SHORT COMMUNICATION

# Exflagellation Responses of Cultured *Plasmodium falciparum* (Haemosporida: Plasmodiidae) Gametocytes to Human Sera and Midguts of Anopheline Mosquitoes (Diptera: Culicidae)

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J. Med. Entomol. 31(5): 767–769 (1994)

**ABSTRACT** The process of exflagellation was quantified for cultured *Plasmodium falciparum* gametocytes exposed to human sera and midgut homogenates from six vector species of *Anopheles* mosquitoes. Neither serum factors related to malaria exposure nor factors in the midguts of taxonomically diverse anophelines had significant inhibitory effects on the exflagellation of *P. falciparum* microgametocytes. Therefore, differences in vector competence among anopheline species most likely are caused by vector–parasite interactions occurring after microgametogenesis.

**KEY WORDS** *Plasmodium falciparum*, *Anopheles*, exflagellation

THE PROCESS BY which anopheline mosquitoes become infected with malaria parasites is initiated when mature gametocytes are ingested in the blood meal. Once in the mosquito midgut, mature male gametocytes undergo exflagellation, a dramatic physiologic process also known as microgametogenesis. Exflagellation is a prerequisite to the successful fertilization of *Plasmodium* macrogametes in the mosquito. Factors in the blood meal influencing microgametogenesis (reviewed by Carter & Graves 1988) include gas tension (Bishop & McConnachie 1956), pH (Nijhout & Carter 1978), and temperature, which is the most critical parameter (Sinden & Smalley 1976, Ogwan'g et al. 1993).

Our preliminary studies using cultured *Plasmodium falciparum* (NF54 strain) indicated that additional factors affecting exflagellation may be critical to the fertilization process in mosquitoes. For example, exflagellation intensities varied from one to 20 exflagellations per field when cultures of *P. falciparum* gametocytes were exposed to different groups of pooled sera from nonmalaria exposed volunteers from the United States (unpublished data). This observation may be important because the choice of sera used for experiments could be a key limitation for successfully infecting mosquitoes with *P. falciparum* in the laboratory.

Other parameters may operate to inhibit exflagellation within the mosquito midgut. Variation in ookinete infection rates and densities has been observed for six species of anopheline mosquitoes, irrespective of gametocyte densities and the maturity of gametocytes (Vaughan et al. 1994). We considered that there may be differences in midgut "exflagellation factors" (Nijhout 1979) among these six anopheline species, which serve to block or suppress exflagellation and, thus, limit vector competence.

To address the impact of these two factors on microgametogenesis, we established a simple system for testing how exflagellation may be affected by human sera and midgut homogenates of anopheline mosquitoes.

## Material and Methods

Mature *P. falciparum* (NF54 strain) gametocyte cultures (50% hematocrit) with eight or more exflagellating bodies per five microscopic fields (400×) were used for the bioassay of human sera and mosquito midgut homogenates. Sera were from 30 healthy adults from the United States who had never been exposed to malaria, 30 children (1–9 yr old) from a malaria endemic area with low-level transmission on the coast of Kenya (Mbogo et al. 1993), and 24 adults living in an area of high-level transmission in Kisumu, western Kenya (Beier et al. 1990). Three preparations of midgut homogenates from six anopheline species, *Anopheles gambiae* Giles (G-3), *A. stephensi* Liston (Pakistan), *A. dirus* Peyton & Harrison, *A. arabiensis* Patton (GMAL), *A. freeborni* Aitken (Maryville), and *A. albimanus*

This study followed the National Institutes of Health guidelines regarding the use of human volunteers.

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**Table 1. Exflagellation responses of *Plasmodium falciparum* when gametocytes were mixed with either human sera from different donors or midgut homogenates from six species of anopheline mosquitoes**

| Type effect | % Deviation from control | Human sera | | | *A. albimanus* | *A. arabiensis* | *A. dirus* | *A. freeborni* | *A. gambiae* | *A. stephensi* |
|---|---|---|---|---|---|---|---|---|---|---|
| | | U.S. adults ($n$ = 30) | Kenyan children ($n$ = 30) | Kenyan adults ($n$ = 24) | | | | | | |
| Enhancing | >50 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 20–50 | 1 | 1 | 0 | 4[a] | 4[a] | 0 | 4[a] | 4[a] | 4[a] |
| No effect | 0–20 | 5 | 3 | 2 | 2[b] | 2[b] | 6[a,b] | 2[b] | 2[b] | 2[b] |
| No effect | 0–20 | 17 | 16 | 13 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 20–50 | 7 | 11 | 9 | 0 | 0 | 0 | 0 | 0 | 0 |
| Blocking | >50 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a] Twenty mosquito midguts homogenized in 20 μl of complete medium and M199.
[b] Twenty mosquito midguts homogenized in 20 μl of normal saline.

Weidemann, were made by crushing 20 whole unfed mosquito midguts in 20 μl of: M199 medium (Gibco, New York), normal saline (Sigma, St. Louis, MO), and complete medium containing human sera and sodium bicarbonate (Ponnudurai et al. 1982). Each 20-μl sample of serum was incubated at 37°C for 1 hr before testing with the gametocyte cultures. The midgut samples (20 μl) were kept on ice then transferred to a 37°C incubator after homogenization for 15 min before testing. The 37°C incubation ensured that there was no bias caused by temperature. A serum sample from an adult living in the United States and media used for preparation of midgut homogenates were used as controls in the assessment of the relative effects of human sera and midguts of six anopheline species on the exflagellation, respectively.

Four microliters of the mature gametocyte culture (50% hematocrit) and 4 μl of sera were pipetted onto a clean glass slide, mixed well, and covered with a coverslip (18 by 18 mm). This yielded a uniform monolayer of blood cells. Slides were left at room temperature (24 ± 1°C) for 20 min and exflagellating bodies were observed under phase contrast at 400× magnification. A similar procedure was used for testing mosquito midgut homogenates, except that the slides were observed at 8 min after mixing, because initial testing indicated that mosquito midgut homogenates activated the exflagellation process after 5 to 8 min (unpublished data). A count of exflagellating bodies per 15 fields (randomly selected) was made within 2 min. Each test sera or mosquito midgut homogenate was assayed twice using different gametocyte cultures along with the same control sera. If test counts deviated more than 50% from the control, they were retested a third time.

Exflagellation activity was standardized against the controls as follows: $d = (a - b)/b \times 100$, where $d$ = percentage of deviation of exflagellation count in reference to control serum/media; $a$ = average count of exflagellating bodies in test serum or midgut homogenate; $b$ = average count of exflagellating bodies in control serum or media.

**Statistical Analysis.** Using SPSS (SPSS, Chicago, IL), the exflagellation activity of the three groups of sera was analyzed by analysis of variance (ANOVA).

## Results and Discussion

None of the human sera blocked or enhanced the exflagellation of *P. falciparum* microgametocytes >50% relative to control sera (Table 1). Retests on those sera blocking >30% of the exflagellation activity yielded inconsistent results, indicating that such deviations were caused by inherent variation among gametocyte cultures. On average, most of the sera exhibited a 0–30% reduction of exflagellation activity compared with the control serum. One reason for this apparent lack of difference among sera groups may be differences in pH. The pH of the control sera was 7.9, whereas the pH of the test sera ranged from 7.2 to 7.8. Although these differences may be significant for systems like *P. gallinaceum* (Nijhout & Carter 1978), it is more likely that even subtle changes in temperature contributed to the variation observed in exflagellation responses to sera (Ogwan'g et al. 1993).

Overall, the exflagallation activity of the three groups of sera did not vary significantly (ANOVA, $F = 2.8$; df = 2, 81; $P = 0.135$). An interesting observation was that the Kenyan sera, from individuals presumably with varying degrees of natural immunity against sexual stages of malaria parasites, had similar effects on the exflagellation of *P. falciparum* gametocytes as the nonimmune sera from the United States. Hence, the process of exflagellation of *P. falciparum* microgametocytes may not be affected by diverse serum factors present in humans. Our test, however, only measured the effect of the sera on exflagellation activity and did not address whether the sera interfered with microgamete function or mosquito infection.

Similarly, tests involving midgut homogenates from the six anopheline species demonstrated little variation in exflagellation response (Table 1). Tests with midgut samples yielded higher exflagellation counts than control samples without midgut homogenate. These results were expected because work by Nijhout (1979) on *P. gallinaceum* showed that mosquito exflagellation factors present in the midguts of *Aedes aegypti* (L) stimulated exflagellation. The lack of variation in the exflagellation response of microgametocytes to midguts of taxonomically diverse anopheline species indicated that exflagellation in the mosquito vector probably proceeds in a similar manner regardless of the vector species. Thus, differences in vector competence among anopheline species most likely are caused by vector–parasite interactions occurring after microgametogenesis.

## Acknowledgments

We thank Paul Converse for providing nonimmune human serum samples and Jefferson Vaughan for his assistance and comments on this manuscript. Chandana Mendis was supported by a postdoctoral research training grant from the United Nations Development Program/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. Support was provided by the National Institutes of Health grant AI29000.

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*Received for publication 17 November 1993; accepted 11 May 1994.*