FASCIOLA GIGANTICA: STUDIES OF THE TEGUMENT AS A BASIS FOR THE DEVELOPMENTS OF IMMUNODIAGNOSIS AND VACCINE

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Abstract. The tegument of bile-dwelling *Fasciola gigantica* is the interfacing layer that helps the parasite to maintain its homeostasis, and evade the hostile environment, including the host's immune attacks. The tegument is a syncytial layer about 10 mm thick, that is formed by the fusion of cytoplasmic processes of tegument cells, whose soma lie underneath the two muscle layers. The surface of the tegument is highly folded and invaginated into numerous ridges, pits and spines, which help to increase the surface area of the tegument for the absorption and exchanging of molecules, as well as for attachment. The outer membrane covering the tegument is a trilaminate sheet about 12 nm thick, and coated with a carbohydrate-rich glycocalyx layer that also bears high negative charges. Some host molecules may also be adsorbed onto this layer. These unique characteristics enable the parasite to evade the antibody-dependent cell-mediated cytotoxicity (ADCC) reaction exerted by the host. The outer membrane and glycocalyx is continuously replaced by the reserved membrane synthesized and stored in secretory granules of tegument cells, that are transported via cell processes towards the tegument by microtubules. The basal membrane of the tegument is trilaminate and invaginated to form membrane infoldings with closely aligned mitochondria. The tegument cytoskeleton is composed of a highly cross-linked network of 4-6 nm knobby microtrabecular fibers, bundles of intermediate filaments, microtubules that splay out from the tegument cells' processes. Major proteins of the cytoskeleton are actin, paramyosin and tubulin. The flukes' antigens that can elicit strong immunological responses in animal hosts are synthesized and released mainly from the tegument and the cecum. The majority of antigens derived from the surface membrane and the tegument are of MW 97, 66, 58, 54, 47 and 14 kDa, while those released from the cecum are cysteine proteases of MW 27, 26 kDa. Monoclonal antibodies have been raised against some of these antigens, and have been employed in immunodiagnosis of the infection. From the protection conferred to animal models and the *in vitro* killing assays of young parasites by specific antibodies, candidate vaccines could be selected from these antigens, such as, an antioxidant enzyme, glutathione-S-transferase, the digestive enzyme cysteine proteases, the surface-tegument proteins, such as fatty acid binding protein (14kDa), membrane proteins (at 66 kDa), as well as muscle protein paramyosin, and hemoprotein. Ongoing research have been directed at deciphering the genetic codes and the syntheses of some of these antigens by recombinant DNA technology.

INTRODUCTION

Fascioliasis, a disease that infects both domestic and wild animals, is one of the major tropical diseases that afflict both the temperate and tropical regions of the world. The causative parasites in the temperate regions is *Fasciola hepatica,* while in tropical region is *F. gigantica.* Fascioliasis causes significant economic loss estimated at US $ 2,000 million per annum from its effect on domestic and economic animals (Boray, 1985). The disease can also cross infect humans, and there are reports of increasing incidence world wide (Chitchung *et al,* 1982; Maurice, 1994; Anon, 1995). The prevalent rates are as high as 30-90% in Africa, 25-90% in Indonesia (Edney and Muchlis, 1962; Soesetya, 1975; Fabiyi, 1987). In Thailand the prevalence rates in cattle and buffaloes are 4-24%, with the highest incidents in the north and northeast and lowest incidents in the South (Pholpark and Srikitjakara,1989; Sukhapesna *et al,* 1990; 1994). It is clear that the disease is a major impediment to economic progress, which is exacerbated in the less developed countries, particularly towards small-scale farmers, who cannot mobilize limited resources to control, not to mention eradicate the disease.

Fascioliasis could be partially controlled by periodic treatments of the animals in the endemic area with a repertoire of drugs, among which triclabendazole was reported to be highly effective (Sukhapesna *et al,* 1992), eventhough incidence of resistance to this drug have been repeatedly reported

(Overend and Bower, 1995). In view of the cost and the possible mutation of the parasites which could compromise the drug's action, a better alternative would be the development of vaccines which could either completely prevent the infection or arrest the development of the parasite at certain stage of its life cycle, or even partially reducing the fecundity of infecting adult parasites.

PARASITES' LIFE CYCLE AND HOST IMMUNOLOGICAL RESPONSES

The sound understanding of the parasites' life cycles together with their biology and hosts' immunological responses are the two most important corner stones for devising any rational vaccine. The life cycle of *F. gigantica,* which is putatively a single parasite that causes fascioliasis in Thailand, was studied in detail by Chompoochan *et al* (1976). The whole cycle needs about 158-175 days for completion. The prepatent period of *F. gigantica* eggs was 8-14 days, after which eggs are hatched into miracidia, whose longevity in water is about 24 hours. Miracidia penetrate intermediate snail hosts, *Radix rubiginosa,* and develop into sporocysts and rediae within 7-8 days. Cercariae develop from germ cells inside rediae and leave the snail hosts in 35-49 days from the time of miracidial penetration. Within 30-45 minutes, the cercariae settle on leaves of nearby grass or water plants, on which they shed the tails and transform into cysts. Metacercariae continue to develop inside the cysts and may lay dormant up to 60 days. After being ingested by animals or human, excysted juvenile flukes penetrate the gut wall and travel towards the liver via peritoneal cavity. Upon reaching the destination, the flukes penetrate the capsule and burrow themselves into liver parenchyma, and eventually reside in the biliary tree where they develop into adults and begin to lay eggs. Eggs are first found in feces of the cattle and buffaloes between 16-18 weeks and in sheep about 11 weeks after the infection, and reach the peaks about 24-28 and 17-26 weeks, respectively (Thammasart *et al,* 1996). The ensuing histopathological changes are marked by the progressive biliary cirrhosis due to the fibrosis and calcification of bile ducts. This liver failure leads to diarrhea, losses of appetite, weight and vigor (Sukhapesana *et al,* 1994), and meat or milk productions. The heavily infected animals, particularly the young ones, could succumb and die.

There are ample evidence which demonstrate that animals hosts mount both humoral and cell-mediated immune responses against parasites. Antibody against *F. gigantica* infection in cattle and buffaloes were first detected at 2-4 weeks after the infection, and reached the peaks around 16 to 20 weeks before declining slowly (Thammasart *et al,* 1996). It is noticeable that the detection of antibody preceded the finding of eggs in feces by 12 to 14 weeks. This information attests that the devising of immunodiagnostic methods for detecting the early infection is one of key strategy that could benefit the monitoring and early treatment of the disease. Apart from causing antibody formation, the parasites can elicit cascades of antibody-dependent cell-mediated cytotoxic (ADCC) reactions. Similar to the cases of schistosomiasis (reviewed by Sher and Coffman, 1992; Maizels *et al,* 1993; Capron and Capron, 1994), newly excysted juvenile *F. hepatica* could be coated and killed by antibodies and variety of host immune effector cells that attach to the tegument, including eosinophils, neutrophils, macrophages and mast cells, during their migration through the host's peritoneal cavity (Rajasekariah and Howell, 1977; Kelly *et al,*1980; Doy and Hughes, 1982; Hughes, 1987). The release of hydrolytic enzymes and production of oxygen free radicals and nitric oxides by these effector cells on, or in the immediate vicinity of the tegument are the primary action that kill young parasites (James and Gleven, 1989; Smith, 1989; Golenser and Chevion, 1993; Liew and O' Donnell, 1993; Wynn *et al,* 1994). By contrast, the adult parasites hardly appear affected by the host immune reactions. This may be due to their fairly sequestered residence in the bile duct which has lower immunological activities, though the bile itself contains emulsifying agents and certain level of IgA antibody. Adult parasites may also possess counteracting reactions against oxygen free radicals and nitric oxides which are catalysed by a series of detoxifying or antioxidant enzymes, such as, superoxide dismutase, glutathione-S-transferase and glutathione peroxidase. In schistosomes, these enzymes are more concentrated in adult tissue than juveniles (Mei and Loverde, 1997). Adult parasites can also evolve an evasion mechanism by which their tegument can avoid the attachment of immune effector cells, and thus damage from ADCC reaction. In addition, they can release certain immunomodulating factors that compromise the cell-mediated immune response of the hosts. Studies showed that T cell proliferation and IL-2 production in cattle,

sheep and rats were suppressed during the course of infection by *F. hepatica* (Oldham and Williams, 1985; Zimmerman *et al,* 1983; Cervi *et al,* 1996). Thus any successful vaccine candidate should be directed primarily at preventing the infections by juvenile parasites, which have not yet develop any viable evasion mechanism.

TEGUMENTAL STRUCTURE AND EVASION MECHANISM

The tegument is the interfacing layer between parasites and hosts that helps the parasites to maintain their homeostasis which is essential for their survival in the hostile biliary environment. Judging from its remarkable structural characteristics, which will be elaborated later, the tegument is probably playing key roles in the absorption and exchange of nutritive and waste molecules, and the regulation of osmolarity as well as ionic equilibrium between the interior of parasites' bodies and the surrounding fluid. In addition, evasion mechanisms, including many of the parasites' counteracting reactions against hosts' immune attacks, are probably generated in large part by the tegument. Any compromise on the integrity and normal functioning of the tegument such as the treatment by certain drugs, could facilitate and accelerate the killing action of hosts' immune effector cells. Thus one can perceive a delicate balance between counteracting actions between hosts' immunological responses and parasites' evasion mechanisms as a forever ongoing exercise. The complete understanding of structural organization and the roles played by the tegument is hence crucial in devising any rational vaccine.

Observations of the adult parasite surface by scanning electron microscope (SEM)

When adult *F. gigantica* were observed under scanning electron microscope (Fig 1A-D) the tegument surface was characterized by the presence of numerous spines, except in the areas around the oral and ventral suckers. These spines were closely spaced and varied in shape and size depending upon the body parts. Some, especially those on the antero-ventral and lateral sides of the body, were large with serrated edges and directed backward. 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 which in turn were covered with small ridges invaginated with pits. The surface of the spines themselves was also highly ridged and pitted. 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, and each of them has dome shape, some with cilia on top. Large groups of papillae (up to 10-15 per group) tended to concentrate on the ventero-lateral aspect of the body.

Observations of the tegument by transmission electron microscope (TEM)

Under TEM (Fig 1E-H), the tegument could be subdivided into 4 layers, based on the concentration of the organelles and the density of the cytoplasmic matrix. The outermost layer was a thin strip representing cross sections of ridges and invaginated pit, which together appeared like microvilli (Fig 1E; 1G). 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 30x200 nm) embedded within the cytoskeletal network, and some were fused with the overlying membrane (Fig 1G).

The second layer was a thin strip of cytoplasm that contained a high concentration of discoid bodies, lysozomes and spherical or ovoid bodies (Fig 1G, H). Spherical or ovoid bodies (about 160x190 nm) contained homogeneously dense matrix surrounded by the trilaminate membrane. Some of them were fused with the surface membranes at the bottoms of the pits, and hence probably released their content to form part of the glycocalyx coating the exterior of the surface membrane. Lysozomes were large dense spherical bodies (about 400 nm in diameter) that were arranged in rows in the inner part of the second layer.

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. It contained evenly distributed discoid as well as spherical bodies, but with much lower concentrations than in the first two layers (Fig 1E).

The fourth layer was the basal zone where there

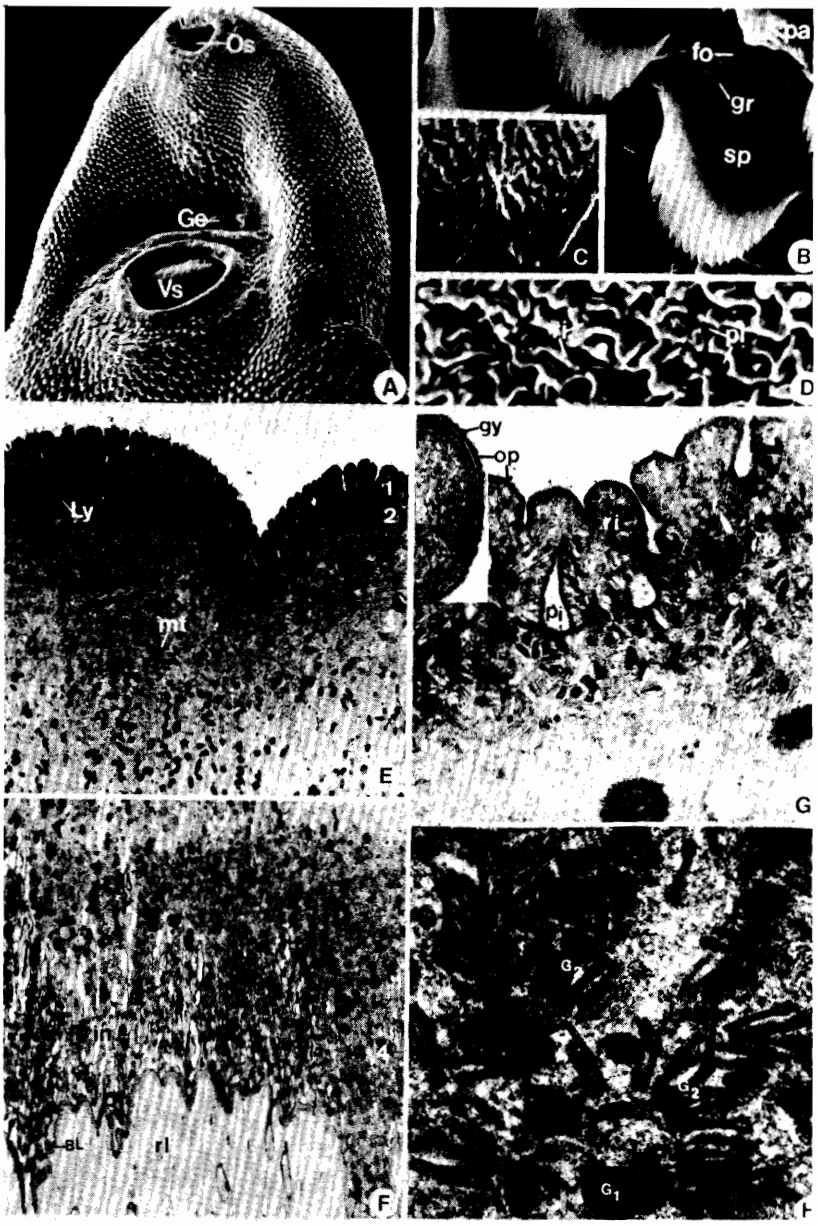

Fig 1–A-D) Scanning electron micrographs, showing the surface characteristics of adult *F.gigantica*. In A the anterior half of on an adult parasite showing oral (Os), ventral suckers (Vs), genital opening (Ge), and the surface studded with spines.In B and C, the spines have serrated edges with folded covering of the edges and spines main bodies. In D, the surface between spines is highly folded by ridges (ri) and pitted (pi).

E,F) Transmission electron micrographs showing the division of the tegument across its thickness into 4 layers (1-4) according to the following characteristics: the cross sections of ridges and pits in layer 1; the high concentration of tegumental granules and lysosomes (Ly) in layer 2; the high concentration of mitochondria in layer 3, and the extensive basal membrane in foldings in layer 4.

G,H) The first and second layers showing cross sectional profiles of ridges (ri) and pits (pi), and the high concentration of dense spherical granules (G1), ellipsoid granules (G2), and mottled spherical granules (G3) in layer 2. In set showing the trilaminate surface membrane (Op) coated with a thick layer of glycocalyx (Gy).

were infoldings of the basal plasma membrane. The basal plasma membrane rested on the thick basal lamina which was coupled to the former by series of hemidesmosomes. Underneath the basal lamina was the reticular lamina that connected the tegument to the underlying muscle layers. There were numerous processes of tegument cells traversing the reticular lamina outwardly to join up with the tegument (Fig 1F). The tegument cells themselves lie in rows underneath the muscle layer, and there appears to be one type of tegument cell that is capable of producing all types of tegumental bodies.

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

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 (Sobhon *et al,* 1994). 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 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 overlaying surface membrane in *Schistosoma mansoni* (Hockley and McLaren,1973; McLaren, 1980) or through semi-permanent membrane channels joining between the membraneous bodies and the surface membrane in *S. japonicum* and *S. mekongi* (Sobhon *et al,* 1984; Sobhon and Upatham, 1990).

The synthesis and packaging of tegumental bodies were carried out by the Golgi complex-RER system of the tegumental cells. It is still debatable whether the synthesis of all types of bodies is carried out in a single or several cell types. In *F. hepatica,* Hanna (1980a,b,c) reported that there were at least 3 cell types responsible for the production of T_1 and T_2 bodies in adult, and T_0 in metacercariae and newly excysted juvenile parasites. In contrast our preliminary observation (Sobhon *et al,* 1994) demonstrated that in *F. gigantica* there was only one cell type responsible for the syntheses of all kinds of tegumental bodies, while those in metacercariae and newly excysted juveniles have not yet been studied. Following their syntheses in the tegument cells, tegumental bodies were transported to the tegument by the propelling action of microtubules localized in the cells' process and the second and third layers of the tegument cytoplasm. In schistosomes and *Opisthorchis viverrini,* it has been clearly demonstrated that this translocation was mediated by microtubules, since the treatment with tubulin-depolymerizing drugs disrupted this transport process (Wilson and Barnes, 1974; Sobhon and Upatham, 1990; Sobhon and Apinhasamit, 1996). It can be concluded, therefore, that the synthesis/secretory activity of the tegumental cells is the major source of replenishment and turn over of the surface membrane and tegument. This continuous process helps to repair damaged surface membrane as well as fending off the immune attacks by hosts. It remains to be proven whether under the influence of the latter insults, the process of membrane synthesis and replenishment is accelerated.

SOURCES OF PARASITE ANTIGENS

Antigens of *F. gigantica* that causes antibody formation in hosts are generated and released principally from the tegument and the cecum as demonstrated by immunoperoxidase detection method (Sobhon *et al,* 1996).

Tegument

The immuno-staining of the tegument exhibited four distinctive characteristics: the intense undulating 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 (Fig 2).

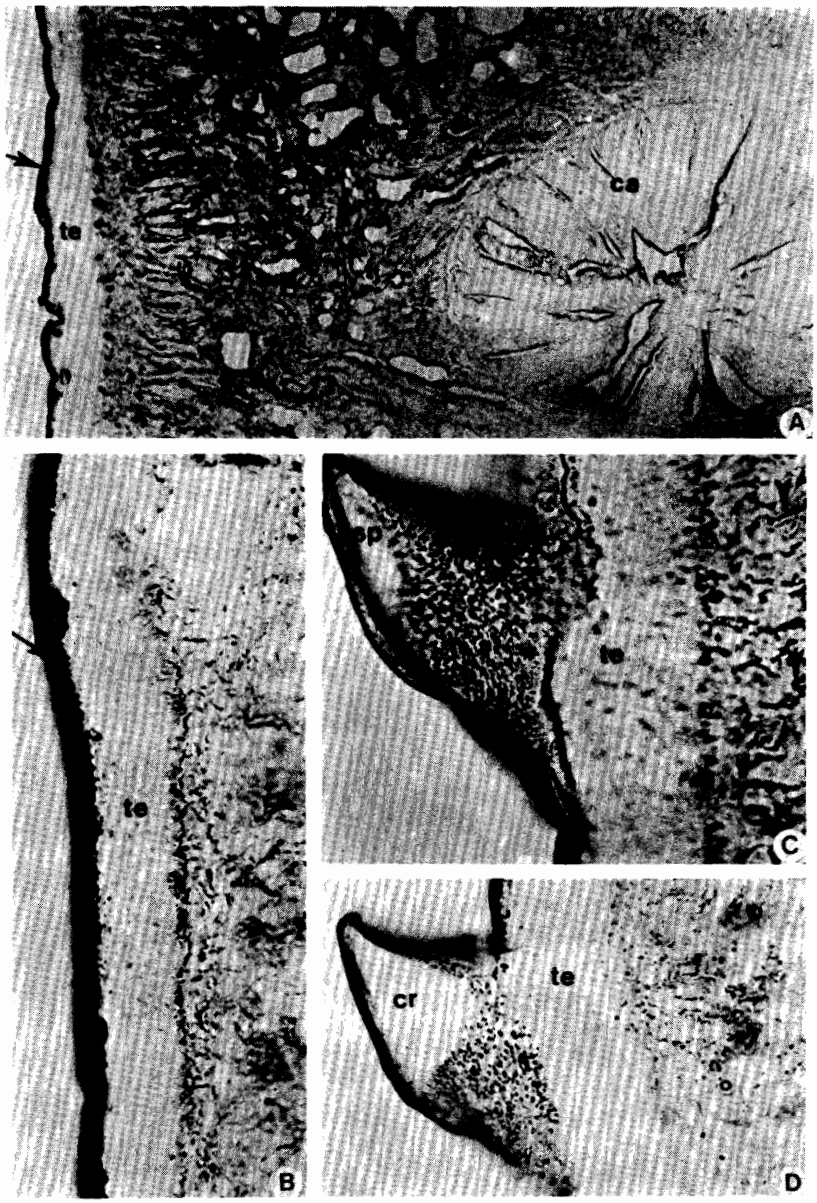

Fig 2–A) A cross section of an adult *F.gigantica* stained with immunoperoxidane technic, demonstrating the presence of dense deposit that indicate the locations of antigens on the surface membrane and outer rim (arrow) of the tegument (Te), and the apical membrane of cecal epithelial cells (Ca).
B) A high power micrograph of the tegument showing dense deposit on surface membrane and the narrow rim (arrow) of tegument (Te).
C,D) The surface of a spine is densely stained with granules of dense deposit (in C), while the spine crystalline matrix (Cr-in D) is unstained.

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 2A,B). This narrow zone should correspond to the deeply-stained outer margin of the tegument's cytoplasm, containing the first and second layers of the tegumental cytoplasm as revealed by TEM observations. 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 and held in reserved within the two types of granules.

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. Within the tegument, both types of granules were scatterred throughout which corresponded to the patterns of distribution of brownish granules in the tegument exhibited in the immuno-stained tegument.

It was reported that eventhough portions of the surface membrane covering spines were intensely stained, the spine crystalline matrix themselves were not stained (Fig 2C,D). This implies that spines'material was 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 cecum

The immuno-staining of the cecum demonstrated that antigens were concentrated mainly in the luminal content as well as in the cecal epithelial cells. The staining of the cecal content, in most cases, was 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 exocystosed into the lumen.

Antigens from the cecum might be the most abundant among the excretory-secretory (ES) antigens, considering the mass of highly branching cecum in the adult parasites. *F. gigantica* lacks a 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 cecum, that is pervasive throughout the parasite's body. 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 cecal contents were also major antigens that were released from the parasites. Some of those so called ES antigens were proven to be the digestive enzyme cysteine protease (Dalton and Heffernan, 1989; Rege *et al,* 1989; Yamasaki, *et al,* 1989; 1992; McGinty *et al,* 1993; Smith *et al,* 1993; Dowd *et al,* 1994; Wijffels *et al,* 1994a). Direct evidence that the cecal 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 homogenized whole body of the adult parasites showed that there were approximately 21 detectable bands, ranging in molecular weights (MW) from 110 to 14 kDa (Fig 3A). Eleven of these bands at MW 97, 86, 66, 64, 58, 54, 47, 38, 35, 19 and 14 kDa were present in the surface tegument (ST) 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 kDa, and lightly stained bands at 66, 64, 58 and 54 kDa. The latter group was also observed in the ST fraction, while the former group was not (Sobhon *et al,* 1996). Hence it is believed that 27 and 26 kDa proteins were most likely derived from the deeply-localized tissue, such as cecum 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 are believed to be cysteine proteases (Kiatpathomchai *et al,* 1995). These enzymes were generally detected in other *Fasciola* species, such as *F. hepatica* (Dalton and Heffernan, 1989; Rege *et al,* 1989; McGinty *et al,* 1993; Smith *et al,* 1993; Dowd *et al,* 1994; Wijffels *et al,* 1994a) and in *Schistosoma* species (Rege, *et al,* 1992; Gotz and Klinkert, 1993). They are probably used in the digestion of hosts' tissues, such as epithelia of the bile ducts, and blood cells for the parasites to feed on. The enzymes could also be acting inside the parasites' bodies to break down the ingested material.

In immunoblotting analysis, 14 from 21 bands in the whole body fraction 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 kDa. 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 kDa (Sobhon *et al,* 1996). 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 kDa. The former group were most likely the tegument-associated antigens, while the latter group were cecal-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* (1995), 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 kDa, 38-28 kDa, 17 kDa, 15 kDa, 13kDa and 12 kDa. 160 kDa 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 kDa (versus 66-54 kDa reported by us) and 38-28 kDa (versus 27-26 kDa reported by us). It is also reported that antigens at 64-54 kDa might be possible candidates for serodiagnosis of fascioliasis in cattle. The work by our group also reported that antigens at MW 66, 58, 54 kDa were more species-pecific than the cecal-associated antigens at 27-26 kDa (Viyanant *et al,* 1997a,b).

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 kDa (Sobhon and Upatham, 1990; Sobhon *et al,* 1992). *S. mansoni* also showed common antigens with these species at MW 97, 86, 68, 50 and 38 kDa (Taylor *et al,* 1981). These antigens were believed to be mainly the surface and tegument-associated antigens because similar pattern were obtained when the parasites were isotopically labeled with ^{125}I and antigenic bands analysed by immunoprecipitation (Sobhon *et al,* 1987). 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 cecal content.

PRODUCTION OF MONOCLONAL ANTIBODIES (mAB)

Our group has attempted to produce monoclonal antibodies (mAB) against *F. gigantica* antigens (Fig 3B,C) and found that mAB obtained from mice immunized with ES antigens recognized epitopes present in 54, 27, and 26 kDa proteins (Viyanant *et al,* 1997a). As mentioned earlier 27, 26 kDa proteins are the major antigens from the cecum that are detected in the excretory/secretory fluid of adult parasites which is also reported to be the case in *F. hepatica* (Fagbemi and Hillyer, 1992; Wijffels *et al,* 1994a). Since these antigens have been proven to be a family of cysteine protease enzymes (Kiatpathomchai *et al,* 1995) that are widely dis-

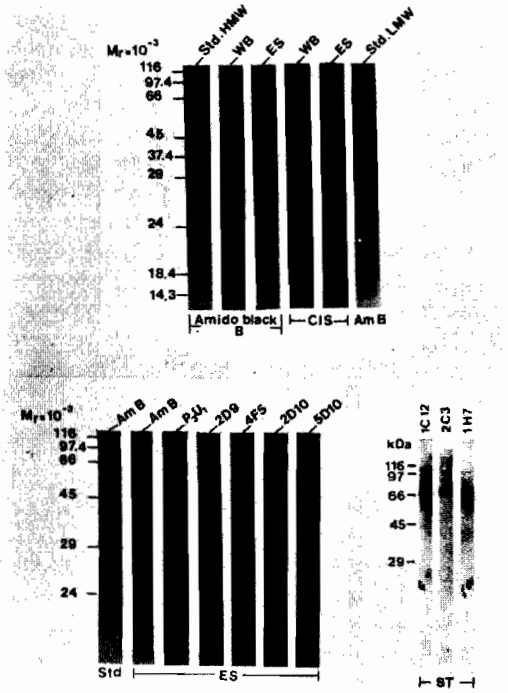

Fig 3–A) Proteins from adult *F.gigantica* separated on 12.5% SDS PAGE, blotted on to nitrocellulose papers : whole body (WB), excretory-secretory (ES) antigens stained with Amide black dye (Am B), and immunblotted with cow infected serum (CIS).
B) Immunoblot of ES antigens against monoclonal antibodies from various clones of hybrid cells immunized by ES antigens (12D9, 4F5, 2D10, 5D10), and control culture medium (P3U1). Positive bands are at 26, 27 kDa (2D9, 4F5) and 54, 58 kDa (2D10, 5D10). Lanes 1 and 2 are standard molecular weigths (Std), and ES stained with Amide black.
C) Immunoblot of surface and tegument antigens (ST) against monoclonal antibodies from various clones of hybrid cells immunized by ST antigens (1C12, 2C3, 1H7), indicates major positive band at 66 kDa.

tributed in trematode parasites including schistosomes, it is doubtful that their lack of specificity could be used for immunodiagnosis of fascioliasis. A monoclonal antibody that recognized a more specific surface tegumental antigen at 66 kDa could also be produced. This mAB has been used in ELISA-based immunoassay for detecting circulating antigens in the blood of cattle with up to 87% sensitivity (Viyanant *et al,* 1997b). In addition to their immunodiagnostic protential, the parasite killing capabilities of these mAB against juvenile parasites are still being tested both *in vitro* and *in vivo.*

VACCINE DEVELOPMENT STRATEGY AND CANDIDATE ANTIGENS

The rationale of vaccine design consists of 3 major strategies: first to identify parasites' antigens that are significantly different and do not cross react with those of hosts. These antigens may be molecules that are essential for maintaining the structural and functional integrity of parasites; or enzymes that are catalysing key reactions which are needed by parasites for acquiring nutrients, or for repairing damage done by various insults. Secondly, the molecules of choice should be able to elicit strong immunological responses in hosts, such that migrating juveniles could be immobilized or killed when they pass into the hosts' tissues. And thirdly, bulk synthesis of the antigens of choice should be feasible through the applications of recombinant DNA cloning technics.

Most vaccines may, however, not be as ideal as desired, because fasciola parasites are large and complex animals with substantial capacity to tolerate any insults and capability to repair themselves. Even partial vaccines that could impair the penetration and migration of newly excysted juveniles or those that could reduce the fecundity of adult parasites would have benificial effect for both the infected individual animals, and the proliferation of parasites in the endemic area. Recent research indicate that future prospect for the control of fascioliasis by immunological intervention appears brighter than previously thought. Candidate antigens from *F. hepatica* and *F. gigantica* that have shown vaccine potential during trials in cattle, sheep and rats are as follows:

Fatty acid binding proteins (FABP)

FABP are probably a group of surface or membrane-associated proteins with molecular weights ranging from 12-14 kDa. These proteins were first isolated from *F. hepatica* by affinity chromatography using rabbit antisera against *S. mansoni* whole-worm extract (Hillyer *et al,* 1977). Thus they are proteins that share common epitopes between two trematode species. Furthermore, in schistosomes

they were found to be a major fraction with fastest migration in 12.5% SDS-PAGE gel, and their intensity is lessen by chloroform extraction (Sobhon and Upatham, 1990). These proteins were shown to confer cross protection for calves against *F. hepatica* (Hillyer, 1985; Hillyer *et al,* 1987), and for mice and hamsters against infection by schistosomula of *S. mansoni* (Hillyer, 1979; Hillyer *et al,* 1988a,b). A cDNA library has been made for these proteins from *F. hepatica* (Rodriquez-Perez *et al,* 1992; Chicz, 1994), and an almost identical group of proteins have been characterized and expressed in *E. coli* (Smooker *et al,* 1997). This protein fraction confers significant protection (31%) against *F. gigantica* infection in cattle (Estuningsih *et al,* 1997). It is, therefore, quite optimistic to say that FABPs are one of the most promising vaccine candidate that could confer protection against at least two species of major parasitic trematodes.

66 kDa surface membrane protein

Our group has discovered major antigens from the surface of tegument of adult *F. gigantica* at 66, 58, 54 kDa (Sobhon *et al,* 1996, Viyanant *et al,* 1997b). The 66 kDa antigens has been specifically localized and found to be concentrated in the surface membrane. And as already mentioned mAB has been produced against this antigen (Viyanant *et al,* 1997b). It is possible that this antigen is the major protein of the surface membrane and its potential as vaccine candidate should be explored, since hosts immunological attacks elicited by this protein may result in the disruption of the membrane and the tegument, which in turn could injure and kill the parasites.

Paramyosin

Another structural molecule that exhibits potential as a vaccine candidate is paramyosin, a muscle protein with molecular weight at 97 kDa. Paramyosin is one of the major proteins that ubiquitously present in invertebrate muscle cells, including that of trematodes (Laclette *et al,* 1991; Kalina and McManus, 1993). This protein is also found to be one of the cytoskeletal protein of the tegument in *Schistosoma* species (Matsumoto *et al,* 1988; Sobhon and Upatham, 1990). Paramyosin could be easily purified and obtained in large quantity, and it is known to induce high level of protection in mice infected with *S. mansoni* or *S. japonicum* (Pearce *et al,* 1988; Flanigan *et al,* 1989; Gressman *et al,* 1990; Ramirez *et al,* 1996). It is possible that the protective action of antiparamyosin is its damaging effect on the cytoskeletal component of the tegument as well as on the muscular layers that lie underneath. Consequently, this might lead to the impairment of the tegumental function and the decrease motility, which may affect the survival and migration of parasites, especially the juvenile stages. Library of cDNA for schistosome's paramyosin has been constructed and expressed in *E. coli* (Yang *et al,* 1992). While the protective action of paramyosin has been proven in schistosomiasis, it has not yet been proven positive in fascioliasis, especially against *F. gigantica.*

Glutathione-S-transferase (GST)

An antioxidant enzyme, glutathione-S-transferase (GST) is another antigen that could be considered for vaccine candidate. GST actually consists of a large family of dimeric isoenzymes, whose monomeric units have molecular weight at 24-29 kDa. They are widely distributed in both animals and plants, in mammalian as well as parasites' tissues. GSTs comprise at least 4-5 main classes (Mannervik, 1985; Meyer *et al,* 1991). GSTs catalyse reactions that mop up oxygen free radicals and peroxides, hence preventing them from damaging parasites' surface. GSTs protective action has been shown in rats and mice against infection by *S. mansoni* and *S. japonicum* (Smith *et al,* 1986; Balloul *et al,* 1987; reviewed in Brophy and Pritchard, 1994), and against *F. hepatica* in sheep (Sexton *et al,* 1990) and cattle (Morrison *et al,* 1996). Evidently, cross protection by GSTs from the two species of parasites is also possible which implies that GSTs from different species may share common epitopes. Eventhough it has been shown recently that in one breed of cattle GSTs cannot confer protection against *F. gigantica* (Estuningsih *et al,* 1997), the potential of these molecules as vaccine candidates in other ruminants could not be ruled out, and should be exhaustively studied.

Cysteine proteases

Cysteine proteases at 26-27 kDa is the main components of excretory/secretory fluid of both species of fasciola (Dalton and Hefferman, 1989;

Yamasaki, Aoki and Oya,1989; Smith *et al,* 1993; McGinty *et al,* 1993; Wijffels *et al,* 1994a,b; Kiatpathomchai *et al,* 1995; Sobhon *et al,* 1996). These enzymes are localized in the lumen and cecal epithelial cells (Sobhon *et al,* 1996; Creney *et al,* 1996; Viyanant *et al,* 1997a), and hence they presumbly are the secretory product of epithelial cells. cDNA library of fasciola cysteine proteases have been isolated, and to date a total of 6 complete sequences have been reported (Yamasaki and Aoki, 1993; Heussler and Dobbelaere, 1994; Wijffels *et al,* 1994a). Cysteine proteases are intensely immunogenic in cattle and sheep (Kiatpathomchai *et al,* 1995; Wijffels *et al,* 1994b) and have been used for immunodiagnosis with fairly high level of specificity (Yamasaki *et al,* 1989; Fagbemi and Goubadia, 1995; Silva *et al,* 1996). *F. hepatica* cysteine proteases have been tested as possible vaccine candidate in sheep and cattle. However, rather than decreasing the worm numbers fecal egg count were significantly decreased (Wijffels *et al,* 1994b; Dalton *et al,* 1996). Cysteine proteases from *F. gigantica* have also been tested in cattle, however, neither reduction of worm burden nor egg count was detected (Estuningsih *et al,* 1997).

Hemoglobin-like antigen (Hemoprotein)

Recently a heme-containing protein has been detected in ES materials from adult *F. hepatica* (McGinty and Dalton, 1995). This is a high molecular weight protein at more than 200 kDa, with ability to bind oxygen molecules. Hence its role in concentrating and transporting oxygen to various tissues of the parasites' body is essential, especially in the low oxygen bile environment (Tielens *et al,* 1984). The high vaccine potential of this protein has been demonstrated, particularly in mixture with cysteine proteases (Dalton *et al,* 1996). The combined vaccine can greatly reduce the parasite fecundity, perhaps due to the decrease amount of nutrient materials and oxygen that must be delivered to the ovary and Mehlis gland for the egg shell formation (Bjorkmann and Thorsell, 1963; Dalton *et al,* 1996). Though this vaccine candidate cannot kill parasites and reduce the worm burden, the reduction of their fecundity help to alleviate the damaging effect on the hosts' liver, and may indirectly reduce or even eliminate parasites from the infected areas.

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REFERRENCES

Anonymous Control of foodborne trematode infections. *WHO Tech Rep Ser* 1995; 849.

Balloul JM, Grzych JM, Pierce RJ, Capron A. A purified 28,000 dalton protein from *Schistosoma mansoni* adult worms protects rats and mice against experimental schistosomiasis. *J Immunol* 1987; 138: 3448-53.

Bjorkmann N, Thorsell W. On the fine morphology of the egg shell globules in the vitelline glands of the liver fluke (*Fasciola hepatica*). *Exp Cell Res* 1963; 32: 153-6.

Boray JC. Flukes of domestic animals. In: Gaafar SM, Howard WE, Marsh RE eds. Parasites, pests and predators. New York: Elsevier, 1985: 179-218.

Brophy PM, Pritchard DI. Parasitic helminth glutathione-S-transferase: An update on their potential as targets for immuno- and chemotherapy. *Exp Parasitol* 1994; 79: 89-96.

Capron M, Capron A. Immunoglobulin E and effector cells in schistosomiasis. *Science* 1994; 264: 1876-77.

Cervi L, Robinstein H, Masih DT. Involvement of excretion-secretion products from *Fasciola hepatica* inducing suppression of the cellular immune responses. *Vet Parasitol* 1996; 61: 97-111.

Chicz RM. Submitted to the protein sequence database, August 1994 : Accession A44638.

Chitchung S, Ratananikom N, Mitranun W. *Fasciola hepatica* in human pancreas : A case report. *J Parasitol Trop Med Assoc Thai* 1982; 5: 113.

Chompoochan T, Apiwattanakorn B, Seniwong V, Penpairatkul S. Study on the life cycle of liver fluke of cattle in Thailand. *J Thai Vet Med Assoc* 1976; 27: 43-7.

Creney J, Wilson L, Dosen M, Sandeman RM, Spithill TW, Parsons JC. *Fasciola hepatica*: irradiation-induced alterations in carbohydrate and cathepsin-B protease expression in newly excysted juvenile liver fluke. *Exp Parasitol* 1996; 83: 202-15.

Dalton JP, Hefferman M. Thiol proteases released in vitro by *Fasciola hepatica*. *Mol Biochem Parasitol* 1989; 55: 161-6.

Dalton JP, McGonigle S, Rolph TP, Andrews SJ. Induction of protective immunity in cattle against infection with *Fasciola hepatica* by vaccination with cathepsin L proteases and hemoglobin. *Infect Immun* 1996; 64: 5066-74.

Doy TG, Hughes DL. *In vitro* cell adherence to newly excysted *Fasciola hepatica*: failure to effect their subsequent development in rats. *Res Vet Sci* 1982; 32: 118-20.

Dowd JA, Smith AM, McGonigle S, Dalton JP. Purification and characterization of a second cathepsin L proteinase secreted by the parasitic trematode *Fasciola hepatica*. *Eur J Biochem* 1994; 223: 91-8.

Edney JM, Muchlis A. Fascioliasis in Indonesian livestock. *Commun Vet* 1962; 6: 49-52.

Estuningsih SE, Smooker PM, Wiedosari E, *et al.* Evaluation of antigens of *Fasciola gigantica* as vaccine against tropical fasciolosis in cattle. *Int J Parasitol* 1997 (in press).

Fabiyi JP. Production losses and control of helminths in ruminants of tropical regions. *Int J Parasitol* 1987; 17: 435-42.

Fagbemi BC, Hillyer GV. The purification and characterization of a cysteine protease of *Fasciola gigantica* adult worms. *Vet Parasitol* 1992; 43: 223-32.

Fagbemi B, Goubadia EE. Immunodiagnosis of fascioliasis in ruminants using a 28-kDa cysteine protease of *Fasciola gigantica* adult worms. *Vet Parasitol* 1995; 57: 309-18.

Flanigan TP, King CH, Lett RR, Nanaduri J, Mahmoud AA. Induction of resistance to *Schistosoma mansoni* infection in mice by purified parasite paramyosin. *J Clin Invest* 1989; 83: 1010-14.

Golenser J, Chevion M. Implication of oxidative stress and malaria. In: Aruoma OI. Free radicals in tropical diseases. Switzerland: Harwood Academic Publishers, 1993: 53-80.

Gotz B, Klinkert MQ. Expression and partial characterization of a cathepsin B-like enzyme (Sm31) and a proposed "haemoglobinase" (Sm32) from *Schistosoma mansoni*. *Biochem J* 1993; 290: 801-6.

Gressman Z, Ram D, Markovica A, *et al.* *Schistosoma mansoni*: stage-specific expression of muscle-specific gene. *Exp Parasitol* 1990; 70: 62-71.

Hanna REB. *Fasciola hepatica*: Glycocalyx replacement in the juvenile as a possible mechnism for protection against host immunity. *Exp Parasitol* 1980a; 50: 103-14.

Hanna REB. *Fasciola hepatica*: An immunofluorescent study of antigenic changes in the tegument during development in the rat and the sheep. *Exp Parasitol* 1980b; 50: 155-70.

Hanna REB. *Fasciola hepatica*: Autoradiography of protein synthesis, transport and secretion by the tegument. *Exp Parasitol* 1980c; 50: 297-304.

Heussler VT, Dobbelaere DA. Cloning of protease gene family of *Fasciola hepatica* by the polymerase chain reaction. *Mol Biochem Parasitol* 1994; 64: 11-23.

Hillyer GV. *Schistosoma mansoni:* reduced worm burdens in mice immunized with isolated *Fasciola hepatica* antigens. *Exp Parasitol* 1979; 48: 287-95.

Hillyer GV. Induction of immunity in mice to *Fasciola hepatica* with *Fasciola/Schistosoma* cross-reactive defined immunity antigen. *Am J Trop Med Hyg* 1985; 34: 1127-31.

Hillyer GV, del Llano de Diaz A, Reyes CN. *Schistosoma mansoni*: acquired immunity in mice and hamsters using antigens of *Fasciola hepatica*. *Exp Parasitol* 1977; 42: 348-55.

Hillyer GV, Haroun EM, Hernandez A, De Galanes MS. Acquired resistance to *Fasciola hepatica* in cattle using a purified adult worm antigen. *Am J Trop Med Hyg* 1987; 37: 363-69.

Hillyer GV, De Galanes MS, Rosa MI, Montealegre F. Acquired immunity in schistosomiasis with purified *Fasciola hepatica* cross-reactive antigens. *Vet Parasitol* 1988a; 29: 265-80.

Hillyer GV, Rosa MI, Alicea H, Hernandez A. Successful vaccination against murine *Schistosoma mansoni* infection with a purified 12 kDa *Fasciola hepatica* cross-reactive antigen. *Am J Trop Med Hyg* 1988b; 38: 103-10.

Hockley DJ, McLaren DJ. *Schistosoma mansoni*: change in the outer membrane of the tegument during development from cercaria to adult worm. *Int J Parasitol* 1973; 3: 13-25.

Hughes DL. Fasciola and fascioloides. In: Soulsby EJL, ed. Immune response in parasitic infection: immunology, immunopathology, and immunoprophylaxis. Vol II. Trematodes and cestodes. Florida, USA: CRC Press, 1987: 91-114.

Itagaki T, Sakamoto T, Itagaki H. Analysis of *Fasciola* sp antigen by enzyme-linked immunotransfer blot using sera from experimentally and naturally infected cattle. *J Vet Med Sci* 1995; 57: 522-13.

James SL, Gleven JA. Macrophage cytotoxicity against schistosomula of *Schistosoma mansoni* involves arginine-dependent production of reactive nitrogen intermediates. *J Immunol* 1989; 143: 4208-12.

Kalina B, McManus DP. An IgE (Fc gamma) binding protein of *Taenia crassiceps* (Candida) exhibits sequence homology and antigenic similarity with schistosome paramyosin. *Parasitology* 1993; 106: 289-96.

Kelly JD, Campbell NJ, Dineen JK. The role of the gut in acquired resistance to *Fasciola hepatica* in the rat. *Vet Parasitol* 1980; 6: 359-67.

Kiatpathomchai K, Chaichomelert S, Kusamran T, *et al.* Protein antigens of cattle liver-fluke *Fasciola gigantica.* In: Biopolymer and bioproducts : structure, function and applications. 11th FAOBMB Symposium. Bangkok: Samakkhisan 1995.

Laclette JP, Landa A, Arcos L, Williams K, Davis AE, Shoemaker CB. Paramyosin is the *Schistosoma mansoni* (Trematoda) homoloque of antigen B from *Taenia solium* (Cestoda). *Mol Biochem Parasitol* 1991; 44: 287-95.

Liew FY, O'Donnell CA. Immunology of leishmaniasis. *Adv Parasitol* 1993; 32: 161-259.

Maizels RM, Bundy DA, Selkirk ME, Smith DF, Anderson RM. Immunological modulation and evasion by helminth parasites in human population. *Nature* 1993; 365: 797-805.

Mannervik B, Alin P, Guthenberg H, *et al.* Identification of three classes of cytosolic glutathione-S-transferase common to several mammalian species: correlation between structural data and enzymatic properties. *Proc Nat Acad Sci USA* 1985; 82: 7702-6.

Matsumoto Y, Perry G, Levine RJC, Blanton R, Mahmoud AAF, Aikawa M.Paramyosin and actin in schistosomal tegument. *Nature* 1988; 333: 76-8.

Maurice J. Is something lurking in your liver? *New Scientist* 1994; 1917: 26-31.

McGinty S, Dalton JP. Isolation of *Fasciola hepatica* hemoglobin. *Parasitology* 1995; 111: 209-15.

McGinty A, Moore M, Halton DW, Walker B. Characterization of the cysteine proteases of the common liver fluke *Fasciola hepatica* using novel, active-site directed affinity labels. *Parasitology* 1993; 106: 487-93.

McLaren DJ. *Schistosoma mansoni*: The parasite surface in relation to host immunity. New York : John Wiley and Sons Research Studies Press, 1980.

Mei H, Loverde PT. *Schistosoma mansoni* : The development regulation and immunolocalization of antioxidant enzymes. *Exp Parasitol* 1997; 86: 69-78.

Meyer DJ, Coles B, Pemble SE, Gilmore KS, Fraser GM, Ketterer B. Theta, a new class of glutathione transferase purified from rat and man. *Biochem J* 1991; 274: 409-14.

Morrison CA, Colin T, Sexton JL, *et al.* Protection of cattle against *Fasciola hepatica* infection by vaccination with glutathione-S-transferase. *Vaccine* 1996; 14: 1603-12.

Oldham G, Williams L. Cell mediated immunity to liver fluke antigens during experimental *Fasciola hepatica* infection of cattle. *Parasite Immunol* 1995; 17: 503-16.

Overend DJ, Bower FL. Resistance of *Fasciola hepatica* to triclabendazole. *Aust Vet J* 1995; 72: 275-6.

Pearce EJ, James SL, Hieny S, Lanar DE, Sher A. Induction of protective immunity against *Schistosoma mansoni* by vaccination with schistosome paramyosin (Sm97), a nonsurface parasite antigen. *Proc Nat Acad Sci USA* 1988; 85: 5678-82.

Pholpark M, Srikitjakara L. The control of parasitism in swamp buffalo and cattle in north-east Thailand. In: International Seminar on Animal Health and Production Services for Village Livestock, Khon Kaen, Thailand,1989: pp 244-9.

Zhou Y, Podesta RB. Effect of serotonin (5HT) and complement C3 on the synthesis of the surface membrane precursors of adult *Schistosoma mansoni. J Parasitol* 1989; 75: 333-43.

Rajasekariah GR, Howell MJ. The fate of *Fasciola hepatica* metacercariae following challenge infection of immune rats. *J Helminthol* 1977; 51: 289-94.

Ramirez BL, Kurtis JD, Wiest PM, *et al.* Paramyosin - a candidate vaccine antigen against *Schistosoma japonicum. Parasite Immunol* 1996; 18: 49-52.

Rodriguez-Perez J, Rodriguez-Medina JR, Garcia-Blanco MA, Hillyer GV. *Fasciola hepatica*: molecular cloning, nucleotide sequence and expression of a gene encoding a polypeptide homologous to a *Schistosoma mansoni* fatty acid binding protein. *Exp Parasitol* 1992; 74: 400-7.

Rege AA, Herrera PR, Lopez M, Dresden MH. Isolation and characterization of a cysteine protease from *Fasciola hepatica* adult worm. *Mol Biochem Parasitol* 1989; 35: 89-96.

Rege AA, Wang W, Dresden MH. Cysteine proteases from *Schistosoma haematobium* adult worms. *J Parasitol* 1992; 78: 16-23.

Sexton JL, Milner AR, Panacco M. Glutathione-S-transferase: Novel vaccine against *Fasciola hepatica* infection in sheep. *J Immunol* 1990; 145: 3905-10.

Sher A, Coffma RL. Regulation of immunity to parasites by T cells and T cell-mediated cytokines. *Ann Rev Immunol* 1992; 10: 385-409.

Silva MLS, Dacosta JMC, Dacosta AMV, *et al.* Antigenic components of excretory-secretory products of adult *Fasciola hepatica* recognized in human infections. *Am J Trop Med Hyg* 1996; 54: 146-8.

Smith DB, Davern KM, Board PG, Tiu WU, Garcia EG, Mitchell GF. Mr 26,000 antigen of *Schistosoma japonicum* recognized by resistant WEHI 129/J mice is a parasite glutathione-S-transferase. *Proc Nat Acad Sci USA* 1986; 83: 8703-7.

Smith AM, Dawd AJ, McGonigle S, *et al.* Purification of a cathepsin L-like protease secreted by adult *Fasciola hepatica. Mol Biochem Parasitol* 1993; 62:1-8.

Smith NC. Review article: The role of free radicals in the expulsion of primary infection of *Nippostrongylus brasilliensis*. *Parasitol Res* 1989; 75: 423-38.

Smooker PM, Hickford DE, Vaiano S, Spithill TW. Isolation, characterization and expression of cDNA encoding a *Fasciola gigantica* fatty acid binding protein. *Exp Parasitol* 1997; (in press).

Sobhon P, Upatham ES, Mclaren DJ. Topography and ultrastructure of the tegument of adult *Schistosoma mekongi*. *Parasitology* 1984; 89: 511-21.

Sobhon P, Upatham ES. Snail hosts, life-cycle, and tegumental structure of oriental schistosomes. UNDP/World Bank/ WHO Special Programme for Research and Training in Tropical Diseases, 1990.

Sobhon P, Panuwatsuk W, Tharasub C, Upatham ES, Kusamran T, Viyanant V. Identification of surface antigens in *Schistosoma japonicum* (Chinese & Philippine) by immunoprecipitation. Proceeding of His Majesty's Fifth Cycle Commemorative Conference of USAID Science Research Award Grantees, Nakhon Pathom Thailand 1987: 237-50.

Sobhon P, Upatham ES. Viyanant V, Kusamran T, Mohthong V, Anantavara S. Identification and localization of surface antigens in adult *Schistosoma japonicum* and *Schistosoma mekongi*. National Research Council, USA, Aquaculture and Schistosomiasis. Proceedings of a network meeting held in Manila, Philippines, August 6-10, 1991. Washington, DC: National Academy Press, 1992: pp178-95.

Sobhon P, Dangprasert T, Saitongdee P, Wanichanon C, Upatham ES. Surface topography and ultrastructure of the tegument of adult *Fasciola gignatica*. *Elec Micros Soc Thai* 1994; 8: 36-45.

Sobhon P, Apinhasamit W. *Opisthorchis viverrini* : The effect of colchicine and cytochalasin B on the adult tegument. *Southeast Asian J Trop Med Public Health* 1996; 27: 312-7.

Sobhon P, Anantavara S, Dangprasert T, *et al*. *Fasciola gigantica*: Identification of adult antigens, their tissue sources and possible origins. *J Sci Soc Thailand* 1996; 22: 143-62.

Soesetya RHB. The prevalence of *Fasciola gigantica* infection in cattle in East Java, Indonesia, *Malaysian Vet J* 1975; 6: 5-8.

Sukhapesna V, Tantasuvan D, Sarataphan N, Sangiumluksana S. A study on epidemiology of liver fluke infection in buffaloes. *Thai J Vet Med* 1990; 20: 527-34.

Sukhapesna V, Sarataphan N, Tantasuvan D. Anthelmintic activity of triclabendazole against *Fasciola gigantica* in cattle and buffaloes. Bangkok: Thai Veterinary Medical Association Conference, 1992: 53.

Sukhapesna V, Tantasuvan D, Sarataphan N, Imsup K. Economic impact of fascioliasis in buffalo production. *J Thai Vet Med Assoc* 1994; 45: 45-52.

Tielens AG, van den Heuvel JM, van den Bergh SG. The energy metabolism of *Fasciola hepatica* in the final host. *Mol Biochem Parasitol* 1984; 13: 301-7.

Taylor JW, Hayunga EG, Vannier WE. Surface antigens of *Schistosoma mansoni*. *Mol Biochem Parasitol* 1981; 3: 157-68.

Thammasart S, Chompoochan T, Prasittirat P, Nithiuthai S, Taira N. Dynamics of ELISA titers in cattle, buffaloes and sheep infected with *Fasciola gigantica* metacercariae. *J Trop Med Parasitol* 1996; 19: 13-23.

Viyanant V, Upatham ES, Sobhon P, *et al*. Development and characterization of monoclonal antibodies against excretory- secretory antigens of *Fasciola gigantica*. *Southeast Asian J Trop Med Public Health* 1997; Vol 28 (supp 1): 128-33.

Viyanant V, Krailas D, Sobhon P, *et al*. Diagnosis of a circulating antigen using a monoclonal antibody. *Asia Pacific J Allerg Immunol* 1997b (in press).

Wijffels GL, Panaccio M, Salvatore L, Wilson L, Walker LD, Spithill TW. The secreted cathepsin L-like protease of the trematode, *Fasciola hepatica*, contains 3-hydroxyproline residues. *Biochem J* 1994a; 299: 781-90.

Wijffels GL, Salvatore L, Dosen M, *et al*. Vaccination of sheep with purified cysteine proteases of *Fasciola hepatica* decreases worm fecundity. *Exp Parasitol* 1994b; 78: 132-48.

Wilson RA, Barnes PE. The tegument of *Schistosoma mansoni*: Observation on the formation, structure and composition of cytoplasmic inclusions in relation to tegument function. *Parasitology* 1974; 68: 239-58.

Wynn T, Oswald IP, Eltoum IA. Elevated expression of Th1 cytokines and nitric oxide synthase in the lungs of vaccinated mice after challenge infection with *Schistosoma mansoni*. *J Immunol* 1994; 153: 5200-9.

Yamasaki H, Aoki T, Oya H. A cysteine protease from the liver fluke *Fasciola* spp; purification, characterization, localization and application to immunodiagnosis. *Jpn J Parasitol* 1989; 38: 373-84.

Yamasaki H, Kominami E, Aoki T. Immunocytochemical localization of a cysteine protease in adult worms of the liver fluke *Fasciola* sp. *Parasitol Res* 1992; 78: 574-80.

Yang W, Waine GJ, Sculley DG, Liu X, McManus DP. Cloning and partial nucleotide sequence of *Schistosoma japonicum* paramyosin: a potential vaccine candidate against schistosomiasis. *Int J Parasitol* 1992; 22: 1187-91.

Zimmerman GL, Kerkvliet NI, Brauner JA, Cerro JE. Modulation of host immune responses by *Fasciola hepatica*: reponses by peripheral lymphocytes to mitogens during liver fluke infection of sheep. *J Parasitol* 1983; 69: 473-7.