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In vitro Melanin Deposition on Microfilariae of *Brugia pahangi* and *B. malayi* in Haemolymph of the Mosquito, *Armigeres subalbatus*

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Abstract

Live microfilariae (Mf) of *Brugia pahangi* and *B. malayi* were melanized in haemolymph samples taken from 1-day-old female adults of *Armigeres subalbatus* that had been injected with *Aedes* saline supplemented with sucrose. In cell-free haemolymph prepared by centrifugation, live Mf were only slightly melanized, while heat-killed Mf were strongly melanized. Thus, haemocytes, fat body cells, or other precipitable components in haemolymph play an important role in the melanization response against live Mf. Haemagglutinins in haemolymph may also play a role in the melanization response, since stachyose, a haptenic sugar against *Ar. subalbatus* haemagglutinin, inhibited melanin deposition.

Key words: Melanization, filaria, nematoda, mosquito, Diptera, *in vitro*

Introduction

The mosquito, *Armigeres subalbatus*, is an efficient host for filarial larvae of *Brugia pahangi*, while the mosquito is not a good host for the larvae of *B. malayi* and shows remarkable melanization responses against the larvae in the thorax (Wharton, 1962) or in the thorax and abdomen (Yamamoto *et al.*, 1985). Mechanisms underlying the melanization responses in the haemocoel of *Ar. subalbatus* are not understood. *In vitro*, melanin is deposited on heat-killed microfilariae (Mf) of *B. pahangi* in haemocyte-free haemolymph (Ogura, 1987). However, ultrastructural observation of response against intrathoracically inoculated Mf of *Dirofilaria immitis* in the mosquitoes, *Aedes trivittatus* and *Ae. aegypti*, indicates that haemocytes are important (Forton *et al.*, 1985; Christensen and Forton, 1986). The results of present *in vitro* experiments using *B. pahangi*, *B. malayi* and *Ar. subalbatus* also show that haemocytes, fat body cells or other precipitable components in haemolymph play important role(s) in the melanization responses against live Mf. The experiments, moreover, suggested that haemagglutinins in haemolymph may play a role in the melanization response.

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Materials and Methods

Parasites

B. pahangi and *B. malayi* (Che-ju strain) were maintained in *Meriones unguiculatus*. Sheathed microfilariae (Mf) were collected by pipette from Hanks' balanced salt solution (HBSS) containing both Mf and haemolysed blood cells treated with phosphate buffered NH_4Cl solution. Mf gathered were washed 3 times with HBSS. Heat-killed Mf (abbreviated as hk-Mf) were prepared by heating Mf at 95°C for 5 min. Mf and hk-Mf suspensions were adjusted to contain approximately 200 Mf per 1 μl of HBSS.

Collection of haemolymph sample

Ar. subalbatus (Rendaiji strain) for this study were reared and maintained as previously described (Ogura, 1986). Each 1-day-old female adult was injected with 1 μl of *Aedes* saline (Hayes, 1953) supplemented with sucrose (15%) or other sugars as previously described

(Ogura, 1987). The injected mosquitoes were kept at 20°C for 15 min, and then chilled on ice for 15 min. Then, their abdominal integuments were torn with forceps on a polyvinyl chloride board. Haemolymph on the board was sucked with a glass-capillary tube (1 mm in diameter and 30 mm in length) with or without absorbent cotton (2 mm in height from lower end of tube), which had been soaked with *Aedes* saline supplemented with the sugar used for injection. The lower end of the tubes with absorbent cotton were sealed with putty and the tube was centrifuged at 6,000 g for 3 min to yield cell-free haemolymph samples. Cell-free haemolymph samples for haemagglutinating tests were kept at −80°C until needed.

In vitro melanin deposition on Mf

Five μl of the haemolymph sample or cell-free haemolymph sample (obtained from 7 – 10 mosquitoes) were dropped onto a polyvinyl chloride board (20 x 20 mm). One fourth μl of Mf or hk-Mf suspension was added and suspended using a blunt iron needle in the haemolymph sample or cell-free haemolymph sample. The boards were kept in a wet chamber at 15°C for 24 hr before observation of melanin deposition.

Haemagglutinating test

Haemagglutinating activities in cell-free haemolymph samples were measured using microtiter V-plates as previously described (Ogura, 1986) with the exception that human 0 erythrocytes were suspended in phosphate buffered saline supplemented with bovine serum albumin V (1%) instead of phosphate buffered saline.

Results

In vitro melanin deposition on Mf in haemolymph sample and cell-free haemolymph sample

When live Mf of *B. pahangi, B. malayi* and hk-Mf of *B. pahangi* were incubated in haemolymph samples taken from *Ar. subalbatus* which had been injected with 1 μl of *Aedes* saline supplemented with sucrose (15%) (abbreviated as haemolymph sample with 15% sucrose), average percentages of Mf with melanin deposits out of total Mf per drop of haemolymph sample were 24.0, 55.1 and 95.9%, respectively (Table 1) (Fig. 1A). Many various-sized spherical components with melanin (smaller than 20 μm in diameter) were also observed in the drops of haemolymph samples with 15% sucrose (Fig. 1A and B).

Table 1 *In vitro* melanin deposition on Mf of *B. pahangi, B. malayi* and heat-killed Mf of *B. pahangi* in haemolymph and cell-free haemolymph samples taken from *Ar. subalbatus*

Mf used	% Mf with melanin deposits in a drop of sample (Average ± SD)	
	Haemolymph	Cell-free haemolymph
B. pahangi	24.0±17.5 (12/15)	1.5±1.3 (10/13)
B. malayi	55.1±18.6 (10/13)	5.8±7.7 (10/15)
Hk-Mf of *B. pahangi*	95.9± 7.0 (10/15)	84.2±7.7 (10/13)

Haemolymph samples were collected from 1-day-old female adults injected with 1 μl of *Aedes* saline supplemented with sucrose (15%).
Cell-free haemolymph samples were obtained by centrifuging haemolymph sample at 6,000 g for 3 min.
0.25 μl of HBSS containing 50 Mf was suspended in a drop (5 μl) of the sample on vinyl chloride board and then kept at 15°C for 24 hr.
Percentage of Mf with melanin deposits was obtained from a drop of sample which did not show whole darkening.
Perentheses show numbers of drops without whole darkening per all drops tried.

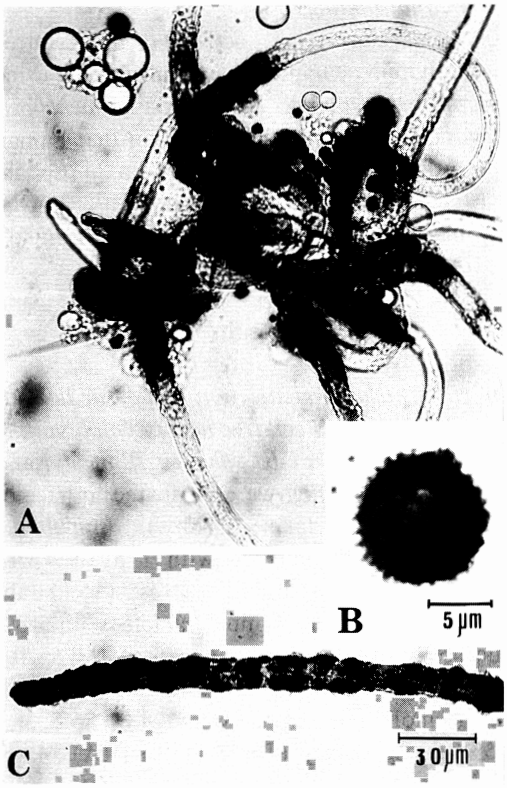

Fig. 1 A: *B. malayi* Mf melanized in haemolymph sample collected from 1-day-old female adults of *Ar. subalbatus* injected with *Aedes saline* supplemented with sucrose (15%).
B: Spherical component with melanin observed in haemolymph sample with 15% sucrose.
C: Anterior half of heat-killed Mf of *B. pahangi* melanized in cell-free haemolymph sample of *Ar. subalbatus*.

When those Mf were incubated in cell-free haemolymph sample with 15% sucrose, average percentages of Mf with melanin deposits were 1.5, 5.8 and 84.2%, respectively (Table 1) (Fig. 1C). Differences among the percentages obtained from the incubations of live Mf of the same species in the haemolymph sample and cell-free haemolymph sample were statistically significant at <0.001 level by t-test, respectively.

Effect of stachyose on haemagglutinating activities in cell-free haemolymph samples

Haemagglutinating activities in cell-free haemolymph samples with 15% (438 mM) sucrose were high (Titer: 2^{-5} to 2^{-7}). Whole darkening in haemolymph sample was inhibited by stachyose. Haemagglutinating activities in cell-free haemolymph samples with 5% (75 mM) stachyose and 10% (292 mM) sucrose, with 10% (150 mM) stachyose and 5% (146 mM) sucrose, and with 15% (225 mM) stachyose were 2^{-4}, 2^{-2} and 2^{-1} to 2^{-2} respectively in haemagglutinin titer (Fig. 2).

Effect of stachyose on in vitro melanin deposition on Mf in haemolymph samples

Average percentage of *B. malayi* Mf with melanin deposits was 63% in haemolymph sample with 15% sucrose. Average percentages of *B. malayi* Mf with melanin deposits in haemolymph samples with 10% stachyose and 5% sucrose, and with 15% stachyose, were 5.6%, and 1.5%, respectively (Fig. 2). Differences between percentages of the former and the latter two were statistically significant at <0.001 level by t-test.

When hk-Mf of *B. pahangi* were incubated in haemolymph sample with 15% sucrose, 85.7% of hk-Mf were melanized. Percentages of hk-Mf with melanin deposits decreased significantly when the concentration of stachyose was over 10% ($P<0.01$ level by t-test) (Fig. 2).

Discussion

In insect haemolymph, specific components on surfaces of organisms, such as β-1,3-glucans and lipopolysaccharides, activate prophenoloxidase (pro PO), and the resultant phenoloxidase (PO) oxidizes and polymerizes phenols such as tyrosine and DOPA to form melanin (reviewed by Söderhäll and Smith, 1986). The haemolymph also begings melanin formation soon after bleeding. This phenomenon interferes with *in vitro* melanin deposition on the foreign materials in haemolymph kept at room temperature for more than 30 min (Götz, 1986). In the previous study (Ogura, 1987), however, *in vitro* melanin deposition on hk-Mf of *B. pahangi* occurred clearly in haemolymph sample taken from 1-day-old female adults of *Ar. subalbatus* injected with

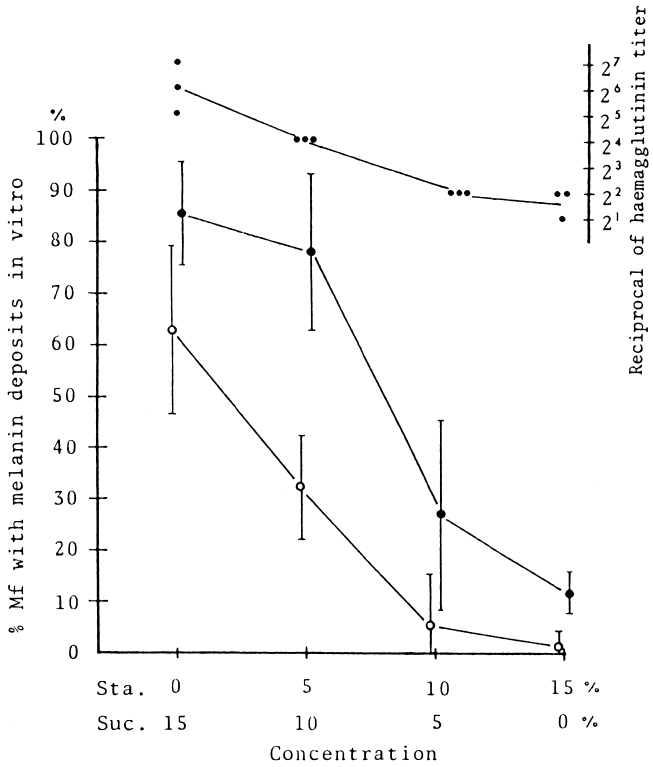

Fig. 2 Concentration effect of stachyose on haemagglutinating activity, melanin deposition on live Mf of *B. malayi* and melanin deposition on heat-killed Mf of *B. pahangi* in haemolymph sample of *Ar. subalbatus*.
A mosquito was injected with 1 μl of *Aedes* saline supplemented with 15% sucrose (suc.), with 5% stachyose (sta.) and 10% suc., with 10% sta. and 5% suc. or with 15% sta. Each large dot represents the results obtained from 10 haemolymph samples without whole darkening (○—○: Live Mf of *B. malayi* ●—●: hk-Mf of *B. pahangi*). Each small dot represents each result of haemagglutinating test.

Aedes saline containing trehalose (15%) (abbreviated as haemolymph sample with 15% trehalose). In the present study, sucrose instead of trehalose was used as a supplement, since a cane sugar factor which is one of impurities of sucrose efficiently suppresses spontaneous activation of proPO in haemolymph of the silkworm, *Bombyx mori* (Ashida, 1981). The results indicated that melanin deposition on live Mf of *B. pahangi, B. malayi* and hk-Mf of *B. pahangi* occurred in haemolymph sample with 15% sucrose. However, little melanin was deposited on live Mf in cell-free haemolymph sample with 15% sucrose, even though hk-Mf of *B. pahangi* were melanized under the same condition. Haemolymph sample with 15% sucrose contains haemocytes, fat body cells and the other precipitable components while cell-free haemolymph sample does not contain such cells or components. One or more of such cells or components, therefore, seem(s) to play important role(s) in melanin deposition on live Mf.

Granulocytes, a type of haemocyte, contain proPO in the wax moth, *Galleria mellonera* (Ratcliffe and Rowley, 1979), and crystal cells contain proPO in the vinegar fly, *Drosophila melanogaster* (Rizki and Rizki, 1979). It seems,

however, that cell-free haemolymph sample with 15% sucrose contains proPO and surface of hk-Mf of *B. pahangi* activates the enzyme, since hk-Mf were melanized in the cell-free haemolymph sample. One or more of the precipitable components, including haemocytes and fat body cells, may change the surface structure of live Mf to that appearing in hk-Mf and which then activates proPO. Possibly, resultant PO adhered to the live Mf in a fashion similar to that reviewed by Götz (1986), and melanin was deposited on the live Mf.

In haemolymph sample with 15% sucrose, moreover, melanin was formed in or on the various-sized spherical components. Possibly, the components with melanin adhered to the live Mf and ruptured in a fashion similar to that proposed by Forton *et al.* (1985).

It has been suggested that there are two groups of materials in activation of proPO in insect haemolymph: one requires haemocytes for the induction of PO activity and the other one induces PO activity by acting directly upon proPO system (Ashida, 1981). Live Mf and hk-Mf used for the present study may belong to the former and the latter group, respectively. It has been reported that haemocytes may play important roles in melanization responses against intrathoracically inoculated live Mf of *D. immitis* in *Ae. trivittatus* and *Ae. aegypti* (Christensen and Forton, 1986; Harris *et al.*, 1986).

B. malayi larvae sometimes are melanized without haemocyte attachment in thoracic muscle of *Ar. subalbatus* (Kobayashi *et al.*, 1986). *D. immitis* larvae are melanized within Malpighian tuble cells of *Ae. trivittatus* without direct participation of haemocytes (Christensen, 1981). A certain intraorganic or intracellular component may change surface structures of such larvae and the structures changed may activate proPO which exists intrinsically or infiltrates there. When *B. pahangi* Mf are taken by *Anopheles quadrimaculatus* orally, Mf are first melanized and then enclosed by plasmatocytes in haemocoel (Chen and Laurence, 1985). Roles of haemocytes would not be important in the melanization responses, if surface structures of Mf were damaged seriously in the mid-gut or during passing through mid-gut wall into the haemocoel.

Haemagglutinin in haemolymph of *Ar. subalbatus* binds to stachyose and to a lesser extent N-acetylated sugars (Ogura *et al.*, 1985). *Ar. subalbatus* haemagglutinin may be involved in melanization responses against live Mf of *B. pahangi* and *B. malayi* occurring in haemocoel (Ogura, 1986). Present study also suggests that the haemagglutinin may play some role in the melanization responses against not only live Mf of *B. malayi* but also hk-Mf of *B. pahangi*.

More study needs to be done to elucidate the mechanisms underlying the melanization responses against Mf in the haemocoel of *Ar. subalbatus*.

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