Preparation and characterization of monoclonal antibodies against ES antigens of *Trichinella spiralis*

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Summary

Hybridomas secreting monoclonal antibodies (MAbs) against excretory-secretory (ES) antigen of *Trichinella spiralis* muscle larvae were produced. The 12 monoclones (designated 3A11, 7D9, 7F6, 5A7, 6H7, 39F3, 38H2, 37E7, 35B9, 29A11, 39C9 and 39A6) secreted IgM, IgG3 and IgG2b, respectively. Western blot using *T. spiralis* ES antigens showed that ten of 12 MAbs recognized the bands between 119.8 – 19.3kDa (mainly 68.4 – 28.7kDa) and two MAbs (3A11, 37E7) did not recognize any protein constituents of ES antigens. Nine of 12 MAbs also recognized the larval antigens of *T. nativa*, *T. pseudospiralis* and *T. nelsoni* at 40 days post-infection (dpi). No cross-reactions were found with the somatic antigens of adult worms of *P. westermani*, *C. sinensis*, and *T. solium* cysticercus. However, three (3A11, 6H7 and 39A6) were cross-reacted with the somatic antigens of adult worms of *S. japonicum*. Immunofluorescent location showed that the nine MAbs reacted with the cuticle and internal organs of *T. spiralis* larvae. Additionally, five and eight MAbs generated in this study can recognize the early antigens of pre-encapsulated *T. spiralis* larvae at 11 dpi and 13 dpi, respectively. The generation and characterization of the MAbs against *T. spiralis* ES antigens provide foundation for the development of specific early serodiagnostic techniques for trichinellosis.

Keywords: *Trichinella spiralis*; MAbs; muscle larvae; excretory-secretory (ES) antigens; mice

Introduction

Trichinellosis is a serious parasitic zoonosis which is widely distributed in the world. Humans acquire the disease by ingesting raw or insufficiently cooked meat of pigs or other animals containing the *Trichinella* larvae. The adult worms live in the duodenal and jejunal mucosa of flesh-eating animals and humans, while the larvae live in

skeletal muscles of the same hosts. *Trichinella* spp. infection has been documented in domestic animals (mainly pigs) and wildlife in 43 (21.9 %) and 66 (33.3 %) countries around the world, respectively. Human trichinellosis has been documented in 55 (27.8 %) countries around the world (Pozio, 2007). In China, 15 outbreaks of human trichinellosis, consisting of 1,387 cases and four deaths, were reported in southwestern China during 2004 – 2009 (Cui *et al.*, 2011). So, trichinellosis is a major food-borne zoonosis with health, social, and economic impacts in China.

However, the diagnosis of trichinellosis is rather difficult because its clinical manifestations are nonspecific. The main disadvantage of serological test (such as ELISA) for the detection of anti-*Trichinella* antibodies is the occurrence of a high rate of false negative results during the early stage of infection, as it has shown that the maximum positive rate of 100 % of ELISA in detecting anti-*Trichinella* antibodies was not reached until at least 1 to 3 months after human infection with the parasite (Bruschi *et al.*, 1990; Wang *et al.*, 1998; Gamble *et al.*, 2004). Therefore, it is important to produce murine monoclonal antibodies (MAbs) that can be used for the development of serodiagnostic assay to detect the circulating antigens for trichinellosis.

Several MAbs have been prepared against the newborn larvae, muscle larvae or adults of *T. spiralis* (Arriaga *et al.*, 1995; Boireau *et al.*, 1997; Romarís *et al.*, 2001; De-La-Rosa *et al.*, 2001). However, immunodiagnostic methods developed using these MAbs had problems relating to sensitivity, specificity, and reproducibility. The ES antigens of *T. spiralis* muscle larvae have been shown to be useful antigens for producing MAbs for use in the development of immunodiagnostic techniques for trichinellosis (Zhu *et al.*, 1990; Srimanote *et al.*, 2000; Li & Ko 2001; Zumaquero-Ríos *et al.*, 2012). The aim of the present study was to generate and characterize MAbs against ES antigens of *T. spiralis* muscle larvae, which would

provide a foundation for the development of specific immunodiagnostic techniques for the specific early diagnosis of trichinellosis.

Materials and methods

Parasites

The *Trichinella* isolate used in the study was *T. spiralis* (ISS534), obtained from domestic pigs in Nanyang city of Henan Province, China. This isolate was maintained in Kunming mice at 6–8-month intervals in our department (e.g., Department of Parasitology, Medical College, Zheng-zhou, University) and muscle larvae were recovered from infected mice by digestion of carcasses as described previously (Li *et al.*, 2010). For comparative purposes, *T. nativa* (T2, ISS10), *T. pseudospiralis* (T4, ISS13) and *T. nelsoni* (T7, ISS29) obtained from the *Trichinella* Reference Center (IRC; Rome, Italy) were used in this study. Adult worms of tissue-dwelling helminths (*Paragonimus westermani*, *Clonorchis sinensis* and *Schistosoma japonicum*), and *Teania solium* cysticercus were stored at -20 °C in our laboratory.

Experimental animals and cell line

Six-week-old BALB/c mice were purchased from the Experimental Animal Center of Henan province. SP2/0 mouse myeloma cells were obtained from Zhengzhou Autobio Biological Engineering Company, China. Cells were cultured in RPMI-1640 with L-glutamine, nonessential amino acids and 10 % fetal bovine serum (FBS). Myeloma cells were cultured in a constant temperature incubator, which is programmed to maintain a temperature of 37 °C and humidity of 98 %, in an atmosphere of 5 % CO₂.

Preparation of ES antigens

The ES antigens of *T. spiralis* muscle larvae were prepared as described previously (Gamble *et al.*, 1988; Wang *et al.*, 2012b). In brief, after being washed thoroughly in sterile phosphate-buffered saline (pH7.4), the larvae were again washed four times in serum-free RPMI-1640 medium supplemented with 100 U penicillin/ml and 100 µg streptomycin/ml. The larvae were incubated in the same medium at concentration of 5 000 larvae/ml for 18 h at 37 °C in 5 % CO₂. After incubation, larvae were removed by centrifugation (6 000×g for 25 min at 4 °C), the media contained the ES products were filtered through a 0.2 µm membrane into a 50-ml conical tube, then centrifuged at 4 °C, 15,000×g for 30 min. The supernatant contained ES was concentrated by a vacuum concentration and freeze-drying (Heto Mxi-Dry-Lyo, Denmark). The protein concentration of ES antigens (1.72 mg/ml) was assayed by the method described by Bradford (1976), and then samples were aliquoted and kept at -80 °C before use.

Immunization

Six female BALB/c mice were intraperitoneally immunized with 50 µg of ES antigens and complete Freund's adjuvant, followed by three boosts with 25 µg of ES antigens alone at

an interval of 3 weeks. At 5 and 7 weeks after the first immunization, mice were bled and the titers of serum samples were determined, respectively. The mice were boosted once with 50 µg antigens at 3 days before cell fusion.

Production of hybridoma cell lines

The mice with the highest polyclonal antibody titer were sacrificed, and aseptically splenectomized. Splenocytes were isolated, and fused with SP2/0 myeloma cells by the addition of 50 % polyethylene glycol (PEG 4 000) (Roche, Basel, Switzerland) in RPMI-1640 (Kohler & Milstein, 1975). The fused cells were suspended in RPMI 1640 containing 1 % hypoxanthine aminopterin thymidine (HAT) (Sigma) and 20 % fetal calf serum (Gibco, Carlsbad, CA), and then were dispensed into 96-well cell culture plates (Corning, NY). The plates were then incubated at 37 °C in a CO₂ incubator with 5 % CO₂ and 98 % relative humidity for 10 to 14 days until hybridoma colonies were observed. At the end of the incubation, the supernatants of cell culture were isolated and screened by indirect enzyme-linked immunosorbent assay (ELISA) as described below. Positive cultures were used in cloning, expanded, and frozen. Cloning was performed by limiting dilution technique (Kennett *et al.*, 1980).

Preparation and purification of MAbs against ES antigens of *T. spiralis*

Isolated clones were intraperitoneally injected into pristine (Sigma) primed BALB/c mice and the ascitic fluid was harvested 10 days later, by the time an adequate volume accumulated. The ascites were centrifuged at 1 500 g for 10 min. Then, the MAbs was purified by using caprylic acid at 4 °C for 1 h (McKinney and Parkinson, 1987). The precipitate was centrifuged at 10 000 g for 20 min at 4 °C. The pellet was resuspended and dialyzed in PBS. The MAb isotype was determined by using the Mouse Monoclonal Antibody Isotyping Kit (Sigma, USA).

ELISA assay

MAbs from the hybridoma culture supernatants and ascites were assayed by an indirect ELISA procedure (Wang *et al.*, 2006). Briefly, each well of polystyrene 96-well plates (Corning, NY) was coated with 100 µL ES antigens of a concentration of 5 µg /mL in 0.05M bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. After the incubation, the plates were washed three times with PBS containing 0.05 % Tween-20 (PBST). After blocking with 3 % skimmed milk in PBST at 37 °C for 1 h, the hybridoma supernatants (without dilution) or ascitic fluid (serially dilution) were added to each well (100 µL/well) and incubated at 37 °C for 1 h. After three washings with PBST, HRP-conjugated goat anti-mouse IgG or IgM (Sigma, USA) was added and incubated at 37 °C for 1h. The reactions were detected by addition of the substrate O-phenylenediamine dihydrochloride (Sigma, USA) plus H₂O₂ and stopped with 50 µL/well of 2M H₂SO₄. Quantification of the reactions was determined by absorbance at 490 nm with an ELISA reader (TECAN, Austria).

SDS-PAGE and Western blotting

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described (Nöckler *et al.*, 2009; Wang *et al.*, 2011). Briefly, ES antigens of *T. spiralis* muscle larvae were diluted with loading buffer (250 mM Tris-HCl pH 6.8, 50 % glycerol, 10 % SDS, 5 % 2-mercaptoethanol, 0.5 % bromophenol blue) up to a concentration of 15 µg/lane and denatured at 95 °C for 10 min. After cooling, the proteins were separated by SDS-PAGE on 12 % acrylamide separating gel and 5 % acrylamide stacking gels (83×73×1.0 mm) in a Mini-PROTEAN 3 Cell electrophoresis unit (Bio-Rad, USA) at 120 V for 2.5 h. After electrophoresis, the gel was stained with 0.25 % Coomassie brilliant blue R-250 for 4 h, and then bleached with the eluate (100 ml acetic acid, 50 ml ethanol, 850 ml dH₂O). A second gel was prepared with the same proteins. After electrophoresis, proteins were transferred at 20 V for 25 min onto nitrocellulose membrane by blotting the gel in a trans-blot SD transfer cell with the electrode buffer recommended by the supplier (Bio-Rad, USA; Wang *et al.*, 2013a). Blocking was done with 5 % skimmed milks in TBST at 4 °C overnight. The strips were then probed with MABs at 37 °C for 1 h. After washing, the strips were incubated at 37 °C for 1 h with HRP-conjugated goat anti-mouse IgG or IgM (Sigma, USA), and finally with 3, 3'-diaminobenzidine tetrahydrochloride (Sigma, USA). The reaction was finally stopped by washing the strips with distilled water. Finally, the molecular sizes of the bands were evaluated by comparison with the molecular size ladder using a gel documentation system (GeneGenius, Syngene, USA) and related software (GeneSnap 6.08 and GeneTools, Syngene, USA).

Immunolocalization

Immunofluorescent test (IFT) was used to the immunolocalization of MABs in the parasite. The fresh diaphragm muscles of mice experimentally infected with *T. spiralis* at 11, 13, 20 and 40 days post infection (dpi) were fixed in 10 % neutral buffered formalin for 24 h, embedded in paraffin wax, and cut into sections of 4 µm thick, and then tissue sections were processed by a graded series of alcohols and rehydrated in distilled water. The immunofluorescent staining was performed by the method described by Cui *et al* (1999). In brief, the tissue sections were blocked with 3 % H₂O₂ diluted in PBS for 20 min, washed three times with PBS. After blocking with 5 % normal goat sera, the tissue sections containing larvae were incubated with MABs at a dilution of 1:100 for 1h, then washed and incubated with a 1:200 dilution of FITC-labeled goat anti-mouse IgG (Biotechnology Company of Zhongshan Goldenbridge, China). The tissue sections were re-dyed with 0.3 % propidium iodide (Sigma, USA) for 15min, and examined under fluorescent microscope (Olympus, Japan). In order to observe the recognition of larval surface by MABs, the whole larvae at 40 dpi were also used in IFT. The procedures of IFT with whole larvae were the same as above, but the larvae were not blocked with H₂O₂ or goat sera nor re-dyed with 0.01 % Evans blue.

Specificity of the MABs

The specificity of the MABs was also assayed by IFT. The frozen sections with 4 µm thick of adult worms of *Paragonimus westermani*, *Clonorchis sinensis* and *Schistosoma japonicum*, and *T. solium* cysticercus were prepared. The skeletal muscles of mice experimentally infected with *T. nativa*, *T. pseudospiralis* and *T. nelsoni* at 40 dpi were also embedded in paraffin wax and cut into sections. The IFT was performed as mentioned above.

Results

Antibody titer and subtype of MABs

ELISA assays of culture supernatants from each well showed that 12 wells (3A11, 7D9, 7F6, 5A7, 6H7, 39F3, 38H2, 37E7, 35B9, 29A11, 39C9 and 39A6) were positive to ES antigens of *T. spiralis* muscle larvae. Seven clones of MABs belonging to IgG3, one clone of MABs belonging to IgG2b, and four clones of MABs belonging to IgM were produced against ES antigens (Table 1). The titers of MABs in culture supernatant and ascites determined by ELISA ranged from 1:1280 to 1:6 400 and, 1:1.28×10⁵ to 1:3.3×10⁶, respectively.

Western blot analysis

SDS-PAGE analysis of *T. spiralis* muscle larval ES antigens revealed ten bands with molecular weight from 119.8 kDa to 14.37 kDa, of which three were the main band with molecular weight of 55.65 kDa, 50.41 kDa, and 43.61 kDa (Fig. 1). Western blotting of ES antigens showed that ten (7D9, 7F6, 5A7, 6H7, 39F3, 38H2, 35B9, 29A11, 39C9 and 39A6) of 12 MABs recognized the bands between 119.8-19.3kDa (mainly 68.4 – 28.7kDa), two MABs (3A11, 37E7) did not recognize any protein bands of ES antigens (Fig. 2 and Table 1).

Fig. 1. SDS-PAGE of the excretory-secretory (ES) antigens of *T. spiralis* muscle larvae. Proteins were separated in 12% polyacrylamide gels under reducing condition, the gel was stained with Coomassie brilliant blue R-250, *M* protein marker with low molecular weights, *1* ES antigens of *T. spiralis* muscle larvae.

Fig. 2. Western blotting analysis of the ES antigen of *T. spiralis* muscle larvae with MABs.

Ten MABs (Lane 2-6, 8, 10-13) recognized with the main protein bands (68.4 to 19.2 kDa) of ES antigens; two MABs (Lane 1 and 9) did not recognize any protein bands of ES antigens. *M* Protein marker with low molecular weights; 1-6 MABs 3A11, 7D9, 7F6, 5A7, 6H7 and 39F3; 8-13 MABs 38H2, 37E7, 35B9, 29A11, 39C9 and 39A6, 7 Sera of the infected mice; 14 Sera of mice immunized with ES antigens. 15 Sera of normal mice.

Immunolocalization

The IFT results of whole larvae at 40 dpi showed that nine MABs (7D9, 7F6, 5A7, 39F3, 38H2, 35B9, 29A11, 39C9 and 39A6) recognized the larval cuticle and exhibited the bright green fluorescence at the external surface of the larvae (Fig. 3 and Table 1).

The results of immunofluorescent location showed that the above nine MABs reacted with muscle sections of the infected mouse at different time intervals after infection,

the immunofluorescent staining was predominantly located on the cuticles and internal organs of the larvae (Fig. 4 and Table 1). Of the nine MABs, five MABs (7F6, 6H7, 35B9, 29A11 and 39A6) recognize the infected muscle sections as early as 11 dpi. At 13 days, additional three MABs (5A7, 38H2 and 39C9) also can recognize these sections. At 20 and 40 dpi, all the nine MABs reacted with the infected muscle sections.

Fig. 3. Immunofluorescent staining of *T. spiralis* muscle larvae at 40 days post infection with MABs. *A* (3A11), *E* (6H7) and *H* (37E7) showing the three MABs that did not recognize the external surface of the larvae; *B* (7D9), *C* (7F6), *D* (5A7), *F* (39F3), *G* (38H2), *I* (35B9), *J* (29A11), *K* (39C9) and *L* (39A6) showing the nine MABs that recognized the external surface of the larvae.

Fig. 4. Immunofluorescent staining of *T. spiralis* infected mouse muscle sections at different time post infection with MAbs. The bright green fluorescence exhibited at the cuticles and internal organs of the larvae when the MAbs was positively reacted with the sections. A (7D9), B (7F6), C (5A7), D (6H7), E (38H2), F (35B9), G (29A11), H (39C9), I (39A6). 1-4 represent the muscle sections of *T. spiralis* infected mice at 11, 13, 20 and 40 days post infection, respectively.

Table 1. Characteristics of MAbs against of ES antigens of *T. spiralis* muscle larvae

MAbs	Subtypes	ES antigens reacted (kDa) by Western blot	Recognition of surface of whole larvae at 40 dpi	Reaction with infected muscle sections at days post infection				Cross-reaction with other helminths				Reaction with larvae of		
				11	13	20	40	P*	C*	S*	T*	T2	T4	T7
3A11	IgM	No	—	—	—	—	—	—	—	+	—	—	—	—
7D9	IgM	120~19.3	+	—	—	+	+	—	—	—	+	+	+	+
7F6	IgG3	58.3~28.7	+	+	+	+	+	—	—	—	+	+	+	+
5A7	IgM	97.2~19.4	+	—	+	+	+	—	—	—	+	+	+	+
6H7	IgG3	49	—	+	+	+	+	—	—	+	+	—	+	+
39F3	IgM	48.3	+	—	—	—	—	—	—	—	—	—	—	—
38H2	IgG3	53.8~23.7	+	—	+	+	+	—	—	—	+	+	+	+
37E7	IgG3	No	—	—	—	—	—	—	—	—	—	—	—	—
35B9	IgG3	52.3~23.3	+	+	+	+	+	—	—	—	+	+	+	+
29A11	IgG2b	126~19.9	+	+	+	+	+	—	—	—	+	+	+	+
39C9	IgG3	56~23.4	+	—	+	+	+	—	—	—	+	+	+	+
39A6	IgG3	48.1~28.7	+	+	+	+	+	—	—	+	+	+	+	+

p*= *Paragonimus westermani*; C*=*Clonorchis sinensis*; S*= *Schistosoma japonicum*; T*= *T. solium* cysticercus; dpi= days post infection; + positive reaction; - negative reaction

P*= *Paragonimus westermani*; C*=*Clonorchis sinensis*; S*= *Schistosoma japonicum*; T*= *T. solium* cysticercus., dpi= days post infection; + positive reaction; - negative reaction

Specificity of the MABs

The results of IFT also showed that the 12 MABs prepared in this study were not cross-reacted with the somatic antigens of adult worms of *P. westermani*, *C. sinensis* and the sections of *T. solium* cysticercus (cysticercus cellulosae). However, three (3A11, 6H7 and 39A6) of 12 MABs were cross-reacted with the somatic antigens of adult worms of *S. japonicum*.

Out of the 12 MABs against ES antigens of *T. spiralis* muscle larvae, nine MABs (7D9, 7F6, 5A7, 6H7, 38H2, 35B9, 29A11, 39C9 and 39A6) were cross-reacted with sections of mouse muscles infected with *T. nativa*, *T. pseudospiralis* and *T. nelsoni* at 40 dpi, in which the MAB 6H7 only recognized the encapsulated species of the genus *Trichinella* (*T. spiralis*, *T. nativa* and *T. nelsoni*) and did not recognize the non-encapsulated *T. pseudospiralis*.

Discussion

Since monoclonal antibody technique was invented by Kohler and Milstein in 1975, it has been widely used in the research on parasitic diseases (Harnett *et al.*, 1997; Chen *et al.*, 2010). In the production of MABs, the choice of immunogen is the key step. The ES antigens of *T. spiralis* muscle larvae are the larval excretory and secretory products which come mainly from the stichosome, distribute throughout in various tissues of host, and are easily targeted by the immune system. So, the ES antigens are the important functional antigens of *T. spiralis* (Dea-Ayuela & Bolas-Fernandez, 1999; Wang *et al.*, 2013b, 2014). The ES antigen collected from muscle larvae cultivated in vitro for 24 h had the highly specific for the serodiagnosis of trichinellosis (Gamble *et al.*, 1988).

In this study, the ES antigens collected from the muscle larvae maintained in vitro for 18h were used to prepare the MABs. A total of twelve MABs against ES antigens of *T. spiralis* muscle larvae were obtained, of which 7 MABs recognize only the antigens of *Trichinella* spp. and have no cross-reaction with other tissue-dwelling helminths (*P. westermani*, *C. sinensis*, *S. japonicum*, and *T. solium* cysticercus), the results indicated that these seven MABs have the high *Trichinella* genus-specificity, which may be related with the common antigens among the different species of *Trichinella* genus (Kapel *et al.*, 1998; Kapel & Gamble, 2000). Acute paragonimiasis, clonorchiasis and schistosomiasis are common zoonosis in China and their main clinical manifestations are fever and eosinophilia which are similar with that of acute trichinellosis (Wang *et al.*, 1998). When the ES antigens, their antigenic components (40 – 70 kDa), and even the synthetic or recombinant antigens of *T. spiralis* were used to serodiagnosis of trichinellosis, the cross-reaction with the above helminthiasis occurred sometimes (Mahannop *et al.*, 1995; Gamble *et al.*, 2004; Wang *et al.*, 2009). The generation and characterization of the seven specific MABs against *T. spiralis* ES antigens provide foundation for the development of specific serological diagnostic techniques for trichinellosis.

The immunofluorescent location showed that the nine MABs

generated in this study reacted with the surface of the whole larvae isolated at 40 dpi and the surface as well as internal tissues of sectioned parasites at 20 dpi and 40 dpi, indicating their epitopes were localized at the surface and internal tissues of the larvae. The results are similar to those obtained by McLaren *et al.* (1987). However, because the ES antigens of *T. spiralis* muscle larvae have many immunodominant epitopes, most of the MABs against ES antigens can recognize more than one protein band (Zhu & Bell, 1990; Romaris *et al.*, 2001; Zumaquero-Rios *et al.*, 2012). Additionally, five and eight MABs generated in this study can recognize the early antigens of the *T. spiralis* pre-encapsulated larvae in the infected muscle sections at 11 dpi and 13 dpi, respectively. Although the reaction of five MABs with early antigens of *T. spiralis* larvae in the sections at 11 dpi was weak, the further sandwich ELISA with mAb 35B9 and 5A7 demonstrated that the detection rate of *T. spiralis* circulating antigens in serum samples of the infected mice at 11 dpi was only 33.33 % (5/15) and 100 % (20/20), respectively (Wang *et al.*, 2012a; Liu *et al.*, 2013), suggesting that some *T. spiralis* antigenic epitopes present in infected muscle tissue sections at 11 dpi, which were recognized by mAb 35B9 and 5A7, were also present in early circulating antigens. The results suggested that these MABs have the potential values for early serodiagnosis of trichinellosis by sandwich ELISA assay to detect the circulating antigens in serum samples of the infected humans and animals.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 81271860 and No. 81371843).

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RECEIVED JANUARY 29, 2014

ACCEPTED JULY 16, 2014