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Specific Monoclonal Antibodies to *Fasciola gigantica*

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SUMMARY **Monoclonal antibodies (mAbs) were produced against soluble metabolic products (excretory/secretory; ES) of the liver fluke, *Fasciola gigantica*. The ES antigen was obtained from spent culture medium in which the adult flukes had been maintained *in vitro*. From two cell fusions, the mAbs produced were exclusively associated with either IgG or IgM isotypes. When screened against a panel of homologous and heterologous parasite antigens by indirect ELISA, two mAbs were highly specific for *F. gigantica*. The remainder cross-reacted extensively with other parasites. Results from immunoblotting and immunofluorescence exhibited two patterns of reactivity. The first group of mAbs which recognized the multiple bands between > 14.4 - 27.5 kDa gave extremely bright fluorescence over all major muscular systems, vitelline gland, testes and intestinal caeca. The second group which reacted with the 185 kDa band showed a bright fluorescence over a thinner muscular layer underlying the tegument, intestinal caeca and testes.**

Fascioliasis is a disease caused by hermaphroditic trematodes of the genus *Fasciola*, of which *F. hepatica* and *F. gigantica* are the most common. In Thailand, the disease caused by *F. gigantica* is one of the public health problems.[1-2] Humans are usually infected by the ingestion of aquatic plants that harbor the infectious metacercaria. Diagnosis of fascioliasis is complicated by the fact that symptoms often occur during early infection, yet the flukes mature and lay eggs only after eight weeks of infection, after which parasitological diagnosis is feasible by the identification of eggs in the feces. However, false fascioliasis can occur when *Fasciola* eggs are found in the stool following the ingestion of infected liver. Moreover, repeated stool examinations are usually required for the detection of eggs and in many cases, no eggs are recovered.[3] The eggs are unembryonated and practically indistinguishable from those of *Fasciolopsis buski* and *Echinostoma* spp. Early diagnosis is important so that treatment may be instituted before irreparable damage to the liver has occurred. To date, serodiagnostic tests are helpful[4-7] and numerous antigens have been used for anti-*Fasciola* antibody detection. The excretory-secretory (ES) antigen is the reliable antigen for serodiagnosis of fascioliasis,[6, 8-11] but cross reactions were evident in sera infected with other parasites. Therefore, the production of monoclonal antibody (mAb) is required for the purification of the specific epitopes from crude antigens by affinity chromatography. In the present study, we described the specific mAbs against the *F. gigantica* ES (FGES) antigen and identified the reactive antigens by immunoblotting and immunofluorescence.

MATERIALS AND METHODS

Parasite

Adult *F. gigantica* were obtained from infected bovine livers col-

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lected from Khon Kaen province, Thailand. The worms were washed with sterile saline and identified as *F. gigantica* according to previously described criteria. [12-13]

Preparation of antigens

Adult FGES antigen was prepared from spent culture medium (RPMI 1640 [Gibco, USA]) containing 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mM tosylamide-2-phenylethyl-chloromethylketone (TPCK), 1 mM of L-trans-3-carboxy-oxiran-2-carbonyl-L-leucylagmatine (E-64), 100 units/ml penicillin G and 100 µg/ml streptomycin in which the worms had been maintained for 6 hours at 37°C under 5% CO_2 in air. After incubation, the worms were removed and the collected spent medium was centrifuged 10,000 x *g* at 4°C for 30 minuntes. The medium was concentrated by ultrafiltration using Amicon YM3 membrane filter (Grace Co., USA) and dialyzed against distilled water containing the same protease inhibitors. The protein content of this FGES was also determined by the standard method.[14] Crude somatic antigens to be used for checking cross-reactivity of the mAbs were prepared from many other parasites as previously described.[15] These preparations included: crude extracts of protozoa, namely *Entamoeba histolytica* trophozoite obtained from growth in axenic cultures,[16] *Leishmania donovani* promastigotes grown *in vitro* cultures,[17] blood stage cultured *Plasmodium falciparum*[18] and tachyzoites of *Toxoplasma gondii* obtained from experimentally infected mice. Heterologous antigens were also prepared from various helminths obtained from different sources, namely *Taenia saginata* segments, adults *Ascaris lumbricoides*, *Echinostoma ilocanum*, *E. revolutum*, filariform larvae of *Strongyloides stercoralis* and human hookworms from stools of infected humans; *E. malayanum*, *Haplorchis taichui* and *Opisthorchis viverrini* adult worms from experimentally infected hamsters; *Brugia malayi* adult worms from experimentally infected jirds; *Angiostrongylus cantonensis* adult worms from experimentally infected rats; *Dirofilaria immitis* and canine adult hookworms from naturally infected dogs; *Cysticercus cellulosae* from naturally infected swine; *Paragonimus heterotremus* adult worms from experimentally infected cats; *Schistosoma mekongi*, *S. mansoni* and *Hymenolepis nana* adults worms, muscle larvae of *Trichinella spiralis* and advanced third stage of *Gnathostoma spinigerum* larvae from experimentally infected mice.

Monoclonal antibodies production

Ten adult BALB/c mice (6 weeks old) were immunized intraperitoneally with 60 µg of FGES antigen in 0.2 ml of normal saline (0.85 % NaCl in H_2O) emulsified in complete Freund's adjuvant. Booster immunizations, spleen cell harvests and their fusion with Sp 2/0 myeloma cells, culture of the resultant hybridomas and their cloning by limiting dilution were carried out according to the techniques previously described by Chaicumpa *et al.*[19]

Immunological techniques

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting and indirect ELISA were done as previously described.[15,20-22] The classes and subclasses of mAbs were determined after cloning by indirect ELISA using different rabbit anti-mouse immunoglobulin classes/subclasses (BIO-RAD, Bio-Rad Laboratories, USA). The immunofluorescent antibody test (IFAT) was performed as previously described.[23]

RESULTS

Recovery of antibody secreting hybridomas

Supernatant fluids from hybridomas grown in 96-well plates were screened for antibody activity by indirect ELISA using FGES antigen. The supernatants of 15 wells with growing cells reactive with *F. gigantica* were obtained from two identical fusions. The cells from these wells were then transferred to grow in 24-well tissue culture plates and the culture supernatants of these cells were then retested for antibody activity against FGES antigen. It was found that cells from 7 wells produced antibodies which gave an OD reading much higher than 0.5 against FGES antigen. They were then tested for specificity by an indirect ELISA using a panel of heterologous antigens. Results from these experiments showed that antibodies secreted by cells of the 7 wells exhibited four patterns of reactivity. The culture supernatants from 2 clones namely 21 C10 and 13D7 were found to be highly specific for *F. gigantica* (Group 1). One clone namely 6B6 produced antibody which was reactive with antigens of *A. cantonensis*, *A. lumbricoides*, canine hookworms, *D. immitis*, *E. malayanum*, *H. taichui*, *H. nana*, *O. viverrini*, *P. heterotremus*, *T. saginata* and *T. spiralis* (Group 2). Culture fluid from one clone namely 18B6 cross reacted with the antigens from *A. cantonensis*, canine hookworms, *D. immitis*, *P. heterotremus* and *T. saginata* (Group 3). The remaining 3 clones (2C6, 6 D8 and 4E6) cross-reacted with all of

Table 1. Isotypes and immunoblotting patterns of monoclonal antibodies

Category	Original clones	Isotypes	Immunoblotting
F. gigantica specific	21C10	IgG_1 (κ)	multiple bands >14.4-27.5 kDa
	13D7	IgM (κ)	multiple bands >14.4-27.5 kDa
Cross-reactive	6D8	IgM (κ)	multiple bands >14.4-27.5 kDa
	4E6	IgM (κ)	multiple bands >14.4-27.5 kDa
	2C6	IgM (κ)	multiple bands >14.4-27.5 kDa
	6B6	IgM (κ)	single band at 185 kDa
	18B6	IgG_3 (κ)	single band at 185 kDa

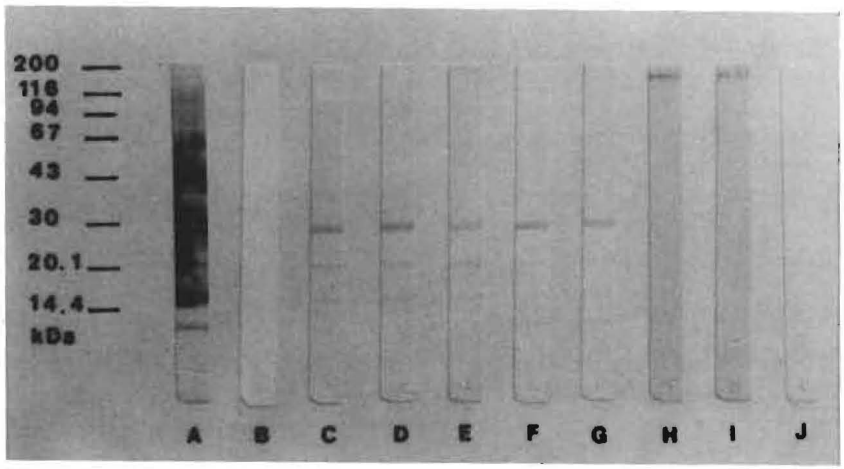

Fig. 1. Immunoblotting pattern of FGES antigen electrophoresed in the presence of SDS, electroblotted on nitrocellulose membrane and allowed to react with various antibody preparations. Lanes A-B = patterns of immunoblot after reacted with immune mouse serum (A) and normal mouse serum (B). Lane C-I = immunoblot patterns of mAbs from clones 21C10, 13D7, 6D8, 4E6, 2C6, 6B6 and 18B6 respectively. Lane J = negative control (medium used for culturing myeloma cells).

the panel of heterologous antigens (Group 4).

When these mAbs were typed, the heavy chain isotypes were found to exclusively belong to IgG_1, IgG_3 and IgM, respectively (Table 1). Regardless of their specificity and heavy chain isotypes, these mAbs were found to possess a kappa type of light chain.

Identification of immunoreactive component by immunoblotting

Monoclonal antibodies with strong ELISA activity for FGES antigen from seven clones (Table 1; Figure 1) were characterized further by SDS-PAGE and immunoblotting. The results exhibited two different reactive patterns against FGES antigen. Five of seven mAbs from clones namely 21C10, 13D7, 6D8, 4E6 and 2C6 were found to react with the multiple bands of relative molecular mass between >14.4-27.5 kDa. The remaining 2 mAbs from clones namely 6B6 and 18B6 reacted only with the 185 kDa band.

Anatomical specificity of the mAbs detected by IFAT

The seven mAbs used in the immunoblotting were also tested for their anatomical specificities against the adult worm sections by IFAT (Figure 2). Five mAbs (21C10, 13 D7, 6D8 4E6 and 2C6) recognizing the multiple bands between > 14.4 - 27.5 kDa gave extremely bright fluorescence over the surface tegument, all the major muscular systems, vitelline gland, testes, and intestinal caeca. The remaining 2 mAbs (6B6 and 18B6) which reacted with the 185 kDa showed a bright fluorescence over the thinner muscular layer underlying the tegument, intestinal caeca and testes.

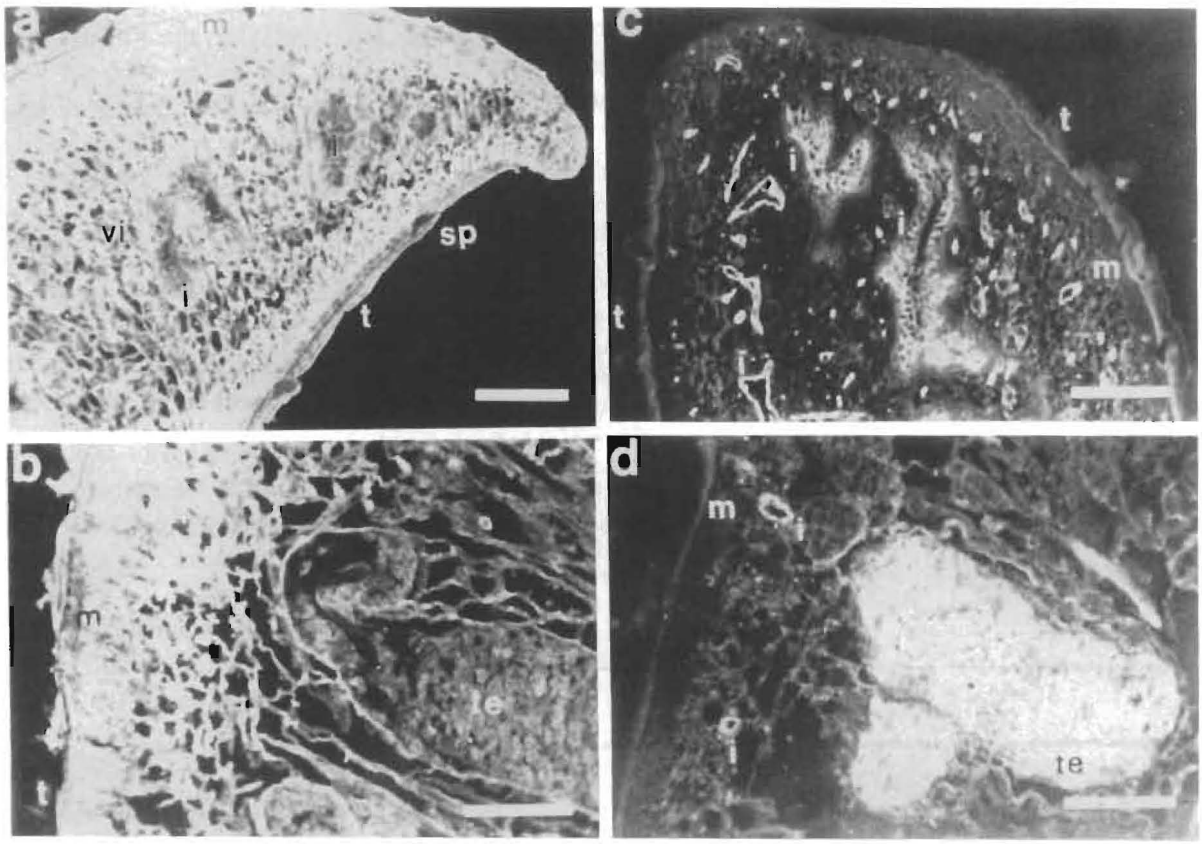

Fig. 2. Indirect immunofluorescence reactivities of localized antigens of *F. gigantica* sections stained with monoclonal antibodies 21C10 (panel a-b) and 6B6 (panel c-d). Surface tegument (t), all muscular systems (m), vitelline glands (vi),testes (te) and intestinal caeca (i) stained brightly with 21C10. Thin muscular layer underlying the tegument (m), intestinal caeca (i) and testes (te) also fluoresced with 6B6. (Scale bar = 100 mm ; sp = spine).

DISCUSSION

From a total of two fusions, a large proportion of IgM mAbs has been obtained. The production of IgM mAbs were unexpected since these were produced from spleen cells taken from mice repeatedly immunized with the parasite antigens. One reason why a large proportion of IgM mAbs was obtained could be due to T independent antigen, such as carbohydrates or nucleic acids which seldom induce a secondary response.[24] Immunoblotting patterns showed multiple reactive bands by a large proportion of mAbs suggested that these bands represent proteins with similar epitopes or they are break down products of the same protein.

The production of mAbs to *Fasciola* spp. have been reported with based on *F. hepatica*. Hanna and Trudgett[25] and Hanna *et al.*[26] produced mAbs against tegumental and internal organs of *F. hepatica*. Yamasaki *et al.*[27] produced mAbs against a 27 kDa protease of an unknown species of *Fasciola*. Solano *et al.*[28] produce mAbs against some ES products. Recently, Fagbemi [29] developed the mAbs reactive with a 28 kDa protease of *F. gigantica*. In this study, a large proportion of mAbs to FGES antigen which reacted with the multiple bands at <14.4-27.5 kDa was produced. These mAbs were reactive with more intensely band at approximate molecular mass of 27.5 kDa. This finding agreed with the previous developments of the mAbs reacted with a 28 kDa protease[29] and

it is possible that our mAbs might be reactive with protease enzymes. The slight difference in the relative molecular mass of the reactive band has thought to be attributed to different methods of antigen preparations or the estimate deviation of the relative molecular mass.

The FGES antigen which represent proteins released in the *in vitro* culture of adult flukes composed mainly of two prominent bands at relative molecular mass of 26 and 27 kDa.[7] These two bands were also detected in immunoblotting of ES antigen and were believed to be cysteine protease.[7] The protease of parasitic helminths composed of very important fractions which serve functions in immunodiagnosis[30] and immunity evasion.[31] In addition, the cysteine protease of *F. gigantica* has been also shown to enhance the specificity and sensitivity for serodiagnosis of fascioliasis in ruminants.[32] From above discussion, it is possible to use our specific mAbs in the affinity purification of parasite specific antigen for serodiagnosis of fascioliasis. Moreover, the mAbs might be used in detection of parasite products in serum or stool samples.[33-34]

From these studies, the two specific monoclonal antibodies (21 C10 and 13D7) recognized target epitopes over the surface tegument, all the muscular systems, vitelline glands, testes and intestinal caeca. However, these target epitopes were different from the mAbs specific for the *Fasciola* spp. protease reported previously,[27] since the strong fluorescence was only observed in the epithelial cells and microvilli of the intestine of the *Fasciola* spp. adult.

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