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Research Note

In vitro Melanin Deposition on Heat-killed Microfilariae of *Brugia pahangi* in Haemolymph of the Mosquito, *Armigeres subalbatus*

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Defense reactions against filarial larvae in the haemocoel of mosquitoes are commonly characterized by encapsulation and melanization of the larvae (Ho *et al.*, 1982). A recent ultrastructural study of the encapsulation of *Brugia pahangi* in *Anopheles quadrimaculatus* revealed that filarial larvae are first enclosed in an acellular electron dense material ("melanin"), which is then covered by plasmatocytes (Chen and Laurence, 1985). Forton *et al.* (1985) and Christensen and Forton (1986) also found haemocyte involvement in encapsulation of *Dirofilaria immitis* in *Aedes trivittatus* and *Ae. aegypti*. Generally observed process is as follows: First, lysis of haemocytes occurs at or near the surface of filarial larvae. Then, melanin formed in the area of lysed haemocytes and haemocyte remnants adhere to the larvae. Finally, a double membrane-like structure surrounds the encapsulated filarial larvae. In this paper, a simple method of *in vitro* melanin deposition was used in order to clarify the role of haemocytes in melanin deposition on *B. pahangi* in *Armigeres subalbatus*. Experiments using heat-killed microfilariae indicated that melanin deposition can occur without direct participation of haemocytes, though haemocytes might be involved in melanin synthesis.

Sheathed microfilariae (Mf) of *B. pahangi* were isolated from the blood of *Meriones unguiculatus* using agarose gel as previously described (Ogura and Kobayashi, 1986). They were washed three times with Hanks' balanced salt solution and once with distilled water. The Mf suspension was heated at 95°C for 5 min, and 0.5 μl of the suspension which contained approximately 100 Mf was dropped onto a glass-slide. The glass-slides were dried at room temperature and then used for tests of *in vitro* melanin deposition on dead filarial larvae.

Ar. subalbatus (Rendaiji strain) for this study was reared and maintained as previously described (Ogura, 1986). Since there is no enough haemolymph in adult mosquitoes to be easily collected, female adults were injected 30 min before bleeding with 2 μl of *Aedes* saline (Hayes, 1953) to increase haemolymph volume. Since trehalose is a common sugar in the haemolymph of insects (reviewed by Wigglesworth, 1972), an experiment was conducted to determine whether or not *in vitro* melanin deposition could occur when haemolymph viscosity was increased by the injection of *Aedes* saline supplemented with trehalose. Each of 1-day-old female adults was injected with 2 μl of 10, 15, 20 or 25% trehalose dissolved in *Aedes* saline. Injections were done with a microsyringe and a fine glass needle inserted through the ventral intersegmental membrane between the 6 and 7 abdominal segments. The needle track was sealed with alon alpha A (Sankyo, Ltd., Tokyo). The dorsal abdominal integument of a mosquito was torn with forceps near heat-killed Mf (abbreviated as hk-Mf) placed on a

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glass-slide. A blunt iron needle was used to suspend hk-Mf in the spilt haemolymph and then the glass-slide was held in a wet chamber at 25°C for 30 min before observation of melanin deposition. Mf torn in pieces during this procedure were excluded from the count.

To prepare haemocyte-free haemolymph, 10 1-day-old female adults were injected with 2 μl of *Aedes* saline supplemented with trehalose (15%) 30 min before collection of haemolymph. They were torn on a glass-slide, and then the spilt haemolymph was sucked up with a glass-capillary tube (1 mm in diameter and 20 mm in length) that was previously filled with absorbent cotton 2 mm in long from the lower end in order to prevent fat bodies mixing with the haemolymph. The lower end of the capillary tube was then sealed with putty, and the tube was centrifuged at 6,000 g for 5 min in a cold room (4°C). Two μl of the supernatant was pipetted onto the hk-Mf on a glass-slide.

In 3 replicates, *in vitro* melanin deposition occurred on more than 82% of the hk-Mf in haemolymph samples taken from 1-day-old adults that were injected with *Aedes* saline (Fig. 1).

Results of 3 replicates showed obvious melanin deposition on more than 90% of the hk-Mf in haemolymph samples added with 10 or 15% trehalose. Melanin was deposited on 35.6, 38.9 and 73.2% of the hk-Mf in each haemolymph sample with 20% trehalose and on 20.8, 36.6 and 46.9% of the hk-Mf in each haemolymph sample with 25% trehalose.

The next experiment was conducted to determine whether the frequency of *in vitro* melanin deposition on hk-Mf would change in the haemolymph from female adults of various age, that were injected with 2 μl of *Aedes* saline with 15% trehalose (Fig. 2). More than 90% of the hk-Mf had melanin deposits in haemolymph from 0 and 1-day-old adults, while only 6.3% or

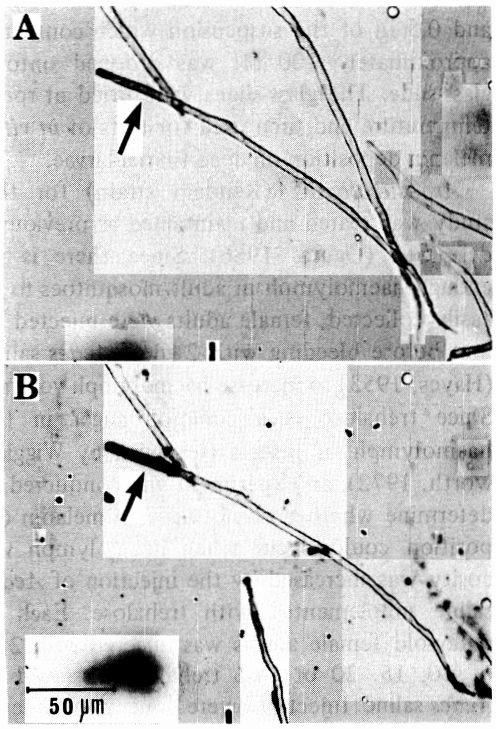

Fig. 1 *In vitro* melanin deposition on heat-killed microfilariae of *Brugia pahangi* in haemolymph taken from *Armigeres subalbatus* injected with *Aedes* saline 30 min before bleeding.
A: 5 min incubation; B: 15 min incubation
Arrows show the same portion of the same microfilaria.

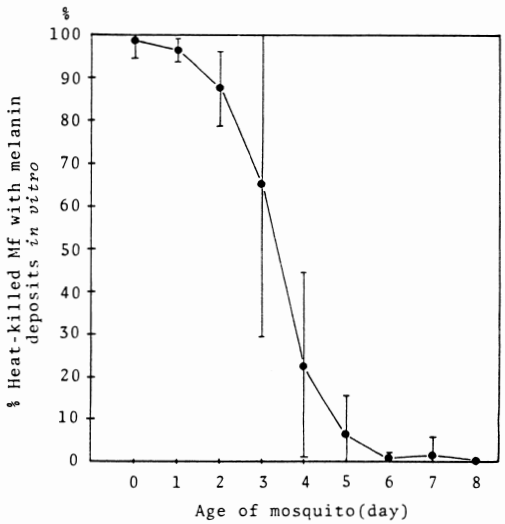

Fig. 2 Changes in the frequency of heat-killed microfilariae of *B. pahangi* with melanin deposits in haemolymph taken from the female adults of *Ar. subalbatus*. Each dot represents the average percentage obtained from 6 glass-slides.

less hk-Mf were melanized in haemolymph of 5-day and older adults. Mosquito's age affected melanization responses to filarial larvae as shown by Christensen *et al.* (1986) and Ogura (1986). It is possible that the change in *in vitro* melanin deposition was due to a decrease in the volume of intrinsic haemolymph, a decrease in the activity of phenoloxidase in haemolymph, or a decrease in the activity of a certain mediator between hk-Mf and melanin synthesis.

The final experiment tested whether or not melanin is deposited on hk-Mf in haemocyte-free haemolymph. In haemocyte-free haemolymph prepared from 1-day-old adults, melanin was deposited on more than 93% of the hk-Mf on 3 glass-slides (Fig. 3).

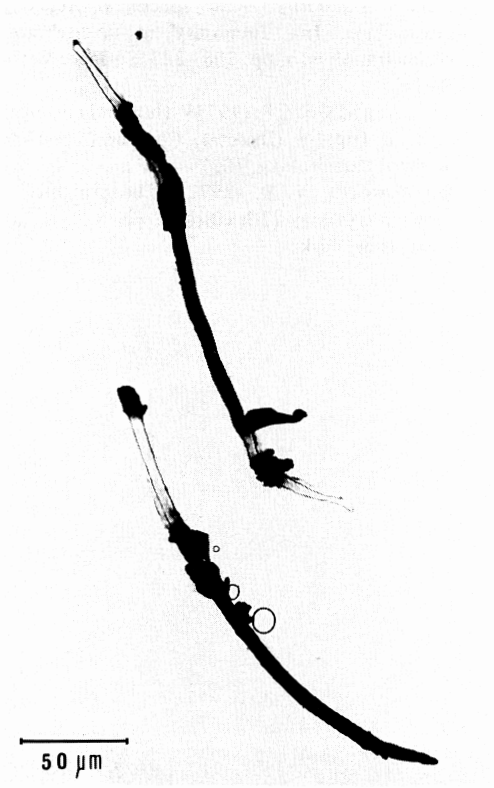

Fig. 3 *In vitro* melanin deposition on heat-killed microfilariae of *B. pahangi* in haemocyte-free haemolymph prepared from *Ar. subalbatus* injected with *Aedes* saline supplemented with trehalose 30 min before collection of haemolymph.

Phenoloxidases oxidize and polymerize phenols such as tyrosine and DOPA to form melanin, and foreign materials that enter the haemocoel of insects may activate these enzymes as a defense response (reviewed by Nappi, 1975; Lipke, 1975). The source of phenoloxidases, the process of enzyme activation and the deposition of melanin after synthesis have not been fully clarified (reviewed by Söderhäll and Smith, 1986). Melanin deposition on metazoan parasites in dipterans such as mosquitoes may be due to the humoral responses of non-cellular components of haemolymph (reviewed by Lackie, 1981). Defense reaction against entomogenous fungi occurring in the haemocoel of dipterans is also thought to be humoral encapsulation, as shown in *in vitro* experiments (Vey and Götz, 1975). These views are supported by the present study since melanin deposition on heat-killed Mf occurred in haemocyte-free haemolymph of mosquitoes. Melanin seems to be deposited on foreign materials such as dead filarial larvae without direct participation of haemocytes, although the haemocytes might be involved in melanin synthesis.

In this study, trehalose was added to *Aedes* saline to increase haemolymph viscosity. Trehalose or impurities, however, may suppress acute activation of prophenoloxidase in haemolymph *in vitro* in the same way as a cane sugar factor suppresses the activation of the enzyme in haemolymph of the silkworm, *Bombyx mori* (Ashida, 1981). Slight modifications of the present method made it possible to analyze a part of mechanisms underlying the susceptibility of *Ar. subalbatus* to *Brugia* spp. and revealed that the precipitable components in haemolymph play an important role in melanization responses to live Mf of *B. pahangi* and *B. malayi* (Ogura, in preparation).

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References

1) Ashida, M. (1981): A cane sugar factor suppressing activation of prophenoloxidase in haemolymph of the silkworm, *Bombyx mori*. Insect Biochem., 11, 57–65.
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) Christensen, B. M. and Forton, K. F. (1986): Hemocyte-mediated melanization of microfilariae in *Aedes aegypti*. J. Parasitol., 72, 222–225.
4) Christensen, B. M., LaFond, M. M. and Christensen, L. A. (1986): Defense reactions of mosquitoes to filarial worms: Effect of host age on the immune response to *Dirofilaria immitis* microfilariae. J. Parasitol., 72, 212–215.
5) Forton, K. F., Christensen, B. M. and Sutherland, D. R. (1985): Ultrastructure of the melanization response of *Aedes trivittatus* against inoculated *Dirofilaria immitis* microfilariae. J. Parasitol., 71, 331–341.
6) Hayes, R. O. (1953): Determination of a physiological saline for *Aedes aegypti* (L). J. Econ. Ento., 46, 624–627.
7) Ho, B.-C., Yap, E.-H. and Singh, M. (1982): Melanization and encapsulation in *Aedes aegypti* and *Aedes togoi* in response to parasitization by a filarioid nematode (*Breinlia booliati*). Parasitology, 85, 567–575.
8) Lackie, A. M. (1981): Immune recognition in insects. Develop. Comparative Immunol., 5, 191–204.
9) Lipke, H. (1975): Melanin in host-parasite interaction. In "Invertebrate immunity" (Maramorosch, K. and Shope, R. E. ed.), pp. 327–336, Academic Press, London.
10) Nappi, A. J. (1975): Parasite encapsulation in insects. In "Invertebrate immunity" (Ditto), pp. 293–326.
11) Ogura, N. (1986): Haemagglutinating activity and melanin deposition on microfilariae of *Brugia pahangi* and *B. malayi* in the mosquito, *Armigeres subalbatus*. Jpn. J. Parasitol., 35, 542–549.
12) Ogura, N. and Kobayashi, M. (1986): A method for isolation of exsheathed microfilariae of *Brugia pahangi*. Jpn. J. Parasitol., 35, 257–259.
13) Söderhäll, K. and Smith, V. J. (1986): The prophenoloxidase activating system: The biochemistry of its activation and role in arthropod cellular immunity, with special reference to crustaceans. In "Immunity in invertebrates" (Brehélin, M. ed.), pp. 208–223, Springer-Verlag, Berlin.
14) Vey, A. and Götz, P. (1975): Humoral encapsulation in Diptera (Insecta): Comparative studies *in vitro*. Parasitology, 70, 77–86.
15) Wigglesworth, V. B. (1972): The principles of insect physiology (7th edition). pp. 827, Halsted Press, New York.