ALTERATIONS OF THE SURFACE TEGUMENT OF *OPISTHORCHIS VIVERRINI* EXPOSED TO PRAZIQUANTEL *IN VITRO* AND *IN VIVO*

S. SIRISINHA, S. PUENGTOMWATANAKUL, P. SOBHON*, P. SAITONGDEE*, P. WONGPAYABAL*, V. MITRANONDE*, P. RADOMYOS**, D. BUNNAG** and T. HARINASUTA**

Departments of Mirobiology and *Anatomy, Faculty of Science, and **Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.

INTRODUCTION

There are numerous reports on the efficacy of praziquantel in the treatment of cestode and trematode infections. Its effectiveness in the treatment of patients with clonorchiasis and opisthorchiasis has been well documented (Bunnag and Harinasuta, 1980, 1981; Ambroise-Thomas *et al.*, 1981; Horstmann *et al.*, 1981; Loscher *et al.*, 1981; Supanvanich *et al.*, 1981; Rim *et al.*, 1981). Ultrastructural changes induced by praziquantel have been studied extensively, particularly in schistosomes (Becker *et al.*, 1980; Mehlhorn *et al.*, 1981). Tegumental alterations have also been reported for *Clonorchis sinensis* (Rim *et al.*, 1980; Kim *et al.*, 1981; Mehlhorn *et al.*, 1983). On the other hand, studies with *Opisthorchis viverrini* is limited (Mehlhorn *et al*, 1983). In most of these studies, ultrastructural changes commonly observed included the formation of tegumental bubbles (Mehlhorn *et al.*, 1983). The purpose of this study was to examine in detail by scanning elecronmicroscopy the effects of praziquantel on the tegumental surface of *O. viverrini* both under *in vitro* and *in vivo* conditions.

MATERIALS AND METHODS

Parasites and hosts: Metacercariae of *O. viverrini* used for stock infections were obtained from naturally infected cyprinoid fish collected from Khon Kaen province in the north-eastern part of Thailand. Whole fish were minced in a homogenizer and incubated at 37°C for 1 hour with continuous stirring in 1.5% pepsin solution (BDH Chemicals Ltd., Poole, England) containing 20 ml of concentrated hydrochloric acid per liter. At the end of the incubation period, the metacercariae were recovered by passing the digested material through 3 graded sieves down to the mesh size of 53 μm. The metacercariae obtained were suspended in 0.85% NaCl. Adult, golden Syrian hamsters weighing 100-120 g were infected by gastric intubation with appropriate number of metacercariae depending on experimental protocol. Under these conditions adult flukes could be recovered from the biliary system within 3-4 weeks (Tuti *et al.*, 1982). These flukes could be maintained in Earle's basal medium (BME, GIBCO, Grand Island, N.Y., U.S.A) and remained metabolically active for at least 2 weeks.

Adult flukes were also available from patients with opisthorchiasis. Normal adult flukes were recovered from canulated bile duct of patients with biliary obstruction as well as from the bile of patients who had undergone surgical operation of the gall bladder. The flukes were also obtained from feces of patients treated with praziquantel. These patients were given praziquantel tablets (40 mg/kg body weight); 45 ml of saturated magnesium sulfate and 2 glasses of warm water were given 4 hours later. Stools collected during the next 4-6 hours were pooled,

suspended in large volume of saline and allowed to sediment at room temperature. The sediment was resuspended and the process was repeated several times until the supernatant fluid was clear. The flukes were finally separated from fecal debris and only those which were grossly undamaged and still alive were taken for further study. The whole process took approximately 1½ to 2 hours. Flukes collected directly from the bile of a patient who was operated 24 hour after similar drug treatment were used in SEM and compared with those obtained from the feces.

Scanning electron microscopy (SEM): Flukes were fixed in 2.5% glutaraldehyde in 0.25M cacodylate-calcium acetate buffer pH 7.4 for at least 3-4 hours at 4°C. The fixed specimens were then washed and post-fixed in 0.1% osmium tetroxide in the same buffer, dehydrated in increasing concentration of ethanol and critical-pointed dried in Hitachi HCP-2 apparatus, using liquid CO_2 as a transitional medium. The specimens were sputtered with gold and viewed under the Hitachi S-430 scanning electron microsope operating at 20 kV.

Praziquantel: Praziquantel tablet and powder used were provided by Dr. P. Andrews (Institut fur Chemotherapie, Bayer AG, Wuppertal, Federal Republic of Germany). The powder was dissolved in small volume of ethanol and then diluted with BME to a required concentration before use in the *in vitro* study. The final concentration of ethanol never exceeded 1%, a concentration which had no detectable effect on either the activity or the ultrastructure of the flukes (unpublished observation).

RESULTS

In vitro study: When adult *O. viverrini* were exposed to various concentrations of praziquantel *in vitro*, eg., from 0.01 µg/ml to 100 µg/ml, they became paralyzed almost instantaneously. The flukes contracted almost immediately following exposure to the drug, the latent period varied with the concentrations used. However, if the flukes were kept longer in the praziquantel solution, they began to relax and finally had an elongated thread-like appearance, particularly obvious was the distance between the two suckers.

In SEM, the tegumental surface of normal adult flukes freshly obtained from experimentally infected hamsters were in general similar to those from untreated patients. The surface was covered with stubby microvilli (Plate 1). Structures with morphological appearance similar to "sensory papillae" found in other trematodes (Mehlhorn *et al.*, 1983) were found scattered between these stubby microvilli and were particularly numerous in an area surrounding the ventral sucker (Plate 1A). These structures remained unchanged after parasites had been maintained *in vitro* for at least 1 week. In contrast, marked changes were observed following a 2-hour exposure to 20 µg/ml praziquantel at 37°C. Tegumental bubbles appeared all over the surface, particularly on the ventral surface adjacent to the sucker (Plate 2A). Bubbles with various sizes could be readily observed, some of which ruptured and resulting in crater-like pits surrounded by raised edges (Plate 2 B). In some other areas, particularly in flukes with prolonged exposure to praziquantel, normal organization of the entire surface could not be recognized (Plate 2 C, D). On occasions, the damage was so severe that pieces of tegument had sloughed off, and frequently exposing the underlying structure which could represent the basal lamina layer of the tegument.

In vivo study: SEM studies were carried out using specimens collected from both experimentally infected hamsters and patients with opisthorchiasis. Hamster infected with 100 metacercariae for at least 2 months

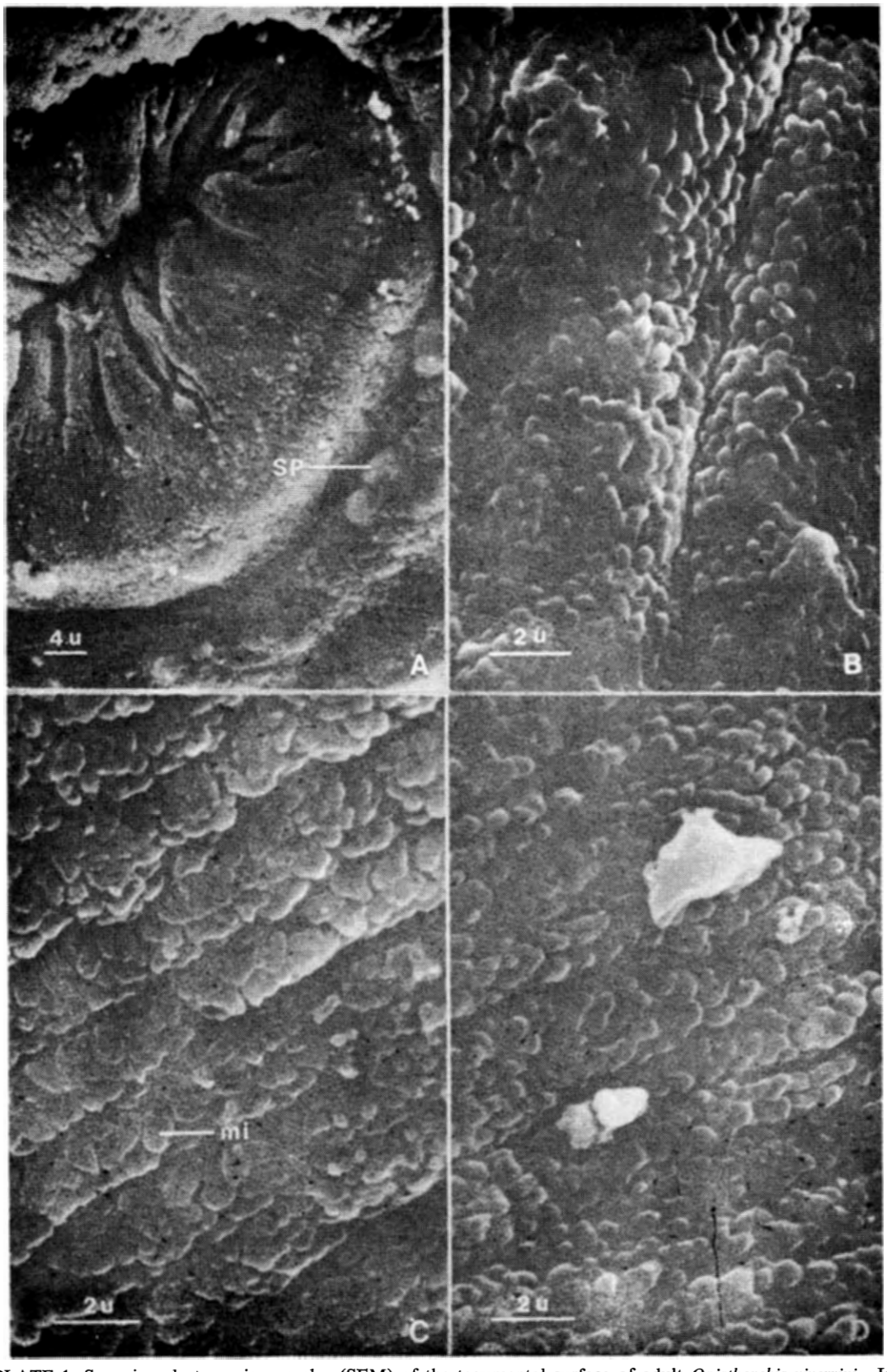

PLATE 1. Scanning electronmicrographs (SEM) of the tegumental surface of adult *Opisthorchis viverrini*. In general, the surface appears corrugated and has numerous closely packed microvilli (mi). Sensory papillae (SP) are seen throughout the entire surface, particularly high concentration on the laterodorsal aspect of the body and around ventral sucker (VS).

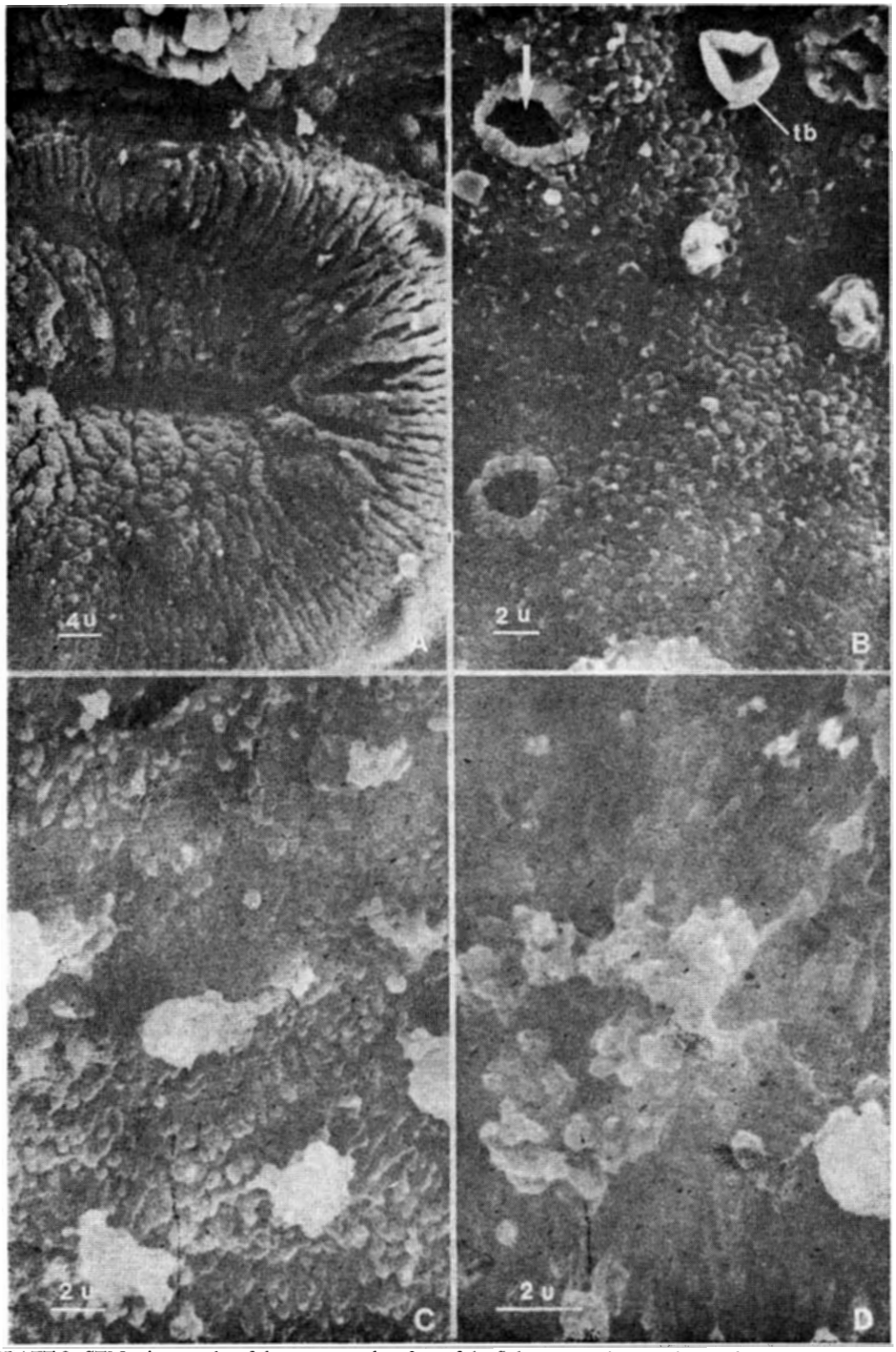

PLATE 2. SEM micrographs of the tegumental surface of the flukes exposed to praziquantel *in vitro*. The flukes were incubated at 37°C for 2 hours in 20 μg/ml praziquantel prior to being fixed for SEM examination. Tegumental bubbles (tb) are seen over the entire body. Some of these rupture resulting in crater-like lesions (Arrow in B). In some flukes, the normal organization of the surface could not be recognized (C & D).

were given by intragastic tube, a single dose of praziquantel suspension at approximately 350 mg/kg body weight. Four hours later, the animals were sacrificed and the flukes were removed from biliary system as previously described (Tuti *et al.*, 1982). Although these worms were still alive at the time of collection, they appeared to be partially paralyzed. Like those observed in the *in vitro* treatment, numerous tegumental bubbles appeared, particularly in areas adjacent to both suckers (Plate 3 A) and randomly on the ventral side (Plate 3 B). In most areas, the normal organization of stubby microvilli and sensory papillae could not be recognized. The bubbles were so numerous in other areas together with debris which might be due to the sloughing of the tegumental components (Plate 3 C). The damage might be so extensive in some flukes as to result in a peeling of the surface tegument. It should be noted that in the center of these badly damaged surface, numerous small micronodules appeared (Plate 3 E); and they were also present at the periphery of the lesion (Plate 3 D). Although the nature of these micronodules could not be ascertained, it may be that they represent regenerative components which appear during the reparative phase of the flukes.

Similar changes (Plate 4) were noted on the flukes recovered from the feces and bile of patients treated with praziquantel. Many flukes were found to be elongated in shape as those noted in the *in vitro* study. However, in addition to the lengthening of area between the two suckers, there was also a ballooning structure similar to that previously reported for *C. sinensis* by Rim (1982). In SEM the tegumental bubbles were again observed and they were so numerous that at times appeared to coalesce, forming "microridges" over large surface areas (Plate 4 B and 4 C). Crater-like lesions could be seen ono ccasions (Plate 4 A). In some flukes, large patches of surface tegument peeled off (Plate 4 D). It should be noted here again in this area, structures with appearance similar to micronodules seen in flukes taken from treated infected hamsters could be readily observed.

DISCUSSION

The present study clearly demonstrates that the tegument of *O. viverrini* is sensitive to praziquantel as has been shown for many other trematodes including the closely relatd *C. sinensis*, (Rim *et al.*, 1980; Kim *et al.*, 1982; Mehlhorn *et al.*, 1983). Alterations of the surface tegument as demonstrated by SEM in the present study were similar, whether the flukes were exposed to the drug under the *in vitro* or *in vivo* conditions. Using transmission electromicroscopic study, other investigators have noted that vacuolization appeared very early following exposure to praziquantel at concentration as low as 0.01 μg/ml (Mehlhorn *et al.*, 1983). It is most likely that these vacuoles would eventually protrude onto the surface giving rise to tegumental bubbles seen during the later stage of exposure. The results present in this study show that upon prolonged exposure, both under the *in vitro* or *in vivo* conditions, these bubbles many rupture giving rise to crater-like lesions on the surface tegument. Moreover, these lesions may form close to one another and may be so extensive as to cause a large area of the surface tegument to peel off, exposing the underlying basal lamina layer. Surface alterations of the flukes recovered from the feces of patient treated with praziquentel as shown in Plate 4 were similar to those noted with the flukes recovered directly from the bile of a patient who was operated. Similar ultrastructural changes in the tegument of flukes exposed to praziquantel under different conditions strongly indicate that these lesions were caused by the drug and not by technical artefact. Because the flukes taken from infected hamster were fixed at the

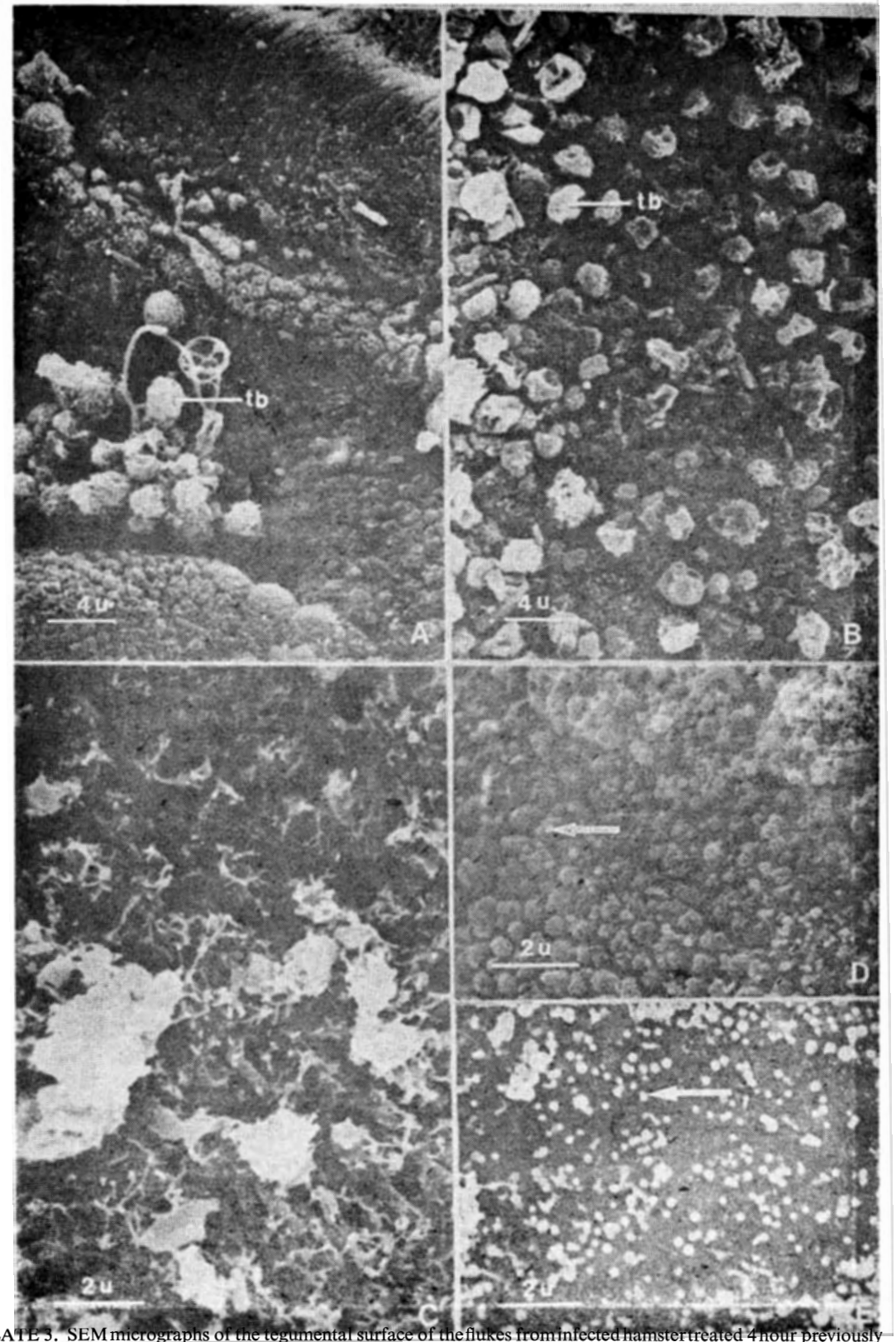

PLATE 3. SEM micrographs of the tegumental surface of the flukes from infected hamster treated 4 hour previously with 350 mg/kg body weight. Various sizes of tegumental bubbles (tb) are seen around the sucker (A) and over the entire body (B). Some tb are formed close to one another and appear to coalesce (C). In and around the smooth and presumably previously badly damaged surface, numerous micronodules (arrows in D and E) appear, some of these appear to be formed on the surface of apparently normal microvilli (D).

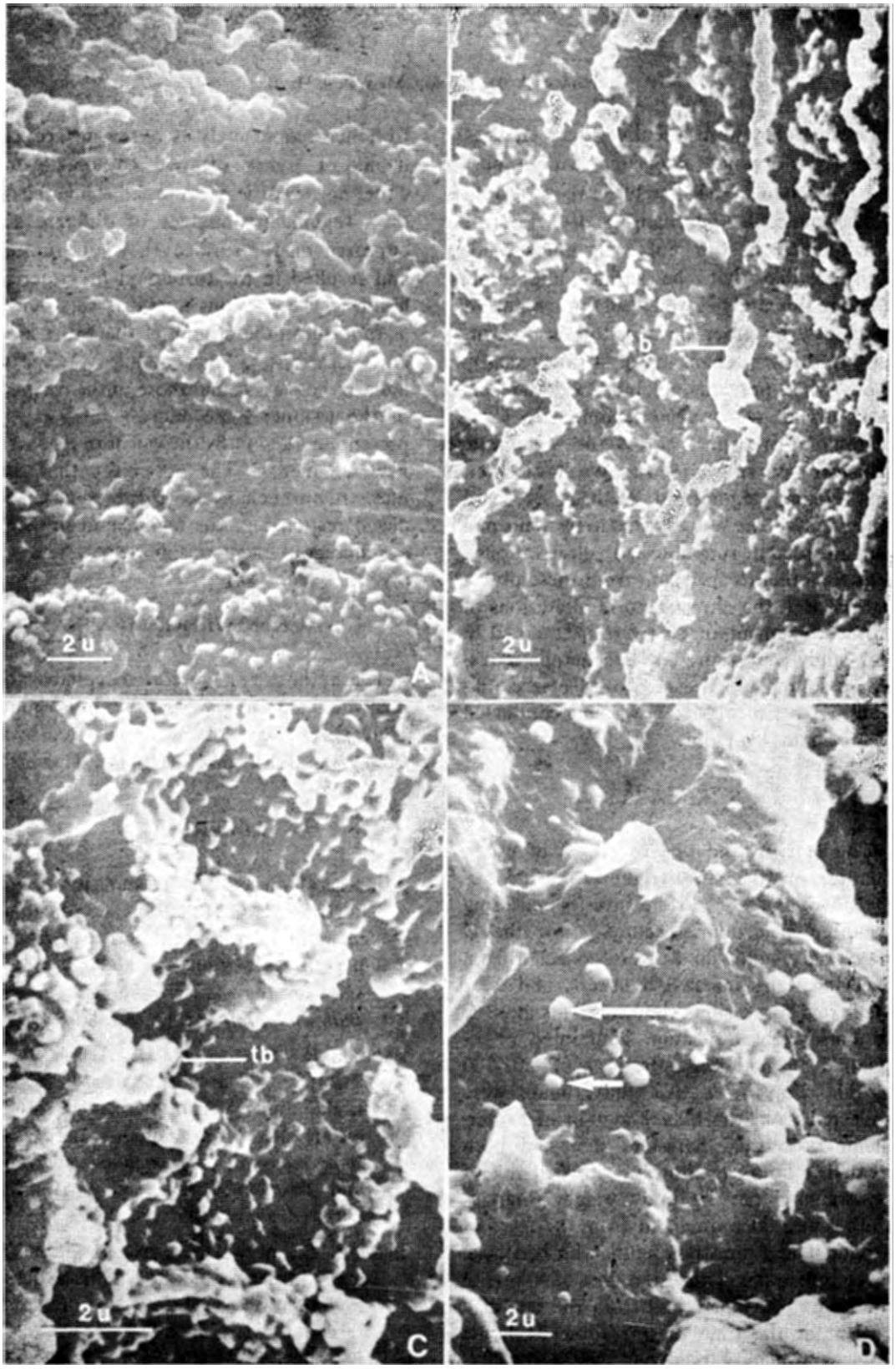

PLATE 4. SEM micrographs of the tegumental surface of the flukes recovered from the feces of a patient who was treated with praziquantel 3-4 hour previously. Tegumental bubbles (tb) are seen all over the entire body. In most flukes these tb form close to one another and coalesce to form microridges on the surface (B and C). Some of these bubbles rupture forming crater-like lesions (A). Micronodules are seen on the surface of the denuded area (Arrow in D).

time when they were still alive, these changes were therefore not associated with a post mortem change that might have occurred.

The molecular mechanism of action of praziquantel is still not well understood. It is possible that the surface alterations noted in this study as well as in those reported previously by other investigators are secondary to other mechanism (s). It was recently shown that praziquantel increased the permeability of *O. viverrini* tegument to Ca^{++} binding or transport across the membrane (Ruenwongsa *et al.*, 1983). We have noted that similar tegumental changes occurred when *O. viverrini* was exposed to fresh normal human serum (unpublished observations). It is tempting to speculate from these observations that the ultrastructural alterations of surface tegument of *O. viverrini* as noted in this study may represent a generalized response following exposure to adverse conditions. Experiments are now being conducted to elucidate this point.

SUMMARY

The *in vitro* and *in vivo* effects of praziquantel on the ultrastructural surface of *Opisthorchis viverrini* were investigated using scanning electronmicroscopy. For the *in vitro* study, adult flukes were collected from experimentally infected hamsters, and were incubated for various time intervals at 37°C in Earle's basal medium containing praziquantel at final concentrations of 0.01-100 µg/ml. For the *in vivo* study, flukes were collected from the biliary system of experimentally infected hamsters that had been treated 4 hours previously with 350 mg of praziquantel per kg body weight (mg/kg). Flukes were also obtained from the feces of a patient with opisthorchiasis who had been given praziquantel once at a dose of 40 mg/kg 4-6 hours previously and from the bile of a patient at the time of operation 24 hours after praziquantel treatment. Scanning electronmicroscopic analyses of the surface teguments of flukes exposed to praziquantel either *in vitro* or *in vivo* showed similar changes. Tegumental bubbles of different sizes appeared on the surface; they later ruptured and resulted in the formation of crater-like lesions. These lesions might be so extensive as to result in the peeling of the entire areas. On occasions, "micronodules" appeared later in these areas and those at the periphery of the lesions; these micronodules may represent an attempt by the worm to regenerate new tegument. The possibility that these ultrastructural changes may represent a generalized response of the tegumental surface to an obnoxious agent was discussed.

ACKNOWEDGEMENTS

The authors are grateful to collaboration received from Dr. E.S. Upathum of Mahidol University. This work was supported by the Wellcome Trust and Mahidol University.

REFERENCES

AMBROISE-THOMAS, P., GOULLIER, A. and WEGNER, D.G.H., (1981). Le praziquantel dans le traitement des distomatoses hepatiques extreme-orientales a *Clonorchis sinensis ou Opisthorchis viverrini. Bull. Soc. Path. Exot., 74 :* 426.

BECKER, B., MEHLHORN, H., ANDREWS, P., THOMAS, H. and ECKERT, J., (1980). Light and electronmicroscopic studies on the effect of praziquantel on *Schistosoma mansoni, Dicrocoelium dendriticum* and *Fasciola hepatica* (Trematoda) *in vitro. Z. Parasitenke., 63* : 113.

BECKER, B., MEHLHORN, H., ANDREWS, P. and THOMAS, H., (1981). Ultrastructural investigations on the effect of praziquantel on the tegument of five species of cestodes. *Z. Parasitenke., 64* : 257.

BUNNAG, D. and HARINASUTA, T., (1980). Studies on the chemotherapy of human opisthorchiasis: I. Clinical trial of praziquantel. *Southeast Asian J. Trop. Med. Pub. Hlth., 11* : 528.

BUNNAG, D. and HARINASUTA, T., (1981). Studies on the chemotherapy of human opisthorchiasis. III. Minimum effective doses of praziquantel. *Southeast Asian J. Trop. Med. Pub. Hlth., 12* : 413.

HORSTMANN, R.D., FELDHEIM, W., FELDMEIER, H. and DIETRICH, M., (1981). High efficacy of praziquantel in the treatment of 22 patients with Clonorchis/Opisthorchis infections. *Tropenmed. Parasit., 32* : 157.

KIM, S.S., KIM, S.J. and RIM, H.J., (1982). Electron-microscopic studies on the effect of praziquantel to *Clonorchis sinensis. Korea Univ. Med. J., 19* : 91.

LOSCHER, T., NOTHDURFT, H.D., PRUFER, L., von Sonnenburg, F. and Lang, W., (1981). Praziquantel in clonorchiasis and opisthorchiasis. *Tropenmed. Parasit. 32* : 234.

MEHLHORN, H., BECKER, B., ANDREWS, P., THOMAS, H. and FRENKEL, J.K., (1981). *In vivo* and *in vitro* experiments on the effects of praziquantel on *Schistosoma mansoni*. A light and electron microscopic study. *Arzneim. Forsch., 31* : 544.

MEHLHORN, H., KOJIMA, S., RIM, H.J., RUENWONGSA, P., ANDREWS, P., THOMAS, H. and BUNNAG, D., (1983). Ultrastructural investigations on the effects of praziquantel on human trematodes from Asia: *Clonorchis sinensis, Metagonimus yokogawai, Opisthorchis viverrini, Paragonimus westernmani* and *Schistosoma japonicum. Arzneim. Forsch., 31* : 91.

RIM, H.J., PARK, H.Y., KIM, S.J. and LEE, S.H., (1980). Morphological observations on *Clonorchis sinensis* obtained from rats treated with praziquantel. *Korean J. Parasit., 18* : 192.

RIM, H.J., (1982). Current results on chemotherapy of clonorchiasis and paragonimiasis. *New Med. J., 2* : 155.

RUENWONGSA, P., HUTADILOK, N. and YUTHAVONG, Y., (1983). Stimulation of Ca^{++} uptake in the human liver fluke *Opisthorchis viverrini* by praziquantel. *Life Sc., 32* : 2529.

SUPANVANICH, S., SUPANVANICH, K. and PAWABUT, P., (1981). Field trial of praziquantel in human opisthorchiasis in Thailand. *Southeast Asian J. Trop. Med. Pub. Hlth., 12* : 598.

TUTI, S., VICHASRI, S. and SIRISINHA, S., (1982). Effect of culture media on production of excretory-secretory products and egg output of *Opisthorchis viverrini in vitro. J. Parasit., 68* : 892.