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Haemagglutinating Activity and Melanin Deposition on Microfilariae of *Brugia pahangi* and *B. malayi* in the Mosquito, *Armigeres subalbatus*

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Key words: Haemagglutinin, melanization, filaria, *Brugia pahangi*, mosquito, *Armigeres subalbatus*

Introduction

Haemagglutinating activity in haemolymph has been demonstrated in many invertebrate species (reviewed by Olafsen, 1986) including several mosquitoes (Ogura *et al.*, 1985a, b). The roles of haemagglutinins, however, have not been clarified, though they are generally assumed to be involved in a primitive immune system (Komano *et al.*, 1980). Despite numerous reports of defense reactions to filarial larvae occurring in the haemocoel of mosquitoes (Christensen, 1981; Ho *et al.*, 1982; Yamamoto *et al.*, 1985; Forton *et al.*, 1985; Chen and Laurence, 1985; Kobayashi *et al.*, 1986), mechanism underlying such defense responses have not been elucidated. In a recent ultrastructural study of these reactions (Chen and Laurence, 1985), it was shown that microfilariae in the haemocoel of a mosquito are first enclosed by an acellular electron dense capsule ("melanin") which is then covered by plasmatocytes. This paper reports a study on haemagglutinin in haemolymph of the mosquito, *Armigeres subalbatus*, and its possible role in the defense reaction to filarial nematodes such as *Brugia pahangi* and *B. malayi*.

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Materials and Methods

Mosquito

Ar. subalbatus (Rendaiji strain) used is susceptible to *B. pahangi* and refractory to *B. malayi* (Che-ju strain) (Kobayashi *et al.*, 1981). Larvae were reared on mouse pellets CA-1 (Clea, Ltd., Tokyo), and adults were reared on a 5% sucrose solution at 26 ± 1°C under a 16 light: 8 dark photoregime. Female adults were starved for 6 hr before collection of body fluid.

Parasites

B. pahangi and *B. malayi* (Che-ju strain) were those maintained in *Meriones unguiculatus*. Sheathed microfilariae (Mf) for haemagglutinin absorption test were isolated from the blood of *M. unguiculatus* through agarose gel according to the method of Ogura and Kobayashi (1986) and washed three times with phosphate buffered saline (PBS: 0.15M NaCl, 0.01M Na_2HPO_4/KH_2PO_4, pH 7.2). Mf for injection into mosquitoes were collected by pipette from Ca^{2+} free Hanks' balanced salt solution (HBSS) containing both Mf and haemolyzed blood cells treated with phosphate buffered NH_4Cl solution, since there was a possibility that viability of Mf became lower during passing through agarose gel.

Collection of mosquito body fluid, estimation of protein concentration and haemagglutinating test

A small incision was made with a fine needle

through the thoracic wall of pupae or the abdominal wall of adults. Fifty incised female pupae or 100 incised female adults were put into each centrifuge tube devised by Mack and Vanderberg (1978), and the tubes were centrifuged at 23 g for 5 min (pupae) or at 380 g for 10 min (adults) under refrigeration at 4°C. Body fluid at the bottom of the centrifuge tubes was gathered, kept at −80°C until centrifuged at 3,500 g for 5 min at 4°C just before using. Protein concentration in the body fluid was estimated by the method of Bradford (1976). Haemagglutinating activity in the body fluid was measured on a microtiter V-plate. Twenty-five μl of human O erythrocyte suspension, adjusted to contain 60,000 to 65,000 erythrocytes per μl of PBS, was added to each well, which previously held 25 μl of a serial two-fold dilution of body fluid. The plate was shaken vigorously on a microtiter mixer for 5 min and kept at 4°C for 2 hr before the results were read. In the haemagglutinin absorption test, 30 μl of body fluid with 2^{-2} haemagglutinin titer was added to 30 μl of PBS containing Mf and incubated at 15°C for 2 hr. Then, the suspension was centrifuged at 3,500 g for 5 min at 4°C and 50 μl of the supernatant was mixed with 25 μl of erythrocyte suspension on the microtiter plate. The plate was kept at 4°C for 2 hr before the results were read.

Injection of Mf and sugar mixture solution into mosquitoes

Two-day old female pupae and 1 to 8-day old female adults were injected with 0.2 μl of Ca^{2+} free HBSS containing 20 to 50 Mf through the dorsal intersegmental membrane between the 6 and 7 abdominal segments with a microsyringe and a fine glass needle. Pupae injected were left intact in a wet chamber. Adults injected were ligated between thorax and abdomen, both head and thorax were cut off and the isolated abdomens were held in the wet chamber. Two-day old female adults were injected with 0.6 μl of sugar mixture solution through the ventral intersegmental membrane between the 7 and 8 abdominal segments. They were allowed to feed on distilled water for 6 hr, injected with Mf and then made into the isolated abdomens by the aforementioned method. These pupae and isolated abdomens were dissected 24 hr after injection in order to examine the number of Mf to which melanin* deposited. To make sugar mixture solution containing 250 mM of each sugar, a mixture of stachyose and N-acetylated sugars was dissolved respectively in 0.32N NaOH solution or distilled water if the mixture included or was devoid of N-acetylneuraminic acid (NeuNAc). A sugar mixture solution of lower concentration was made by diluting the 250 mM solution with distilled water.

For the simultaneous injection of both sugar mixture and Mf, Mf were suspended in RPMI 1640 supplemented with foetal bovine serum (FBS) (20%), stachyose (30 mM), N-acetyl D-galactosamine (GalNAc) (30 mM), N-acetyl D-glucosamine (GlcNAc) (30 mM), N-acetyl D-mannosamine (ManNAc) (30 mM) and NeuNAc (30 mM), which was adjusted to pH 7.0 to 8.0 by adding 1N NaOH. Approximtely 5 μl of the Mf suspension with 20 to 50 Mf was injected into the abdominal haemocoel of 2-day old female adults. They were made into the isolated abdomens and dissected 4 days after injection.

After injection, the needle track was sealed with alon alpha A (Sankyo, Ltd., Tokyo) in these experiments.

Results

Developmental change of haemagglutinating activity

Protein concentrations in pupal and adult body fluid were between 12.2 and 13.3 μg/μl of body fluid and did not greatly vary between pupae and each age of adult (Fig. 1). Haemagglutinin titers of body fluid were 2^{-6} in the pupal stage and gradually decreased to 2^{-2} in 4 and 8-day old adults (Fig. 1).

*The term "melanin" is used in this paper according to Christensen (1981), though nature of black pigments on Mf has not been clarified fully.

Haemagglutinin absorption test

Haemagglutinating activity in body fluid was completely lost after incubation with PBS containing 4800 live Mf of *B. pahangi* per μl or 2400 and 4800 dead Mf of *B. pahangi* per μl (Fig. 2) (Table 1).

Developmental change of melanin deposition on inoculated Mf in Ar. subalbatus

When mosquitoes were injected with live Mf of *B. pahangi*, average percentage of Mf with melanin deposits out of total Mf per mosquito was 100% in pupa, over 50% in 1 to 3-day old adults and less than 20% in 4 to 8-day old adults (Fig. 3). When mosquitoes were injected with live Mf of *B. malayi*, average percentage of Mf with melanin deposits was 100% in pupae and over 60% in 1 to 8-day old adults. Injection of dead Mf of *B. pahangi* resulted in over 90% of Mf having melanin deposits in pupae and 1 to 8-day old adults.

Effect of sugars on melanin deposition on inoculated Mf in Ar. subalbatus

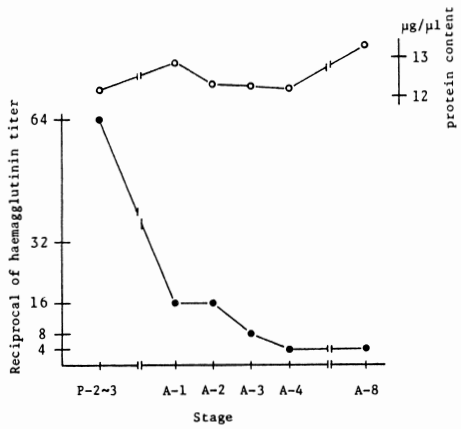

Fig. 1 Changes in haemagglutinating activity and protein concentration in body fluid of *Ar. subalbatus* by stage (each black dot indicates 2 or 3 cases occurring within 3 replicates and each open dot indicates average of 3 replicates).
P-2 to A-8: 2-day old pupa to 8-day old adult

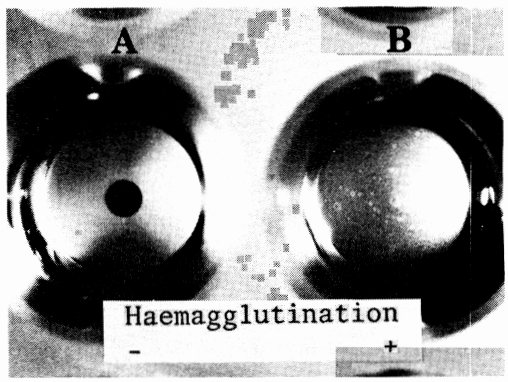

Fig. 2 *Ar. subalbatus* haemagglutinin absorption by *B. pahangi* Mf. A: Human 0 erythrocytes which precipitated after incubation with haemagglutinin absorbed by 4800 live Mf/μl. B: Erythrocytes which aggregated after incubation with control haemagglutinin.

Table 1 Effects of *B. pahangi* Mf on haemagglutinating activity in body fluid collected from female adults of *Ar. subalbatus*

Mf used for absorption	No. of Mf per μl	Haemagglutination	Mf used for absorption	No. of Mf per μl	Haemagglutination
Live Mf	4800	−, −, −	Dead Mf	4800	−, −, −
	2400	(+), (+), (+)		2400	−, −, −
	1200	+, +, (+)		1200	(+), +, +
	600	+, +, +		600	+, +, +
Control		+, +, +	Control		+, +, +

−: Clear dot was formed. (+): Haemagglutination was weaker than control. +: Diffuse mat was formed.
30 μl of body fluid showing haemagglutinin titer 2^{-2} was mixed with 30 μl of PBS containing Mf and incubated at 15°C for 2 hr. Haemagglutinating activity in 50 μl of the mixture was assayed by adding 25 μl of erythrocyte suspension at 4°C. Live Mf heated at 95°C for 5 min become dead Mf.

A preliminary experiment showed that *Ar. subalbatus* haemagglutinin (titer: 2^{-2}) was inactivated by a sugar solution consisting of stachyose (25 mM), GalNAc (25 mM), GlcNAc (25 mM), ManNAc (25 mM) and NeuNAc (25 mM). The same mixture was therefore tested for an effect on deposition of melanin on Mf in mosquitoes. Twenty-four hr after injection of live Mf of *B. pahangi* following injection of sugar solution, average percentage of Mf with melanin deposits to total Mf per isolated abdomen was 53.5, 61.9, 50.2 and 1.6% in isolated abdomens provided with 0.9% NaCl solution, 150, 200 and 250 mM of the sugar solution, respectively (Fig. 4).

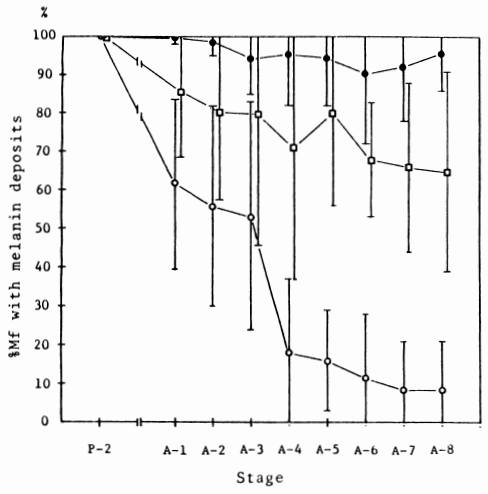

Fig. 3 Changes in average percentages of Mf with melanin deposits out of total Mf per mosquito in *Ar. subalbatus*. Each dot or square represents the result obtained from dissection of 9 to 11 mosquitoes injected with Mf (○—○: *B. pahangi* Mf, □—□: *B. malayi* Mf and ●—●: Dead Mf of *B. pahangi*).

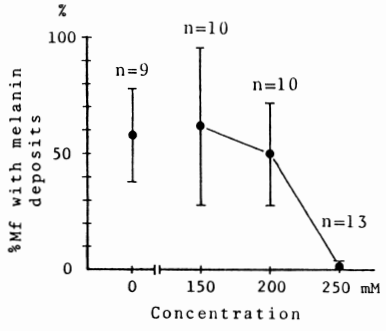

Fig. 4 Concentration effect of mixture of stachyose, GalNAc, GlcNAc ManNAc and NeuNAc on melanin deposition on *B. pahangi* Mf placed in abdominal haemocoeles of 2-day old female adults of *Ar. subalbatus*. A mosquito was injected with 0.6 μl of test solution and with 0.2 μl of Ca^{2+} free HBSS containing 20 to 50 Mf and then dissected 24 hr after injection. Dots show the average percentage of Mf with melanin deposits out of total Mf per isolated abdomen (n: no. of abdomens dissected).

By the same method, the effect of sugar mixtures on melanin deposition on *B. pahangi* Mf, *B. malayi* Mf and dead Mf of *B. pahangi* was examined. Average percentage of both *B. pahangi* and *B. malayi* Mf with such deposits in isolated abdomens given the sugar solution consisting of stachyose (250 mM), GalNAc (250 mM), GlcNAc (250 mM), ManNAc (250 mM) and NeuNAc (250 mM) was significantly lower than those in isolated abdomens provided with 0.9% NaCl solution ($P < 0.002$ level by t-test) (Table 2). Average percentage of both *B. pahangi* and *B. malayi* Mf with melanin deposits in isolated abdomens provided with sugar solution from which one component of the aforementioned mixture had been deleted were also significantly lower than those in controls ($P < 0.05$ level by t-test) (Table 2). In the case of dead of Mf of *B. pahangi*, however, melanin deposition on Mf occurred irrespective of the presence or absence of sugar mixture.

Effect of sugar mixture on development of B. pahangi and B. malayi larvae in abdominal haemocoel of Ar. subalbatus

When isolated abdomens were provided with both sugar mixture and *B. pahangi* Mf or with *B. pahangi* Mf alone, average percentage of microfilari-form larvae with melanin deposits out of total larvae per abdomen was 11.3 and 45.2%, respectively (Table 3). When the abdomen were provided with both sugar mixture and *B. malayi* Mf or with *B. malayi* Mf alone, average percentages of microfilari-form larvae with melanin deposits were 31.0 and 65.5%, respectively. In these abdomens, average per-

Table 2 Effect of mixture of stachyose and N-acetylated sugars on melanin deposition on Mf of *B. pahangi* and *B. malayi* placed in abdominal haemocoels of 2-day old female adults of *Ar. subalbatus*

	% Mf with melanin deposits in an isolated abdomen (Average ± SD)		
	Mf of *B. pahangi*	Mf of *B. malayi*	Dead Mf of *B. pahangi*
Sugar mixture*	1.6 ± 2.9 (13)	0.7 ± 2.1 (9)	81.3 ± 13.6 (11)
Sugar mixture devoid of;			
Stachyose	9.1 ± 17.8 (10)	25.6 ± 29.3 (10)	
GalNAc	7.1 ± 9.9 (10)	22.8 ± 21.2 (9)	
GlcNAc	4.8 ± 7.3 (10)	13.6 ± 10.1 (11)	
ManNAc	16.1 ± 19.3 (10)	7.3 ± 4.6 (11)	
NeuNAc	9.9 ± 17.1 (11)	44.7 ± 27.7 (14)	
0.6 μl of 0.9% NaCl	53.3 ± 27.6 (9)	74.3 ± 26.8 (7)	99.1 ± 2.2 (6)

*Mixture consisted of 250 mM stachyose, 250 mM GalNAc, 250 mM GlcNAc, 250 mM ManNAc and 250 mM NeuNAc.
A mosquito was injected with 0.6 μl sugar mixture and 0.2 μl Ca^{2+} free HBSS containing 20 to 50 Mf and then ligated behind the thorax. Isolated abdomens were dissected 24 hr after injection of Mf. Parentheses show number of isolated abdomens dissected.

Table 3 Effect of mixture of stachyose and N-acetylated sugars on the larval development of *B. pahangi* and *B. malayi* in isolated abdomens of *Ar. subalbatus*

Injection		% Microfilari-form[a] larvae per isolated abdomen (Average ± SD)		% Developing larvae[b] per isolated abdomen (Average ± SD)	
		Intact	With melanin deposits	Intact	With melanin deposits
B. pahangi	Sugar mixture	40.3 ± 19.5	11.3 ± 17.6	45.0 ± 17.2	3.4 ± 6.1 (9)
	Control	8.4 ± 10.9	45.2 ± 35.7	44.5 ± 32.5	1.9 ± 3.2 (10)
B. malayi	Sugar mixture	13.6 ± 12.6	31.0 ± 34.4	46.9 ± 29.0	8.5 ± 12.4 (10)
	Control	13.5 ± 14.8	65.5 ± 39.2	19.8 ± 23.3	1.2 ± 2.5 (11)

Isolated abdomen was provided with approximate 5 μl of RPMI 1640 supplemented with FBS (20%), GalNAc (30 mM), GlcNAc (30 mM), ManNAc (30 mM), NeuNAc (30 mM) and 20 to 50 Mf or supplemented with FBS and 20 to 50 Mf as control. isolated abdomens were dissected 4 days after injection.
a) Microfilari-form larvae: Body width was less than 8 μm.
b) Developing larvae: Body width was 8 μm or more.
Parentheses show numbers of isolated abdomens dissected.

centage of developing larvae over 8 μm in body width was respectively 46.9 and 19.8%, and the differences between these percentages was statistically significant at < 0.05 level by t-test.

Discussion

Haemagglutinating activity of body fluid from *Ar. subalbatus* was high in pupae and decreased gradually in adults after eclosion. Haemagglutinating activity in haemolymph of the silkworm, *Bombyx mori,* also varies with the stage of development, and the *Bombyx* haemagglutinin may be adsorbed to the surface of larval tissue destined for decomposition or to developing adult tissues (Suzuki and Natori, 1983). Haemagglutinin in *Ar. subalbatus* may play the same role intrinsically during reconstruction of tissues. *Ar. subalbatus* haemagglutinin also seems to attach to surface of Mf, as suggested by loss of the haemagglutinating activity when *Ar. subalbatus* body fluid and *B. pahangi* Mf were coincubated. *Ar. subalbatus* haemagglutinin binds to stachyose and to a lesser extent N-acetylated sugars such as GalNAc, GlcNAc, ManNAc and NeuNAc (Ogura *et al.,* 1985a). In comparison, various lectins of plant origin cohere to GlcNAc and glucose or mannose that are present on the sheath surface of *B. pahangi* Mf (Furman and Ash, 1983), and to GlcNAc on that of *B. malayi* Mf (Kaushal *et al.,* 1984). Haemolymph of the American cockroach, *Periplaneta americana,* strongly agglutinates freshly-hatched oncospheres of *Hymenolepis diminuta* (Lackie, 1981). Haemolymph of the locust, *Shistocerca gregaria,* and of *P. americana* agglutinates trypanosomatid flagellates, *Trypanosoma brucei* and *Leishmania hertigi* (Ingram *et al.,* 1984). Therefore, *Ar. subalbatus* haemagglutinin would be expected easily to attach to the surface of Mf which invade into *Ar. subalbatus* haemocoel.

When Mf were injected into *Ar. subalbatus,* the frequency of melanin deposition on *B. pahangi* Mf was high in pupae and 1 to 3-day old adults and low in 4 to 8-day old adults. This trend of frequency was in good conformity to the developmental change of haemagglutinating activity in the mosquito's body fluid. Injection of haptenic sugars against the haemagglutinin into *Ar. subalbatus* decreased melanin deposition on live Mf of both *B. pahangi* and *B. malayi* Mf. Moreover, after 4 days of incubation of the sugars and *B. pahangi* or *B. malayi* Mf in abdominal haemocoels of *Ar. subalbatus,* the number of microfilari-form larvae with melanin deposits decreased in both species and, there was an increase in number of developing *B. malayi* larvae.

If *Ar. subalbatus* haemagglutinin attaches to Mf, the quantity of haemagglutinin adhering to Mf may have a certain relation with the melanin deposiiton on Mf. Competition between haptenic sugars against *Ar. subalbatus* haemagglutinin and surface sugars of Mf may reduce attachment of the haemagglutinin to Mf, resulting a reduction of melanin deposition on Mf. The competition may also allow limited larval development of filariae even in refractory mosquitoes. In the European crayfish, *Astacus astacus,* cell walls of yeast, *Saccharomyces cerevisiae,* were encapsulated by haemocytes and eventually melanized (Söderhäll *et al.,* 1979); the authors suggested that lectin-like proteins conjunct to phenoloxidase might be involved in melanization.

In *Ar. subalbatus, B. pahangi* Mf develop into infective larvae, and *B. malayi* Mf do not. Dark pigmented 1st stage larvae of *B. malayi* are observed in thoracic muscle (Wharton, 1962) or in both thorax and abdomen (Yamamoto *et al.,* 1985) of *Ar. subalbatus.* Defense reactions against Mf in the abdominal haemocoel of *Ar. subalbatus* have been reported by Kobayashi *et al.* (1986) as follows: first, Mf are enclosed in a dark brown capsule; second, many round shaped host cells attach to the capsule; third, the adhered cells flatten to become the outer layer. Such defense reactions are remarkable against *B. malayi* (Yamamoto *et al.,* 1985). In this study, moreover, it is shown that pupae and early aged adults of *Ar. subalbatus* have a defense reaction against

B. pahangi Mf injected into the abdominal haemocoel. Continued investigation will elucidate the role of haemagglutinin in defense reactions.

Summary

Haemagglutinating activity of body fluid collected from *Ar. subalbatus* was high at the pupal stage and decreased gradually after emergence. This activity was lost after incubation with *B. pahangi* Mf at 15°C for 2 hr. The frequency of melanin deposition of *B. pahangi* Mf in *Ar. subalbatus* was high in pupae and 1 ro 3-day old adults and low in 4 to 8-day old adults. Since number of *B. pahangi* and *B. malayi* Mf with melanin deposits in abdominal haemocoeles of *Ar. subalbatus* was decreased after injection of haptenic sugars against the haemagglutinin, haemagglutinin may mediate defense reaction of *Ar. subalbatus* to Mf.

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オオクロヤブカ *Armigeres subalbatus* における赤血球凝集活性と *Brugia pahangi* および *B.malayi* ミクロフィラリアへのメラニンの沈着について

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オオクロヤブカの体液中には赤血球凝集素が含まれており，その凝集活性は蛹期に高くて羽化後漸次減少する．この凝集活性を示す体液を *B.pahangi* ミクロフィラリア（Mf）を含んだPBSと15°C下で2時間インキュベートした場合にその活性が消失したので，オオクロヤブカ体液の赤血球凝集素は赤血球のみならずMfへも付着すると推察された．一方，様々な発育段階のオオクロヤブカ雌個体の腹部体腔内へ *B.pahangi* Mfを注入した場合，メラニンの沈着したMfの全Mfに対する割合は蛹では100%，羽化後3日目までの成虫では50%以上で羽化後4日から8日目までの成虫では20%以下であった．さらに，オオクロヤブカ体液の赤血球凝集素に対するハプテンにあたる数種糖の混合物を注入したオオクロヤブカ雌成虫腹部体腔内においては，メラニンの沈着した *B.pahangi* および *B.malayi* Mfの割合が著しく減少した．これらの結果から，オオクロヤブカのMfに対するメラニン沈着を伴なう防御反応において，体液中の赤血球凝集素が何らかの役割を果している可能性が示唆された．