Abstract. Our understanding of the biology of malaria parasite liver stages is limited because of the lack of efficient in vitro systems that support the exo-erythrocytic (EE) development of the parasite. We report the development of a new hepatocyte line (HC-04) from normal human liver cells. The HC-04 cells have proliferated in hormone-free medium for more than 200 passages. The cells were hyperdiploid, resembled liver parenchymal cells, and synthesized major liver-specific proteins and enzymes. Using Plasmodium falciparum and P. vivax sporozoites harvested from salivary glands of infected mosquitoes, we showed that HC-04 cells supported the complete EE development of these two most prevalent human malaria parasites. The EE parasites attained full maturation as shown by their infectivity to human erythrocytes. The infection rates of the liver cells were estimated to be 0.066% and 0.041% for P. falciparum and P. vivax, respectively. As the first human hepatocyte line known to support complete EE development of both P. falciparum and P. vivax, HC-04 will provide an experimental model that can be used for studying the biology of liver stage malaria parasites.

INTRODUCTION

To alleviate the increasing burden of malaria, there is an urgent need for vaccines and new drugs. Development of successful drugs and vaccines requires a better understanding of parasite biology. Insights on the molecular and cellular biology of the parasite from the complete Plasmodium falciparum genome sequence have led to the identification of new targets for drugs and vaccines. Since hepatocyte infection is the first natural step in the establishment of a malaria infection, vaccines for pre-erythrocytic stages aiming to prevent blood stage infection are important components of multivalent vaccines that target different developmental stages of the parasite. However, advance in this direction is impeded by the scarcity of our knowledge about the antigenic repertoire of liver stage parasites.

Since the landmark discovery of the exo-erythrocytic (EE) development of mammalian malaria parasites in the liver by Shortt and Garnham, most of our knowledge about the EE development of mammalian parasites has been derived from studies using animal malaria models. Sporozoites, after being injected by an infected anopheline mosquito, are rapidly cleared from the blood circulation by the liver, where they invade hepatocytes and undergo EE schizogony. The homing of sporozoites to the liver is mediated by the interactions between sporozoite proteins and proteoglycans on the surface of Kupffer cells, which the sporozoites use to traverse the sinusoidal cell layer. Once inside the parasitophorous vacuole of an infected hepatocyte, the sporozoite transforms into a trophozoite, which later undergoes schizogony to produce numerous merozoites. In the case of P. vivax and P. ovale, the development of certain trophozoites is arrested at earlier stages to form hypnozoites, which are responsible for relapses of the disease. Currently, the mechanism of hypnozoite development is not understood and no specific molecular markers are available for detecting hypnozoites. The importance of liver stages in initiating erythrocyte infection has spurred enormous interests in vaccine development, which aims to prevent infection of the liver cells or inhibit the maturation of liver stages. Especially since the early exciting finding of induction of sterile immunity in humans by immunization with irradiated sporozoites, numerous studies have been carried out to examine host immune responses to the liver stage parasites. However, the structures, functions, and expression of liver stage antigens still remain poorly understood.

In vitro systems have been developed to study the EE stages of malaria parasites. The rodent malaria parasite P. berghei has been used as a model for EE stages. The P. berghei sporozoites are relatively indiscriminate of the cells for invasion, and the entire EE cycle can be completed in a human lung cell line, a human hepatoma cell line, human HeLa cells, and mouse hepatocyte lines. As for human and primate malaria parasites, the sporozoites readily infect human and primate primary hepatocyte cultures with complete EE development. However, primary hepatocytes do not grow continuously in culture and need to be isolated from liver. Therefore, model systems have been developed using human hepatocyte lines. The human hepatoma cell line, HepG2-A16, is able to support the complete EE cycle of P. berghei and several strains of P. vivax. However, P. falciparum could not achieve complete maturation in this cell line. Calvo-Calles and others reported development of the P. falciparum liver stages beyond the uninucleate stage in the human hepatoma cell lines hUH-1, but maturation of the liver stages were not observed. Subsequently, a Thai human hepatoma cell line, HHS-102, has been established, which supports the complete EE cycle of P. falciparum. However, infectivity of this cell line by P. falciparum sporozoites was low (0.009%), and it is unknown whether P.
vivax could grow in this cell line. To improve sporozoite infectivity, we have established a hepatocyte line from normal human liver tissue and demonstrated that it supports the complete EE cycle for the human malaria parasites *P. falciparum* and *P. vivax*.

**MATERIALS AND METHODS**

**Establishment of a human hepatocyte line.** All research involving human subjects was reviewed and approved by the Ethics Committee of the Thai Ministry of Public Health and the Human Subjects Research Review Board of the United States Army. To establish a human hepatocyte line, a small piece of normal liver tissue (2.5 x 2.5 x 2.0 cm) free of hepatitis A, B, and E was obtained from Ramathibodi Hospital (Bangkok, Thailand). The tissue was obtained under the therapeutic operation of a hepatoma patient and the remaining normal tissue surrounding the lesion was selected for cell isolation and initiation of the primary culture. The tissue was washed twice in cold phosphate-buffered saline (PBS) supplemented with an antibiotic cocktail (100 μg/mL of streptomycin, 100 units/mL of penicillin, and 50 μg/mL of amphotericin B). Perfusion was performed with a HEPES buffer (0.01 M HEPES, pH 7.3, 0.13 M NaCl, 0.002 M KCl, 0.73 mM NaHCO3, 0.01 M glucose, 0.5 mM EGTA) to remove red blood cells. Isolation of hepatocytes was performed essentially as described by Kono and others.3 Briefly, the liver tissue was cut into small pieces and incubated at 37°C for 30 minutes in HEPES buffer containing 0.05% collagenase, 0.34% CaCl2, and 0.025% trypsin. Hepatocytes were released by mincing the digested liver tissue with a syringe plunger against a 70-μm nylon cell strainer (Falcon™; Becton Dickin-son, Franklin Lakes, NJ). The hepatocytes were washed three times with minimal essential medium (MEM) (Invitro-gen, Carlsbad, CA) supplemented with 2% fetal bovine serum (FBS) and resuspended in complete culture medium (CCM, equal volumes of MEM and F12 media with 10% FBS) supplemented with 10 μg/mL of insulin, 10−6 M thyrotropin releasing factor, 10 μg/mL of epidermal growth factor, 10−7 M glucagon, 10−6 M hydrocortisone, 0.5 μg/mL of linoleic acid, 10 mM nicotinamide, 2 mM L-glutamine, 10−7 M sodium selenite, 1 × MEM essential amino acids (Invitrogen), 200 μM pyruvic acid, 100 μg/mL of streptomycin, and 100 units/mL of penicillin. Hepatocytes (5 × 105−1 × 106) in 2 mL of CCM were seeded in a 25-cm2 flask and incubated at 37°C in an atmosphere of 5% CO2. Culture medium was changed daily until a colony of proliferating cells was observed. For subculture, 90% confluent flasks were washed with PBS, digested with 0.25% trypsin at 37°C for 5 minutes, and washed with CCM. Cells (5 × 108) in 2 mL were seeded in a 25-cm2 flask and the medium was changed every other day. Viability of the cells was checked by staining with 0.1% trypan blue.

**Characterization of the hepatocyte line.** The morphology of the hepatocytes was routinely examined under a phase-contrast microscope. Metaphase chromosome spreads were prepared from the cultured cells by a modified cytogenetic protocol for short-term culture of bone marrow or peripheral blood.34 G-banded slides were obtained with Giemsa stain. The karyotypic descriptions were according to the International System for Cytogenetic Nomenclature.35 To determine the growth kinetics of the cell line, liver cells were plated at a density of 5 × 103 cells/25-cm2 flask at passages 5, 8 and 9. Cells were harvested at 12, 24, 36, 48, 72, 96, 120, 144, and 168 hours after plating and counted with a hemacytometer.

**Protein analysis.** Albumin, α-fetoprotein, and transferrin synthesis and secretion were detected by Western blot.36 Briefly, equal amounts of proteins in spent culture medium from different passages were separated by electrophoresis in 10% sodium dodecyl sulfate–polyacrylamide gels and blotted onto nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in PBS, pH 7.4. Polyclonal rabbit antibodies against specific proteins (Sigma, St. Louis, MO) were used at a dilution of 1:2,000 and secondary horseradish peroxidase–conjugated goat anti-rabbit IgG antibodies were used at a dilution of 1:5,000. Blots were developed using the TMB substrate (One component; Kirkegaard and Perry Laboratories, Gaithersburg, MD).

**Liver enzyme activities.** The activity of glucose-6-phosphatase in the hepatocytes homogenate, an enzyme characteristic of hepatocytes, was determined by measuring phosphate release after incubation with glucose-6-phosphate. Cytochrome P-450 1A-related enzyme activities in the microsomal fractions of the hepatocyte homogenates were measured using the ethoxyresorufin O-dealkylation (EROD) and methoxyresorufin O-dealkylation (MROD) reactions with the Cytochrome P450 1A Fluorescence Detection kit (Sigma). Enzyme activities were compared between this hepatocyte line and HepG2 cell line with or without pretreatment for 24 hours with 10 μM dibenz[a,h] anthracene as the inducing agent. Protein concentrations in the cell homogenates were determined using the Bradford method37 with bovine serum albumin as the standard. Enzyme activities were expressed as picomoles of substrates converted per minute per milligram of protein.

**Sporozoite preparation.** *Anopheles dirus* A (Bangkok colony) from the Entomology Department, Armed Forces Research Institute of Medical Sciences were fed on blood containing gametocytes of *P. falciparum* or *P. vivax* (CSP type VK210) by membrane feeding.38 Sporozoites were aseptically dissected from the salivary glands of infected mosquitoes 16–21 days after blood feeding. The sporozoites were pooled in harvesting medium (MEM and F12 media supplemented with 200 μg/mL of streptomycin and 200 units/mL of penicillin).

**Infection of HC-04 cells with Plasmodium sporozoites and culture of EE parasites.** All sporozoite infection experiments were preformed with HC-04 cells after 100 passages in continuous culture. The HC-04 cell line was transferred from a 25-cm2 flask into 96-well plates at a density of 5 × 105 cells/well two days before sporozoite inoculation. For each experiment, wells of hepatocytes in triplicates were used unless otherwise stated. Approximately 2–5 × 104 sporozoites were introduced into each well and incubated with hepatocytes for four hours. The cells were then rinsed with medium to remove uninvaded sporozoites. Subsequently, the medium was changed daily. Liver cells were harvested on day 4, 7, 10, 14, 21, and 28 after sporozoite inoculation by digestion with 0.25% trypsin at 37°C for five minutes. The cells were washed with CCM before being spread as a monolayer on microscopic slides using cytopsin slides (ThermoShandon, Pittsburgh, PA). Slides were fixed for 10 minutes with either methanol for Giemsa staining or cold acetone for indirect immunofluorescent assays (IFAs). For IFA staining of *P. fal-
*P. falciparum*, monoclonal antibodies against *P. falciparum* heat shock protein (Hsp) 70 (kindly provided by Dr. F. Zavala, Johns Hopkins University, Baltimore, MD) and a hyperimmune human serum against *P. falciparum* asexual blood stages were used at dilutions of 1:2 and 1:1, respectively. For IFA of *P. vivax*, monoclonal antibody to circumsporozoite protein (CSP) type VK210 (Kirkegaard and Perry Laboratories) was used at a concentration of 10 μg/mL. Secondary antibodies were fluorescein isothiocyanate–conjugated antimouse or anti-human IgG (Dako, Glostrup, Denmark). The slides were counter-stained with 0.01% Evans blue. To estimate the infection rates, the number of infected hepatocytes and the total numbers of hepatocytes on each slide were counted on day 4.

**Co-culture with erythrocytes and merozoite invasion.** Infection of red blood cells with liver merozoites produced by sporozoite-infected HC-04 cells was performed to demonstrate that the *in vitro* hepatic schizonts could achieve full maturity in HC-04 cells. Freshly prepared human O+ red blood cells were added to each well of sporozoite-infected HC-04 cells at a 2% hematocrit on day 3, 6, 9, 13, 20, and 27 after sporozoite infection. The red blood cells used for *P. vivax* infection were prepared from cord blood containing high proportions of reticulocytes (7–12%). At 24–48 hours after co-incubation, the red blood cells were harvested and thin blood smears were prepared, stained with Giemsa, and examined for asexual stage parasites.

**RESULTS**

**Establishment of a human hepatocyte line.** Approximately 1 × 10⁶ liver cells were used for the initiation of the primary culture. Cell proliferation was observed after culturing the cells for one month, and first subculture was performed on day 47. As of December 2002, this hepatocyte line had been continuously cultured through 195 passages. For each passage, 2–25 vials of cells were cryopreserved. This cell line, hitherto referred to as HC-04, has since been maintained in our laboratory for infection by malaria sporozoites.

**Characterization of HC-04.** HC-04 cells morphologically resembled liver parenchymal cells (Figure 1A). Most of the cells have a polygonal shape with granular cytoplasm under the phase-contrast microscope. The growth curve measured at passages 5, 8, and 9 indicated that at a cell density of 5 × 10⁵ cells/25-cm² culture flask, the cells had a doubling time of approximately 24 hours. Cytogenetic analysis of metaphase chromosomes for 20 cells from passage number 8 and 95 showed that HC-04 cells had a hyperdiploid karyotype with chromosome counts ranging from 47 to 50 (Figure 1B). Consistent abnormality was observed for chromosomes 1, 6, and 15.

**Protein secretion and enzyme activities.** We have evaluated the proper functioning of the subcultured hepatocytes by detection of synthesis and secretion of liver-specific proteins (Figure 2 and Table 1). Using cells from different passages, we detected α-fetoprotein, albumin, and transferrin in the supernatant of the culture by immunoblotting (Figure 2). Albumin and transferrin levels were relatively consistent in different passages, but the levels of α-fetoprotein decreased through serial passages and the protein was undetectable in the culture supernatant by passage 167. Glucose-6-phosphatase activity of HC-04 cells was estimated to be 3.13 nmol/minute/mg of protein, which is similar to that reported for other liver cell lines (Table 1). Normally, cultured hepatocytes have lower glucose-6-phosphatase activity than in normal liver tissues.

Cytochrome P450 enzyme activities can be lost during liver cell differentiation *in vitro*. To determine whether the HC-04 cell line retained these enzyme activities, we determined the EROD and MROD activities associated with the cytochrome P450 1A family. The HC-04 and HepG2 cells have similar levels of basal EROD and MROD activities (Table 1). Upon induction by the polycyclic aromatic hydrocarbon compound dibenz[a,h] anthracene, the HC-04 cell line had almost doubled its EROD and MROD activities compared with no remarkable changes in the HepG2 cell line.

**Development of *P. vivax* liver parasites in HC-04.** Giemsa staining detected developing trophozoites of various sizes at four days (Figure 3A and B). Large trophozoites and schizonts were seen on day 10 (Figure 3D and E). Most of the parasites developed with close proximity to the nuclei, which appeared unaltered in infected cells. No clear transparent space was observed surrounding the developing parasites. The early EE stages were further verified by IFA with antibody to CSP, which detected trophozoites of various sizes at 3, 5, and 7 days after sporozoite inoculation (Figure 3G–I). The fluorescence was prominent on the EE parasites and was also dispersed in the cytoplasm of the infected hepatocytes. As the parasites further developed in the cells, the intensity of the fluorescent staining diminished. The development of liver stage of *P. vivax* was generally asynchronous. Small parasites...
These small EE stages were also confirmed by IFA with antibody to CSP. They resembled hypnozoites that were observed in vivo and in a liver cell line after infection with *P. vivax* sporozoites.

We conducted three experiments to determine the total number of liver stage parasites for each well that initially had $5 \times 10^4$ hepatocytes. An average of 20.4 liver stage parasites was observed per well on day 7. By day 28, most of the cells observed were presumably hypnozoites and its number was approximately 55% of the total number of parasites observed on day 7, which is similar to the proportions of hypnozoites for other tropical strains.

We observed the infection of the hepatocyte line by *P. falciparum* sporozoites. The *P. falciparum* was observed per well on day 7. By day 28, most of the cells observed were presumably hypnozoites and its number was approximately 55% of the total number of parasites observed on day 7, which is similar to the proportions of hypnozoites for other tropical strains. Hepatocyte growth curves were obtained for cell passage 16, 32, and 162 under the experimental conditions in 96-well plates, which were similar. Before becoming confluent (approximately day 2), cells had doubling time within 37 hours. Afterwards, growth of the hepatocyte culture slowed down considerably and cell population doubled on approximately day 10. On day 28, the number of cells/per well approached approximately one million. No significant cell detachment and loss (less than 1.2%) were observed before day 14. On days 21 and 28, the cell detachment rates ranged from 1% to 9.5% among experiments.

### Development of *P. falciparum* liver parasites in HC-04

Using similar methods, we observed the infection of the hepatocyte line by *P. falciparum* sporozoites. The *P. falciparum* was observed per well on day 7. By day 28, most of the cells observed were presumably hypnozoites and its number was approximately 55% of the total number of parasites observed on day 7, which is similar to the proportions of hypnozoites for other tropical strains. Hepatocyte growth curves were obtained for cell passage 16, 32, and 162 under the experimental conditions in 96-well plates, which were similar. Before becoming confluent (approximately day 2), cells had doubling time within 37 hours. Afterwards, growth of the hepatocyte culture slowed down considerably and cell population doubled on approximately day 10. On day 28, the number of cells/per well approached approximately one million. No significant cell detachment and loss (less than 1.2%) were observed before day 14. On days 21 and 28, the cell detachment rates ranged from 1% to 9.5% among experiments.

### Summary of characteristics of HC-04 and HepG2 cells

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HC-04</th>
<th>HepG2</th>
</tr>
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<tbody>
<tr>
<td>Albumin</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>α-Fetoprotein</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Transferrin</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Glucose-6-phosphatase (nmol/min/mg of protein)</td>
<td>3.13</td>
<td>6.38</td>
</tr>
<tr>
<td>Cytochrome P-450 1A family enzyme activities EROD (pmol/min/mg of protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>32.04</td>
<td>33.11</td>
</tr>
<tr>
<td>Induced</td>
<td>72.53</td>
<td>27.56</td>
</tr>
<tr>
<td>MROD (pmol/min/mg of protein)</td>
<td>46.04</td>
<td>36.76</td>
</tr>
<tr>
<td>Basal</td>
<td>72.05</td>
<td>30.41</td>
</tr>
</tbody>
</table>

*ND = not determined. EROD = ethoxyresorufin O-dealkylation; MROD = methoxyresorufin O-dealkylation.*
EE stages developed much more synchronously than those of \textit{P. vivax}. Large trophozoites were seen at four days and late trophozoites and schizonts were seen at 7–14 days after infection (Figure 4A–F). The EE parasites were unambiguously detected by IFA using a monoclonal antibody to Hsp70 on day 5 (Figure 4B) and a hyperimmune human serum to erythrocytic \textit{P. falciparum} stages on day 14 (Figure 4F). In six experiments, with each well initially containing $5 \times 10^4$ hepatocytes, an average of 19.9 liver stage parasites were observed on day 4.

\textbf{Maturation of \textit{P. falciparum} and \textit{P. vivax} liver stages.} To demonstrate that hepatic schizonts could achieve full maturity in HC-04 cells and release infective merozoites, red blood cells were added to the culture on different days after sporozoite inoculation. For both parasite species, erythrocyte infection was not observed on day 4. For \textit{P. falciparum}, ring-stage parasites were first observed on day 7 (Figure 5A), concomitant with the observation of liver stage schizonts. Infection of erythrocytes was observed from 7 days through 28 days after sporozoite inoculation, suggesting continuous maturation of liver schizonts during this period. Maturation of liver schizonts and infection of erythrocytes were most frequently observed during days 7–14. For \textit{P. vivax}, the schizonts matured more slowly, with erythrocyte infection first being observed only on day 10 (Figure 5B). Afterward, erythrocyte infection was continually observed through the end of the experiments (on day 28). Thus, the hepatocyte line established from normal human liver could support complete development of liver stages of the two human malaria parasites.

\textbf{DISCUSSION}

Sporozoites of malaria parasites, after being inoculated into the bloodstream of mammalian hosts, infect hepatocytes to initiate the EE cycle. Transformation of sporozoites into an early EE form can be completed in a cell-free medium, but further development of the parasite is arrested and may require components from the host cells.\textsuperscript{41} The complete EE cycle of human malaria parasites has been achieved in human primary liver cells\textsuperscript{21–23,27–30} and human hepatoma cell lines\textsuperscript{19,31,32} However, different human hepatoma cell lines varied greatly in their ability to support EE development of \textit{P. vivax} and \textit{P. falciparum}. Those that supported EE development of \textit{P. falciparum} were either inconsistent or with low infection rates, and their ability to support the development of other human malaria parasites was not characterized.\textsuperscript{19,32} In this regard, HC-04 is the only hepatocyte line established from normal human liver cells that could support complete EE development of the two most prevalent human malaria parasites.

The infection rates of the \textit{in vitro} cultured cells by malaria sporozoites and the transformation rates of the sporozoites into EE stages are generally low. The average transformation rate for \textit{P. falciparum} sporozoites to liver parasites estimated on the basis of six experiments was 0.13\%. This is much lower than the reported transformation rates of rodent malaria
sporozoites in human HepG2 cells (3–8%) or in cell-free medium (−13%). Using a standard procedure, we have obtained an average infection rate of 0.066% of HC-04 hepatocytes by P. falciparum sporozoites (based on day 4 observations from six experiments), which is much higher than those obtained with primary cultures of human hepatocytes and human hematoma cells.32,42 In comparison, the transformation rates for P. vivax was even lower and the results from three experiments showed an average of 20 of 50,000 sporozoites transformed into liver EE stages. However, this may reflect biologic distinctions of P. vivax parasites because similar rates were reported for both tropical and temperate P. vivax strains.28,43 Furthermore, the generally low infection rates by sporozoites may reflect the heterogeneous nature of the cultured hepatocyte populations in susceptibility to sporozoite infection because these infection experiments were carried out using non-cloned cells. In addition, a slight reduction in α-fetoprotein secretion was observed at later passages, suggesting a change in the cultured hepatocytes through serial passages. Cloning of the HC-04 cells is underway to compare differences among cultured cells and to improve sporozoite infection rates.

The EE development of both human malaria parasites in HC-04 cells was asynchronous with parasite maturation still being seen on day 28, as shown by the infection of co-incubated erythrocytes. Although it has not been directly tested in vivo, this asynchrony has normally been observed during in vitro culture of malaria EE stages.32 In our experiments, merozoite release from P. falciparum liver schizonts was observed as early as on day 7. This earliest time of EE parasite maturation in vitro is consistent with the in vivo 6.5-day minimum EE cycle for P. falciparum,44 suggesting that P. falciparum development in HC-04 cells resembled its in vivo development. In comparison, maturation of P. vivax liver stages was first observed on day 10, which is consistent with the development of other tropical P. vivax strains in HepG2 cells.28 In addition, P. vivax developed more asynchronously than P. falciparum, and early trophozoite stages were still observed when the experiments were terminated on day 28. In addition to this asynchrony, the size of the schizonts from the infected HC-04 cells were much smaller (rarely > 15 μm) than those developed in human primary hepatocyte culture (10–40 μm)22 and those obtained in vivo from human on day 5 (30–50 μm).4 As a result, these schizonts contained much fewer merozoites. Despite these abnormalities, the EE parasites of both species could reach full maturity in HC-04 cells to produce merozoites infective to erythrocytes. Although such abnormalities are often associated with in vitro EE cultures, they may indicate that the culture conditions were not optimal for the parasite growth and multiplication. In our experiments using 96-well plates, we observed slowing down of the hepatocyte growth after the culture became confluent, which may also affect the development of the EE parasites.

One unique feature of P. vivax and P. ovale infection is formation of hypnozoites in the host liver, which are responsible for the relapses of malaria. In chimpanzees inoculated with P. vivax sporozoites, small (5–6 μm) and non-dividing parasites were persistently observed, which were considered as hypnozoites that were developmentally arrested.40 In human HepG2 cells infected with P. vivax sporozoites, similar non-dividing parasites, presumably hypnozoites, were produced. For tropical strains such as the Cheson strain from Papua New Guinea and the ONG strain from Indonesia, the proportions of primary EE schizonts and hypnozoites were approximately equal,28 whereas a temperate zone strain from North Korea with long incubation period for relapses differentiated mostly into hypnozoites after invading HepG2 cells.29 Similarly, Shu and others examined hypnozoite formation in P. vivax strains from temperate climates and found that the hypnozoite rates were positively correlated with the latitudes of the parasite origins.43 In our experiments, early trophozoites of small sizes (<5 μm) were clearly visible by IFA with antibodies to CSP and its proportion (55%) was similar to those of tropical P. vivax strains.28 It will be interesting to determine whether these dormant cells can be reactivated to complete the EE cycle at later times.

The developmental biology of liver stages of malaria parasite life cycle is poorly understood largely because of the lack of suitable in vitro culture systems. In this regard, the development of a hepatocyte line from normal liver cells that supports the complete development of the hepatic stages of the two most prevalent malaria species will facilitate comparative studies of the EE stages of the two parasites. Although the infection rates of the cultured hepatocytes have been improved with the new cell line, efforts are still needed for further improvement to provide sufficient experimental materials for studying EE gene expression and identifying novel liver stage vaccine candidates. In addition, selection of more susceptible clones to sporozoite infection from the hepatocyte line and design of more accurate and high throughput methods for EE parasite detection may provide a convenient system for the screening of potential antimalarial drugs against liver stage parasites, especially hypnozoites.

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