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Develope Monoclonal Antibody against Foot-and-mouth Disease Virus A Type*

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Abstract: In order to develop an anti-FMDV A Type monoclonal antibody (mAb), BABL/c mice were immunized with FMDV A type. Monoclonal antibodies (mAbs) 7B11 and 8H4 against Foot-and-mouth disease virus (FMDV) serotype A were produced by fusing SP2/0 myeloma cells with splenocyte from the mouse immunized with A/AV88. The microneutralization titer of the mAbs 7B11 and 8H4 were 1024 and 512, respectively. Both mAbs contain kappa light chains, the mAbs were IgG1. In order to define the mAbs binding epitopes, the reactivity of these mAbs against A Type FMDV, were examined using indirect ELISA, the result showed that both mAbs reacted with A Type FMDV. These mAbs may be used for further vaccine studies, diagnostic methods, prophylaxis, etiological and immunological research on FMDV. Characterization of these ncindicated that prepared anti-FMDV A mAbs had no cross-reactivity with Swine Vesicular Disease (SVD) or FMDV O, Asia1 and C Type antigens. Their titers in abdomen liquor were $1:5\times10^6$ and $1:2\times10^6$, respectively. 7B11 was found to be of subtype IgG_1, 8H4 was classified as IgG_{2b} subtype. The mAbs prepared in this study, are specific for detection of FMDV serotype A, and is potentially useful for pen-side diagnosis.

Key words: Foot-and-mouth disease virus (FMDV) A Type; Monoclonal antibody (mAb); Neutralizing activity

Foot-and-mouth disease (FMD) is a highly contagious vesicular disease of cloven-hoofed animals. Foot-and-mouth disease is the most economically significant infection of domestic livestock. It causes production losses, particularly to the dairy and pig industries and is a major constraint in the international trade of live animals and their products. Its pathogen is foot-and-mouth disease virus (FMDV) which is a member of the genus *Aphthovirus* and of the family *Picornavidae* and includes seven serotypes. Serotype A is widely distributed and very active in the Middle East and Asia [8].

Monoclonal antibodies (mAbs) have been widely used in infectious diseases research, diagnosis and therapy. Compared to the tests based on polyclonal anti-sera, diagnostic tests with mAbs always have

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higher specificity, accuracy and efficiency[10]. In this study, we report the generation of an anti-FMDV A Type mAbs. Two mAbs 7B11 and 8H4 were screened after immunization of mice with A Type FMDV. The specificity, stability, titers and neutralization activity of the mAbs were analyzed.

MATERIALS AND METHODS

Viruses and cells

The A/AV88 virus strain (isolated in Russia in 1958) and FMDV O, Asia1 and C serotypes and swine vesicular disease virus (SVDV) used in this study were reference strains 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）supplement 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 A Type FMDV

Baby hamster kidney 21 cells (BHK-21) were used for all strains of FMDV. The virus infected BHK-21 cells were cultured in Glasgow's MEM (Alpha MEM was used for LK cells) supplemented with 2 mmol/ L glutamine and 50 mg/mL genta-mycin. Viruses were harvested 24 h post-infection and clarified by centrifugation at 3 500 r/min for 30 min. About 10 mmol/L 2-bromo- ethylamine hydrobromide (BEI) was used to inactivate viruses for 24 h at 37℃. 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 5-6 weeks old were immunized subcutaneously with 20 mg of purified A Type VP1 protein in an equal volume of complete Freund's adjuvant[5]. 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. The two immunized mice used for each fusion were sacrificed by overdose anaesthesia. A single-cell splenocyte suspension was obtained for fusion. 1640 with 10% fetal bovine serum was used for fusion and subcloning. Immunized spleen cells were fused with myeloma cells at 5–10:1 ratio in the presence of 50% polyethylene glycol (Merck). The cells were plated out in semisolid medium (Stem Cell) and incubated at 37℃ in a humidified 5% CO_2 atmosphere[2]. 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 isotype of three mAbs was determined by adding 25 μL of the cell culture supernatant containing 7B11 and 8H4, 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. Detection of bound mAb was 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.

ELISA

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

Western blot analysis

Purified (Saturated ammonium sulfate and Sephacry S-300HR) 7B11 and 8H4 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 for 1 h at 37℃ with TBS. 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 37℃. 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[3].

Neutralization assay

The standard procedure for microneutralization (MN) assay for the detection of mAb neutralization activity were followed with certain modifications[4]. In general, serial diluted mouse ascitic fluid and equal volume of A/AV88 (100 $TCID_{50}$) were mixed and incubated at 37℃ for 1 h. Each virus/diluted mAb mixture was then applied to eleven wells of a 96-well microtiter plate. BHK-21 cells 5×10^4 per well were also added. After incubation for 2 days at 37℃, the fifty percent end point of neutralization titers for A/AV88 were calculated. The amount of virus actually used per well should contain 100 $TCID_{50}$. Neutralization assays were repeated at least two times[6].

mAbs characteristic analysis

The specificity, stability and titers of mAbs were examined by ELISA (the procedure as described previously). For the specificity, the two mAbs were reacted with O, A and C serotypes of FMDV and SVDV. The stability and titers of prepared mAbs were analysed by ELISA in different passaged generations.

RESULTS

Purification of A Type FMDV protein

The A Type FMDV was purified by one-step affinity purification using glutathione Sepharose 4B as described in Refs [7,9,12]. The presence of recombinant protein in the eluted fractions was confirmed by SDS–PAGE. The recombinant A Type FMDV was 34 kDa, which accounted for 30 % of total protein in *E. coli* lysates (Fig. 1).

mAb production

In this study, two mAbs (7B11 and 8H4) were obtained. All of them were produced from the mice abdominal cavity. SDS-PAGE showed that the molecular weights of the heavy chain and light chain were about 45.0 and 25.0 kDa (Fig. 2A), which was consistent with the predicted molecular weight, and reacted to VP1 protein specifically with Western blot (Fig. 2B).

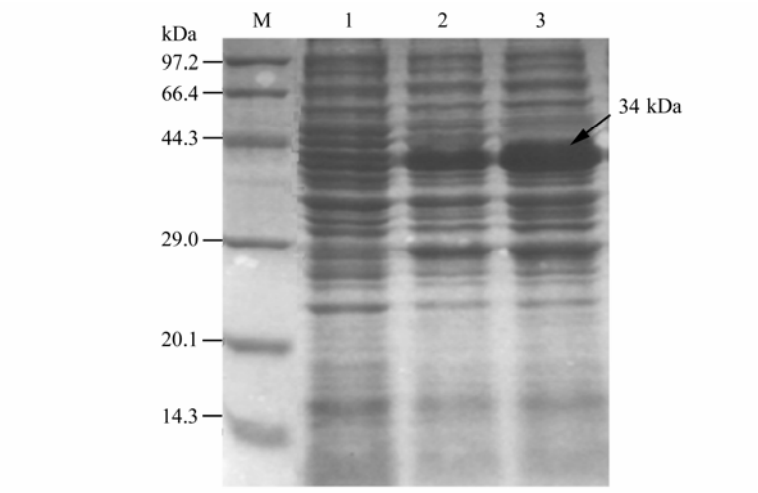

Fig.1. Analysis of expressed products VP1 protein by SDS-PAGE. Lane 1, Recombinant before induced with IPTG; 2, Recombinant induced with 0.6 mm IPTG; 3, Recombinant induced with 0.8 mm IPTG; M, Low molecular weight.

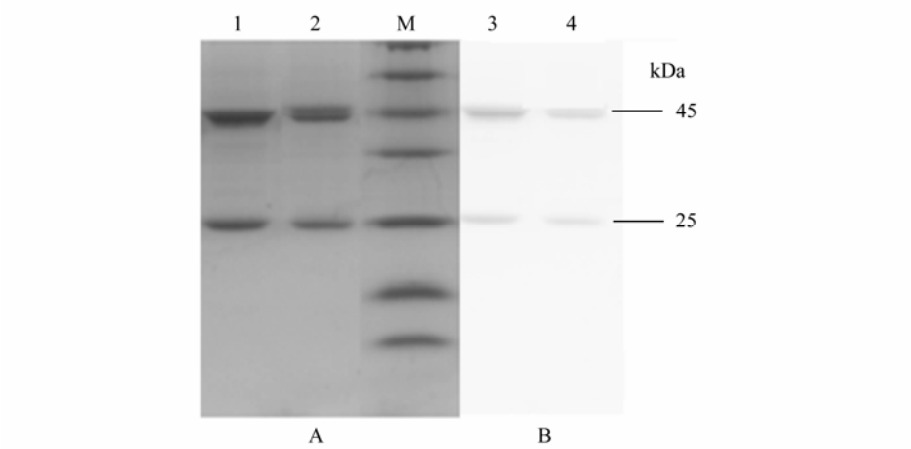

Fig.2. SDS-PAGE (A) and western-blot (B) analysis of purified of mAbs. Lane 1 and 3, mAbs 7B11; 2 and 4, mAbs 8H4; M, Protein molecular weight markers.

Neutralization assay

In the neutralization assay, the result indicated that prepared mAbs could inhibit virus strain A/AV88 from infecting BHK-21 cells (Table 1). When the mAbs 7B11 dilution ratio reached 1:1024, 50% of the cells still survived; indicationg that the titers of mAbs were more than 1:1024. For mAbs 8H4 when the dilution ratio reached 1:512, 50% cells still survived; this showed that the titers of mAbs were more than 1:512.

Cross-reaction

In this test, FMDV O, 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 SVD and FMDV O, Asia1 and C Type antigens (Table 2).

Isotype identification

In the isotype test, 7B11 was found to be from IgG1 whereas 8H4 belonged to IgG2b. As shown in Table 3, the ascites titer of mAbs was between 1-2×10^5. In the stability test, the titers of prepared mAbs were invariably maintained when passaged to thirty generations (as shown in Table 3). All of these results showed that the developed mAbs possessed good specificity and high titers.

Table 1. Detection of mAbs neutralization titers by virus neutralization test

mAb type	Dilution ratio				
	1:64	1:128	1:256	1:512	1:1024
7B11	+	+	+	+	+
8H4	+	+	+	+	-
Blank control	<1:4			<1:4	
Test control	Virus control —			Cell control +	

"+" means at least 50% BHK-21 cell didn't appear cytopathic effect (CPE); "-" means almost 100% BHK-21 cell appeared CPE; Virus control: A/AV88 virus were inoculated in BHK-21 cells; Cell control: BHK-21 cell control,without inoculation A/AV88 virus.

Table 2. The results of the cross-reaction test

	A Type VP1	A Type FMDV	O FMDV	Asia1 FMDV	C FMDV	SVDV
7B11	+	+	—	—	—	—
8H4	+	+	—	—	—	—

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

Table 3. The ascites titer and stability of the screened monoclonal antibodies

mAb	Subtype	Ascites titer	Passages		
			10th generation	20th generation	30th generation
7B11	IgG_1	5×10^5	1:20 000	1:20 000	1:20 000
8H4	IgG_{2A}	2×10^5	1:51 200	1:51 200	1:51 200

DISCUSSION

In this study we purified the serotype A Type FMDV inactivated virus, vaccinated BABL/c mouse with purified A Type FMDV antigen, fusion spleen cell and SP2/0 cell and screened two strains mAbs. Before fusing to the cell, the antibody titer of the immunized BALB/c mouse showed a titer as high as 1024, which was a good preparation for this test. In this study, we also found that using expressed protein immunized BALB/c is a good strategy to prepare mAb, since this method would avoid the possibility of live virus escaping from laboratories and other places[11].

Two anti-FMDV Asia1 type mAbs, 7B11 and 8H4, were raised and characterized. ELISA and Western blot analysis showed that the prepared mAbs were highly specific to A Type FMDV and did not cross-react with SVD and FMDV O, Asia1 and C Type antigens. Moreover, prepared mAbs were shown to have an excellent neutralizing activity against A Type FMDV. The neutralizing activity of prepared mAbs were at least 1:1024 and 512 (as shown in Table 1, Table 2 and Table 3). Because of this good quality, the prepared mAbs can be used to develop various FMDV Type-independent tests for diagnosis and analysis of the antigen epitopes of FMDV serotype A.

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