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The Effect of Exogenous Haemagglutinin on *in vitro* Melanin Deposition on Microfilariae of *Brugia pahangi* in Haemolymph of the Mosquito, *Armigeres subalbatus*

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Abstract

The encapsulation and melanization of microfilariae (Mf) of *Brugia pahangi* in haemolymph of the mosquito, *Armigeres subalbatus*, were studied *in vitro*. Heat-killed microfilariae (hk-Mf) were heavily melanized in both haemolymph samples and cell-free haemolymph samples of the mosquitoes of which the haemagglutinin titer had been increased by injection of Bacto-Phytohemagglutinin P (PHA-P). Heavy melanization of hk-Mf also occurred in haemolymph samples of the mosquitoes when PHA-P was added after collection of haemolymph. Induction of melanin deposition by PHA-P was inhibited by N-acetyl-D-galactosamine which is a haptenic sugar against PHA-P. Many lumps of melanin deposited on live Mf in cell-free haemolymph samples prepared from the mosquitoes injected with PHA-P as densely as on hk-Mf. Haemagglutinin seems to be one of the mediators between parasitic nematodes and the prophenoloxidase systems of host insects.

Key words: Haemagglutinin, lectin, melanization, filaria, mosquito, *in vitro*

Introduction

When mosquitoes ingest the pathogens of viral, malarial or filarial diseases and recognize them as foreign substances, the mosquitoes interfere with the proliferation or development of the pathogen through certain defense reactions. Melanization of filarial larvae occurring in the haemocoel is a typical model of mosquito defense reaction (reviewed by Christensen, 1986). Ultrastructural studies on a filaria-mosquito system have revealed two different interpretations of the melanization process; melanization of microfilariae (Mf) is a humoral encapsulation (Chen and Laurence, 1985) or is mediated by certain haemocytes responsible for melanin synthesis (Forton *et al.*, 1985; Christensen and Forton, 1986). Recent *in vitro* studies on the melanization (Chen and Laurence, 1987 a, b) report that melanization of *Brugia pahangi* Mf in *Anopheles quadrimaculatus* is a humoral encapsulation. Another *in vitro* study (Ogura, in press b) suggests that melanization of *B. pahangi* Mf in *Armigeres subalbatus* involves humoral encapsulation and the interaction between Mf and various-sized spherical components in the haemolymph. These discrepancies may be dissolved by outlining mechanisms underlying the melanization responses. For this purpose, mediators between a system of melanin synthesis (reviewed by Söderhäll and Smith, 1986) in the mosquito and Mf as a foreign substance must be found. In a series of *in vivo* and *in vitro* study on melanization of Mf in mosquitoes (Ogura, 1986; in press a, b), it has been shown that *B. pahangi* Mf are easily melanized in the haemolymph of female pupae and young adults of *Ar. subalbatus* of which the haemagglutinin titer is high but melanization is less frequently observed in the haemolymph of 8-day-old adults of which the titer is low. The present study, therefore, was undertaken to examine whether the addition of exogenous haemagglutinin into haemolymph of 8-day-old adults of *Ar. subalbatus* with low haemagglutinin titer

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can elicit *in vitro* melanization of both heat-killed and live Mf of *B. pahangi*.

Materials and Methods

Mosquito and parasite

Ar. subalbatus (Rendaiji-strain) was reared and maintained as previsouly described (Ogura, 1986). Sheathed *B. pahangi* Mf were collected from the blood of *Meriones unguiculatus* and heat-killed Mf (hk-Mf) were prepared as previously described (Ogura, in press b).

Preparation of solution and suspension

Bacto-Phytohemagglutinin P* (PHA-P) (Difco, USA) was dissolved in sterilized distilled water according to the prescription to give approximately 1% in 0.85% saline. PHA-P was diluted with *Aedes* saline (Hayes, 1953) supplemented with bovine serum albumin V (BSA) (Nakarai, Japan) (1%). *Aedes* saline containing BSA (1%) was sterilized by passage through 0.2 μm pore size Sartorius membrane filters. The pH of this solution was approximately the same as that of PHA-P. Zymosan A (Sigma, USA) was suspended in *Aedes* saline (10 mg/ml). The suspension was centrifuged at 6,000 g for 5 min to yield zymosan-supernatant. Ten-fold dilution of the supernatant with *Aedes* saline containing BSA (1%) was also used. N-acetyl-D-galactosamine (GalNAc) and D-galactose (Gal) (Nakarai, Japan) were dissolved in PHA-P.

Preparation of haemolymph sample and cell-free haemolymph sample

Each of 8-day-old female adults was injected with 1 μl of PHA-P or *Aedes* saline containing BSA (1%) by previously described method (Ogura, in press a). They were kept at 20°C and 0°C for 25 and 5 min, respectively, and then their abdominal integuments were torn with forceps on a polyvinyl chloride board. Spilt haemolymph was sucked up with a glass-capillary tube (1 mm in diameter and 30 mm in length) and was used as haemolymph samples with PHA-P or with BSA. To prepare cell-free haemolymph sample, haemolymph from 20 mosquitoes, which had been injected with 1 μl of PHA-P or *Aedes saline* with BSA (1%), was sucked up with a glass-capillary of which the lower end was filled with absorbent cotton as previously described (Ogura, in press b). The lower end of the glass-capillary was sealed with putty. The capillary was centrifuged at 6,000 g for 3 min and 5 μl of the supernatant was used as a cell-free haemolymph sample with PHA-P or with BSA.

In vitro incubation of Mf in haemolymph samples

Hk-Mf were suspended in Hanks' balanced salt solution (HBSS) to give 200 Mf per μl. Hk-Mf were also suspended in PHA-P, in *Aedes* saline with zymosan and in *Aedes* saline with BSA (1%) to give 10 Mf per μl. 0.25 or 5 μl of the suspension containing 50 hk-Mf was mixed with a drop (5 μl) of haemolymph sample or cell-free haemolymph sample which was placed on a polyvinyl chloride board (2 x 2 cm).

For the inhibition test, hk-Mf were suspended in PHA-P supplemented with sugar or in PHA-P without sugar. 5 μl of the suspension containing 50 hk-Mf was mixed with a drop (5 μl) of haemolymph sample with BSA.

Live Mf were suspended in HBSS to give 200 Mf per μl, and 0.25 μl of the suspension was mixed with a drop (5 μl) of cell-free haemolymph sample with PHA-P or with BSA.

Collection of haemolymph and preparation of Mf-incubation were carried out in a cold room (4°C). Hk-Mf and live Mf were suspended in the haemolymph samples placed on polyvinyl chloride board, using a blunt iron needle. The polyvinyl chloride boards were placed in a wet chamber and kept at 20°C for 3 hr before observation of melanin deposition.

Results

Effect of PHA-P on in vitro melanin deposition on hk-Mf

**Haemagglutinin titers measured by Human 0 erythrocytes were between 2^{-10} and 2^{-12}. PHA-P was only one lectin eliciting intense melanization of hk-Mf among 4 lectins tested (e.i., ConA, PHA-P, WFA and WGA showing 2^{-10} haemagglutinin titer).*

Table 1 Effect of Bacto-Phytohemagglutinin P (PHA-P) on *in vitro* melanization of heat-killed microfilariae (hk-Mf) of *Brugia pahangi* in haemolymph sample of the 8-day-old female adults of *Armigeres subalbatus*

Donor injected with;	Kind of haemolymph sample	%Hk-Mf with melanin deposits (Average±SD)	
		Hk-Mf with small lumps of melanin	Hk-Mf with large lumps of melanin
PHA-P	intact	9.4±7.4	71.0±10.0 (10)
BSA	intact	1.4±2.5	1.9±6.1 (10)
PHA-P	cell-free	1.5±2.1	66.2±15.1 (5)
BSA	cell-free	15.8±15.6	0 (5)

Each mosquito was injected with 1 μl of PHA-P or with *Aedes* saline supplemented with bovine serum albumin V (BSA) (1%) and then their haemolymph was collected. 0.25 μl of HBSS containing 50 hk-Mf were mixed with 5 μl of the haemolymph sample. Melanization was observed 3 hr after incubation at 20°C. Parentheses show numbers of haemolymph samples incubated.

In haemolymph samples with PHA-P and with BSA, average percentages of hk-Mf with large lumps of melanin in the total hk-Mf per a drop of haemolymph sample were 71.0 and 1.9%, respectively (Table 1) (Fig. 1-A). In the haemolymph sample with BSA, various-sized spherical components with melanin (smaller than 20 μm in diameter) were observed though most hk-Mf were not melanized (Fig. 1-B).

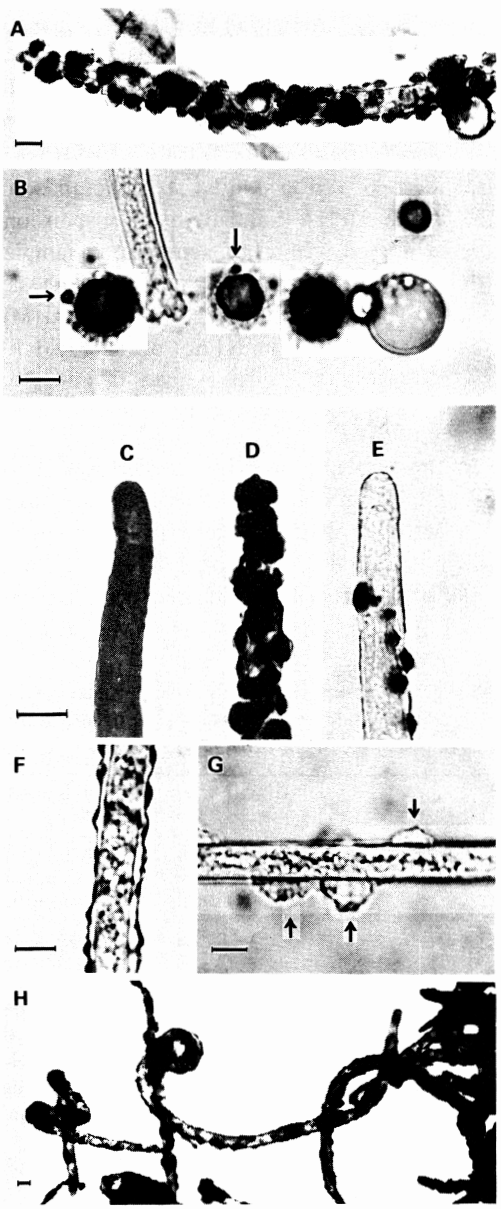

Fig. 1 A: Melanin deposition on heat-killed Mf of *B. pahangi* in haemolymph sample of the 8-day-old female adults of *Ar. subalbatus* injected with Bacto-Phytohemagglutinin P (PHA-P).
B: Spherical components with melanin in haemolymph sample of the 8-day-old female adults injected with *Aedes* saline containing bovine serum albumine (BSA) (1%). Arrows show spherical components in company with additional small spherical component.
C·D: Melanin deposition on hk-Mf in cell-free haemolymph sample prepared from the 8-day-old female adults injected with PHA-P.
E: Melanin deposition on hk-Mf in cell-free haemolymph sample from the 8-day-old female adults injected with *Aedes* saline containing BSA (1%).
F: Melanin deposition on hk-Mf in haemolymph sample with BSA where zymosan was added. Tiny lumps of melanin scattered on surface of hk-Mf.
G: Translucent substances (Arrows) on hk-Mf in haemolymph sample with BSA where PHA-P and GalNAc were added.
H: Melanin deposition on live Mf of *B. pahangi* in cell-free haemolymph sample prepared from the 8-day-old female adults injected with PHA-P.
All bars represent 5 μm.

In cell-free haemolymph samples with PHA-P, the average percentage of hk-Mf with intense melanin deposition was 66.2% and both homogeneous (Fig. 1-C) and rugged (Fig. 1-D) melanin were found around the hk-Mf (Table 1). In cell-free haemolymph samples with BSA, some small lumps of melanin appeared in 15.8% of the hk-Mf (Table 1) (Fig. 1-E).

Effect of PHA-P and Zymosan on in vitro melanin deposition on hk-Mf

When hk-Mf suspended in PHA-P were incubated with the haemolymph samples with BSA, 71.2% of the hk-Mf were heavily melanized. In the control preparation, 4% of the hk-Mf were slightly melanized (Table 2). When hk-Mf were suspended in various-kinds of zymosan solutions without PHA-P and then the suspensions were incubated with the haemolymph samples with BSA, tiny lumps of melanin were deposited on approximately 20% of the hk-Mf, and heavy melanization did not occur (Fig. 1-F).

Effect of GalNAc on melanin deposition induced by PHA-P

In the presence of PHA-P added with 800, 400 and 200 mM GalNAc, average percentages of hk-Mf with plenty of melanin deposits were 0, 24.6 and 29.8%, respectively (Table 3). And lumps of translucent substance were often observed on the hk-Mf (Fig. 1-G). In the presence of PHA-P added with Gal and with no sugar, 42.0 and 85.6% of the hk-Mf were heavily melanized (Table 3).

Table 2 Effect of PHA-P on *in vitro* melanization of hk-Mf of *B. pahangi* in haemolymph sample of *Ar. subalbatus*.

Hk-Mf suspended in	%Hk-Mf with melanin deposits (Average±SD)	
	Hk-Mf with small lumps of melanin	Hk-Mf with large lumps of melanin
PHA-P	13.2±15.5	71.2±20.5 (5)
Aedes saline with BSA	4.0±6.6	0 (5)

5 μl of hk-Mf suspension was mixed with 5 μl of haemolymph sample taken from the 8-day-old female adults injected with *Aedes* saline containing BSA (1%). Melanization was observed 3 hr after incubation at 20°C.
Parentheses show the number of haemolymph samples incubated.

Effect of PHA-P on in vitro melanin deposition

Table 3 Effect of N-acetyl-D-galactosamine (GalNAc) on *in vitro* melanin deposition on hk-Mf of *B. pahangi* in haemolymph sample of *Ar. subalbatus*

Hk-Mf suspended in PHA-P supplemented with;	%Hk-Mf with melanin deposits (Average±SD)	
	Hk-Mf with small lumps of melanin	Hk-Mf with large lumps of melanin
800 mM GalNAc	23.7±10.6	0 (6)
400 mM GalNAc	26.5±24.6	24.6±38.6 (6)
200 mM GalNAc	33.2±9.9	29.8±18.6 (6)
800 mM D-galactose	23.6±22.0	42.0±19.8 (5)
No sugar	6.7±11.7	85.6±17.5 (6)

GalNAc or D-galactose was dissolved in PHA-P and hk-Mf were suspended in the solution.
5 μl of the hk-Mf solution was mixed with 5 μl of haemolymph sample taken from the 8-day-old female adults injected with 1 μl of *Aedes* saline added with BSA (1%). Observation was carried out 3 hr after incubation at 20°C.

Table 4 *In vitro* melanin deposition on live Mf of *B. pahangi* in cell-free haemolymph sample prepared from *Ar. subalbatus* injected with PHA-P or dilutions of PHA-P.

Donor injected with;	%Mf with melanin deposits (Average±SD)	
	Mf with small lumps of melanin	Mf with large lumps of melanin
PHA-P, Original solution	1.4±1.4	90.8±6.3 (5)
8-fold dilution*	1.2±1.7	92.7±9.0 (5)
16-fold dilution*	6.7±7.5	87.5±9.1 (5)
32-fold dilution*	27.4±34.1	10.5±8.9 (5)
Aedes saline with BSA	6.4±7.9	4.5±5.5 (5)

Cell-free haemolymph samples were prepared from the 8-day-old female adults injected with 1 μl of PHA-P or *Aedes* saline containing BSA (1%). 0.25 μl of HBSS containing 50 Mf was added into 5 μl of the cell-free haemolymph sample. Incubation time was 3 hr. Parentheses show the numbers of cell-free haemolymph samples incubated. *Dilution of PHA-P was used soon after preparation.

on live Mf

In cell-free haemolymph samples with PHA-P, and 8-, 16- and 32-fold dilutions of PHA-P, average percentages of live Mf with many large lumps of melanin were 90.8, 92.7, 87.5 and 10.5%, respectively (Table 4) (Fig. 1-H). 4.5% of the live Mf were melanized heavily in cell-free haemolymph samples with BSA.

Discussion

Defense reactions against parasitic nematodes occurring in the haemocoels of insects have been studied for a long time (Salt, 1963; Poinar, 1974; Nappi, 1975; Götz, 1986). A general defense reaction is a encapsulation (reviewed by Götz, 1986). The encapsulation is divided into cellular encapsulation and humoral encapsulation. The former encapsulation is initiated by the attachment of granular haemocytes on a foreign substance. The cells partly disintegrate and cause melanin formation and then many haemocytes, mainly plasmatocytes, accumulate to form multicellular capsule. Humoral encapsulation is caused by non-cellular components responsible for melanin formation in haemolymph but haemocytes often accumulate on the melanized foreign substance. The later encapsulation is often observed in Diptera such as the Chironomidae and Culicidae.

The knowledges of melanin formation in haemolymph of arthropod have been accumulated rapidly. Briefly, β-1, 3-glucans or glucans of lipopolysaccharides on the surface of foreign substances such as zymosan activate a serine protease. Active serine protease changes prophenoloxidase (proPO) to phenoloxidase (PO) which oxidizes and polymerizes phenols to form melanin. This activation process produces sticky or attaching proteins, which, on the whole, is called the activation of proPO system (reviewed by Söderhäll and Smith, 1986). The activation of the proPO system might be triggered by the agglutinins binding non-self materials to the coagulation cells (Ratcliffe, 1986).

The haemagglutinin titers in haemolymph of *Ar. subalbatus* were high at pupal stage and gradually decreased after eclosion, and this change was comparable to that of melanization rate of live *B. pahangi* Mf injected into the mosquitoes (Ogura, 1986). *Ar. subalbatus* haemagglutinin adhered to the surface of both live and heat-killed Mf of *B. pahangi* (Ogura, 1986), agglutinated human (A, B, O type), rabbit, rat and jird erythrocytes and bound to stachyose or to a lesser extent N-acetylated sugars (Ogura, *et al.*, 1985). PHA-P also agglutinates erythrocytes of all human blood groups as well as those of many animals (Nungster and Halsema, 1953) and binds to GalNAc (Borberg *et al.*, 1966). The similarity in haemagglutination between *Ar. subalbatus* haemagglutinin and PHA-P led to the present study.

Many lumps of melanin densely deposited on the many hk-Mf both in haemolymph samples and cell-free haemolymph samples with PHA-P but not in samples with BSA. Hk-Mf were also melanized in haemolymph samples with BSA when PHA-P was added into it. Hk-Mf were not melanized heavily in haemolymph samples with BSA where zymosan, a proPO activator, was added. Particles of Zymosan A were melanized in cell-free haemolymph samples with PHA-P but not in that with BSA prepared from the 8-day-old adults (Ogura, unpublished observation). Hk-Mf, therefore, seem to have the same character as zymosan and to be melanized by mixture of PHA-P and proPO system.

In the presence of GalNAc which is a haptenic sugar against PHA-P, induction of melanin deposition by PHA-P was inhibited, and lumps of a translucent substance were often observed on the surface of hk-Mf. This results suggest that GalNAc inhibits adhesion of proPO systems to both hk-Mf and the translucent substances or GalNAc inhibits activation of proPO systems adherent to both hk-Mf and the translucent substances. The translucent substances are derived from the various-sized spherical components arising from the haemocytes (Ogura, in preparation). In *in vitro* encapsulation studies using *B. pahangi* and *An. quadrimaculatus* (Chen and Laurence, 1987 a, b),

transparent capsule material from acellular source is first deposited on the surface of Mf and then Mf are melanized. Different origins of the translucent or transparent materials might be due to various differences in experimental conditions.

In a previous study (Ogura, in press b), live Mf of *B. pahangi* were melanized in the haemolymph sample but slightly in the cell-free haemolymph sample prepared from the 1-day-old adults of *Ar. subalbatus* injected with *Aedes* saline added with sucrose (15%). In the present study, live Mf of *B. pahangi* were well melanized in the cell-free haemolymph sample with PHA-P prepared from even the 8-day-old adults. These results suggest that certain precipitable components in haemolymph are involved in the release of PHA-P like haemagglutinins which facilitate melanization.

Haemagglutinins could exist as heterogenous-multisubunit proteins in haemolymph and also exist on the surface of haemocytes (reviewed by Olafsen, 1986). Haemagglutinins would separate into populations according to the predominantly expressed binding site (Olafsen, 1986). In the silkworm, *Bombyx mori*, β-1, 3-glucan receptor and peptidoglycan receptor are present as separate entities within proPO activating system (Yoshida *et al.*, 1986). Therefore, the following hypothesis of melanization process was suggested (Fig. 2).

Live Mf would cause release of haemagglutinins from certain precipitable components in haemolymph. The haemagglutinins would cohere to the Mf, to proPO systems in haemolymph and to spherical components in company with both proPO systems and substrates for PO in haemolymph. Smaller spherical components with both proPO systems and the substrates would be produced from the larger spherical components. Complexes of haemagglutinin and proPO system would bind to the haemagglutinins which previously cohered to the Mf. At this time, certain structural change would occur in the haemagglutinins and proPO would turn to PO. Haemagglutinins on the various-sized spherical components also would cohere to the haemagglutinins on the Mf, and

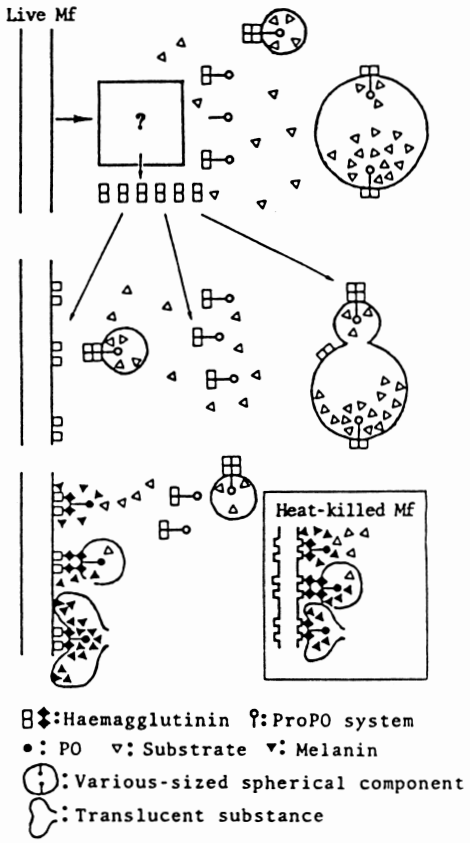

Fig. 2 Hypothetical scheme of process of melanin deposition on filarial larvae in haemocoel of mosquito.

proPO in or on the components would turn to PO, and finally the components would rupture. Then many lumps of melanin would be formed around the Mf. Surface of hk-Mf itself would cause structural change in haemagglutinins, resulting in melanization in haemolymph with fewer haemagglutinins. Whether or not Mf are melanized would depend on the binding specificity and quantity of haemagglutinin and on the surface structure of Mf.

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