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Direct and indirect immunosuppression by a malaria parasite in its mosquito vector

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Malaria parasites develop as oocysts within the haemocoel of their mosquito vector during a period that is longer than the average lifespan of many of their vectors. How can they escape from the mosquito's immune responses during their long development? Whereas older oocysts might camouflage themselves by incorporating mosquito-derived proteins into their surface capsule, younger stages are susceptible to the mosquito's immune response and must rely on other methods of immune evasion. We show that the malaria parasite *Plasmodium gallinaceum* suppresses the encapsulation immune response of its mosquito vector, *Aedes aegypti*, and in particular that the parasite uses both an indirect and a direct strategy for immunosuppression. Thus, when we fed mosquitoes with the plasma of infected chickens, the efficacy of the mosquitoes to encapsulate negatively charged Sephadex beads was considerably reduced, whether the parasite was present in the blood meal or not. In addition, zygotes that were created *ex vivo* and added to the blood of uninfected chickens reduced the efficacy of the encapsulation response. As dead zygotes had no effect on encapsulation, this result demonstrates active suppression of the mosquito's immune response by malaria parasites.

Keywords: immunosuppression; insect immunity; malaria; *Aedes aegypti*; *Plasmodium gallinaceum*

1. INTRODUCTION

Although malaria parasites use a variety of strategies to escape their vertebrate host's immune response (Zambrano-Villa *et al.* 2002), analogous strategies in their mosquito vector have not yet been identified. In a first attempt at describing the influence of malaria parasites on the efficacy of the mosquito's immune response, we recently showed that the immune encapsulation response of the mosquito *Aedes aegypti* is less effective after a blood meal infected with malaria parasites, in particular at the time when the parasite has developed into the ookinete or early oocyst (Boëte *et al.* 2002). As these are the stages that are most sensitive to the mosquito's encapsulation response (Collins *et al.* 1986; Vaughan *et al.* 1992; Gouagna *et al.* 1998), the reduced efficacy of encapsulation in infected mosquitoes could be the result of the parasite's active strategy to suppress the mosquito's immune response. Alternatively, it could be a consequence of the parasite's effect on the quality of the blood in its vertebrate host, as the immunocompetence of mosquitoes depends on a sufficient blood meal (Chun *et al.* 1995; Schwartz & Koella 2002).

Thus, malaria parasites may increase their survival in the mosquito host indirectly, by reducing the blood quality of their vertebrate host, or directly, by actively suppressing the mosquito's immune response. To distinguish between these possibilities and thus to determine whether the parasite actively suppresses the mosquito's immune response, we measured the mosquito's encapsulation response with an experimental design enabling us to examine independently the direct effect of the parasite and the indirect effect of infected blood.

2. MATERIAL AND METHODS

We used *Plasmodium gallinaceum* strain 8A, its vector *Ae. aegypti* and the domestic race of its natural host, 3–4-week-old white leghorn chickens (*Gallus gallus domesticus*). The chickens were maintained according to European Union guidelines. The colony of *Ae. aegypti* was derived from a natural Senegalese population in 1999 and maintained with a population size of several hundred females 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 maintained at 28 ± 0.5 °C and 70 ± 5% relative humidity with a 12 L : 12 D cycle. We hatched several thousand larvae synchronously by flooding eggs under reduced pressure for 20 min, added the larvae to 1 l of demineralized water in a plastic pan and fed them with a standard amount of food Tetra-Min (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).

The experiment was run in three replicates (and we controlled for the differences among replicates in all statistical analyses, see below). In each replicate, we used three chickens, of which we infected two with an intravenous injection of 0.5 ml of infected blood (*ca.* 15% parasitaemia). In each replicate, we bled one of the infected chickens 6 days after infection to obtain infected

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Table 1. Test statistics (analysis of variance) for the comparison of mortality among treatments.
(An analysis of variance analysed the arcsine-transformed proportion of mosquitoes that survived in each treatment. Replicate was included in the analysis as a random factor.)

source	d.f.	sum of squares	*F*	*p*
replicate	2	0.0609	11.673	0.079
status of zygote	1	0.0219	8.378	0.102
plasma status	1	0.0045	1.743	0.318
replicate × zygote	2	0.0203	3.885	0.205
replicate × plasma	2	0.0510	9.776	0.093
zygote × plasma	1	0.0742	28.445	0.033
error	2	0.0052		

plasma (parasitaemia in the three replicates: 1, 2 and 4% red blood cells infected), bled the other infected chicken to obtain gametocytes and produce zygotes (parasitaemia: 3, 5 and 10%), and bled the uninfected chicken to obtain uninfected plasma and red blood cells. The zygotes were prepared *in vitro* as described previously (Kaushal *et al.* 1983). We killed *ca.* 50% of the zygotes by heating them for 1 h at 56 °C. In the other two chickens, the plasma was separated from the red blood cells by centrifugation at 2000 r.p.m. for 10 min and then maintained at 37 °C. The red blood cells from the infected chicken were discarded; those from the uninfected chicken were divided into four parts to form the basis of the blood meal. To each part we added plasma from the infected or from the uninfected chicken and added live or dead zygotes, ensuring that the red blood cell density was the same in all samples, to create the four treatments. Each blood meal treatment was presented to mosquitoes on an artificial membrane feeder (containing 1.5 ml with *ca.* 5×10^4 zygotes) for *ca.* 20 min. Mosquitoes that had not obtained a full blood meal were discarded.

We estimated the parasite's effect on the encapsulation response (see § 3) at its ookinete stage, 24 h after infection. We evaluated the indirect effect of the parasite (through its consequences on the chicken host's immune response) by comparing the encapsulation responses of *Ae. aegypti* mosquitoes fed with a blood meal composed of uninfected red blood cells supplemented with the plasma derived from either a healthy chicken or from one infected with *P. gallinaceum*. We estimated the direct effect of the parasite by supplementing the blood meal with either live or with heat-killed malaria zygotes. All other factors were standardized: the concentration of red blood cells was the same in all blood meals, and the mosquitoes were reared under identical conditions, were used only if they pupated 8 days after their eclosion and were fed 3 days after emergence. The mosquito's size, measured by the length of its wings, could not be controlled, and was therefore included as a potential confounder in all of our statistical analyses.

We stimulated the encapsulation melanization response by inoculating a negatively charged CM-25 Sephadex bead into the mosquito's thorax. The beads ranged between 40 and 120 μm in diameter; we selected, by visual inspection, the smallest ones for inoculation. They were rehydrated in saline solution containing 1.3 mM of NaCl, 0.5 mM of KCl, 0.2 mM of $CaCl_2$ 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 one 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, we dissected mosquitoes that were able to fly in a mixture of saline solution and 0.01% methyl green (Paskewitz & Riehle 1994) and scored the degree of melanization of their beads according to three groups: no visible melanization, patchy melanization (i.e. leaving unmelanized areas on the bead) or complete melanization.

The three levels of the melanization response were analysed with an ordinal logistic analysis that included the condition of the blood plasma (from a healthy or an infected chicken), the status of the zygote (alive or dead) and their interaction, and that controlled for the three replicates (replicate was included as a random factor). As mentioned previously, we included wing length (calculated as the mean length of the two wings, measured from the distal end of the allula to the tip of vein R3) as a potential confounding factor.

The use of beads generally has the disadvantage that some mosquitoes die before being assayed (see § 3). 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 blood and zygote status were analysed with an analysis of variance. Both statistical analyses were performed with JMP version 4.0 (http://www.jmpdiscovery.com).

3. RESULTS

Two days after being inoculated, 125 out of the 164 mosquitoes survived and were able to fly. The mortality of the mosquitoes was influenced by the interaction between the zygote's status and the type of plasma (table 1). Thus, if the plasma came from an infected chicken, live zygotes increased mortality: 20% of the mosquitoes fed with dead zygotes died, whereas 28% of the ones fed with live zygotes died. By contrast, live zygotes decreased mortality, if the plasma was taken from a healthy chicken (dead zygotes, 35%; live zygotes, 19%).

Our analyses of the remaining mosquitoes showed that the encapsulation response of mosquitoes feeding on a blood meal composed of plasma from an infected chicken and live zygotes was compromised, with less than 20% of the beads melanized, whereas the mosquitoes fed on healthy blood containing dead zygotes melanized 58.8% of the bead at least partly (17.6% completely). Live zygotes reduced the efficacy of the melanization response (figure 1). Thus, out of the 30 mosquitoes that had fed on the healthy blood supplemented with live zygotes, only 10% melanized the bead partly (and none of them completely). In addition, infected plasma reduced the efficacy of the

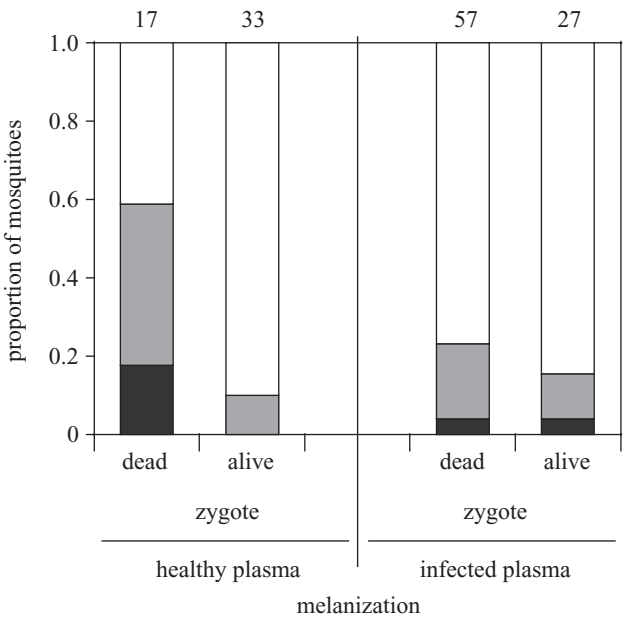

Figure 1. Melanization response against negatively charged Sephadex beads injected into the mosquito's thorax 1 day after the blood meal. Each bar represents the proportion of mosquitoes that melanize a bead to different degrees (no melanization, open bar; patchy melanization, grey bar; complete melanization, black bar). The numbers of mosquitoes are given above each bar.

Table 2. Test statistics (logistic analysis) for the comparison of the degree of melanization of beads injected into the thorax of mosquitoes.
(Melanization was categorized as three levels (no, patchy or complete melanization) and analysed with an ordinal logistic analysis that controlled for the size (wing length) of the mosquitoes and for replicate (which was included in the analysis as a random factor).)

source	d.f.	log-likelihood χ^2	p
replicate	2	2.065	0.356
wing length	1	0.240	0.001
status of zygote	1	12.606	< 0.001
plasma status	1	1.668	0.197
replicate × zygote	2	2.446	0.294
replicate × plasma	2	1.288	0.525
zygote × plasma	1	3.998	0.046

melanization response (figure 1). Out of the 52 mosquitoes that had fed on dead zygotes together with the plasma from an infected chicken, only 23.1% of the mosquitoes melanized the bead partly (3.8% completely). Thus, the status of the zygote and of the blood interacted to determine the efficacy of the melanization response (table 2). These results were similar in each of the three replicates (table 2).

4. DISCUSSION

Our results confirm our previous study (Boëte *et al.* 2002) by showing that infection by malaria parasites seriously compromises the efficacy of the mosquito's encapsulation response. Closer inspection of the results suggests that the parasite suppresses the mosquito's immune response with a direct and an indirect mechanism. Thus, the presence of live zygotes in a healthy blood meal decreased the proportion of (partly) melanized beads by a factor of *ca*. 6. This reduction in melanization ability (which was due only to the presence of live parasites) suggests a direct and active role of the malaria parasite during its ookinete stage in the immunosuppression of its mosquito vector. The mechanism for this active immunosuppression is unknown, but might resemble the mechanisms known from other parasites. The bacterium *Yersinia pestis* (Juris *et al.* 2002), for example, produces Yop effectors (Rho GTPase-activating proteins and protein tyrosine phosphatase), which alter the cytoskeleton of their host's cells in a way that inhibits phagocytosis. A similar mechanism is used by some parasitoids, which suppress the encapsulation response of their insect hosts by modifying the microtubule arrangement in the haemocytes so that they change their morphology and lose surface adhesion, and thus lose their encapsulation ability (Rizki & Rizki 1990, 1992). It is therefore tempting to speculate that a similar mechanism is also used by *Plasmodium* in its immunosuppression process, which would indicate a remarkable degree of convergent evolution in host–parasite relationships. An alternative mechanism could be that the parasite blocks the expression of a leucine-rich-repeat immune gene, as it has recently been shown that silencing one of these, *LRIM1*, in *Anopheles gambiae* prevents the melanization of *P. berghei* (Osta *et al.* 2004).

The indirect mechanism is suggested by our observation that the plasma of infected chickens reduced the efficacy of the melanization response by approximately threefold. As we used only the plasma of infected chickens, this indirect suppression is likely to be a consequence of the chicken's immune response to infection for the insect's immune response. There is increasing evidence that insect and vertebrate immune defences share a common ancestry (Barillas-Mury *et al.* 2000). In what way the chicken's immune response could interfere with the mosquito's is unknown, but immune effector molecules, including specific IgG antibodies, can pass from the vertebrate blood meal into the mosquito's haemolymph (Lackie & Gavin 1989; Vaughan *et al.* 1990), suggesting non-described and potentially complex interactions between the two immune systems.

Although the immune response was suppressed directly and indirectly, the combined effect of the two mechanisms was no more effective than the direct suppression alone (figure 1). Indeed, the effect of direct suppression was slightly stronger than the combined effect, showing that the two mechanisms do not work in an additive fashion.

Note that the combined evaluation of the data on the mosquitoes' mortality and encapsulation response make it unlikely that our conclusions are biased by the fact that surviving mosquitoes have a more efficient immune response *per se*. More interestingly, they suggest an alternative mechanism for the malaria parasite's virulence in mosquitoes (Ferguson & Read 2002). Indeed, in our experiment, mosquitoes that were infected with live zygotes but had been fed on uninfected plasma survived

better than those fed with dead zygotes. We therefore suggest that the reduced survivorship often noted for infected mosquitoes (Ferguson & Read 2002) might not be caused solely by a direct effect of the infection. Rather, mosquito mortality was highest when the encapsulation response was strongest (healthy plasma and dead zygotes), which suggests that the reduced survivorship is at least partly due to a cost of the mosquito's immune response. This would conform to several studies showing a cost of mounting an immune response (Fellowes *et al.* 1999; Ahmed *et al.* 2002), and in particular to the observation that the activation of the immune system of undernourished bumblebees with inoculated latex beads reduces their lifespan (Moret & Schmid-Hempel 2000). This could be due to the cytotoxic immune molecules that are involved in resistance, as these are known to have detrimental effects in an open circulatory system (Schmid-Hempel 2003). In other words, immunosuppression, at least at intensities of infection that do not cause too much direct damage, may increase not only the parasite's, but also the mosquito's, survival by reducing the cost of the immune response. Indeed, as the mortality of mosquitoes fed with dead zygotes (uninfected, with an intact immune response) was higher than the mortality of infected, immunosuppressed mosquitoes, our results suggest (at least if other immune responses involved in regulating parasite density show similar costs) that some degree of immunosuppression of the encapsulation response could be beneficial to the host.

Overall, our results show that malaria parasites can suppress the encapsulation response of their mosquito vectors with at least two mechanisms. First, they modify the blood of their vertebrate host in a way that decreases the efficacy of the insect immune response. As we considered only the plasma of infected chickens, this indirect suppression is probably a consequence of the chicken's immune response to infection on the insect's immune response. In addition, although this effect was not evaluated with our experimental design (as it was held constant), parasite-induced anaemia could contribute to a suppressed immune response in natural infections, as the encapsulation response is efficient only in blood-fed mosquitoes (Chun *et al.* 1995; Schwartz & Koella 2002). Second, malaria parasites directly suppress the immune response of their mosquito vector, as they reduce its efficacy even if the blood meal contains the plasma of an uninfected chicken. As the parasite remains attached to the intestinal tube in the abdomen, while the beads were inoculated into the thorax, the reduced efficacy suggests that malaria parasites actively and systemically suppress their mosquito vector's immune response.

Malaria parasites use many strategies to maximize their survival probability within the mosquito vector and the probability of being transmitted. Previously, we have shown that the parasite manipulates the mosquito feeding behaviour in a way that reduces insect mortality and maximizes parasite transmission (Koella *et al.* 1998, 2002; Koella 1999). We demonstrate that the parasite also manipulates the mosquito's immune response. This not only directly enables the parasite to survive, but also reduces the vector's mortality associated with its immune reaction. This confronts the vector with an evolutionary trade-off between the deleterious effects of being infected and the cost of mounting an effective immune response. Thus, despite a cost of parasitism, balanced fitness effects may constrain the hosts' evolutionary response in a way that enables the parasite's development to proceed unhindered.

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