DEVELOPMENT AND CHARACTERIZATION OF MONOCLONOL ANTIBODIES AGAINST EXCRETORY-SECRETORY ANTIGENS OF *FASCIOLA GIGANTICA*

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Abstract. Monoclonal antibodies (MAbs) directed against *Fasciola gigantica* excretory - secretory (ES) antigens were developed from BALB/c mice. Four were selected for further study, from the panel of hybridomas. The antigen specificities of these MAbs were characterized and localized by enzyme-linked immunoeletrotransfer blot (EITB) and immunoperoxidase technique. The target epitopes of these MAbs are 66 kDa protein (MAb 2D10), 66 and 27-26 kDa proteins (MAbs 5D10 and 4F5) and 27-26 kDa protein (MAb 2D9). MAb 2D9 reacted to the antigenic components of the luminal content and epithelial cell lining the cecum, whereas MAb 2D10 reacted specifically to the antigens of the tegument and surface membrane. It was found that all MAbs cross-reacted to various degrees with the antigens extracted from *Schistosoma mansoni, S. mekongi, S. spindale* and *Paramphistomum* spp. However, when MAbs were diluted to 1:100 or 1:400 significant reduction of their cross-reactivities was observed.

INTRODUCTION

Liver fluke infection in animals due to *Fasciola gigantica* is an important veterinary problem that causes a great deal of economic loss throughout the tropical region of the world. Identification and characterization of the parasites antigens are needed in order to develop efficient immunodiagnostic methods and/or vaccines against the infection. In recent years, several authors have recognized the role of excretory-secretory products released from the parasites (ES antigens) as important in eliciting the antibody formation in the infected animals, and these antigens were also detectable in the host circulation and feces. (Langley and Hillyer, 1985; Espino *et al*, 1990; 1992; Espino and Finlay, 1994). Therefore, they have been considered as possible candidates for immunodiagnosis or vaccine against facioliasis (Espino *et al*, 1990, 1992; Solano *et al*, 1991; Ruiz-Navarrette *et al*, 1993).

The possession of common antigens or cross reactions among antigens of *Fasciola* and other trematode parasites species has been demonstrated by several investigators (Hillyer and Serrano, 1983; Hillyer, 1985; Yagi *et al*, 1986; Haroun and Hillyer, 1988). It is anticipated that immunodiagnostic methods using monoclonal antibody-based systems would help to avoid the problem of cross-reactivities. In the present study, we have produced four monoclonal antibodies (MAbs) against ES antigens of *F. gigantica* and attempted to characterize and localize them against their respective immunoreactive molecules by immunoperoxidase technic. Cross-reactivities of these MAbs with antigens from other common species of cattle and human trematodes have also been investigated.

MATERIALS AND METHODS

Preparation of antigens

Live, intact adult *F. gigantica* were obtained from the bile ducts of naturally infected cattle at local abattoirs. The parasites were washed three times with Hanks' Balanced Salt Solution (HBS) containing 100

U/ml penicillin and 100 mg/l streptomycin to remove the host blood, bile and contaminating microorganisms. Three to five *F. gigantica* adults were maintained under axenic conditions in a sterile Petridish (6 cm in diameter) containing 20 ml of Minimum Essential Medium (MEM) with 100 U/ml penicillin and 100 mg/l streptomycin at room temperature for 72 hours. Throughout this duration, the viability of the parasites was checked regularly and those that appeared inactive were removed. The medium was collected and replaced every 3-4 hours and stored at -20 °C. To obtain the excretory - secretory (ES) antigens, the collected medium was centrifuged at 1500*g* for 20 minutes at 4 °C to remove eggs and debris. The supernatant was concentrated by lyophilization, and the ES antigen was disolved in double distilled water and dialyzed in 0.01 M phosphate buffer saline (PBS), pH 7.2, at 4-6 °C for 48-72 hours, until the clear solution was obtained. The antigen solution was centrifuged at 5,000*g* at 4 °C for 20 minutes. Protein concentration was determined by Lowry's method (Lowry *et al*, 1951). The ES antigen solution was sterilized by filtering through a Millipore 0.22 µm and stored at -70 °C. The tegumental antigens were prepared according to the method described by Viyanant *et al*, 1993.

Production of monoclonal antibodies (MAb)

Six to eight weeks old BALB/c mice were used for production. Each animal was immunized by subcutaneous inoculation at four sites with 100 µg of ES antigens mixed with equal amount of complete Freund's adjuvant. The second and third immunization were given at two-week intervals by the same route, with the same amount of antigen, but with incomplete Freund' s adjuvant. The last immunization was given via intraperitoneal route two weeks after the third, with 400 µg of antigen in normal saline. The antibody titers were determined by enzyme - linked immunosorbent assay (ELISA) at 4-6 days after each immunization. Only mice with antibody titers higher than 1:400 were used for cell fusion. Splenic cells taken from immunized mice were fused with P3/x63-Ag8 mouse myeloma cells using polyethylene glycol (MW 1,500, BDH) as previously described (Viyanant *et al*, 1991). MAbs were typed by ELISA using Mouse MonoAb-ID Kit (ZYMED, California, USA)

Enzyme - linked immunoelectrotransfer blot (EITB)

In order to identify the immunogenic molecules in *F. gigentica*, EITB was performed between ES antigens as well as crude extract of whole body (WB) antigens and the experimentally infected bovine serum (ICS). Stable clones of MAbs which showed high ELISA activity were also used for characterization of the specific immunogenic molecules in the ES antigens. EITB was performed as described previously by Viyanant *et al* (1993).

Localization of the immunoreactive molecules

Two MAbs were used for localization of the antigenic molecules in the parasites' tissues by immunoperoxidase technic as previously described by Viyanant *et al* (1993). However, frozen sections of adult *F. gigantica* cut with cryostat were used in this study. Infected bovine serum diluted 1:50 and 10% fetal calf serum were used as positive and negative controls, respectively.

Cross-reactivities of MAbs with other parasite antigens

Indirect ELISA was used for studying the cross-reactivities of MAbs. The antigens used in this study were cruded extracts *of Schistosma mansoni, S. spindale, S. mekongi* and *Paramphistomum* spp. The parasite antigens (0.75 µg) were used for coating the polystyrene microtiter plate, and then allowed to react with various dilutions of MAb from supernatant culture fluid. The ELISA technic was performed essentially as described by Srivatanakul *et al* (1985).

RESULTS

Selection of hybridomas

A total of 128 clones of antibody-secreting hybridomas were obtained from three fusions. Culture fluids from hybridomas grown in 24-well plates were screened for antibody activity by indirect ELISA, using ES antigens at the concentration of 50 µg ml^{-1}. Reading of at least 0.3 optical density units (OD) above the background was used as the criterion for positive antibody secretion.

Identification of immunoreactive components of ES antigens.

Moniclonal antibodies which had high reactivities against ES antigens were characterized further by EITB. The results of EITB between whole body (WB) as well as ES antigens and ICS indicate the presence of five major immunogenic bands at molecular weights 97,66 and 27-26 kDa, respectively (Fig 1). The antigenic molecules at 27-26 kDa are very prominent in ES antigens. When monoclonal antibodies were used, three patterns of reactive bands were exhibited. MAb 2D10 (IgM) reacted strongly with antigenic molecules at 66 kDa, MAb 2D9 (IgG_1) reacted specifically with 27-26 kDa molecules, and MAb 5D10 (IgM) and MoAb 4F5 (IgM) reacted with both 66 and 27-26 kDa molecules (Fig 1). All the immunogenic bands detected by using these of MAbs were present in the EITB pattern of reaction between whole body antigens (WB) and ICS. It should also be mentioned that clearly distinct bands at 27-26 kDa were observed in the EITB pattern of reaction ES antigens and ICS.

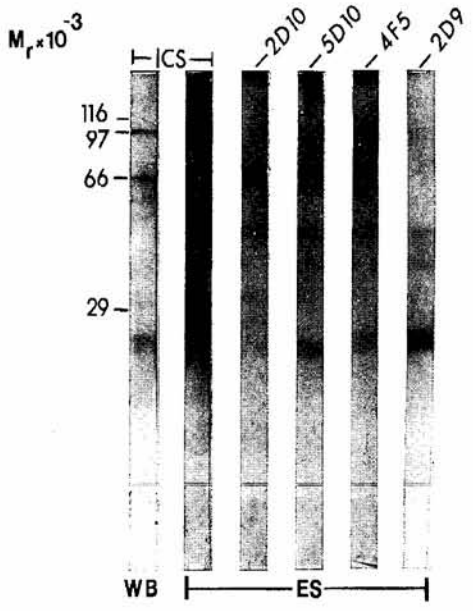

Fig 1 - Result of enzyme-linked immunoelectrotransfer blot of 2D10, 5D10, 4F5 and 2D9 monoclonal antibodies. ICS = infected cow serum; WB = extract of whole body; ES = excretory-secretory antigens.

Localization of the immunoreactive 66 and 27-26 kDa components by immunoperoxidase technic

MAb 2D9, which reacted specifically with the ES antigens at 27-26 kDa, stained intensely the luminal content as well as epithelial cells lining the cecum. The latter also showed positively stained granules in their cytoplasm (Fig 2B, C). MAb 2D10, which reacted with an antigen at 66 kDa, exhibited light staining of the tegument and its surface membrane while it showed no staining of the cecal lumen and its epithelium (Fig 2A). In contrast, the control sections that were treated similarly but without MAbs showed no staining in either tegument or cecum.

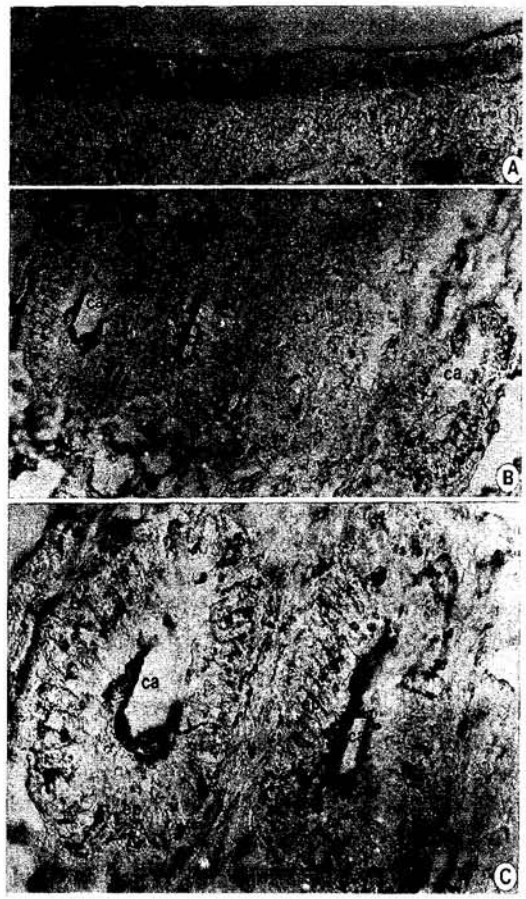

Fig 2 - Anatomical localization of the *F. gigantica* antigenic target recognized by MAb 2D10 (A) and 2D9 (B,C). te = tegument; ca = cecum; ep = epithelium.

Cross-reaction of MAbs with other parasite antigens

Sixteen MAbs which have been found to give high ELISA readings were used for cross-reactivity study. All of the 16 clones were found to cross-react to varying degrees with the panel of the parasite antigens tested. However, when dilution titers of MAbs were decreased to 1:100 and 1:400 significant reduction in their cross-reactivities were observed, especially with MAbs 2D10, 5D10, 2D9 and 4F5. The mean ELISA values (OD) of the above four MAbs are shown in (Fig 3). It should also be mentioned that all of these 16 MAbs reacted strongly with all antigens used for specificity testing at dilution titers of 1:10 and 1:100.

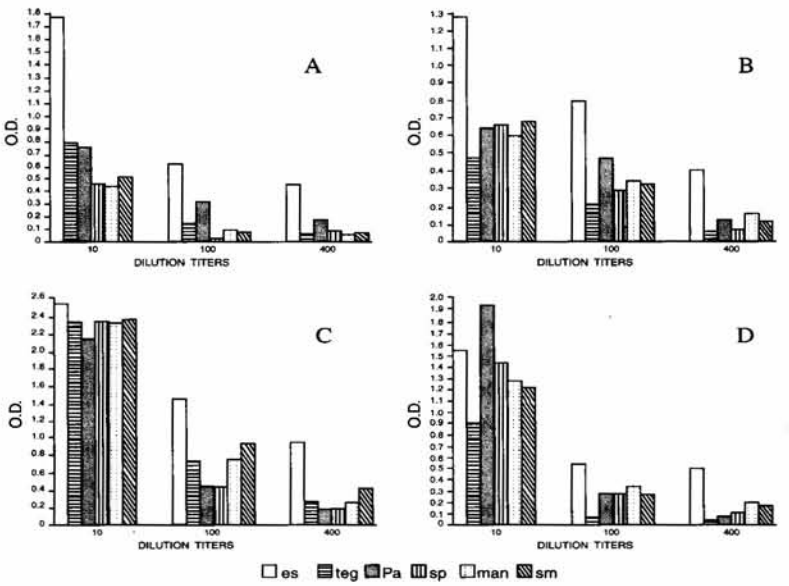

Fig 3 - Mean ELISA values of cross-reactivities studies between MoAbs 2D10 (A), 5D10 (B), 2D9 (C) 4F5 (D) and ES antigens (es), tegumental antigens (teg), *Paramphistomum* spp (pa), *S. spindale* (sp), *S. mansoni* (man), *S. mekongi* (sm).

DISCUSSION

Results in this study demonstrated that the monoclonal antibodies obtained from mice immunized with ES antigens of *F. gigantica* recognized epitopes present in 66 and 27-26 kDa molecules secreted by the parasites. These ES antigens are probably composed of molecules released from the continuous turnover of the glycocalyx coating the tegument surface membrane, as well as some enzymes released from the cecum. Various groups of investigators have found that ES antigens could form immune complexes with hosts antibodies to *Fasciola* infection (Lehner and Sewell, 1980; Santiago, 1986; Solano *et al*, 1991). Thus, ES antigens may not only have an important role in the induction of protective immunity but contains antigens useful for immuno-diagnosis of fascioliasis.

By using EITB, we could identify a group of ES antigens which were recognized by infected bovine serum (ICS), and the four MAbs reported here recognized different group of epitopes on the ES antigens. Of special interest are IgM 2D10 and IgG_1 2D9 MAbs which specifically recognized 66 and 27-26 kDa polypeptides, which react strongly with all ICS tested. This group of polypeptides has also been reported as the major antigenic component in ES antigens of *F. hepatica* (Irving and Howell, 1982; Santiago and Hillyer, 1986). The immunoperoxidase staining pattern of MAb against 27-26 kDa antigens suggested that these immunoreactive components are present principally in the cecal lumen and cecal

epithelium. Preliminary biochemical analysis indicates that the 27-26 kDa proteins contain antigens which might be the cysteine protease enzyme synthesized and secreted from the cecal epithelium (unpublished data). The 27-26 kDa cysteine protease enzyme reported in this study could not be distinguished from the 26-28 kDa protease purified from homogenization of adult *F. hepatica* worms by Fagbemi and Hillyer (1992). The protease enzyme is probably used by the parasites for digesting nutritive materials derived from their hosts. Since this enzyme is highly immunogenic, it is being investigated for uses in immunodiagnosis and vaccine development for fascioliasis (Fagbemi and Hillyer, 1992; Wijffels *et al*, 1994).

The epitope recognized by IgM 2D10 MAb was detected in the tegument, particularly on its surface which might represent the surface membrane. This 66 kDa molecule was not as prominent as 27-26 kDa antigen in both whole body and ES preparations when reacted with either ICS or MAbs. Nevertheless, its presence is definite and could also be considered as another potential candidate antigen to be used for immunodiagnosis.

Many investigators have shown that immunological cross-reactivities exist between antigens of *Fasciola, Schistosoma, Paragoniums* and some other parasite species (Hillyer and Serrano, 1983; Yagi *et al*, 1986; Haroun and Hillyer, 1988; Solano *et al*, 1991). Our results similarly demonstrated that MAbs against ES antigens of *F. gigantica* strongly cross-reacted with antigens extracted from *S. mansoni, S. mekongi, S. spindale* and *Paramphistomum* spp. The immunological cross-reactivities between *Fasciola* and other parasite species may reduce the accuracy of immunodiagnostic assay for fascioliasis. However, from our experiments we could show that all four selected MAbs exhibited significantly decreased cross-reactivities when the antibody titers were decreased to 1:100 or 1:400. These four MAbs are currently being investigated further for their potential uses in immunodiagnosis of animal fascioliasis.

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