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PLASMODIUM FALCIPARUM: PURIFICATION OF THE VARIOUS GAMETOCYTE DEVELOPMENTAL STAGES FROM *IN VITRO*–CULTIVATED PARASITES

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Abstract. Cultivated *Plasmodium falciparum* gametocytes reach maturity *in vitro* in approximately 14–16 days, during which they pass through five morphologically distinct developmental stages. Purification of the earlier developmental stages has not been previously reported. We have modified the standard discontinuous Percoll gradient method for the separation of stage IV and V gametocytes to obtain enriched preparations of those and the earlier *P. falciparum* gametocyte stages. In contrast to the stages II, III, and IV, the mature stage V gametocytes from our gradient readily transformed into gametes. Such preparations may be useful in research studies on the mechanisms that underlie gametocytogenesis.

Several previously reported techniques for the purification of mature *Plasmodium falciparum* gametocytes exploit the differences in sedimentation rates and buoyant densities of uninfected erythrocytes and erythrocytes infected with the various stages of the parasite.[1,2] The major limitation of these techniques is their inability to maintain physiologic osmolality throughout the purification procedure and avoid cell damage.[3] Percoll, a colloidal solution of polyvinylpyrrolidone-coated silica particles, can be used to overcome this problem of osmotically induced cell damage because of its low osmolality, 10 mOsm at 1.13 g/ml.[4] A Percoll step gradient centrifugation method has been successfully used to obtain highly purified preparations of viable mature *P. falciparum* and *P. yoelii* gametocytes.[5]

The addition of fresh erythrocytes to *P. falciparum* cultures leads to loss of synchrony.[6,7] Synchrony can be restored by the addition of DNA inhibitors during gametocytogenesis.[8] However, inhibitors such as mitomycin C and chloroquine are toxic to stages I and II gametocytes. Mature gametocytes that have been synchronized by the addition of mitomycin C cannot be used immediately for research studies and membrane feeds because gametogenesis is inhibited for 24–48 hr after removal of the drug.[8] To avoid this transient drug toxicity, Ponnudurai and others synchronized gametocytes in an automated culture system by using gelatin flotation and N-acetyl glucosamine treatment.[9]

Despite the fact that methods for synchronization and purification of the mature sexual stages of *P. falciparum* cultures have been separately described, these two techniques have not been combined to obtain purified fractions of the early gametocyte stages. This is a crucial requirement for research because the events that regulate gametocytogenesis are still in question. Our ability to study the early sexual stages, soon after morphologic differentiation, may provide insight into this intriguing aspect of cell cycle regulation. We have modified the Percoll step gradient technique to obtain enriched fractions of almost all the stages of *P. falciparum* gametocytes from *in vitro* culture.

MATERIALS AND METHODS

Culture of gametocytes. *Plasmodium falciparum* strain NF54 was cultured *in vitro* according to the Ifediba and Vandeberg[10] modification of the Trager and Jensen method.[6] Ten cultures (0.2% parasitemia, 6% hematocrit of blood group O Rh^+ human erythrocytes) were set in 25-mm^3 canted neck flasks. The flasks were aerated with a gas mixture (92% N_2, 5% O_2, and 3% CO_2) and incubated at 37°C with daily media changes. The parasitemia of the cultures were monitored by microscopic examination of Giemsa-stained thin films. When the parasitemia reached 5–6%, the cultures were pooled and fresh red blood cells and media were added to bring the parasitemia to 0.5% at 6% hematocrit. Five milliliters of this suspension were then dispensed into each of ten 25-cm^3 canted neck flasks. The flasks were incubated at 37°C with daily medium changes for three days. On the third day, the 10 culture flasks were pooled again and 5 ml of the pooled suspension was dispensed into 10 other flasks. The following day (Day 4) and every three days thereafter (Day 7, 10, 13 and 16), the gametocytes of two flasks were harvested on a Percoll gradient while the contents of the remaining flasks were pooled and redistributed.

Purification of gametocytes. Incomplete medium (ICM) was prepared by the addition of HEPES (5.94 g/L) and hypoxanthine (50 mg/L) to RPMI 1640 medium with glutamine and sterilized through a 0.2-μ filter. Percoll (1.13 g/ml) was made isosmotic by the addition of one volume of 10× ICM to nine volumes of stock Percoll solution (90%). This solution was then diluted with 1× ICM to make 80%, 65%, 50% and 35% Percoll solutions. Two milliliters of each of these diluted solutions were run slowly down the side of a tilted 15-ml test tube, starting with the heaviest (80%), to form the layered gradient.

The gametocytes from the two selected flasks were harvested by removal of the spent medium and addition of 5 ml each of ICM. The resultant suspensions were pooled in a 15-ml test tube and centrifuged at 1,500 × *g* for 5 min. The pellet was washed twice with ICM and the final pellet was suspended in two volumes of ICM. Two milliliters of the final gametocyte suspension was carefully layered over the discontinuous Percoll gradient and centrifuged at 1,450 × *g* for 10 min.

Initial experiments had indicated that gametocytes were suspended at the 35%/50% interphase of the gradient (F2 band). This band was carefully removed and washed three times with ICM. Thin films were prepared, Giemsa-stained, and examined under the microscope. Photographs were taken at 1,000× magnification under oil.

Testing for exflagellation. Exflagellating medium was prepared by the addition of 25 mM sodium bicarbonate and 10% (v/v) heat-inactivated human serum to ICM. The pH of the medium was adjusted to 8.0 with 1M NaOH. Five milliliters of washed F2 gametocytes were suspended in the exflagellation medium, placed under a vaseline-rimmed cover glass and examined by phase contrast microscopy (400× and 1,000× magnification).

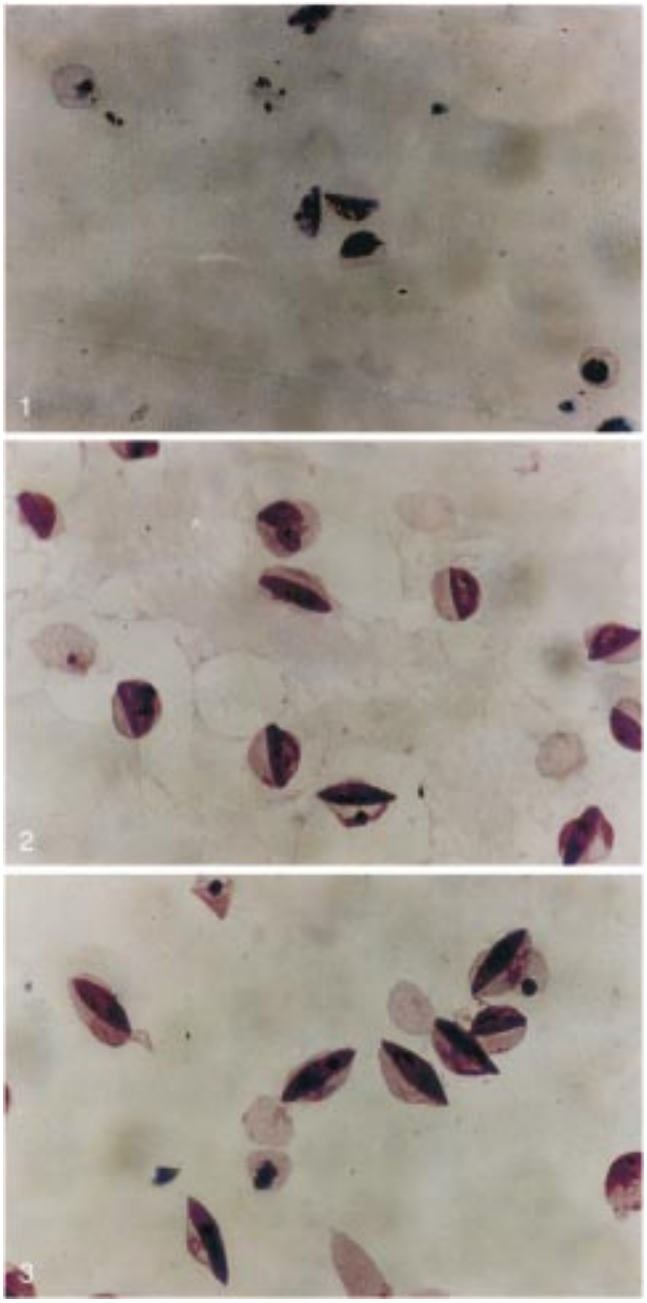
RESULTS

Figure 1 shows a Giemsa-stained thin film of day 4 *P. falciparum* cultures from the F2 fraction of a Percoll gradient. The oat grain shape of the gametocytes is indicative of stage II gametocytes of the classification of Hawking and others.[11] Similarly processed F2 fractions from day 7 and day 10 cultures are shown in Figures 2 and 3, respectively. The day 7 gametocytes have lost their oat grain shape and are more segment-like (stage III), whereas the day 10 gametocytes are spindle-shaped with pointed poles (early stage IV). Even though most of the gametocytes from day 13 cultures, (Figure 4), were spindle-shaped, their poles had started to show the blunting characteristic of late stage IV gametocytes. None of the microgametocytes obtained from day 13 and younger cultures formed microgametes when placed in exflagellating medium. In contrast, crescent-shaped F2 microgametocytes from the day 16 cultures (Figure 5) readily transformed into gametes (Figure 6) when resuspended in exflagellating medium.

DISCUSSION

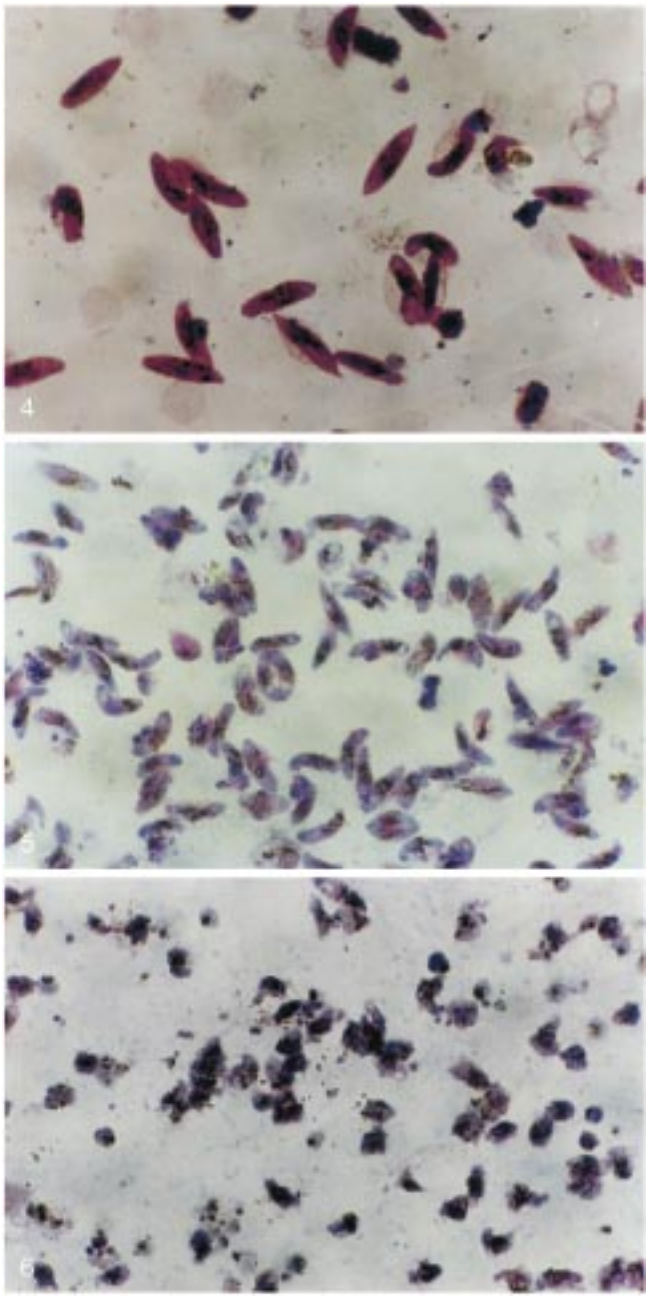
Gametocytes, like most dividing cells, go through a four-phase cycle. During the G1 phase, which immediately follows mitosis (M phase), the cell prepares to synthesize its DNA. Synthesis of DNA then occurs in the S phase and is followed by another growth phase, G2. The five morphologically distinct stages of *Plasmodium* gametocytes identifiable in culture are represented in the figures. Even though a classic cell cycle does not exist in *Plasmodium* gametocytogenesis,[12,13] gametocyte stages 1, 2 and 3 are generally associated with G1, S, and G2A, respectively. Stage 4 and 5 gametocytes, on the other hand, are associated with the G2B phase of the cell cycle.[7] The later gametocyte stages are easily distinguished from asexual parasite stages by unique parasite and red blood cell morphologic characteristics.[7,9] When seen in stained smears of infected blood, these blunt end crescent-shaped gametocytes are predictive of *P. falciparum* infection.

Gametocytogenesis in *P. falciparum* has an unusually prolonged G2B phase (> 10 days). The persistence of inactive mature gametocytes in the circulation increases the chances of uptake by a vector mosquito and subsequent transmission of the disease. During most of this G2B phase, mature gametocytes are not engaged in DNA, RNA, and protein synthesis.[7,8] They have completed the maturation process and will transform into gametes when activated. Stage 4 and 5 gametocytes that are routinely enriched from culture are best suited for studies of gametogenesis.[14–16] In contrast, commitment to gametocytogenesis may occur prior to the preceding schizogony. Even though the events that regulate initiation and maturation of gametocytes in culture commence in prestage I gametocytes, they may extend into stage 2, 3, and 4 gametocytes.



FIGURES 1–3. **1,** gametocytes isolated from a Percoll gradient (fraction 2) of four-day-old *Plasmodium falciparum* cultures. The oat grain shape of the cells are indicative of stage II gametocytes. **2,** gametocytes isolated from a Percoll gradient (fraction 2) of seven-day-old *P. falciparum* cultures. The segment shape of the parasites is reminiscent of stage III gametocytes. **3,** gametocytes isolated from a Percoll gradient (fraction 2) of 10-day-old *P. falciparum* cultures. Parasites are now spindle-shaped with pointed poles, reminiscent of early stage IV gametocytes. Gametocytes from gradients were washed with incomplete medium. (Giemsa stained, original magnification × 1,000.)

We have outlined modifications to existing techniques that allow synchronized fractions of all stages of *P. falciparum*



FIGURES 4–6. **4,** gametocytes isolated from a Percoll gradient (fraction 2) of 13-day-old *Plasmodium falciparum* cultures. Spindle-shaped gametocytes with blunt poles are characteristic of late stage IV gametocytes. **5,** gametocytes isolated from a Percoll gradient (fraction 2) of 16-day-old *P. falciparum* cultures. Gametocytes are now crescent-shaped, a characteristic of stage V gametocytes. **6,** mature gametocytes (16-day culture) were purified on a Percoll gradient, washed with incomplete medium, and resuspended in exflagellation medium. Within 10–15 min, crescent-shaped gametocytes transformed into rounded macrogametes and flagellated microgametes. Gametocytes from gradients were washed with incomplete medium. (Giemsa stained, original magnification × 1,000.)

gametocytes, except stage 1, to be obtained from *in vitro* culture. Modifications included the substitution of incomplete medium for Tris-buffered saline during the entire period during which the parasites are on the discontinuous Percoll gradient. In this manner, gametocytes were in constant nutritive and electrolyte balance throughout the purification process. Even though DNA inhibitors (mitomycin C, chloroquine) have been used to produce enriched fractions of *P. falciparum* gametocytes from *in vitro* culture, the toxicity of these drugs to young gametocytes precluded their use over the density centrifugation techniques. Our studies used multiple cycles of pooling and redistribution of the parasites to ensure uniformity in culture conditions and synchrony of gametocytes. This modified Percoll gradient centrifugation technique may provide the parasite stages needed for the study of cell cycle regulators in *Plasmodia* and other dividing cells.

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