VIROLOGICA SINICA, October 2012, 27 (5):316-319
DOI 10.1007/s12250-012-3261-x

Brief Report

Generation of Monoclonal Antibodies against Non-structural Protein 3AB of Foot-and-Mouth Disease Virus*

Tong Lin, Junjun Shao, Huiyun Chang✉, Shandian Gao, Guozheng Cong and Junzheng Du

State Key Laboratory of Veterinary Etiological Biology, National Foot and Mouth Disease Reference Laboratory, Lanzhou Vetersinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou 730046, China

To identify linear epitopes on the non-structural protein 3AB of foot-and-mouth disease virus (FMDV), BABL/c mice were immunized with the 3AB protein and splenocytes of BALB/c mice were fused with myeloma Sp2/0 cells. Two hybridoma monoclonal antibodies (mAbs) cell lines against the 3AB protein of foot-and-mouth disease virus (FMDV) were obtained, named C6 and E7 respectively . The microneutralization titer was 1:1024 for mAb C6, and 1:512 for E7. Both mAbs contain kappa light chains, and were of subclass IgG2b. In order to define the mAbs binding epitopes, the reactivity of these mAbs against FMDV were examined by indirect ELISA. The results showed that both mAbs can react with FMDV, but had no cross-reactivity with Swine Vesicular Disease (SVD) antigens. The titers in abdomen liquor were 1:5×10^6 for C6 and 1:2×10^6 for E7. In conclusion, the mAbs obtained from this study are specific for the detection of FMDV, can be used for etiological and immunological researches on FMDV, and have potential use in diagnosis and future vaccine designs.

Foot-and-Mouth disease virus (FMDV); Monoclonal antibody (mAb); 3AB

Foot-and-mouth disease (FMD) is a highly contagious and the most important animal disease caused by foot-and-mouth disease virus (FMDV). FMDV is an RNA virus belonging to the *Aphthovirus* genus within the family *Picornaviridae* and has seven distinct serotypes (O, A, C, SAT1-3 and Asia 1) and numerous subtypes[10]. Once outbreak of the disease takes hold it can severely impact livestock industry and cause huge economic losses in affected countries or regions. Therefore, vaccination of susceptible animals is one of the major strategies and is extensively used to control and prevent occurrence of FMD in developing countries where the disease most frequently occurs. However, this has raised a new problem of how to distinguish between infected and vaccinated animals in herds after immunization. The identification of infected animals is important to facilitate monitoring animal health in a herd, and to help the control and eradication of the disease in countries where FMD is endemic and controlled by preventive vaccination. Vaccinated animals principally produce antibodies against structural proteins (SPs) as the vaccines are based on partially purified killed virus, while a true FMDV infection induces antibodies to both the structural proteins (SPs) and non-structural proteins (NSPs) [8,11]. Thus, a serological survey for antibodies to NSPs of FMDV can be used to differentiate infected from vaccinated animals in a herd[1,11]. Based on this premise, several ELISA tests based on regions of the NSP 3ABC have been developed to differentiate infected and vaccinated animals in a herd [2,3,9].

There are nine FMDV non-structural proteins which are primarily involved in replication and assembly functions. In this work we investigated the ability of monoclonal antibodies (mAbs) to the non-structural protein 3AB to distinguish natural infection and immune response to vaccination. In this study, an *E.coli* system was used to express the 3AB epitope and specific mAbs targeting the 3AB protein were generated. The mAbs showed high specificity and sensitivity, and have potential use in future laboratory diagnostics.

MATERIALS AND METHODS

Preparation 3AB protein and cells

3AB protein from the 3AB gene of FMDV O/China/99 isolate and expressed in an *E.coli* expression system as a recombinant protein attached to His-tag using the pET-30a(+) expression system (Novagen)[8,10,12].

The A/AV88 virus strain (isolated in Russia in 1958) and

Received: 2012-05-31, Accepted: 2012-09-12
* Foundation items: State Key Projects of Transgene Program (No.2011ZX08011-004; 2009ZX08007-008B; 2009ZX08006-002B).
✉ Corresponding author.
Phone: +86-931-8342359, Fax: +86-931-8342359,
Email:changhuiyun@126.com

FMDV O, Asia1 and C serotypes and swine vesicular disease virus (SVDV) used in this study were obtained from the National Foot and Mouth Disease Reference Laboratory, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

Sp2/0 cells were purchased from ATCC (Manasa, VA) and were cultured in EX-CELL® CD Hybridoma Medium (sigma) supplemented with 10 % fetal calf serum. BHK-21 cells were preserved by LVRI and cultured in DMEM supplement with 10 % fetal calf serum.

Complete Freund's adjuvant, incomplete Freund's adjuvant, Horseradish peroxidase, Tetramethylbenzidine and Dimathyl sulfoxide (DMSO) were purchased from Sigma Chemical Co., USA.

Preparation of FMDV

Baby hamster kidney clone 21 (BHK-21) cells were used for FMDV replication. The virus infected BHK-21 cells were cultured in Glasgow's MEM supplemented with 2 mmol/L glutamine and 50 g/L gentamicin. Viruses were harvested after 24 h post-infection and collected by centrifugation at 3500 r/min for 30 min. About 10 mmol/L 2-bromo-ethylamine hydrobromide (BEI) was used to inactivate viruses for 24 h at 37 ºC. After 24 h, any remaining BEI was inactivated using sodium thiosulphate to a final concentration of 2%[1].

Mice immunization and generation of hybridoma cells

Female BALB/c mice of 5–6 weeks old were immunized subcutaneously with 20 mg of purified 3AB protein in an equal volume of complete Freund's adjuvant[7,13]. Three identical boosters emulsified in incomplete Freund's adjuvant were given at 4 week intervals. Mice were boosted with the same antigen in PBS by intraperitoneal injection 3–4 days before fusion. After three rounds of immunized the mice were euthanized. Immunized splenocytes were then obtained, and fused with SP2/0 cells at 5–10:1 ratio in the presence of 50% polyethylene glycol (Merck). 1640 medium with 10% fetal bovine serum was used for fusion and subcloning. The cells were plated out in semisolid medium (Stem Cell) and incubated at 37 ºC in a humidified 5% CO_2 atmosphere. After 2 weeks, single colonies were transferred to 96-well culture plates. Hybridoma supernatants were screened using an indirect ELISA (developed by our Lab). The positive hybridomas were subcloned using the limiting dilution technique. The mAb isotyping was performed using a mouse monoclonal antibody isotyping kit (Isostrip, Roche).

Isotying and isoelectric focusing (IEF)

The isotypes of two mAbs were determined by adding 25 μL of the cell culture supernatant containing C6 and E7, respectively, with 200 μL assay buffer to wells coated with each of the rabbit anti-mouse antibodies from the mouse MonoAb ID kit (Sigma) against IgG1, IgG2a, IgG2b, IgG3, IgA and IgM. Bound mAbs were detected by goat anti-rabbit IgG-HRP conjugated antibody (Sigma). IEF was performed in IEF gel pH 3–7 (Invitrogen). Electrophoresis was run following the manufacturer's protocol[14].

ELISA

A 96-well flat-bottomed plate was coated with serially diluted inactivated FMDV cell virus. Plates were then washed three times with PBS (containing 0.05% Tween 20), and then blocked with[6] 1 % BSA at 37 ºC for 2 h. Two mAbs were added (100 ng/well) and incubated at 37 ºC for 2 h. After washing with PBST, an HRP-conjugated goat anti-mouse IgG (Sigma) was used for detection.

Western blot analysis

Purified (Saturated ammonium sulfate and Sephacry S-300HR) C6 and E7 were subjected to SDS-PAGE according to the standard method and transferred to 450 nm nitrocellulose membranes (Bio-Rad) for 1 h. The blots were then blocked with Tris-buffered saline (TBS) containing 0.1% (w/v) casein for 2 h at room temperature. After washing three times with TBS, the blots were incubated with TBS for 1 h at 37 ºC. The membranes were washed three times with TBS and incubated with goat anti-mouse IgG conjugated to HRP (Bio-Rad Laboratories, Hercules, CA) for 1 h at 37ºC. Following the washes with TBS, the plates were incubated with the enzyme substrate solution containing 0.5 mg/mL 4-chloro-1-naphthol, 0.15% (v/v) hydrogen peroxide and 25% (v/v) methanol[5].

mAbs characteristic analysis

The specificity, stability and titers of mAbs were examined by ELISA (using the same procedure as described in above). For investigating specificity, the two mAbs were reacted with O, A and C serotypes of FMDV, but no reaction with SVDV was observed. The stability and titers of prepared mAbs were analysed by ELISA in different passaged generations.

RESULTS

Purification of 3AB protein

The 3AB was purified by one-step affinity purification using glutathione Sepharose 4B as described elsewhere[4,10,12]. The presence of recombinant protein in the eluted fractions was confirmed by SDS-PAGE. The recombinant 3AB protein weight was 33 kDa, which accounted for 30 % of total protein in *E. coli* lysates (Fig. 1).

mAb production

In this study, two mAbs (C6 and E7) were obtained from the weights of the heavy chain and light chain were about 45.0 mice

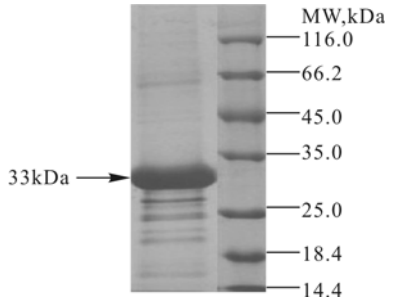

Fig.1. Analysis of FMDV O type by SDS-PAGE.

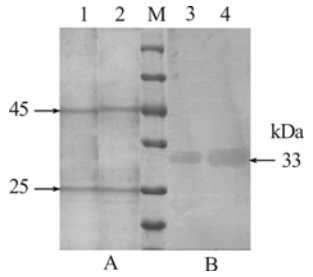

Fig. 2. Analysis of purified mAbs by SDS-PAGE and Western-blot. Lane 1 is mAb C6 SDS-PAGE; lane3 is C6 Western-blot ; lane 2 is SDS-PAGE and lane 4 is Western-blot of mAb E7; M, Protein molecular weight markers.

abdominal cavity. SDS-PAGE showed that the molecular and 25.0 kDa (Fig. 2), which was consistent with the predicted molecular weight, and reacted to the 3AB protein specifically with Western blot (Fig. 2).

Cross-reaction

In this test, FMDV O, A, Asia1 and C Type and swine vesicular disease (SVD) were used to detect the specificity of prepared mAbs. The results indicated that no cross reaction was found with SVDv. The mAbs reaction abilities with FMDV O, Asia1,A and C Type antigens were shown in Table 1.

Isotype identification

In the isotype test, C6 and E7 were associated with IgG2b. As shown in Table 2, the ascites titers of mAbs were between 1-2×10^6. In the stability test, the titers of prepared mAbs were consistently maintained when passaged to thirty generations (as shown in Table 2). All of these results showed that the developed mAbs possessed good specificity and high titers.

DISCUSSION

In this study we vaccinated BABL/c mouse with purified 3AB protein, then the spleen cells were taken out and fused with Sp2/0 cells to generate mAbs. Two mAb stains were screened out. Before fusing spleen cells to Sp2/0 cells, the antibody titer of the immunized BALB/c mouse was found to be as high as 1:1024, which was an indication of good preparation. In this study, we also found that using expressed 3AB protein immunized BALB/c mice is a good strategy to prepare mAbs, since this method avoids the possibility of live virus escaping from laboratories[6].

Two anti-FMDV 3AB protein mAbs, C6 and E7, were raised and characterized. ELISA and Western blot analysis showed that the obtained mAbs were highly specific to FMDV and did not cross-react with SVD antigens.

Table 1. Specific test results of selected mAbs

	O	A	Asia1	C	3AB	SVDV
C6	+	+	+	+	+	-
E7	+	+	+	+	+	-

Note: "+" Positive reaction; "-"Negative reaction. (according to previous experiments, an OD value more than 0.4 is considered positive, less than 0.4 is considered negative.)

Table 2. Isotype identification and titer

	IgG1	IgG2a	IgG2b	IgG3	IgA	IgM	titer
C6	-	-	+	-	-	-	1:5×10^6
E7	-	-	+	-	-	-	1:2×10^6

Note: " + " Positive reaction; " - " Negative reaction.

Presently, FMD is a highly contagious and devastating zoonotic disease seriously threatening public health that causes significant financial losses. Rapid and effective diagnoses as well as proper preventive measures are particularly important. Monoclonal antibodies prepared in this experiment were identified as anti-3AB protein monoclonal antibodies and have an important application value. Use of these monoclonal antibodies to develop corresponding detection techniques will be the next steps to allow their use in the near future for the diagnosis and distinction between of infection and vaccination and control of FMDV vaccine quality.

References

1. **Barteling S J.** 2004. Modern inactivated foot-and mouth disease vaccines: historical background and key elements in production and use. In: **Foot-and-Mouth Disease: Current Perspectives**, Sobrino F, Domingo E, ed. England: Horizon Bioscience, pp305–333.
2. **Freiberg B, Hohlich B, Haas B, *et al***. 2001. Type-independent detection of foot-and-mouth disease virus by monoclonal antibodies that bind to amino-terminal residues of capsid protein VP2. **J Virol Methods,** 92:199–205.
3. **Guo H, Liu Z, Sun S**, ***et al***. 2005. Immune response in guinea pigs vaccinated with DNA vaccine of foot-and-mouth disease virus O/China99.**Vaccine**, 23:3236–3242.
4. **Hema M, Nagendrakumar S B, Yamini R, *et al.*** 2007. Chimeric tymovirus-like particles displaying foot-and-mouth disease virus non-structural protein epitopes and its use for detection of FMDV-NSP antibodies. **Vaccine,** 25: 4784–4794.
5. **Jackson T, King A M, Stuart D I, *et al.*** 2003. Structure and receptor binding. **Virus Res**, 91:33–46.
6. **Lee H S, Heo E J, Jeoung H Y, *et al.*** 2009. Enzyme-Linked Immunosorbent Assay Using Glycoprotein and Monoclonal Antibody for Detecting Antibodies to Vesicular Stomatitis Virus Serotype New Jersey. **Clin Vaccine Immunol**, 16(5):667–671.
7. **Lin T, Du J Z, Shao J J, *et al***. 2009. Application of VP1 Protein to Develop Monoclonal Antibody against Foot-and-mouth Disease Virus Asia1 Type. **Virol Sin,** 24(3):215–220.
8. **Lu Z J, Zhang X L, Fu Y F, *et al.*** 2010. Expression of the major epitope regions of 2C integrated with the 3AB non-structural protein of foot-and-mouth disease virus and its potential for differentiating infected from vaccinated animals. **J Virol Methods,** 170:128–133.
9. **Mohapatra J K, Pandey L K, Sanyal A, *et al.*** 2011. Recombinant non-structural polyprotein 3AB-based serodiagnostic strategy for FMD surveillance in bovines irrespective of vaccination. **J Virol Methods,** 177(2) :184–192.

10. **Priyadharshini M L, Balamurugan V, Prabhudas K**, ***et al***. 2007. Expression of 3AB protein of foot and mouth disease virus in *Pichia pastoris*. **Indian J Biotechnology,** 6:329–335.
11. **Rosas M F, Vieira Y A, Postigo R, *et al.*** 2008. Susceptibility to viral infection is enhanced by stable expression of 3A or 3AB proteins from foot-and-mouth disease virus**. Virology,** 380:34–45.
12. **Shao J J, Chang H Y, Lin T, *et al.*** 2011. Expression and utilization of 3AB nonstructural protein of foot-and-mouth disease virus in *Escherichia coli*. **Chin J Biotechnol,** 27(2): 180−184. (in Chinese)
13. **Smith D B.** 2000. Generating fusions to glutathione S-transferase for protein studies. **Methods Enzymol,** 326:254–270.
14. **Song M Y, Park S K, Kim C S, *et al.*** 2008. Characterization of a novel anti-human TNF-α murine monoclonal antibody with high binding affinity and neutralizing activity. **Exp Mol Med**, 40(1):35–40.