REGULATION OF THE RATE OF ASEXUAL GROWTH AND COMMITMENT TO SEXUAL DEVELOPMENT BY DIFFUSIBLE FACTORS FROM IN VITRO CULTURES OF PLASMODIUM FALCIPARUM

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Abstract. The mechanism of switching to sexual differentiation (gametocytogenesis) of Plasmodium falciparum appears to be controlled by stochastic mechanisms that are sensitive to environmental conditions. In any given conditions, only a proportion of genetically identical parasites will become committed to sexual development. We used an experimental co-culture system to detect the presence of diffusible molecules from asexually replicating bloodstream parasites in culture that can stimulate their own growth and replication, and constitutively inhibit sexual conversion via diffusible molecules. These observations support the model that for P. falciparum, the sexual pathway of development is the default, and that constitutive repression of the sexual pathway permits asexual multiplication to occur in the bloodstream of the human host.

INTRODUCTION

A proportion of intra-erythrocytic bloodstream forms of the human malaria parasite Plasmodium falciparum cease asexual cycling and differentiate into specialized sexual stages known as gametocytes. These are responsible for transmission by surviving and establishing infection in the female Anopheles mosquito following ingestion of an infected blood meal. Evidence from field observations and from experimental in vitro systems indicate that many factors are capable of modulating the rate of commitment to gametocytogenesis of this parasite. These include the DNA synthesis inhibitors and other classes of anti-malarial drugs, cholera toxin, and somatostatin-like hormone. Further circumstantial evidence suggests that commitment to sexual development may be linked to the dynamics of asexual growth; studies of natural infections and in vitro experimentation have both shown that sexual conversion appears to correlate with a high asexual parasitemia. In accordance with this is the observation that cAMP had a stimulatory effect on sexual commitment only when added to cultures that have slowed down asexual growth. Together, these findings suggest that environmental influences may affect sexual differentiation via the dynamics of asexual growth.

There are two basic models for the way in which altered asexual parasite growth may govern the mechanisms controlling the switch to sexual differentiation. The developmental switch of each parasite may be coupled to its own growth dynamics by an internal metabolic indicator (e.g., ATP/ADP ratio), or each parasite may be influenced by the growth dynamics of the local parasite population via a diffusible parasite growth indicator. These two models are not mutually exclusive; both may have input.

There is also evidence for association of sexual commitment with the activation of specific signal transduction pathways; both protein kinase A and protein kinase C-dependent pathways, as well as the heterotrimeric G-proteins, are implicated. This activation may be responsible for the relay of signals from various environmental stimuli, ultimately resulting in the morphologic changes associated with sexual development. To date, the sensor mechanisms involved in detecting the changes in the environment or growth parameters have not been addressed. However, evidence has been published that supports the possibility of the involvement of diffusible molecules in P. falciparum communication: asexual bloodstream parasites were shown to secrete a somatostatin-like hormone and to respond to host-derived diffusible molecules; and dynamic interactions were demonstrated between genotypes of the rodent malaria P. chabaudi in mixed infections, although the latter are more likely to be due to immune cross-reaction.

In this study, we tested if asexually replicating P. falciparum in culture contributes to controlling its own growth and sexual development through diffusible molecules. By co-culturing pairs of parasite populations across semi-permeable membranes, we were able to measure the effect of diffusible factors from the presence of a second culture on 1) asexual replication and division, 2) metabolic growth rate, and 3) the rate of commitment to gametocyte production. Our findings demonstrate the existence of self-regulation of both P. falciparum asexual growth and sexual differentiation in culture via diffusible molecules.

MATERIALS AND METHODS

Parasite culture. Cultures of P. falciparum cloned line 3D7 were maintained according to the method of Trager, with modifications previously described. To synchronize parasite cultures, trophozoites were selected by flotation on Plasmagel (Fresenius Medical Care, Hamburg, Germany) 24 hours before setting up experiments with ring-stage parasites.

Co-culture arrangement. One day after purification of erythrocytes parasitized with mature asexual stages on Plasmagel, triplicate 0.4-mL test cultures were set up in Nunc (Roskilde, Denmark) 10-mm tissue culture insert wells with a 0.2-μm pore Anopore membrane base (SPI Supplies, West Chester, PA). Each group of three wells was supported within a 20-mL environmental culture set up at a parasitemia of 0–4% in a 140-mm diameter petri dish. Molecules were thus free to diffuse between environmental and test cultures, while the membrane contained the cells and parasites. Containment was confirmed by the absence of parasites within the erythrocyte environmental culture after extensive co-culture with...
asexually replicating parasites in the test culture wells. Asexual growth in environmental cultures was monitored by analysis of Giemsa-stained smears and remained in log-phase growth for the duration of the replication and growth measurements (48 hours). For the extended (five days) co-culturing (before measuring the rate of commitment to sexual development), parasite growth in environmental cultures was maintained in log-phase during co-culture by substitution of all environmental cultures with fresh starting cultures when the parasitemia of any one reached 10%. The effect of the presence of environmental cultures of varying starting parasitemia on test culture parasite growth, replication, and the proportion committing to sexual development was measured. Ninety-five percent confidence intervals were calculated for replicates within each experiment and results between independent replicate experiments were compared using the two-way analysis of variance (ANOVA). This system is advantageous over the traditional system of adding growth-conditioned medium to the cultures because the effect of any diffusible factors that are short-lived will not be detected using conditioned medium (see Discussion).

Parasite replication rates and growth measurement. Parasite replication was monitored by counting the parasitemia from Giemsa-stained smears of the cultures and expressed as a percentage calculated from the number of infected erythrocytes per 1,000 erythrocytes. To obtain a measure of the rate of growth of parasites in culture, incorporation of $^3$H-hypoxanthine into nucleic acids was measured over a fixed time period. Twelve hours after co-culturing was initiated, 2.5 μCi/mL of $^3$H-hypoxanthine was added for an additional period of 24 hours. Samples (50 μL) of each resuspended test culture were harvested using a cell harvester, and incorporation of $^3$H-hypoxanthine into cell nucleic acids was measured by liquid scintillation counting.

Quantification of the rate of switching to sexual development. The proportion of parasites that committed to sexual development was determined for a single generation of parasites after co-culturing for five days as follows. The cultures were removed from the test wells and the infective merozoites were captured by overlaying on a monolayer of erythrocytes for eight hours, before washing them off as described previously and in Figure 1. This resulted in a single cohort of 0–8-hour-old parasites being isolated from an asynchronous culture that may already contain gametocytes of various ages. Monolayer culture was continued for 36 hours to allow the parasites that were developing sexually to express the sex-specific antigen Pfpg16.$^{23}$ Cells in the monolayer were then resuspended using a pastette. Sexual and asexual parasites were fixed, differentially stained using 2G7, a monoclonal antibody specific for Pfpg16 (kindly provided by Dr. Richard Carter, University of Edinburgh, Edinburgh, Scotland), and 0.05 μM of DNA-specific fluorescent stain SYTO™ 24 (Molecular Probes Europe BV, Leiden, The Netherlands), and quantified by flow cytometry as described previously.$^7$

In three of the eight repeat experiments, the cumulative production of gametocytes was measured by omitting the monolayer selection and simply assessing test cultures for the proportion of sexual parasites after eight days of co-culturing.

RESULTS

Asexual growth and replication. We postulated that diffusible factors from the presence of replicating parasites may influence asexual growth and replication of *P. falciparum*. To investigate this, a system of co-culturing was used as detailed in Figure 1 and the Materials and Methods, which allows macromolecules to diffuse between the environment culture and the test culture, but which retains cells and parasites on a 0.2-μm porous membrane. The parasitemia of the test culture in the presence and absence of replicating environment culture was monitored by analysis of Giemsa-stained smears. Incorporation of $^3$H-hypoxanthine into nucleic acids was measured in the test cultures over the period from 12 to 36 hours after co-culturing was initiated. Asexual growth in the environment cultures was also monitored by analysis of Giemsa-stained smears and remained in log-phase for the dura-

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**FIGURE 1.** Experimental design for quantifying conversion to sexual development of *Plasmodium falciparum* in co-culture experiments (see Methods and Methods for co-culture arrangement and synchronization of parasites by monolayer infection).
tation of the replication and growth measurements. Since the first gametocytes were not detected in our test cultures until 5–7 days after co-culturing was initiated, gametocyte conversion did not interfere with these measurements of the rate of asexual growth.

Figure 2A shows that $^3$H-hypoxanthine incorporation into *P. falciparum* test cultures was stimulated by the presence of asexually replicating parasites in the environment culture. Stimulation peaked at a parasitemia of 2% in the environment culture (mean ± 95% confidence interval = 194 ± 7 cpm/hour) compared with uninfected erythrocytes (84 ± 3 cpm/hour). As the starting parasitemia in the environment was increased to 4%, the stimulation of growth of the test cultures was still apparent (143 ± 16 cpm/hour) relative to the uninfected erythrocyte environment, but it was significantly less than with the 2% starting parasitemia.

Pairwise data points each representing the mean growth of three test cultures in an environment of uninfected erythrocytes or a 2% starting parasitemia were pooled from six independent repeat experiments (Table 1). These were analyzed by two-way ANOVA and showed that the growth rate of *P. falciparum* as measured by hypoxanthine incorporation was significantly increased (mean stimulation factor = 1.51) when cultured in the presence of asexually replicating parasites ($P < 0.01$). This demonstrates that a diffusible factor from asexually replicating parasites in the environmental culture stimulated the growth of *P. falciparum*.

Similarly, in the same experiments, monitoring asexual parasitemia of the test cultures by analysis of Giemsa-stained slides showed that the presence of asexually replicating parasites in the environment stimulated asexual reproduction of parasites in the test cultures. This effect also reached a maximum at an initial parasitemia of 2% in the environment culture (Figure 2B). Analysis of the pooled data from six experiments (Table 2) by two-way ANOVA verified that the test culture parasitemia was higher after co-culture in the presence of replicating parasites (mean stimulation factor in an environment with an initial parasitemia of 2% = 1.62; $P < 0.01$).

**Sexual commitment.** To detect the action of diffusible molecules derived from the presence of infected erythrocytes in the control of commitment to sexual development, we measured the proportion of a single generation of *P. falciparum* 3D7 parasites converting to sexual development after five days of co-culturing either in the presence of uninfected erythrocytes or asexually replicating parasite environment cultures. The co-culture arrangement was performed as shown in Figure 1 and in the Materials and Methods.

A co-culture time of five days was chosen since our experience of gametocyte culture shows that the most significant wave of gametocytes normally appears seven days after initiation of culture if no further erythrocytes are added. The merozoites that give rise to those gametocytes will then be produced five days after initiation of the culture. Our experiments were designed to investigate the effect of co-culturing on this single wave of conversion. When co-culturing was continued for a longer period of time, both asexual growth and gametocyte production in the test culture were highly variable. The reason for this is that when we initiated cultures at a parasitemia of 2%, the asexual parasitemia peaks around day 6 (in the cell line we used) before decreasing to a level that we cannot predict. It follows that subsequent recovery and further gametocyte production are also unpredictable. Test cultures were seeded in triplicate at parasitemias of both 0.1% and 2% in the presence of environment cultures at parasitemias of 0%, 0.1%, and 2%. While test cultures were allowed to replicate unchecked, asexual replication was main-
Asexual growth of Plasmodium falciparum measured by $^3$H-hypoxanthine incorporation in co-culture experiments*

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* For each experiment, the mean growth of three replicate test cultures in the presence and absence of 2% parasitemia in the starting environment culture is shown. RBCs = red blood cells.
† Results from the experiment shown in Figure 2A, n = 6.

Data presented show that in culture, diffusible factor(s) from log-phase asexually cycling blood stages of P. falciparum can stimulate their own growth and replication (Figure 2 and Table 1). Since it has been previously shown that the products of lysed erythrocytes alone do not stimulate parasite replication,24 our results indicate that the presence of the parasite is essential for this regulation of P. falciparum growth and that this is a density-dependent process. An alternative explanation for this result is that it is an effect of nutrient depletion in the test culture by the presence of the larger environment culture. This seems unlikely, since nutrient loss would be expected to reduce, rather than stimulate growth. Encouragingly, quantitative polymerase chain reaction monitoring of experimental human infections appears to support the existence of density-dependent stimulation of parasite growth in vivo during the early phases of infection; in one study, growth accelerated from logarithmic (days 1–4) to an above logarithmic rate (days 6–8) in all five subjects.25 This parasite-induced growth stimulation may or may not involve a growth factor-receptor system or any signaling molecule unique to the parasite. For instance, it has been demonstrated previously that the growth capacity of the culture environment may be increased by presence of either a feeder layer or the reducing agent β-mercaptoethanol.26 Therefore, P. falciparum may be improving the culture (or local in vivo) environment via a diffusible molecule, thus effecting increased growth.

When the starting parasitemia in the environment culture was higher (2%), asexual stimulation of growth in the test cultures was only evident over short periods of co-culture (24–48 hour). Beyond that period, asexual growth in the test culture (as measured by hypoxanthine incorporation) was not significantly stimulated by the presence of parasites in the environment. This is likely to be due to density-dependent growth inhibition as the culture approaches the stationary phase of growth acting to counter the parasite-induced stimulation of growth. The existence of such a density-dependent growth inhibitory component in P. falciparum culture is well-recognized; during standard culture of malaria parasites we observe density-dependent repression of growth that limits parasitemia to approximately 10–15% of the erythrocyte population. This threshold is even lower in newly adapted

**DISCUSSION**

TABLE 2

Asexual replication measured by analysis of Giemsa-stained slides*

<table>
<thead>
<tr>
<th>Experiment</th>
<th>RBCs</th>
<th>2% 3D7</th>
<th>Ratio of 2% 3D7 to RBCs</th>
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* For each experiment, the mean growth of three replicate test cultures in the presence and absence of 2% parasitemia in the starting environment culture is shown. RBCs = red blood cells.
† Results from the experiment shown in Figure 2B, n = 6.

The effect of the presence or absence of asexually replicating parasites in the environment culture on the rate of commitment to gametocyte production of a single generation of parasites from the test cultures is shown in Figure 3A. Test (T) parasite cultures showed an approximately three-fold decrease in commitment to sexual development when co-cultured in the presence of asexually replicating parasites (proportion of parasites committing to sexual development in an environment [E] of uninfected erythrocytes was 0.1% T + 0% E = 0.112 ± 0.01; 2.0% T + 0% E = 0.110 ± 0.02 compared with the presence of asexually replicating parasites: 0.1% T + 0.1% E = 0.042 ± 0.006, 0.1% T + 2.0% E = 0.046 ± 0.007, 2.0% T + 0.1% E = 0.039 ± 0.002, 2.0% T + 2.0% E = 0.031 ± 0.004, see Figure 3A). Thus, diffusible factor(s) derived from the presence of asexually growing parasites in the environment cultures repressed commitment to sexual development. A similar level of repression of sexual commitment was imposed by an environment of asexually replicating parasites seeded at both 0.1% and 2% ring-stage parasites.

Furthermore, the data from nine independent experiments was pooled and analyzed by two-way ANOVA (Table 3). This showed that the proportion of parasites switching to sexual development in an environment of asexually replicating parasites was decreased significantly (mean repression factor = 2.32) compared with parasites in an environment of uninfected erythrocytes ($P < 0.01$). These experiments included measurements of commitment to sexual development for both a single generation of P. falciparum and cumulative production of gametocytes over the entire co-culture period (three experiments). This confirms the inhibition of gameto-
isolates. Its existence is also supported by our data from the titration of asexual stimulation (Figure 2): as the density of parasitemia in the environment culture was increased beyond the 2% optimum for stimulation to 4%, the magnitude of stimulation of asexual growth in test cultures (above the 0% control) decreased.

Repression of gametocytogenensis by the presence of asexually growing parasites maintained in log-phase was demonstrated both for a single generation of parasites after five days of co-culture (Figure 3) and for cumulative gametocyte production over a period of eight days (Table 3). No proportional correlation was evident between the starting density of the environment cultures and the rate of sexual commitment measured in the test cultures; parasite densities of both 0.1% and 2% repressed sexual commitment equally, indicating that the factor responsible for repression saturates at a low parasite density.

Several possible mechanisms can be envisaged connecting the two observed activities of log-phase asexually replicating *P. falciparum*: stimulation of asexual growth and repression of sexual development. Either two independent molecules may effect the asexual stimulation and repression of sexual development, or a single molecule may be responsible. While the asexual stimulation appears to be the result of a molecule released from parasitized erythrocytes, the repression of sexual development (measured after five or eight days of co-culture) may be a secondary result caused by the altered growth and metabolism of the parasites. This may or may not involve a second diffusible molecule; instead it may rely upon internal regulatory mechanisms within each individual parasite.

Using a similar co-culture set-up, Williams demonstrated stimulation of sexual conversion by parasite cultures that had started to produce gametocytes, and also by media conditioned by such cultures. We hypothesize that the mechanism for this was as follows: the environment culture of Williams and the conditioned media taken from it contained diffusible factor(s) responsible for density-dependent inhibition of growth, since it was already producing gametocytes. This is supported by the observation that cultures of *P. falciparum* approaching peak parasitemia, and thus in stationary growth phase, encourage maximum gametocyte development. In the co-culture and conditioned medium experiments of Williams, this would have inhibited the asexual growth of the

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**Figure 3.** Effect of the presence or absence of asexually replicating parasites in the environment culture on the rate of commitment to gametocyte production of a single generation of *Plasmodium falciparum*. A. Triplicate co-cultures of *P. falciparum* were set up as described in the Materials and Methods and Figure 1. Starting parasitemias are given for the test and environment cultures (T + E) on the x-axis (+ 0 indicates that the environment culture consisted of unparasitized red blood cells [rbc] at a hematocrit of 5%). After five days of co-culture, all test cultures were harvested and the sexual or asexual destiny of a single cohort of invading merozoites was determined as described in the Materials and Methods. This destiny is shown on the y-axis as the proportion of parasites that convert to sexual development and gives a measure of the sexual conversion rate of the cultures at the time of harvest. Error bars represent 95% confidence intervals. B. Mean parasitemia for the environment cultures starting at parasitemias of 0.1% (-○-) and 2.0% (-●-) during the course of co-culture, as determined by microscopic examination of Giemsa-stained smears of the cultures.
parasites in the test culture through sharing of the culture media. In light of the above interpretation of our own experimental results, we would also predict that more parasites would then be released from asexual growth-dependent sexual repression.

Thus, as the growth of the parasite slows for whatever reason, e.g., lack of nutrients, immune activity, application of antimalarial drugs, oxidative stress, build-up of metabolic waste products, or some other density-dependent factor, sexual repression may be lifted (Figure 4). Production of transmission forms will therefore be maximized in response to increasing opposition to vigorous asexual growth and should increase the probability of successful transmission under difficult circumstances for asexual growth. If as all the evidence suggests, conversion to sexual development occurs at maximum parasitemia, then we may expect to see the observed association between the presence of gametocytes in the bloodstream (after development in sequestered sites) and a decrease in asexual parasitemia during in vivo infection.28–31 A more complete description of the dynamics of asexual growth and commitment to sexual development may therefore help to understand the mechanisms that lead to an increase in gametocytogenesis and transmission when parasites are exposed to sub-lethal doses of antimalarial drugs such as chloroquine, which act to repress asexual growth.6,30

While previous work has shown that gametocytogenesis is encouraged in cultures when the parasitemia is highest27 and that diffusible factors from cultures in this growth state encourage gametocytogenesis,27 this report provides evidence for the mechanism underlying these observations. It illustrates more clearly the relationship between asexual growth and gametocytogenesis: parasites in log-phase growth stimulate their asexual growth via diffusible factors and gametocytogenesis is repressed; when asexual parasite density reaches a critical level, nutritional or other diffusible factors limit parasite growth and the repression on gametocytogenesis is lifted. In this model, the sexual pathway is the default developmental pathway with asexual blood stage replication providing a large mass of potentially transmissible parasites for a longer period of time. Erythrocytic schizogony is not present in related genera Leucocytozoon and Haemoproteus, suggesting that this life cycle feature evolved after the divergence of Plasmodium from these genera, as it appears to have done at least three times in the apicomplexan lineage.32

Although the static in vitro culture method for P. falciparum falls short of replicating the conditions within the human host, it should not be assumed that all molecules used as signals would be removed or greatly diluted by circulation. Since the maturing parasitized erythrocytes adhere in deep tissues, the local flow conditions may be low enough for short distance communication. This more favorable scenario for signaling would be encouraged by partial or complete blockage of small capillaries by rosette-forming erythrocytes and may even be a pre-requisite for switching to sexual development.

Our unsuccessful attempts to isolate and further characterize the diffusible molecule(s) responsible for stimulation of growth of P. falciparum in culture suggest that they are highly labile; conditioned media stored overnight (either snap-frozen or chilled in the presence or absence of protease inhibitors) could not replicate the stimulatory effect on growth of the presence of asexually replicating parasites across a permeable membrane. A proteomic analysis of parasites undergoing commitment to sexual differentiation may allow us to identify the molecules involved in these controlling events of P. falciparum development.
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