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ScienceDirect
Veterinary Parasitology 144 (2007) 371–374
veterinary parasitology
www.elsevier.com/locate/vetpar

Short communication

Penetration of the mosquito midgut is not required for *Brugia pahangi* microfilariae to avoid the melanotic encapsulation response of *Armigeres subalbatus*

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Received 3 August 2006; received in revised form 15 September 2006; accepted 29 September 2006

Abstract

Insect vectors of disease have the capacity to respond to, and prevent further development of, parasites and pathogens using a response known as melanotic encapsulation. The naturally-occurring *Armigeres subalbatus-Brugia* spp. system provides an excellent way to investigate melanotic encapsulation and immune recognition in a mosquito host, because *Brugia malayi* microfilariae (mf) acquired via a blood meal are rapidly melanized in the body cavity of *Ar. subalbatus*, but *Brugia pahangi* mf evade or suppress the immune response and develop normally into infective stage larvae. Previous studies have suggested that *B. pahangi* mf are changed in some manner in the process of exiting the mosquito gut, thereby facilitating escape from, or suppression of, the melanotic encapsulation response. By inoculating mosquitoes with parasites, thus circumventing the midgut, we show that ~88% of *B. pahangi* mf escape the melanotic encapsulation response while approximately 90% of inoculated *B. malayi* mf are melanized. Methods to isolate parasites for this procedure are described. These results mimic those observed in *Ar. subalbatus* against *Brugia* spp. mf that are ingested following blood feeding, and demonstrate that midgut penetration is not required for *B. pahangi* mf to avoid the melanotic encapsulation response of *Ar. subalbatus*.

Keywords: *Brugia malayi*; *Brugia pahangi*; *Armigeres subalbatus*; Melanization; Blood-isolated; Microfilaria

1. Introduction

Melanotic encapsulation is an immune response employed by mosquitoes to destroy protozoan and metazoan parasites, including malaria parasites and filarial worms that cause lymphatic filariasis and dog heartworm (Christensen et al., 2005; Beerntsen et al., 2000). Efforts to study melanotic encapsulation often have involved the isolation of microfilariae (mf) of *Brugia* spp. and *Dirofilaria immitis* from vertebrate blood via a series of distilled water washes. These mf then are injected intrathoracically into mosquitoes, thereby initiating a melanization response (Christensen et al., 1984). Microfilariae that have been washed with saline and then subjected to a filtration technique prior to injection into a mosquito also elicit a melanization response, although it is not as robust (Beerntsen et al., 1989).

The naturally-occurring *Armigeres subalbatus-Brugia* spp. system provides an ideal way to investigate immune recognition as well as the melanotic encapsulation response, because it circumvents the inoculation process and avoids a wound healing response that

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doi:10.1016/j.vetpar.2006.09.037

incorporates many of the same components and pathways involved in a melanization response against mf (Lai et al., 2002). In this system, *B. malayi* mf are rapidly melanized in the hemocoel of *Ar. subalbatus*, but *B. pahangi* mf evade or suppress the immune response and develop normally into infective stage larvae (Yamamoto et al., 1985; Beerntsen et al., 1989).

This latter observation, coupled with those involving the inoculation of mf isolated via distilled water or saline washes, suggested that penetration of the mosquito midgut changes *B. pahangi* mf in some manner to facilitate escape from, or suppression of, the melanotic encapsulation response (Sutherland et al., 1984; LaFond et al., 1985; Christensen et al., 1987; Damian, 1997). In this study, we use *B. malayi* and *B. pahangi* mf from cat and dog blood, respectively, isolated using a modified filtration technique, to test the hypothesis that midgut penetration is prerequisite for immune evasion. When isolated and injected into mosquitoes, *B. malay*i mf are melanized, but most *B. pahangi* mf evade the melanotic encapsulation response, mimicking the situation that exists when mosquitoes are infected with a blood meal and the mf subsequently penetrate the midgut prior to entering the mosquito hemocoel. This result suggests that *B. pahangi* mf do not need to penetrate the *Ar. subalbatus* midgut to avoid or suppress the melanization response.

2. Material and methods

2.1. Mosquitoes

Ar. subalbatus were maintained as previously described (Beerntsen et al., 1989). Adult mosquitoes were maintained at 27 °C and 80% relative humidity with a 16 h light and 8 h dark cycle. Three to 7 day old female mosquitoes were used for these experiments.

2.2. Isolation of microfilariae

B. malayi mf were isolated from feline blood and *B. pahangi* mf were isolated from canine blood. Blood containing *B. pahangi* or *B. malayi* mf was filtered and concentrated using Millipore mixed cellulose esters filters (5.0 μm, white, SMWP, 25 mm). Briefly 1.0 ml of blood containing mf was added to 9.0 ml of physiologic (*Aedes*) saline (Hayes, 1953) and the mix was passed through a 10.0 ml syringe connected to a filter holder containing a pre-wetted filter. The filter then was washed with an additional 5.0–10.0 ml of *Aedes* saline before being removed and placed in a watch glass containing sufficient *Aedes* saline to keep the filter hydrated. After the desired amount of blood had been filtered, mf were washed off of filters using a Pasteur pipet and saline. These combined mf were brought to 10.0 ml in saline and filtered one final time. The mf then were washed off of the final filter into a watch glass kept on ice. After isolation, mf motility was confirmed for both species as a preliminary measure of mf viability.

2.3. Inoculation of microfilariae and mosquito dissection

Approximately 20 mf were aspirated into a finely drawn capillary tube and inoculated intrathoracically into a single mosquito using methods previously described (Christensen, 1981). At 48 h post-inoculation (PI), inoculated *Ar. subalbatus* were dissected in a drop of *Aedes* saline on a microscope slide as described by Beerntsen et al. (1989). Slides were examined at 100–200× using phase-contrast optics. The presence of melanotic deposits on all or part of a microfilaria was considered a positive reaction. A portion of the inoculated mosquitoes was held for 14 days PI, at which time the prevalence and mean intensity of third-stage larvae were determined. Experiments were performed in triplicate using independent populations of mf and mosquitoes. Microfilaria melanization data were analyzed using a z-test and L_3 infection prevalences were analyzed using a Fisher exact test.

3. Results and discussion

At 48 h PI, the mf-inoculated mosquitoes were dissected and melanization rates assessed (Fig. 1). Using this isolation approach, approximately 90% of *B. malayi* mf were melanized while only ∼12% of *B. pahangi* mf were melanized ($z = 16.035$; P-value < 0.001). On day 14 PI, the prevalence rates of infective third-stage larvae (L_3s) were 0% (0/50) and 100% (44/44), with mean intensities of 0 and 11.0 L_3s

Table 1
Percentage of *Ar. subalbatus* infected and mean parasite intensity of third-stage larvae in mosquitoes inoculated with *B. pahangi* or *B. malayi* microfilariae

Parasite species	Percentage infected[a]	Mean parasite intensity ± S.E. (range)
B. pahangi	100 (44/44)[b]	11.0 ± 0.799 (1–22)
B. malayi	0 (0/50)	0

[a] Fisher exact test: $P < 0.001$.
[b] Number of mosquitoes harboring infective-stage larvae/number of mosquitoes inoculated and surviving 14 days PI.

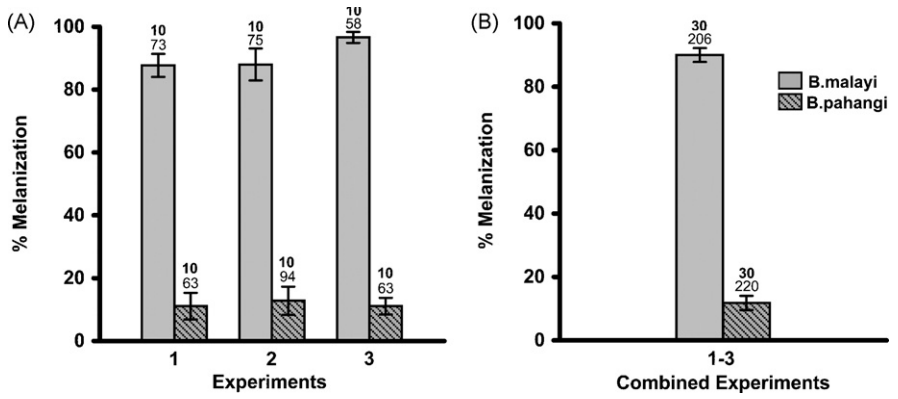

Fig. 1. Melanization response at day 2 post-inoculation in *Ar. subalbatus* inoculated with *B. malayi* or *B. pahangi* microfilariae (mf) isolated from blood. *B. pahangi* mf are melanized at a significantly lower rate than *B. malayi* mf. Data shown are for each individual experiment (A) as well as for the combined experiments (B). Range bars indicate standard error of the mean. Above the range bars, the top number indicates the number of mosquitoes examined; the bottom number indicates the number of mf recovered. All comparisons of the two parasite species are significant with a *P*-value < 0.001; $z = 16.035$ for the combined experiments.

for mosquitoes inoculated with *B. malayi* and *B. pahangi*, respectively (Table 1). The presence of L_3s in injected mosquitoes demonstrated that *B. pahangi* mf, unlike *B. malayi* parasites, were able to avoid the melanotic encapsulation response of *Ar. subalbatus* and develop successfully into infective stage larvae. In contrast, mf isolated from mammalian host blood using a distilled water method resulted in large proportions of melanized mf of both *B. malayi* (~90% by Day 2 PI) and *B. pahangi* (~80% by Day 2 PI) (Beerntsen et al., 1989). Beerntsen et al. (1989) also showed that ~65% of *B. pahangi* ($n = 28$ mosquitoes examined) and 82% of *B. malayi* mf ($n = 25$ mosquitoes) isolated from blood using a Metricel® (Pall/Gelman) filter resulted in a significantly lower rate of melanization at day 3 PI for *B. pahangi* as compared with *B. malay*i ($P < 0.018$). Since this paper was published, further modifications of the filtration technique have now consistently and reliably resulted in low melanization rates of inoculated *B. pahangi* by *Ar. subalbatus*, similar to those observed against mf acquired via feeding on a *B. pahangi*-infected vertebrate host.

It appears that differences in the membrane filter composition and fewer washes with *Aedes* saline have resulted in a gentler isolation method as evidenced by the reduction in melanization of the *B. pahangi* mf. In Beerntsen et al. (1989), a Metricel membrane composed of polyvinylidene chloride was used to isolate the mf followed by several washes with *Aedes* saline until the filtrate was clear in color. In this current research, a membrane made of mixed cellulose esters was used and the mf were washed only twice with *Aedes* saline.

Previous reports have suggested that following midgut penetration, *B. pahangi* mf may have altered surface charges or may have acquired midgut antigens on their surface, thereby enabling them to avoid immune recognition and the subsequent melanotic encapsulation response (Sutherland et al., 1984; LaFond et al., 1985; Christensen et al., 1987; Beerntsen et al., 1989). The results reported herein indicate that *Ar. subalbatus* respond to inoculated blood-isolated mf, which have never been exposed to the midgut environment, in the same differential manner as is observed against mf that are acquired via blood feeding on an infected mammalian host and that subsequently penetrate through the midgut. In this report, most blood-isolated *B. pahangi* mf avoid melanization and develop into L_3s whereas *B. malayi* are melanized and fail to develop into infective stage parasites within *Ar. subalbatus*. Therefore, an inherent difference exists in the presentation of these two species of parasites to the mosquito immune system. Consequently, with the availability of genome projects and functional genomics tools for mosquitoes and filarial worms, including *B. malayi* (Williams et al., 2000; Mongin et al., 2004; Severson et al., 2004; Whitton et al., 2004), it is now an ideal time to revisit the possible explanations as to how parasites evade the immune response within the mosquito. Towards this end, studies designed to assess transcriptional and protein differences between these two morphologically and biologically similar filarial worms are in progress.

In summary, the use of a less disruptive *Aedes* saline/Millipore filter isolation method ensures that the response in *Ar. subalbatus* against each *Brugia* spp. will be the same as that which occurs following blood feeding. More importantly, this research demonstrates that midgut penetration is not required in order for *B.*

pahangi mf to avoid the melanotic encapsulation response of *Ar. subalbatus*.

Acknowledgements

Parasites used in this study were provided by the Filariasis Research Reagent Resource Center (Contract No. N01AI30022), which is funded by the NIAID, NIH, Department of Health and Human Services. We thank Dr. Ron Tessman for statistical analysis advice.

References

Beerntsen, B.T., James, A.A., Christensen, B.M., 2000. Genetics of mosquito vector competence. Microbiol. Mol. Biol. Rev. 64, 115–137.

Beerntsen, B.T., Luckhart, S., Christensen, B.M., 1989. *Brugia malayi* and *Brugia pahangi*: Inherent difference in immune activation in the mosquitoes *Armigeres subalbatus* and *Aedes aegypti*. J. Parasitol. 75, 76–81.

Christensen, B.M., 1981. Observations on the immune response of *Aedes trivittatus* against *Dirofilaria immitis*. Trans. R. Soc. Trop. Med. Hyg. 75, 439–443.

Christensen, B.M., Forton, K.F., Lafond, M.M., Grieve, R.B., 1987. Surface changes on *Brugia pahangi* microfilariae and their association with immune evasion in *Aedes aegypti*. J. Invertebr. Pathol. 49, 14–18.

Christensen, B.M., Li, J., Chen, C.C., Nappi, A.J., 2005. Melanization immune responses in mosquito vectors. Trends Parasitol. 21, 192–199.

Christensen, B.M., Sutherland, D.R., Gleason, L.N., 1984. Defense reactions of mosquitoes to filarial worms: Comparative studies on the response of three different mosquitoes to inoculated *Brugia pahangi* and *Dirofilaria immitis* microfilariae. J. Invertebr. Pathol. 44, 267–274.

Damian, R.T., 1997. Parasite immune evasion and exploitation: reflections and projections. Parasitol. 115, S169–S175.

Hayes, R.O., 1953. Determination of a physiological saline for *Aedes aegypti* (L.). J. Econ. Entomol. 46, 624–627.

LaFond, M.M., Christensen, B.M., Lasee, B.A., 1985. Defense reactions of mosquitoes to filarial worms: potential mechanism for avoidance of the response by *Brugia pahangi* microfilariae. J. Invertebr. Pathol. 46, 26–30.

Lai, S.C., Chen, C.C., Hou, R.F., 2002. Immunolocalization of prophenoloxidase in the process of wound healing in the mosquito *Armigeres subalbatus* (Diptera: Culicidae). J. Med. Entomol. 39, 266–274.

Mongin, E., Louis, C., Holt, R.A., Birney, E., Collins, F.H., 2004. The *Anopheles gambiae* genome: an update. Trends Parasitol. 20, 49–52.

Severson, D.W., Knudson, D.L., Soares, M.B., Loftus, B.J., 2004. *Aedes aegypti* genomics. Insect Biochem. Mol. Biol. 34, 715–721.

Sutherland, D.R., Christensen, B.M., Forton, K.F., 1984. Defense reaction of mosquitoes to filarial worms: role of the microfilarial sheath in the response of mosquitoes to inoculated *Brugia pahangi* microfilariae. J. Invertebr. Pathol. 44, 275–281.

Whitton, C., Daub, J., Quail, M., Hall, N., Foster, J., Ware, J., Ganatra, M., Slatko, B., Barrell, B., Blaxter, M., 2004. A genome sequence survey of the filarial nematode *Brugia malayi*: repeats, gene discovery, and comparative genomics. Mol. Biochem. Parasitol. 137, 215–227.

Williams, S.A., Lizotte-Waniewski, M.R., Foster, J., Guiliano, D., Daub, J., Scott, A.L., Slatko, B., Blaxter, M.L., 2000. The filarial genome project: analysis of the nuclear, mitochondrial and endosymbiont genomes of *Brugia malayi*. Int. J. Parasitol. 30, 411–419.

Yamamoto, H., Kobayashi, M., Ogura, N., Tsuruoka, H., Chigusa, Y., 1985. Studies on filariasis. VI. The encapsulation of Brugia malayi and B. pahangi larva in the mosquito, Armigeres subalbatus. Japanese J. Sanit. Zool. 36, 1–6.