatography following the manufacturer's instructions.

ELISA

Indirect ELISA was performed in 96-well flat-bottom plates (Thermo Scientific, Waltham, MA). The plates were coated overnight at 4°C with 100 ng/well of antigen in 50 μL of 1X PBS, washed three times with 1X PBS, and blocked with 1% bovine serum albumin (BSA) in 1X PBS. Test samples were added (100 μL/well) and incubated at 25°C for 1 h, and the wells were washed three times with 1X PBS containing 0.05% Tween-20 (PBST). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was diluted in 1XPBS containing 1% BSA and added to each well (100 μL/well), and then incubated at 25°C for 1 h. The plates were washed three times with PBST, and incubated with 100 μL/well of tetramethylbenzidine (TMB) peroxidase substrate (Sigma-Aldrich, St. Louis, MO) without light at 25°C for 15 min. The reaction was stopped by adding 50 μL of H_2SO_4 to each well and incubated for 5 min. The absorbance was determined at 450 nm using a Benchmark plus plate reader (Bio-Rad, Hercules, CA) using RPMI 1640 media as a blank. Positive ELISA results were defined as those yielding A450 values greater than O.D 1.0.

Immunofluorescence microscopy

ZZ-R cells (fetal goat tongue epithelium cell, kindly gifted by Dr. Mattias Lenk, CCLV, FLI, Germany) was maintained with Iscove's Dulbecco modified Eagle medium (DMEM) and Ham's F12 medium at 1:1, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycinin in a humidified chamber. ZZ-R cells were seeded onto sterile cover slips the day before infection in 24-well plates. ZZ-R cells were infected by FMDV type A or type O viruses with 0.1 multiplicity of infection (MOI) for 18 h, washed three times with 1X PBS, and fixed with 4% paraformaldehyde (Sigma-Aldrich) in 1X PBS at 25°C for 10 min. Cells were washed three times with 1X PBS and permeabilized with 0.1% Triton X-100 for 5 min. The cells were blocked with 0.5% BSA in 1X PBS for 1 h and incubated with hybridoma culture supernatant or with 1:100 and 1:200 diluted rabbit polyclonal antibody at room

temperature for 1 h. The cells were washed three times with 1X PBS and incubated with 1:100 diluted FITC-conjugated goat anti-mouse or anti-rabbit IgG (Molecular Probes, Carlsbad, CA). The cover slips were mounted on glass slides using Vectashield mounting medium (Vector Laboratories, Peterborough, United Kingdom), and cell images were captured under the fluorescence microscope (Nikon Eclipse).

Virus

For the immunofluorescence assay, the FMDV isolate of O serotype (O/SKR/5/2010) collected from Gyeongbuk province of Korea in 2010, and the isolate of A serotype (A/Pocheon/KOR/2010) collected from Gyeonggi province of Korea in 2010 were used for infection against ZZ-R cells, as described above. The exact information about VP1 sequences of those two viruses is available in the Genebank with accession nos. JQ070321 and GU441855 for O/SKR/5/2010 and A/Pocheon/KOR/2010, respectively.

Immunoblotting

HEK293 (human embryonic kidney cell line) cells were maintained in DMEM (Gibco-BRL Rockville, MD) supplemented with 10% FBS and 1% penicillin/streptomycin. HEK 293 cells were seeded onto 6-well plates and transfected with 0.5 μg plasmids expressing type A VP1 or type O VP1 genes for 24 h. Cell were harvested and lysed with lysis buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 m EDTA, 1 mM PMSF, 10 mM NaF, 0.1 mM $NaVO_3$). Thirty μg of whole cell lysates were loaded to 12% reducing SDS-PAGE. The gels were transferred to Immobilin P (Millipore, Bedford, MA), a polyvinylidene fluoride (PVDF) membrane, using the semi dry method. The membrane was blocked with TBS (20 mM Tris-HCl [pH 7.6], 150 mM NaCl) containing 5% skim milk and 0.5% Tween-20 at room temperature for 1 h, and incubated with primary antibody (hybridoma culture supernatant of type A VP1/Type O VP1 or 1:3000 diluted rabbit polyclonal type A VP1/type O VP1 serum) at room temperature for 2 h, and washed two times with TBS containing 0.1% Tween-20. The membrane was incubated with 1:20,000 diluted HRP-conjugated goat anti-mouse (Thermo Scientific, Waltham, MA) or anti-rabbit (Thermo Scientific) at room temperature for 1 h. The membrane was washed three times with TBS containing 0.1% Tween-20 and developed using enhanced chemiluminescence (ECL) solution (Santa Cruz Biotechnology, Santa Cruz, CA) to detect their specific signals.

Results

Production of mouse monoclonal and rabbit polyclonal antibodies

To generate mouse monoclonal and rabbit polyclonal antibody, VP1 regions encoding 212 aa (type A) or 213 aa (type O) was cloned into *E. coli* expression vector pET28a harboring 6-histidine tag. Firstly, proteins were expressed in *E. coli* under the stimulation of 0.1 mM IPTG. Most of the proteins were shown to be present in inclusion bodies. Although we tried to make VP1 protein soluble by changing the induction temperature, IPTG concentration, and host, most of the proteins were still insoluble (data not shown). Thus, we tried to renaturate recombinant proteins after purification using the denaturation method. However, most of the proteins form aggregates during the renaturation process. Alternatively, we tried to make VP1 recombinant protein soluble using in-chromatography renaturation method because proteins having an unstable structure in solution tend to associate with each other to form aggregate. Thus VP1 recombinant proteins containing 6-histidine tag were solubilized using 6 M guanidine-HCl, bound to nickel affinity chromatography, and washed with guanidine-omitted buffer of a 20 bed volume (20 mM KH_2PO_4, 500 mM NaCl, 2 mM β-mercaptoethanol [pH 7.8]). The eluted recombinant proteins with 300 mM imidazole did not form aggregate (data not shown). The amount of protein obtained using in-chromatography renaturation method was checked on the SDS-PAGE (Fig. 1). Densitometirc analysis showed that protein purity was over 90%. The affinity-purified proteins were used to immunize two rabbits for generating polyclonal antibodies. In addition, to raise mouse monoclonal antibodies, the affinity purified VP1 recombinant proteins were immunized to four BALB/c mice. The mice were immunized four times; after three boosting injections, the mouse serum antibody titer was assessed by indirect ELISA. Splenocytes showing the highest serum titer against recombinant VP1 were fused with SP2/0 myeloma cells to form hybridomas, as described in the Methods section. The culture supernatants were screened using indirect ELISA and Western blot analysis, and positive hybridoma cells showing high titer values were sub-cloned by standard limiting dilution. The screening processes were finalized with three stable cell lines—1E3, 1G3, and 2F6 in type A VP1—and three stable cell lines—3C4, 2G9, and 4D3 in type O VP1. Isotyping using a commercial kit, as described in the Methods section, showed that all of them were identified as IgG1, kappa (data not shown). The properties were further characterized by Western blot and immunofluorescence staining analyses.

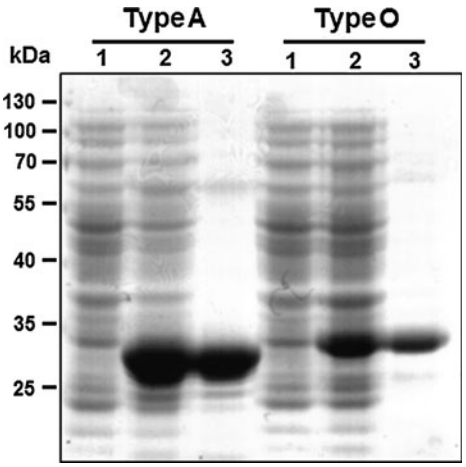

FIG. 1. Purification of FMDV type A and type O antigens. The samples were analyzed on Coomassie blue-stained SDS-PAGE. Lane 1, uninduced BL21 (DE3) lysates; lane 2, 0.1 mM IPTG induced lysates; lane 3, purified VP1 proteins (5 μg).

Western blot analysis

The obtained mouse monoclonal antibodies and rabbit polyclonal antibodies were tested to analyze their specificity

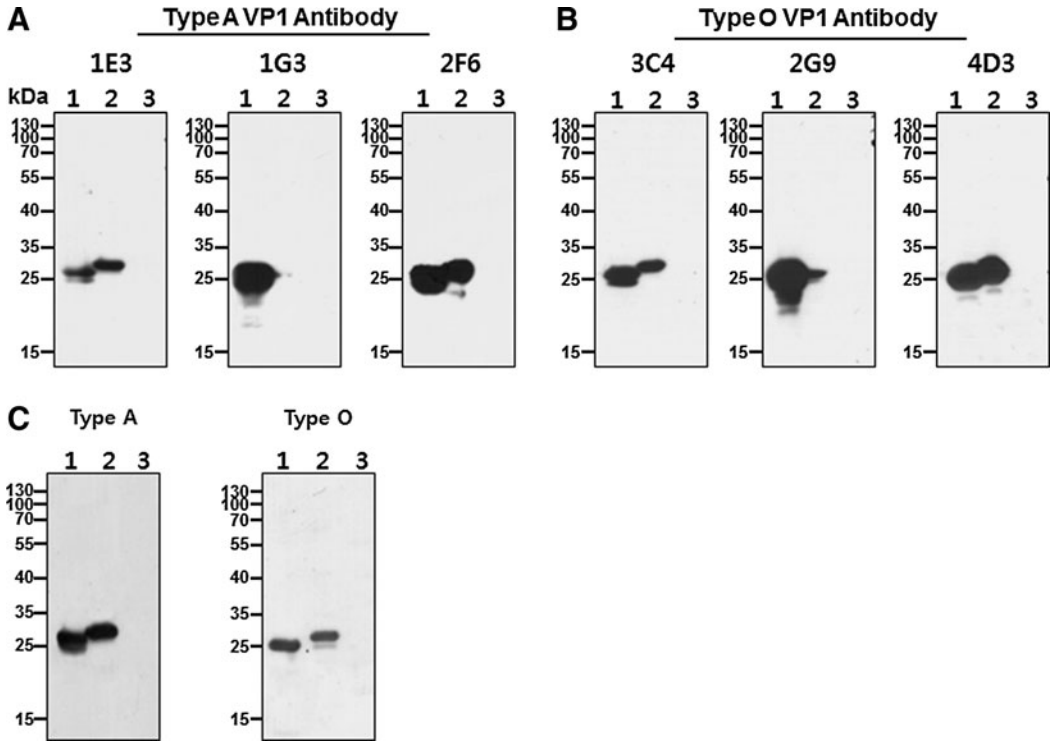

FIG. 2. Western blot analysis using mouse monoclonal and rabbit polyclonal antibodies. HEK293 cells were transiently transfected with pcDNA3 empty vector, pcDNA3-type A VP1 or pcDNA3-type O VP1. 30 μg of protein extracts were loaded to 12% SDS-PAGE and blotted with the indicated hybridoma culture supernatants (**A**, **B**) and with 1:3000 diluted rabbit polyclonal antiserum (**C**). Lane 1, pcDNA3-type A VP1 transfected; lane 2, pcDNA3-type O VP1 transfected; lane 3, pcDNA3 empty vector.

for immunoblot application. HEK293 cells transfected with empty vector and type A VP1 or type O VP1 construct were analyzed by performing Western blotting. Mouse monoclonal anti-type A VP1 or anti-type O VP1 antibodies readily detected exogenously expressed VP1 proteins (Fig. 2A, B). The 1G3 clone among clones generated against type A VP1 showed only specificity to type A VP1, whereas 1E3 and 2F6 showed reactivity in both type A and type O VP1 (Fig. 2A). In the case of monoclonal antibodies generated against type O VP1, there are no clones exclusively specific to type O VP1. All clones were shown to be reactive with both type A and type O VP1 (Fig. 2B). There are no cross-reactive clones with endogenous HEK293 proteins, which implies that all clones generated are specific to type A VP1 or type O VP1 of FMDV. The rabbit polyclonal antibody could readily detect ectopically over-expressed VP1 of FMDV in both types A and O. There was no cross-reactivity in the whole cell lysates transfected with the empty vector, which means that polyclonal antibody could be efficiently applicable for the Western blot analysis (Fig. 2C).

Characterization of antibodies by immunofluorescence staining

We assessed whether the generated antibodies against type A or O VP1 could be available for the application of immunofluorescence staining. IBRS-2 cells were infected by type A or O FMDV with 0.1 multiplicity of infection (MOI) for 18 h, fixed with 4% paraformaldehyde, and incubated with six different hybridoma supernatants or with the polyclonal antibody, as described in the Methods section. Immunofluorescence staining showed that the 1E3, 1G3, and 2F6 clones generated against type A VP1 were reactive with type A FMDV, and the 1E3 and 2F6 clones were also reactive with type O FMDV, whereas the 1G3 clone did not recognize type O FMDV (Fig. 3A). This is in agreement with the results from Western blot analysis, as shown in Figure 2A. In analysis using monoclonal antibodies generated against type O VP1, the 3C4, 2G9, and 4D3 clones were reactive in both type A and O FMDV infected cells (Fig. 3B). In addition, we assessed the FMDV reactivity of polyclonal antibodies generated against types A and O VP1 using immunofluorescence staining. Polyclonal antibodies were also shown to be applicable for the immunofluorescence staining (Fig. 3C). Taken together, the immunofluorescence staining using monoclonal and polyclonal antibodies generated against type A or O VP1 showed that the antibodies worked very well in this application and the infected FMDV is mainly localized in cytoplasm.

Discussion

The Republic of Korea had been free of foot-and-mouth disease (FMD) since 2002 until the outbreaks of FMD of serotypes A followed by serotype O from 2010 to early 2011.[16] In particular, the serotype O that occurred in the province of Gyeongbuk in November 2010 spread nearly nationwide (except in the provinces of Cheonla and Jeju) and caused enormous economic loss and devastation across the country. To increase the resources for developing highly specific and sensitive new diagnostics, we produced and validated

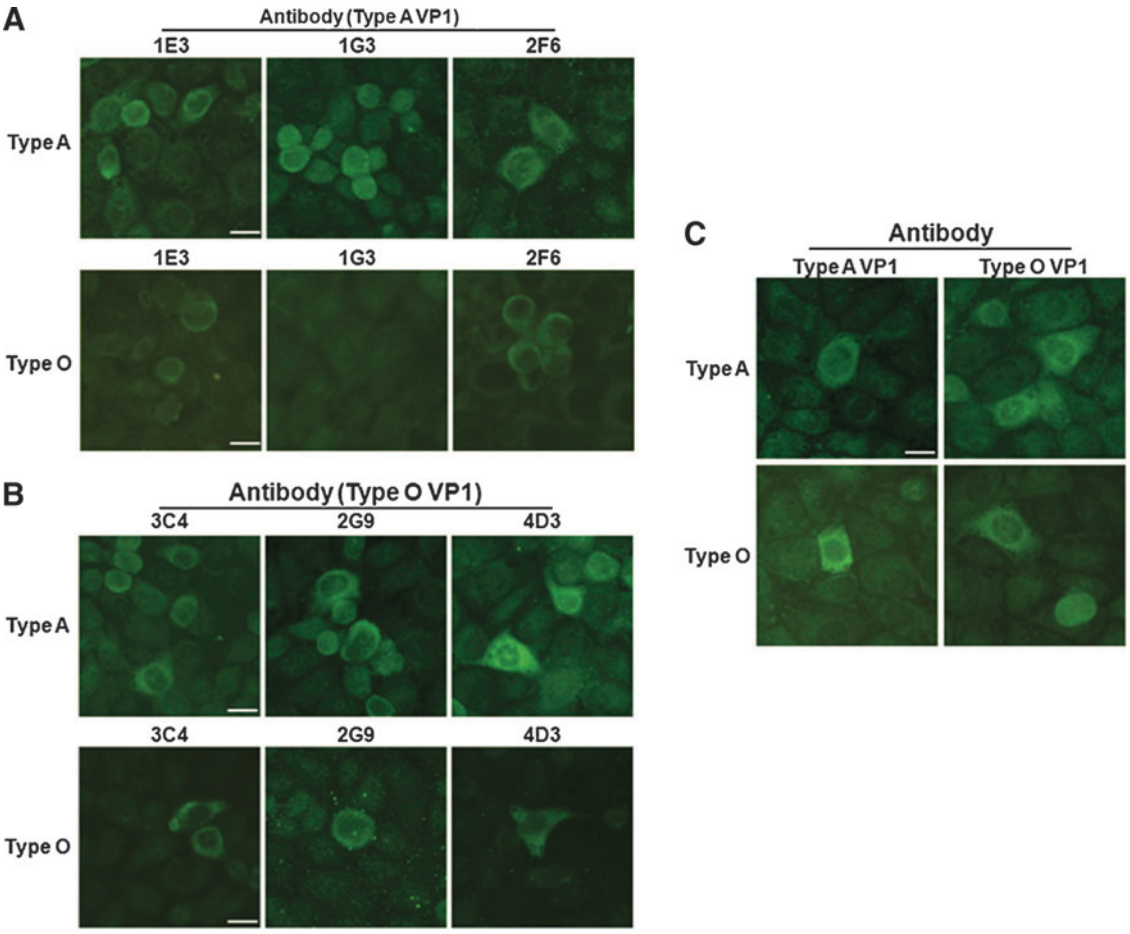

FIG. 3. Immunofluorescence staining analysis. FMDV type A or type O virus (0.1 MOI) were infected to ZZ-R cells and stained with the each indicated mouse hybridoma supernatants generated against type A VP1 (**A**), type O VP1 (**B**), or 1:500 diluted rabbit polyclonal serum (**C**). Scale bar, 50 μm.

monoclonal and polyclonal antibodies applicable to early diagnosis of FMDV.

FMDV VP1 protein is synthesized as part of one single polypeptide and processed by self-contained 3C protease to be one of the four structural proteins VP4, VP2, VP3, and VP1. Across different serotypes, a major antigenic site of FMDV has been identified around amino acid residues 140–160 of VP1 forming the G-H loop in three-dimensional conformation.[17–19] Because the RGD ligand in the G-H loop interacts with cellular integrin receptors, substantial neutralizing antibodies are elicited against those residues.[20,21] Thus, a peptide vaccine including those residues has been developed.[4,22] In addition, carboxy terminus (residues 200 to 213) of that protein has been reported to contribute the antigenicity of FMDV. Thus we cloned and purified VP1 protein using the in-chromatography renaturation method. The identity in amino acids in VP1 between FMDVs of serotypes A (JF792355) and O (JF916985) used for protein expression and production of monoclonal antibody in this study was about 72.4%. The type A virus A/Pocheon/KOR/2010 used for immunofluorescence assay was very close to the virus A/HuBWH/CHA/2009 (JF792355), to which the expressed VP1 protein was referred, with the identity of 98.5% in the amino acid sequence in VP1. Our results showed that most antibodies generated against VP1 of FMDV A and O types have cross-reactivity to each other (Fig. 2A, B), which may imply that the conserved regions between VP1 of FMDV A and O types were highly antigenic. The antigenicity analysis using Protscale tool (http://kr.expasy.org/tools/protscale.html) shows that VP1 of FMDV type A is broader in the scope of antigenicity than VP1 of FMDV type O (data not shown). This is consistent with our results because even if the VP1 of FMDV type O was used as antigen, the generated clone was also reactive with VP1 of FMDV type A, as well as VP1 of FMDV type O (Fig. 2B). The rabbit polyclonal antibodies did not show any difference in the recognition of VP1 of FMDV types A and O.

Taken together, we generated monoclonal and polyclonal antibodies to recognize the VP1 of FMDV type A or type O, and one of them was type A-specific. These antibodies could be applicable for the development of an ELISA or lateral flow device for detecting FMD-specific antibodies or antigens by further characterization.

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Author Disclosure Statement

The authors have no financial interests to disclose.

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