J.Sci.Soc.Thailand, 22(1996) 143-162

FASCIOLA GIGANTICA: IDENTIFICATION OF ADULT ANTIGENS, THEIR TISSUE SOURCES AND POSSIBLE ORIGINS

PRASERT SOBHON,[a] SIRINART ANANTAVARA,[a] TUNYARAT DANGPRASERT,[a] ARDOOL MEEPOOL,[a] CHAITIP WANICHANON,[a] VITHOON VIYANANT,[b] SUCHART UPATHAM,[b] THANIT KUSAMRAN,[c] TASSANEE CHOMPOOCHAN,[d] SUREE THAMMASART,[d] PIYANUT PRASITTIRAT[d]

[a] *Department of Anatomy, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand.*

[b] *Department of Biology, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand.*

[c] *Department of Biochemistry, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand.*

[d] *Institute of Animal Health and Production, Department of Livestock Development, Ministry of Agriculture and Co-operatives, Phaholyothin Road, Bangkhaen, Bangkok 10900, Thailand.*

(Received April 26, 1996)

ABSTRACT

Fasciola gigantica *is a major parasitic disease of cattle, water buffaloes and sheep. In Thailand it costs economic damages in terms of meat, milk production and labour losses, estimated at 300-350 million bahts per year. Convenient and reliable immunodiagnostic methods based on the detections of either antibodies or antigens in the blood of infected animals should be devised in place of the identification and counting of eggs in faeces. Before reaching such goals certain basic information concerning the parasite's biology must be known. In the present study, using immunoblotting technique we have identified major antigens in adult parasites as those originated from the tegument at molecular weights (MW) 66, 58, 54, 47 kD, and those from the caecum at MW 27, 26 kD. The two tissue sources of antigens have been studied by light and electron microscopies, as well as immuno-microscopic detection methods, in order to understand the distribution, relative amount, as well as possible origins of the antigens in the tissues. The tegument-associated antigens were concentrated in the tegument surface membrane, the outer rim of the tegument cytoplasm and tegument cells. The caecal-associated antigens were present in the lumen and the apical cytoplasm of caecal epithelial cells, and thus were probably digestive enzymes secreted in the form of zymogen granules.*

INTRODUCTION

Fasciola gigantica is one of the major parasitic diseases that can infect water buffaloes, cattle and sheep. It is prevalent in Thailand and countries in the tropical regions. Cases of human infection have also been reported in the endemic regions.[1-3] The parasite is transmitted by the snail intermediate host, *Radix rubiginosa*, which is widely distributed in a variety of habitats, such as rice fields, ponds, reservoirs, canals, dams, etc. The rates of prevalence of the parasite vary considerably, ranging from 0% to 33%, with the mean rates of prevalence of 8.9% in buffaloes and 13.9% in cattle.[4] The disease, fascioliasis, results in reduced weight gain (3%), in death of young (3 to 10%) and adult (3%) animals, and in low production of milk (16 to 24%). This liver fluke infection has caused substantial loss in adult buffaloes and cattle throughout the north and northeastern parts of Thailand,[5-8] which was estimated to cost approximately 312.50 million bahts in 1986 by the Department of Livestock Development, Ministry of Agriculture and Co-operatives.

At present, the definitive diagnosis of fascioliasis is by the demonstration of the presence of *F. gigantica* eggs in the faeces. However, the faecal examination technique is unpleasant, tedious, time-consuming, and requiring a well trained microscopist for interpretation, which may not be practical for use in remote areas. More suitable assays technique that are economical, easy to perform, have high sensitivities and reliabilities should be developed in place of the egg counting method. Immunodiagnostic assays, either for circulating antigens or antibodies, should be the methods of choice. However, before such goals could be reached, there must be basic information on the identities and characters of parasites' antigens that could be utilized in immunodiagnoses. In the present report we have identified major antigens from the adult parasites, studied their tissue sources as well as their possible origins at the ultrastructural level.

MATERIALS AND METHODS

1. Sources of parasites

Adult *Fasciola gigantica* were collected by dissecting livers and gall bladders of infected cow and water buffaloes, killed at the local abattoirs. The flukes were washed carefully to remove contaminating blood and tissue debris with several changes of Hanks' balanced salt solution, before being processed for further studies.

2. Immune sera

The immune sera were obtained from 1-year-old cows infected orally with 500 or 1,000 metacercariae per animal. Immune sera were collected at intervals from 12-20 weeks after the infection, when the antibody titers were highest, as tested by microwell ELISA. Non immune sera were collected from cows proven to be free from the infection by *F. gigantica*.

3. Preparations of *Fasciola gigantica* antigens

3.1 Excretory-secretory (ES) antigens

Freshly collected worms were incubated in Minimal Essential Medium (MEM) at room temperature for 6-8 h. Periodically, the MEM medium was collected and replaced whenever its color changed from pink to yellow. The collected solution was centrifuged at 10,000 rpm, 4 °C for 1 h, lyophilized and kept at -70 °C until further use.

3.2 Surface and tegument (ST) antigens

Fresh worms collected as above were washed in several changes of 10 mM Tris-HCl buffer, pH 8.0. Then the worms were extracted with 1% Triton X-100 inTris-HCl buffer, at room temperature, for 20-30 min, and the degree of extraction of the tegument was monitored under the microscope. After complete extraction, the solution was collected, centrifuged, lyophilized and kept as described in section 3.1.

3.3 Somatic (SA) antigens

The remaining bodies of the extracted worms from section 3.2 were washed in several changes of 10 mM PBS buffer, pH 7.2, and then put into the tissue grinder tube. The buffer was then added into the tube which was placed in an ice bath. Then the worms were homogenized, sonicated, centrifuged, lyophilized and kept as described in section 3.1.

3.4 Whole body (WB) antigens

Freshly collected adult worms were prepared as described in section 3.3, and the WB antigens were kept at -70 °C until further use.

4. Procedures for SDS-PAGE

All separating gels were casted from 12.5% acrylamide, and each gel slab was 0.75 mm thick by 5.5 cm long. After the solution was poured, the mixture was gently overlayed with about 1 ml of distilled water and allowed to stand for 1 h for complete polymerization. The stacking gel of 5% acrylamide was poured onto the top of the separating gel after removing the covering water and residual unpolymerized gel mixture, and the assembled glass plates were mounted in the LKB Midget electrophoresis unit. A teflon strip was inserted into the slab before the polymerization of the stacking mixture occurred, in order to make ten slots near the edge of the gel for loading samples and standard molecular weight proteins into the gel. Then the stacking gel was allowed to polymerize for 30-45 min.

Protein antigens (as prepared in section 3), with concentrations determined according to Lowry's method,[9] were dissolved and mixed in the loading buffer (2.5 ml 1.25 M Tris-HCl pH 6.8, 1.0 g SDS, 2.5 ml 2-mercaptoethanol, 5.8 ml glycerol and 5 mg Bromophenol blue) by vortexing and boiling for 2 minutes in a water bath, and then cooled to room temperature before loading into the gel. Thus protein samples (about 40 μg/slot) and the standard molecular weight proteins (β-galactosidase-116 kD, phosphorylase B-97.4 kD, bovine albumin-66 kD, egg albumin-45 kD, pepsin-34.7 kD, carbonic anhydrase-29 kD, trypsinogen -24 kD, β-lactoglobulin-18.4 kD and lysozyme-14.3 kD) were loaded into the slots, and the slab gel was run with Tris-glycine electrode buffer, pH 8.3 at 20 mA constant D.C. current until the dye front reached the bottom of the gel. At the end of the run, the gel was stained for at least 1 h with 0.15% (w/v) Coomassie

brilliant blue R-250 in 50% (v/v) methanol and 10% (v/v) acetic acid at room temperature. The gel was then destained and dried in a slab gel dryer.

5. Procedures for immunoblotting

5.1 Electrophoretic transfer of proteins onto nitrocellulose (NC) paper

The sheet (6 x 8 cm^2) of NC paper (Amersham, Hybond™ RPN.303C) was briefly wetted with distilled water and laid on filter papers on one-half of the blotting cassette. The wet gel from section 4 was placed on the NC sheet and care was taken to remove all air bubbles. Another filter paper and one half of the cassette were placed on top of the stack, and the two halves of the cassette were locked together. The blotting cassette was inserted into the LKB electrophoretic chamber containing 700 ml buffer with the gel facing the anode. The transferring buffer was a mixture of 25 mM Tris, 192 mM glycine and 20% (v/v) methanol at pH 8.3. Proteins were transferred from gels onto the sheet by applying 200 mA constant current for 1 h. When the transfer was completed, the gel and the NC sheet were carefully removed from the cassette, dried and stained with 0.1% Amido Black in 50% (v/v) methanol and 10% (v/v) acetic acid. The NC paper was then destained with several changes of 50% (v/v) methanol and 10% (v/v) acetic acid. The paper was placed on filter paper after destaining, and air-dried.

5.2 Immunoenzymatic staining of the NC sheet containing protein antigens

In addition to the above staining, some nitrocellulose sheets were stained with diluted (1:5) non immune or immune cow sera in 3% BSA/TBS (3% bovine serum albumin in 10 mM Tris-HCl buffer, 150 mM NaCl, pH 7.4), for 12 h or overnight at 4 °C. The sheets were washed with buffer, incubated with 3% BSA/TBS for 1 h, followed by diluted (1:200) rabbit antibovine IgG-HRP, for 2 h, and then stained with 3,3'-diaminobenzidine (DAB) + H_2O_2 (30mM DAB + 0.1% H_2O_2 in 50 mM Tris-HCl buffer, 150 mM NaCl, pH 7.4), for 15 min, at room temperature. The membranes were gently washed with double distilled water, and placed on filter papers for air drying.

The photographs of dried gels and blotted nitrocellulose membranes from sections 4, 5 were taken and the distances of the migration of protein bands were measured. The relative mobility (R_f) of the standard proteins against the reference front of the tracking dye were determined, and their molecular weights plotted against their R_f values. Molecular weights of proteins of interest were estimated from this standard curve.

6. Tissue preparations for light microscopic studies

6.1 Paraffin-embedding technique

Both unextracted and extracted *Fasciola gigantica* worms were fixed with 2% paraformaldehyde in 0.1 M phosphate buffer saline, pH 7.4, for 24 h, dehydrated in graded ethanol and then embedded in paraffin. Blocks of specimens were sectioned at 4-5 μm thick, stained with Hematoxylin and Eosin, and examined in a light microscope.

6.2 Cryo-embedding technique

Pieces of adult worms were immediately fixed with 2% paraformaldehyde in 0.1 M phosphate buffer saline, pH 7.4, for 2 h, then embedded in Tissue Tek O.C.T. medium at -30 °C. The frozen tissues were cut at 4 to 12 μm thick in a cryostat microtome, and the sections were picked up on gelatin-coated slides, and stained with immunoperoxidase as follows. The cryosections were treated with 3% H_2O_2 in methanol to inhibit endogenous peroxidase, and then stained with non immune or immune sera from section 2. The staining with primary antibodies was followed by biotinylated rabbit anti-bovine IgG, Z-avidin and biotinylated peroxidase complex, and DAB solution.

7. Tissue preparation for transmission electron microscopic (TEM) studies

The 1 mm^3 pieces of parasites' tissues were fixed with Karnovsky's fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer saline, pH 7.4), at 4 °C for 4 h, post-fixed in 1% osmium tetroxide in 0.1 M PBS for 1 h, and finally in aqueous solution of 0.5% uranyl acetate containing 45 mg/ml sucrose, pH 5.5. The tissues were then dehydrated in increasing concentrations of ethanol, infiltrated in propylene oxide, and then embedded in Araldite 502. Thin sections were cut with Sorvall MT-2 ultramicrotome, collected on copper grids, doubly stained with uranyl acetate and lead citrate, and examined under a Hitachi H-300 TEM, set at 75 kV.

8. Tissue preparation for scanning electron microscopic (SEM) studies

8.1 Conventional SEM

Adult worms were fixed and dehydrated as described in section 7, and critical-point dried in a Hitachi HCP-2 apparatus, using liquid CO_2 as drying medium. The specimens were then coated with Pt-Pd in a Hitachi E-102 ion sputter, and examined under a Hitachi S-2500 SEM set at 30 kV.

8.2 Backscattered imaging of immunogold labels

The whole worms were fixed with 0.25% buffered glutaraldehyde for 2 h, and rinsed with 0.1% glycine in phosphate buffer saline, pH 7.4. Then they were incubated with immune sera, biotinylated rabbit anti-bovine IgG, and streptavidin-gold, and post-fixed in 2.5% buffered glutaraldehyde for 1 h, rinsed in 7% buffered sucrose, dehydrated in graded ethanol, and critical-point-dried in a Hitachi HCP-2 apparatus. The specimens were lightly coated with carbon in a Hitachi HUS-5GB vacuum evaporator and examined under Hitachi S-2500 SEM using Robinson backscattered detector.

RESULTS

By using immunoenzyme histochemistry (Avidin-Biotin-HRP method) as described in Materials and Methods (section 6.2) the brownish reaction products were deposited at the sites where antigens were localized and produced. Such evidence was, therefore, used to indicate the site of antigenic production in the parasites' tissues. And it was observed that the two major tissues that exhibited significant deposit of reaction products were the tegument and its associated-tegumental cells, and the caecal lumen and its epithelium (Fig. 6, 7). Therefore, the patterns of distribution of the brownish reaction products in frozen sections were correlated with the normal structural components and characteristics of the tegument and the caecum as revealed by the light, scanning and transmission electron microscopic observations.

1. Normal structure of the adult tegument and the caecum

1.1 Light microscopic observations

A fully grown adult *F.gigantica* has a leaf-like and lanceolate shape with the body length about 30-35 mm, and the width at the mid region about 8-10 mm. The body is very thin, as internal organs could be readily visualized when the parasite was whole-mounted and spread on the glass slide (Fig. 1). Major organs that were clearly exhibited consisted of the bifurcated digestive tracts or caeca which branched extensively throughout the body (Fig. 1A, 1B), the pale mid-line bladder, the centrally-located testes, and the bilaterally-situated vitellaria whose major ducts were connected with the anteriorly-located oviduct and uterus (Fig. 1A).

The cross section of the body, which was stained with Hematoxylin and Eosin, showed numerous cross sectional profiles of tubes and tubules (Fig. 2A). The single large tube in the center was the bladder, while the two large lateral tubes were the main trunks of the caeca. The smaller tubes and tubules were branches of the caeca and the bladder, both of which were extensive and permeated all parts of the interior of the body, with branches of the bladder tended to concentrate more on the dorsal aspect while those of the caeca in the middle region. The two sets of tubules could be distinguished by the height of the lining epithelia, which tended to be thin squamous type in the bladder, and cuboidal or columnar type with extensive apical branching in the caecum (Fig. 2A, 5A).

The body was covered by a syncytial layer of the tegument which was about 8-10 μm thick, with numerous spines embedded throughout its matrix (Fig. 2B, 2C). The central and widest part of the tegument and the main bodies of the spines were lightly stained, while the outer rim of the tegument and tips of the spines were intensely stained. Similarly, the narrow basal zone, which should represent the basement membrane was also deeply stained (Fig. 2B, 2C). The tegument rested on the layer of connective tissue called reticular lamina, which connected the former to the underlying and deeply stained two muscular layers (Fig. 2B, 2C). Tegumental cells were located underneath the muscles and sent their processes between the muscle cells outwardly to join up with the tegument (Fig. 2C).

1.2 Observations of the adult parasites' surface by scanning electron microscope (SEM)

When adult *F. gigantica* were observed under scanning electron microscope the tegument surface was characterized by the presence of numerous spines, except in the areas around the oral and ventral suckers (Fig. 3A, 3B). These spines were closely spaced and varied in shape and size depending upon the body parts.

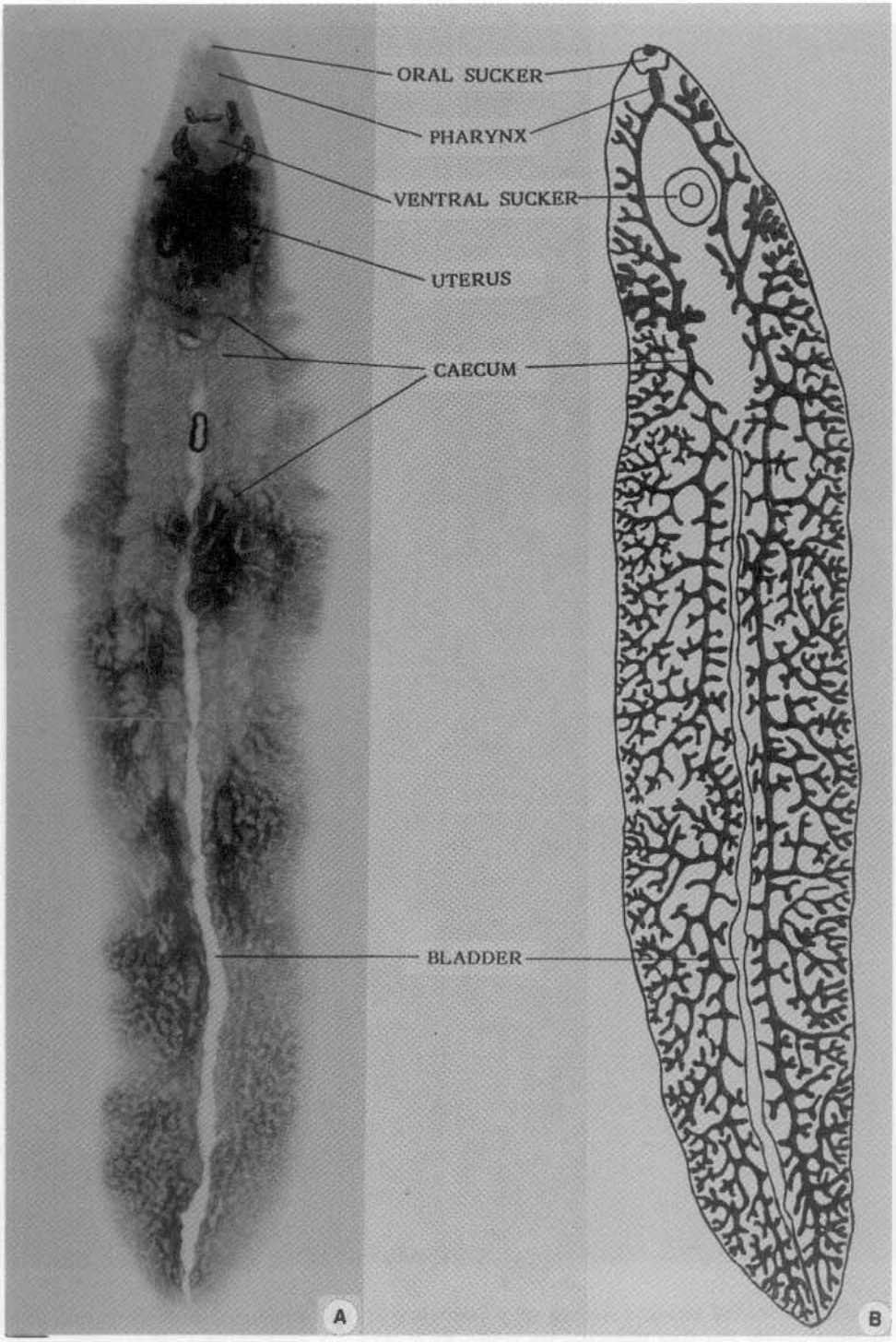

Fig. 1 (A) Whole mount of an adult *F. gigantica* fixed and stained with 1% osmium tetroxide overnight, illustrating various visible organs. (B) Drawing from *camera lucida,* demonstrating the extensive branching of the caecum.

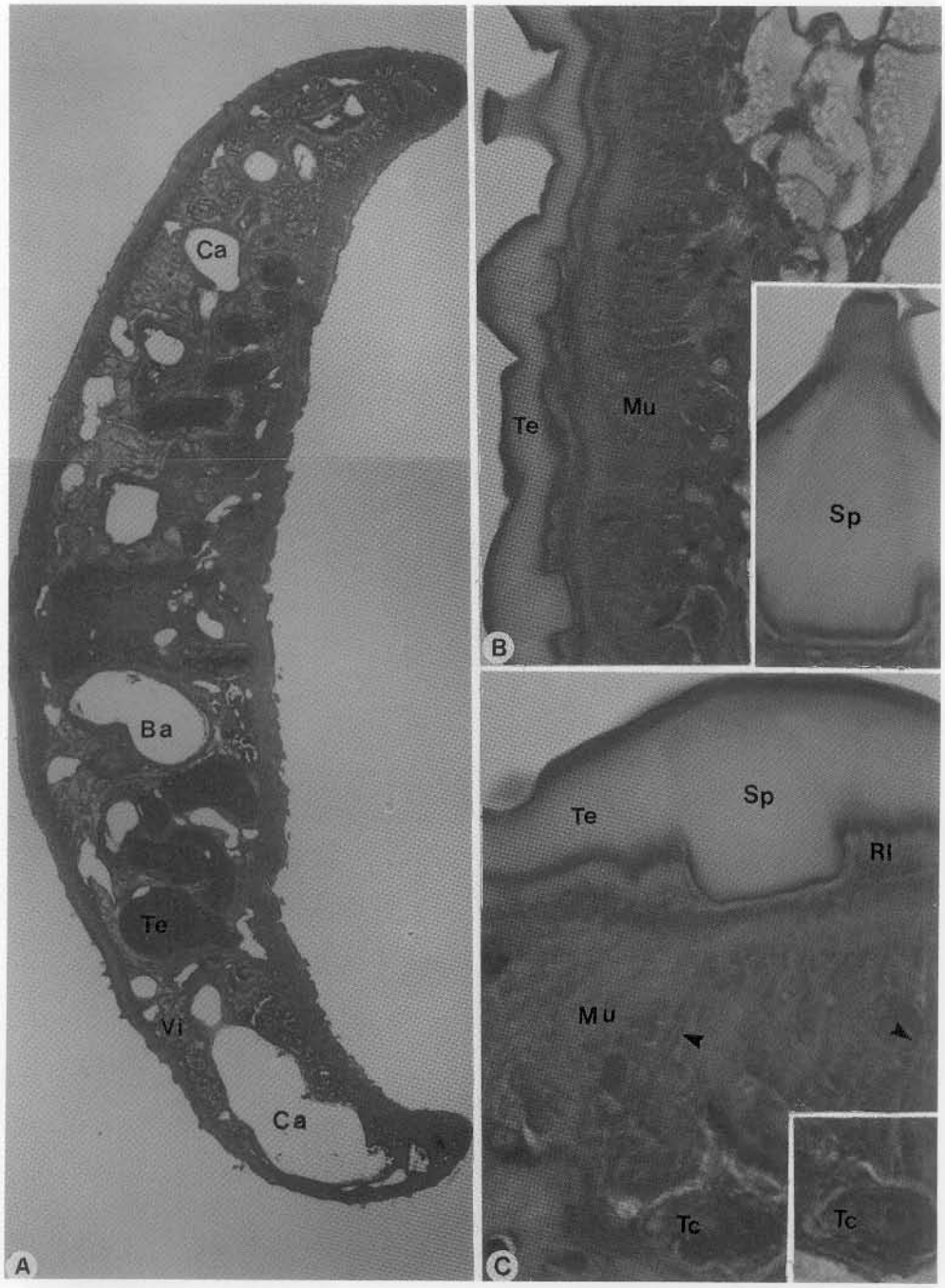

Fig. 2 Paraffin-embedded sections stained with Hematoxylin and Eosin: (A) A cross section of an adult parasite's body showing various-sized tubes, Ba-bladder, Ca-caecum; and other organs such as, Te-testes, Vi-vitelline gland. (B, C and their insets) Cross sections showing tegument (Te), spines (Sp), tegument cells (Tc), and their processes (arrows) running between muscle layers (mu).

Some, especially those on the antero-ventral and lateral sides of the body, were large (Fig. 3B, 3C) with serrated edges and directed backward (Fig. 3B). Others on the postero-ventral and dorsal sides tended to be smaller with no serrated edges (Fig. 3E). The areas between spines appeared corrugated with series of grooves and folds (Fig. 3B) which in turn were covered with small ridges invaginated with pits (Fig. 3D). The surface of the spines themselves was also highly ridges and pitted (Fig 3C). Thus the surface area of the parasites were vastly increased by these structural characteristics. Groups of sensory papillae were also seen in the areas between spines (Fig. 3E), and each of them has dome shape, some with cilia on top (Fig. 3F). Large groups of papillae (up to 10-15 per group) tended to concentrate on the ventero-lateral aspect of the body.

1.3 Observations of the tegument by transmission electron microscope (TEM)

Under TEM, the tegument could be divided into 4 layers, based on the concentration of the organelles and the density of the cytoplasmic matrix (Fig. 4). The outermost layer was a thin strip representing cross sections of ridges and invaginated pits (Fig. 4A, 4B), which together appeared like microvilli. These ridges were covered by trilaminate outer membrane and their interior contained a dense network of cytoskeleton fibers. There were numerous pale-stained discoid bodies (about 30 x 200 nm) embedded within the cytoskeletal network, and some were fused with the overlying membrane (Fig. 4B).

The second layer was a thin strip of cytoplasm that contained a high concentration of discoid bodies, lysozomes and spherical or ovoid bodies (Fig. 4A, 4B). Spherical or ovoid bodies (about 160 x 190 nm) contained homogeneously dense matrix surrounded by the trilaminate membrane. Some of them were fused with the surface membranes at the bottom of the pits, and hence probably released their content to form part of the glycocalyx coating the exterior of the surface membrane (Fig. 4B). Lysozomes were large dense spherical bodies (about 400 nm in diameter), that were arranged in rows at the inner part of the second layer (Fig. 4A).

The third layer was the widest zone of the tegument cytoplasm that contained a high concentration of mitochondria and dense scaffold of the cytoskeletal network (Fig. 4C). It contained evenly distributed discoid as well as spherical bodies, but with much lower concentrations than in the first two layers.

The fourth layer was the basal zone where there were infoldings of the basal plasma membrane (Fig. 4E). The basal plasma membrane rested on the thick basal lamina which was coupled to the former by series of hemidesmosomes (Fig. 4E). Underneath the basal lamina was the reticular lamina that connected the tegument to the underlying muscle layers (Fig. 4F). There were numerous processes of tegument cells traversing the reticular lamina outwardly to join up with the tegument.

Spines appeared as triangular crystalline lattice, whose interior was tightly packed (Fig. 4D). Their bases were firmly anchored to the basal lamina, while their peripheral boundaries adjoining the tegument cytoplasm exhibited no special condensation or anchoring fibers.

1.4 Observations of the caecum by transmission electron microscope

Under TEM apical branching of epithelial cells was remarkable, and consisted of the branching the cells' apical cytoplasm which gave rise to numerous cone-shape projections, which were in turn adorned with long and freely floating stereocilia (Fig. 5A-5C). All epithelial cells contained abundant rough endoplasmic reticula (RER) that completely surrounded nuclei which contained mostly euchromatin. In most cells, RER were highly dilated with flocculent material filling their cisternae, and with numerous mitochondria insinuating between them (Fig. 5B, 5C). This type of cells usually contained numerous highly condensed zymogen granules that tended to concentrate in the apical cytoplasm (Fig. 5C). Zymogen granules that lied close to the surface were exocytosed to the lumen, where upon taking up more water appeared enlarged and porous (Fig. 5B, 5C). There were also evidence that the highly dilated RER close to the surface became fused with the apical membrane, so that the flocculent material in their cisternae were directly released into the lumen of the caecum (Fig. 5D).

2. Localization of antigens in frozen sections of the adult parasites by the immuno-staining

2.1 Tegument

In the control section, where the non immune serum was used in place of immune serum, there was no reaction product on neither the tegument nor the caecal epithelium and its lumen (Fig. 6A, 6B). The only brownish areas were the vitelline glands, where there might be endogenous peroxidase that could not be completely inhibited by prior treatment with 3% H_2O_2. Spines were numerous and appeared completely translucent (Fig. 6A).

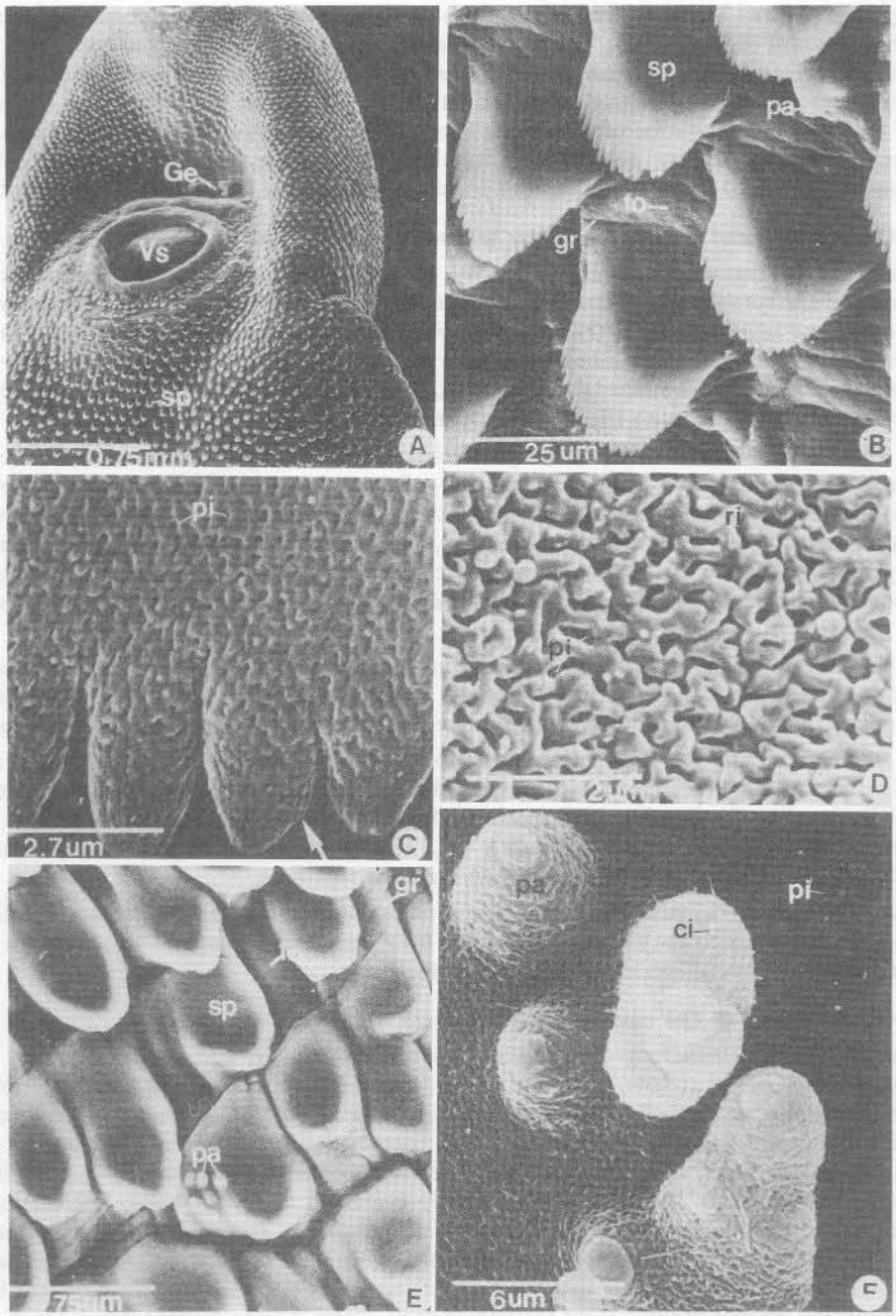

Fig. 3 SEM micrographs of the surface of adult parasites: (A) showing the anterior end with oral sucker (Os), ventral sucker (Vs), genital pore (Ge), and the surface roughened with spines (Sp). (B, C) High magnification of large spines on the anterior-half of the body, showing serrated edges (arrow). Areas between spines are highly corrugated with folds (fo) and grooves (gr). (D) High magnification of a fold where the surface appears as network of small ridges (ri) permeated by pits (pi). (E) Surface from the posterior half of the body, showing smaller and rounder spines (sp) with no marked serrated edge, and a group of papillae (pa) in between. (F) A group of papillae, with each papilla (pa) appears as a small dome, and some are capped with cilia (ci).

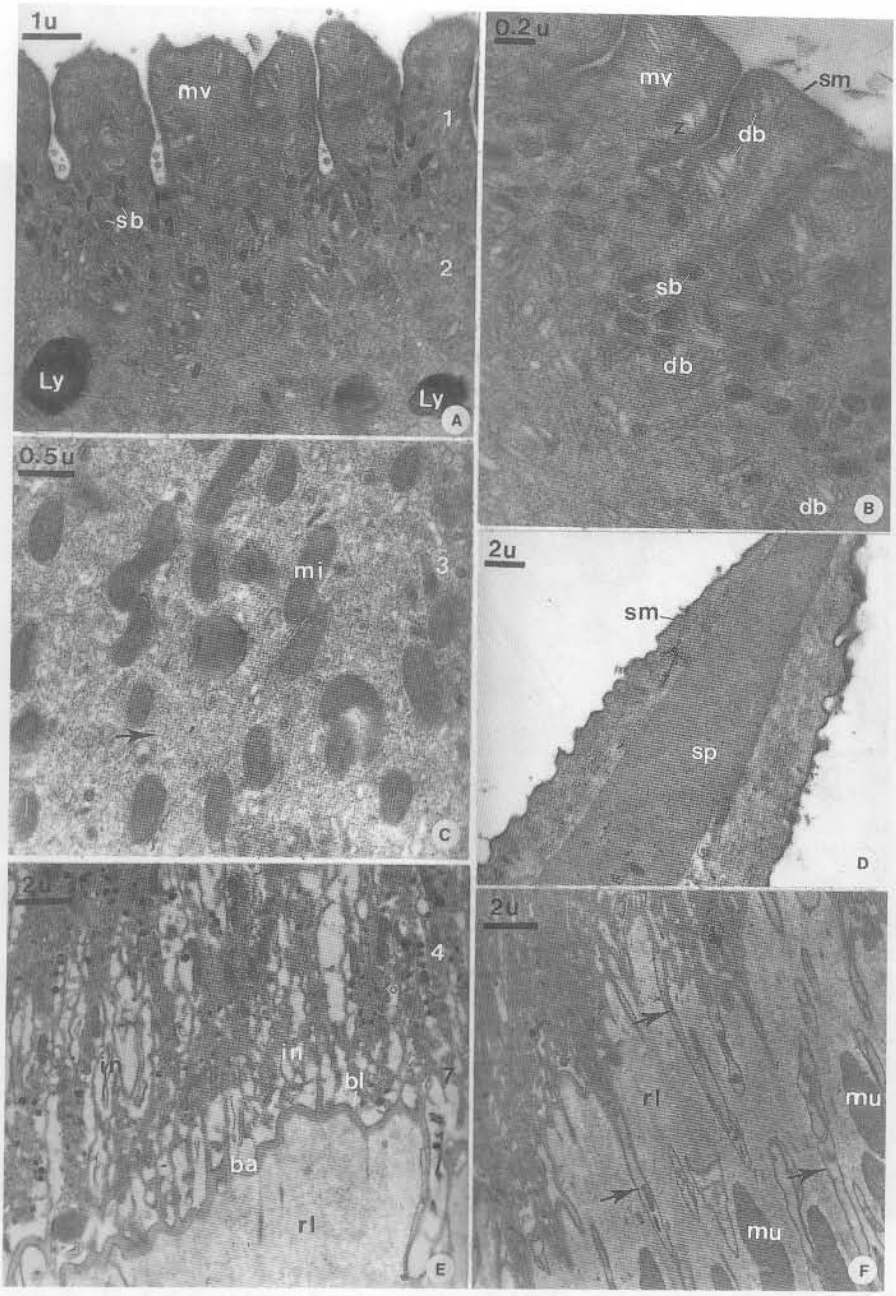

Fig. 4 TEM micrographs of the tegument of adult parasites: (A, B) showing the first (1) and second (2) layers with high concentrations of discoid (db) and spherical (sb) bodies, and a row of lysosomes (Ly). The surface membrane (Sm) is trilaminate and covered with a thin layer of glycocalyx. (C) The third (3) or middle layer, showing a high concentration of mitochondria (mi) enmeshed in a dense cytoskeletal network (arrow). (D) A spine, showing a dense crystalline-lattice core surrounded by the tegument cytoplasm. (E) The fourth (4) or basal layer, showing complex and extensive infoldings (In) of the basal plasma membrane (bl) which is attached to the thick layer of basal lamina (ba). (F) A layer of connective tissue, reticular lamina (rl), linking between the muscle layer (mu) and the basal lamina. Numerous processes of tegument cells (arrow) traverse this layer to join up with the tegument.

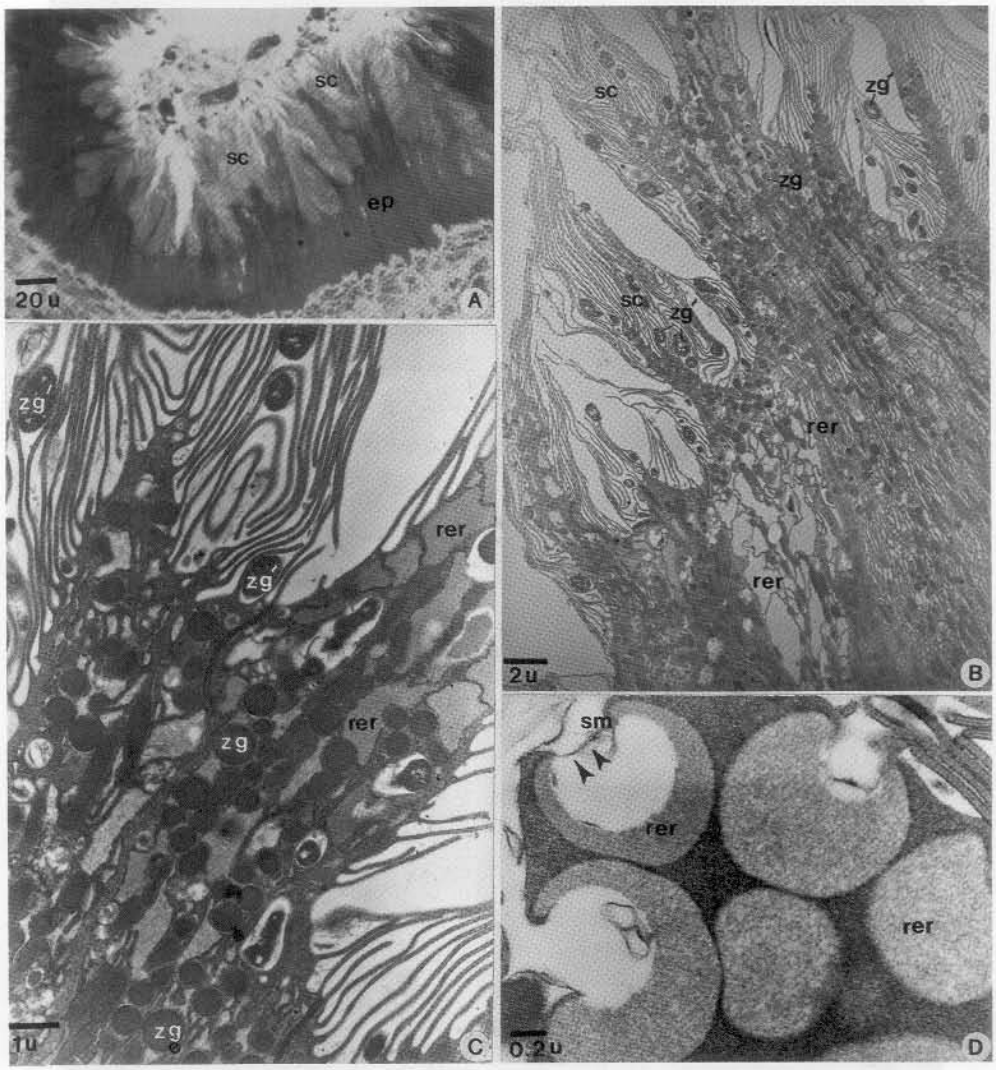

Fig. 5 (A, B) A light (A) and low-power TEM (B) micrographs of caecal epithelial cells, showing branchings of the apical cytoplasm which are covered by long stereocilia (sc). Exocytosed zymogen granules (zg') appear enlarged and porous. (C) High-power micrograph of the apical cytoplasm showing dilated rough endoplasmic reticulum (rer), unexocytosed (zg), and exocytosed (zg') zymogen granules. (D) Cross section of the apical cytoplasm, showing the release of the content in the rough endoplasmic reticulum directly to the exterior.

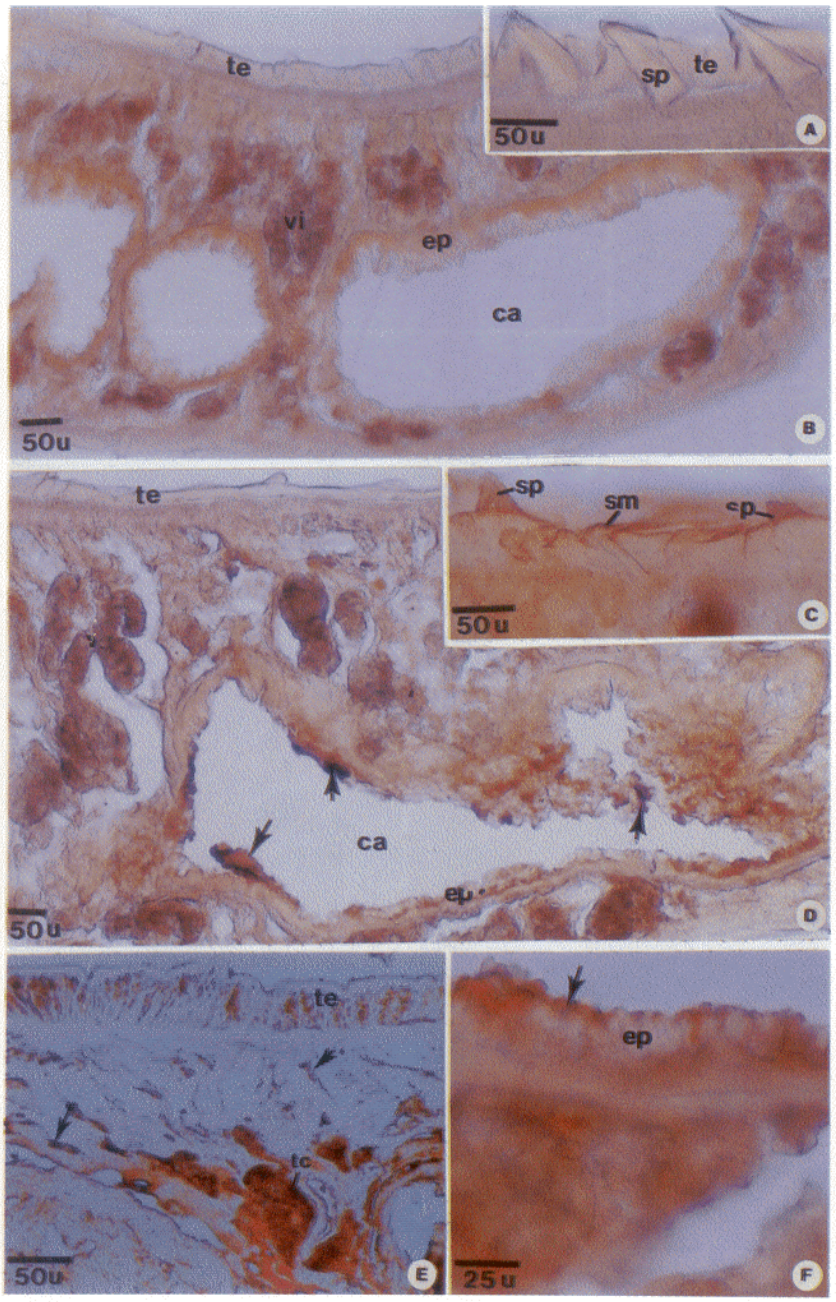

Fig. 6 Immunostaining of 4-5 μm-thick frozen sections of adult parasites: (A, B) Control section stained with non immune serum, showing pale caecum (ca), tegument (te), and spine (sp), while vitelline glands (vi) show some brownish hue. (C, D) In sections stained with immune sera, the brownish reaction products appear on the surface membrane (sm), the spines (sp), the caecal lumen (arrow) and its epithelial cells (ep). (E) An intense brown stain appears on the tegument cell (tc), its processes running between muscle cells (arrows), and granules in the tegument (te). (F) The staining of caecal epithelial cells (ep), showing brown reaction products in the apical cytoplasm of the cells (arrows).

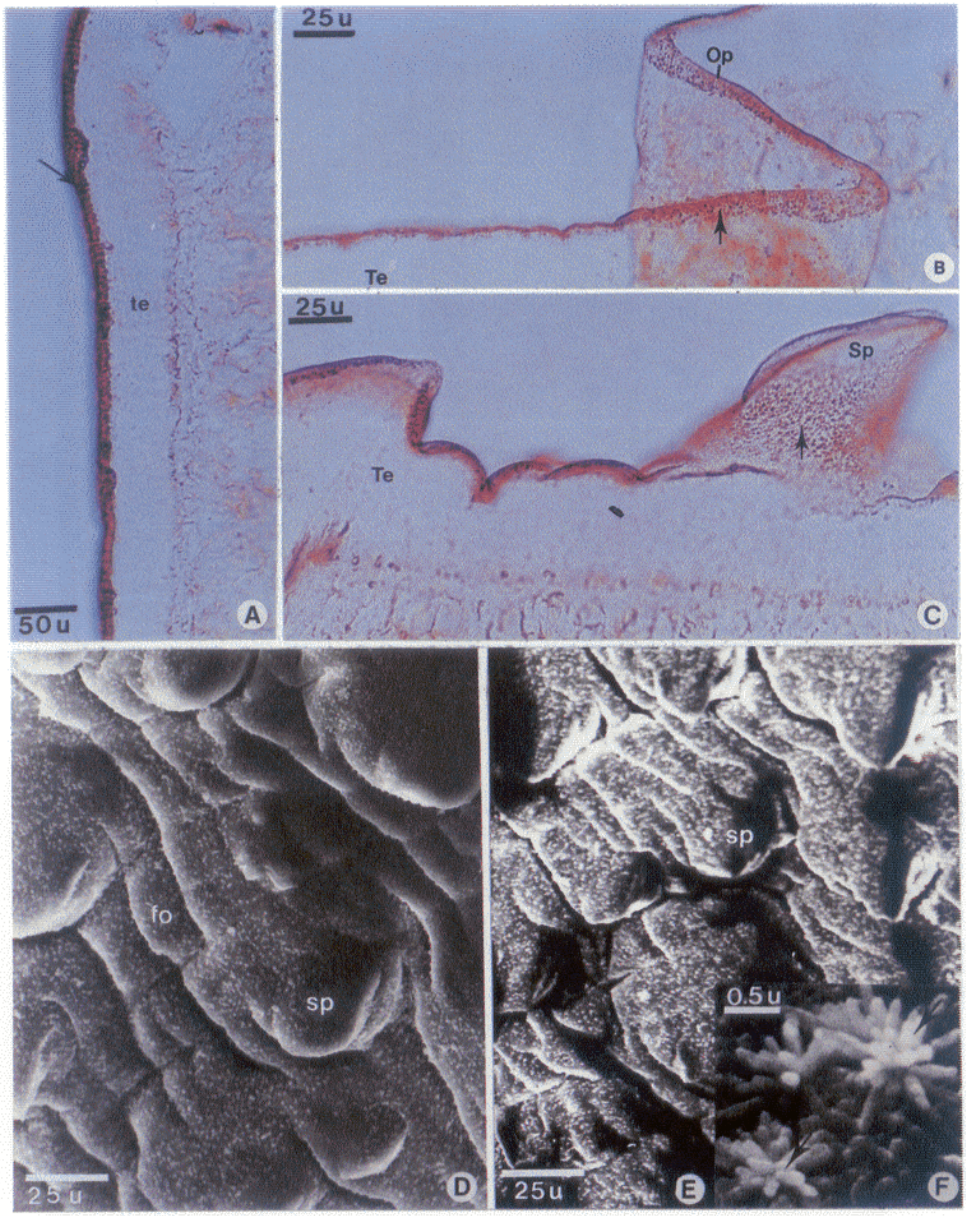

Fig. 7 (A, B, C) Immuno-staining of 12 μm-thick sections, showing intense brownish stain concentrated on the outer rim of the tegument cytoplasm (A-arrows). In tangential sections (B, C), reaction products appear as brownish dots spread evenly on the surface of the tegument (op) and the spine (C-arrow), whereas the spine crystalline matrix (sp) is unstained. (D, E, F) Immunogold labelling of the surface of adult parasites as shown by secondary electron imaging (D) and backscattered electron imaging (E). The labels appear as white spots that are evenly spread on the surface; and at high magnification (F) each spot is an aggregate of cigar-shaped gold particles (arrow).

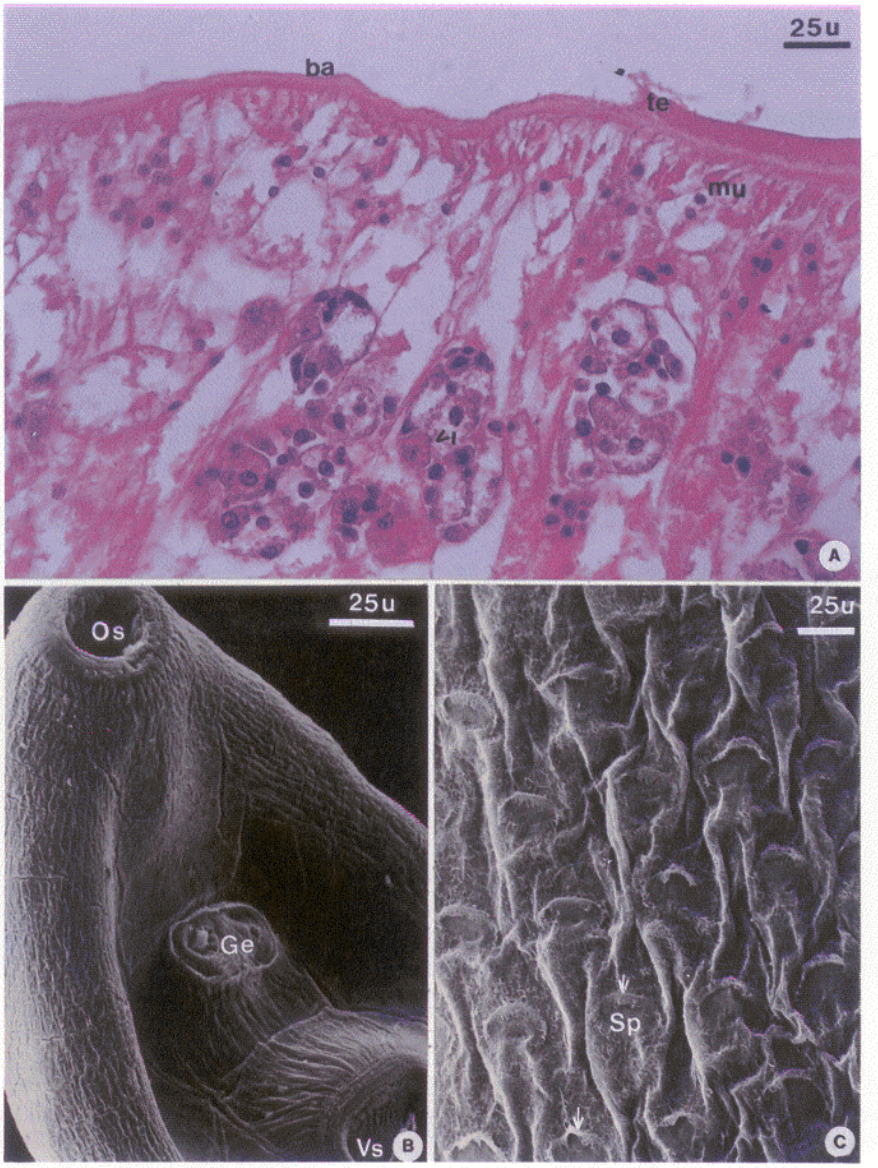

Fig. 8 (A) A paraffin-embedded section of an adult parasite, extracted with 1% Triton X-100 for 30 minutes, showing the stripping of most of the tegument layer (te) down to the level of basal lamina (ba) which is still intact. The interior of the parasite appears slightly extracted. (B, C) SEM micrographs of the extracted parasites, showing still largely intact basal lamina, except at the sites where spines were formerly attached (arrow).

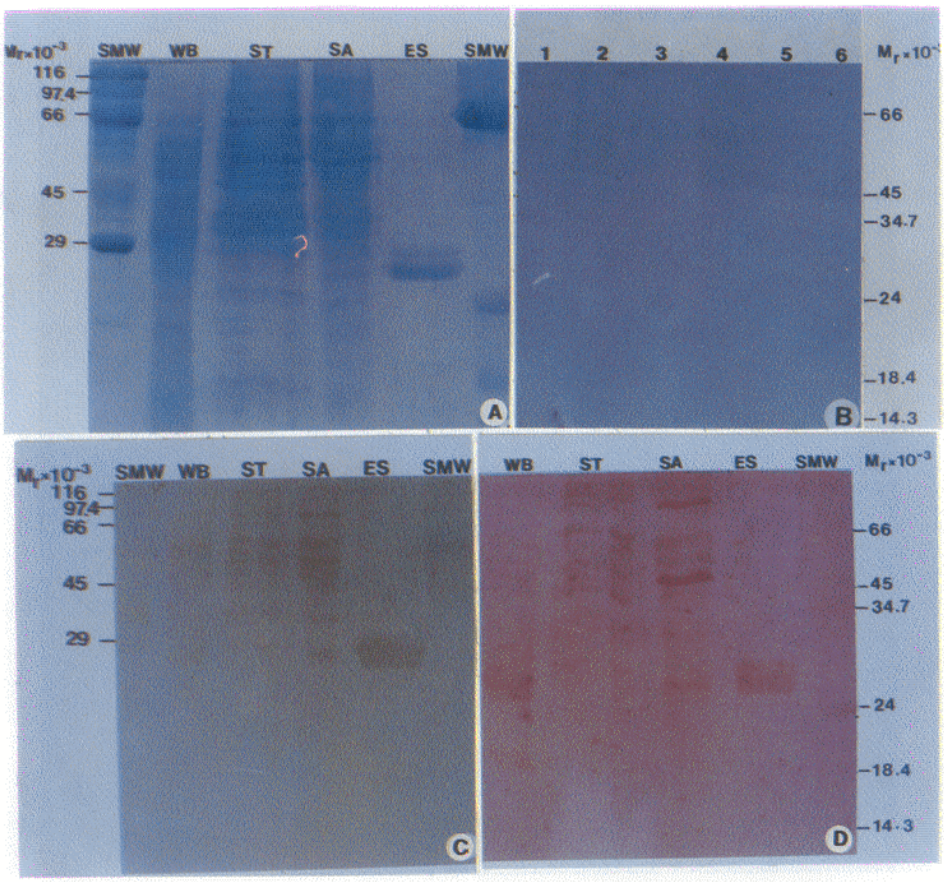

Fig. 9 (A) 12.5% SDS-PAGE of proteins from various fractions of adult parasites stained with Coomassie blue: lanes WB-whole body, ST-surface and tegument, SA-somatic, ES-excretory and secretory proteins. SMW are standard molecular weights. (B) Immunoblotted NC paper of the above protein fractions stained with non immune bovine serum, biotinylated rabbit anti-bovine IgG and avidin-biotin HRP complexes. Lanes 1, 6=SMW, 2=WB, 3=ST, 4=SA, 5=ES. (C, D) Immunoblots of the above protein fractions stained with immune bovine sera in place of non immune sera showing various antigenic bands.

In the frozen sections stained with immune sera, the brownish reaction products occurred in the tegument and the caecum. In the tegument, the brownish stain appeared as an intense undulating line on its surface (Fig. 6C, 6D), and the outer surface of spines' tips were also intensely stained (Fig. 6C). At high magnification, there were dense brownish granules spreading throughout the tegument cytoplasm, and in the bodies of tegumental cells lying beneath the muscle layer (Fig. 6E). In thick sections (Fig. 7A-7C), the densely stained line on the outer rim of the tegument was actually a narrow strip of the outermost cytoplasm underneath the surface membrane. Numerous fine and brownish granules were evenly scattered throughout the width of the tegument cytoplasm, but they tended to concentrated in the narrow strip described above (Fig. 7A, 7C).

On the surface of a rather thick and tangentially cut sections (~12 μm) the stain appeared as evenly scattered brownish dots (Fig. 7B). The outer surface of the spines was also covered with evenly dispersed brownish dots, but the spines' crystalline bodies were not stained, as witnessed in the grazing section through the spines' interior (Fig. 7C). Intense brownish lines undulating through the muscle layers were also clearly observed (Fig. 6E, 7C).

2.2 Caecum

In addition to the tegument, the positive reaction products of the immuno-staining was also detected within the lumen of the caecum and its lining epithelium (Fig. 6D, 6F). The brownish products that were present in the lumen were either tightly adhered to the apical surface of the epithelium or lying within the partially digested food content unattached to the epithelial cells' surface (Fig. 6D). On the epithelial cells themselves the products were mostly deposited in the apical cytoplasm (Fig. 6F). The remaining parts of the parasite's body stroma were unstained, except that vitelline glands showed light brownish color which was much less intense than the positive reaction products and with different shade of brown color (Fig. 6D).

2.3 SEM observation of the surface of the tegument labelled by immunogold technique

Adult parasites were treated with immune sera, followed by biotinylated rabbit antibovine antibody and streptavidin-gold, to demonstrate the location of the antigens and their distribution on the tegument surface. When viewed by using both secondary and back-scattered electron detectors, gold particles could be seen as bright spots in contrast to the dark background. However, the contrast appeared much better in the back-scattered mode than in the secondary mode (Fig. 7D, 7E). The gold particles were evenly distributed throughout the surface, except on the tip of spines and sensory papillae where gold particles were fewer in number and appeared less concentrated. At high magnification (Fig. 7F), each spot of gold label was actually composed of several cigar-shaped gold particles in a tightly packed aggregate; and these aggregates were fairly uniform in size as well as being evenly spaced (Fig. 7E, 7F).

3. Identification and characterization of antigens in adult parasites

3.1 Different types of antigens

From the above descriptions, it is clear that most antigens were derived from the tegument and the caecum. Therefore, this experiment was designed to collect different types of antigens; the first of which was the excretory-secretory antigens (ES antigens) which were released from the parasites kept in *in vitro* culture. This groups of antigens might be similar to those released under the natural condition, where infecting parasites might release their antigens into the hosts' blood and bile. The second group of antigens were extracted from the surface and the tegument by Triton X-100, which was designated as ST antigens. The third group was the somatic antigens remaining after the Triton X-100 extraction and designated as SA antigens. And the fourth is the homogenized whole body extract which was called whole body (WB) antigens.

3.2 Extraction of parasites with Triton X-100

When examining the paraffin-embedded extracted parasites' sections by the light microscope, it was revealed that most of the tegument was stripped off, down to the level of the basement membrane which still remained as an intact layer (Fig. 8A). Muscle, tegumental cells as well as caecal epithelium remained unextracted; while stromal cells and vitelline cells exhibited slight extraction.

Observing such extracted parasites by scanning electron microscope revealed that, large flaps of tegument were stripped off rather cleanly (Fig. 8B, 8C). However, the tegument covering the rims of the

suckers were still attached, probably because it was more tightly bound to the underlying tissues. On the areas where there was a total extraction of the tegument (Fig. 8C), the layer of basal lamina still remained largely intact. The only torn spots were the sites where the bases of spines were formerly attached to. Meshwork of small fibrils were seen still adhering to the areas of basal lamina between the spines (Fig. 8C).

From the above observations, it was, therefore, concluded that the major portion of the extracted antigens were derived from the tegument.

3.3 Analyses of antigens by 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Analyses of four groups of antigens by 12.5% SDS-PAGE gels stained with Coomassie Blue and blotted NC papers stained with Amido Black showed that the lanes containing WB and SA antigens exhibited the most numerous protein bands, which were quite similar in pattern (Fig. 9A). There were approximately 21 bands at molecular weights (MW) 110, 97, 95, 86, 66, 64, 58, 54, 50, 49, 47, 38, 35, 28, 27, 26, 24, 22, 20, 19 and 17 kilo-Dalton (kD). In the lanes that contained ST antigens, there were approximately 11 bands that were similar to those in WB and SA antigens. These bands were at MW 97, 86, 66, 64, 58, 54, 47, 38, 35, 19 and 17 kD. The lane that contained ES antigens showed the least number of protein bands, with the two most prominent bands at MW 27, 26 kD, and only few light bands at higher molecular weights at 66, 64, 58 and 54 kD. By contrast, in lanes containing ST and SA antigens 27, 26 kD bands were extremely light, while 66, 64, 58 and 54 kD bands were quite intense.

In immunoblotting analysis, proteins separated in the SDS-PAGE gel were electroblotted onto NC papers, stained serially with non immune or immune sera, biotinylated rabbit anti-bovine antibody, avidin and biotinylated-HRP. NC papers that were stained with the non immune sera exhibited no positive bands (Fig. 9B), whereas those that were stained with immune sera showed mostly similar bands with different intensities (Fig. 9C, 9D). Generally, there were 14 positive bands in lanes that contained WB and SA antigens, and they were at MW 97, 86, 66, 64, 58, 54, 50, 47, 38, 35, 28, 27, 26 and 20 kD. The most intensely stained bands were at 97, 66, 58, 54 kD and moderately intense bands at 64, 50, 47 and 26 kD, while the remaining bands were lightly stained.

In the ST lane, there were also 11 positive bands of antigens at MW 97, 86, 66, 64, 58, 54, 47, 38, 35, 19 and 17 kD. The most intense bands were at 66, 58, 54, and 47 kD. In contrast there were only 7 bands in ES lane, with the most intense ones at MW 27, 26 kD, and the lightly stained bands at MW 66, 64, 58 and 54 kD.

DISCUSSION

By using immune sera to detect the sites of antigen formation, the deposit of brownish reaction products implied that the major antigen-producing tissues were the tegument and the caecum. Antigens from these two tissues were most likely natural antigens that were released into the hosts' circulation and stimulated antibody production in the hosts. The patterns of brownish staining were unique in both tissues and should correspond to particular characteristics of the tissues' morphologies.

The tegument

The immuno-staining of the tegument exhibited four distinctive characteristics: the intense undulating line on the outer rim of the tegument, the fine brownish granules being evenly distributed throughout the width of the tegument, the brownish attenuated processes between muscle cells, and the staining of the tegument cells' soma.

At high magnification, it was revealed that the intensely stained outer rim was composed of a narrow zone of tightly packed brownish granules, as well as the deeply stained outermost thin line (Fig. 6C, 7A-7C). This narrow zone should correspond to the deeply-stained outer margin of the tegument's cytoplasm, as observed in the Hematoxylin and Eosin-stained light microscopic sections (Fig. 2B, 2C), and to the first and second layers of the tegumental cytoplasm as revealed by the TEM observations (Fig. 4A, 4B). It was, therefore, interpreted that the deeply-stained outer thin line was actually the surface membrane and its coating of glycocalyx, while the thicker outer brownish rim was the layers 1 and 2 of the tegument's cytoplasm which have high concentrations of discoid and spherical granules. In other words, the immuno-staining reflected the antigenic content in the existing surface membrane and its constituents that were

stored or held in reserved within the two types of granules. Earlier detailed electron microscopic observations by our group showed strong evidence that both the discoid and spherical bodies might contain the content that were contributed to the formation of the surface membrane.[10] The discoid bodies were actually vesicles of trilaminate membrane that were invariably fused with the surface membrane, and that most of them were concentrated in the layer 1 of the tegumental cytoplasm immediately underneath the membrane. Likewise, the spherical bodies might contribute both their dense matrix and surrounding membrane to the formation of the surface membrane and its glycocalyx coating, by fusing themselves with the latter at the bottoms of the invaginated pits. In *Fasciola hepatica* similar features were described, where the components of the adult surface membrane was contributed by T1 and T2 vesicles that replaced the juvenile form tegumental bodies (T0).[11-13] In schistosomes, the dynamics of membrane synthesis and renewal have been well documented. The so called membraneous bodies, which were spherical in shape, contained stack of presynthesized membrane held in reserve. Later they were added *in toto* to the surface membrane by direct fusion of the bodies with the overlying surface membrane in *S. mansoni*[14,15] or through semi-permanent membrane channels joining between the membraneous bodies and the surface membrane in *S. japonicum* and *S. mekongi*.[16,17]

Furthermore, the SEM observations of immunogold labelling of the parasites' surface by backscattered electron detector demonstrated that there were indeed abundant amount of antigens that were scattered evenly on the surface membrane (Fig. 7D-7F). Most of these antigens are believed to be the integral proteins of the membrane, and they were orientated such that their major epitopes were pointed outwards on the external side of the surface membrane. Such epitopes might be bound to polyvalent antibodies from immune sera, and, inturn, to immunogold labels. It is also remarkable that gold labels appeared as aggregates rather than isolated particles. This implies the "grouping" of antigen molecules rather than an even dispersal of such molecules within the thickness of the membrane. Such grouping of antigens may represent functional units of membrane proteins with certain but not yet identified role. In other words, membrane antigens in *F. gigantica* may be rigidly bounded and definitely localized within the surface membrane rather than being floating freely around as in many cases of general membrane antigens of mammalian cells.[18-20]

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 *via* their processes towards the tegument. Eventhough direct evidence such as gold labelling of the cells' soma is still lacking, it is believed that antigens were concentrated in the discoid and spherical bodies that were produced in the cells' soma. It was revealed by earlier TEM observations of the adult *F. gigantica* tegument that both types of granules were synthesized within a single cell type by the Golgi complex-RER system. Then they were transported to the tegument *via* the cells' extensive branching towards the tegument. This transport process was mediated by the action of microtubules that were aligned in bundles within branches of the cells.[10] Within the tegument, both types of granules were evenly scattered throughout which corresponded to the pattern of distribution of brownish granules in the tegument exhibited in the immuno-stained tegument (Fig. 6D, 6E). However, eventually both types of granules became highly concentrated in the layers 1 and 2 of the tegumental cytoplasm.

It is noticeable that eventhough portions of the surface membrane covering spines were intensely stained, the spine crystalline matrix themselves were not stained. This implies that spines' material were not antigenic by nature, or more likely that spines were not shed and turned over like the surface membrane, hence their content were not released into the hosts' circulation to stimulate the antibody production.

The caecum

The immuno-staining of the caecum demonstrated that antigens were concentrated mainly in the luminal content as well as in the caecal epithelial cells. The staining of the caecal content, in most cases, were very intense and could represent the secreted products of epithelial cells in mixture with the food content. On the other hand the immuno-staining of the apical zone of the epithelial cells' cytoplasm superimposed with the region where zymogen granules were mostly concentrated, and rough endoplasmic reticulum were highly dilated. Therefore, the antigens that were detected by the immuno-staining could be the enzymatic content already packed within zymogen granules as well as those still in the cisternae of rough endoplasmic reticulum, and those that were already exocytosed into the lumen. It is possible that there might be two types of secretory products from the epithelial cells since there appeared to be two modes of secretion: the exocytosis of zymogen granules (Fig. 5B, 5C), and the direct release of content in the dilated cisternae of the rough endoplasmic reticulum through the fusion of the latter with the apical membrane (Fig. 5D).

Antigens from the caecum might be the most abundant among the excretory-secretory (ES) antigens, considering the mass from the branching of caecum in the adult parasites. *F. gigantica* lacks circulatory system but the conveyance of digested nutrients to every part of the parasite's body is carried out directly by the extensive branching of the caecum, from the very large trunks to small tubules that are pervasive throughout the parasite's body (Fig. 1). The nutrient molecules are absorbed by the layer of epithelial cells and passed directly to the surrounding tissues. In other species of helminth parasites, particularly *F. hepatica*, the caecal content were also one of the major antigens that were released from the parasites. Some of those so called ES antigens were proven to be the digestive enzyme cysteine protease.[21-28] Direct evidence that the caecal antigens are the content of zymogen granules could be provided by the immunogold labelling of the zymogen granules in the Lowicryl-embedded sections which is still under investigation by our group.

Antigens of the adult parasites

Analysis of proteins from the homogenized whole body of the adult parasites showed that there were approximately 21 detectable bands, ranging in molecular weights (MW) from 110 to 17 kD. Eleven of these bands at MW 97, 86, 66, 64, 58, 54, 47, 38, 35, 19 and 17 kD were present in the tegument fraction which was extracted from the parasites' bodies by Triton X-100. Most of these are believed to be proteins associated with the tegument cytoplasm and the surface membrane, because both light and electron microscopic examinations revealed that the basement membrane underlining the tegumental layer was generally still intact, and pieces of the tegument were cleanly separated from the basement membrane. Besides, there was little extraction of the underlying tissues, such as, muscles, gut, excretory, reproductive tissues, as well as stromal tissues of the parasites' bodies. In contrast the excretion-secretion (ES) fraction which represented the proteins released in the *in vitro* culture were composed mainly of two prominent bands at MW 27 and 26 kD, and lightly stained bands at 66, 64, 58 and 54 kD. The latter group was also observed in the ST, while the former group was not. Hence it is believed that 27 and 26 kD proteins were most likely derived from the deeply-localized tissue, such as caecum which also continuously released its content to the exterior. The two proteins have been purified and characterized by our group for their amino acid composition and sequence, and were believed to be cysteine proteases.[29] These enzymes were generally detected in other *Fasciola* species, such as *F. hepatica*,[21,22,25-28] and in *Schistosoma* species.[30,31] They are probably used in the digestion of hosts' tissues, such as epithelia of the bile ducts, and blood cells. The enzymes could be acting both inside as well as outside the parasites' bodies.

In immunoblotting analysis, there were in total 14 from 21 bands in the whole body fraction that were antigenic, while all 11 bands of tegumental-associated proteins in ST fraction were antigenic. Among the latter, the major antigens, judging from the staining intensity, were 4 bands at MW 66, 58, 54 and 47 kD. These bands were also detected in the immunoblot pattern of ES fraction, albeit they were very lightly stained, in comparison to the major and more intensely stained bands at MW 27 and 26 kD. Hence, in *F. gigantica* we have found that major antigens in adult parasites were the group at high MW at 66, 58, 54, 47 and the group at low MW at 27 and 26 kD. The former group were most likely the tegument-associated antigens, while the latter group were caecal-associated antigens. These data on *F. gigantica* were the first to be reported by our group. In comparison, there have been considerable work on *F. hepatica*. Itagaki *et al.*,[32] using enzyme-linked immunotransfer blot probed by sera from experimentally and naturally infected cattle, have found that the major antigens of adult *Fasciola* sp. were at 64-52 kD, 38-28 kD, 17 kD, 15 kD, 13kD and 12 kD. And 160 kD antigens were detected only by sera from the early stage infection. The lower molecular weights antigens reported by these authors were within the same ranges of MW as reported in our findings, especially at MW 64-52 kD (versus 66-54 kD reported by us) and 38-28 kD (versus 27-26 kD reported by us). It is also reported that antigens at 64-54 kD might be possible candidates for serodiagnosis of fascioliasis in cattle. The work by our group[33] also reported that antigens at MW 66, 58, 54 kD were more specie-specific than the caecal-associated antigens at 27-26 kD.

In *Schistosoma* species, especially *S. japonicum* and *S. mekongi*, analysis by immunoblotting showed that there were 15-20 bands of antigens at MW 205, 158, 128, 116, 110, 105, 97, 86, 76, 68, 64, 56, 54, 50, 45, 43, 38, 28 and 26 kD.[17,34] *S. mansoni* also showed common antigens with these species at MW 97, 86, 68, 50 and 38 kD.[35] These antigens were believed to be mainly the surface and tegument-associated antigens because similar pattern were obtained when the parasites were isotopically labelled with ^{125}I and antigenic bands analysed by immunoprecipitation.[36] It is most likely that isotopic label of living parasites could only attach to the external facet of the surface membrane, and that little would gain excess to the internal tissues such as caecal content.

From the above discussion it can be concluded that the pattern of antigens in helminth parasites are similar; that is, the two major sources of antigens released from parasites are from the tegument and the caecum. However, in *Fasciola* species, including *F. gigantica,* the majority of released antigens are from the caecum, while in *Schistosoma* species they are mainly derived from the tegument and the surface membrane.

ACKNOWLEDGEMENT

This project received financial support from National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency (Project # CPT 89B-1-05-173).

REFERENCES

1. Kachintorn, U., Atisook, K., Tesjaroen, S., Lertakyamanee, N. and Plengvanit, U. (1988) ***J. Med. Assoc. Thailand* 71**, 451.
2. Tesana, S., Pamarapa, A. and Sio, O. T. (1989) ***Southeast Asian J. Trop. Med. Pub. Hlth.* 20**, 447.
3. Cho, S. Y., Yang, H. N., Kong, Y., Kim, J. C., Shin, K. W. and Koo, B. S. (1994) ***Am. J. Trop. Med. Hyg.* 50**, 349.
4. Sukhapesna, V., Tuntasuvan, D., Sarataphan, N. and Imsup, K. (1989) ***J. Thai. Vet. Med. Assoc.* 40**, 13 (in Thai).
5. Dissamarn, R. (1955) ***J. Thai. Vet. Med. Assoc.* 6**, 64 (in Thai).
6. Dissamarn, R. (1961) ***Bull. Int. Epizoot.* 56**, 1002.
7. Usanakornkul, S. and Sukhapesana, V. (1980) ***Buffaloes Production in Small Farms ASPAC, FFTC,*** Taiwan, pp. 167.
8. Loehr, K. F. (1982) ***Annual Report***. Northeast Veterinary Research and Diagnosis Centre,Tra Phra, Khon Kaen, Thailand.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) ***J. Biochem.* 193**, 265.
10 Sobhon, P., Dangprasert, T., Saitongdee, P., Wanichanon, C. and Upatham, E. S. (1994) ***J. Elec. Mic. Soc. of Thailand* 8**, 36.
11. Hanna, R. E. B. (1980) ***Exp. Parasitol* 50**, 103.
12. Hanna, R. E. B. (1980) ***Exp. Parasitol* 50**, 155.
13. Hanna, R. E. B. (1980) ***Exp. Parasitol* 50**, 297.
14. Hockley, D. J. and McLaren, D. J. (1973) ***Int. J. Parasitol.* 3**, 13.
15. McLaren, D. J. (1980) ***Schistosoma mansoni: the Parasite Surface in Relation to Host Immunity***. Research Studies Press, John Wiley & Sons Ltd, New York.
16. Sobhon, P., Upatham, E. S. and McLaren, D. J. (1984) ***Parasitol.* 89**, 511.
17. Sobhon, P. and Upathum, E. S. (1990) ***Snail Hosts, Life-cycle, and Tegumental Structure of Oriental Schistosomes***. UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.
18. Molday, R. S., Dreyer, W. J. and Rembaum, A. (1975) ***J. Cell Biol.* 64**, 75.
19. De Harven, E., Leung, R. and Christensen, H. (1984) ***J. Cell Biol.* 99**, 53.
20. Stump, R. F., Pfeiffer, J. R., Schneebeck, M. C., Seagrave, J. C. and Oliver, J. M. (1989) ***Amer. J. Anat.* 185**, 128.
21. Dalton, J. P. and Heffernan, M. (1989) ***Mol. Biochem. Parasitol.* 35**, 161.
22. Rege, A. A., Herrera, P. R., Lopez, M. and Dresden, M. H. (1989) ***Mol. Biochem. Parasitol.* 35**, 89.
23. Yamasak, H., Aoki, T. and Oya, H. (1989) ***Japan J. Parasitol.* 38**, 373.
24. Yamasaki, H., Kominami, E. and Aoki, T. (1992) ***Parasitol. Res.* 78**, 574.
25. McGinty, A., Moor, M., Halton, D. W. and Walker, B. (1993) ***Parasitol.* 106**, 487.
26. Smith, A. M., Dowd, A. J., McGonigle, S., Keegan, P. S., Brennan, G., Trudgett, A. and Dalton, J. P. (1993) ***Mol. Biochem. Parasitol.* 62**, 1.
27. Dowd, A. J., Smith, A. M., McGonigle, S. and Dalton, J. P. (1994) ***Eur. J. Biochem.* 223**, 91.
28. Wijffels, G. L., Salvatore, L., Dosen, M., Waddington, J., Wilson, L., Thompson, C., Campbell, N.,

Sexton, J., Wicker, J. and Bowen, P. (1994) ***Exp. Parasitol.*** **78**, 132.
29. Kiatpathomchai, K. (1995) Purification and characterization of liver-fluke *Fasciola gigantica* antigens, ***M.Sc. Thesis in Biochemistry***, Faculty of Graduate Studies, Mahidol University.
30. Gotz, B. and Klinkert, M. Q. (1993) ***Biochem. J.*** **290**, 801.
31. Rege, A. A., Wang, W. and Dresden, M. H. (1992) ***J. Parasitol.*** **78**, 16.
32. Itagaki, T., Sakamoto, T. and Itagaki, H. (1995) ***J. Vet. Med. Sci.*** **57**, 511.
33. Ratanapongleka, D. (1996) Production and characterization of monoclonal antibodies against tegumental antigens for the detection of *Fasciola gigantica* (Cobbold, 1856) infection, ***Ph.D. Thesis in Biology***, Faculty of Graduate Studies, Mahidol University.
34. Sobhon, P., Upatham, E. S., Viyanant, V., Kusamran, T., Mohthong, V. and Anantavara, S. (1992) ***National Research Council, U.S.A. Aquaculture and Schistosomiasis: Proceeding of a Network Meeting Held in Manila, Philippines*** (August 6-10, 1991), pp. 178, National Academic Press, Washington, D.C.
35. Taylor, J. W., Hayunga, E. G. and Vannier, W. E. (1981) ***Mol. Biochem. Parasitol.*** **3**, 157.
36. Sobhon, P., Panuwatsuk, W., Tharasub, C., Upatham, E. S., Kusamran, T. and Viyanant, V. (1987) ***Proceeding of His Majesty's Fifth Cycle Commemorative Conference of USAID Science Research Award Grantees***, Nakorn Pathom, Thailand (July 24-26), pp. 237.