FULL PAPER *Immunology*

Study on the Porcinophilic Foot-and-Mouth Disease Virus I. Production and Characterization of Monoclonal Antibodies against VP1

Ivan-Chen, CHENG[1)], Shu-Mei, LIANG[2)], Wen-Jane, TU[3)], Chi-Min, CHEN[1)], Siou-Yu, LAI[1)], Yung-Chih, CHENG[2)], Fan, LEE[3)], Tien-Shine, HUANG[3)] and Ming-Hwa, JONG[3)]

[1)]Division of Animal Medicine, Animal Technology Institute, P.O. Box 23, Chunan, Miaoli, 35099, [2)]Institute of Bioagricultural Sciences, Academia Sinica, No. 128 Academia Road, Section 2 Nankang, Taipei, 11529 and [3)]Department of Hog Cholera, Animal Health Research Institute, Council of Agriculture, No. 376 Chung-Cheng Road, Tamsui, Taipei, 25158, Taiwan

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ABSTRACT. Monoclonal antibodies (MAbs) reported here were produced against the porcinophilic foot-and-mouth disease virus (FMDV) that caused the devastating swine disease on 1997 in Taiwan. A panel (25) of MAbs were found to react with VP1 of O/Taiwan/97 (O/97) by ELISA with various potencies. The biological identities of these VP1 reacting MAbs, such as neutralization activity, isotype and capability to distinguish between two serotype O FMDVs, O/97 and O/Taiwan/KM1/99 (O/99), were further analyzed. Eleven out of the total eighteen O/97 neutralizing MAbs were able to neutralize heterologous O/99. Eight O/97 neutralizing and five non-neutralizing MAbs could differentiate two serotype O FMDVs by immunofluorescence assay (IFA) implied that these thirteen MAbs recognized O/97 specific epitope(s). Furthermore, reactivities of the VP1 reacting MAbs with a 29 amino acids synthetic peptide (P29) representing the βG-βH loop of VP1 were analyzed by ELISA and fourteen were found positive. MAb clone Q10E-3 reacting strongest with VP1 and P29, neutralizing both but not differentiating two serotype O viruses suggested that the antibody binding site might involve the RGD motif and its C terminal conserved region on βG-βH loop. MAbs with diverse characters presented in this study were the first raised against porcinophilic FMDV. The complete set of MAbs may be used for further studies of vaccine, diagnostic methods, prophylaxis, etiological and immunological researches on FMDV.

KEY WORDS: ELISA, foot-and-mouth disease virus, monoclonal antibody, neutralizing activity, VP1.

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All cloven-hoofed species are susceptible to foot-and-mouth disease (FMD) that is considered the most contagious animal disease. The outbreaks of FMD in Argentina, Europe, Asia, South Africa and Uruguay [8] have brought to world attention the devastating effects of the disease in a naïve population and economic costs of control and eradication. The swine-adapted strain of FMD virus which occurred in Taiwan in 1997 killed more than 4 million pigs, hit related industries heavily and made Taiwan losing FMD free status [27]. To regain the free status, an eradication campaign has been implemented since then.

The FMD virus, O/Taiwan/97 (O/97), produced high morbidity and mortality in swine but did not affect cattle [5]. The distinct host tropism was demonstrated that the mutated 3A coding region of O/97 played a major role in the attenuation of this virus in bovine [3]. Besides, limited biologic character of this porcinophilic virus was known. With the high specificity of antigen (Ag) recognition, monoclonal antibody (MAb) has been applied on the field of determination of antigenic epitope, diagnostic development, vaccine research and studies of viral pathogenesis for years. Therefore, production of MAbs against the porcinophilic FMDV would be beneficial to both basic and applied researches.

This paper described the production of a panel of MAbs against porcinophilic O/97 strain FMDV. Their reactivities with VP1 were determined primarily because VP1 possessed three out of five neutralizing antibody binding sites identified so far [4, 13] and attracted more interest than the other three structural proteins. N-terminal residues 43 and 44 on βB-βC loop, the βG-βH loop and the C-terminal residues onVP1 are recognized as neutralizing antigenic site 3, 1 and 5 respecitvely. The βG-βH loop of VP1, including an Arg-Gly-Asp (RGD) motif at its apex, binds to RGD-dependent integrins of host cell and is involved in neutralization of viral infectivity [12, 14, 17]. It is recognized as a major antigenic and less conformation-dependent site of FMDV [26]. Therefore, a synthetic peptide with RGD motif at the center region and the O/97 βG-βH loop sequence, spanning the amino acid residues of 131–159 of VP1 [25], was used further to map the antigenic site of all the VP1 reacting MAbs. Other biological characters, such as isotype, neutralizing activity and differentiation capability, of these MAbs were also examined and discussed in this study.

MATERIALS AND METHODS

Cell line and viruses: BHK-21 cells, a cell line of baby hamster kidney, were used to propagate FMDV stock and were used in the immunofluorescent assay (IFA) and neutralization assay. PK-15 cells, a cell line of pig kidney, were used in IFA of swine vesicular disease virus (SVDV). Cell lines were cultured in MEM (Minimum Essential Medium) medium containing 5% fetal calf serum (FCS) in a 3.5% CO_2 incubator at 37°C. Two virus isolates of serotype O, the O/97, O/Taiwan/KM1/99 (O/99), and SVDV were used in this study. The O/97 virus used as the immunogen, target of IFA and neutralization assay is a porcinophilic virus strain, which does not infect ruminants in natural route [5].

The O/99 virus, isolated from the Chinese yellow cattle in Kinmen Island during the 1999 outbreaks [11], was used as the target in IFA to study the specificity of MAbs. A SVDV Taiwanese field isolate was a target in IFA for characterizing the MAbs.

Immunogen and immunization: The O/97 virus used as the immunogen was titrated for infectivity containing a titer of 10^7 $TCID_{50}$/0.1 m*l*. Two-month-old female BALB/c mice kept in Animal Health Research Institute P3 laboratory were immunized with 10^7 $TCID_{50}$ infectious FMDV in 0.1 m*l* MEM each time at 3-to 4-week intervals except the last booster. The first and second immunizations were intraperitoneal injections. The third immunization and last injections three days before fusion were given to the anesthetic mice via intrasplenic route [10]. Animals were bled and sera neutralization titers were examined to monitor the individual immune response.

Monoclonal antibodies: Splenocytes from mouse showed the highest virus neutralization (VN) titers to O/97 were fused with SP2/0-Ag14 myeloma cells to produce the hybridoma according to the standard protocol [9]. Hybridoma secreting antibodies (Abs) against O/97 virus were selected by IFA on acetone-fixed Ag plate described as the following. Positive hybridoma was cloned three times by the limiting dilution method. The maximum number twenty monoclonal hybridomas were selected and named individually from one parental hybridoma on the basis of positive IFA. One monoclonal hybridoma was picked randomly from a parental to inoculate intraperitoneally into pristine-primed BALB/c mice to produce ascitic fluid containing MAb. The hybridoma cultural supernatant and ascitic fluid were collected for subsequent characterization. Isotypes of MAbs from hybridoma cultural supernatant were analyzed by IsoStrip® Isotyping kit (Roche, IN U.S.A.) according to the manufacturer's recommendation. This study was designed to use a minimum number of animals and approved by an independent review board organized by the institute to ensure that local legal and ethical requirements for care and use of laboratory animals were complied with.

Neutralization assay: VN assay was performed according to the OIE manual [19]. Briefly, the titers of VN were determined in a microdilution test using the BHK-21 cells. Serial dilutions of sera samples were incubated with a virus dose of 100 $TCID_{50}$ of the O/97 virus. Microplates were incubated at 37°C for 2 days before being examined microscopically for CPE. The fifty percent end point of neutralization titers were calculated using the method of Reed-Muench [21]. The procedures of microneutralization (MN) assay [7] for the detection of MAb neutralization activity were followed with modification. In principle, both of serial diluted mouse ascitic fluid and equal volume of 100 $TCID_{50}$ of the O/97 or O/99 virus prepared with 2% FCS in MEM were mixed and incubated at 37°C for 1 hr. Each virus/diluted MAb mixture was then applied to eleven wells of a 96-well microtiter plate. BHK-21 cells 5×10^4/0.1 m*l*/well were added as well. After incubation for 2 days at 37°C, CPE was scored under microscope first and IFA with pooled MAbs obtained in this study was further applied to confirm the results. The fifty percent end point of neutralization titers for O/97 or O/99 were calculated. The amount of virus actually used per well should contain 100 $TCID_{50}$ within an acceptable range (e.g. 35–350 $TCID_{50}$) from back titration control. Neutralization assays were repeated at least two occasions.

Immunofluorescent assay: The IFA was performed on Ag plates which were constituted of BHK-21 monolayer infected by 4×10^3 $TCID_{50}$/well O/97 and incubated for 6–8 hr till the appearance of CPE under microscope examination. The Ag plates were then fixed by icy cold 70% (v/v) acetone and kept at –70°C for storage. The preparation of Ag plates of O/99, and SVDV were similar to that of O/97. The Ag plates were thawed and washed three times with phosphate buffered saline (PBS) before adding 50 μl hybridoma cultural supernatant or diluted mouse ascitic fluid for IFA. After room temperature incubation for 2 hr, plates were washed 3 times with PBS and Fluorescein-conjugated goat IgG to mouse IgG, IgA, IgM (ICN Pharmaceuticals, OH U.S.A.) at 1:1,000 dilution was added 50 μl/well. One hr room temperature incubation was followed, the plates then were washed as mentioned above and observed under inverted microscope. The highest dilution of mouse ascitic fluid gave the Ag plate a strong and evident (+++) fluorescence of positive cell at 100 magnification was recorded as the IFA dilution titer.

Assay for VP1 and P29 antibody titer by ELISA: The recombinant VP1 (rVP1) capsid protein was expressed in *E. coli*, purified and refolded. Also, a polypeptide P29 (NGSSKYGDTSTNNVRGDLQVLAQKAERTL), representing the amino acid residues 131–159 of VP1 of the FMDV O/97, was synthesized as described previously by Wang *et al.* [25]. rVP1 and P29 were then used respectively in ELISA tests as an Ag source. To determine the titer of anti-rVP1 or P29, 96-well microtiter plates were coated with rVP1 or P29 (20 μg/m*l* in coating buffer containing 45 mM $NaHCO_3$, 18.2 mM Na_2CO_3, at pH 9.6; 50 μl/well). After incubation at 4°C overnight, the plates were blocked with 100 μl of 5% (w/v) skim milk in PBS, and washed three times with PBS containing 0.1% (v/v) Tween-20 (PBST). Ascitic samples in serial dilution were added to the wells (50 μl/well) and incubated for 1 hr at 37°C. Plates were then washed three times with PBST, and treated with biotinylated horse anti-mouse IgG Abs (Vector; 1:3,000 dilution in PBS; 50 μl/well) for 1 hr at 37°C. Subsequently, the plates were washed thoroughly with PBST, and avidin DH and biotinylated horseradish peroxidase H complex (Vector; 1:3,000 dilution in PBS; 50 μl/well) were added. After incubation for 1 hr at room temperature, the wells were then washed, enzyme substrate 3,3',5',5-tetramethylbenzidine (TMB, Vector; 50 μl/well) was added and the reaction was carried out at room temperature for 20 min. Finally, an equal volume of 1 N H_2SO_4 solution was added to stop the reaction and the absorbance at 450 nm was measured by an ELISA reader. The titer was defined as the reciprocal of the dilution that resulted in an absorbance value 50% of total value obtained from subtracting maximum absorbance with

background absorbance.

RESULTS

Monoclonal antibodies: The BALB/c mouse showed VN titer at 1,024 when it was sacrificed on the fusion day. Using the IFA assay, 67 parental hybridoma cells with positive reaction against O/97 virus were frozen for further cloning. The maximum number of monoclone selected was twenty from one parental hybridoma due to the high fusion rate. Three hundred and four monoclonal hybridomas were obtained from forty-two parental hybridomas by two sequential limiting dilution. However, the effort to gain a positive clone in at least three cloning trials from the other twenty-five parental hybridomas was failed. One monoclonal hybridoma from each forty-two parental was chosen randomly as its representative and the ascitic fluid was produced for further characterization. The same batch of MAb was tested on the O/99 Ag plate and the results were recorded (Table 1). None of the MAb showed positive IFA reaction on SVDV Ag plate (data not shown). All MAbs had κ light chain isotype and different IgG isotypes of heavy chain (Table 1).

Neutralization assay: The neutralization activities with O/97 and O/99 of those 25 VP1 reacting MAbs were shown as MN titers (Table 1). Eighteen MAbs which were classified into clusters 1 to 6 neutralized the infectivity of O/97 virus with various potencies. Seven MAbs restricted to neutralize homologous O/97 while the other eleven MAbs neutralized both O/97 and heterologous O/99 FMDV. Additionally, seven MAbs from cluster 7 to 9 behaved as non-neutralizers to both strains.

Immunofluorescence assay: The O/97 or O/99 IFA titer of each MAb/ascites was determined based on the highest dilution of ascites to give strong IFA reaction on O/97 or O/99 Ag plate. The MAbs IFA titers of O/97 were between

Table 1. Characterization of MAbs against O/Taiwan/97 isolate

Cluster #	MAb Name	Heavy chain isotype	MN titer[a)]		IFA +++ titer[b)]		ELISA titer[c]	
			O/Taiwan/97	O/Taiwan/KM1/99	O/Taiwan/97	O/Taiwan/KM1/99	Anti-rVP1	Anti-P29
1	N3C-3	G1	160	320	12,000	16,000	1,542	2,330
	N7H-2	G1	640	640	50,000	4,000	1,005	–
	Q11C-15	G1	2,560	2,560	32,000	32,000	1,019	–
2	Q10E-3	2a	10,240	5,120	16,000	16,000	606,000	205,280
	S2D-2	2a	12,800	10,240	20,000	8,000	4,097	1,202
	S10F-1	2a	25,600	10,240	60,000	16,000	3,069	1,338
	P12D-1	2a	1,280	640	10,000	32,000	1,282	–
3	S12G-6	2a	3,200	–	40,000	–	22,440	1,595
	P8C-5	2a	320	–	60,000	–	6,215	–
	M4F-16	2a	1,280	–	16,000	–	5,510	–
	Q5C-10	2a	2,560	–	16,000	–	1,451	3,119
4	N10E-1	2b **2a**	12,800	640	40,000	–	14,305	–
	P11A-15	2b **G3**	160	–	40,000	–	4,009	–
5	P2B-1	G3	2,560	320	16,000	8,000	4,712	1,683
	P3B-3	G3	2,560	640	20,000	8,000	3,463	–
	T7C-3	G3	3,200	320	16,000	8,000	1,106	1,050
6	O4A-1	G3	80	–	20,000	–	4,478	1,156
	O4D-1	G3	80	–	60,000	–	1,000	1,658
7	P9E-1	G1	–	–	16,000	8,000	25,099	–
	S3G-1	G1	–	–	16,000	8,000	4,342	1,325
8	R3C-10	G1	–	–	32,000	–	7,921	1,775
	S8B-1	G1	–	–	32,000	–	1,347	–
9	T5H-12	2b	–	–	40,000	–	23,755	5,690
	Q2B-13	2b	–	–	32,000	–	6,497	–
	S7C-10	2b	–	–	40,000	–	3,478	3,164

a) "–" Meant the MN titer of ascites less than 80 fold dilution.
b) "–" Meant the dilution of ascites to give strong IFA reaction less than 2,000 fold.
c) "–" Meant the ELISA titer of ascites less than 1,000 fold dilution.

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10,000 and 60,000 fold. In general, the MAbs IFA titers of O/99 were lower than that of O/97. Those MAbs had titers under 2,000 fold were scored as negative.

VP1 and P29 ELISA: The anti-rVP1 and P29 antibody (Ab) titer of MAb/ascites greater than 1,000 was considered positive in this study. Twenty-five MAbs out of fourty-two showed positive results with various anti-rVP1 Ab titers (Table 1). Fourteen MAbs, composed of ten O/97 neutralizers and four non-neutralizers, showed positive P29 reaction with diverse Ab titers. Eight O/97 neutralizers and three non-neutralizers did not react with P29 (Table 1). Five clones–Q10E-3, S12G-12, N10E-1, P9E-1 and T5H-12–with anti-rVP1 titers greater than 10,000 on VP1 ELISA were considered excellent rVP1 responders in this study. MAb Q10E-3 showed extremely strong reaction on P29 ELISA in contrast to the other 13 responders only reacted with P29 mildly–ELISA titer lower than 5,000 in general.

For the convenience of data presentation, twenty-five MAbs with rVP1 reactivity were first divided by neutralizing activity with homologous O/97 then grouped by the order of their heavy chain isotype. The reactivity with heterologous O/99 on IFA was applied to further categorize the MAbs with the same isotype. Nine clusters were finally classified (Table 1). MAbs in the same cluster were ranked by the anti-rVP1 Ab titer and followed by anti-P29 Ab titer to illustrate the possible relationship between Ab binding site and βG-βH loop.

Clusters 1 to 6 were O/97 neutralizing MAbs. Although with different P29 ELISA activities, cluster 1 MAbs were all isotype G1, neutralized and recognized O/99 virus. Cluster 2 MAbs were isotype 2a, neutralized heterologous serotype O FMDV while exhibited great variation in anti-P29 activity. Cluster 1 and 2 MAbs all failed to differentiate homologous from heterologous FMDV on IFA. Cluster 3 MAbs were isotype 2a, but O/99 non-neutralizers and non-reactors. Their anti-P29 ELISA titers were varying. Cluster 4 MAbs were isotype 2b, non-reactors of heterologous virus on IFA and P29 tests, but of different O/99 neutralizing capability. Cluster 5 MAbs were isotype G3, neutralized and reacted to O/99, weak- or non-reactors of P29. Cluster 6 MAbs carried isotype G3, weakly neutralized homologous O/97 and reacted to P29, neither MN nor IFA activity for O/99 was detected. MAbs from clusters 7 to 9 did not neutralize homologous O/97 and possessed various P29 activities. Clusters 7 and 8 MAbs were all isotype G1. The later could differentiate two serotype O viruses on IFA but the former not. Cluster 9 MAbs were isotype 2b, O/99 IFA non-responders, while exhibited various P29 activities.

DISCUSSION

Three major procedures, immunization of live virus, intrasplenic injection and screening by IFA were undertaken in this study to develop a maximum varieties of MAbs for research and application in the future. A panel of new mouse MAbs, raised against an unique isolate of porcinophilic FMDV O/97 was obtained finally on one fusion task. Although BALB/c mice were not the natural host of FMD, it still replicated FMDV for a maximum of 60 hr [23] which made it a perfect bio-reactor to generate MAbs against all structural and non-structural viral proteins expressed. Neutralizing Ab titer of the immunized BALB/c mouse did show a high titer as 1,024 and gave a good start in this study. Basically, the Abs produced by live virus immunized BALB/c should be similar to the Abs produced by the natural host. Besides, MAbs originated from live virus immunization strategy would favor the selection of escaped mutants for the purpose of mapping the neutralizing antigenic sites in the long run. Therefore, the MAbs generated here would fit well to the needs of studies on viral relatedness, vaccine strain choice and so on.

Intrasplenic immunization was proven to give a strong response and resulted in a high frequency of specific MAbs [10]. To pick up the positive hybridoma secreting immunoglobulin against the immunogen, the IFA on susceptible BHK monolayer infected by optimum dose of FMDV was chosen to screen the parental and monoclonal hybridoma cells. The existence of both FMDV-infected and normal BHK cells with their distinct morphology on IFA Ag plates clearly demonstrated that Ab either bound to viral or cellular components and favored the selection of FMDV specific MAb. Moreover, the antigenicity of viral protein kept by acetone, the fixative on the plate, was almost the same as that of the live virus thus beneficial to be recognized by the elicited MAbs. MAbs in ascites reported in this study were of high (from 10,000 to 60,000) O/97 IFA titers. MAb showing negative IFA result on O/99 Ag plate implied that this MAb possessed O/97 specific binding site.

A well established VP1 ELISA was utilized to analyze these fourty-two MAbs primarily. Synthetic peptide P29 representing the sequence of βG-βH loop was employed further to examine the activities of twenty-five VP1 reacting MAbs. The positive results of fourteen MAbs on P29 ELISA indicated that their MAb binding sites located on the βG-βH loop region. Those eleven P29 negative MAbs recognized antigenic epitope(s) outside the βG-βH loop *vice versa*.

The possible antigenic sites of five selected MAbs reacting with VP1 strongly are discussed. Firstly, Q10E-3 was the strongest rVP1 and P29 reacting MAb among all, it neutralized both O/97 and heterologous O/99 efficiently but could not distinguish those two strains. It is suggested that Q10E-3 must bind to the conserved neutralizing antigenic site shared by the two serotype O FMDVs. The sequence alignment of VP1 a.a. 131–159 of O/97 and O/99 [15, 24] shows 72% identities (21/29) on Table 2 to illustrate the possible binding sites of MAbs. The antigenic epitope recognized by Q10E-3 would lie somewhere from a.a. residues 143 tending to the C terminus of P29 due to residues 143–159 region not only containing the RGD motif but characteristic of high sequence identities (16/17) between this 2 serotype O FMDVs. Nevertheless, O/97 and O/99 show seven differences in the interval between positions 131 and 142 situated on the N-terminal region of P29. Amino acid

Table 2. Alignment of VP1 a.a. 131–159 (P29) of O/Taiwan/97 and O/Taiwan/KM1/99

O/Taiwan/97	131	NGSSKYGDTSTNNV**RGD**LQVLAQKAERTL	159
O/Taiwan/KM1/99	131	NGNCKYGESPVTNV**RGD**LQVLAQKAARTL	159
identities: 72% (21/29)			

substitution on viral surface protein usually causes heterogenicity and affects Ab recognition [18]. Being a non-neutralizing MAb and the P29 second-best reactor, T5H-12 binding site would sit on somewhere βG-βH loop but exclude the RGD motif. The antigenic site recognized by T5H-12 would be mainly the sequence heterogenic region, such as a.a. 131–142, on P29 as a result of not reacting with the heterologous O/99 FMDV on IFA.

In view of the negative reactivity for P29, the Ab binding site of neutralizing MAb N10E-1 should be irrelevant to the βG-βH loop. Binding on one of the other two VP1 conformational antigenic sites—the βB-βC loop and C terminal residues—might be responsible for the neutralizing activity of N10E-1. It is suggested that N10E-1 may bind to the sequence heterologous region between O/97 and O/99 because of not reacting with heterologous O/99 on IFA. The Ab binding site of P9E-1 is indeed nothing to do with the 3 identified neutralizing antigenic sites on VP1 due to the incompetence of neutralization and P29 reactivity. However, positive result of IFA with heterologous O/99 indicated that the P9E-1 binding site located on the conserved region between this 2 serotype O viruses. Lastly, the O/97 neutralizer but heterologous O/99 non-neutralizer S12G-6 reacted with P29 weakly and did not react with O/99 on IFA. It suggested that the neutralization binding site of S12G-6 was O/97 specific and might irrelevant to βG-βH loop.

Except the five MAbs discussed above, S2D-2 and S10F-1 have about the same level of neutralizing activities for both O viruses as Q10E-3 has but two hundreds fold less on Ab titer of VP1 and P29 reacting capabilities than Q10E-3 displays (Table 1). In the other word, neutralizing activities of these 3 MAbs are roughly the same, but their binding capability with rVP1 and P29 are significantly different. Current analysis hardly explained the phenomenon that S2D-2 and S10F-1 showed much weaker responses with rVP1 and P29 on ELISA than Q10E-3 did. Further studies, such as mapping the amino acid sequences of complementarity-determining regions (CDRs) of Q10E-3, S2D-2 and S10F-1, or analyzing the sequences of MAb escaped mutants will answer why Q10E-3 bears the dramatically high rVP1 and P29 ELISA reactivities and find out the antigenic site for each MAb.

It is generally accepted that the specific humoral immune response not only the most important factor in conferring protection from FMDV infection but also an critical *in vitro* parameter of evaluating the efficacy of vaccine. In this respect, strong correlation between virus neutralizing Ab and protection for bovines, one of the main target species, was documented [20]. Five neutralizing antigenic sites have been identified as well since then with the aim of developing more effective vaccines [22]. Studies on delineating those neutralizing sites have mainly involved the sequencing of escape mutant viruses especially the O, A and C serotypes produced after selection with murine neutralizing MAbs. It was recently shown that the mouse recognized similar antigenic features to those seen by bovines [2]. Murine MAbs therefore play an important role on vaccine development. Interestingly, pig did not responded to epitopes on the carboxyl terminus end as efficiently as the ruminant did [1]. Question follows whether the Abs repertoire responded to FMDV by the pig are the same as that by the mouse. Further characterization on the eighteen neutralizing MAbs presented here may answer the question particularly with regard to strain O/97, a swine adapted FMDV, and merit to the relevant vaccine development.

According to the study on serotype C FMDV, the epitopes recognized by ten non-neutralizing MAbs raised against two different isolates of type C FMDV were conserved in all 46 isolates analyzed [16]. In our study, five of the seven non-neutralizing MAbs could differentiate the two serotype O FMDV Taiwan isolates. It indicated that the epitopes of O/97 recognized by these five non-neutralizing MAbs were not conserved in O serotype. The discrepancy of non-neutralizing MAbs performance between our results and the previous ones deserves further investigation, such as, the examination of more serotype O isolates to find out the truth of the difference.

Phagocytosis is another well-known mechanism other than neutralization to defend the viral infection by the humoral immunity. In this respect, the *in vitro* neutralizing activity does not completely reflex the Abs which contribute to the viral opsonization process preceding to the phagocytosis. Also, the neutralizing activity defined by the *in vitro* assay might not play an essential role in the mechanism of immunity induced by whole inactivated FMDV vaccines [6]. All of the above implied that non-neutralizing Abs might confer the point of *in vivo* protection to FMDV infection. In this point of view, those non-neutralizing MAbs identified in our study have niche. Once the contribution of non-neutralizing MAb-mediated mechanisms of FMDV become appreciated and quantified, improved correlates of evaluating all the neutralizing and non-neutralizing activity of FMDV can be devised to permit the replacement of current *in vivo* potency testing. Moreover, frequent operation on live virus always brings a serious concern of bio-hazardousness. Not only the challenge potency test, the going *in vitro* neutralization assay also handles live virus routinely. Should neutralizing and non-neutralizing MAbs obtained from our study be applied on a new design of *in vitro* assay, like competition ELISA, for detecting the complete humoral immunity of host animal. It will fit perfectly the practical needs of bio-security and assessment of the potency of

FMDV vaccination.

The continuous and complete analysis on the twenty-five MAbs reported here and the other seventeen rVP1 non-reactors (data not included), some of them carrying neutralizing activity, will reveal the biological characters of O/97 thoroughly. The number and location of its neutralizing antigenic sites and the relevance will then be answered and beneficial to the development of the vaccine and diagnostics furthermore.

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