[Jpn. J. Parasitol., Vol. 40, No. 4, 358–362, August, 1991]

Effect of Sheath on *in vitro* Melanization of Microfilariae of *Brugia malayi* and *B. pahangi*

MUTSUO KOBAYASHI AND HISASHI YAMAMOTO

(Accepted for publication; July 17, 1991)

Abstract

In *in vitro* melanization of microfilariae (Mf) of *Brugia* spp. using pupal haemolymph of mosquitoes, *Armigeres subalbatus,* Mf of *Brugia malayi* with sheath are less melanized than those of *B. pahangi.* After artificial exsheathment of Mf, *B. malayi* showed strong melanization, while *B. pahangi* showed a very weak reaction. These results entirely agree with the data of melanizing responses in experimental infection of *Ar. subalbatus* with *B. malayi* and *B. pahangi.* Surface properties of cuticles of Mf from both filarial species seem to be important in eliciting the melanization responses of *Ar. subalbatus.*

Key words: *in vitro* melanization, microfilaria, exsheathment, *Armigeres subalbatus, Brugia pahangi, B. malayi.*

Introduction

Numerous reports have been published on the melanization and/or encapsulation of the filarial larvae in mosquitoes (Burton, 1963; Oothman *et al.*, 1974; Christensen, 1981; Yamamoto *et al.*, 1985). However, the mechanisms by which melanin is deposited on the surface of parasites is largely unknown. In experimental infection of *Armigeres subalbatus* with *Brugia malayi,* about 60% of ingested microfilariae (Mf) were melanized in the abdominal haemocoel and the larvae which migrated to thoracic muscles degenerated during 2 or 3 days post-infection (Yamamoto *et al.*, 1985; Kobayashi *et al.*, 1986a). On the other hand the larvae of *B. pahangi,* that is a sibling species of *B. malayi,* normally developed to infective larvae and the number of melanized larvae was a few (Yamamoto *et al.*, 1985). Mf of *B. malayi* and *B. pahangi* are morphologically similar to each other and definitive identification of both species is based on the distribution pattern of acid phosphatase activity of Mf (Redington, *et al.*, 1975; Yen & Mak, 1978; Kobayashi, *et al.*, 1987). But in the mosquito, *Ar. subalbatus,* these Mf are clearly recognized each other by the defense system (Kobayashi and Yamamoto, 1988). Recently, it is clearly shown that humoral encapsulation (melanization) first takes place around Mf in the haemocoel of mosquitoes and then haemocytes start to attach to and spread on the surface of melanotic capsules (Chen and Laurence, 1985; Kobayashi *et al.*, 1986b; Chen, 1988) although some evidences of direct participation of mosquito haemocytes are suggested (Forton *et al.*, 1985). It was also reported that heat-killed Mf preincubated with pupal mosquito haemolymph supplemented with phenylthiourea (PTU, phenoloxidase inhibitor) were strongly melanized *in vitro* when they were incubated with supernatant of thermocoagulated haemolymph which was not supplemented with PTU (Kobayashi, *et al.*, 1988). The results show that prophenoloxidase (proPO) or activated phenoloxidase (PO) attach to the surface of Mf in the first incubation without any participation of haemocytes, and in the second incubation of Mf with supernatant of thermocoagulated haemlymph they are melanized *in vitro.*

In *in vitro* melanization Mf of *B. pahangi* constantly showed a strong reaction comparing with those of *B. malayi.* This *in vitro* reaction

Department of Medical Zoology, Dokkyo University School of Medicine, Mibu, Tochigi, 321-02, Japan.
小林睦生　山本　久（獨協医科大学医動物学教室）

clearly conflicts with the results observed in mosquito, *Ar. subalbatus* which showed a strong melanization reaction against *B. malayi* (Yamamoto *et al.*, 1985). The present work was carried out to study the effect of exsheathment of Mf on *in vitro* melanization.

Materials and Methods

Haemolymph of mosquito

Haemolymph of mosquito was collected from 1-day old female pupae of *Ar. subalbatus* (406 strain) by the centrifugation method according to Ogura *et al.* (1985) with a modification, *i.e.* an addition of 3 to 5 mg of PTU to centrifugation tubes. The supernatant of haemolymph was stored in a freezer at –80°C until used.

Collection of microfilariae

Microfilariae (Mf) of *B. malayi* and *B. pahangi* were collected from the infected jirds by injecting wormed Hanks balanced salt solution (HBSS) into the peritoneal cavity and then sucking it out. The fluid containing Mf was incubated for 30 min in plastic Petri dishes to eliminate peritoneal cells and then Mf were collected into centrifugation tubes by Pasteur pipettes. These Mf were washed with HBSS by repeated centrifugation and finally washed with distilled water. An aliquot of the Mf suspension was smeared on glass slides and the rest of Mf was kept in a freezer at –30°F for later use.

Artificial exsheathment of microfilariae

Mf of *B. malayi* were treated with papain (0.5% in Dulbecco's PBS pH 7.0) for 60 min and ones of *B. pahangi* were treated with 2.0% papain solution for 60 min at 37°C. In these conditions above 90% of Mf exsheathed. The exsheathed Mf were washed with distilled water by centrifugation and an aliquots of Mf suspension were smeared on the glass slide.

In vitro melanization of sheathed and artificially exsheathed microfilariae

About 10 μl of haemolymph was applied on the glass slides previously smeared with sheathed and exsheathed Mf and then incubated for 2 hrs at 20°C in a wet chamber. The haemolymph on the glass slides was found to have no formation of blackish pigment during this incubation. Thereafter, the glass slides were fully washed with PBS and then the smeared Mf were incubated with 20 μl of 3,4-dihydroxyphenyl-L-alanine (DOPA, 0.02 M in PBS) for 1 hr at 20°C in the wet chamber. Melanin formation on the surface of Mf was observed under a microscope and the degree of melanization was scored on a scale of 0–3. Microfilariae showing no evidence of melanization were scored 0; those with less than half of their surface covered were scored 1; those with more than half of their surface covered were scored 2; and completely melanized Mf were scored 3.

Results

In vitro melanization of sheathed and exsheathed microfilariae of Brugia malayi and B. pahangi

Results of *in vitro* melanization of sheathed and exsheathed Mf of both *Brugia* species were shown in Table 1. In sheathed Mf of *B. pahangi* about 40 percent of Mf showed a strong melanin deposition, which were covered with melanin more than half of their surface, but artificially exsheathed Mf were melanized weakly and about 50 percent of Mf showed no evidence on melanization. On the contrary sheathed Mf of *B. malayi* showed a extremely weak melanization and about 80 percent of sheathed Mf showed no evidence on melanization. No sheathed Mf of *B. malayi* showing strong melanization over scale 2 were observed in these experiments. In the exsheathed Mf of *B. malayi* they showed a strong melanin deposition (Fig. 1) and about 65 percent of Mf were covered with melanin more than half of their surface. These results entirely agree with the data of melanizing responses in experimental infection of *Ar. subalbatus* with *B. malayi* and *B. pahangi.*

Discussion

In the experimental infection of the mosquito, *Ar. subalbatus* with *B. pahangi,* Mf penetrated into abdominal haemocoel show a weak melani-

Table 1 Effect of exsheathment of microfilariae of *Brugia malayi* and *B. pahangi* on *in vitro* melanization

Microfilariae (Mf)	No. of experiments	Total no. of Mf observed	Degree of melanization* 0	1	2	3
Brugia malayi						
Sheathed Mf	5	268	209	59	0	0
Exsheathed Mf†	5	161	0	56	60	45
Brugia pahangi						
Sheathed Mf	6	637	81	308	47	201
Exsheathed Mf‡	7	495	254	201	21	19

* The degree of melanization was scored on a scale of 0–3.
0: microfilariae showing no evidence on melanization
1: those with less than half of their surface covered
2: those with more than half of their surface covered
3: completely melanized Mf
† Microfilariae were treated with papain (0.5% in PBS) for 60 min.
‡ Microfilariae were treated with papain (2.0% in PBS) for 60 min.

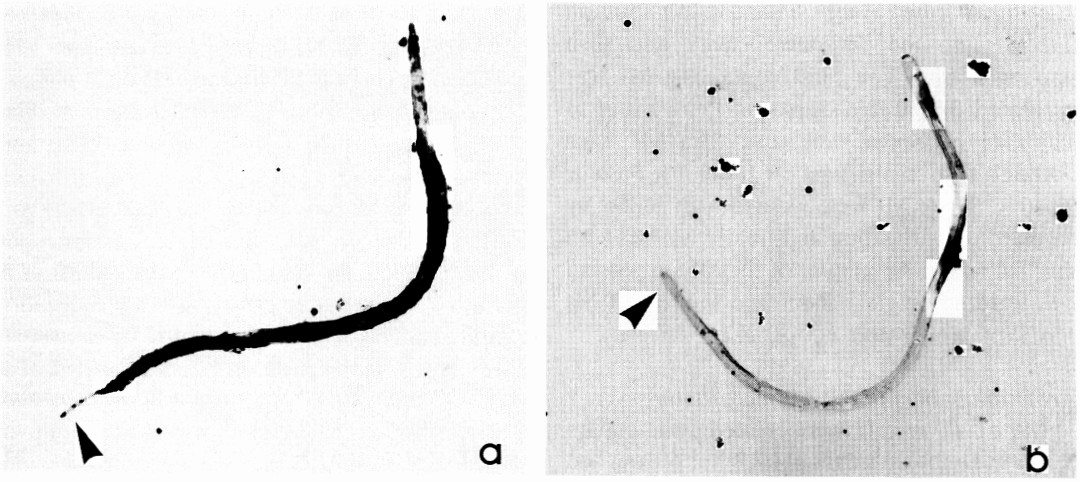

Fig. 1 *In vitro* melanization of artificially exsheathed and sheathed microfilariae (Mf) of *Brugia malayi*.
a) Strongly melanized exsheathed Mf
b) Weakly melanized sheathed Mf
Arrowheads show tail of sheathed and exsheathed microfilariae.

zation during 24 hrs post-infection. Conversely Mf of *B. malayi* are strongly melanized in the haemocoel of the mosquito (Yamamoto *et al.*, 1985; Kobayashi *et al.*, 1986). However, the results of *in vitro* melanization using sheathed Mf of both *Brugia* species showed that Mf of *B. pahangi* were more strongly melanized as compared with those of *B. malayi*. Artificially exsheathed Mf of *B. pahangi* showed weak melanization, however, those of *B. malayi* were strongly melanized *in vitro*. These results completely agree with the data of melanizing

responses in experimental infection of *Ar. subalbatus* with *B. pahangi* and *B. malayi*.

Recently it is shown that the exsheathment of Mf of *Brugia* spp. mainly take place in the haemocoel of mosquito, not in the midgut (Yamamoto *et al.*, 1983; Christensen and Sutherland, 1984; Chen and Shih, 1988). In the abdominal haemocoel of *Ar. subalbatus* sheathed Mf of *B. pahangi* could be strongly melanized, but quick exsheathment in the haemocoel of mosquito may avoid the defence responses. Conversely sheathed Mf of *B. malayi* could be weakly melanized in the haemocoel, but after exsheathment they are strongly melanized.

It is plausible that some differences in surface properties of the cuticle of *B. pahangi* and *B. malayi* Mf are present. The carbohydrates on the surface of sheathed Mf of *B. malayi* is reported by Kaushal *et al.* (1984), but detailed work on exsheathed Mf has not been done. In *B. pahangi* sheathed Mf were stained with FITC-labeled wheat germ lectin (WGA) but exsheathed Mf treated with papain and sonication did not react with this lectin (Furman and Ash, 1983). Recently, Zahedi *et al.* (1990) shows that calcium-exsheathed Mf of *B. malayi* also reacted with FITC-conjugated WGA. These results means that surface carbohydrates of cuticle in both brugian nematodes are clearly different. Same difference is also observed in our experiments (unpublished data). The differences in *in vitro* melanization of sheathed Mf from both species which showed both strongly positive reaction with WGA are not able to elucidate simply at a moment, because elicitor or trigger of proPO activating system in mosquitoes and the mechanism of proPO or PO attachment are not fully understood.

In the present results exsheathed Mf of *B. malayi* are strongly melanized *in vitro* and almost all of exsheathed Mf of *B. pahangi* are not melanized. These results will be valuable to understand defence responses of the mosquito to exsheathed Mf of *B. malayi* and *B. pahangi*.

References

1) Burton, G. J. (1963): Encapsulation of *Wuchereria bancrofti* in seven species of mosquitoes in British Guiana. Am. J. Trop. Med. Hyg., 12, 870–876.
2) Chen, C. C. and Laurence, B. R. (1985): An ultrastructural study on the encapsulation of microfilariae of *Brugia pahangi* in the haemocoel of *Anopheles quadrimaculatus*. Int. J. Parasitol., 15, 421–428.
3) Chen, C. C. (1988): Further evidence of both humoral and cellular encapsulations of sheathed microfilariae of *Brugia pahangi* in *Anopheles quadrimaculatus*. Int. J. Parasitol., 18, 819–826.
4) Chen, C. C. and Shih, C. M. (1988): Exsheathment of microfilariae of *Brugia pahangi* in the susceptible and refractory strains of *Aedes aegypti*. Ann. Trop. Med. Parasitol., 82, 201–206.
5) Christensen, B. M. (1981): Observation on the immune response of *Aedes trivittatus* against *Dirofilaria immitis*. Trans. Roy. Soc. Trop. Med. Hyg., 75, 439–443.
6) Christensen, B. M. and Sutherland, D. R. (1984): *Brugia pahangi*: Exsheathment and midgut penetration in *Aedes aegypti*. Trans. Am. Microsc. Soc., 103, 423–433.
7) Forton, K. F., Christensen, B. M. and Sutherland, D. R., (1985): Ultrastructure of the melanization responses of *Aedes trivittatus* against inoculated *Dirofilaria immitis* microfilariae. J. Parasitol., 71, 331–341.
8) Furman, A. and Ash, L. R. (1983): Analysis of *Brugia pahangi* microfilaria surface carbohydrates: comparison of the binding of a panel of fluoresceinated lectins to mature in vivo-derived and immature in utero-derived microfilariae. Acta Tropica, 40, 45–51.
9) Kobayashi, M., Ogura, N. and Yamamoto, H. (1986a): Studies on filariasis VIII: Histological observation on the abortive development of *Brugia malayi* larvae in the thoracic muscles of the mosquitoes, *Armigeres subalbatus*. Jpn. J. Sanit. Zool., 37, 127–132.
10) Kobayashi, M., Ogura, N., Tsuruoka, H., Chigusa, Y. and Mishima, S. (1986b): Studies on filariasis VII: Histological observation on the encapsulated *Brugia malayi* larvae in the abdominal haemocoele of the mosquitoes. *Armigeres subalbatus*. Jpn. J. Sanit. Zool., 37, 59–65.
11) Kobayashi, M., Yamada, K. and Yamamoto, H. (1987): Acid phosphatase activity in the hybrid microfilariae between *Brugia malayi* and *B. pahangi*. Jpn. J. Parasitol., 36, 430–432.
12) Kobayashi, M., Yamada, K. and Yamamoto, H. (1988): *In vitro* adhesion of enzyme(s) related to melanin formation in the pupal mosquito haemolymph to the surface of *Brugia pahangi* microfilaria. Jpn. J. Sanit. Zool., 39, 143–146.
13) Kobayashi, M. and Yamamoto, H. (1988): Defense mechanisms of mosquitoes. Host defense, 5, 257–265. (in Japanese with English summary)

14) Kaushal, N. A., Simpson, A. J. G., Hussain P. and Ottesen, E. A. (1984): *Brugia malayi*: Stage-specific expression of carbohydrates containing N-acetyl-D-glucosamine on the sheathed surfaces of microfilariae. Exp. Parasitol., 58, 182–187.
15) Ogura, N., Kobayashi, M. and Yamamoto, H. (1985): Haemagglutinating activity in fluid collected from the mosquito, *Armigeres subalbatus,* by centrifugation method. Dokkyo J. Med. Sci., 12, 217–221.
16) Oothman, P., Simpson, M. G. and Laurence, B. R. (1974): Abnormal development of a filarial worm, *Brugia patei* (Buckley, Nelson and Heisch), in a mosquito host, *Anopheles labranchiae atroparvus* von Thiel. J. Helminthol., 48, 161–165.
17) Redington, B. C., Montgomery, C. A., Jervis, H. R. and Hockmeyer, W. T. (1975): Histochemical differentiation of the microfilariae of *Brugia pahangi* and sub-periodic *Brugia malayi*. Ann. Trop. Med. Parasitol., 69, 489–492.
18) Yamamoto, H., Ogura, N., Kobayashi, M. and Chigusa, Y. (1983): Studies on filariasis. II. Exsheathment of the microfilariae of *Brugia pahangi* in *Armigeres subalbatus*. Jpn. J. Parasitol., 32, 287–292.
19) Yamamoto, H., Kobayashi, M., Ogura, N., Tsuruoka, H., Chigusa, Y. (1985): Studies on filariasis VI: Encapsulation of *Brugia malayi* and *B. pahangi* larvae in the mosquito, *Armigeres subalbatus*. Jpn. J. Sanit. Zool., 36, 1–6.
20) Yen, P. K. F. and Mak, J. W. (1978): Histological differentiation of *Brugia, Wuchereria, Dirofilaria* and *Breinlia* microfilariae. Ann. Trop. Med. Parasitol., 72, 157–162.
21) Zahedi, M., Denham, D. A. and Ham, P. J. (1990): Surface lectin binding characteristics of developing stages of *Brugia* in *Armigeres subalbatus*: II *Brugia malayi*. Japan. J. Trop. Med. Hyg., 18, 285–293.