Fasciola gigantica: Ultrastructure of the Adult Tegument

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ABSTRACT The tegument of adult *Fasciola gigantica* can be divided into four layers based on ultrastructural characteristics. The first layer includes ridges and pits which are covered by a trilaminate membrane about 8 nm thick, underlined by a dense lamina about 15 nm thick. The membrane is coated externally by the glycocalyx which consists of two layers: the inner dense homogeneous layer about 10-15 nm, and the outer fibrillar layer about 100-300 nm thick which is intensely stained with ruthenium red. The cytoplasm is composed of densely-packed microtrabecular network, and contains many ovoid granules (G_1) whose size is about 90 x 180 nm, and numerous discoid granules (G_2) whose size is about 40 x 250 nm. G_1 contain dense ruthenium red-positive matrix while G_2 contain translucent matrix, and both are surrounded by a trilaminate membrane. G_1 close to the surface invariably exocytose their content into bottoms of the pits, while some G_2 are fused and have their membrane joined up with the surface membrane. It is, therefore, suggested that G_1 contribute to the formation of glycocalyx while G_2 are the main contributor to the surface membrane. The second layer of the tegument is a narrow zone of cytoplasm that contains high concentrations of G_1, G_2 granules and lysosomes. The third layer is the widest middle portion of the tegument which contains numerous and evenly distributed mitochondria. Both G_1 and G_2 granules are present but in much fewer number than in the first and second layers. The fourth layer is the innermost zone that rests on and couples with the 120-140 nm thick basal lamina. Its cytoplasm is loosely packed and contains numerous infoldings of the basal plasma membrane which have mitochondria in close association. It contains fairly large numbers of G_1 and G_2 granules which are produced and transported to the tegument by one type of tegumental cells lying in rows underneath the muscular layers. Spines in the tegument are numerous, each is a wedge-shaped crystalline structure with the lattice spacing about 4 nm, and its rootlets are firmly implanted in the basal lamina.

KEYWORDS: *Fasciola gigantica*, tegument, ultrastructure, transmission electron microscopy.

INTRODUCTION

Fasciolosis due to *Fasciola gigantica* causes significant economic loss in animal production in the tropics. The disease can also infect humans, and there are reports of increasing incidence worldwide.[1] The prevalence of infection of animal are as high as 30-90% in Africa, 25-90% in Indonesia.[2-4] In Thailand the prevalence of infection in cattle and buffaloes are 4-24%, with the highest incidence in the North and Northeast, and the lowest in the South.[5,6] Hence, the disease is one of the major impediments to economic progress in developing countries. Fasciolosis could be partially controlled by periodic treatment of animals in endemic areas with drugs, among which triclabendazole was reported to be highly effective, even though resistance has been observed.[7] In view of the cost and possible emergence of drug resistance, a better preventive measure would be the development of vaccines which could either completely prevent the infection or arrest the development of the parasites at certain stages of their life cycle.

The tegument of the parasite is one of the major targets for vaccines since it produces and releases a number of antigens that can stimulate the immune responses in hosts.[6,8] Furthermore, the tegument plays roles in maintaining the parasites' homeostasis, such as the absorption and exchange of nutritive and waste molecules, and the regulation of ionic equilibrium between the interior of the parasites and the surrounding host fluid.[9] A complete understanding of the structural organization of the tegument is hence crucial in developing any rational drugs or vaccines that can damage the parasites through their actions

on the tegument. Up to now, all work on the tegument ultrastructure have been carried out in *Fasciola hepatica*.[10-13] Though the basic structure of the tegument of *F. gigantica* is expected to be similar to that of *F. hepatica*, there is evidence that the two species exhibit differences in their resistance to drugs and potential vaccine candidates.[14] Hence there could be variations in the tegument ultrastructure that have not yet been observed. In this study, we report the tegument ultrastructure of adult *F. gigantica*.

Materials and Methods

Specimen Collection

Adult *F. gigantica* were collected from the bile ducts and gallbladders of cattle and water buffaloes killed at the abattoirs. The flukes were washed several times in 0.85% normal saline before being transferred into Minimum Essential Medium (MEM) with three changes. The flukes were kept in MEM supplemented with 10mg/ml penicillin and 100 unit/ml streptomycin until prepared for TEM.

Conventional Transmission Electron Microscopy (TEM)

The adult flukes were sliced into thin strips while being fixed in 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer containing 0.1 M calcium acetate, pH 7.2, at 4°C for 2 h. Then they were washed three times with the same buffer, post-fixed in 1 % osmuim tetoxide in 0.1 M sodium cacodylate buffer, pH 7.2, at 4°C for 3 h, and washed in distilled water. Finally, the specimens were fixed in 0.5% aqueous solution of uranyl acetate, pH5, containing 45mg/ml sucrose, for 30 min, at 4°C. The specimens were washed three times in distilled water, then dehydrated in graded series of ethanol (50-100%), for 20 min at each step. Subsequently, they were infiltrated twice in propylene oxide for 20 min each; and the solution was later sequentially replaced with the mixture of propylene oxide and Aradite-502 at the ratio of 2:1 for 1 h and 1:2 overnight at room temperature. Finally, the specimens were infiltrated with pure Aradite for at least 12 h at room temperature, and then polymerized at 45°C and 60°C for 2 days each. Thin section were cut and collected either on naked or formvar-coated 200-mesh copper grids, and stained with methanolic 1.0% uranyl acetate and lead citrate, for 30 min each. The sections were viewed in a Hitachi H-300 TEM, operating at 75 kV.

Ruthenium Red Staining

Ruthenium red is a low-mol.wt polycationic compound which binds electrostatically to polyanions such as proteoglycans and sialoglycoproteins.[15] This method is used to exhibit glycocalyx and the structure that contains negatively-charged acidic carbohydrates.[16,17] Another group of parasite specimens was thus stained with ruthenium red.[15] Briefly, the specimens were fixed and stained for 1 h at room temperature in a solution containing equal proportions of 4% glutaraldehyde in 0.2 M cacodylate buffer pH 7.2, and 1500 ppm aqueous solution of ruthenium red (Polysciences Co). After washing three times with the same buffer, the flukes were post-fixed for 3 h at room temperature in a solution containing equal proportions of 2 % osmium tetroxide in 0.2 M cacodylate buffer pH 7.2, and an aqueous solution of 1500 ppm ruthenium red. The specimens were rinsed briefly with the same buffer, and then dehydrated and processed for TEM as already described. Both unstained and counterstained sections with uranyl acetate and lead citrate were examined in TEM.

Results

Ultrastructure of the Tegument

When examined in cross section, the tegument can be divided into four layers based on the presence of various organelles and the density of the tegumental cytoskeleton (Fig 1A-C; 2A, B). The first and outer most layer is the microvillus-like zone which actually represents cross sections of ridges or microfolds intervened by oblong pits as visualized in SEM.[6] The crevices or grooves between major folds may run deep down in the tegument, such that in these areas the surface membrane of the ridges are compressed together (Fig 2B). The cytoplasm of the first layer consists of tightly packed microtrabraculae of very thin filaments (Fig 2C, D), and contains moderate number of granules. In conventional TEM preparation the surface membrane appears trilaminate with 8 nm in thickness, and coated on the exterior by a thin layer of homogeneous glycocalyx about 10-15 nm in width (Fig 2D; 3C). By contrast, in ruthenium red-stained sections, glycocalyx appears as a fibrillar layer with thickness as much as 100-300nm (Fig 4D, E). On the cytoplasmic side, the surface membrane is underlined by a lamina of condensed cytoplasm about 15 nm thick (Fig 2D).

The second layer is a narrow zone of cytoplasm under the first layer, which is characterized by the presence of a high concentration of tegumental granules and lysosome-like bodies (Fig 1A; 2B, C).

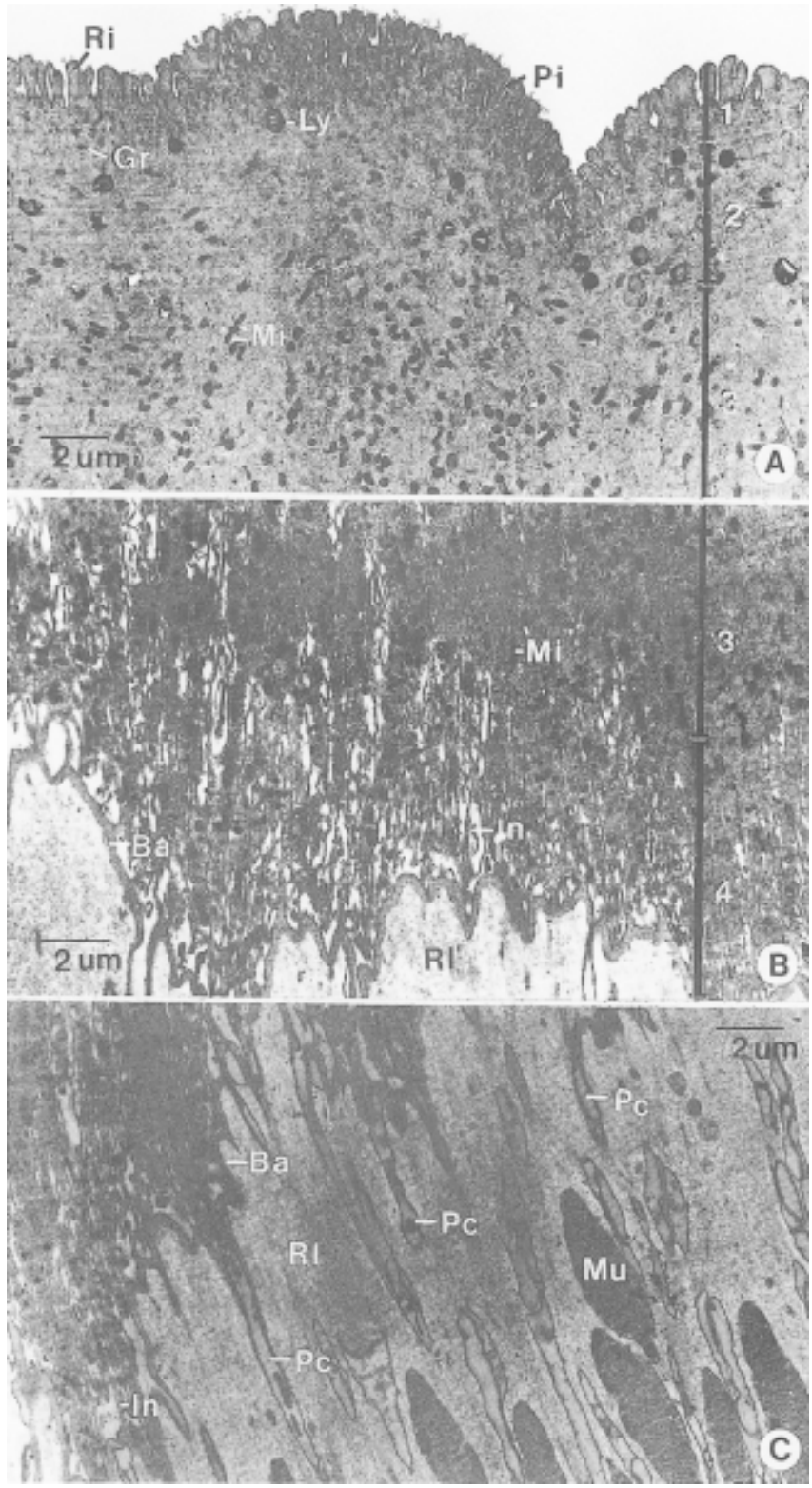

Fig 1. A, B) Low power conventional TEM micrographs illustrating the division across the width of the adult *F. gigantica* tegument into 4 zones (1, 2, 3, 4). Notice the cross sections of ridge (Ri) and pits (Pi) in zone 1, high concentration of granules (Gr) and lysosomes (Ly) in zone 2, mitochondria (Mi) in zone 3, basal plasma membrane infoldings (In) in zone 4.
C) A TEM micrograph showing basal (Ba), reticular (Rl) laminae and muscle (Mu) of the body wall traversed by tegumental cells' processes (Pc).

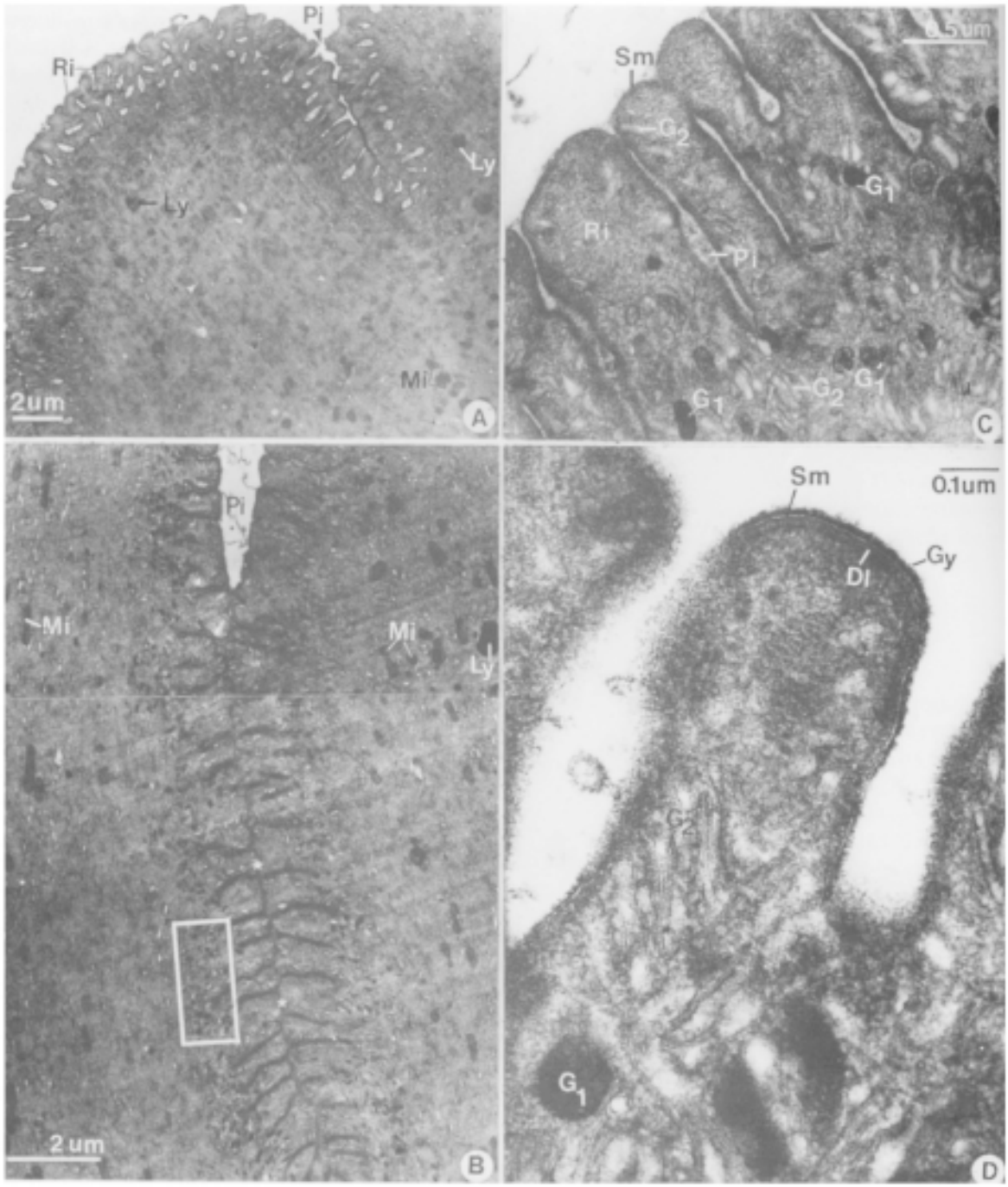

Fig 2. A, B) Zone 1 and 2 of the tegument, exhibiting cross sections of ridges (Ri) and pits (Pi) and deep crevices (stars). Zone 2 has high concentrations of tegumental granules (rectangle) and lysosomes (Ly).
C, D) Medium and high magnifications of zone 1 exhibiting trilaminate surface membrane (Sm) underlined by dense lamina of cytoplasm (Dl), and covered by thin glycocalyx (Gy). A large member of G_2 granules are present in this zone while there are very few G_1 granules.

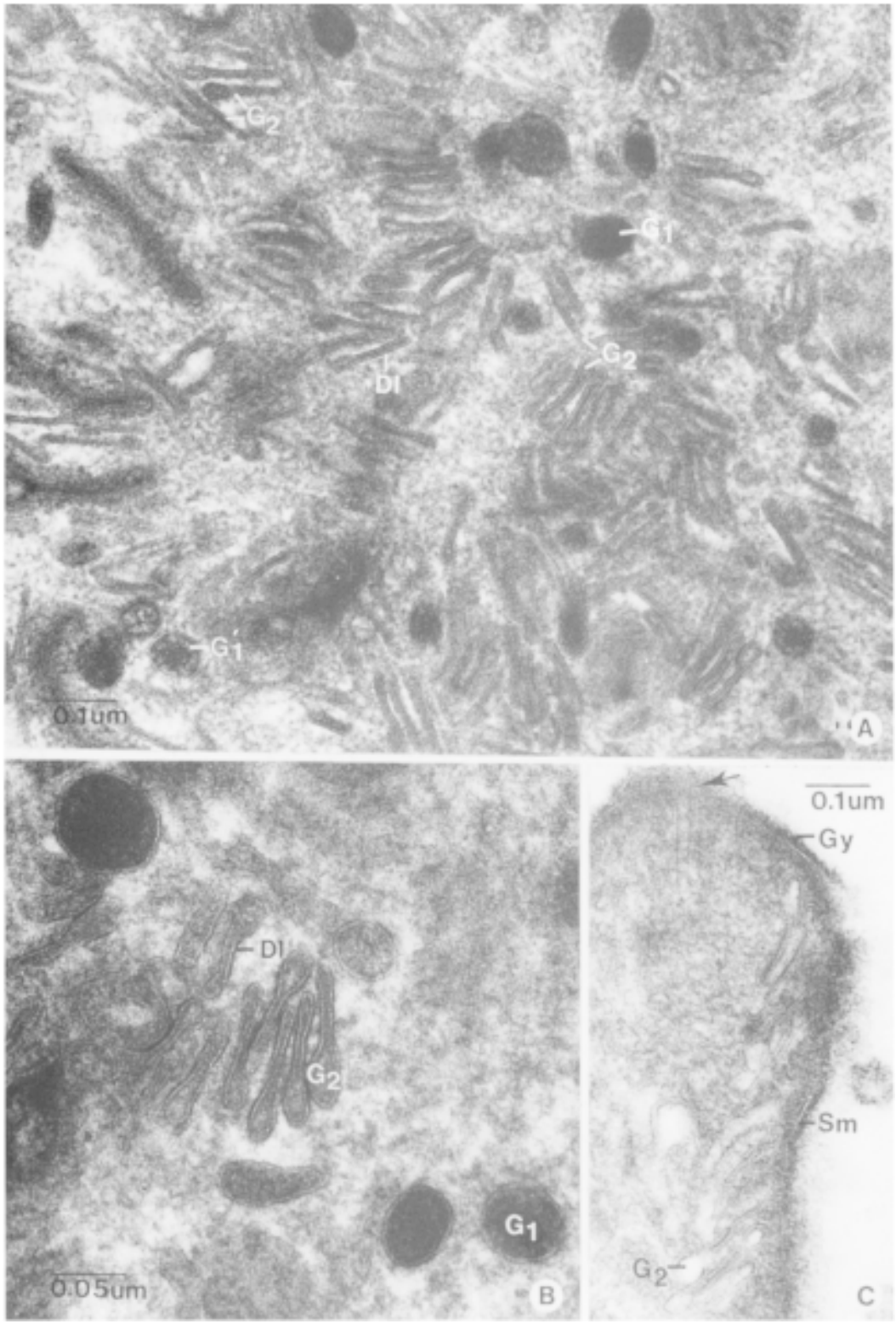

Fig 3. A, B) High magnifications of zone 2, (taken from boxed area in Fig. 2B) illustrating a high concentration of G_2 granules and less numerous G_1 granules. Most G_1 contain homogeneously dense matrix, while some (G_1') contain dark fibrous matrix. G_2 have dumbbell shape and contain pale matrix, and their membrane is surrounded by dense lamina (Dl).
C) A high magnification micrograph of a ridge showing the fusion of some G_2 with the surface membrane (arrow).

At high magnifications there appear to be two types of tegumental granules (Fig. 2C; 3A). The first type (G_1) is a dense ovoid granule that is measured approximately 90 nm wide by 180 nm long. G_1 contains homogeneously dense ruthenium red-positive matrix and is surrounded by a trilaminate membrane (Fig 2C, D; 3A, B; 4A, D). Some G_1 granules appear very close to the surface membrane at the bottoms of the pits (Fig 2D; 4B, C), and a few appear to join with the membrane and exocytose their content into the pits (Fig 4A-C). The second type is a discoid granule (G_2) which is measured about 40 nm wide by 250 nm long. This granules is surrounded by a trilaminate membrane, and at the central part its membrane is closely apposed, while both ends become enlarged such that the granule assumes a mild dumbbell shape (Fig 2D; 3A, B). G_2 granule contains light homogeneous matrix, while the cytoplasmic side of its membrane has a thin dense lamina lining (Fig 3B). While G_2 are more numerous than G_1, both are concentrated in this zone. A large number of G_2 flow up to the first layer to join up with the membrane lining the sides of ridges and pits (Fig 2D, 3C). The second layer also contains lysosome-like bodies arranged in rows parallel to the surface (Fig 1A; 2A, B).

The third layer is the middle and widest zone of the tegumental cytoplasm (Fig 1A, B). It consists of uniformly packed microtrabecular network, and contains numerous mitochondria but only few lysosomes (Fig 1A, B). G_1 and G_2 tegumental granules are evenly distributed throughout the layer, and appear less concentrated than in the second layer. Mitochondria have dense matrix and only few cristae running parallel to their longitudinal axes.

The fourth or basal layer rests on the basal and reticular laminae (Fig 1B, C). Its cytoplasm contains loosely-packed microtrabecular network and long narrow lightly-stained channels, running vertically towards the surface of the tegument. At high magnification these channels are actually oblong spaces between the tortuous infoldings of the basal plasma membrane. The basal plasma membrane is trilaminate and coupled by hemidesmosomes to the basal lamina, whose homogeneous matrix continues to fill the narrow spaces between the infoldings. G_1 are the most frequently observed granules in the third layer, while G_2 are relatively scarce.

Spines

Spines are the most prominent feature of the adult tegument. Each spine has the main part of its body embedded within the whole thickness of the tegument with its apical part jutting out from the surface (Fig 5A, B). The spine is composed of a wedge-shaped crystalline structure, with each spacing between the lattice about 4 nm (Fig 4C, D). The tip of the spine is covered only by the surface membrane, while the edges are covered by thin sheet of tegumental cytoplasm that are invaginated. The edge of the spine and adjoining cytoplasm have no specialized coupling other than the presence of a rather compact mass of cytoplasm that contains relatively high concentration of mitochondria (Fig 5C, E). In contrast, at the basal end the crystalline lattices are fragmented into "rootlets" that are firmly embedded in the matrix of the basal lamina (Fig 5F)

The Basal and Reticular Laminae

The basal lamina is a thin layer of fairly dense matrix about 120-140 nm in width, whose components are made of closely-packed fine filaments enmeshed within a gel-like ground substance (Fig 1B; 5F). The filaments are so fine and tightly packed together that they are hardly resolved individually even at high magnification. The basal lamina is tightly adhered to the tegument's basal plasma membrane, and its matrix continues to fill the spaces between the basal membrane infoldings (Fig 1B). Small plaques of hemidesmosomes distributed at irregular intervals help to couple the tegument's basal membrane and the lamina together. On its internal surface the basal lamina binds with the reticular lamina (Fig 1B, C; 5F) whose major components are uniform fibers similar in character to the reticular fibers present in the basement membranes of higher vertebrates.

Tegumental Cells

Tegumental cells lie in rows underneath the longitudinal muscle layer, and send their processes containing bundles of microtubules outwards between the muscle cells to joint up with the tegument (Fig 1C). Each cell has a large round vesicular nucleus containing a thin strip of heterochromatin along the inner surface of the nuclear envelope, and a few small blocks scattered within the interior of the nucleus, while most of the remaining chromatin appears as euchromatin. The nucleus also has a very prominent nucleolus (Fig 6A). The cytoplasm contains numerous mitochondria, rough endoplasmic reticulum, free polysomes and few areas of Golgi complexes (Fig 6A-C). Both G_1 and G_2 tegumental granules could be observe within a single cell (Fig 6B, C); however, G_2 are the more numerous and they are closely aggregated near the Golgi complexes (Fig 6A). In addition to these

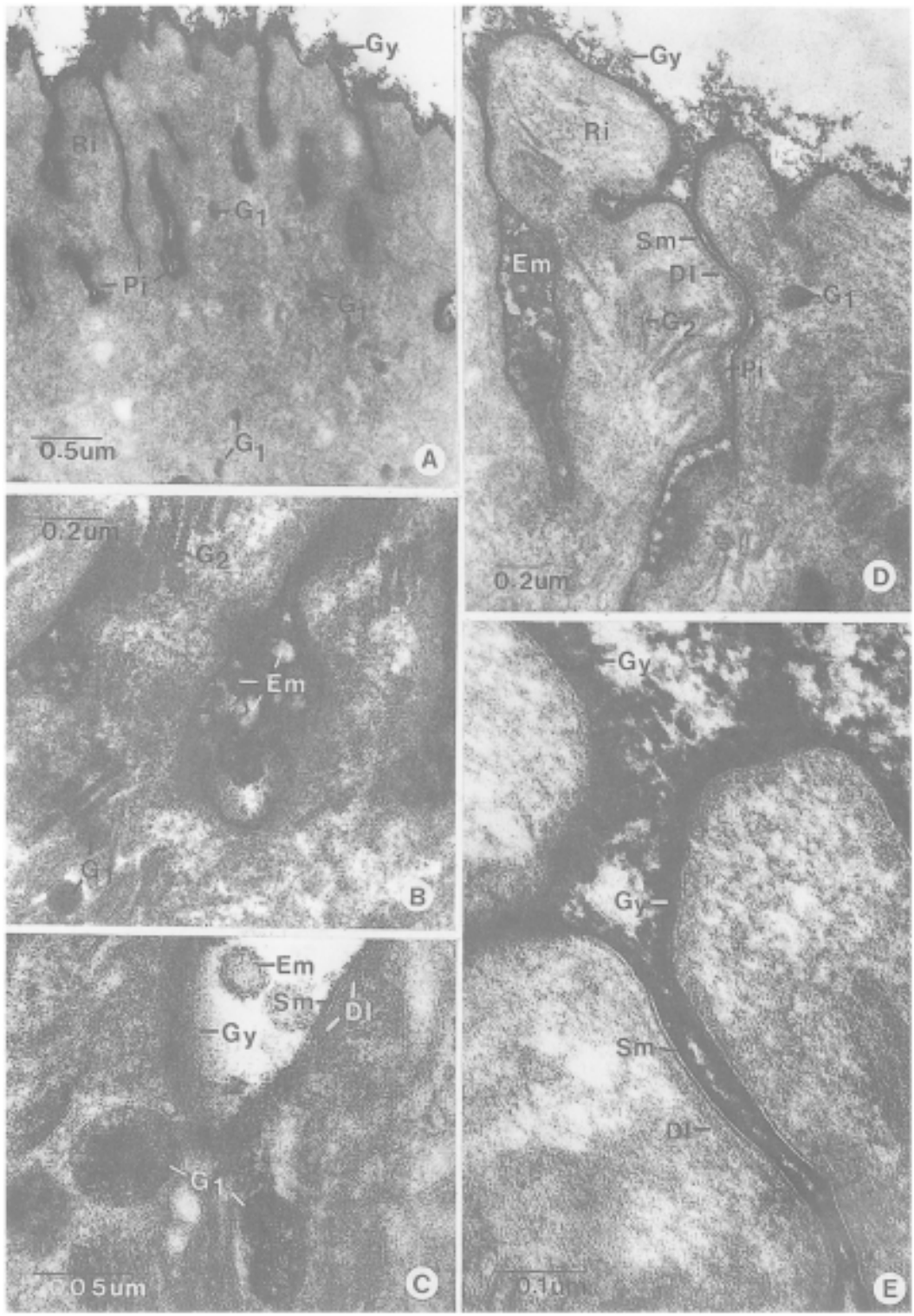

Fig 4. A, B, C) Micrographs of ruthenium red-fixed sections, uncounterstained (A) and counterstained with lead citrate and uranyl acetate (B, C), illustrating intense staining of ruthenium red on the glycocalyx (Gy) lining the pits (Pi) and the ridges (Ri), and G_1 granules closed to the surface. B, C indicate the exocytosis and release of matrix (Em) of G_1 granules into the pit.
D, E) The counterstained ruthenium-fixed sections illustrating thick (100-300nm) non uniform glycocalyx on the surface of a ridge, and exocytosed matrix of G_1 in the pit (Em). The surface membrane (Sm) and underlining dense lamina (Dl) are clearly seen.

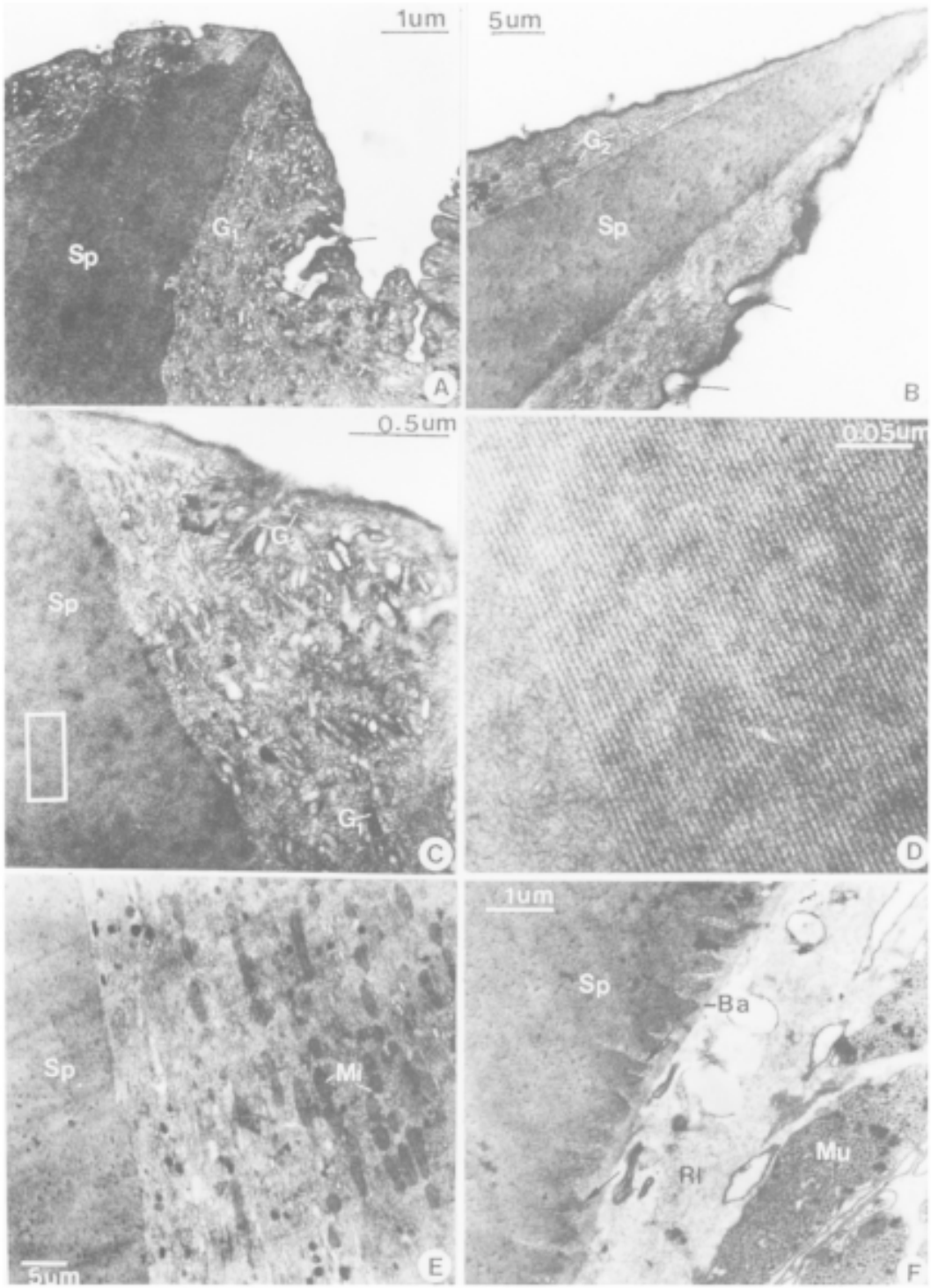

Fig 5. A, B) Micrographs of the spines (Sp) whose tips are covered by the surface membrane and whose edges by tegument cytoplasm that are invaginated (arrow).
C, D, E) The middle of the spines showing crystalline structure with lattice space about 4nm apart (boxed area in C, D). The side of the spine is covered by dense cytoplasm containing numerous mitochondria (Mi).
F) The base of a spine (Sp) which is fragmented into rootlets (arrow) that are embedded in matrix of the basal lamina (Ba).

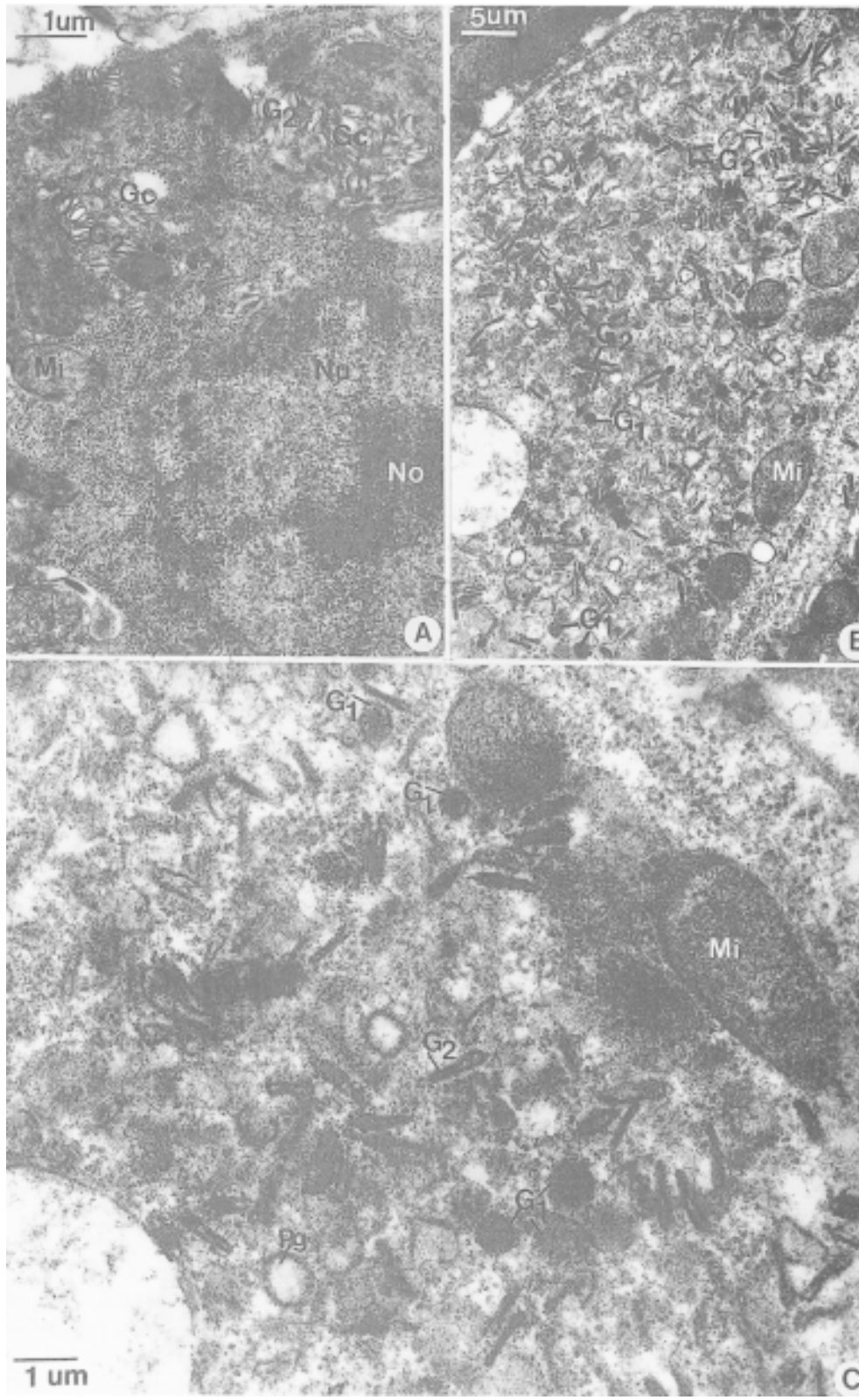

Fig 6. A, B, C) Micrographs of tegumental cells showing the nucleus (Nu) containing mostly euchromatin and prominent nucleolus (No). The Golgi complex areas (Gc) contain high concentration of G_2 granules. In the cytoplasm (in B, C) both G_1 and G_2 granules are present within the same cell, with G_2 predominate in number.

tegumental granules, there are spherical-shaped granules whose content are very lightly stained, and some may actually appear empty (Fig 6B, C). These granules could be the precursor of G_1 whose matrix has not yet been highly concentrated.

Discussion

Ultrastructure of the Tegument

Trematode parasites that live in the mammalian hosts' circulation or biliary system need to absorb nutrient molecules from either the blood or bile, as demonstrated in schistosomes' tegument which can absorb substantial amount of small nutrient molecules such as glucose, fructose and amino acids.[18,19] Simultaneously, the tegument of these parasites can also protect them from hosts' immune attacks by evolving evasion mechanisms that include the rapid turn over of the surface membrane to prevent the attachment of immune effector cells, and by immune mimickry or disguise through the adsorption of hosts' antigens onto the parasites' surfaces. In addition, the fluid environment in the host body, especially the bile, is vastly different in terms of ionic composition from that of the parasites' bodies. Hence another important role of the tegument is the regulation of ions and fluid balance, which will keep the homeostatic equilibrium within the parasites' bodies. Adult *F. gigantica* tegument, as reported in the present study, exhibits all the ultra-structural features to subserve these critical functions.

The First Layer

Based on their ultrastructural features the tegument of adult *F. gigantica* could be divided into four layers of specialized functions. The first and outermost region of the tegument contains highly folded parts, namely, ridges and pits. The membrane covering these structures is trilaminate and coated with a substantial layer of glycocalyx, a typical feature found in all bile and lumen-dwelling trematodes, including, *F. hepatica*[20-22] *Clonorchis sinensis*[23] and *Oposthorchis viverini*.[24] The vast amount of membrane covering the ridges and pits helps to increase the surface area for absorption and exchanging of materials. Glycocalyx may provide the first line of defense because of its substantial thickness and insolubility. In conventional TEM, glycocalyx appears only as a thin layer (about 10-15 nm in thickness) which may be due to the failure of the fixative to preserve the glycocalyx in its entirety. By contrast, when the parasites were simultaneously treated with ruthenium red which acts as both stain and fixative[15-17] the glycocalyx appears as thick as 100-300 nm, and consists of two definite layers, ie, the thin inner homogeneous layer apposed to the surface membrane and the thick outer fibrillar layer. The latter may be more labile and could be preserved only by extra treatment with ruthenium red. Glycocalyx may be quite resistant to the emulsifying action of the bile and, therefore, able to confer a certain degree of protection to *F. gigantica*. High affinity to ruthenium red is indicative that glycocalyx of adult *F. gigantica* is highly negatively charged. The anions present may be contributed mainly by sialic acids, as it has been shown that ruthenium red can bind strongly to this sugar.[17] The presence of abundant negative charges on the surface could be another factor that helps to defend the parasites against hosts' immune attacks by repelling the attachment of the hosts' immune effector cells, which also bear high electronegativity on their own surfaces.[11,25] Moreover, the presence of a large quantity of large negatively-charged molecules, like glycoproteins, may be instrumental in retaining and concentrating small molecules including sugars and amino acids and various ions. Thus, the glycocalyx may be viewed as a hydrated shell around the parasites' bodies that helps to protect as well as concentrate nutrient molecules, in order to make them readily available for the absorption by the tegument.

Glycocalyx is probably derived from G_1 granules present in abundance within the second layer of the tegument. Ruthenium red stain shows similar binding to the glycocalyx as well as the matrix of G_1 granules lying close to the surface. These granules invariably join up with the surface membrane at the bottom of the pits, and exocytose their matrix into the lumen of pits. This material could be later incorporated into the surface membrane, thus forming part of the new glycocalyx. In *F. hepatica*, similar granules which were termed T_0 by Hanna,[26] were also thought to be contributing to the formation of glycocalyx in metacercaria and juvenile parasites, particularly during their invasive migration through the host's tissues. Once *F. hepatica* juveniles reach their final destination and take up permanent residence in bile ducts of the liver T_0 granules were replaced by a similar set of adult-type granules (T_1). In comparison to T_0, the number of T_1 in adult tegument is drastically decreased, while another set of so call T_2 granules became the majority of tegumental granules in adult. Each T_2 granule is surrounded by a trilaminate membrane and associated thin layer of glycocalyx that express adult-type antigens on the surface.[26-28] These adult antigens are much less immunogenic than juvenile

antigens.[26,28] In contrast to *F. hepatica*, we have shown that in the tegument of adult *F. gigantica* G_1 granules, which are probably equivalent to T_1 granules, are still quite numerous; and the ruthenium red staining implicates that the secretion of these granules into the pits may form the major part of glycocalyx.

The surface membrane of an adult *F. gigantica* is trilaminate and supported internally by a thin lamina of dense cytoplasm, while the cytoplasm underneath the lamina is composed of tightly packed microtrabeculae and bundles of larger fibrils. Within this interstice there are numerous G_2 granules that may be equivalent to T_2 granules of *F. hepatica* tegument. In fortuitous sections several G_2 granules were seen joined up with the surface membrane, and evert their cisternal side outwards, thus turning into the exterior surface of the tegument's surface membrane, which may later become coated with the glycocalyx material released from G_1 granules. The matrix inside the cisternae of G_2 granules could become part of the thin homogenous layer of glycocalyx that may be coupled with the more fibrillar components derived from the secretion of G_1 granules. On the cytoplasmic side, which is now turned into the cytoplasmic face of the surface membrane, the dense cytoplasm covering the membrane of the G_2 granules could coalesce and form the dense lamina underlining the surface membrane. The surface membrane and associated glycocalyx of the adult *F. gigantica* tegument probably maintain a high turnover rate, since there are always a vast amount of G_2 granules accumulated in the first and the second layers of the tegument, as well as a still relatively large number of G_1 granules in the second layer. Such rapid turnover and renewal of the surface membrane could be a part of the mechanism for self defense against the detergenic action of bile and the host's immune attacks, as it has been shown that bile also contains copious amount of antibodies, especially IgA.[29]

The Second Layer

The most prominent characteristic of this layer is the presence of a very high concentration of G_1 and G_2 granules, particularly G_2 granules. Therefore, this layer could be viewed as the storage area for the new membrane (G_2) and glycocalyx (G_1) material. Another prominent feature of this layer is the presence of a large number of lysosomal granules, which implies that there may be absorption of large molecules via the endocytotic pathway within the tegument. In addition to nutritive substances, some of these large molecules could be antibodies forming immune complexes with the surface antigens. Once attached to the surface membrane these large molecules or immune complexes could be internalized and broken down by the fusion with lysosomes, which could be another part of parasites' defense mechanism against the hosts' immune attacks.

The Third Layer

This is the widest layer of the tegument. Its cytoskeleton is less tightly packed, and it has the highest concentration of mitochondria which are evenly distributed throughout the layer. G_1 and G_2 granules are present but appear to be much fewer than in the second layer. This layer may, therefore, be involved mainly in supplying energy to other layers. The concentration of mitochondria and their positioning within the tegument of this parasite are quite different from that of schistosomes and a human liver fluke, *O. viverrini*. In schistosomes most of mitochondria are concentrated in the basal layer,[30] while in *O. viverrini* they are localized close to the surface plasma membrane.[24] The distribution of energy for various metabolic processes in the tegument of these two species could be carried out by these eccentrically-located mitochondria because of the relative thinness of their tegument. In contrast, *F. gigantica* has a much thicker tegument because of the worms' very large size. The increased energy requirement dictates that the parasites' tegument must possess a very large number of mitochondria that are positioned strategically in the middle zone, so that energy could be sufficiently distributed to other layers.

The Fourth Layer

This layer exhibits the most unique features in having highly convoluted infoldings of the basal plasma membrane, with closely-associated mitochondria. These features resemble the basal cytoplasm of ion-transport epithelium in mammals, such as, the kidney proximal and distal tubules. Based on these similarities the fourth layer could be involved in the transport of ions which will help to maintain the ionic equilibrium within the parasites' bodies with regards to the bile.

Tegumental Cells

In *F. hepatica*, Hanna[26] and Burden et al[13] reported that there is one type of cell which synthesize T_0 granules in metacercaria and in the very early juvenile stage, while the parasites are migrating through the hosts' abdominal cavities. T_0 cells transform into T_1 cells which produce granules with similar feature but with greater density when the

parasites reach the liver. Both T_0 and T_1 granules were thought to give rise to the juveniles', and later adults' glycocalyx, that enables the parasites to evade the killing by hosts' immune responses. When parasites become established in the bile duct at about 12 weeks there is another type of tegumental cells which produce T_2 granules, which are the main contributor to the formation of the adult parasites' surface membrane and glycocalyx. These T_2 cells supersede T_1 cells, and as a result the number of T_1 granules in the tegument of the adult parasites is drastically decreased. In *F. gigantica* the number of G_1 granules in the adult tegument is still relatively high in comparison with the T_1 granules of *F. hepatica*. In contrast to the two types of tegument cells reported in the adult *F. hepatica* we observed only one type of cells in adult *F. gigantica*. These cells produce both G_1 and G_2 granules, even though the productions of G_2 predominate in the adult stage. Both types of granules are found in close association with microtubules within the processes of the cells that extend outwards to join up with the tegument. Hence, we believe that these granules are translocated form cell soma to the tegument by the sliding action of microtubules.

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