The *in vitro* effect of albendazole, ivermectin, diethylcarbamazine, and their combinations against infective third-stage larvae of nocturnally subperiodic *Brugia malayi* (Narathiwat strain): scanning electron microscopy

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**ABSTRACT:** Scanning electron microscopy (SEM) was employed to observe the effects of ivermectin (IVM), diethylcarbamazine (DEC), and albendazole (ALB) alone, and the drugs in combination (ALB+IVM and ALB+DEC) against infective third stage larvae (L3) of nocturnally subperiodic (NSP) *Brugia malayi* (Narathiwat strain) *in vitro*. IVM, at a concentration of 10⁻⁴ M, killed L3 within 1-2 h. The SEM data showed damage to the L3 surface and loss of regular cuticular annulations. The cuticles were grooved in the middle region of the body. In comparison with normal L3 before treatment with IVM, the cuticular surface showed transversed striations with periodic annulations. The result demonstrated that IVM showed a larvicidal activity against L3 of NSP *B. malayi* cultivated *in vitro*. Compared with those larvae in the control group, the treated larvae had no morphological changes in the cuticular surface at the head, body, and tail regions after cultivation with all drugs alone, and in their combinations at a concentration of 10⁻⁵ M for 7 d. In this system, and at that concentration, only the larvae cultured with ALB alone remained highly motile. Although no morphological changes had been observed by SEM, those drugs used alone (IVM and DEC) and in combinations (ALB+IVM and ALB+DEC), reduced larval motility throughout the experiments at a concentration of 10⁻⁵ M. The minimum lethal concentration (MIC) of IVM against NSP *B. malayi* was 10⁻⁴ M. *Journal of Vector Ecology* 29 (1): 101-108. 2004.

**Keyword Index:** *Brugia malayi*, ivermectin, diethylcarbamazine, albendazole, scanning electron microscopy (SEM), nocturnally subperiodic.

**INTRODUCTION**

Chemotherapy is one of the most strategic tools used for reducing microfilaremia in humans. Diethylcarbamazine (DEC) and ivermectin (IVM) are the only widely accepted drugs available for the control of onchocerciasis and filariasis. Given as a single dose at 6-12 mo intervals, they effectively suppress or reduce the microfilariae that cause clinical manifestations, i.e. ocular lesions in onchocerciasis, lymphoedema, or elephantiasis in lymphatic filariasis (Karbwang et al. 1997, Michael et al. 1996, Whitworth et al. 1996). Albendazole (ALB) appears to be a particularly good drug to use against lymphatic filariasis because it not only clears microfilaraemias but also has some adulticidal activity on the filarial parasites and clears intestinal helminths (WHO 1996). Mass treatment with either an annual single dose of antifilarial drugs or through fortification of salt with DEC is now recommended to interrupt transmission (ALB (400 mg for all ages)+DEC (6 mg/kg body weight) and ALB+IVM (200 mg/kg body weight)) (Ottesen et al. 1997). IVM, a derivative of avermectin B, is a macrocyclic lactone and fermentation product of *Streptomyces avermitilis*. The mechanism of action of IVM is unknown. However, it appears to prevent the release of intrauterine microfilaria. It has also been shown to mimic the actions of the neurotransmitter, gamma aminobutyric acid (GABA), which induces paralysis in infective larvae, but is inactive on microfilariae (Deverre et al. 1989). A high dose of IVM suppresses microfilaremia in lymphatic filariasis (Richards et al. 1991). DEC has been a drug of choice for filariasis for more than 40 years. The mechanism of action of DEC against adult filariae is not understood, but it may be involved in muscular paralysis. It has been shown as a microfilaricidal against *Onchocerca volvulus*.
Scanning electron microscopy (SEM) of adult, infective larvae and microfilaria of \textit{Brugia malayi} has been examined in several studies (Chew et al. 1983, Franz and Lenze 1982, Zaman 1987, Zaman and Narayanan 1986, Lim et al. 1983, Choochote et al. 1987, Maleewong et al. 1987). In addition, the ultrastructure surface morphology of nocturnal subperiodic (NSP) \textit{B. malayi} strain from Narathiwat province, southern Thailand, has been studied (Choochote et al. 1987). There has also been evidence of efficacy when using drug combinations in the management of microfilaria due to lymphatic filariasis (Ottesen et al. 1997, Karam and Ottesen 2000).

The action of these drugs against infective larvae should be observed for effectiveness both in vivo and in vitro. Although the filaricidal drugs affect the parasite in vivo, the possible action site of these drugs against the infective larvae or third stage larva (L3) in the in vitro cultivation examined by SEM has not been studied yet. Thus, the present study was undertaken to examine the external morphology structures of the L3 of NSP \textit{B. malayi} (Narathiwat strain) after exposure to filaricidal drugs, i.e. IVM, DEC, and ALB alone, and the drug combinations in the in vitro cultivation by SEM.

**MATERIALS AND METHODS**

The infective third-stage larvae (L3) of nocturnally subperiodic (NSP) \textit{Brugia malayi} were obtained from laboratory-reared \textit{Aedes togoi} that had been fed on the blood from microfilaraemic cats 12 d previously. Mass colonization of \textit{Ae. togoi} and rearing of mosquitoes followed the techniques described by Choochote (1981). The whole bodies of 12-d post-infection mosquitoes were dissected using the aseptic technique (Suwan et al. 1993). Mass dissection was carried out in Hank Balance Salt Solution (HBSS) with antimicrobial agents (10,000 unit/ml of penicillin G, 10,000 mg/ml of streptomycin disulfate, and 25 mg/ml of amphotericin B) in a sterile petri dish (diameter 30 mm).

The in vitro cultivation was followed by a simple technique modified by Tippawangkosol et al. (2002). The culture media consisted of a 1:1 (v/v) mixture of NCTC-135 (Sigma) and Iscove’s modified Dulbecco’s medium (IMDM; Sigma) supplemented with 20% heat inactivated fetal bovine serum (NI-FBS) and a mixture of antimicrobial agents. Fresh L3 were washed by repeatedly transferring to fresh media with a mixture of antibiotic agents in a culture petri dish. Then the L3 were pipetted into each well of a 24-well culture dish that contained 0.9 ml of culture media (NI-FBS) and 0.1 ml of filaricidal drugs alone, e.g. ivermectin (IVM), albendazole (ALB), and diethylcarbamazine (DEC), and their combinations (ALB+IVM and ALB+DEC) at concentrations of $10^{-3}$ to $10^{-6}$ M. Each experiment was performed in four replicates.

Plates were placed in a candle jar (a glass desiccator equipped with stopcock and white candle). By this method, an atmosphere with low oxygen ($90\% \text{N}_2, 5\% \text{O}_2, 5\% \text{CO}_2$) was produced. The candle jar was incubated at $37^\circ \text{C}$ in an incubator for 7 d. Motility of the L3 was examined daily under dissecting microscopy for 7 d. The larvicidal activity of the test drugs was evaluated in terms of the relative motility (RM) value (Kiuchi et al. 1987). A smaller RM value indicates stronger larvicidal activity, and when all larvae died this value was 0. A minimal lethal concentration (MLC) was determined as the lowest concentration giving an RM of 0 after 24 h of incubation. The RM value was calculated by using the motility score. The motility of larva was recorded using an arbitrary score of 3 (highly active), 2 (moderately active), 1 (less active), and 0 (immobile for at least 10 sec). The results were expressed as the average motility score with respect to the exposure time of motile parasites in treated and untreated groups. For the scanning electron microscopy (SEM) study, the larvae were fixed overnight in 2.5% glutaraldehyde in 0.1 M phosphate buffer solution (PBS) pH 7.4 at 4°C, washed with PBS (10 min, 2 changes), and then post fixed (1 h) in 1% osmium tetroxide at room temperature. Dehydration was performed via a passage through a long ethanol series, i.e. 30%, 50%, 70%, 80% (10 min), 95% (15 min, 2 changes) and followed by absolute ethanol (10 min, 2 changes). The specimens were finally dried by a critical point dryer, mounted on stubs, sputter coated with gold, and examined at 42 KV in a JEOL MED JSM 840-A SEM.

**RESULTS AND DISCUSSION**

The surface morphological structure of untreated larvae after cultivation in this study (Figures 1-6) was similar to that described previously by Maleewong et al. 1987, although the L3 in the previous study were not cultivated in vitro. The surface morphological structure of the L3 treated with and without drugs is shown in Figures 1-16. The L3 in the previous study was dissected from the same species of mosquito (\textit{Aedes togoi}) and then examined directly for surface structures by SEM. The result indicated that the L3 in this study could not molt to a further stage during 7 d cultivation in vitro. Our recent study reported that a simplification of the in vitro culture system promoted molting of the L3 to the fourth-stage larva after 7 d cultivation (Tippawangkosol et al. 2002).

SEM showed the head region of untreated larva after 7 d cultivation (Figure 1). The mouth was circular-
Figures 1-6. Scanning electron microscopy of NSP *Brugia malayi* infective third stage larvae after culturing in NI-FBS medium for 7 d. (1) Head region with circular mouth (M), four smaller inner papillae (IP), four spherical-shaped outer papillae (OP). (2) Tail end showing anus (A) and ventrolateral papillae (VLP). (3) Crescent-shaped anus with a raised anterior border. (4) Three caudal papillae; two VLP and one dorsoterminal papilla (DTP). (5) Body cuticular surface around head region. (6) A higher magnification of body cuticular surface at the middle of the body showing the transversed annulations.
Figures 7-12. *In vitro* cultivation of NSP *Brugia malayi* infective third stage larvae for 7 d in NI-FBS medium with drugs alone. (7) Body cuticle around head region showing outer papilla (OP), inner papilla (IP) and mouth (M) after culturing with albendazole (ALB 10^{-5} M). (8) Tail end showing two ventrolateral papillae (VLP) and dorsoterminal papilla (DTP) after culturing with diethylcarbamazine (DEC 10^{-5} M). (9) Body cuticle around head region and (10) body cuticle at the middle of the body after culturing with ivermectin (IVM 10^{-5} M) showing transversed annulations. (11) Body cuticle at the middle of the body and (12) a higher magnification showing many grooves of body cuticle and loss of regular cuticular annulations after culturing with IVM 10^{-4} M.
shaped. There were eight cephalic papillae arranged in two concentric circles. The four smaller inner papillae were nipple-like protuberances, and the four bigger outer papillae were spherical-shaped. Apart from these oral papillae, the two amphids could not be seen clearly. The tail end showed the crescent-shaped anus with a raised anterior border and three caudal papillae (Figures 2 and 3). The two ventrolateral caudal papillae were seen clearly and were rather similar in size, and they appeared spherical in shape, whereas the dorsoterminal papillae were round and rudimentary in appearance (Figure 4). There were numerous transversed annulations on the cuticular surface (Figures 5 and 6), except at the anterior and posterior ends. After treatment with IVM and DEC alone, at a concentration of $10^{-5}$ M, and combinations of ALB+DEC and ALB+IVM at a concentration of $10^{-5}$ M, the larvae gradually reduced larval motility throughout the experiment compared with those in the control group (Figure 17). Except for ALB, at that concentration the larvae showed high active movement for 7 d (Figure 17). After treatment with drugs alone (ALB, DEC and IVM) and in their combinations (ALB+DEC and ALB+IVM), at a concentration of $10^{-5}$ M, SEM showed normal surface morphological structures of the whole body of this larvae(Figures 7-10 and 13-16).

Observations on the surface topography of L3s of NSP *B. malayi* before and after treatment with the drugs in this study indicated that the morphological structures of both larvae were generally in agreement with the descriptions of previous investigations on *B. malayi* larva (Maleewong et al. 1987, Zaman and Narayanan 1986, **Figures 13-16**. *In vitro* cultivation of NSP *Brugia malayi* infective third stage larvae for 7 d in NI-FBS medium with a drug combination. (13) Tail end showing two ventrolateral papillae (VLP) and dorsoterminal papilla (DTP) and (14) body cuticle in the middle of the body after culturing with albendazole/diethylcarbamazine (ALB/DEC $10^{-5}$/10$^{-5}$ M) showing transversed annulations. (15) Head region showing the normal structure of outer papilla (OP), inner papilla (IP) and mouth (M), and (16) body cuticle in the middle of the body showing transversed annulations of body cuticle after culturing with albendazole/ivermectin (ALB/IVM $10^{-5}$/10$^{-5}$ M).
However, IVM at a high concentration, (10^-4 M), began to kill L3s within 1-2 h, as observed under dissecting microscopy (Figure 17), and SEM study showed damage to the surface of L3 (Figures 11 and 12). They also lost regular cuticular annulations, and the cuticles were grooved in the middle region of the body. The minimal lethal concentration (MIC) of IVM against *B. malayi* L3s cultivated *in vitro* was 10^-4 M. The effect of this drug was unknown, but it could be diffused through the cuticular surface of the parasite.

The loss of regular annulations in this parasite by some monoclonal antibodies was previously studied by SEM (Tan et al. 1989). The study of the *in vitro* IVM uptake by adult male *Onchocerca ochengi*, a cattle parasite closely related to *O. volvulus*, showed that the epicuticle had an irregular surface, which greatly increased the absorptive surface of the worm examined under SEM and transmission electron microscope (TEM) (Cross et al. 1998). Yates and Higashi (1986) reported the effect of a vaccination with 60 cobalt radiation attenuation *B. malayi* (L3) in jird and TEM, which showed that a high number of eosinophil were present around and within the damaged larvae structure to the L3 cuticle. The study by Bandi et al. (1999) showed the effect of tetracycline in blocking embryo development in *B. pahangi* and *Dirofilaria immitis*, in which embryo degeneration was documented by TEM. Hamann et al. (1990) reported that the eosinophil granule proteins were toxic to microfilariae of *B. pahangi* and *B. malayi* in their *in vitro* study. Many ultrastructural studies on *B. malayi* have been examined to determine the effect of many agents against the parasite in order to learn the mechanism of action and target region, with an aim to controlling and eradicating lymphatic filariasis.

The study of a possible action site of filaricidal drugs for killing larvae can only be performed *in vitro*, where all direct responses, symptoms and specific organs, can be observed. However, we could not investigate the direct responses to the drugs because there are many factors involved with *in vivo* study, such as the pharmacokinetics and pharmacodynamics of the drugs. IVM, at a concentration of 10^-3 M, only reduced larval motility, while this drug at a high concentration (10^-4 M) killed this larva in the *in vitro* study. However, the effectiveness of IVM, DEC, ALB, and their combinations in the *in vitro* study was correlated with other recent *in vivo* studies (Dunyo et al. 2000, Horton et al. 2000, Ismail et al. 2001, Shenoy et al. 2000, Supali et al. 2002). Although the morphological structure appearance could not be seen by SEM when using IVM and DEC alone, and in combinations with ALB at a concentration of 10^-5 M, these drugs appeared to disturb the motility of the parasite. Further action of these drugs will be examined by TEM in order to observe the abnormality in the organelles.

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