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Studies on Filariasis II: Exsheathment of the Microfilariae of *Brugia pahangi* in *Armigeres subalbatus*

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Microfilariae of some filarial species such as *Wuchereria bancrofti*, *Brugia malayi* and *B. pahangi* are enclosed within the egg shell which forms a tubular sheath around the larval body (Devaney and Howells, 1979). It has been reported that the sheath is cast in the midgut of the insects and then unsheathed microfilariae burrow into the midgut wall to enter the abdominal haemocoel in susceptible mosquitoes (Aoki, 1971a). In fact, cast-off sheath of *W. bancrofti* microfilariae is found among the ingested erythrocytes in the midgut of the vector mosquitoes (Wilkocks and Manson-Bahr, 1972).

It has been also presented that the exsheathment of *B. pahangi* microfilariae takes place almost immediately on their arrival to the midgut of *Anopheles quadrimaculatus* and those which emerge from the ingested blood mass are usually devoid of sheath (Esslinger, 1962). However, the details of *in vivo* exsheathment in *Brugia spp.* have not been fully understood, though it is known that the cast-off of the sheath occurs within a few hours after the infective blood meals among some species of susceptible mosquitoes (Ewert, 1965).

In this paper, the authors present the result of the observations on the exsheathment of *B. pahangi* microfilariae in *Armigeres subalbatus*, one of the susceptible mosquitoes to the parasite. The results suggest the strong possibility that the exsheathment of the microfilariae takes place in the abdominal haemocoel rather than in the midgut.

Materials and Methods

Parasite

A mongolian jird, *Meriones unguiculatus*, infected with *B. pahangi* was used for the source of blood meal. The average number of microfilariae in blood was 50.7 per cmm. After brief anaesthesia by ethylether, the jird was fixed on a fixing apparatus to expose to the mosquitoes.

Mosquitoes

Armigeres subalbatus, Rendaiji strain, were reared at 26±1 C and 60±5% RH in the insectarium. Mean infective rate of the mosquitoes on the 9th day after infective blood meal was 100%.

Dissection of mosquitoes and an outline of in vitro cultures

Ten or 30 minutes after infective blood

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meal, the abdomen of the mosquito was dissected without tearing the midgut in a drop of distilled water on a slide. The head, thorax and midgut were taken out and then the remainder was crushed. Then, the specimens were covered with a coverslip for examining the presence of the sheath under a microscope.

The abdomen of mosquito was dissected in Ringer-Tyrode's solution containing penicillin (200 units/ml) and streptomycine (100 μg/ml), within 30 minutes of infective blood meal. The intact midgut taken out carefully from a mosquito was submerged in Grace's insect tissue culture medium (GMA) which had been dropped on a hollow slide glass previously. Both the thorax and abdomen of mosquitoes were dissected at 2 hours after feeding and the intact alimentary canal was placed in GMA in the same way.

The culture vessel, hollow slide glass, was kept at 26±1 C under the light for 2 hours, then the larvae which emerged from the midgut were collected on a slide glass t oobserve the presence or absence of the sheath. GMA was supplemented with 20% heat-inactivated foetal bovine serum and the antibiotics were also added at the concentrations mentioned above.

Injection of microfilariae into mosquitoes

About 10 μl of blood of a jird infected with *B. pahangi* was transferred to a hollow slide containing Ringer-Tyrode's solution. After 1 hour, microfilariae wriggling on the bottom were sucked by a capillary tubule and transferred again to another hollow slide, in which they were kept for another 1 hour. Then, they were injected into abdominal haemocoel of the female *Ar. subalbatus* by two ways, using a capillary tubule pointed at head. One group of the mosquitoes whose thoraco-abdominal junction were ligated by a fine thread and the other one without ligation were received an innoculant of approximately 0.8 μl GMA containing 30 to 50 microfilariae individually. The former was kept at 26 C for 12 hours and then was subjected to the examination of the sheath, while the latter was dissected for examining the presence of the 3rd stage larvae on the 10th day after injection.

On the other hand, collected microfilariae were packed in a glass tubule, 10 mm long, 1.0 mm in diameter, sealed both side, as a control and it was kept at 26 C for 12 hours. Then, all the microfilariae were examined whether the exsheathment had occurred spontaneously.

Results

Dissection of mosquitoes after the infective blood meal

As shown in Table 1, 68 (83.9%) out of 81 which were recovered from abdominal

Table 1 Sheathed larvae* of *Brugia pahangi* in the abdominal haemocoel of *Armigeres subalbatus* dissected 10 minutes and 30 minutes after an infective blood meal

Time after infective blood meal	No. (%) of sheathed larvae per larvae recovered			
	In each abdominal haemocoel			Total
10 minutes	22/29 (75.8), 17/19 (89.4),	6/7 (85.7), 4/4 (100)	19/22 (86.3),	68/81 (83.9)
30 minutes	2/8 (25.0), 6/31 (19.3)	7/50 (14.0),	1/7 (14.2),	16/96 (16.6)

* Sheathed larvae are the larvae which have just come out from the midgut wrapped in the sheath.

haemocoel 10 minutes after infective blood meal were found to be in sheath, that is, apparently microfilariae. Concerning to the terminology of the larvae that just came out of the midgut and that were still in sheath will be discussed later.

In case of 30 minutes-dissection 16 (16.6%) out of 96 were sheathed.

Results obtained by the in vitro cultures of the infected midgut

The rate of microfilariae per larvae recovered from each culture vessel ranged from 22.5 to 98.5% in the culture of exposed midgut taken out from mosquitoes within 30 minutes after an infective blood meal. After all, 276 (64.9%) out of 425 were found sheathed as seen in Table 2.

In those of alimentary canals of 2 hours after infection, it ranged from 10.5 to 93.7%. Forty (42.1%) out of 95 were apparently microfilariae, *i.e.*, sheathed, as a whole.

Injection of microfilariae into mosquitoes

It was found that only 4 (2.0%) out of 200 microfilariae were devoid of sheath among those collected from the bottom of a hollow slide containing GMA. As shown in Table 3, it was also confirmed that the microfilariae kept in sealed glass tubules for 12 hours showed only 1.4% exsheathment.

Microfilariae which had been inoculated into the abdominal haemocoels of the ligated abdomens showed exsheathment of 45.3% on 12 hours post-injection.

Among 30 mosquitoes injected with 30 to 50 microfilariae into the haemocoel without ligation, 25 mosquitoes were alive

Table 2 Sheathed larvae of *Brugia pahangi* emerged from the midgut of *Armigeres subalbatus* to the artificial medium at 2 hours culture

Time until setting of *in vitro* culture	No. (%) of sheathed larvae per larvae recovered from the medium			
	In each culture vessel			Total
Within 30 minutes of blood meal	7/31 (22.5), 25/38 (65.7), 9/10 (90.0),	70/140 (50.0), 32/46 (69.5), 21/23 (91.3),	26/46 (56.5), 16/20 (80.0), 70/71 (98.5)	276/425 (64.9)
Two hours after blood meal	2/19 (10.5), 9/20 (45.0),	2/12 (16.6), 9/19 (47.3),	3/9 (33.3), 15/16 (93.7)	40/95 (42.1)

The term of the sheathed larvae is explained in Table 1.

Table 3 Exsheathment of *Brugia pahangi* microfilariae in the haemocoel of the isolated abdomen by ligation between thoraco-abdominal junction of *Armigeres subalbatus*

	No. (%) of exsheathed larvae per larvae recovered			
	In each abdominal haemocoel			Total
Isolated abdomen	11/46 (23.9), 20/54 (37.0), 15/25 (60.0),	12/36 (33.3), 19/40 (47.5), 19/29 (65.5),	6/17 (35.2), 9/15 (60.0), 24/36 (66.6)	135/298 (45.3)
Glass tubule	0/58 (0.0), 5/106 (4.7)	0/196 (0.0),	2/120 (1.6),	7/480 (1.4)

Thrity to 50 microfilariae were injected into each isolated abdomen. Results were read 12 hours after injection of microfilariae.

Table 4 The 3rd stage larvae obtained from *Armigeres subalbatus* injected with microfilariae of *Brugia pahangi* which were collected from blood of *Meriones unguiculatus*

No. of mosquitoes injected	No. of the 3rd stage larvae obtained from each mosquito	Average
30(5)*	0, 0, 0, 0, 0, 0, 0, 1, 1, 1, 2, 2, 2, 3, 3, 3, 3, 4, 5, 5, 5, 7, 7, 10, 12	3.04

Each mosquito was injected with 30 to 50 microfilariae.
Figure in parentheses* shows the number of mosquitoes died before 10 days after injection.

on 10 day post-injection. Number of the 3rd stage larvae per mosquito are shown in Table 4. It was found that 3.04 (ranging 0 to 12) larvae in average could complete their development in the mosquitoes when microfilariae were injected.

Discussion

In the abdominal haemocoel of *Armigeres subalbatus* 10 minutes after an infective blood meal with *Brugia pahangi* microfilariae, about 80% of the larvae have been observed as microfilariae, judging from the presence of the sheath. The term, *microfilariae*, has been commonly used in the larvae found in the peripheral blood and/or tissues of the definitive host. On the other hand, there have been some confusions on the term which should be used exactly for the larvae of the very early stage in the intermediate host. For example, microfilariae, microfilari-form larvae, sheathed microfilariae and so on have been adopted in many literatures to describe those found in the midgut and/or abdominal haemocoel just after coming out of the midgut. In the present study, it was found that many larvae wrapped in the sheath, apparently looked like microfilariae, could come out of the midgut.

The authors' opinion is that the term "microfilariae" should be used only in the stage of the definitive host. It seems reasonable to consider that the microfilariae which once emerged from the mosquitoes' midgut, regardless to sheathed or not, might have already started their development. Therefore, the authors prefer the term "sheathed larvae" to indicate specifically those that just came out of the midgut and still wrapped in the sheath, tentatively. As the term "sheathed larvae" will remain different opinions, the matter should be considered and a decision be made in near future.

On the dissection of mosquitoes 30 minutes after infective blood meal, 85% of the larvae, recovered from haemocoel had already exsheathed (Table 1). This result suggests that the exsheathment takes place in the abdominal haemocoel, and that sheathed larvae found early in the haemocoel may abort their development and some other larvae which exsheathed in the midgut just prior to penetrate it could begin further development. In the *in vitro* culture of the infected midgut, about a half of the larvae piecing out through the midgut wall were provided with sheath irrespective of the time after infective blood meal (Table 2). It was also found that the exsheathment of microfilariae had occurred in 45.3% at the abdominal haemocoel of the mosquitoes ligated at the thoraco-abdominal junction (Table 3).

Summarizing these results obtained, it may be concluded that the exsheathment of microfilariae of *B. pahangi* ingested by

Ar. subalbatus takes place in the abdominal haemocoel. A finding that the injected microfilariae into mosquitoes developed to the 3rd stage strongly support this suggestion (Table 4).

Ewert (1965) reported the presence of sheathed larvae of *B. pahangi* in the thorax and abdominal haemocoel of *Anopheles quadrimaculatus, Aedes aegypti* and *Ae. albopictus.* Therefore, the sheathed larvae of *B. pahangi* may also come out of the midgut even in other species, such as *Ae. togoi* and *Culex pipiens.*

In vitro exsheathment of microfilariae in *Wuchereria bancrofti, B. malayi, B. pahangi* and *Litomosoides carinii* has been studied by some authors (Weinstein, 1963; Katamine and Aoki, 1970; Aoki, 1979a, 1979b; Devaney and Howells, 1979). However, the factors responsible for inducing exsheathment *in vivo* have not been elucidated (Devaney and Howells, 1979).

Results obtained from the present experiments indicate the importance of abdominal haemocoel for *in vivo* exsheathment of *B. pahangi* microfilariae.

Summary

1) Microfilariae of *Brugia pahangi* taken by *Armigeres subalbatus* could penetrate the midgut wall without casting off their sheath. On the dissection of 10 minutes after infective blood meal, 68 (83.9%) out of 81 were sheathed.

2) In the short time *in vitro* cultures of the infected midgut which were taken out of mosquitoes, about a half of the larvae were found sheathed in the medium.

3) Among the microfilariae injected into the abdominal haemocoel which had been ligated at the thoraco-abdominal junction, some 45% of them exsheathed during 12 hours post-injection.

4) Average number of 3.04 3rd stage larvae were found from the batch of 25 mosquitoes that had been injected with 30 to 50 microfilariae into haemocoel.

5) From the results, it is clarified that the exsheathment of microfilariae of *B. pahangi* ingested by *Ar. subalbatus* takes place in the abdominal haemocoel of this species.

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糸状虫症に関する研究 II. *Brugia pahangi* のミクロフィラリアのオオクロヤブカ体内における脱鞘について

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Brugia pahangi に感染させたスナネズミにオオクロヤブカを吸血させ，吸血後10分および30分後に解剖し，腹腔内に遊出した幼虫の鞘の有無を調べた．前者ではその 83.9% が，後者では 15.0% が被鞘しており，一見ミクロフィラリアと変わらなかった．そこで，吸血後30分以内および２時間後に蚊を解剖して，その中腸を無傷のまま取り出し，20% 牛胎児非働化血清を添加したグレース培養液に沈めて幼虫を遊出させた．この場合，前者では 64.9%，後者では 42.1% が被鞘していた．一方，無吸血蚊の胸腹間を結紮した上，腹腔内にミクロフィラリアを注入して，12時間後に腹腔内の幼虫を観察したところ，平均 45.3% は脱鞘していた．対照として，同じロットのミクロフィラリアを毛細管に封入し12時間後に観察した．この群では平均1.4% しか脱鞘がみられなかった．また，感染スナネズミの末梢血から集めたミクロフィラリアを毛細管を利用して 30～50 隻ずつ30匹の蚊の腹腔内に注射し，10日間飼育した群では25匹の蚊がそれまで生存し，蚊一匹当り平均 3.04 のIII期幼虫を保有していた．

以上の結果から，1)従来，一般に考えられていたように幼虫は蚊中腸内で脱鞘して出て来るのではなくてオオクロヤブカに取り込まれたミクロフィラリアの多くは脱鞘することなく中腸から脱出する．2) *in vitro* の実験でもミクロフィラリアは中腸壁を高率に被鞘したまま脱出することが確認された．また結紮した腹腔内では高率にミクロフィラリアの脱鞘が起っていることがわかった．しかし，対照としてとったガラス毛細管内に12時間保存したミクロフィラリアでは脱鞘は極めて小数であった．3) 腹腔内に人工的に注入されたミクロフィラリアの一部はIII期幼虫に迄発育することが出来る．即ち，ミクロフィラリアは蚊の中腸内で必ずしも脱鞘をしないことがわかった．1)～3)を総合して考察すると，*Brugia pahangi* とオオクロヤブカの組合せによる実験系では，ミクロフィラリアは高率に被鞘したまま腹腔内に脱出すること，そしてこれらはその後正常に発育してIII期幼虫にまで達するものと考えられた．また，腹腔内の条件が幼虫の脱鞘に関与している可能性が推察された．

一方，脱鞘しないままで腹腔内に脱出している幼虫は外見上ミクロフィラリアそのものである．従って従来の如く，中腸内での脱鞘以降をI期幼虫と呼称すれば，著者らの観察した幼虫はミクロフィラリアとしか呼びようがない．従って，本論文では，とりあえずこれらを “sheathed larvae” として区別した．しかし，この用語が最適とも思われないので近い将来に用語の統一をはかる必要がある．