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Three Monoclonal Antibodies Specific to *Gnathostoma spinigerum*

WICHIT ROJEKITTIKHUN, SUSUMU SAITO, TAKAO YAMASHITA, TADASHI WATANABE, PRASERT SETASUBAN[1] AND FUJIRO SENDO

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Abstract

Spleen cells from BALB/c mice immunized with crude soluble antigen of the advanced third-stage larvae of *Gnathostoma spinigerum* (GsAL3) were fused with P3-X63-Ag8.653 myeloma cells. Three specific monoclonal antibodies (mAbs: FS-3D11, SS-5H5 and SK-6C4) were obtained. All were IgG_1 and showed no cross-reactivity with 11 other parasite antigens including *Angiostrongylus cantonensis, Ascaris suum, Dirofilaria immitis, Toxocara canis, Trichuris vulpis, Clonorchis sinensis, Fasciola gigantica, Schistosoma japonicum, Diphyllobothrium latum, Taenia solium* (*Cysticercus cellulosae*), and *Trichinella spiralis* (muscle larvae). The ascitic antibody titers of FS-3D11, SK-6C4 and SS-5H5 against crude GsAL3 antigen by ELISA were 1:50,000, 1:75,000 and 1:1,000,000, respectively. It is suggested that the antigen recognized by FS-3D11 and SS-5H5 is a carbohydrate moiety, and that recognized by SK-6C4 is a protein.

Key words: Monoclonal antibody, *Gnathostoma spinigerum*

Introduction

Human gnathostomiasis is a disease primarily caused by the larval and immature stages of the roundworm *G. spinigerum*. Diagnosis is mainly based on clinical manifestations of intermittent migratory circumscribed swellings, a previous history of consuming raw or improperly cooked fish or meat, and discovery of the migrating parasite (Daengsvang, 1986). A number of immunodiagnostic tests have also been applied, but results were not satisfactory because of insensitivity and lack of specificity (Suntharasamai *et al.*, 1985; Dharmkrong-at *et al.*, 1986; Maleewong *et al.*, 1988; Anantaphruti, 1989). To remedy these shortcomings, our purpose was to produce monoclonal antibodies specific to *G. spinigerum* antigens and to purify the antigen(s) by means of these mAbs. In this article, we describe three mAbs that are specific to *G. spinigerum*.

Department of Parasitology, Yamagata University, School of Medicine, Yamagata 990-23, Japan.
[1]*Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand.*
ウイチット・ロジキッティクン　斉藤　奨　山下隆夫　渡辺　正　仙道富士郎（山形大学医学部寄生虫学教室）
プラサート・セタスバン（マヒドール大学熱帯医学部蠕虫学教室）

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Materials and Methods

Crude *G. spinigerum* larval antigen. Advanced third-stage larvae of *G. spinigerum* (GsAL3) were obtained from laboratory infected mice, 3 to 4 weeks post-infection, thoroughly washed several times with normal saline and distilled water, then lyophilized and kept at –20°C until use. The dried larvae were homogenized in phosphate buffered saline (PBS) and sonicated 5 times with a Handy Sonic UR-20P (Tomy Seiko Co., Ltd., Japan) for 30 sec each, at 4°C. After centrifugation at 10,000 g for 2 cycles of 40 min each at 4°C, the supernatants were collected and stored at –60°C. The protein concentration of this *Gnathostoma* AL3 extract was determined using the Bio-Rad Protein Assay (BIO-RAD, U.S.A.).

Crude heterologous parasite antigens. Whole worm extracts of adult *A. cantonensis, A. suum, D. immitis, T. canis, T. vulpis, C. sinensis, F. gigantica, S. japonicum, D. latum,* and the larvae

of *T. solium* (*C. cellulosae*) and *T. spiralis* (muscle larvae) were prepared and stored as described above. The protein content of each parasite preparation was measured using a Bio-Rad Protein Assay Kit.

Production of hybridoma cell lines. Eight-week-old BALB/c mice were immunized intraperitoneally with 100 μg of crude soluble GsAL3 antigen in 0.2 ml PBS emulsified with an equal volume of Freund's complete adjuvant (DIFCO Laboratory, U.S.A.). Two weeks later, they were immunized with the same antigen mixed with 0.2 ml Freund's incomplete adjuvant (DIFCO). Mice with high antibody titers one week after the second injection were then boosted intravenously with the same amount of antigen without adjuvant a week afterwards. Spleen cells of the immunized animals were harvested 3 days after the booster injection, and fused with P3-X63-Ag8.653 myeloma cells in the presence of 45% polyethylene glycol 4000 (Nakarai Chemicals Ltd., Japan) (Gefter *et al.*, 1977; Kipps and Herzenberg, 1986). Cloning of antibody-producing hybridomas was performed as described by Oi and Herzenberg (1980). Isotyping of the putative mAbs was performed by double immunodiffusion (Ouchterlony and Nilsson, 1986) using class and subclass-specific rabbit anti-mouse immunoglobulins (Serotec Ltd., England).

ELISA. Details of the method were described elsewhere (Yamashita *et al.*, 1989). All ELISA values were expressed as net OD, which was the OD value of a mAb or immunized serum minus the OD value of the respective negative control, that was, ascitic fluids of BALB/c mice injected with P3-X63-Ag8.653 or normal BALB/c mouse serum.

SDS-PAGE and Western blot analysis. Polyacrylamide gel electrophoresis was performed in the presence of sodium dodecyl sulphate (Laemmli, 1970) using 5% stacking gel and 12.5% separating gel. The antigen samples (final concentration of approximately 3 mg/ml) were boiled at 100°C for 5 min in a final 2% and 5% concentration of SDS and 2-mercaptoethanol, respectively, and were then loaded onto the gel. The SDS-PAGE resolved components were electrophoretically blotted (Towbin *et al.*, 1979) onto an Immobilon (Millipore Corp., U.S.A.) transfer membrane. After blotting, the membrane was cut into strips which were then treated with antibody and developed as described previously (Yamashita *et al.*, 1989).

Treatment of *G. spinigerum* AL3 antigen with periodate and trypsin. Determination of the molecular nature of the antigen reactive with mAb was accomplished by treating with sodium metaperiodate (WAKO, Japan) or trypsin 1:250 (DIFCO), a proteolytic enzyme. The methods of Woodward *et al.* (1985), and Bhattacharya *et al.* (1990) were used with slight modifications. Briefly, 100 μl of GsAL3 antigen (3 μg/ml) in 0.05 M carbonate buffer, pH 9.6, were used to coat each well of an ELISA multi-plate (Sumitomo Co., Ltd., Japan). The plate was incubated at 37°C for 2 hr and then at 4°C overnight. Periodate concentrations ranging from 1 to 50 mM were added to sets of coated wells. Oxidation was carried out in 50 mM sodium acetate buffer, pH 4.5, for 1 hr at room temperature in the dark and the reaction was stopped by adding 20 mM sodium sulfite. The plate was then blocked with 1% glycine for 30 min and washed 3 times with PBS-Tween 20. After treating with primary and secondary antibodies, the extent of binding was assayed colorimetrically using 0-phenylenediamine (OPD) (WAKO) in the presence of 0.003% H_2O_2. The reaction was stopped by addition of 8 N H_2SO_4. The optical density was measured at 492 nm with a Titertek Multiskan Spectrophotometer (Flow Laboratories, U.S.A.).

For treatment with trypsin, sets of antigen-coated wells (0.3 μg/well) were exposed to varying concentrations of the enzyme (1–100 μg/ml) for 30 min at room temperature. Controls were treated with PBS, pH 7.6. The reaction was stopped by adding an equal amount of trypsin inhibitor (SIGMA Chemical Co., U.S.A.).

Results

Establishment of hybridomas producing specific mAbs. Out of 280 culture wells from a single fusion experiment, 265 were found to contain hybrid colonies. Among 144 wells, 107

(74.3%) were positive for antibodies against GsAL3, as screened by ELISA. Of these 107 antibody-secreting hybridomas, 20 were selected for cloning by 3 limiting dilutions. Cloning efficiency was approximately 25 to 50%. Three hybridoma cell lines producing specific mAbs designated FS-3D11, SS-5H5 and SK-6C4 were isolated. The isotypes of these mAbs were all IgG_1. The ascitic antibody titers of FS-3D11, SK-6C4 and SS-5H5, as measured by ELISA, were 1:50,000, 1:75,000 and 1:1,000,000, respectively (Fig. 1).

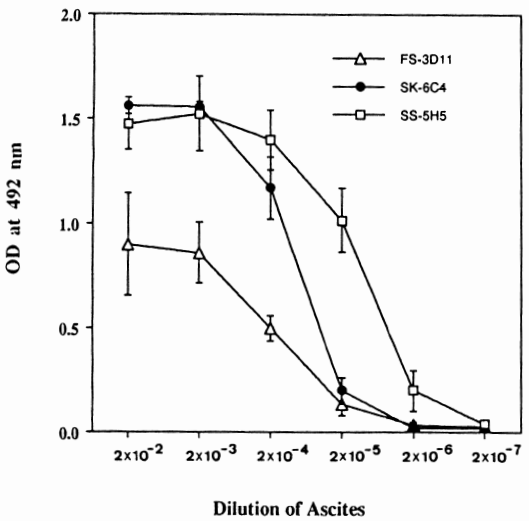

Fig. 1 ELISA titers of monoclonal antibodies to *G. spinigerum* AL3 antigen. Data are the average OD of duplicate samples from each of 3 separate experiments.

Specificity of these 3 mAbs was determined by ELISA using GsAL3 and 11 other heterologous parasite antigens. No cross-reactivity was observed at mAb dilutions that gave approximately 1 OD value when GsAL3 was used as the test antigen (Table 1), whereas the corresponding immunized sera at dilutions that gave the same OD value with GsAL3 reacted with antigens of parasites other than *G. spinigerum*.

SDS-PAGE and Western blot analysis. Western blot analysis of GsAL3 with these 3 mAbs revealed that FS-3D11 recognized antigenic components from about 30 to 170 KD in MW while SS-5H5 and SK-6C4 recognized more complex constituents of about 22 to 170 KD and 16 to 170 KD, respectively (Fig. 2). Numerous bands were observed with GsAL3 treated with SS-5H5 (lane 4). However, when probed with SK-6C4, 3 prominent bands at approximately 16, 38, and 86 KD were demonstrated (lane 3).

Table 1 Reactivities of immune sera and monoclonal antibodies to various parasite antigens

Parasite antigens	OD at 492 nm			
	Immune sera (1 : 3,000)	mAbs		
		FS-3D11 (1 : 500)	SK-6C4 (1 : 5,000)	SS-5H5 (1 : 50,000)
G. spinigerum	1.04 ± 0.03	0.88 ± 0.04	1.24 ± 0.11	1.13 ± 0.07
A. cantonensis	0.25 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
A. suum	0.30 ± 0.06	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
D. immitis	0.08 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
T. canis	0.44 ± 0.12	0.00 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
T. spiralis	0.02 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
T. vulpis	0.02 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C. sinensis	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
F. gigantica	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
S. japonicum	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C. cellulosae	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
D. latum	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

OD values are the average of duplicate samples from 3 separate experiments.

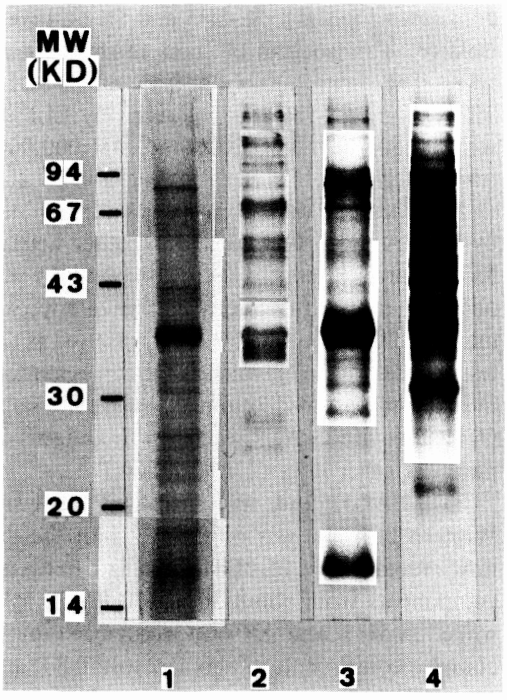

Fig. 2 Western blot analysis of *G. spinigerum* AL3 antigen recognized by monoclonal antibodies. The antigen is stained with CBB (lane 1), treated with FS-3D11 (1:200, lane 2), SK-6C4 (1:1,000, lane 3), and SS-5H5 (1:10,000, lane 4).

Sensitivity of GsAL3 antigen to periodate and trypsin treatment in terms of reactivity with the three mAbs. Treatment of the antigen with periodate decreased the binding of GsAL3 to FS-3D11 and SS-5H5 but not to SK-6C4 (Fig. 3). An increase in the periodate concentration (up to 50 mM) lowered the binding of GsAL3 to FS-3D11 and SS-5H5. The greatest reduction in binding occurred at a concentration of 10 mM periodate, and more than 85% inhibition was seen at 5 mM. In contrast, the SK-6C4 antigen was sensitive to trypsin although other determinants recognized by FS-3D11 and SS-5H5 were only slightly affected (Fig. 4).

Discussion

In this study, we obtained 3 mAbs designated FS-3D11, SS-5H5 and SK-6C4 which are specific to *G. spinigerum*. No cross-reactivity was

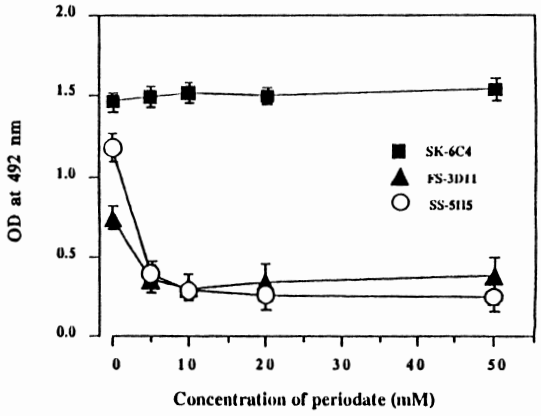

Fig. 3 Effect of periodate oxidation of *G. spinigerum* AL3 antigen on binding with monoclonal antibodies by ELISA. Data are the average OD of quadruplicate wells from 3 assays.

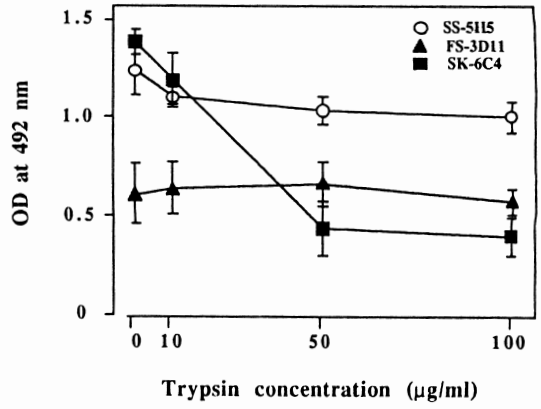

Fig. 4 Effect of trypsin digestion of *G. spinigerum* AL3 antigen on binding with monoclonal antibodies by ELISA. Data are the average OD of quadruplicate wells from 3 experiments.

observed with these mAbs when tested against antigens prepared from 11 other helminths. To our knowledge, this is the first report of mAbs specific to *G. spinigerum*. We found that they recognize a wide range of GsAL3 components ranging in molecular mass from about 16 to 170 KD. However, the reaction pattern differs between SK-6C4, and both FS-3D11 and SS-5H5. SK-6C4 mainly recognizes a 38 KD molecule which is the main band seen after protein staining. This antibody also reacted with other

molecules, the reason for which is unknown. Both FS-3D11 and SS-5H5 gave numerous bands by Western blotting. MAb recognition of various determinants on the antigenic molecules has also been reported by other authors (Yamashita *et al.*, 1989; Bhattacharya *et al.*, 1990) who found the antigenic epitopes to be carbohydrate moieties.

After oxidation of GsAL3 with periodate, binding of the antigen to FS-3D11 and SS-5H5 decreased, although binding to SK-6C4 was unaffected. In contrast, after digestion with trypsin, a decrease in binding to SK-6C4 was observed, but not to FS-3D11 and SS-5H5. These results suggest that antigenic determinants recognized by the 2 mAbs which reacted similarly are carbohydrate whereas that recognized by SK-6C4 is a protein. Abrogation of antigen reactivity with SK-6C4 was only partial with trypsin treatment. This would be expected if the antigenic determinant consists of trypsin sensitive and non-sensitive components.

False-positives and cross-reactions pose major problems in immunodiagnosis. Cross-reactivities arise when crude parasite extracts are used as antigen (Gamble and Graham, 1984a). Purification of specific antigens is a step toward overcoming this problem (Su and Prestwood, 1990). The application of partially purified hybridoma-derived antibodies for isolation of specific parasite antigens has been extensively studied. Several reports have been published on the successful use of mAbs with their affinity-isolated antigens for immunodiagnosis of various helminthic infections, both in human and other species (Mitchell *et al.*, 1981; Mitchell *et al.*, 1983; Gamble and Graham, 1984a; Gamble and Graham, 1984b; Maddison *et al.*, 1989; Fu and Carter, 1990; Su and Prestwood, 1990). Torian *et al.* (1987) stated that the specificity of a mAb is potentially very useful in diagnosis. In the present study, SK-6C4 was shown to be specific as indicated by the very low ELISA values obtained when high concentrations were used to test heterologous antigens (unpublished observation). The applicability of SK-6C4 in the serodiagnosis of gnathostomiasis spinigera is currently under investigation in our laboratory.

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