Research Note

In vitro adhesion of enzyme(s) related to melanin formation in the pupal mosquito haemolymph to the surface of *Brugia pahangi* microfilaria

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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; Kobayashi *et al.*, 1986a, b). However, the manner in which melanin is formed on the larval surface is not understood. There are two hypotheses: 1) the haemocytes initiate the melanin formation (Forton *et al.*, 1985), and 2) humoral encapsulation takes place around the larvae followed by cellular participation (Chen and Laurence, 1985; Kobayashi *et al.*, 1986a). Humoral encapsulation is known only in certain dipteran insects such as Culicidae and Chironomidae and the reaction is correlated with the low count of haemocytes in the haemolymph. In Insecta or Crustacea melanin is known to be synthesized from aromatic amino acids such as tyrosine by phenoloxidase which is produced by an activation of prophenoloxidase. It is probable that the activation of prophenoloxidase in the mosquitoes follows the pathway proposed by Ashida and Söderhäll (1984), that is, the activation is initiated by β -1,3-glucans with the aid of serine pro-

tease. If prophenoloxidase was activated in the circulating haemolymph of the mosquitoes when the parasites invaded them, a total melanization of the haemolymph would occur. However, this has never been seen in the haemolymph of *Armigeres subalbatus*, though a strong encapsulation was observed around microfilariae (Mf) of *Brugia malayi* (Yamamoto *et al.*, 1985).

From an ultrastructural observation of the melanization processes in *Culex pipiens* larvae to the nematode, *Neoplectana carpocapsae*, Poinar and Leutenegger (1971) proposed that melanin is formed from components in the noncellular portion of the haemolymph coagulated on the surface of the nematode. Other ultrastructural observations of encapsulated Mf in the mosquitoes strongly suggest that capsule materials contain melanin (Chen and Laurence, 1985; Forton *et al.*, 1985). Vey and Götz (1975) also observed that *in vitro* humoral encapsulation occurred on entomogenous fungi, a certain foreign body and nematodes in the larval *Chironomus* haemolymph. Kobayashi *et al.* (1986a) described the adhesion of a light brown substance to the *B. malayi* Mf in the abdominal haemocoel of *Ar. subalbatus* within 30 min post-infection. To date, however, the chemical natures of such capsules adhered to the surface of the invading parasite during the initial processes of humoral encapsulation have not been analyzed. The present study was carried out to learn the participation of enzymes in the haemolymph in the melanin formation on the surface of Mf.

Brugia pahangi Mf were collected from the infected birds by injecting warmed 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 leucocytes from the host. Then Mf were collected by centrifugation after washing twice with HBSS. Packed Mf were resuspended in distilled water and were killed in a hot water bath (95°C) for 5 min. An aliquot of the Mf suspension was smeared on albuminized glass slides and the rest was kept in a freezer at -30°C for later use. Haemolymph of 2-day old female pupae of *Ar. subalbatus* (406 strain) was collected by

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the centrifugation method according to Ogura *et al.* (1985) with some modifications, that is, an addition of a small amount of phenylthiourea (PTU) to centrifugation tubes and spinning at 1,500 *g* for 5 min in a second centrifugation to eliminate cells or cellular components. Five to 6 μ l of the supernatant was immediately put on the glass slides previously smeared with Mf as described above. These had been kept in a wet chamber for 2 hr at room temperature before the second incubation for pigment deposition on the Mf. Haemolymph on the glass slides was found to have no formation of blackish pigment during this preincubation. Thereafter, the glass slides were washed twice with HBSS and covered with 5–6 μ l supernatant of thermocoagulated haemolymph which had been prepared by heating at 95°C for 5 sec. They were incubated again for 1 hr in a wet chamber at room temperature, and melanin formation occurring on the Mf was observed under a microscope. Fully and/or partially melanized Mf were counted as positive (Fig. 1). When Mf were incubated with both PTU-treated and thermocoagulated haemolymph, 63.2% of Mf was melanized during the second incubation (Table 1). In a control experiment of Mf incubated with PTU-treated haemolymph and HBSS, a small number of Mf (7.7%; 10/130) showed a positive reaction though partially deposited pigment on Mf was very thin as compared with that on Mf incubated with thermocoagulated haemolymph. When Mf on the glass slides were incubated with PTU-treated haemolymph and then heated at 95°C for 20 sec no pigment formation on the Mf was observed during the second incubation with the thermocoagulated haemolymph. Mf incubated with PTU-treated haemolymph were not pigmented during the second incubation with thermocoagulated haemolymph previously added with a small amount of PTU. These data suggested that the heat unstable enzyme(s) related to melanin formation adhered to the surface of Mf during the preincubation and that a melanization reaction on the Mf surface occurred with the addition of substrate(s) or heat stable factor(s) during the second incubation.

Coomassie brilliant blue (CBB) was used

Fig. 1 *In vitro* melanization of heat-killed microfilaria of *Brugia pahangi* incubated with the supernatant of thermocoagulated haemolymph of pupal mosquito, *Armigeres subalbatus*.

Table 1 Adhesion of enzyme(s) related to melanin formation in the haemolymph collected from pupal mosquitoes, *Armigeres subalbatus* to the surface of heat-killed microfilariae of *Brugia pahangi* *in vitro*.

Condition of reaction	No. of Mf observed	Rate of melanization (%)
PTU-Haemo.* and TC-Haemo.**	155	63.2
PTU-Haemo. and HBSS***	130	7.7
PTU-Haemo., Heat-treatment**** and TC-Haemo.	70	0.0
TC-Haemo. only	149	0.0

* Preincubation of heat-killed Mf on glass slides with PTU-treated haemolymph of pupae for 2 hr.

** Incubation with supernatant of thermocoagulated (TC-) haemolymph of pupae for 1 hr.

*** Incubation with HBSS for 1 hr.

**** After preincubation glass slides were heated at 95°C for 20 sec.

to detect attached substances around Mf in the preincubated specimens. Mf on the glass slides were incubated with PTU-treated haemolymph for 2 hr, washed twice with HBSS, and stained with 0.05% CBB solution for 10 min. Although Mf themselves were stained light blue, the majority showed a heavy blue color on their surface (Fig. 2). This indicates that some proteinous sub-

Fig. 2 Adhesion of proteinous substances stained with Coomassie Brilliant Blue to the surface of heat-killed microfilaria of *Brugia pahangi*.

Arrows show heavy blue deposition on the surface of microfilaria.

stances in the haemolymph attach to the surface of Mf, and therefore strongly suggests that these substances may be the enzyme(s) associated with melanin formation or proteinous substances containing prophenoloxidase or active phenoloxidase. In the present experiments the authors did not determine whether the enzyme(s) adhering to the surface of Mf during the preincubation was prophenoloxidase or active phenoloxidase. Another experimental model is needed to definitely resolve this. In the isolated haemolymph of *Chironomus* larvae foreign organisms were initially completely enclosed by a soft sticky material, then hardened, and finally brown pigmentation appeared (Götz, 1986). The substances detected in the present study might be analogous to the materials observed in *Chironomus*. Haemagglutinin activity of the fluid collected from the pupae of *Armigeres subalbatus* was high and the frequency of pigment deposition of the inoculated *B. pahangi* Mf was also high in the pupal stage (Ogura, 1986). There is some possibility that haemagglutinin mediates the attachment of enzyme(s) to the surface of Mf, although the initial process of enzyme adhesion is still obscure. Chen and Laurence (1987a, b) reported that transparent materials were deposited on the surface of *B. pahangi* Mf *in vitro* using haemolymph of *Anopheles quadrimaculatus* in the presence of phenoloxidase inhibitor. However, in their paper it was not clear whether the transparent materials contained an enzyme(s) related to

melanin formation. In the present experiments, *in vitro* adhesion of enzyme(s) to Mf using L-DOPA as a substrate showed that these Mf were melanized the same as those incubated with thermocoagulated haemolymph (unpublished data).

In this report the authors have shown the adhesion of an enzyme(s) possibly related to melanin formation on the surface of Mf without apparent participation of the cells in the haemolymph of mosquitoes.

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摘 要

In vitro における蚊体液中のメラニン形成 に關与する酵素のマイクロフィラリア 体表への付着

オオクロヤブカの雌蛹より遠心法によって集められた体液と、熱処理で殺した *Brugia pahangi* のマイクロフィラリア (Mf) とをスライドガラス上で2時間反応させた。反応後スライドガラスをハanks液 (HBSS) で2回洗浄し、加熱凝固させた体液上清と1時間反応させたところ、全体の63.2% (98/155) のMfにメラニンの沈着が起こった。2回目の反応をHBSSで行った場合は全体の7.7% (10/130) のMfに淡褐色の被囊が観察されたが、加熱凝固させた体液上清のみとの反応、第二の反応系にフェニルチオウレアを添加した場合、また、最初の2時間の反応後スライドガラスを95°C, 20秒間加熱した後、加熱凝固体液上清と反応させた場合には色素の沈着がまったく起こらなかった。以上の結果より最初の反応でメラニン形成に關与する酵素または酵素を含む蛋白質のMf体表への付着が起こり、第二の反応では加熱凝固体液の上清中に含まれている基質によってメラニンの沈着が起こったものと考えられる。