Plasmodium falciparum: Release of Circumsporozoite Protein by Sporozoites in the Mosquito Vector

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BEIER, J. C., VAUGHAN, J. A., MADANI, A., AND NODEN, B. H. 1992. *Plasmodium falciparum:* Release of circumsporozoite protein by sporozoites in the mosquito vector. *Experimental Parasitology* 75, 248–256. The release of circumsporozoite (CS) protein by *Plasmodium falciparum* sporozoites was investigated to identify factors regulating this process within infected *Anopheles gambiae* mosquitoes. The potential for sporozoites to release CS protein *in vitro* was not dependent upon their site-specific developmental stage (i.e., mature oocysts, hemolymph, salivary glands), their duration in the vector, or their exposure to mosquito-derived components such as salivary glands or hemolymph. The capacity of sporozoites to release CS protein was depressed by mosquito blood feeding during periods of sporozoite migration to the salivary glands, but the effect was only temporary and those sporozoites already in the glands were not affected. Free CS protein in the salivary glands was present in 93.3% of 45 infective mosquitoes. Sporozoites from these same, individual mosquitoes were also tested *in vitro* for CS protein release. In both cases, the amount of soluble CS protein increased as a function of sporozoite density but the total amount of CS protein per sporozoite became progressively less with increasing numbers of sporozoites. Further experiments showed that sporozoite contact with increasing amounts of soluble CS protein caused a down-regulation of CS protein release. Thus, a primary factor regulating the production and release of CS protein by sporozoites is their contact with soluble CS protein within the mosquito. © 1992 Academic Press, Inc.

INDEX DESCRIPTORS AND ABBREVIATIONS: Malaria; Sporozoite; Circumsporozoite protein; Mosquito; *Plasmodium falciparum; Anopheles gambiae;* Circumsporozoite (CS); *Anopheles (An.); Plasmodium (P.);* Medium 199 (M199); Bovine serum albumin (BSA); Enzyme-linked immunosorbent assay (ELISA); Picogram (pg); Blocking buffer (BB); Nonidet 40 (NP40).

INTRODUCTION

Malaria infections in humans are initiated when sporozoites are inoculated during blood feeding by anopheline mosquitoes. In the vector, sporozoites remain viable for up to several weeks and are not depleted by mosquito blood feeding (Shute 1945). Sporozoites depend upon exogenous sugars and amino acids (Mack and Vanderberg 1978) and effectively enhance transmission by altering the normal salivary functions of the mosquito (reviewed by James and Rossignol 1991). Mechanisms by which sporozoites survive, maintain their infectivity, and respond to microenvironmental changes during long periods within the vector are unknown.

An important behavior of sporozoites is their ability to release CS protein, an immunodominant protein that occurs on the sporozoite surface (reviewed by Nussenzweig and Nussenzweig 1985). In the mosquito, CS protein is first expressed during oocyst development (Boulanger *et al.* 1988; Hamilton *et al.* 1988). Sporozoites release CS protein as they migrate to and reside in the salivary glands (Posthuma *et al.* 1989; Golenda *et al.* 1990). Motile sporozoites (Vanderberg 1974) release trails of CS protein as they glide *in vitro* (Stewart and Vanderberg 1988) but CS protein release also occurs independently of gliding, in response to temperature, albumin, and serum (Stewart and Vanderberg 1991). Sporozoites also release CS protein in the verte-

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brate host as they differentiate into exo-erythrocytic stages (Suhrbier *et al.* 1988; Atkinson *et al.* 1989).

Stewart and Vanderberg (1991) have shown that *Plasmodium berghei* sporozoites release CS protein in solution under conditions simulating those encountered by sporozoites in the vertebrate host. Their findings on the physiological basis of CS protein release by sporozoites illustrate the functions of CS protein in sporozoite motility, the circumsporozoite precipitation reaction, and the invasion of target cells.

This study investigated the capacity of *P. falciparum* sporozoites to release CS protein in response to conditions encountered in the mosquito host. The overall goal was to identify factors affecting CS protein release by sporozoites in the vector and to explore the potential adaptive significance of this process for parasite survival in the vector.

MATERIALS AND METHODS

Anopheles gambiae (G-3 strain) were infected with the NF54 strain of *P. falciparum* according to standard methods (Ponnudurai *et al.* 1982). Fed mosquitoes were provided with 5% Karo syrup solution and were maintained in an insectary at 27°C and 70% relative humidity.

Salivary glands of mosquitoes were dissected in M199 (GIBCO Laboratories, Grand Island, NY), transferred to a 200-µl glass tissue grinder (Kontes, Vineland, NJ) held on ice, and pools of glands from 10 to 50 mosquitoes were ground gently to free sporozoites from glands. Sporozoites were diluted to a volume of 100 to 400 µl with M199, transferred to a 2.0-ml polypropylene vial coated with a dry spray Teflon lubricant (Elmers Slide-All, Borden, Inc., Columbus, OH), and centrifuged at 2000*g* (5000 rpm) for 15 min at 4°C. Supernatants were discarded to remove any soluble CS protein associated with sporozoite samples. The pellet (10–15 µl) containing intact, viable sporozoites was resuspended in M199 (100 to 400 µl). Sporozoites in 10-µl aliquots were added to a hemocytometer and counted at 400× phase contrast.

Procedures for measuring the physiological release of CS protein by sporozoites were a modification of methods described by Stewart and Vanderberg (1991) for *P. berghei* sporozoites. Desired numbers of sporozoites in 5 to 15 µl M199 were added to M199 containing 3% w/v BSA (Sigma Chemical Co., St. Louis, MO) (50–60 µl) in Teflon-coated 2.0-ml vials; the test volume was always 65 µl. Some experiments involved mixing sporozoites in M199–BSA containing a substance under study or M199 alone. Suspensions were mixed and incubated for 2 hr at either 27 or 37°C. This was sufficient time for *P. falciparum* sporozoites to release maximal levels of CS protein, as determined by preliminary experiments and described previously for *P. berghei* sporozoites (Stewart and Vanderberg 1991). Following incubation, sporozoites were centrifuged at 2000*g* for 15 min at 4°C; conditions which consistently removed >99% of the sporozoites from the supernatant. Fifty-five microliters of supernatant were removed, mixed with 55 µl of a boiled casein blocking buffer (Wirtz *et al.* 1989) containing 0.5% NP40 (Sigma Chemical Co.), and samples were stored frozen at −20°C.

Supernatant samples were tested by a *P. falciparum* sporozoite ELISA (Wirtz *et al.* 1987b) using the 2A10 monoclonal antibody (Zavala *et al.* 1983). This assay can detect CS protein at a sensitivity level of 25 sporozoite equivalents. Negative control samples on each plate included four 50-µl samples of M199–BSA. Positive control samples on each microtiter plate included eight twofold dilutions (from 100 pg to <1 pg per 50 µl) of R32tet32, a recombinant CS protein (Young *et al.* 1985) normally used as a positive control and quantitation standard in this ELISA (e.g., 100 pg of R32tet32 is equivalent to 400 sporozoites) (Wirtz *et al.* 1987a). Sample absorbance values were converted to picogram (pg) quantities of CS protein by regression equations determined for relationships between the eight dilutions of R32tet32 and corresponding absorbance values (405 nm) 30 min after the addition of substrate. Quantities of CS protein per sample were multiplied by the appropriate correction factor to account for supernatants remaining in vials and the total sample volume.

Preliminary experiments were performed to ensure that the test system was indeed detecting soluble-form CS protein. Briefly, the amount of CS protein in supernatants from low-speed centrifugation (2000*g*) of sporozoite samples was not reduced by high-speed centrifugation at 100,000*g* for 1 hr. Second, the centrifugation process alone did not affect levels of CS protein in supernatants. Supernatants collected from samples of 5000 sporozoites after each of three centrifugations at 2000*g* did not contain significant quantities of CS protein. Third, sporozoites which were killed by treatment with 1% formalin did not release CS protein.

The following experiments involved quantifying the release of CS protein by sporozoites using the test system described above:

The release of CS protein by sporozoites was evaluated in relation to incubation times and temperatures. Triplicate samples of 10,000 sporozoites were incu-

bated at either 4, 27, or 37°C for either 1 or 2 hr, and levels of CS protein were determined for supernatant samples.

To examine the effect of sporozoite maturity on CS protein release, sporozoites from different anatomical sites (i.e., mature oocysts, hemolymph, salivary glands) were obtained from a single batch of infected mosquitoes on Day 14 postinfection and processed as follows. Mosquito hemocoels, containing ca. 0.5 µl hemolymph, were perfused with 2 µl M199 (Vaughan and Azad 1988) to collect sporozoites in the hemolymph. Salivary glands were excised and washed three times to remove any contaminating hemolymph sporozoites. Midguts were excised, washed three times in M199, and gently ground to free sporozoites from mature oocysts. The output of CS protein for sporozoites from the three anatomical sites was determined as described above, with sample incubation for 2 hr at 37°C. Similarly, the effect of sporozoite aging on CS protein release was determined by testing sporozoites from the salivary glands on Days 16 and 26 postinfection.

To determine the effects of mosquito blood feeding on sporozoite release of CS protein, batches of infected *An. gambiae* (Days 11, 13, 18, and 19 postinfection) were fed by membrane feeding on 1:1 mixtures of normal human sera and washed human red cells. Unfed mosquitoes from each batch served as controls. Sporozoites from each batch were obtained twice, from 1 to 5 days after feeding and tested for the release of CS protein as described above, with sample incubation for 2 hr at 37°C.

Background levels of free CS protein in the salivary glands of individual, infective mosquitoes were determined by washing dissected salivary glands in 100 µl of M199, transferring and grinding the glands in 65 µl of M199, pipetting the material to a Teflon-coated vial (held on ice), centrifuging, and processing supernatants as described above. Sporozoite pellets were resuspended in 70 µl M199-BSA. Sporozoite loads, the number of sporozoites in the salivary glands, were determined by counting sporozoites from 5-µl aliquots (Beier *et al.* 1991a). Resuspended sporozoite samples in 65 µl M199-BSA were incubated for 2 hr at 27°C, the holding temperature of infected mosquitoes, and tested to determine the amount of CS protein released in relation to numbers of sporozoites incubated.

Various substances were added to the incubation media to examine their effects on sporozoite release of CS protein. These included BSA, casein, salivary gland lysates from uninfected *An. gambiae*, hemolymph perfusates from uninfected *An. gambiae*, and sporozoite-produced soluble CS protein from salivary glands of infective *An. gambiae*. The CS protein output of sporozoites incubated in these substances was compared with the CS protein output of equivalent numbers of sporozoites incubated in M199 alone.

In all of these experiments, sample incubation times were for 2 hr at 37°C.

Comparisons between the amount of CS protein released by sporozoites and other variables involved regression analysis and analysis of variance. Statistical tests done on data transformed to $\log_{10}$ or $\log_{10}(n + 1)$ values to normalize variance are indicated in the results. Results of statistical tests are shown only for those tests where α levels were less than 0.05.

RESULTS

The amount of CS protein released increased with temperature when sporozoites were incubated for either 1 hr ($F = 19.16$; $df = 2,6$; $P = 0.0025$) or 2 hr ($F = 26.35$; $df = 2,6$; $P = 0.0011$) (Fig. 1). At each of the temperatures, 4, 27, and 37°C, sporozoites incubated for 2 hr did not release significantly more CS protein than those incubated for only 1 hr.

Sporozoites obtained from the salivary glands, the hemolymph, and from mature oocysts on Day 14 postinfection did not differ significantly in their ability to release CS protein (Fig. 2). Similarly, there were no differences in the amount of CS protein released by sporozoites obtained from salivary glands on either Day 16 or Day 26 postinfection.

A decrease in the amount of CS protein released by salivary gland sporozoites was observed when mosquitoes were blood fed
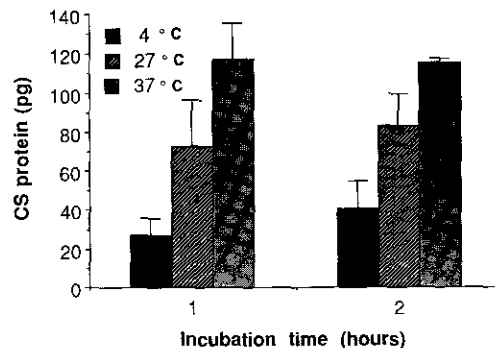

FIG. 1. Release of CS protein by *Plasmodium falciparum* sporozoites incubated at 4, 27, or 37°C for either 1 or 2 hr. Mean plus 1 SD measurements of CS protein in supernatants are shown for triplicate samples of 10,000 sporozoites tested at each temperature and incubation period.
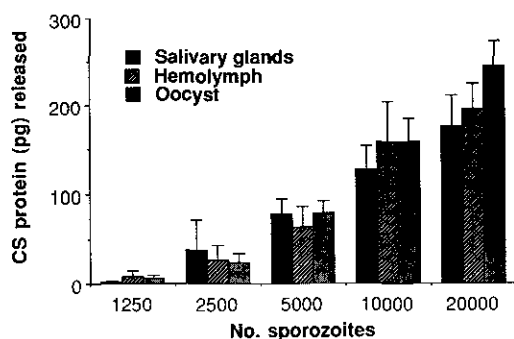
FIG. 2. Comparison of CS protein release by *Plasmodium falciparum* sporozoites obtained from either the salivary glands, the hemolymph, or mature oocysts of *Anopheles gambiae* on Day 14 postinfection. Mean plus 1 SD measurements of CS protein in supernatants are shown for five concentrations of sporozoites from salivary glands, hemolymph, and oocysts which were incubated, in replicates of 3 to 6, for 2 hr at 37°C.

during periods when sporozoites were being released from mature oocysts and migrating through the hemolymph to the salivary glands (e.g., Days 11 and 13) (Table I). Sporozoites from mosquitoes fed on Day 11, 1 day before sporozoites were in the salivary glands, showed a 30% reduction in CS protein release on the second day after feeding but sporozoite release of CS protein returned to normal levels by the fifth day after feeding. Sporozoites from those fed on Day 13, 1 day after sporozoites first reached the salivary glands, showed a 61% reduction in CS release on the day following blood feeding. The potential for sporozoites to release CS protein was not affected when mosquitoes were fed on either Day 18 or Day 19 postinfection.

Free CS protein in the salivary glands was detected in 93.3% of 45 individual, infective *An. gambiae* containing a geometric mean of 1858 sporozoites (range, 13–14,289). Quantities of free CS protein were proportional to sporozoite loads ($r = 0.85$; $df = 43$; $P < 0.0001$) (Fig. 3a). Sporozoites from the same 45 mosquitoes were then tested for their potential to release CS protein. There were corresponding increases in the release of CS protein with increasing

numbers of sporozoites ($r = 0.78$; $df = 43$; $P < 0.0001$) (Fig. 3b). Overall, mean (± 1 SD) quantities of CS protein free in the salivary glands (116.0 ± 104.3 pg) were two times greater than that released by sporozoites during a 2-hr incubation (61.2 ± 68.0) ($F = 8.74$; $df = 1,88$; $P = 0.0041$).

Calculations of the amount of CS protein per sporozoite indicated that sporozoites from the 45 infective mosquitoes varied in their output of CS protein. There were inverse relationships between sporozoite loads, and both the amount of free CS protein per sporozoite in the salivary glands ($r = -0.60$; $df = 43$; $P < 0.0001$) and the amount of CS protein released per sporozoite during incubation ($r = -0.54$; $df = 43$; $P < 0.0001$). Table II illustrates decreasing levels of CS protein per sporozoite with increasing numbers of sporozoites, when samples from the 45 mosquitoes were divided according to four classes of sporozoites ranging from <100 to >5000.

The possibility that sporozoites release CS protein in response to either background levels of free CS protein or to normal mosquito-derived substances (i.e., salivary gland lysates or hemolymph) was investigated. Incubating samples of 10,000 salivary gland sporozoites in increasing concentrations of soluble-form CS protein from salivary glands of infective mosquitoes caused a 19-fold decrease in the amount of CS protein released per sporozoite (Fig. 4). A second, comparable experiment yielded similar results, with a 14-fold decrease in CS protein release per sporozoite over four concentrations of soluble CS protein tested. In contrast, the amount of CS protein released by sporozoites was not affected when samples of 10,000 sporozoites were incubated with increasing concentrations (30, 100, and 200 μg protein/ml) of salivary gland lysates or hemocoel perfusates obtained from uninfected mosquitoes. Similarly, the same concentrations of BSA or casein had no effect on CS protein release.

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TABLE I

Release of CS Protein by *Plasmodium falciparum* Sporozoites after Infected *Anopheles gambiae* Were Fed
by Membrane Feeding on Mixtures of Normal Human Sera and Human Erythrocytes

Blood meal days postinfection[a]	Sporozoites tested days after blood meal	Mean ($\pm$1 SD) pg CS protein in sporozoite supernatant	
		Unfed group	Blood-fed group
11	2	137.3 (13.6)	96.1 (15.1)**
	5	142.0 (24.3)	151.4 (43.5)
13	1	134.6 (38.1)	52.2 (10.9)**
	3	84.2 (17.4)	63.3 (10.7)*
18	1	111.3 (24.5)	99.3 (26.1)
	3	102.4 (23.2)	90.8 (17.6)
19	1	94.2 (23.3)	84.0 (18.6)
	3	93.0 (10.1)	80.3 (10.9)

[a] In each of the four groups of infected mosquitoes, sporozoites were first detected in the salivary glands on Day 12 postinfection.

Note. From unfed and fed groups of infected mosquitoes, 6 replicates of 10,000 sporozoites were tested for the release of CS protein by incubating sporozoites in M199–BSA (total volume 65 μl) for 2 hr at 37°C and then testing supernatants by ELISA (see Materials and Methods). Differences in CS protein release by sporozoites from unfed and blood-fed groups of mosquitoes were determined by analysis of variance: *$P < 0.05$; **$P < 0.01$; unmarked are not significantly different.

DISCUSSION

These studies on sporozoite biology addressed the hypothesis that sporozoites respond to conditions in the mosquito host by regulating their release of CS protein. In characterizing and testing the ability of sporozoites to release CS protein, under a range of conditions found in the vector, we have identified environmental, vector-related, and parasite-related determinants of CS protein release. These studies extend previous observations that the release of soluble CS protein is a normal activity of viable sporozoites (reviewed by Stewart and Vanderberg 1991) by considering how and why this process occurs in the vector.

Our experiments on sporozoites of *P. falciparum*, as well as recent studies on *P. berghei* sporozoites (Stewart and Vanderberg 1991), establish that the release of CS protein is a temperature-dependent process. Functionally, this is important because the mosquito host is poikilothermic. Observations that the time to maximal release of CS protein for *P. falciparum* (i.e., 1

hr) was nearly half of that for *P. berghei* (Stewart and Vanderberg 1991) stress that the dynamics of CS protein release may differ among *Plasmodium* species.

Sporozoite release of CS protein is not regulated strictly by vector-related factors. The lack of quantitative differences in the output of CS protein by *P. falciparum* sporozoites from mature oocysts, hemolymph, or salivary glands, even those residing for up to 15 days in the glands, indicates that CS protein release is not governed by the site or duration of sporozoites in the vector. Further, sporozoite contact with different concentrations of mosquito-derived substances, such as salivary glands and hemolymph, neither enhanced nor depressed the normal release CS protein *in vitro*. Essentially, the normal conditions in the mosquito host provide a microenvironment suitable for sporozoite existence but do not exert direct control over the regulation of CS protein release.

Significantly, the blood-feeding behavior of the vector affected sporozoite output of CS protein but only when mosquitoes were

$y = -0.464 + 0.741x$

$r^2 = 0.722$
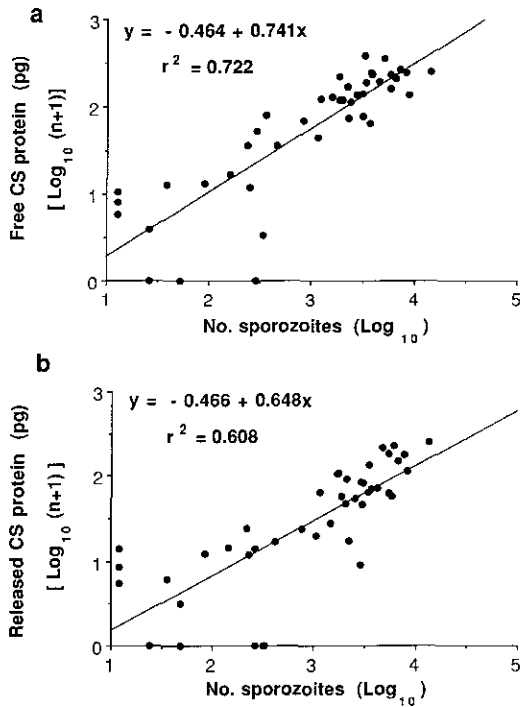
$y = -0.466 + 0.648x$

$r^2 = 0.608$

FIG. 3. Quantities of CS protein either free in the salivary glands (a) or subsequently released during a 2-hr incubation at 27°C (b), in relation to sporozoite loads for 45 individual, infected Anopheles gambiae. Linear relationships and regression equations are shown for $\log_{10}(n + 1)$ transformed quantities of CS protein in relation to $\log_{10}$ transformed numbers of sporozoites from individual mosquitoes.

which cross the hemocoel following a blood meal (Vaughan and Azad 1988) may have hyperstimulated the release of CS protein. However, the reduced capacity for CS protein output was only temporary because sporozoites responded normally within a few days. This is the first evidence that the behavior of the mosquito can affect the physiological condition of Plasmodium sporozoites.

A key observation was that sporozoites responded to background levels of free CS protein by regulating their release of CS protein. There are three lines of evidence to support such a negative feedback mechanism. First, free CS protein in the salivary glands was detected in nearly all of the sporozoite-infected An. gambiae. However, the actual amount of free CS protein in the glands was rather limited, given that the output of CS protein by extracted sporozoites during a 2-hr incubation was nearly half of that observed free in the salivary glands. Thus, sporozoites in the salivary glands have a tremendous potential to release CS protein but this potential is never realized.

Second, cohorts of sporozoites from the salivary glands of individual mosquitoes exhibited a differential release of CS protein. Regardless of whether soluble CS protein was measured in freshly dissected salivary glands or for incubated sporozoites, calculations showed that the amount of CS pro-

fed during periods when sporozoites were being released from oocysts and migrating to the salivary glands. Sporozoite contact with any of the host blood components

TABLE II

Picograms of Plasmodium falciparum CS Protein per Sporozoite in Relation to Numbers of Sporozoites in the Salivary Glands for 45 Individual, Infective Anopheles gambiae Mosquitoes where Soluble CS Protein Content was Determined Both in the Salivary Glands and from the Same Sporozoites Incubated for 2 hr at 27°C

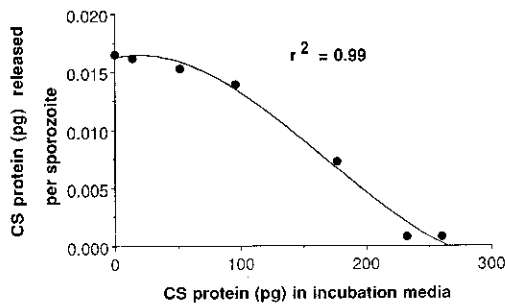| Sporozoites per salivary gland infection	N	Picograms of CS protein per sporozoite	
		Salivary glands mean (±1 SD)	After incubation mean (±1 SD)
<100	9	0.244 (0.265)	0.269 (0.372)
100–1000	9	0.096 (0.078)	0.041 (0.040)
1000–5000	18	0.062 (0.030)	0.029 (0.018)
>5000	9	0.034 (0.016)	0.024 (0.012)

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FIG. 4. Levels of soluble-form CS protein in the incubation media affects the release of CS protein by sporozoites. Triplicate samples of 10,000 sporozoites were incubated in six dilutions of soluble CS protein from the salivary glands of infective mosquitoes and in M199–BSA alone for 2 hr at 37°C. Each point, representing the amount of CS protein released per sporozoite, was determined as the mean quantity of CS protein in samples incubated with sporozoites minus the mean quantity of CS protein in corresponding samples of CS protein incubated without sporozoites, divided by 10,000.

tein per sporozoite was inversely related to the numbers of sporozoites (Table II).

Third, the amount of CS protein released per sporozoite *in vitro* was greatest when there was minimal or no exogenous CS protein in the media (Fig. 4). Beyond a threshold concentration of CS protein, there was a 19-fold decrease in CS protein release by constant numbers of sporozoites. Thus, sporozoites have the ability to regulate their output of CS protein in response to background levels of CS protein. How sporozoites recognize soluble CS protein is unknown.

Sporozoite regulation of CS protein release in the mosquito may be an important adaptive mechanism promoting sporozoite viability. In perspective, sporozoites release CS protein either as a function of their motility or by mechanisms that operate independent of this behavior (Stewart and Vanderberg 1991). Motility-related CS protein release may be critical during periods when sporozoites are migrating from mature oocysts, through the hemolymph, to the salivary glands; soluble CS protein is present throughout the hemocoel (Robert *et*

al. 1988). We propose that as sporozoites invade the salivary glands, the build-up of CS protein is the signal for sporozoites to halt their active motility, and thus their release of CS protein. This is supported by findings that *P. falciparum* sporozoites, residing *in situ* within the salivary glands of older, infected mosquitoes, do not react with anti-CS monoclonal antibodies (Golenda *et al.* 1990). Sporozoites within the salivary glands may be nonreactive because they are metabolically less active and have minimal CS protein on their surfaces.

The regulation of CS protein release may function to prepare sporozoites for transmission in the following way. Salivary gland size and structure promote intimate contact between sporozoites and released CS protein. Over time, sporozoite response to threshold levels of CS protein (see Fig. 4) would promote an equilibrium state of CS protein release. An effective sporozoite energy balance would be achieved through the release of just enough CS protein to maintain background levels of soluble CS protein but not enough to metabolically exhaust the sporozoites. The regulation of CS protein release may be most efficient for those sporozoites from heavily infected salivary glands since CS protein output per sporozoite is inversely related to sporozoite density (Table II). Similarly, as sporozoites decrease in number over time in the salivary glands, the probability that remaining sporozoites may have to release correspondingly more CS protein may be one factor responsible for age-related decreases in sporozoite infectivity (Porter *et al.* 1954). The metabolic state of the small number of sporozoites transmitted at each blood feeding (Beier *et al.* 1991a) may be critical for the initiation of host infections because as sporozoites are transmitted to the vertebrate host they are exposed to increases in temperature and to components in the bloodstream such as albumin, key factors which stimulate motility and CS protein release (Stewart and Vanderberg 1991).

Sporozoites with depleted energy reserves may be less effective in establishing exo-erythrocytic stages.

In conclusion, the release of CS protein by sporozoites is a normal but complex mechanism that appears to be associated with sporozoite survival in the mosquito host. During their residence in the salivary glands, sporozoites regulate their output of CS protein through feedback mechanisms which depend upon parasite density. Maintaining an equilibrium state of CS release in the salivary glands may be an important factor affecting the viability and infectivity of transmitted sporozoites.

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