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Studies on Filariasis V: Exsheathment of *Brugia pahangi* and *B. malayi* Microfilariae in the Mosquitoes, *Aedes aegypti, Ae. togoi, Culex pipiens molestus* and *Armigeres subalbatus*

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Introduction

In some species of filarial worms such as *Wuchereria bancrofti, Brugia malayi, B. pahangi* and *Loa loa,* females produce microfilariae enclosed in a sheath, which is an elongated egg-shell. It has long been believed that these microfilariae cast off sheath in the mid-gut of the intermediate host immediately after ingestion (Faust *et al.,* 1970; Wilcocks and Manson-Bahr, 1971; Denham and McGreevy, 1977). It was reported that *B. pahangi* microfilariae when taken by *Anopheles quadrimaculatus* exsheathed in the ingested blood mass (Esslinger, 1962). In this respect, however, it has been recently reported that exsheathment of *B. pahangi* larvae* in *Armigeres subalbatus* occurred both in the lumen of the mid-gut and in the abdominal haemocoel (Yamamoto *et al.,* 1983). This finding leads to a hypothesis that sheath plays a role of protecting body against various conditions both in the alimentary canal and in the haemocoel.

The present study is aimed at solving question whether the larval exsheathment takes place frequently in the haemocoels of the mosquitoes.

Materials and Methods

Parasites

Mongolian jirds, *Meriones unguiculatus,* infected with *Brugia pahangi* or *B. malayi* (Che-ju strain, Korea) were used as the source of infective blood meal. Jirds infected with *B. pahangi* showed an average microfilaria density of 73.5 and 2.6/cmm of tail blood. Those infected with *B. malayi* showed an average microfilaria density of 1.5/cmm.

Mosquitoes were fed on the jirds which had been previously anesthetized by subcutaneous injection of sodium pentobarbital (Nembutal) at a dose of 40 mg/kg body weight into hypogastric region.

Mosquitoes

Aedes aegypti (Liverpool strain and Red eye-I strain), *Ae. togoi* (Nagasaki strain and Rendaiji strain), *Culex pipiens molestus* and *Armigeres subalbatus* (Rendaiji strain) were used in the experiments. The infective rates

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*Microfilariae signify the 1st stage larvae of the filariae dwelling in the definitive hosts regardless of presence or absence of their sheath (Yamamoto *et al.,* 1983).

of these mosquitoes to the parasites were as follows: to *B. pahangi* 100% in *Ae. aegypti* (Liverpool strain), 81.0% in *Ae togoi* (Nagasaki strain), 3.3% in *Cx. pipiens molestus* respectively; to *B. malayi* 83.9% in *Ae. aegypti* (Red eye-I strain), 90.0% in *Ae. togoi* (Rendaiji strain) and 0.0% in *Ar. subalbatus*, respectively.

These mosquitoes were reared at 26 ± 1°C, 60 ± 5% r.h. and adults were provided with 5% sucrose solution. They were fed on jirds 4–10 days after emergence, with the exception of *Cx. p. molestus* which were engorged after oviposition.

Count of filarial larvae in the mosquitoes

Mosquitoes were dissected 24 hrs after blood meal. Both thorax and blood mass which was taken out from the mid-gut were separately disrupted in 0.65% NaCl solution dropped onto glass slides, then the number of filarial larvae was counted after covering the specimens with coverslips.

Observation of the sheath around the larvae

At certain intervals after blood meal, each abdomen of mosquitoes whose mid-gut had been carefully taken out without tearing it was dissected in 30 μl of distilled water dropped onto a glass slide. Then the specimen was covered with a coverslip to examine existence of sheath around the larvae.

Isolation of filarial larvae from the mid-gut in vitro

The abdominal integument of mosquito was thoroughly removed in Ringer-Tyrode's solution within 30 min following engorgement. Mid-gut, thus exposed, was submerged in 0.5 ml of Grace's insect tissue culture medium (GMA) dropped onto a hollow glass slide. After 2 hrs incubation at 26 ± 1°C in the light, larvae in the medium were gathered on a glass slide for observing presence or absence of the sheath. To both Ringer-Tyrode's solution and GMA were added Penicillin (200 units/ml) and Streptomycine (100 μg/ml). In addition, GMA was supplemented by 20% heat inactivated foetal bovine serum.

Injection of microfilariae into mosquitoes

About 10 μl of tail blood taken from jirds with a heavy infection of *B. pahangi* was mixed with 0.5 ml GMA dropped on to a hollow glass slide. After one hour, microfilariae in the medium were transferred with a capillary glass tubule to another GMA where they were kept for next one hour. Rate of exsheathed microfilariae to all microfilariae collected was 4.8% in this experiment. Thirty to 50 microfilariae in about 0.8 μl GMA was injected into the abdominal haemocoel of 7-day-old female mosquito by a syringe with a fine needle made of a capillary glass tubule. The mosquitoes were reared at 26 ± 1°C, 60 ± 5% r.h. for 10 days and dissected to examine the 3rd stage larvae.

Results

I. Results of the experiments in *Brugia pahangi*

Migration of filarial larvae into thorax

The averages and standard deviations of the filarial larvae recovered from the mid-gut and thorax were shown in Table 1.

In *Ae. aegypti* (Liverpool strain), migration rates of the larvae into thorax were 25.1% when they had been fed on 73.5 mf/cmm jird and 80.9% when fed on 2.6 mf/cmm jird. Those in *Ae togoi* (Nagasaki strain) fed on 73.5 mf/cmm jird or 2.6 mf/cmm jird were 91.7% or 95.5%, respectively. On the other hand, only 1.9% of the larvae (mean 10.5, SD 11.2) were recovered in the thorax of *Cx. pipiens molestus* in spite of the large intake of microfilariae (mean 532.5, SD 226) when fed on 73.5 mf/cmm jird.

The following results were obtained using these mosquitoes having the above characters in filarial migration.

Dissection of mosquitoes immediately after blood meal

Results of dissection of *Ae. aegypti* within 20, 40 and 80 min after feeding were shown in Table 2. A few larvae were recovered from the abdominal haemocoel, among which 0, 15 and 25% larvae retained sheath at each interval of

Table 1 Number of *Brugia pahangi* larvae in the mid-gut and the thorax of *Aedes aegypti, Ae. togoi* and *Culex pipiens molestus* fed on *Meriones unguiculatus* 24 hours after blood meal

Species	Mf-density (Mf/cmm)	No. of mosquitoes dissected	No. of larvae recovered (Average ± SD)	
			In mid-gut	In thorax
Ae. aegypti	73.5	9	99.8 ± 76.7	33.5 ± 25.2 (25.1%)
	2.6	10	1.8 ± 2.0	7.6 ± 3.0 (80.9%)
Ae. togoi	73.5	5	1.5 ± 3.2	16.6 ± 30.6 (91.7%)
	2.6	10	0.4 ± 0.6	8.5 ± 7.2 (95.5%)
Cx. pipiens molestus	73.5	10	532.5 ± 226.0	10.5 ± 11.2 (1.9%)

Percentage in parentheses: No. of larvae in thorax to total larvae.

dissection after feeding. In *Ae. togoi,* on the other hand, escape of the larvae from the mid-gut took place very quickly as seen in Table 2 and the sheath retained larvae was as high as 61.5% when observed 20 min after feeding. The cast-off sheaths were also found in the specimens of the abdomens without mid-gut. In *Cx. p. molestus* which were dissected within 20 min after feeding no larva was observed from the abdominal haemocoels of 10 mosquitoes.

Isolation of filarial larvae from the mid-gut in vitro

In *Ae. aegypti,* majority of the larvae, 97.5% and 68.0% recovered from the medium retained sheath (Table 3). In *Ae. togoi,* 52.9% and 55.2% of the larvae retained sheath. Observation in *Cx p. molestus* was not done in this series of experiments.

Injection of microfilariae into mosquitoes

After extrinsic incubation period in the mosquitoes, which had been injected with microfilariae collected from blood of jirds, some degenerating 1st or 2nd stage larvae or 2nd stage larvae abnormal in appearance were observed in addition to the normal 3rd stage larvae (Table 4).

Table 2 Sheathed larvae of *Brugia pahangi* in the abdominal haemocoel of *Aedes aegypti* and *Ae. togoi*

Species	Time of dissection after blood meal (min)	No. of mosquitoes dissected	No. of sheathed larvae per all larvae recovered
Ae. aegypti	20	5	0/3 (0%)*
	40	5	2/13 (15.4%)
	80	5	2/8 (25.0%)
Ae. togoi	20	13	136/221 (61.5%)

Donor: 73.5 mf/cmm jird.

*Percentage of sheathed larvae in all larvae recovered from the abdominal haemocoels.

In *Ae. aegypti,* 14 out of 53 mosquitoes were alive 10 days after injection of microfilariae and an average number of the 3rd stage larvae per mosquito was 12.7, which would be equivalent to 25% of the injected microfilariae provided that each mosquito had received exactly 50 microfilariae (Table 4). In *Ae.*

Table 3 Sheathed larvae of *Brugia pahangi* emerging from the mid-guts of *Aedes aegypti* and *Ae. togoi* into the culture medium during 2 hours' incubation

Species	Mf-density (Mf/cmm)	No. of Mid-guts incubated	No. of sheathed larvae per larvae recovered from the medium
Ae. aegypti	73.5	5	232/238(97.5%)**
	2.6	13	51/75(49)*(68.0%)**(41.1%)***
Ae. togoi	73.5	7	164/310(52.9%)**
	2.6	10	24/46(26)*(52.2%)**(33.3%)***

*Number in parentheses: No. of larvae recovered from blood masses within mid-guts.
**Percentage of sheathed larvae in all larvae recovered from the medium.
***Percentage of sheathed larvae recovered from the medium to all larvae recovered from both medium and blood masses.

Table 4 The third stage larvae obtained from *Aedes aegypti, Ae. togoi* and *Culex pipiens molestus* injected with *Brugia pahangi* microfilariae collected from blood of *Meriones unguiculatus*

Species	No. of mosquitoes examined	No. of mosquitoes with the 3rd stage larvae	No. of the 3rd stage larvae obtained	
			Total	Average
Ae. aegypti	14	13	178(170)*	12.7(25%)**
Ae. togoi	10	10	177(81)	17.7(35%)
Cx. pipiens molestus	9	8	126(37)	14.0(28%)

Each mosquito was injected with 30 to 50 microfilariae.
*Number in parentheses: No. of both 1st and 2nd stage larvae recovered from mosquitoes.
**Percentage in parentheses: The 3rd stage larvae per microfilariae injected, on the assumption that each mosquito was provided with just 50 microfilariae.

togoi, 10 out of 43 mosquitoes injected with microfilariae were alive same period after injection and an average number of the 3rd stage largae was 17.7, which would be equivalent to 35.4%. In *Cx. p. molestus,* 9 out of 38 mosquitoes survived 10 days after injection and showed an average number of the 3rd stage larvae of 14.0. It is interesting that filarial larvae could complete their cycle even in the refractory mosquitoes, when microfilariae entered through unusual route.

II. Results on the experiments in *Brugia malayi*

Dissection of mosquitoes immediately after blood meal

Ae. aegypti which had been fed on the jird infected with *B. malayi* (1.5 mf/cmm) were dissected at 20, 40 and 80 min after engorgement (Table 5). Only 7 larvae were recovered from the abdominal haemocoels of 30 mosquitoes, among which 1 larva retained sheath. In *Ae. togoi* which were dissected 20 min after engorgement, 35.7% of larvae obtained from 10 abdominal haemocoels retained sheath and

Table 5 Sheathed larvae of *Brugia malayi* in the abdominal haemocoels of *Aedes aegypti, Ae. togoi* and *Armigeres subalbatus*

Species	Time of dissection after blood meal (min)	No. of mosquitoes dissected	No. of sheathed larvae per all largae recovered
Ae. aegypti	20	10	0/0 (172)*(0%)**
	40	10	1/3 (146) (33.3%)
	80	10	0/4 (87) (0%)
Ae. togoi	20	10	5/14(34) (35.7%)
Ar. subalbatus	20	10	16/30(316) (53.3%)

Donor: 1.5 mf/cmm jird.
*Number in parentheses: No. of larvae recovered from blood masses within mid-guts.
**Percentage of sheathed larvae in all larvae recovered from the abdominal haemocoels.

Table 6 Sheathed larvae of *Brugia malayi* emerging from the mid-gut of *Aedes aegypti, Ae. togoi* and *Armigeres subalbatus* into the culture medium during 2 hours' incubation

Species	No. of mid-guts incubated	No. of sheathed larvae per larvae recovered from the medium
Ae. aegypti	12	9/16(40)*(56.3%)**(16.1%)***
Ae. togoi	11	33/49(17) (67.3%) (50.0%)
Ar. subalbatus	11	25/34(67) (73.5%) (24.8%)

Donor: 1.5 mf/cmm jird.
*, **, ***: see Table 3.

in *Ar. subalbatus* sheaths were observed in 53.3% of the larvae obtained from 10 abdominal haemocoels.

Isolation of microfilariae from the mid-gut in vitro

Result is shown in Table 6. In *Ae. aegypti* 56.3% of the larvae which were collected from the medium retained sheath. In the case of *Ae. togoi* and *Ar. subalbatus* sheath retained larvae in the medium were 67.3% and 73.5% respectively. Among three species, escape of the larvae from the mid-gut *in vitro* was the highest in *Ae. togoi* showing 74.2%.

Discussion

Abnormal first stage larvae which retained sheath were observed in the thorax of mosquitoes which had been fed on a cat infected with *B. pahangi* (Schacher, 1962). Sheathed larvae of *B. pahangi* were also observed in the haemocoels of *An. quadrimaculatus, Ae. aegypti* and *Ae. albopictus* (Ewert, 1965).

In these two papers exheathment which takes place in the abdominal haemocoel was not referred to. Yamamoto *et al.* (1983), however, have shown the presence of many sheathed larvae of *B. pahangi* in the abdominal haemocoel of *Ar. subalbatus* immediately after blood meal and that exsheathment of artificially injected microfilariae could occur in the abdominal haemocoel of the mosquitoes. Christensen and Sutherland (1984), moreover, have shown that sheathed larvae of *B. pahangi* and *B. malayi* completely free themselves of the sheath in the haemocoel in the case of

Ae. aegypti. Present observations using *Ae. aegypti, Ae. togoi* together with *Ar. subalbatus* have shown similar results.

In *Ae. aegypti,* migraiton rate of *B. pahangi* larvae to the thorax during 24 hrs after blood meal was lower in the mosquitoes fed on the donors of high microfilaria density. This trend of low migraiton rate when mosquitoes ingested a large number of the larvae was noted by Brenguess and Bain (1972). Therefore, penetration by the sheathed larvae might be due to the density effect in the ingested microfilariae in the mid-gut. Data obtained from the incubation of the mid-guts suggested that this is not the case. In *Ae. togoi,* more than 90% of the ingested *B. pahangi* microfilariae were estimated to migrate into the thorax. Sheathed larvae were also obtained from both abdominal haemocoel and culture media where the mid-guts were incubated.

The fact that about 25 to 35% of the injected *B. pahangi* microfilariae developed to the 3rd stage larvae in *Ae. aegypti* and *Ae. togoi* suggests that injected microfilariae cast off sheath in the haemocoel. Therefore, it is most probable that in naturally infected mosquitoes, *Ae. aegypti* and *Ae. togoi,* exsheathment should take place in the haemocoel as well as in the mid-gut. Observation of the presence of cast-off sheaths in the abdominal haemocoels of *Ae. togoi* supports this speculation.

Sheathed larvae were also found in the abdominal haemocoels of *Ae. aegypti, Ae. togoi* and *Ar. subalbatus* fed on the jird infected with *B. malayi.* In the incubation of their mid-guts, sheaths were found in about 50% of the larvae recovered from culture media. It is obvious that *B. malayi* larvae also penetrate through mid-gut wall to the abdominal haemocoel regardless of presence or absence of their sheath.

As a result, it might be better to put aside the conventional conception that microfilariae penetrate through the mid-gut wall to the abdominal haemocoel after casting off their sheath in the mid-gut.

Summary

The present paper describes some experiments with exsheathment of *Brugia pahangi* and *B. malayi* microfilariae in the abdominal haemocoel of the mosquitoes, *Aedes aegypti, Ae. togoi, Culex pipiens molestus* and *Armigeres subalbatus.*

Sheathed larvae were recovered from the abdominal haemocoel of *Ae. aegypti* and *Ae. togoi* fed on the jirds infected with *B. pahangi.* Sheathed larvae of *B. pahangi* were also found in the medium where the mid-gut of *Ae. aegypti* and *Ae. togoi* had been incubated for 2 hrs. Approximately 40% of the ingested larvae were supposed to penetrate through the mid-gut wall to the haemocoel without casting off their sheath when mosquitoes had been fed on the donors of relatively low microfilaria density (2.6 mf/cmm). About 20% or more of microfilariae which were collected from the donor's blood and injected into the abdominal haemocoel of *Ae. aegypti, Ae. togoi* and *Cx. pipiens molestus* were able to develop to the 3rd stage larvae.

In the experiments of *B. malayi,* sheathed larvae also observed in the abdominal haemocoal of *Ae. aegypti, Ae. togoi* and *Ar. subalbatus.*

These results suggests that exsheathment of the sheathed larvae takes place commonly in the haemocoel as well as in the mid-gut and that they are able to complete their development to the 3rd stage larvae.

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フィラリア症に関する研究V：*Brugia pahangi* および *B. malayi* ミクロフィラリアの *Aedes aegypti, Ae. togoi, Culex pipiens molestus* および *Armigeres subalbatus* 体内における脱鞘

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*Brugia pahangi*感染スナネズミを吸血したネッタイシマカおよびトウゴウヤブカの腹腔から有鞘幼虫が見出された．また，これらの蚊の中腸を培養した培養液中にも有鞘幼虫が観察された．スナネズミ血液から回収した*B. pahangi*ミクロフィラリアをネッタイシマカやトウゴウヤブカおよびチカイエカの腹部体腔に注入したところ，約20%あるいはそれ以上のミクロフィラリアが感染幼虫にまで発育した．*B. malayi*についても，ネッタイシマカやトウゴウヤブカおよびオオクロヤブカに取り込まれたミクロフィラリアの一部は脱鞘することなく中腸壁を穿通して体腔に出ることが確認された．