Reduced efficacy of the immune melanization response in mosquitoes infected by malaria parasites

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SUMMARY

Although the mosquito vectors of malaria have an effective immune system capable of encapsulating many foreign particles, they rarely encapsulate malaria parasites in natural populations. A possible reason for this apparent paradox is that infection by malaria reduces the capability of the mosquito to mount an effective immune response. To investigate this possibility, we blood-fed *Aedes aegypti* mosquitoes on an uninfected chicken or on one infected with *Plasmodium gallinaceum*, and compared the proportions of the infected and uninfected mosquitoes that melanized a negatively charged Sephadex bead injected into the thorax 1, 2 and 4 days after blood-feeding. About 40% of the uninfected mosquitoes, but less than 25% of the infected ones, melanized the bead. The difference between infected and uninfected mosquitoes was most obvious 1 day after infection (at the parasite's ookinete stage), while the difference diminished during the early oocyst stage (2 days after infection) and disappeared at the later oocyst stage (4 days after infection). These results suggest that the parasite can either actively suppress its vector's immune response or that it modifies the blood of its chicken host in a way that reduces the efficacy of the mosquito's immune system. In either case, the reduction of immunocompetence can have important consequences for malaria control, in particular for the current effort being invested into the genetic manipulation of mosquitoes.

Key words: malaria, mosquito, invertebrate immunity, immunosuppression.

INTRODUCTION

Malaria remains one of the successful diseases of humans both in terms of its prevalence and of defying our attempts at control. Contributing to its success is the fact that its transmission fails to be prevented by the natural immune responses of its human host or its mosquito vector. But why has the mosquito's immune encapsulation response, an effective defence mechanism against many eukaryotes and micro-organisms (Richman & Kafatos, 1996), not evolved to resist the invading parasites? The reason is unlikely to be a lack of selection pressure, as parasites reduce the mosquito's reproductive success in at least 2 ways: by reducing fecundity (Hogg & Hurd, 1995) and by increasing mortality (Anderson, Knols & Koella, 2000). One possibility for the lack of an immune response would be that it is too costly. Indeed, in *Drosophila* the encapsulation response reduces competitive ability (Kraaijeveld & Godfray, 1997), in bumblebees it reduces survival (Moret & Schmid-Hempel, 2000), and in the mosquito *Aedes aegypti* it is genetically correlated with important life-history traits such as age at pupation (Koella & Boëte, 2002). In general, however, the cost of the immune response does not appear to outweigh its advantages and to have prevented the evolution of effective immunity, as mosquitoes in natural populations have a well-functioning immune system that is able to encapsulate other foreign particles (Schwartz & Koella, 2002) – but not malaria parasites.

Thus, a more likely explanation for the lack of immune responsiveness against malaria may be that infected mosquitoes mount their immune response less effectively than uninfected ones. Several mechanisms could lead to reduced immunocompetence in infected mosquitoes. First, a lower immune response could result from the general stress that the infection puts on the host. Second, the quantity and quality of infected blood, for example, could differ from uninfected blood in a way that does not permit the immune response to be mounted. This mechanism may indeed seem likely, as the immune response of mosquitoes depends on them having obtained a bloodmeal (Chun, Riehle & Paskewitz, 1995; Schwartz & Koella, 2002). A third mechanism for a lack of immunity in mosquitoes could be active suppression by the parasites (Sinden & Billingsley,

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2001). Such immunosuppression has been studied mostly for several parasites that infect humans, e.g. for HIV (Bloom, Salgame & Diamond, 1992), for the leprosy bacterium *Mycobacterium* (Modlin *et al*. 1986) and for the malaria parasite *Plasmodium falciparum* (Urban *et al*. 1999). For invertebrates, the best known examples of immunosuppression are found in several dipteran and hymenopteran parasitoid species, whose larvae develop within their insect host (Strand & Pech, 1995). These parasitoids inject a poly-DNA virus into the insect host (Beckage, 1998; Vinson, 1990), which shuts down the insect's immune system (Rizki & Rizki, 1990).

Here, we study the effect of infection by the malaria parasite *P. gallinaceum* on the melanization immune response of its mosquito vector *Ae. aegypti*. To do so, we compared the ability of infected and uninfected mosquitoes to encapsulate and melanize Sephadex beads. The use of beads has become a standard and convenient method of assaying the melanization response of mosquitoes (Paskewitz & Riehle, 1994; Chun *et al*. 1995; Gorman & Paskewitz, 1997; Suwanchaichinda & Paskewitz, 1998). In particular, we compared 3 stages of the parasite's development: the late ookinete stage (24 h after blood feeding, as the parasite is crossing the mosquito's midgut wall), the early oocyst stage (48 h) and a more mature oocyst stage (96 h, about half way through the parasite's development). We could thus contrast the stage that is most susceptible to the mosquito's encapsulation response (Collins *et al*. 1986; Vaughan, Noden & Beier, 1992; Gouagna *et al*. 1998) with a stage that appears to avoid recognition by the immune system by incorporating mosquito-derived proteins onto or into their surface capsule (Adini & Warburg, 1999).

MATERIALS AND METHODS

Mosquito rearing

The yellow fever mosquito *Ae. aegypti* is a ubiquitous species in the tropics and subtropics. Our colony was derived in 1999 from a natural population in Senegal and kept at several hundred mosquitoes in each generation in an attempt to maintain genetic diversity at a level close to that of the natural population. For the experiment, mosquitoes were reared in a climate chamber kept at 28 (±0·5) °C and 75 (±5)% relative humidity with a 12 h:12 h light dark cycle. We hatched several thousand larvae synchronously by flooding eggs under reduced pressure for 20 min, added the larvae to 1 litre of demineralized water in a plastic pan and fed them with a standard amount of food TetraMin™ (day 1: 0·04 mg per larva; day 2: 0·06 mg per larva; day 3: 0·12 mg per larva; day 4: 0·24 mg per larva; day 5, 7, 9, …: 0·48 mg per larva). We used only those mosquitoes that pupated at the same age (day 8), so that a possible effect of age at pupation could be eliminated.

Infection by P. gallinaceum

We used *P. gallinaceum* strain 8A and the domestic race of its natural host, 3- to 4-week-old White Leghorn chickens (*Gallus gallus domesticus*). All experimental animals were maintained according to European Union guidelines. The chickens were infected by intravenous injection of 0·5 ml of infected blood (~15% parasitaemia). Four days after infection, the chickens were used to feed (and infect) a total of about 700 mosquitoes. The experiment was run in 2 replicates, each with 1 infected and 1 uninfected chicken. Within each replicate, we allowed mosquitoes (4 or 6 days after emergence) to feed on an infected or an uninfected chicken for about 10 min, and then discarded any individuals that had not obtained a full bloodmeal. In the first replicate, parasitaemia was 11% and gametocytaemia was 0·4%; in the second replicate the corresponding values were 4% and 0·09%. Dissection of 50 surplus mosquitoes that had blood fed on an infected chicken showed that more than 95% of the mosquitoes became infected in both replicates, with geometric mean of 54 oocysts (range 9–155).

Immunocompetence

We tested immunocompetence during 3 stages of the parasite's development: at the late ookinete stage (24 h after blood feeding), at the early oocyst stage (48 h) and at a more mature oocyst stage (96 h). Immunocompetence was assayed as the melanization response against negatively charged CM-25 Sephadex beads. We stimulated the melanization response by inoculating a bead into the mosquito's thorax (this site was chosen for practical reasons). The beads ranged from 40 to 120 μm in diameter; we selected by visual inspection the smallest ones for inoculation. They were rehydrated in saline solution containing 1·3 mM NaCl, 0·5 mM KCl, 0·2 mM $CaCl_2$ and 0·001% methyl green (pH 6·8) (Paskewitz & Riehle, 1994; Gorman & Paskewitz, 1997). We immobilized mosquitoes by chilling them briefly on ice and then inoculated 1 bead with at most 0·3 μl of saline solution into the thorax. Inoculated mosquitoes were kept individually in plastic tubes and supplied with 6% sugar solution. After 48 h, mosquitoes that were able to fly were dissected in a mixture of saline solution and 0·01% methyl green (Paskewitz & Riehle, 1994) and the degree of melanization of their beads was determined. The beads were scored according to 3 groups: no visible melanization, patchy melanization (i.e. leaving unmelanized areas on the bead), or complete melanization.

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Table 1. Test statistics for the comparison of mortality among treatment groups

(Within each group, the percentage survival was arc-sine transformed. The differences among groups were analysed with an analysis of variance.)

Source	D.F.	Sum of squares	*F*	*P*
Replicate	1	$33{\cdot}49 \times 10^{-3}$	0·235	0·635
Age at blood feeding	1	$34{\cdot}22 \times 10^{-3}$	0·240	0·632
Infection status	1	$5{\cdot}85 \times 10^{-3}$	0·041	0·842
Time of inoculation	2	$230{\cdot}05 \times 10^{-3}$	0·807	0·466
Infection * Time of inoculation	2	$50{\cdot}15 \times 10^{-3}$	0·176	0·841
Error	14	$1994{\cdot}98 \times 10^{-3}$		

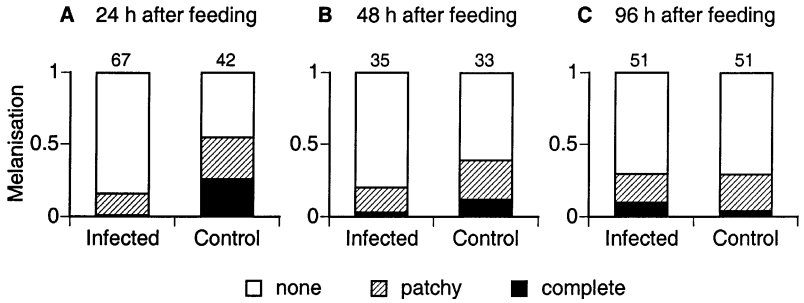

Fig. 1. Melanization response against inoculated beads. Each panel shows the proportion of infected mosquitoes and uninfected controls that melanize a bead to different degrees (no, patchy and complete melanization). (A) Response against beads inoculated 24 h after infection (or blood-feeding), i.e. when the parasite is in its late ookinete stage and most sensitive to the mosquito's melanization response. (B) Inoculation after 48 h, i.e. at an early oocyst stage. (C) Inoculation after 96 h, i.e. at a later oocyst stage. The numbers of mosquitoes are given above each bar.

Statistical analysis

The use of beads has the disadvantage that a fairly large proportion of the mosquitoes dies before being assayed (see Results section). We therefore tested whether a possible association of mortality with infection could have biased our results. The proportion of mosquitoes that survived the inoculation was arcsine-transformed, and its associations with infection status and with time of inoculation after blood-feeding were analysed with an analysis of variance. The 3 levels of the melanization response were analysed with an ordinal logistic analysis that included the infection status and time of inoculation (stage of the parasite) as main factors. The interaction between the two factors indicates stage-specific effects of the parasite. In both analyses, time of inoculation was coded as a nominal factor, as we could not assume that its relationships with mortality and immunocompetence were linear. Two potential confounding factors were included. First, wing length (calculated as the mean length of the 2 wings, measured from the distal end of the allula to the tip of vein R3) was included, as previous studies have shown an effect of body size on immunocompetence (Suwanchaichinda & Paskewitz, 1998). Second, age at blood-feeding was included, as immunocompetence tends to decrease with age (Chun *et al*. 1995; Schwartz & Koella, 2002). The statistical analyses were performed with JMP version 4.0 (http://www.jmpdiscovery.com/).

RESULTS

Of the 599 mosquitoes that we inoculated, only 279 individuals could be used for further analysis. Although the mortality was high (54 %), it was independent of any of the factors of the study (Table 1) and, in particular, it depended neither on infection status (45 % ± 3 % S.E. survival in uninfected mosquitoes, 48 % ± 2·8 % S.E. in infected ones) nor on time of inoculation (45 % ± 3·2 % S.E. for mosquitoes inoculated 24 h after blood-feeding, 37 % ± 3·6 % S.E. after 48 h, 57 % ± 3·7 % S.E. after 96 h).

As described in previous studies (Chun *et al*. 1995; Schwartz & Koella, 2002), immunocompetence of uninfected mosquitoes decreased with the time of the inoculation since blood-feeding (Fig. 1) and tended to increase with wing length (though the effect was not quite statistically significant; Table 2). More importantly in the context of this study, infection by the malaria parasite generally decreased immunocompetence. Among the 126 uninfected females, 60 % showed no sign of melanization, 27 % had patchy melanization and 13 % had completely melanized the bead. Among the 153 infected females, 78 % did not melanize the bead, 17 % had partly and 5 % had completely melanized the bead. The effect

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Table 2. Test statistics for the ordinal logistic analysis of the degree of melanization of beads inoculated into the thorax of mosquitoes

(Melanization was categorized as 3 levels: no, patchy and complete melanization.)

Source	D.F.	Log-likelihood χ^2	P
Replicate	1	5·879	0·015
Wing length	1	3·479	0·062
Age at blood feeding	1	0·670	0·431
Infection status	1	9·929	0·002
Time of inoculation	2	2·911	0·233
Infection * Time of inoculation	2	15·958	<0·001

of infection on immunocompetence strongly interacted with the time of inoculation (Table 2). Thus, if the bead was inoculated 24 h after infection (i.e. at the late ookinete stage), infection decreased the proportion of mosquitoes that at least partly melanized their bead from 55% to 17%; early oocysts (48 h after infection) reduced melanization from 40% to 20%; the later stage of the oocyst (96 h after infection) had no influence on melanization, with 29% infected and uninfected mosquitoes melanizing their beads (Fig. 1).

DISCUSSION

Our results showed that infection by malaria parasites reduces the effectiveness of the encapsulation immune response, in particular at the late ookinete and, to a lesser extent, at the early oocyst stages. While at the later oocyst stage, the parasite had no effect on the proportion of mosquitoes that at least partly melanized a bead, early ookinetes reduced the proportion by a factor of 3. It is striking that the early stages of the parasite are the ones most sensitive to the mosquito's immunity (Collins *et al*. 1986; Vaughan *et al*. 1992; Gouagna *et al*. 1998), so that the selection pressure for immunosuppression in these stages should be more intense than in later oocyst stages. These, in contrast to the sensitive ookinete, appear to avoid recognition by the immune system by incorporating mosquito-derived proteins onto or into their surface capsule (Adini & Warburg, 1999). Such immune evasion by the oocysts would alleviate their need to actively suppress the encapsulation response.

Several factors that could potentially have confounded these results can be ruled out. First, the high mortality induced by inoculation was independent of infection and time of inoculation of the beads. Therefore, we can assume that the mortality did not bias the conclusions. Second, we controlled for the effect of mosquito size. As expected from earlier studies (Suwanchaichinda & Paskewitz, 1998), immunocompetence increased with the wing length of the mosquitoes (though the effect was not quite statistically significant). This is probably due to competition among larvae, which leads to differences in resource availability: individuals with sufficient resources develop into large adults that have stored enough resources for an effective immune response (Suwanchaichinda & Paskewitz, 1998). Third, although earlier studies have shown that the age of a mosquito can influence its immunocompetence (Chun *et al*. 1995; Schwartz & Koella, 2002), mosquitoes infected at either of two ages (4 or 6 days after emergence) did not differ in the degree of melanization. Finally, a previous challenge of the immune response might have exhausted the immune response and thereby weakened or prevented a response against a subsequent challenge, as occurs in bumblebees (Allander & Schmid-Hempel, 2000). In our study, however, no oocysts were found to be encapsulated, so that the initial infection could not have depleted the necessary resources available for the immune response against the bead.

As already mentioned, the reduced efficacy of the immune response in infected mosquitoes might be explained by at least 3 mechanisms. First, a lower immune response could result from the general stress that the infection puts on the host. Second, infection by *Plasmodium* parasites might modify the blood of the chickens in a way that suppresses the immune response in the mosquitoes. The anaemia, for example, of the infected chickens (a decrease of the red blood cells from about 3×10^6 rbc/μl to $2{\cdot}5 \times 10^6$ rbc/μl in the first replicate and to $2{\cdot}9 \times 10^6$ rbc/μl in the second replicate) may decrease the immunocompetence of mosquitoes, as the encapsulation response is more efficient in blood-fed mosquitoes (Chun *et al*. 1995; Schwartz & Koella, 2002). The anaemia, however, is so slight that it seems unlikely to have a major effect on the encapsulation response. Furthermore, specific antibodies (IgG but less so IgM) can pass from the vertebrate into the mosquito's haemolymph (Lackie & Gavin, 1989; Vaughan *et al*. 1990), and these antibodies could possibly interfere with the mosquito's immune response. If this were indeed the case, it might indicate a sophisticated, indirect mechanism, with which the parasite manipulates its mosquito vector's immune response.

Another possible mechanism could be active immunosuppression by the parasite. Thus, the parasite could secrete or excrete molecules that change the microenvironment around the parasite and thus make recognition of the parasite more difficult. Such a mechanism is used, for example, by the nematode *Brugia pahangi* infecting *Ae. aegypti* (Christensen & LaFond, 1986). This mechanism, however, operates only within the immediate surroundings of the parasite and is therefore unlikely to explain our results, as the parasite remains attached

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to the intestinal tube in the abdomen, while the beads were inoculated into the thorax. By contrast, systemic immunosuppression like the one observed here is employed by several dipteran and hymenopteran parasitoid species, whose larvae develop within their insect host (Strand & Pech, 1995). The similarity with their immunosuppression is underlined by the stage-specific inhibition of encapsulation by malaria parasites and by parasitoids. In the malaria system, the mosquitoes infected with early stages (early oocysts and in particular ookinetes) but not with more mature oocysts of the parasite *P. gallinaceum* have lower immunocompetence than uninfected controls. In host–parasitoid systems, the encapsulation of beads inoculated into the haemocoel is only suppressed during the early stages of the parasitoid's development; at later stages immunocompetence is regained, but does not have any detrimental effects for the developing parasitoid larva (Lavine & Beckage, 1996). This suggests that the malaria parasites and parasitoids may have evolved similar mechanisms to avoid the encapsulation response of their hosts.

It is tempting to speculate that immunosuppression could have also been involved in the recent demonstration that malaria ookinetes injected into *Drosophila melanogaster* are not encapsulated and can develop successfully. This lack of encapsulation contrasts strikingly to the observation that the macrophages phagocytose some of the ookinetes, showing that the cellular immune response does not appear to be inhibited (Schneider & Shahabuddin, 2000). Thus, the use of *Drosophila* as the model of choice for the study of innate immune responses (Hoffmann, Reichhart & Hetru, 1996) coupled with the extensive knowledge concerning the mechanisms of immunity in *Drosophila* host–parasitoid relationships (Carton & Nappi, 1997) may open up exciting possibilities to study the molecular basis of suppression by malaria parasites of the mosquito encapsulation response.

We emphasize that, despite several plausible possibilities, the mechanism underlying reduced immunocompetence of infected mosquitoes remains open. But whatever the mechanism, our demonstration that malaria parasites reduce their vector's melanization response will have important consequences for malaria control, in particular for the current effort being invested into the genetic manipulation of mosquitoes. A control program based on genetic manipulation would ideally allow genes controlling the encapsulation mechanism to spread through mosquito populations and thus reduce the transmission of malaria parasites (Collins, 1994). If malaria parasites have generally evolved to suppress their mosquito vector's immune system, the efforts to identify key genes may have to diversify to study not only genes that are involved in mounting the melanization response, but also genes that allow the mosquito to resist the parasite's efforts at immunosuppression. Unfortunately, despite considerable recent progress in our understanding of the interaction between malaria parasites and the mosquito immune system (Dimopoulos *et al*. 1998) and of the genetic basis to encapsulation in the mosquito (Dimopoulos *et al*. 2000; Gorman *et al*. 1996; Zheng *et al*. 1997), research considering immunocompetence in infected mosquitoes, or indeed in any coevolved response by the parasite to the mosquito's immune system, has been neglected. This is underlined by our lack of understanding of species- and line-specific effects. Although, for example, *Anopheles gambiae* that had been selected for refractoriness were able to encapsulate Asian and New World strains of *P. falciparum*, they failed to encapsulate strains from Africa (Collins *et al*. 1986). Such differences within a single species of parasite emphasize the need to expand current research on the study of the mechanisms underlying reduced immunocompetence in infected mosquitoes.

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