OPISTHORCHIS VIVERRINI: THE EFFECTS OF COLCHICINE AND CYTOCHALASIN B ON THE ADULT TEGUMENT

Prasert Sobhon[1] and Wandee Apinhasmit[2]

[1]Department of Anatomy, Faculty of Science, Mahidol University, Bangkok 10400;
[2]Department of Anatomy, Faculty of Dentistry, Chulalongkorn University, Bangkok 10330, Thailand

Abstract. The roles of the tegumental cytoskeleton were tested by treating adult flukes with colchicine and cytochalasin B. Following a short incubation period (10-20 minutes), colchicine disrupted microtubules in the tegumental cells' processes which, in turn, affected the transport of dense granules from the cells' soma to the tegument; as a result some of these granules were fused together to form membrane-bound vacuoles. In addition, at many spots microtrabeculae were also depolymerized, which resulted in the formation of non-membrane-bound vacuoles and the distension of microvilli to form blebs, some of which were disrupted. After prolonged incubation (120 minutes), general breakdown of the tegumental cytoskeleton occurred, and parts of it were sloughed off. In cytochalasin B treatment, the responses were similar to those of colchicine but with less severity. After a short incubation period (10-20 minutes), the microtrabeculae were depolymerized which led to the formation of non-membrane-bound vacuoles in the apical and middle zones of the tegument. Later, the tegumental microvilli were distended to form blebs but no evidence of tegumental sloughing occurred even in prolonged incubation. From these obsevations, it was concluded that microtubules played a role in the translocation of granules from the tegumental cells to the tegument which modulated the synthesis of membrane and glycocalyx, while microtrabeculae were involved in the maintenance of the structure and integrity of the tegument.

INTRODUCTION

The organization of tegumental cytoskeleton in the adult *Opisthorchis viverrini* has been studied by using conventional transmission electron microscopy (TEM) and Triton X-100 extraction (Sobhon and Apinhasmit, 1995). It was demonstrated that the parasite's tegument was composed of microtrabeculae and microtubules. The former formed the scaffold of the cytoplasm by appearing as a highly cross-linked network of knobbed fibers, and the latter formed bundles in the processes of tegumental cells and splayed out into the basal and middle zones of the tegument. The roles and relation of the tegumental cytoskeleton in trematodes and cestodes have been studied by using a variety of compounds known for their capacity to depolymerize various elements of cytoskeleton (Wilson and Barnes, 1974; Borgers and Verheyen, 1976; Bogitsh and Carter, 1980; Etges and Bogitsh, 1985; Zhou and Podesta, 1989; Sobhon and Upatham, 1990). Generally, the cytoskeleton is thought to play important roles in maintaining the tegumental architecture, transportation of tegumental granules from tegumental cells to the tegument, and thus is directly involved with the synthesis and turnover of the outer plasma membrane. In addition, it may exercise some control over the fluidity of the membrane, and hence the position and distribution of membrane components, such as surface antigens. In the present study, the role of the tegumental cytoskeleton of adult *O. viverrini* was investigated by treating adult parasites with colchicine and cytochalasin B, drugs known to depolymerize microtubules (Wilson, 1975) and microfilaments (Lin *et al*, 1980), respectively.

Correspondence: Dr Prasert Sobhon, Department of Anatomy, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand.
Fax: 662-2479880; E-mail:fdenwap@chulkn.chula. ac.th

MATERIALS AND METHODS

Encysted metacercariae of *O. viverini* were obtained from naturally infected cyprinoid fishes collected from Khon Kaen Province in the Northeastern part of Thailand. They were seperated from minced and partially trypsinized fish muscle as described previously (Apinhasmit *et al*, 1993). Adult flukes were collected from adult golden Syrian hamsters, which were infected with 50 metacer-

cariae per animal by gastric intubation twelve weeks previously (Tuti *et al*, 1982).

Freshly collected parasites were divided into two groups; one group was incubated in Minimal Essential Medium (MEM) containing 10 μg/ml of colchicine (CL) (Fisher Co, USA) and the other containing 10 μg/ml cytochalasin B (CB) (Sigma, USA), for the periods of 10, 20, 30, 60, and 120 minutes at 37°C. In control group, flukes were incubated in a similar culture medium devoid of the drugs. Representative samples were taken at the specified periods, and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2, overnight, at 4°C, after which they were processed for scanning (SEM) and TEM electron microscopy using conventional methods.

RESULTS

The surface appearance and ultrastructure of the tegument in control groups

In control groups, in which the drugs were omitted from incubating MEM solution, the surface of parasites exhibited normal topography as described previously (Apinhasmit *et al*, 1993) at all incubation periods (Fig 1A, B). Under TEM observations, the tegument in all control groups exhibited close to normal ultrastructural features as described by our group (Apinhasmit *et al*, 1994) (Fig 1C, D). There were numerous light granules, dense granules, and mitochondria scattering among the network of microtrabeculae in the syncytial tegument (Fig 1C, D). However, after prolonged incubation periods some dense granules began to lose their content (Fig 1D).

SEM observations of changes in the surface in response to colchicine

The changes induced by CL were observed over all the surface. After 10 to 20 minutes incubations, the changes consisted of swelling of microvilli, some of which ballooned out to form blebs, and some were burst to form debris on the surface (Fig 2A). However, a majority of normal-looking microvilli still remained among these blebs (Fig 2A). At 30 minutes incubation, the swelling of microvilli and blebbing became more pronounced

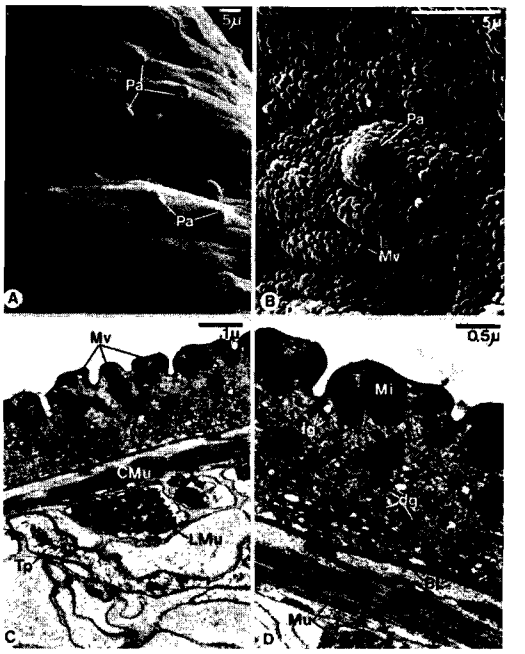

Fig 1–SEM and TEM micrographs of the tegument of adult *O. viverrini* in the control groups, incubated in MEM for 120 minutes.

A) Low magnification of a SEM micrograph of the lateral tegument of the parasite's body exhibiting corrugated surface and numerous sensory papillae (Pa).

B) An enlargement of the area in A, illustrating the numerous normal-looking stubby microvilli (Mv) and sensory papillae (Pa).

C) Low magnification of a TEM micrograph exhibiting the tegument which is formed by fusion of the processes (Tp) of tegumental cells located underneath two layers of muscle (CMu and LMu).

D) An enlargement of the tegument in C, exhibiting its components including, light granules (lg), dense granules (dg), mitochondria (Mi) and a network of microtrabeculae among organelles. The tegument rests on the basal lamina (BL) and two layers of muscles (Mu).

and increasing in number (Fig 2B), and in some areas adjacent blebs were so disrupted that patches of lesion appeared on the tegument (Fig 2C, D). After 60 minutes incubation, the damages were more pronounced; the blebbing and shedding of the tegument became more extensive, so that in some areas the underlying basal lamina were exposed (Fig 2E). After incubation for 120 minutes the

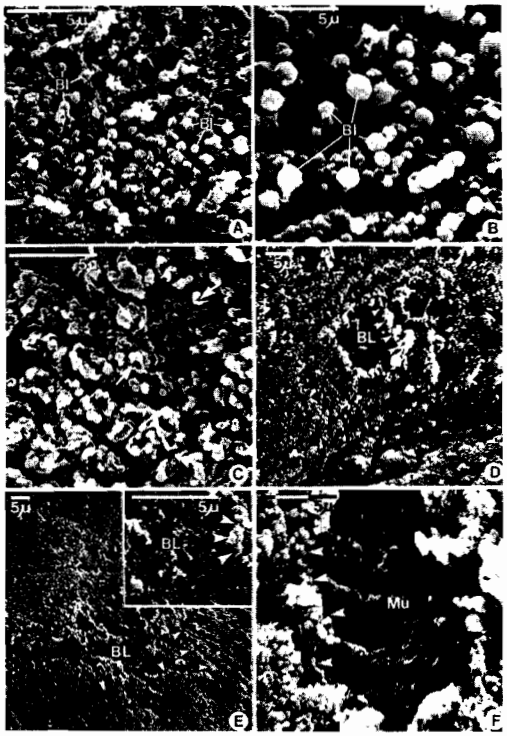

Fig 2–SEM micrographs of the tegument of adult flukes incubated in MEM containing 10 μg/ml of CL.

A) After 10 minutes incubation, microvilli are swollen to form small-sized blebs (B1), and the surface shedding is evident.

B-D) After 30 minutes incubation, the tegument exhibits various-sized spherical blebs (B1), disrupted blebs (arrows), and sloughing (arrow heads) down to the level of the basal lamina (BL).

E) After 60 minutes incubation, large patches of the tegument are sloughed off (arrow heads) exposing the underlying basal amina (BL).

F) After 120 minutes incubation, some areas of the tegument show sloughing of the tegument (arrow heads) down to the level of the underlying musculature (Mu).

damages became even more extensive and large pieces of the tegument were torn off, exposing the basal lamina and the muscle layer underneath (Fig 2F).

TEM observations of the tegument after treatment with CL

After 10 minutes incubation, the tips of some microvilli were distended into blebs (Fig 3A), and small membrane-bound vacuoles were observed in the tegument (Fig 3A). Most dense granules lost their content and became clear vesicles, whereas light granules were swollen but still retained their filamentous content (Fig 3A). After 20 minutes incubation, the surface blebbing and vacuolization appeared throughout all zones of the tegument (Fig 3B). In some areas depolymerization of microtrabeculae occurred and non-membrane-bound vacuoles were formed; and swelling of basal infoldings also began (Fig 3B). After 30 to 60 minutes incubations, the membrane- and non-membrane-bound vacuoles increased in number and size, and the microtrabecular network between them appeared looser (Fig 3C, D). Blebs occurred all over the surface, and some were disrupted to form lesion spots (Fig 3D). After 120 minutes incubation, most of the tegument contained vacuoles, and some parts were totally degenerated and sloughed off, exposing the underlying basal lamina and muscle (Fig 3E, F). Microtubules in the processes of tegumental cells were completely depolymerized and the cells' processes appeared empty (Fig 3F).

Fig 3–TEM micrographs of the tegument of adult flukes incubated in MEM containing 10 μg/ml of CL.

A) After 10 minutes, the tegument microvilli are swollen to form blebs (Bl). A few membrane-bound vacuoles (stars) are formed, with those in or under the blebs appearing larger than others. Most dense granules lose their content and become clear vesicles, whereas light granules are swollen but retain their filamentous content. Inset shows enlargement of membrane-bound vacuoles (stars) and light granules (lg).

B) After 20 minutes, there are increasing vacuolization (stars) in all zones of the tegument, and blebbing (Bl) over most areas of the surface.

C-D) After 30 minutes, numerous large vacuoles (stars) are formed throughout the thickness of the tegument. Some areas exhibit foci of depolymerization of microtrabeculae to form non-membrane-bound vacuoles (Va). The microtrabeculae between vacuoles appear looser, and the cytoplasm is lighter than normal. Surface blebbing (Bl) is present over all the surface and some are disrupted (arrows).

E) After 120 minutes, the tegument is severely damaged. Some regions show detachment of the apical zone of the tegument (double arrow heads), whereas the others exhibit sloughing of the whole tegument (arrow heads) down to the level of the basal lamina (BL).

F) The microtubules in a process of a tegumental cell (Tp) are completely depolymerized and disappear after 120 minutes incubation.

SEM observations of changes in the surface of adult tegument after treatment with CB

After 10 minutes most of the surface appeared unaffected, while only limited regions exhibited the swelling of microvilli, the formation of blebs and shedding (Fig 4A, B). After 20 to 30 minutes, the changes were similar but occurred over more extensive area than at 10 minutes (Fig 4C, D). After 60 to 120 minutes, blebs and surface shedding occurred over the entire surface of the parasites (Fig 3E, F). However, on most parts of the surface there were only few disrupted blebs, and the tegument was not sloughed off (Fig 4F). Generally, the pattern of damages induced by CB was similar to that occurred following the CL treatment, but the degree was less severe.

TEM observations of the tegument after the treatment with CB

The earliest change that could be observed after 10 minutes incubation with CB was the depolymerization of microtrabeculae in the apical and middle zones of the tegument, which resulted in the formation of non-membrane-bound vacuoles (Fig 5A). The surface showed some breakdown and shedding, while most light and dense granules, and microtubules still exhibited normal appearance (Fig 5A). After 30 to 60 minutes incubation, large vacuoles occurred throughout the tegument (Figs 4B-D). Most microvilli were distended, and some

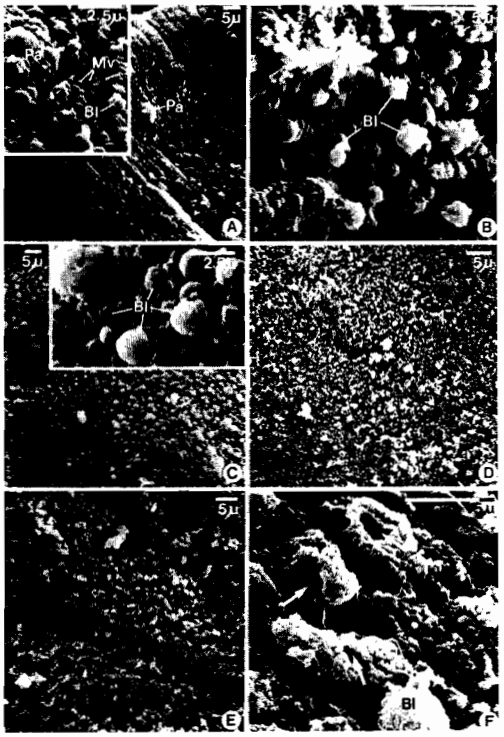

Fig 4–SEM micrographs of the tegument of adult flukes incubated in MEM containing 10 μg/ml of CB.

A-B) After 10 minutes, most areas of the tegument still exhibits normal feature with short stubby microvilli (Mv) and sensory papillae (Pa), but in some limited areas, the tegument shows surface blebbing (Bl) and shedding. Instet shows enlargement of short stubby microvilli (Mv), sensory papillae (Pa) and small-sized blebs (Bl).

C-E) After 20 minutes (C), 30 minutes (D) and 60 minutes (E), the surface changes consist of blebbing (Bl) and shedding, which are similar to that of 10 minutes but cover more extensive area.

F) After 120 minutes, the tegument changes are composed of blebbing (Bl) over most of the surface, and a few blebs are disrupted (arrows).

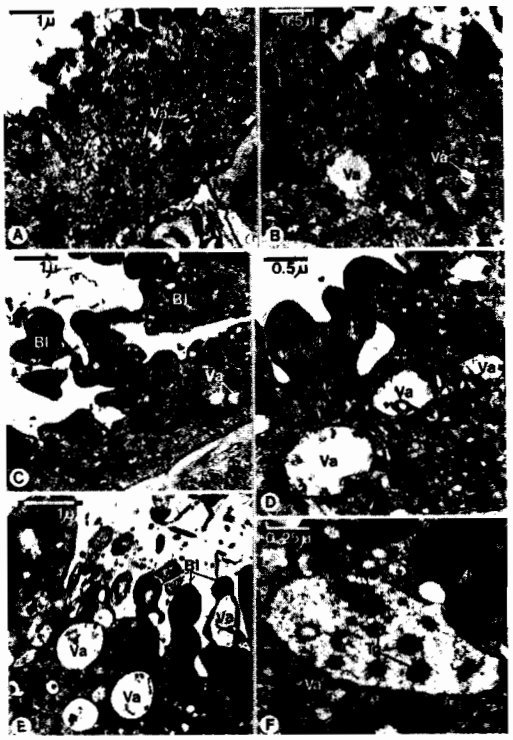

Fig 5–TEM micrographs of the tegument of adult flukes incubated in MEM containing 10 μg/ml of CB.

A) After10 minutes, microtrabeculae in the apical and middle zones of the tegument are depolymerized to form small sized non-membrane-bound vacuoles (Va). The tegumental cytoplasm becomes lighter but most dense and light granules, and microtubules (Mt) still appear intact. The tegument also exhibits some degree of surface shedding.

B-D) After 30 minutes, large vacuoles (Va) are scattered throughout the tegument and the surface shedding is more extensive. Microvilli are distended, with some expanded to form various-sized blebs (Bl).

E) After 120 minutes, the tegumental changes are similar to those of 30 and 60 minutes but cover more extensive area.

F) The higher magnification shows depolymerization of microtrabeculae to form non-membrane-bound vacuoles (Va) after 120 minutes incubation. The vacuole contains a very loose network of microtrabeculae and tegumental granules (Tg) with still denser area of microtrabeculae surrounding the vacuole.

were enlarged to form blebs (Fig 4C). The surface shedding became more extensive (Fig 4B, C). At 120 minutes most vacuoles were of large size and scatterred throughout all zones of the tegument; however, microtrabeculae and tegumental granules in areas between the vacuoles appeared normal (Fig 4E, F). While the blebbing and shedding of the surface were visible, the tegument still maintained its general structure (Fig 4E, F).

DISCUSSION

The roles of the cytoskeleton in the eukaryotic cells have been deduced from the studies using a variety of compounds to depolymerize elements of cytoskeleton (Wilson, 1975; Goldman, 1976; Lin *et al*, 1980; Ken and Wolf, 1988). Based on the same rationale, these drugs have been used to study the teguments of trematode and cestode parasites. It has been reported that the drugs which can depolymerize microtubules also prevent the translocation of secretory granules from tegumental cells to the tegument in several parasitic flatworms (Borgers and Verheyen, 1976; Bogitsh and Carter, 1980; Etges and Bogitsh, 1985; Zhou and Podesta, 1989; Sobhon and Upatham, 1990). Borgers and Verheyen (1976) demonstrated tegumental degeneration in *Hymenolepsis nana* and *Taenia taeniaeformis* when the worms were treated with mebendazole which was believed to have an inhibitory effect on microtubules located in the subtegumental cells and their cytoplasmic processes. It was hypothesized that the inhibition of the microtubule formation prevented the transport of secretory granules from the subtegumental areas to the tegument, where they contributed their content to the repair and maintenance of the tegument. Autoradiographic studies displayed a significant decrease in the amount of [^{3}H]-proline incorporation into the tegument of *S. mansoni* (Bogitsh and Carter, 1980) and of *H. diminuta* (Etges and Bogitsh, 1985) following the treatment with CL. Morphological changes induced by CL were also exhibited in *S. mansoni*, such as, the disappearance of microtubules from the cell processes,the accumulation of discoid bodies and membranous bodies in the tegumental cells (Bogitsh and Carter, 1980). In this species, it was also hypothesized that microtubules in the cell processes facilitated the movement of granules from tegumental cells to the tegument. Further support for this notion was demonstrated by Zhou and Podesta (1989) who treated adult *S. mansoni* with serotonin or complement C_3 following the pre-

incubation with 0.5 mM CL. The microtubules within the cell processes were disrupted, and the increased number of discoid and membranous bodies synthesized in response to serotonin or complement C_3 were accumulated in the cell bodies, instead of being translocated to the tegument as normal. Thus, the authors suggested that the transport of these granules to the tegument was dependent upon the action of microtubules. Recently, Sobhon and Upatham (1990) also demonstrated the effect of CL in schistosomula and adults of *S. japonicum* and *S. mekongi*. The responses to the drug expressed by both species were similar, in that both microtubules and microtrabeculae were depolymerized which resulted in the vacuolization and the general breakdown of the tegument and finally the sloughing of some parts of the tegument. Hence, microtubules might be instrumental in the transport of granules that were involved in the synthesis of the surface membrane and the maintenance of the tegumental structural integrity, from the tegumental cells to the tegument.

In the present study, the responses to CL by the adult *O. viverrini* tegument were studied. Changes were firstly observed at 10 minutes incubation, when some microtubules particularly those in the tegumental cells' processes were depolymerized, and most dense geanules lost their content and turned into clear vesicles, whereas light granules were swollen but still retained their filamentous matrix. It was suggestive that the disruption of microtubules by CL affected the transport of the dense granules from the tegumental cells to the tegument where they usually contribute their content to form the new surface membrane (Apinhasmit *et al*, 1994). Most of the unutilized dense granules might lose their content, and some fused together to form larger membrane-bound vacuoles. After incubation in the drug for 20 minutes, microtrabeculae at many spots were also depolymerized which resulted in non-membrane-bound vacuoles in their places. After 120 minutes incubation, a generalized breakdown of the tegument occurred and some parts were sloughed off. Therefore, the drug also affected the integrity of the tegument in addition to the transport of its granules.

CB has been shown to cause depolymerization of microfilament system within eukaryotic cells (Lin *et al*, 1980). In parasites, such as adult *S. mansoni*, the incubation for 5 hours in a medium containing CB induced the collapse of its tegument (Wilson and Barnes, 1974). Furthermore, when this parasite was exposed to CB followed by serotonin or complement C_{3b}, the discoid and membranous bodies accumulated in the cell bodies similar to the CL treatment, and the tegumental layer was almost completely devoid of these bodies (Zhou and Podesta, 1989). Studies in schistosomula and adults of *S. mansoni* and *S. mekongi* also indicated that CB caused the depolymerization of microtrabeculae which, in turn, resulted in the vacuolization, and eventually the breakdown and detachment of the tegument from the parasites' bodies (Sobhon and Upatham, 1990).

The ultrastructural changes in adult *O. viverrini* tegument after the treatment with CB were similar to that of CL but with less severity. The depolymerization of microtrabeculae occurred first in the apical and middle zones of the tegument after 10 minutes incubation, and this later led to the formation of non-membrane-bound vacuoles. However, in contrast to CL there was no apparent effect on the structure of both types of tegumental granules and microtubules. After 30 minutes incubation large vacuoles occurred throughout the tegument, and most microvilli were distended to form blebs. These changes might be resulted from the weakening of the microtrabecular network and the influx of fluid into the tegument. However, in contrast to CL treatment no tegumental sloughing occurred even in the longer period of incubation.

SEM observations confirmed corresponding changes on the surface of adult *O. viverrini* in response to both CL and CB. These changes consisted of the swelling of microvilli, blebbing, lesion spots which might be formed by the disruption of blebs and eventually the surface shedding. Like in TEM observations the general breakdown of the tegument occurred only in CL treatment, Blebbing might be due to the dilatation of the surface microvilli as the result of the vacuoles formed by the depolymerization of microtrabeculae as demonstrated by TEM. This pattern of changes has also been observed following the treatment of adult *O. viverrini* with anthelminthic drugs, such as praziquantel (Mehlhorn *et al*, 1983; Sirisinha *et al*, 1984) and amoscanate (Sobhon *et al*, 1986), which suggested that one primary mechanism of actions of these drugs might be the destruction of the integrity of the tegument cytoskeletal elements.

ACKNOWLEDGEMENTS

This project was supported partially by grants from Mahidol University and the National Research Council, Ministry of Science, Technology and Environment, Bangkok, Thailand. Appreciation is expressed to Mrs. Wilaiporn Thongmorn for typing the manuscript. We deeply appreciate Assistant Professor Porncharn Saitongdee for her technical assistance and photographic work.

REFERENCES

Apinhasmit W, Sobhon P, Saitongdee P, Upatham ES. *Opisthorchis viverrini*: Changes of the tegumental surface in the newly excysted juvenile, first-week and adult flukes. *Int J Parasitol* 1993; 23 : 829-39.

Apinhasmit W, Sobhon P, Saitongdee P, Menayotin S, Upatham ES. *Opisthorchis viverrini*: Ultrastructure of the tegument of the first-week juveniles and adult flukes. *Int J Parasitol* 1994; 24 : 613-21.

Bogitsh BJ, Carter OS. *Schistosoma mansoni*: Radioautography of colchicine's effect on [^{3}H] proline incorporation into adults *in vitro*. *Exp Parasitol* 1980; 49 : 319-27.

Borgers M, Verheyen A. The role of microtubules in the tegument of cestodes. *J Cell Biol* 1976; 70 : 90a.

Etges DJ, Bogitsh BJ. The effect of colchicine on translocation of incorporated [^{3}H] proline in *Hymenolepsis diminuta*. *J Parasitol* 1985; 71 : 290-6.

Goldman R. The effect of cytochalasin B on concanavalin A induced vacuolization in mouse peritoneal macrophages. *Exp Cell Res* 1976; 99 : 385-94.

Ken J, Wolf P. Presence and distribution of vimentin in cynomolgus monkey trabecular cells. *Anat Rec* 1988; 222 : 309-16.

Lin DC, Tobin K, Grumet M, Lin S. Cytochalasins inhibit nuclei-induced actin polymerization by blocking filament elongation. *J Cell Biol* 1980; 84 : 455-60.

Mehlhorn H, Kojima S, Rim HJ, *et al*. Ultrastructural investigations on the effects of praziquantel on human trematodes from Asia: *Clonorchis sinensis, Metagonimus yokogawai, Opisthorchis viverrini, Paragonimus westermani* and *Schistosoma japonicum*. *Arzneimittelforschung* 1983; 33 : 91-8.

Sirisinha S, Puengtomwatanakul, Sobhon P, *et al*. Alterations of the surface tegument of *Opisthorchis viverrini* exposed to praziquantel *in vitro* and *in vivo*. *Southeast Asian J Trop Med Public Health* 1984; 15 : 95-103.

Sobhon P, Apinhasmit W. *Opisthorchis viverrini*: The tegumental cytoskeleton. *Int J Parasitol* 1995; 25 : 787-96.

Sobhon P, Upatham ES. The tegument cytoskeleton. In: snail hosts, life-cycle, and tegumental structure of oriental schistosomes. UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, Geneva, Switzerland, 1990; 204-73.

Sobhon P, Wanichanond C, Saitongdee P, *et al*. Scanning electron microscopic study of *Opisthorchis viverrini* tegument and its alterations induced by amoscanate. *Int J Parasitol* 1986; 16 : 19-26.

Tuti S, Vichasri S, Sirisinha S. Effect of culture media on production of excretory-secretory products and egg output of *Opisthorchis viverrini in vitro*. *J Parasitol* 1982; 68 : 892-7.

Wilson L. Action of drugs on microtubules. *Life Sci* 1975; 17 : 303-10.

Wilson RA, Barnes PE. An *in vitro* investigation of dynamic processes occurring in the schistosome tegument, using compounds known to disrupt secretory processes. *Parasitology* 1974; 68 : 259-70.

Zhou Y, Podesta RB. Effect on serotonin (5HT) and complement C_3 on the synthesis of the surface membrane precursors of adult *Schistosoma mansoni*. *J Parasitol* 1989; 75 : 333-43.