STIMULATION OF *PLASMODIUM FALCIPARUM* GAMETOCYTOGENESIS BY CONDITIONED MEDIUM FROM PARASITE CULTURES

J. L. WILLIAMS

Department of Preventive Medicine and Biometrics, Uniformed Services University of the Health Sciences, Bethesda, Maryland

Abstract. *Plasmodium falciparum* gametocyte development was examined in erythrocyte monolayer cultures prepared with Cell-Tak®, a cell and tissue adhesive. The monolayers, which were stable for up to 10 days in culture, supported multiple cycles of asexual growth and the development of clusters of stage IV gametocytes. Small numbers of chicken erythrocytes incorporated into the monolayers served as internal reference standards for parasite counts. This permitted quantitative assessment of gametocyte formation under different culture conditions. Gametocyte formation was limited in monolayers grown in standard culture medium but it increased slightly in monolayers cocultured with suspensions of parasitized erythrocytes. The number of gametocytes increased significantly in monolayers grown in parasite-conditioned medium. In both cases the changes resulted from increased numbers of stage II and III gametocytes in the monolayers. These results suggest that parasite conditioned medium contains a factor(s) that stimulates sexual development.

Transmission of malaria begins when blood stage parasites in an infected individual differentiate into microgametocytes or macrogametocytes, the sexual stages of the parasite. The proportion of parasites that develop into gametocytes varies greatly in vivo and in vitro. In humans, factors that influence this include the length of prepatent period, the magnitude of the asexual parasitemia, the immune response of the patient, as well as innate characteristics of the parasites themselves. Due to these numerous, complex influences, the control of gametocytogenesis in vivo remains a mystery.

The development of continuous culture techniques for *Plasmodium falciparum* made it possible to study sexual development of this parasite outside of the human host. Although the dynamics were not the same as in the host, the culture techniques allowed investigators to examine this process in a simpler, more controllable environment. Using these techniques, Carter and Miller demonstrated that environmental conditions modulated gametocytogenesis in *P. falciparum*. Commitment to sexual development was turned off by dilution of parasite cultures with fresh erythrocytes and turned on again after six days in continuous culture. They proposed that changes in the culture medium associated with parasite growth were directly responsible for increasing the rate of gametocyte formation in vitro. The experiments reported in this paper provide the first direct support for this hypothesis.

**MATERIALS AND METHODS**

**Parasite cultures.** The NF 54 strain of *P. falciparum* (obtained from the Department of Entomology, Walter Reed Army Institute of Research, Washington, DC) was maintained in settled erythrocyte cultures. Cultures were seeded at an initial hematocrit of 6% in RPMI 1640 medium supplemented with 10% heat inactivated type O human serum, 25 mM HEPES, 0.2% NaHCO₃, 2 mM glutamine, 50 mg/ml of hypoxanthine, 100 U/ml of penicillin, 100 mg/ml of streptomycin, and 250 pg/ml amphotericin B. Stock cultures were set up with 0.1% parasitemia and maintained in 25-cm² flasks gassed with a mixture of 5% CO₂, 5% O₂, and 90% N₂. The medium was changed daily and new cultures were initiated at 3–4-day intervals.

For conditioned medium, 20-ml cultures were grown in 75-cm² flasks for 5–6 days. Each day during the medium changes, the supernatants were collected and pooled. The pools were clarified by low-speed centrifugation, filtered through a 0.2-µ filter, and stored at −20°C.

**Erythrocyte monolayer preparation.** Erythrocyte monolayers were prepared in Nunc (Naperville, IL) flasks. The flasks were pretreated with Cell-Tak®, a commercially available cell and tissue adhesive derived from the marine mussel, *Mytilius edulis* (Collaborative Biomedical Products, Bedford, MA). The adhesive is a polyphenolic protein containing a tandemly repeated decapetide that binds to many surfaces, including polystyrene as well as infected and uninfected erythrocytes. It was diluted to 35 µg/ml in 0.1 M sodium carbonate, pH 8.0, and 1 ml was added to each flask. The flasks were incubated for 20 min and shaken at approximately 5-min intervals to ensure even coverage of the slides. The slides were rinsed twice with sterile distilled water and once with serum-free medium. A suspension of human erythrocytes was prepared in serum-free medium at 2% hematocrit, 0.1% parasitemia. The cells used to inoculate the erythrocyte suspension were taken from rapidly growing stock cultures to minimize the numbers of multiply infected cells and gametocytes incorporated into the monolayers. When such cells were seen in differential parasite counts, they were scored as a single parasitized cell. Two milliliters of the mixed cell suspension were added to each flask and the flasks were incubated for 20 min without shaking. The unattached cells were resuspended by gentle agitation and removed by aspiration. The monolayers were rinsed three times with serum-free medium and covered with 3 ml of culture medium. The flasks were placed in a humidified gas-tight chamber fitted with sealable access ports (Billups-Rothenberg, Del Mar, CA). The chamber was flushed with the gas mixture described above, sealed, and placed in a 37°C incubator. At the completion of the experiment, the supernatants were aspirated from the cultures and the tops of the flasks were removed and discarded. The monolayers were air-dried, fixed with methanol, and stained with Giemsa. In the cocultivation experiments, erythrocyte monolayers were prepared in adhesive treated six-well tissue culture plates using the technique described for the flasket.

At the end of the experiments, they were air-dried and stained with Giemsa in the wells. The bottoms of the wells, along with the attached monolayers, were cut from the plates with a wire cutter, glued to 2 × 3 inch glass slides, and examined for gametocyte formation.

Whole chicken blood diluted 1:1.2 with Alsever’s solution was obtained from a commercial source (Carolina Biological Supply, Burlington, NC). Before use, the chicken erythrocytes were washed in the same manner as the human erythrocytes. In preliminary studies, the chicken cells were lysed, presumably by heterophile antibodies and complement in the human serum used to supplement the medium. This was prevented by heat inactivation of the serum used in the cultures. Agglutination of the chicken red blood cells was not a problem since the cells were immobilized before they were exposed to the medium. Nonetheless, some of the chicken erythrocytes degenerated during the culture period. These cells were easily recognized by their prominent nucleus and large, oval shape (Figure 1).

Gametocyte counts. The ratio of chicken erythrocytes to parasitized human erythrocytes was determined within 2 hr after preparation of the monolayers. The slides were systematically scanned at a 1,000-fold magnification and chicken and parasitized human erythrocytes were counted. Once 200
monolayers were incubated with inserts containing either into the wells and the plates returned to culture. Control and thin smears were prepared. The inserts were placed back.

leaving approximately 1.7 ml in the wells and 1.3 ml in the fluid level between the wells and the inserts equilibrated the manner described earlier. During the culture period, the plates were placed in the gas-tight chambers and cultured in

ly permeable and 2) the contribution of the erythrocytes in produced a 3-ml culture at 6% hematocrit and 0.1% initial parasitemia. The inserts consisted of a plastic cylinder 1.5 ml of a cell suspension containing 12% erythrocytes at

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0.05).

Cocultivation experiments. Erythrocyte monolayers were prepared in Cell-Tak®-treated six-well tissue culture plates and covered with 1.5 ml of culture medium. Tissue culture inserts (Transwell-Clear®; Costar Scientific Corp., Cambridge, MA) were placed in the wells and filled with 1.5 ml of a cell suspension containing 12% erythrocytes at 0.1% parasitemia. The inserts consisted of a plastic cylinder sealed at one end with a semipermeable (0.4-µ) membrane. This produced a 3-ml culture at 6% hematocrit and 0.1% initial parasitemia, assuming that 1) the membranes were completely permeable and 2) the contribution of the erythrocytes in the monolayers to the total hematocrit was negligible. The plates were placed in the gas-tight chambers and cultured in the manner described earlier. During the culture period, the fluid level between the wells and the inserts equilibrated leaving approximately 1.7 ml in the wells and 1.3 ml in the inserts. Each day the inserts were transferred briefly to a sterile Petri dish and the medium in both the wells and inserts was changed. The cells in the inserts were resuspended and thin smears were prepared. The inserts were placed back into the wells and the plates returned to culture. Control monolayers were incubated with inserts containing either 12% uninfected erythrocytes or culture medium.

RESULTS

Parasite development in erythrocyte monolayers. Erythrocyte monolayers prepared with the adhesive were similar to those prepared with concanavalin A.8-10 Parasitized human erythrocytes and chicken red blood cells appeared to be uniformly distributed throughout the monolayers, surrounded by uninfected human cells (Figure 1A). Although the attached cells generally retained their characteristic biconcave shape, they took on a polygonal appearance in areas where they were densely packed together. They shrank somewhat during fixation, giving rise to small spaces between the cells that were not seen on unfixed slides. Loosely attached cells were crenated immediately after preparation of the monolayers but they resumed a more normal appearance after 1–2 hr in culture. Monolayers prepared with concanavalin A often deteriorated after 3–4 days in culture whereas those prepared with adhesive remained stable for up to 10 days. With prolonged culture periods and daily media changes, some cells around the periphery of the monolayers detached and accumulated toward the middle of the culture vessels. However, erythrocytes in the central areas of the monolayers remained confluent throughout the experiments.

The erythrocyte monolayers supported both asexual and sexual parasite development. Most of the parasites completed schizogony and gave rise to small plaques of erythrocytes infected with ring-stage parasites (Figure 1B). These young parasites also matured and initiated a second round of growth and reinvasion (Figure 1C). Subsequent rounds of parasite growth were less successful with decreasing numbers of parasitized cells around the edges of the plaques.

Gametocytes developed along two pathways. Some of the parasites developed directly into individual gametocytes without going through schizogony. These were detected as isolated stage II gametocytes on the second or third day of culture. The erythrocytes containing these young gametocytes were randomly distributed in the monolayers and surrounded by uninfected cells. With continued cultivation, the parasites developed to stage IV (Figure 1D) and, in some cases, stage V gametocytes. Other parasites completed a round of schizogony and then gave rise to plaques containing stage II gametocytes after 4–5 days of culture (Figure 1E). These gametocytes matured normally, often with 5–6 seen in a cluster (Figure 1F). Late in the culture period (8–10 days), large plaques developed that contained both asexual parasites and young gametocytes (Figure 1G). The gametocytes often appeared to be located close together in discrete areas of the plaques. The plaques usually contained several residual bodies and extracellular parasites at various stages of degeneration suggesting several rounds of growth had taken place.

The total number of gametocytes that developed in the monolayers was so small that determination of the gametocytemia in terms of gametocytes per 100 erythrocytes was impractical. To overcome this limitation, chicken erythrocytes were incorporated into the monolayers as internal reference standards. This made it possible to determine at any time during the culture period the number of gametocytes that developed from a given number of parasites (Figure 2). During the first three days of the culture, the number of gametocytes per 1,000 initial parasitized human erythrocytes increased slowly as individual parasites developed into recognizable gametocytes. The gametocyte count increased more rapidly on the fourth, fifth, and sixth days of culture as gametocyte plaques developed. Thereafter the counts became somewhat erratic as some of the mature forms degenerated and mixed plaques containing both gametocytes and asexual parasites developed. On the final day of culture, the mean ± SD number of gametocytes was 92.7 ± 24.8 per 1,000 initial parasitized cells. Since only one in 1,000 of the human erythrocytes used to prepare the monolayers was parasitized (0.1% parasitemia), the final gametocytemia in the monolayers was approximately 9.3 × 10^{-3}.

Cocultivation of monolayers with parasite cultures. To examine the effect of parasite growth on gametocyte development, monolayer cultures prepared in the bottom of six-well plates. A tissue culture insert containing a suspension
of infected erythrocytes was placed in the wells and cocultured with the monolayers. Asexual parasite growth and gametocyte formation in the inserts is shown in Figure 3. The asexual parasites grew well for 3–4 days and then the growth became static as shown by a decrease in the growth rate and the appearance of degenerating parasites. The growth of the parasites remained poor until the end of the experiment and the parasitemia remained less than 3%. A few young gametocytes were seen in the suspension cultures on the fifth day of culture and their numbers increased on days six and seven.

On the eighth day of culture the monolayers were collected and examined for gametocyte formation. More gametocytes developed in the erythrocyte monolayers grown with the parasite cultures than in those exposed to either uninfected erythrocytes or tissue culture medium (Table 1).
Table 1
Gametocyte development in erythrocyte monolayers*

<table>
<thead>
<tr>
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<th>Parasite cultures</th>
<th>Erythrocytes</th>
<th>Culture medium</th>
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<tbody>
<tr>
<td>Coculture</td>
<td>168.33 ± 18.61</td>
<td>108.67 ± 21.73</td>
<td>83.33 ± 1.53</td>
</tr>
<tr>
<td>Conditioned medium</td>
<td>222.33 ± 23.86</td>
<td>38.00 ± 6.24</td>
<td>29.33 ± 8.02</td>
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* Values are the total number ± 1 SD of gametocytes per 1,000 initial parasites.

The total number of gametocytes in monolayers exposed to the parasite cultures increased 1.5-fold and 2.0-fold over the erythrocyte and medium controls, respectively. The increase over the medium control was significant but the difference between the parasite cultures and the uninfected erythrocytes was not. Differential gametocyte counts demonstrated clear differences in the number of young gametocytes in the monolayers (Figure 4). The number of stage II gametocytes in the monolayers exposed to parasite cultures increased significantly over both of the controls. Although the number of stage III gametocytes also increased, the results were ambiguous. While the increase over the medium control was significant, the increase over the uninfected erythrocytes was not. The numbers of stage IV and stage V gametocytes were unaffected by the various culture conditions.

**Stimulation of gametocytogenesis by parasite conditioned medium.** Conditioned medium from six-day-old parasite cultures had a pronounced effect on gametocyte formation in the monolayers. The total number of gametocytes in the monolayers grown in conditioned medium increased approximately 5.9-fold and 7.6-fold over the uninfected erythrocyte and regular culture medium controls (Table 1). This increase resulted from almost equal numbers of additional stage II and stage III gametocytes (Figure 5). In contrast, the numbers of stage IV and stage V gametocytes did not change. Similar though lesser stimulation occurred in monolayers cultured with parasite conditioned medium collected on the fifth day of culture.

**DISCUSSION**

Experimental studies of the factors that stimulate sexual development in *P. falciparum* are hampered by two problems: 1) the high levels of endogenous gametocyte formation in the control cultures and 2) the use of variable parameters to estimate the number of gametocytes in the cultures. Generally, cultures in such studies are grown for 4–5 days, the putative stimulatory agents are added, and 2–4 days later the gametocytes are counted. Since these conditions promote gametocytogenesis in unstimulated cultures, the increases seen in the treated cultures are usually only 2–4-fold higher than those found in the controls. The results are then calculated as the gametocytemia, either the number of gametocytes per 100 red blood cells or the percentage of erythrocytes infected with gametocytes. As Vanderberg pointed out, these are relative quantification procedures based on the numbers of host erythrocytes in the cultures. Since considerable hemolysis may occur in these cultures, the number of erythrocytes present often varies, both within and between experiments. Thus, care must be taken when comparing experimental results expressed as gametocytemias. The erythrocyte monolayer technique described in this paper effectively circumvents these problems by minimizing background gametocyte formation and providing a fixed internal reference standard for gametocyte counts.

Although the level of background gametocyte formation in unstimulated monolayers was consistently low, it varied somewhat in the experiments reported here. For example, in the experiment shown in Figure 2 the monolayers contained 59.7 ± 14.6 (mean ± SD) gametocytes per 1,000 initial parasitized cells on the eighth day of growth. After the same period of time, the medium controls in the experiments shown in Table 1 contained 83.3 ± 1.5 and 29.3 ± 8.0 gametocytes from an equal number of cells. This variability apparently resulted from differences in the gametocytogenic capacity of the inocula used to prepare the monolayers. Although the NF 54 strain of parasites was chosen for these experiments because of its propensity to produce gametocytes, the number it produced decreased rapidly in our hands due to the regimen used to maintain the stock cultures. To minimize these effects, stabilates were thawed and new stock cultures were initiated at approximately six-month intervals but some variation still occurred. Despite this, the stimula-
FIGURE 5. Gametocyte development in erythrocyte monolayers grown in parasite conditioned medium. The monolayers were prepared in flasks and grown in a 50% dilution of parasite conditioned medium. After eight days of growth, the monolayers were examined for gametocyte formation. RBC = red blood cell. Bars show ± SD.

itory effects of conditioned medium in the last two experiments were easily detectable.

The erythrocyte monolayer technique described in this paper is a modification of the one reported previously by Inselburg and Bruce and others; however, they differed in two important aspects. First, the erythrocytes were attached to the culture vessels with a cell adhesive rather than concanavalin A, a plant lectin. These monolayers are considerably more durable than those made with the lectin. The second modification consisted of the incorporation of chicken erythrocytes into the monolayers as an internal standard for parasite counts. Although a small number of the chicken cells degenerated during the experiments, rate of cell loss appeared to be constant within a given experiment. In subsequent experiments the chicken erythrocytes have been replaced with polystyrene microspheres (4.5-μm diameter) that are inert to culture conditions.

Inselburg and Bruce and others used lectin-mediated monolayers to investigate the models of gametocytogenesis proposed by Carter and Miller. Inselburg noted that most merozoites from a committed schizont developed into gametocytes while some of their siblings continued to grow asexually. On the other hand, Bruce and others argued that commitment of a parasite was an all-or-none event: all of its progeny developed into either gametocytes or asexual trophozoites. In contrast to both of these, Trager and Gill suggested that the developmental fate of individual merozoites depended on the type of erythrocyte that they invaded. These alternative possibilities would give rise to 1) mixed plaques with both gametocytes and asexual parasites, 2) homogenous plaques with only one form of the parasite, or 3) single, isolated gametocytes. All of these were seen in the monolayers reported here, so one cannot rule out any of these developmental schemes. Since none of these possibilities are excluded in the present study, resolution of this issue requires the use of more rigorously controlled experiments with singly infected erythrocytes containing synchronously growing parasites. Consequently, the results of these experiments are reported in terms of total gametocytes per 1,000 initial parasites rather than the number of plaques or isolated gametocytes.

Carter and Miller proposed that changes in the culture medium resulting from parasite growth increased the rate of gametocyte formation in malaria cultures. The results reported here provide the first direct support for this hypothesis. Gametocyte formation was limited in unstimulated erythrocyte monolayer cultures but increased when the cells were cocultured with gametocyte-producing malaria cultures. Exposure of the monolayers to conditioned medium from parasite cultures produced an even more substantial increase in sexual development. In contrast, conditioned medium from uninfected erythrocyte cultures had no effect on sexual development in the monolayers.

The procedures used in these experiments allow one to rule out any effects of a number of factors that reportedly stimulate gametocytogenesis. Particular matter that accumulates in the cultures such as pigment, residual bodies, and degenerating merozoites was removed by centrifugation and filtration. Filtration, along with the freezing and thawing of the media, eliminates the possibility of contamination of the monolayers with parasitized erythrocytes or viable merozoites from the stimulatory cultures. Exogenous agents such as protein kinase activators, immune sera or antibodies, hormones, erythrocyte lysates, or ammonia compounds can be ruled out since they were not used in these studies. A possible exception may be the products released from lysed erythrocytes since hemoglobin was visibly detectable in the parasite-conditioned medium. However, it was also present in the uninfected erythrocyte-conditioned medium, albeit to a somewhat lesser degree. In addition, nutrient depletion does not seem to be an issue since the conditioned media were diluted with equal volumes of fresh, fully supplemented culture medium.

The results presented here suggest that parasite-conditioned medium contains a soluble factor(s) that stimulates gametocytogenesis. The biochemical nature of the factor remains unknown. It may be as simple as the accumulation of metabolic waste products such as lactic acid or as complex
as macromolecules released from lysed and degenerating cells in the cultures. An intriguing possibility is the production of a parasite hormone that specifically stimulates sexual development through an autocrine or paracrine mechanism. Whatever the factor may be, the techniques reported here should provide a powerful tool in its identification and the analysis of the cellular mechanisms it activates to initiate gametocytogenesis.

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Author’s address: J. L. Williams, Preventive Medicine and Biometrics, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814-4799.

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