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Identification of Circulating Antibodies in Fasciolosis and Localization of 66 kDa Antigenic Target using Monoclonal Antibodies

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SUMMARY **We identified three specific circulating antibodies in serum of cattle naturally infected with *Fasciola gigantica*. Two of the antibodies were found to react specifically to 97 and 66 kDa antigenic molecules of adult worm tegumental membrane extract. The third antibody was identified by the reaction with 26-28 kDa molecule of the excretory/secretory antigens. Monoclonal antibody against 66 kDa protein was developed and used for localization of its antigenic target in adult worm frozen sections. The experiment demonstrated that 66 kDa protein is a component on the outer surface membrane and on the membrane lining of the caecal epithelial of adult worm. The 66 kDa antigen was considered as a promising candidate for immunodiagnosis and vaccine.**

The helminthic parasites of humans as well as animals, such as *Schistosoma* and *Fasciola*, are known to release copious amounts of antigens which elicit intense antibody production in the host's circulation. For *Fasciola*, these antigens are mostly released from rapid turn-over of the external covering, called the tegument, which has been shown to contain two distinct types of presumptive secretory vesicle, termed T1 and T2 bodies. Histochemical studies with the electron microscope showed that the vesicles contain glycoproteins resembling in their staining properties of the surface glycocalyx of the tegument.[1] It has been suggested that glycocalyx turn-over may help protect the pre-bile duct flukes against immunological attack.[2] The presence of parasite glycocalyx in the host circulation has been considered as a potential marker for the development of immunodiagnosis method by circulating antigen detection in humans and cattle. The other antigenic molecules that have been considered as potential candidates for immunodiagnosis and vaccine are the excretory/secretory (ES) antigens. ES-antigens contained several enzymes such as glutathione S-transferases,[3] cysteine protenase[4] and cathepsin L protenases.[5] Recently, the method has been successfully developed for detection of the circulating ES-antigens of human and cattle fasciolosis.[6,7]

Considerably less research has been carried out on the nature and potential of antibody against surface tegumental (ST) antigens as a marker for immunological assay or vaccine. In this study, we have demonstrated the presence of three specific antibodies in sera of cattle naturally infected with *Fasciola gigantica* and developed a panel of monoclonal antibodies (MoAbs) against 66 kDa antigen of the surface tegument of adult worm. Anatomical localization of the specific antigens was performed using cryostat sections of adult worm and MoAbs.

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MATERIALS AND METHODS

Preparation of excretory/secretory (ES) antigens

Adult *F. gigantica* were removed from the bile duct and gall bladders of condemned bovine livers at a local slaughterhouse. They were washed three times with Hank's balanced salt solution (HBS) containing 100 U/ml penicillin and 100 mg/l streptomycin to remove all traces of blood, bile and contaminating microorganisms. The ES-antigens were prepared by incubating viable adult worms in Hank's balanced salt solution (Gibco, USA.) at room temperature for 3-6 hours. The parasite eggs in the culture medium were removed by centrifugation at 5,000 x *g* for 20 minutes at 4°C. The supernatant was dialysed in 0.01 M PBS pH 7.2 at 4°C for 24 hours and kept at -20°C until used.

Preparation of surface tegumental (ST) antigens

ST-antigen was obtained by extraction of live adult worms with 1% Triton X-100 in Tris buffer pH 8 for 30 minutes at room temperature. The extraction solution was collected and centrifuged at 5000 x *g* for 20 minutes at 4°C to remove the parasite eggs which may be released during extraction. The supernatant containing ST-antigens was collected and dialysed in 0.01 M phosphate buffer saline (PBS) pH 7.2 at 4°C for 24 hours before it was lyophilized and kept at -20°C until used. Scanning electron microscopy (SEM) was performed to compare the parasites before and after extraction with Triton X-100.

The protein content of all antigens were determined by modified Lowry's method.[8]

Production of monoclonal antibodies against *Fasciola gigantica* ST-antigens

Hybridomas were produced by fusion of spleen cells from mouse immunized with ST-antigens of *F. gigantica* and mouse myeloma cells (P3/x63-Ag8). The hybridoma cells that were found to grow successfully in culture were cloned by limiting dilution methods. Only the hybridoma clones that were found to produce high titer of antibody against ST-antigens were selected for further study. Three MoAbs were used in this study namely 1C12, 2C3 and 1H7, they were typed by ELISA using Mouse MonoAb-ID kit (ZYMED Laboratories, USA).

Enzyme-linked immunoelectrotransfer blot (EITB)

EITB was performed as described previously by Viyanant *et al.*[7] ST-antigens and ES-antigens were separated in SDS-PAGE and their antigenicity was determined by EITB using cow infected sera (CIS) obtained from the pool of serum of naturally infected animals, and MoAbs. Cattle antibodies that reacted with the antigenic molecules were detected by incubation with peroxidase-conjugated rabbit anti-cow immunoglobulin whereas the monoclonal antibodies were detected by peroxidase conjugated rabbit anti-mouse Ig. The reaction was visualized by further incubation in 3,3 diaminobenzidine (DAB) and H_2O_2.

Localization of the immunoreactive molecules

The three MoAbs were used for reaction with the specific antigenic molecules in the frozen section of adult *F. gigantica*. The reaction was demonstrated by Avidin-Biotin method as previously described by Viyanant *et al.*[9] CIS diluted 1:50 and 10% fetal calf serum were used as positive and negative controls, respectively.

RESULTS AND DISCUSSION

Extraction of parasites by Triton X-100

Observation of adult parasites under SEM revealed that the tegumental surface was characterized by the presence of numerous spines, except in the areas around the oral and ventral suckers (Fig. 1A). Examination under higher magnification indicated that spines were closely spaced and varied in shape and size, those on the antero-ventral and lateral sides of the body were large with serrated edges and directed backward (Fig. 1B). Others on the postero-ventral and dorsal sides tended to be smaller with no serrated edges. The areas between spines appeared corrugated with series of grooves and folds. The surface of the spines themselves was also highly ridged and pitted (Fig. 1B).

Examination of the parasite by SEM after extraction with Triton X-100 revealed that large flaps of tegument were stripped off rather cleanly (Fig. 1C). The tegument covering the rims of the sucker which probably bound to the underlying tissue were still attached. Observation under higher magnification revealed total extraction of the tegument in all other areas (Fig. 1D). It was, therefore, concluded that the major portion of the ex-

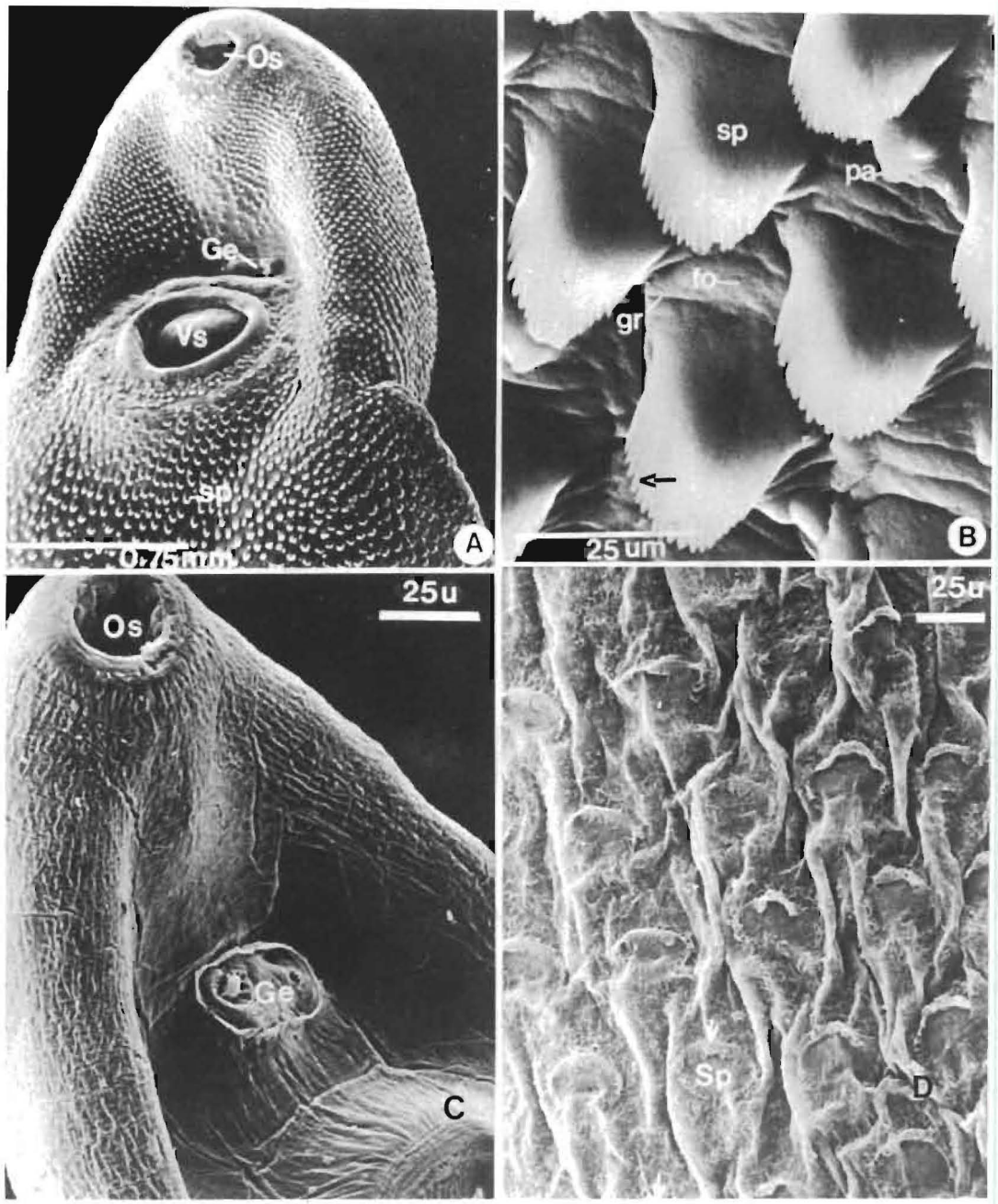

Fig. 1 SEM micrographs of the surface of adult parasites: A, showing the anterior end with oral sucker (Os), ventral sucker (Vs), genital pore (Ge) and spine (Sp). B, high magnification of large spines on the anterior-half of the body, showing serrated edges (arrow), highly corrugated with folds between spines (fo), grooves (gr) and a group of papillae (pa). C, showing the surface of the parasite after extraction with Trion X-100 and D, high magnification of the extracted parasite.

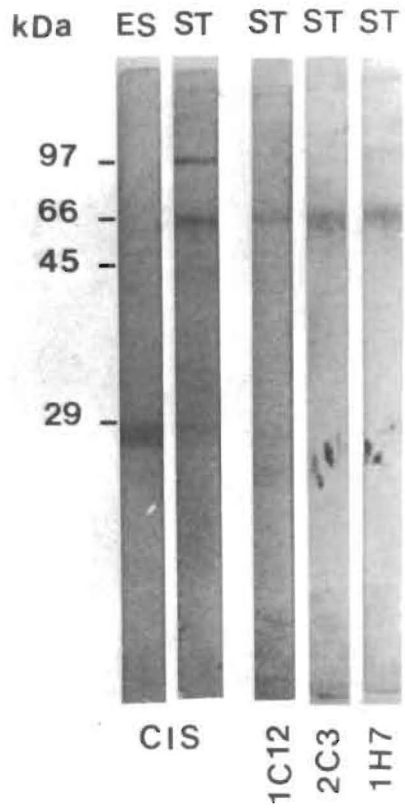

Fig. 2 Immunoelectrotransfer blot pattern of ES-antigens (ES) and ST-antigens (ST) with cow infected sera (CIS) and monoclonal antibodies (1C12, 2C3 and 1H7).

tracted antigens were derived from the tegument.

Identification of specific antibodies in naturally infected animals

The presence of specific antibodies in CIS against the antigenic molecules in ST-antigens and ES-antigens were demonstrated by EITB (Fig. 2). The pattern of EITB between ST-antigens and CIS clearly demonstrated the presence of two specific antibodies which reacted to 97 and 66 kDa antigenic molecules. The third circulating antibody was detected by the reaction between ES-antigens and CIS at 26-28 kDa polypeptide band.

Identification of the above circulating antibodies may be useful for future development of immunodiagnosis and/or vaccine in fasciolosis. The antigenic molecules can be purified and used as the capture antigen for immunodiagnostic method or can be used as candidate vaccine. From the previous study, 97 kDa protein has been identified

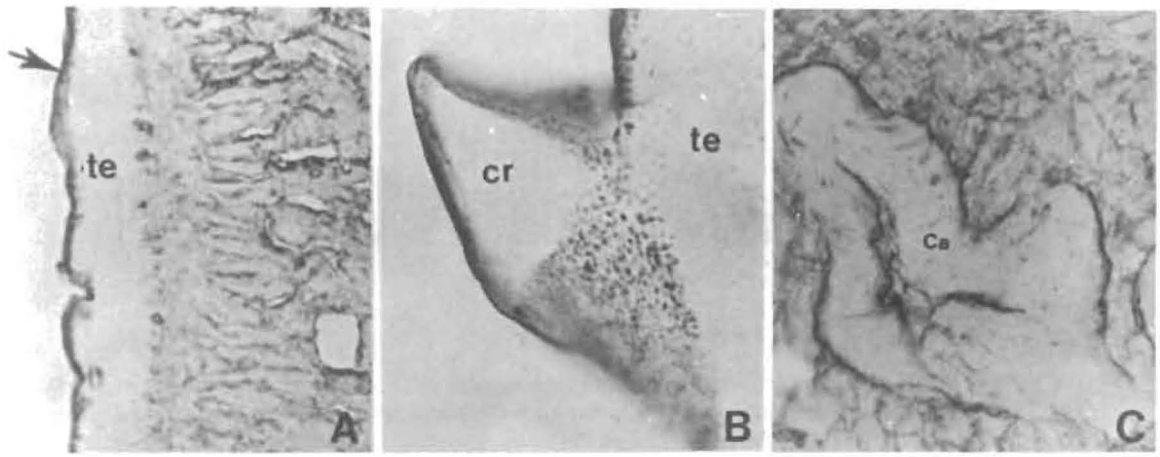

Fig. 3 Light microscopic pattern (x40) for anatomical localization of *F. gigantica* antigenic target recognized by 66 kDa MoAb (A,B,C). Reaction on the surface tegument (Te), crystal lattice (Cr) of the spine is not stained and the reaction on the membrane lining of the caecal epithelial (Ca) of adult worm.

as paramyosin which is a myofibrilla protein located in the muscle and tegument of most species of trematodes. The role of paramyosin for induction of protective immunity has been reported by several workers.[10,11,12] On the other hand, the 26-28 kDa antigenic molecule which was found only in the ES-antigens is a proteinase enzyme of the cysteine family which adult fluke excreted in ES-materials.[13,14] The enzyme, cathepsin L protenase has been used to diagnose infection in ruminants[15] and tested as vaccine.[14]

The third major circulating antibody is the antibody that reacted specifically to 66 kDa protein of ST-antigens. Although the nature of this antigenic molecule has never been thoroughly studied, MoAb against 66 kDa protein has been developed in *Schistosoma* and *Fasciola* parasites and has been successfully used for immunodiagnosis of cattle fasciolosis.[9,10]

Localization of 66 kDa protein by monoclonal antibodies

The three MoAbs (1C12, 2C3 and 1H7) against 66 kDa protein developed from this study were identified as IgM. Immunohistochemical study clearly demonstrated the presence of their specific antigenic molecules in the tissue sections of adult worms. The experiment revealed that the reaction occurred mainly on the tegument of the adult worm which covered its surface and spine as well as cells lining the gut. (Fig. 3). The intense immuno-staining of the tegument cells soma and their branches between muscle cells implicated that most antigens were produced in the cells and transported to the tegument (Fig. 3A). Intense staining was found only on the surface membrane covering the spine, but not in the spine crystalline matrix (Fig. 3B). It is most likely that the spine material were not shed and turned over like the surface membrane, thus their contents were not released into the host's circulation to stimulate antibody production. The result of this experiment indicated that 66 kDa antigen is a component on the outer surface membrane of the adult worm. In addition, there was an intense staining of the narrow zone of cytoplasm just beneath the surface membrane. This might represent the zone of tegumental cytoplasm where tegumental granules were observed to be highly concentrated and these granules were believed to contribute to the synthesis of the surface membrane.

Fig. 3C shows the immunostaining of MoAb on the membrane lining of the caecal epithelial and their basal laminae of the caecal epithelial cells. This suggested that an antigenic epitope similar to 66 kDa was also present in the membrane and basal laminae of caecal epithelial cells. No reaction was observed in the negative control experiments whereas for the positive control the reaction appeared as an intense undulating line on the surface and the outer surface of the spines' tips. The dense staining granules spreading throughout the tegument cytoplasm, and in the bodies of tegumental cells lying beneath the muscle layer were also observed. In addition, the positive reaction of the immuno-staining was also detected within the lumen of the caecum and its lining epithelium.

Further studies on biochemical properties of this antigenic molecule are being performed and will be reported later. The information will be important in understanding the potential use of 66 kDa antigenic molecule and its specific antibody.

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