Am. J. Trop. Med. Hyg., 53(6), 1995, pp. 607–611

COMPLETE DEVELOPMENT OF THE LIVER STAGE OF *PLASMODIUM FALCIPARUM* IN A HUMAN HEPATOMA CELL LINE

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Abstract. *Plasmodium falciparum* parasites develop in the liver before being released into the bloodstream, where they exert the potentially lethal effects characteristic of malaria. Our understanding of the hepatic phase of the life cycle is limited by the parasite's requirement for fresh human liver cells in which to mature. In this work, liver parasites completed their development within a Thai human hepatoma cell line (HHS-102), and the presence of ring-form parasites in erythrocytes overlying the liver cell culture confirmed that an entire liver cycle was completed, culminating in the production of viable blood-stage parasites. The HHS-102 cell line allows investigation of the undefined liver stage of falciparum malaria previously unavailable in the laboratory.

Malaria causes between one and 2.5 million deaths annually.[1] Virtually all malaria deaths are due to the blood stages of *Plasmodium falciparum*, the most lethal of the four human malaria parasite species.[2] There is no evidence that death is regularly associated with species of *Plasmodium* other than *falciparum*. The infection begins when a female anopheline mosquito inoculates sporozoites, which undergo a clinically silent period of development in the liver before entering the bloodstream.[3] Malaria parasites circulating within erythrocytes have been intensively studied; not only can they be visualized by direct microscopy, but those of *P. falciparum* can be maintained in continuous culture.[4–6] Unlike the blood stage, hepatic malaria is relatively inaccessible to study and therefore poorly understood. Liver-stage *P. falciparum* parasites develop in freshly obtained normal human hepatocytes,[7,8] but human liver specimens are difficult to obtain and cannot be maintained in continuous culture. Non-human primates and hepatic cell lines offer alternative approaches, but only incomplete *P. falciparum* parasite maturation occurs within either monkey hepatocytes or the HepG2 human hepatoma cell line.[9,10] A better model would facilitate investigations of hepatic malaria aimed at arresting parasite development in the liver and thus preventing clinical disease and death. A Thai human hepatoma cell line, HHS-102,[11] offered promise as a model able to support the complete development of falciparum hepatic parasites because it was morphologically similar to normal human liver cells in culture.

MATERIALS AND METHODS

The HHS-102 cells were seeded into a six-well tissue culture plate (Costar, Cambridge, MA) at a concentration of 2.0×10^5 cells/well and were maintained in 3 ml/well of a complete culture medium (a 1:1 mixture of RPMI 1640 medium with L-glutamine [Sigma, St. Louis, MO] and minimum essential medium with L-glutamine [Gibco, Grand Island, NY] supplemented with 2.2 mg/ml of sodium bicarbonate, 150 U/ml of penicillin, 150 mg/ml of streptomycin, and 10% fetal bovine serum). The monolayers were incubated until confluent at 37°C in a humidified incubator equilibrated with 5% CO_2 in air.

Laboratory-reared *Anopheles dirus A* mosquitoes, one variety of the *An. dirus* mosquito species complex, were infected by membrane feeding using cultured *P. falciparum* gametocytes from clone A-7 of a Thai isolate, CH-150[12] and maintained at 26–28°C with a relative humidity of 70–80%. On the 21st day after the infective blood meal, sporozoites were aseptically isolated from mosquito salivary glands. One day prior to inoculation of sporozoites, the fetal bovine serum in the complete medium was replaced by heat-inactivated human AB^+ serum in the hepatic cell cultures. Approximately 1.0×10^5 sporozoites were introduced into each well. The medium was changed after a 3-hr incubation, and every 48 hr thereafter.

Detection of liver-stage parasites was performed using Giemsa and immunoperoxidase staining. Giemsa-stained HHS-102 monolayers were examined for *P. falciparum* liver-stage parasites on day 6 and day 12 after sporozoite inoculation. The early stages of liver parasites were detected by indirect immunoperoxidase staining after cytocentrifugation.[13] The HHS-102 culture was trypsinized to achieve a single cell suspension and the cell suspension was counted and diluted with complete culture medium to a concentration of 10^5 cells/ml. A 100-μl aliquot of the diluted cell suspension was centrifuged onto poly L-lysine–coated slides using Cytospin 2 (Shandon, Pittsburgh, PA). After spinning, the slides were air-dried for 20 min and fixed with methanol at −20°C for 10 min. Immunoperoxidase staining was performed on all fixed slides using an avidin-biotin peroxidase complex procedure (OmniTagS® kit; Lipshaw, Detroit, MI). A monoclonal anti-*P. falciparum* circumsporozoite (CS) protein antibody, NFS1 (courtesy of Dr. Yupin Charoenvit, Naval Medical Research Institute, Bethesda, MD) at a 1:4,000 dilution in 1% bovine albumin phosphate-buffered saline was used with 3-amino-9-ethylcarbazol (Lipshaw) as a substrate for the peroxidase enzyme followed by counterstaining with Mayer's hematoxylin. Coverslips were placed on all stained slides and GelTol® (Lipshaw) was used as an aqueous permanent mounting medium.

To determine whether the development of liver-stage parasites could be completed in hepatoma cells, erythrocyte-hepatoma cell cocultures were performed on days 8, 10, and 12 following sporozoite inoculation. Three milliliters of a

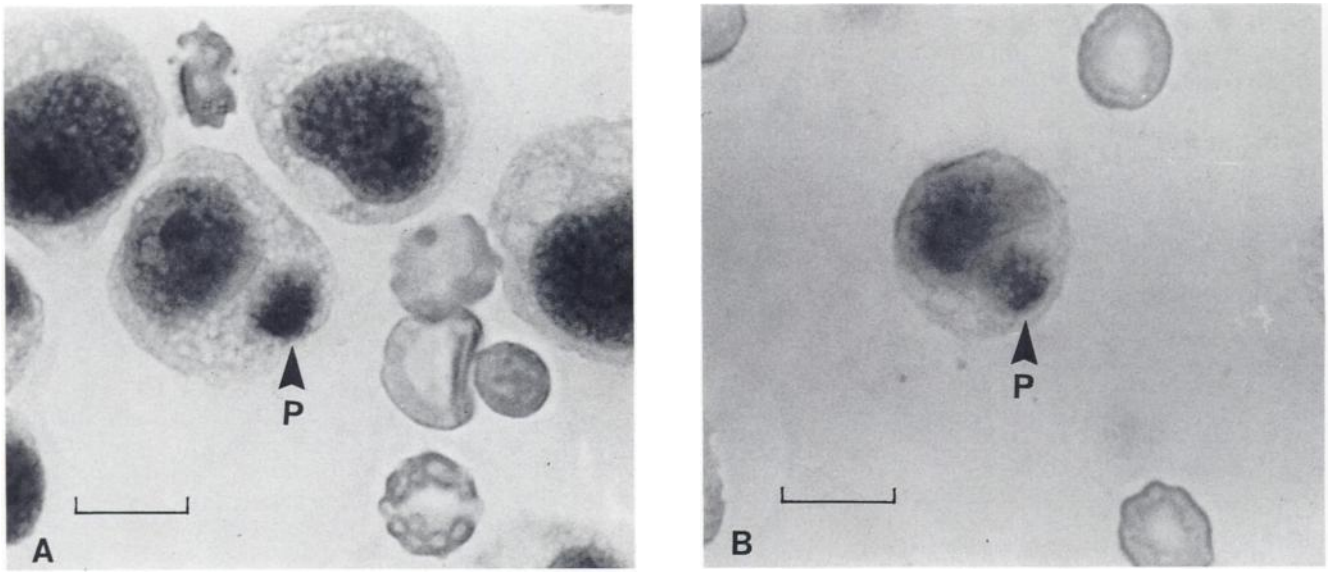

FIGURE 1. Early liver parasites detected by indirect immunoperoxidase staining after cytocentrifugation. Eight-day old liver parasites (P) are seen within HHS-102 hepatoma cells. The parasite in A is stained more intensely than that in B. Bars = 10 μm.

1% suspension of human O^+ erythrocytes in complete medium were introduced into the hepatoma cell cultures to serve as targets for invasion by emerging hepatic merozoites. The erythrocyte-hepatoma cell cocultures were incubated for 6 hr and the target erythrocytes were then removed and processed for in vitro continued culture. The recovered erythrocytes were washed twice in RPMI 1640 medium and adjusted to a 1% suspension in complete culture medium (RPMI 1640 medium supplemented with 5.96 mg/ml of HEPES buffer, 2.2 mg/ml of sodium bicarbonate, and 20% heat-inactivated compatible human serum). One hundred milliliters of cell suspension were dispensed into each microtiter well of a 96-well tissue culture plate (Costar), and the culture was maintained under standard conditions for asexual blood-stage parasite culture.[3] Spent medium was replaced every 48 hr and 100 μl of 1% fresh human erythrocytes in the complete culture medium containing 10% heat-inactivated human AB^+ serum were added every five days. Following removal of the cocultured erythrocytes, the HHS-102 cell monolayers were washed, methanol-fixed, and Giemsa-stained to detect liver-stage parasites by direct microscopy at 20× or 40×.

RESULTS

Hepatoma cells were examined at regular intervals for liver-stage parasites. Liver parasites detected by indirect immunoperoxidase staining eight days after sporozoite inoculation showed variable staining level of CS protein (Figure 1). Giemsa-stained HHS-102 cells 12 days after sporozoite inoculation showed considerable variation in the sizes (5–15 μm) of liver-stage parasites. Some large parasites 10–15 μm in diameter showed partial segmentation (Figure 2A). The rupture of liver-stage parasites was also suggested by clusters of hepatic merozoites, each measuring 1–2 μm in diameter, on top of hepatoma monolayers (Figure 2B).

Rupture of hepatic parasites and invasion of target erythrocytes by released functional merozoites was confirmed by erythrocyte-hepatoma cell coculture on day 8 following sporozoite inoculation. Eight coculture experiments were performed, and intraerythrocytic malaria parasites were produced on each occasion. Several ring-form parasites were seen in Giemsa-stained smears of the recovered erythrocytes made immediately after the 6-hr coculture. Recovered erythrocytes were maintained in continuous blood-stage culture conditions and ring-form parasites were detectable within erythrocytes on days 12 and 19 (Figure 3A). A small number of *P. falciparum* schizonts were also observed in stained blood smears on day 19 (Figure 3B).

DISCUSSION

We provide here the first evidence that all stages of the *P. falciparum* life cycle that occur outside the mosquito can be completed in vitro. Mosquitoes fed on cultured gametocytes produce sporozoites that infected hepatoma cells. In vitro culture of *P. falciparum* sporozoites is possible[14] and is under further investigation. Liver-stage parasites mature and rupture, releasing merozoites capable of infecting erythrocytes. The use of the HHS-102 cell line obviates the impractical requirement for fresh human hepatocytes.

The HHS-102 cell line was selected over three others because its morphology most closely resembled that of normal human liver cells.[11] In culture, HHS-102 cells retain intercellular junctional complexes and canaliculi resembling the bile canaliculi of normal liver cells, and neither overgrow nor pile up at zones of confluence. Several previous attempts both by ourselves and others[10] to achieve complete development of falciparum parasites within HepG2 cells were unsuccessful.

The liver cycle of *P. falciparum* in humans has been reported to occur in as little as 6.5 days.[15] No mature liver-stage parasites were detected on day 6 after sporozoite inoculation, but immature forms in a variety of sizes were

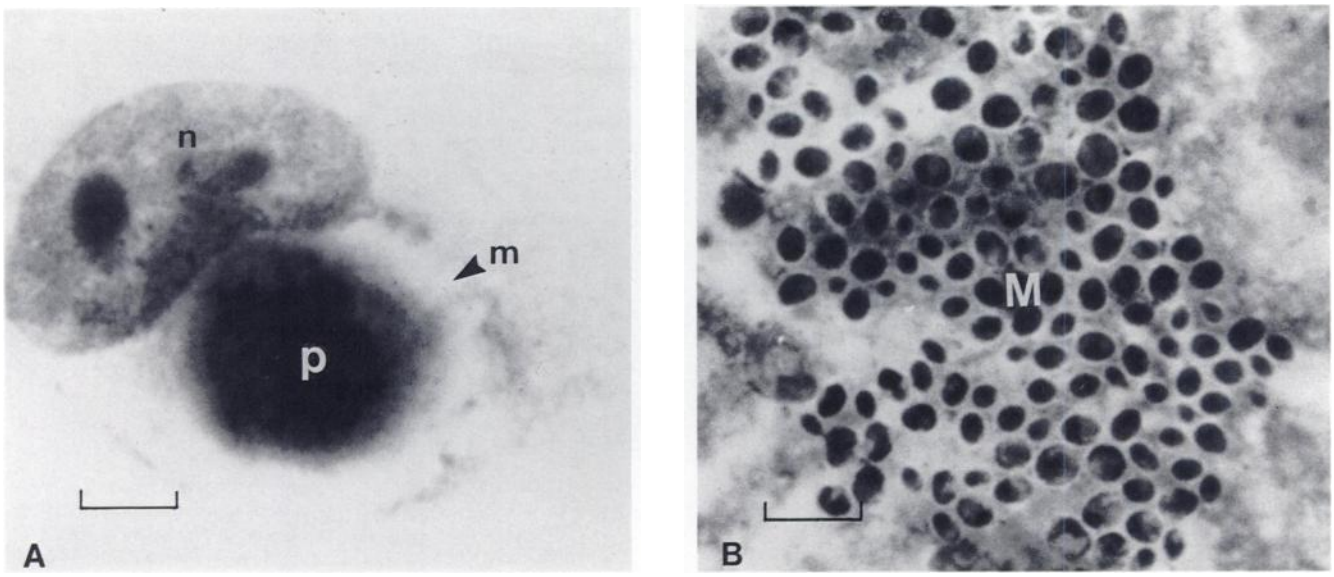

FIGURE 2. Giemsa-stained HHS-102 monolayers after sporozoite inoculation. **A,** a 12-day old parasite (**p**) surrounded by a parasitophorous vacuole membrane (**m**) is seen adjacent to the nucleus (**n**) of an HHS-102 hepatoma cell. **B,** merozoites (**M**) released from ruptured hepatoma cells are seen on top of the HHS-102 hepatoma monolayer 13 days after sporozoite inoculation. Bars = 5 μm.

seen, including forms up to 15 μm in diameter. Variation in the size of liver-stage parasites has also been reported in primary cultures of normal human liver cells infected with *P. falciparum* sporozoites.[8] No parasites larger than 15 μm were found, perhaps because cytocentrifugation causes some large parasites to rupture. Liver-stage parasites were observed using NFS1 monoclonal antibody against CS protein. Circumsporozoite protein can be detected in liver parasites throughout their development[16, 17] and on the surface of liver-stage parasites,[18] but is more abundant in younger forms.[19] Liver parasites eight days after sporozoite inoculation showed variable staining levels of CS protein (Figure 1), which suggests that they had matured at a different rate, with earlier stages staining more intensely than later ones.

Only approximately one in 10,000 hepatocytes contained a parasite. This small infection percentage (0.009%) is not only found with culture of HHS-102 cells, but was also reported with primary cultures of human liver cells.[7] The probable explanation is that only a small percentage of inoculated sporozoites infect HHS-102 hepatoma cells. The CS protein likely to be involved in liver cell recognition[20] probably determines molecular specificity in the invasion of sporozoites into liver cells.[21] The peptides derived from the conserved regions of CS protein bind to liver cells and HepG2 cells.[22]

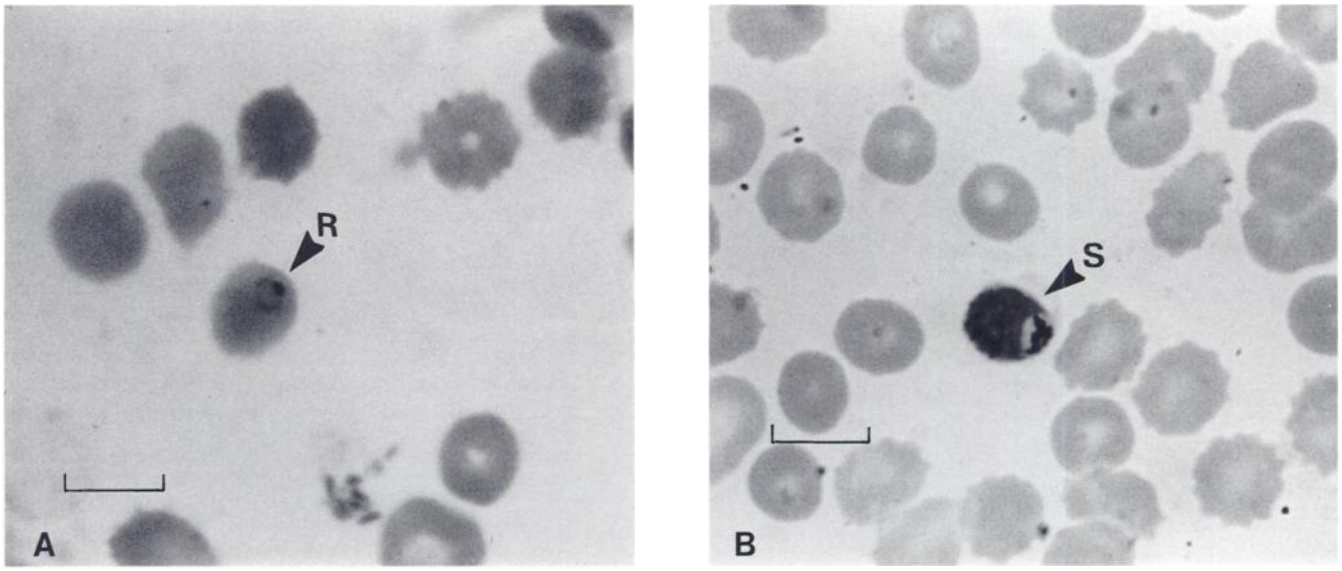

FIGURE 3. Giemsa-stained thin smears of erythrocytes infected by mature liver parasites from infected HHS-102 hepatoma cell cultures. **A,** a ring-form parasite (**R**) found on day 12. **B,** a schizont (**S**) found on day 19. Bars = 10 μm.

Less than 1% of *P. falciparum* sporozoites invade either HepG2 cells[10] or primary human hepatocyte cultures,[7] suggesting that only a few liver cells are capable of recognizing sporozoites. Thus, only a few sporozoites can invade liver cells.

Some large parasites showed partial segmentation, suggesting imminent rupture. Further circumstantial evidence that parasite maturation was completed within HHS-102 cells was provided by the observation of small merozoite-like forms overlying hepatoma monolayers (Figure 2B). This part of the *P. falciparum* life cycle, the liver stage, could not be completed in HepG2 cells.[10] These forms were similar in both size (1–2 μm in diameter) and morphology to those observed in liver schizonts contained within normal human liver cells.[8] Clusters of merozoites can morphologically be mistaken as nonpseudohyphaed yeasts, but yeasts are slightly larger (3 μm in diameter). Fungal contamination was ruled out because these round forms disappeared without the addition of antifungal agents to the medium.

Erythrocyte-hepatoma cell cocultures were performed to determine if these clusters were indeed comprised of viable merozoites. Merozoites produced after as few as eight days following sporozoite exposure invaded cocultured red blood cells. Ring-form trophozoites were seen in Giemsa-stained smears of erythrocytes recovered after a 6-hr exposure to infected hepatoma cells as was previously shown by Mazier and others using primary culture of normal human hepatocytes.[8] The eight-day interval between sporozoite infection and the presence of ring-form parasites within erythrocytes agrees with findings from malaria induced in human volunteers bitten by infected mosquitoes. Patent infection was detected as early as day 8 in one volunteer (Pavanand K and others, unpublished data). The presence of ring forms and schizonts in blood-stage cultures (Figure 3) clearly demonstrated not only that merozoites produced in the hepatoma cell cultures could invade red blood cells in vitro, but also that after invasion the parasites were able to continue growing to maturation. This has previously been described for *P. berghei*.[23]

The observation of hepatoma cells containing malaria parasites of various degrees of maturity, segmented forms, and parasitized erythrocytes confirms that not only has the entire *P. falciparum* hepatic cycle been completed in vitro, but it has produced viable merozoites able to invade erythrocytes. This is the first demonstration that the entire falciparum hepatic cycle from sporozoite through invasion of erythrocytes after hepatocyte rupture can be sustained in vitro. We believe that refinement of culture conditions could increase the yield of blood-stage parasites and permit more ring forms to complete their development to schizonts. This would increase the usefulness of this system for studying the immunology, biology, and chemotherapy of *P. falciparum* liver-stage parasites.

Acknowledgments: We thank Jetsumon Sattabongkot and Nongnuj Maneechai for providing sporozoites, Dr. Yupin Charoenvit for providing the monoclonal anti-*P. falciparum* CS protein antibody NFS1, and Dr. Gary W. Long for valuable advice.

Financial support: This study was supported by the U.S. Army Research and Materiel Command and was performed while C. Karnasuta was a National Research Council Research Associate at the Armed Forces Research Institute of Medical Sciences.

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