PREPARATION OF MONOCLONAL ANTIBODY AGAINST *ANGIOSTRONGYLUS CANTONENSIS* ANTIGEN

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INTRODUCTION

Angiostrongylus cantonensis has been recognized as the main causative agent of human eosinophilic meningitis and meningoencephalitis in areas of Southeast Asia and the Pacific Islands (Rosen *et al.*, 1962). Hundreds cases of this parasitic disease have also been found in Taiwan (Yii *et al.*, 1975; Cheng *et al.*, 1984). The diagnosis of angiostrongyliasis by the characteristic clinical symptoms of patients is difficult because they are often confused with other central nervous disorders. Yii (1976) indicated that only rare cases of human infections were confirmed by the finding of this parasite in the cerebrospinal fluid (CSF). The most reliable diagnosis of this parasitic disease is based on the presence of eosinophils in CSF or on the elevation of antibodies in the blood.

Several immunodiagnostic techniques have been developed to aid in the diagnosing of this parasite infection, as reviewed by Tharavanij (1979). Although immunodiagnosis utilizing extracted worm antigen is a convenient way to diagnose angiostrongyliasis, satisfactory results are not always obtainable because of cross-reactions with other parasitic infections. (Anderson *et al.*, 1962; Kagan and Zaiman, 1964; Suzuki *et al.*, 1975). Köhler and Milstein (1975) developed hybridoma techniques to produce monoclonal antibody. This technique had been applied in various aspects including purification of Toxoplasma antigen with monoclonal antibody by immunoabsoption (Kasper *et al.*, 1983). With this technique, it is possible to use MAb in the affinity purification of parasite-specific antigen to be used in the development of serological tests for specific diagnosis of angiostrongyliasis.

MATERIALS AND METHODS

Preparations of parasite and antigen

The third-stage larvae of *A. cantonensis* were collected from infected African giant snails, *Achatina fulica*, as described by Yen *et al.*, (1987). Rats were sacrified 2 months after individually infected with 100 larvae. Adult worms were picked up from the heart and lung organs. Each BALB/c strain of mice was infected with 50 larvae and on the third week after infection skulls were opened and the brain removed. Juvenile worms were

collected under a dissecting microscope by tearing brain tissue. Worms were detached from the host tissue and washed twice with 0.15M phosphate buffered saline, pH 7.2. After desalting once with distilled water, adult and juvenile worms were pooled respectively and lyophylized and then placed in a deep freeze.

Adult and juvenile worm antigen were prepared according to the modified process of Chaffee *et al.*, (1954). Briefly, lyophilyzed worms were ground in a glass tissue-grinder with triethanolamine buffered saline (TBS), pH 7.2, to extract antigen components. After thoroughly mixing with anhydrous ether, the extracted solution was centrifuged at 30,000 r.p.m. at 4 ° C for 20 minutes. The supernatant was detected for protein content by a protein assay kit (Bio-Rad Co.,) and stored at −20 ° C.

Immunization of mice

Two six-week-old female BALB/c mice, purchased from the School of Medicine, Chen-Kon University, were immunized by intraperitoneal injections with 100 μg *A. cantonensis* adult worm antigen mixed with equal volumes of complete Freund's adjuvant in a total volume of 0.1 ml. A booster dose of 10 μg was given by same route twenty-one days after immunization. The mouse was bled 3 days following the booster dose and the spleen was removed in clean bench as donors of spleen cells for the production of hybridomas.

Preparation of monoclonal antibody

Spleen cells prepared from immunized mouse were fused with NS−1 myeloma cells at a ratio of 10:1 and promoted by the addition of polyethylene glycol (PEG−1500, Boehringer Co.) as described by Harn *et al.*, (1984). Fused cells were dispensed in DMEM containing 15% fetal calf serum (Flow Lab.), glutamine, hypoxanthine, aminopterine and thymidine (HAT, from Sigma Co.) and incubated at 37 ° C in a humid atmosphere of 5% Co_2 in air. The supernatants from wells revealing well growth of hybridomas were detected with *A. cantonensis* adult worm antigen by micro-ELISA. Antibody producing hybridomas were cloned by a limiting dilution technique using BALB/c spleen cells as the feeder layer. Culture medium of cloned hybridomas were tested with several kinds helminthic worm antigen including *A. cantonensis*, *Toxocara canis*, *Ascaris suum*, *Paragonimus westermani*, *Dirofilaria immitis*, *Anisakis* Spp., *Gnathostoma spinigerum* and *Clonorchis sinensis* by micro-ELISA for specific monoclonal antibodies against *A. cantonensis*.

Enzyme linked immunosorbent assay (ELISA).

ELISA was carried out as described by Yen *et al.*, (1984) for the screening of antibody-secreting hybridomas. Briefly, antigens were coated on the surface of wells of a PVC microtiter plate (Falcon Co.). Specific antibodies in the culture medium first reacted with coated antigen and then goat anti-mouse immunoglobulins conjugated with horse radish peroxidase (Nordic Co.). The substrate solution of 0-phenylene diamine and H_2O_2 in citrate buffer were added and the optical density was read out on Titertek Multiscan Colorimeter (Flow Lab.) at 490 nm.

RESULTS

Spleen cells prepared from mice A and B were employed to fuse with NS-1 cells. The successful growth of hybrid cells in the wells of culture plate, as shown in Table 1, were

Table 1

Fusion rate and screening of antibody response to *A. cantonensis*

Spleen donors	Number of wells			
	Cells dispension	Hybridoma	Growing	Antibody positive
A	408	371•	268 @	67
		(90.9)	(65.7)	(25.0)
B	423	378	291	86
		(89.4)	(68.8)	(29.6)
Total	831	749	559	153
		(90.1)	(67.3)	(27.4)

• 3 days after culture
@ 10 days after culture
Percentage in parenthesis

90.9% of mouse A and 89.4% of mouse B observed on the 3rd day after fusion and 65.7% and 68.8% respectively on the 10th day. 25.0% and 29.6% of supernatants from hybridoma growing wells contained antibodies to *A. cantonensis* antigen.

Twelve out of 153 hybridomas with high antibody levels were further cloned, of which 10 clones were picked up on the basis of successful cloning. Cross reactions to helminthic worm antigens other than *A. cantonensis* occurred among two MAb (Table 2). Most of the monoclonal antibodies detected by micro-ELISA were IgG_1.

Four specific IgG_1 monoclonal antibodies designated as A_4 D_8 F_4, B_1 C_6 E_{10}, B_3 F_{10} E_{10} and B_4 D_5 D_7, were selected to react with *A. cantonensis* adult and juvenile worm antigen by micro-ELISA. As shown in Table 3, three monoclonal antibodies only reacted with adult worm antigen, however, one reacted with both adult and juvenile antigens.

DISCUSSION

Based on observations of hybridomas propagated, hybridomas in 22.8% wells ceased in growth or decayed on the 10th day after fusion. Most of these wells were located on the rim of culture plates. Oliver *et al.*, (1983) noted that the "edge effect" in microtitration plates was due to thermal gradients from the peripheral to the central wells of plates. The present study strongly considers the death of hybridomas in the peripheral wells of plates as being related to this effect.

Most of the specific monoclonal antibodies prepared in this study were IgG_1. Probably the most possible type of immunoglobulins secreted by hybridomas was related to immunized schedules. Yen *et al.*, (1987) indicated that the level of specific IgM against *A. cantonensis* adult worm antigen was elevated to the maximum one week after immunization. However, specific IgG_1 reached maximum level three weeks after immunization and boostering 3 days before detection. In this study, BALB/c mice were immunized according to the latter course and served as donors of spleen cells. Secreting IgG_1 of hybridomas was predictable.

Several immunodiagnosis techniques utilizing adult worm antigen of *A. cantonensis* have been successfully tried in the detection of antibody in patients of eosinophilic meningitis or meningoencephalitis due to the infection of this parasite (Kamiya, 1975; Welch *et al.*, 1980; Chen, 1986). As to the host-parasite relationship, humans only play an accidental host of this worm. In reported human cases of *A. cantonensis* infection

Table 2

Screening and determination of class/subclass of specific monoclonal antibodies to *A. cantonensis* adult worm antigen

Class/Subclass	Clones of specific to *A. cantonensis*	Cross react with other helminths antigen
IgG_1	6*	* 2 clones cross react with
IgG_{2a}	1	*Toxocara* spp, *Ascaris suum*,
IgG_{2b}	1	*Anisakis* spp, *Dirofilaria immitis*
IgM	1	and *Clonorchis sinensis* antigens
IgA	1	

Table 3

Determination of IgG_1 monoclonal antibodies specific or cross react to stage development of *A. cantonensis* by enzyme-linked immunosorbent assay

Clones	*A. cantonensis* antigen	
	Adult	Juvenile
$A_4D_8F_4$	1.13 ± 0.03	0.15 ± 0.01
$B_1C_6E_{10}$	1.15 ± 0.04	0.17 ± 0.01
$B_3F_{10}E_{10}$	0.75 ± 0.02	0.16 ± 0.01
$B_4D_5D_7$	0.93 ± 0.04	0.81 ± 0.03
Medium	0.09 ± 0.01	0.08 ± 0.01
Infected serum	1.42 ± 0.03	1.28 ± 0.03

worms rarely reached sexual maturity. Cross and Chi (1982) indicated that it was more important to prepare worm antigen used in serological testing which were usually of the same stage recovered from the human. Recently, Cheng *et al.*, (1988) also demonstrated a higher specificity in the detection of antibodies of human patients and experimental infected animals by using juvenile worm antigen. However, to extract such antigen is costly and time consuming due to a large number of experimental animals used and difficulty in collecting juvenile worms of such a tiny size. Dharmkrongat and Sirisinha (1983) showed that the somatic antigens of the various developmental stages of *A. cantonensis* were highly complex and shared some common antigens. In the running of SDS-polyacrylamide gel electrophoresis, Yen (1988) found that the antigen made from juvenile worms was as complicated as that of the adults. Monoclonal antibodies specific against adult worms antigen or which cross reacted with juvenile worm antigen were obtained in this study. Adult worms of *A. cantonensis* are of a larger size and thus it is easier to collect adequate amounts to prepare antigen. Using this monoclonal antibody to purify the components from adult worm extract which share the antigenicity of juvenile worms can be further studied and applied to serological testing in the future.

SUMMARY

Monoclonal antibodies (MAb) against *A. cantonensis* were produced through fusion of immunised spleen cells from BALB/c mice with NS-1 myeloma cells at a ratio of 10:1. The successful fusion rate on the 3rd day of fusion was 90.1%. Ten MAb were characte-

rised, six of which were IgG_1 and the remaining four were IgG_{2a}, IgG_{2b}, IgM and IgA respectively. Among 6 IgG_1 MAb, four were *A. cantonensis*-specific, of which three reacted to adult worm antigen only and one reacted to both adult worm and juvenile worm antigens. Two other IgG_1 MAb showed cross-reaction with other helminthic antigens of *Toxocara canis*, *Ascaris suum*, *Paragonimus westermani*, *Dirofilaria immitis*, *Anisakis* Spp, *Gnatostoma Spinigerum* and *Clonorchis sinensis*.

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