Scanning Electron Microscopic Observations on Advanced Third-Stage Larva of *Gnathostoma spinigerum* After *in vitro* Exposure to Albendazole Sulphoxide

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Abstract

Gnathostomiasis is the parasitic disease caused by the migration of an advanced third-stage larva of *Gnathostoma spinigerum*. To date, albendazole is claimed to be the effective drug in preventing the reoccurrence of migratory swelling in patients. After being exposed to 1 and 2 μg/ml albendazole sulphoxide (AlbSO) *in vitro*, the parasites moved deteriorately, however, no dead larva was found even exposed to these concentrations for 21 consecutive days. The topographical alterations after 21 days of albendazole sulphoxide exposure are described using a scanning electron microscope. The marked changes in surface morphology were observed in both neck and body regions. The tegumental surface on the neck region was swollen and covered with fuzzy materials, whereas, the spines on the posterior region of the body were dislodged. These changes would probably lead to reduction of intermittent cutaneous migratory swelling in human gnathostomiasis patients.

Key word : Scanning Electron Microscopy, Albendazole Sulphoxide, *Gnathostoma spinigerum*

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Gnathostoma spinigerum Owen, 1836 is a nematode causing human gnathostomiasis in South-east Asia including Thailand[1]. As an accidental host, man is usually infected by the ingestion of raw and/or inadequately cooked freshwater fish or other intermediate hosts that contain the advanced

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third-stage larvae (aL3) of the parasite. The clinical manifestations are most commonly characterized by localized, intermittent, migratory swellings of the skin and subcutaneous tissues, often in association with localized pain, pruritus, and erythema[2]. The damage in many visceral organs may occur due to the migration of the worm to deeper tissues. Much damage to the central nervous system by *G. spinigerum* causes eosinophilic myeloencephalitis and death[1].

Albendazole, an anthelminthic drug, has been shown to be effective for the treatment of human gnathostomiasis[3,4]. In addition, high doses of albendazole (90 mg/kg twice daily) significantly reduced the number of *G. spinigerum* larvae in experiments in mice[5]. Pharmacokinetic studies have shown that albendazole is metabolized rapidly in the liver mainly to the sulphoxide (AlbSO), an active metabolite[6]. Despite the evidence of efficacy; however, the mechanism for the effect of albendazole on gnathostome larvae is still unknown. As far as is known, there have been no studies on the topographical changes which occur in *G. spinigerum* after exposure to AlbSO *in vitro*. Accordingly, a scanning electron microscopic (SEM) study was performed, with attention being paid to the morphology of the tegumental surface of the aL3 stage larvae.

MATERIAL AND METHOD

Gnathostoma spinigerum larval collection and culture

The aL3 of *G. spinigerum* were collected from cysts in the liver of freshwater eels (*Fluta alba*). The larvae were obtained by compression of the liver between two thick transparent glasses. Larvae were removed from the tissue and washed thoroughly in sterile physiologic saline solution (0.85% NaCl), containing 100 units/ml of penicillin G, 100 μg/ml of gentamicin and 100 μg/ml of amphotericin before being placed into culture medium. Sixty larvae were cultured in 35 mm diameter sterile petri dishes (10 larvae/dish) with 2 ml of culture medium of RPMI-1640 (GIBCO laboratories, Life Technologies, Inc., Grand Island, New York) and 10 per cent fetal calf serum (SEROMED Biochrom KG, Leonorenstr, 2-6.D-1000, Berlin, Germany) containing 100 units/ml of penicillin G, 100 μg/ml of gentamicin and 100 μg/ml of amphotericin in each dish. All petri dishes were stored at 37°C under 5 per cent CO_2 in air.

Incubation procedure

Albendazole sulphoxide (Research Institute, Smith Kline & French Laboratories, Ltd., Welwyn Garden City, Herts, United Kingdom) was used in this study instead of albendazole. Since the maximum concentrations of AlbSO in plasma are reportedly very variable (0.45-1.56 μg/ml in most cases of patients with cerebral cysticercosis)[7], the final concentrations of AlbSO 1 and 2 μg/ml in the cultures were chosen to be investigated in this study.

The two petri dishes were used as the controls. Albendazole sulphoxide solution was added to the remaining dishes to obtain the final concentrations of 1 and 2 μg/ml (2 dishes/ concentration). The culture medium as well as AlbSO were changed every 24 h for 21 consecutive days and all petri dishes were maintained at 37°C under 5 per cent CO_2 in air before the incubated larvae were examined for topographical changes by SEM.

Scanning electron microscopy

Following removal, all incubated larvae were briefly washed in 0.1 M phosphate buffer prior to fixation for electron microscopy. Cultured larvae were fixed in a fixative agent consisting of 2.5 per cent glutaraldehyde at 4°C for 24 h. The fixed worms were subjected to postfix in 1 per cent osmium tetroxide and dehydration in a graded alcohol series, followed by acetone and critical-pointed drying. The worms were then mounted on stubs and coated with gold. Ten larvae from each group were viewed with a JEOL-JSM840A scanning electron microscope at an accelerating voltage of 20 kV, and photographed with Kodak® Verichrome Panchromatic film VP 120.

RESULTS

The movement of larvae

Larvae in control groups moved actively with the whole body for all 21 days of the incubation period. When treated with AlbSO 1 and 2 μg/ml, 35 per cent of larvae initially showed reduction of movement on the 11^{th} and 9^{th} day of drug exposure, respectively. All AlbSO-exposed larvae were lethargic on the 21^{st} day; however, no dead worms were found.

SEM observations of larvae incubated in control groups

The surface morphology of aL3 *G. spinigerum* in the control groups were of normal appea-

rance as previously described[8-10]. No changes in surface ultrastructure were observed throughout the experimental period of 21 days. The head bulb is subglobular in shape and bears 4 transverse rows of well-developed single-pointed spines or hooklets (Fig. 1). An individual hooklet has an oblong base pointed at the posterior end. The neck region is posterior to the head bulb which is interrupted by a constriction (Figs. 1, 2). The body follows the neck region and is also covered with several backwardly directed transverse rows of single-pointed spines the same as in the neck region (Fig. 4).

SEM observations of larvae incubated in AlbSO 1 and 2 μg/ml

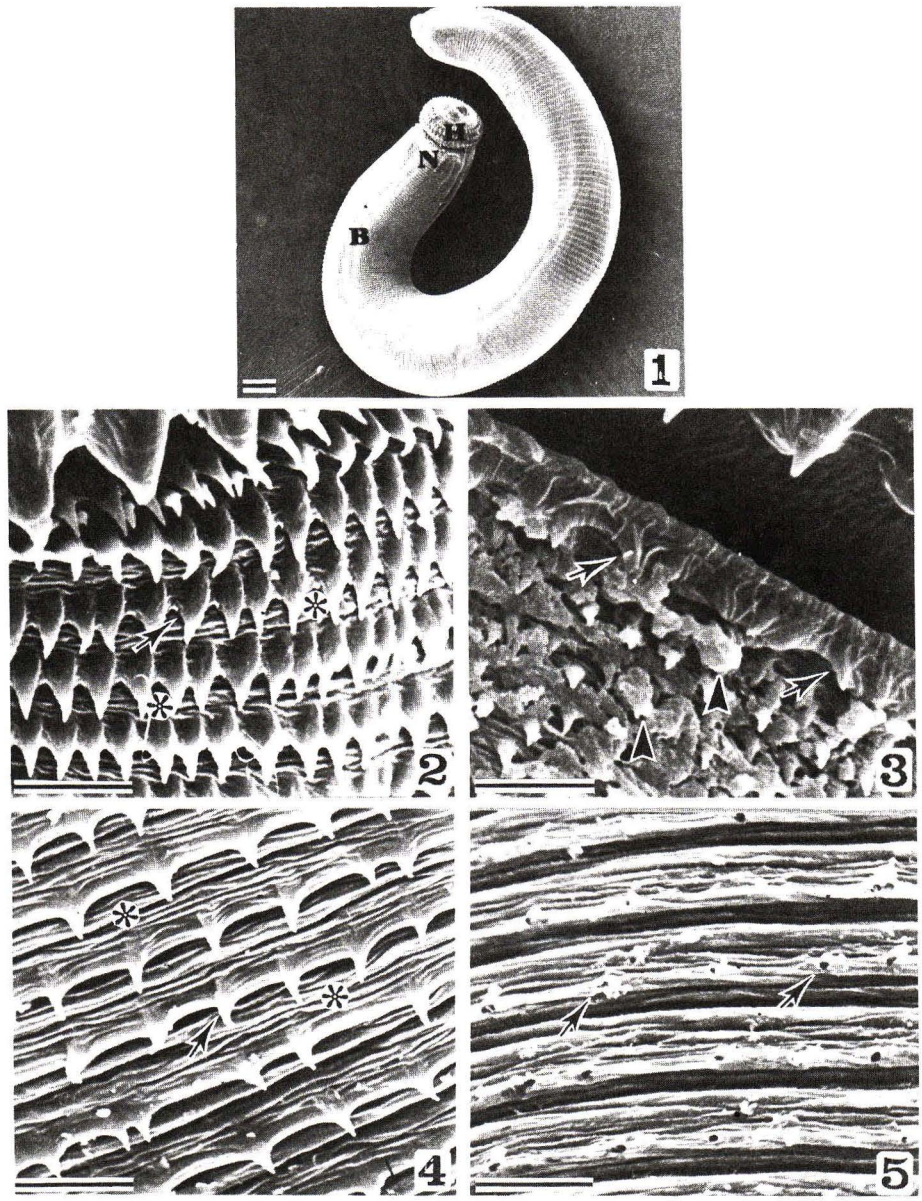
The surface morphology of all aL3 *G. spinigerum* after being exposed to AlbSO 1 and 2 μg/ml for 21 days was altered in the same manner in all exposed larvae (10 in each group). The larvae incubated in the higher concentration suffered similar damage to those in the lower concentration. The noticeable changes were seen in the neck and body regions. The hooklets, by contrast, appeared relatively unaffected. The tegumental surface on each transverse row of the neck region was swollen and externally covered with a coat of fuzzy material (Fig. 3). Some deformity in spine arrangement was apparent. However, a complete loss of tegumental spines on the posterior part of the body occurred in some larvae only following AlbSO 2 μg/ml treatment (Fig. 5).

DISCUSSION

Albendazole may be the effective anthelminthic drug for the treatment of gnathostomiasis. In symptomatic gnathostomiasis patients, albendazole at the dosage of 800 mg/day for 14 days (approximately 15 mg/kg/day) produced the outward migration of gnathostome larvae[4]. However, 94.1 per cent cure rate was seen in the longer period of treatment for 21 days[3]. In contrast, the symptom clearance time of albendazole or placebo treated patients was not different (6.4 and 6.8 days, respectively)[3]. In this regard, the initial disappearance of acute clinical symptoms in gnathostomiasis patients may not be an effect of treatment but rather the normal progression of the disease. The effect of the drug may be to prevent the recurrence of symptoms by inducing the outward migration of larvae or by paralyzing them.

After oral administration of albendazole, no albendazole was detected in plasma but its main active metabolite AlbSO could be measured [7]. Unfortunately, there are no reports of albendazole pharmacokinetics in gnathostomiasis patients. However, in cysticercosis patients treated with albendazole 15 mg/kg/day for 8 days revealed that the maximum plasma levels of AlbSO varied from 0.45 to 2.96 μg/ml. These levels were found between 0.45 and 1.56 μg/ml and the steady state of plasma level of AlbSO was lower than 2 μg/ml in most cases[7]. In our *in vitro* study using 1 and 2 μg/ml of AlbSO incubated with aL3 *G. spinigerum* for 21 days revealed that obvious differences occurred in the surface morphology of worms. The striking tegumental changes were marked swelling and covering with fuzzy materials on the neck region. Perhaps this fuzzy material may result from tegumental sloughing. The detachment of spines on the posterior part of body was observed in some larvae and could be an effect of AlbSO itself. However, detachment might not occur either *in vivo* or *in vitro* during albendazole exposure but the damage may have been enhanced during the process of SEM preparation. The mechanism of swelling on the tegumental surface of *G. spinigerum* in this study is not understood, but might be due to osmotic imbalance as suggested by Apinhasmit and Sobhon[11], who studied the effect of praziquentel in *Opisthorchis viverrini* tegument. The sloughing of the tegument of this fluke was the final sequel of changes in the tegumental's cytoplasm.

As previously mentioned, the striking phenomena produced by the treatment with AlbSO in *G. spinigerum* were the swelling and sloughing of the tegumental surface as well as detachment of spines. Interestingly, this damage was in accordance with results reported by other authors. The effect of albendazole against *Clonorchis sinensis* on the tegument (studied by SEM) and gut microvilli (studied by transmission electron microscope : TEM) revealed that the swelling and adhesion of the projections of both organs appeared 1 h after exposure[12]. Moreover, the detachment of the partial projections also occurred by 72 h. The damage observed on the tegument was identical to that of the gut microvilli. In comparison with our SEM observations of AlbSO-treated gnathostome larvae, larval movement was reduced in some worms after 9 and 11 days of exposure to 2 and 1



Figs. 1-5. Scanning electron micrographs of advanced third-stage larvae of *Gnathostoma spinigerum* showing tegumental surface and spines. Larvae were cultured and exposed to albendazole sulphoxide (AlbSO) 2 μg/ml for 21 consecutive days. 1: Whole body of worm showing head (H), neck (N) and body (B) regions. Scale bar = 100 μm. 2 and 4: Surface (*) and spines (arrow) of worms in control group showing the normal appearance on neck and middle region of body, respectively. 3: Surface of AlbSO-treated worm showing the numerous fuzzy materials (arrow-head) and deformity of spines (arrow). 5: Surface on middle region of AlbSO-treated worm showing the detachment of spines (arrow). Scale bar = 10 μm for figures 2-5.

μg/ml of drug, respectively. However, SEM study was not carried out at those periods of time, and thus the tegumental damages in this study could have occurred earlier. Further daily serial SEM observation needs to be performed.

Besides *C. sinensis*, the effect of *in vitro* albendazole against adult *Ascaris suum*, the pig roundworm, induced prominent ultrastructural changes which included the presence of necrotic dense bodies, with disruption and erosion of the microvilli of the intestinal epithelial cells[13]. Furthermore, ultrastructural changes in *Echinococcus granulosus* induced by albendazole or AlbSO have included rostellar disorganization, formation of numerous blebs on the tegument, loss of the microtriches, detached tegument, loss of hooks on the soma and scolex tegument[14]. Necrosis of the germinal membrane and partial loss of microtriches have also been described in the larval stage of this tapeworm[15-19]. Whether gut microvilli of the aL3 *G. spinigerum* are also affected by AlbSO administration requires further investigation.

Although the function of tegumental spines in *G. spinigerum* is still unknown, they may be involved in movement of the worm as implicated in trematodes[20,21]. If this is so, severe impairment of locomotory function might be expected. This appears to be the case after AlbSO treatment. All larvae exposed to AlbSO in our study were lethargic after 21 days incubation time while those in the control groups were still actively moving. Such disturbance in the tegumental spines would almost certainly lead to the worsening movement, eventually resulting in sluggish and/or paralyzed parasites. In addition, albendazole and AlbSO produce tubulin alterations in small tapeworms, *E. granulosus* protoscolices[22] which are involved in muscle contraction. Perhaps this might be the explanation of reduction in *G. spinigerum* larval movement. These lethargic worms may not be able to produce further migratory swelling after a 21 day-course of albendazole treatment.

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การเปลี่ยนแปลงลักษณะผิวลำตัวของตัวอ่อนระยะที่ 3 ขั้นปลายของพยาธิตัวจี๊ดเมื่อสัมผัสยาอัลเบนดาโซลซัลฟอกไซด์ด้วยกล้องจุลทรรศน์อิเล็กตรอนชนิดส่องกราด

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โรคพยาธิตัวจี๊ดเป็นโรคปรสิตที่เกิดจากการไชตามส่วนต่าง ๆ ของร่างกายภายหลังจากผู้ป่วยได้รับตัวอ่อนระยะที่ 3 ขั้นปลายของพยาธิตัวจี๊ด ในปัจจุบันพบว่ายาอัลเบนดาโซลสามารถทำให้ผู้ป่วยไม่มีอาการบวมเคลื่อนที่กลับมาอีก ในการทดลองนี้ได้นำตัวอ่อนระยะที่ 3 ขั้นปลายของพยาธิตัวจี๊ดมาสัมผัสกับยาอัลเบนดาโซลซัลฟอกไซด์ที่ความเข้มข้น 1 และ 2 ไมโครกรัมต่อมิลลิลิตร ในหลอดทดลองเป็นเวลานาน 21 วัน พยาธิมีการเคลื่อนไหวได้ลดลงเป็นอย่างมากหลังจากสัมผัสยาได้ 9 และ 11 วันตามลำดับ และเมื่อศึกษาลักษณะผิวของพยาธิโดยกล้องจุลทรรศน์อิเล็กตรอนชนิดส่องกราดพบความเปลี่ยนแปลงอย่างมากที่ผิวบริเวณคอและส่วนลำตัว โดยที่ผิวส่วนคอมีการบวมและถูกปกคลุมด้วยผิวที่ถลอก ในขณะที่หนามส่วนท้ายของลำตัวมีการหลุดหายไป การเปลี่ยนแปลงดังกล่าวนี้น่าจะเป็นสิ่งที่ทำให้พยาธิเคลื่อนไหวน้อยลงและผู้ป่วยด้วยโรคพยาธิตัวจี๊ดที่ได้รับยาอัลเบนดาโซลนาน 21 วันไม่มีการบวมเคลื่อนที่อีก

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