Mouse model for exoerythrocytic stages of *Plasmodium falciparum* malaria parasite

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**ABSTRACT** Research on the exoerythrocytic (EE) stages of human malaria parasites has been hindered because of the lack of an easily available suitable animal model. We report here an approach to produce mature EE-stage *Plasmodium falciparum* parasites by using severe combined immunodeficient (scid) mice with transplanted human hepatocytes. Transplantation of human hepatocytes into scid mice (scid-hu-hep), their subsequent intravenous infection with *P. falciparum* sporozoites, and the development of mature liver-stage merozoites was achieved. Immunofluorescent staining of scid-hu-hep kidney tissue sections demonstrated the presence of circumsporozoite protein (early during infection), merozoite surface antigen 1, and liver schizont antigen 1. The scid-hu-hep model can serve as a source of human liver-stage parasites, decreasing the need for nonhuman primates. Use of this model will facilitate characterization of EE-stage antigens and the assessment of stage-specific chemotherapeutic agents and candidate vaccines.

*Plasmodium falciparum* has a complex life cycle involving intracellular and extracellular stages in both the human host and the mosquito vector. Among the different developmental stages of *P. falciparum*, least is known about the hepatic or exoerythrocytic (EE) phase. This stage is a link between the extracellular sporozoite, inoculated by the mosquito vector, and the blood stage, which is responsible for producing disease. Both sporozoite and blood-stage parasites have been extensively studied because, in part, methods for blood-stage cultivation (1) and sporozoite production have been developed. The study of EE-stage parasites has been severely hampered by lack of a suitable in vitro or in vivo model, but it has been facilitated by development of in vitro culture techniques (2). Although the in vitro model is easily manipulable, it is characterized by a low infection rate (0.02–0.05%) (2, 3) and culture parameters that may not mimic in vivo conditions. While nonhuman primates, and in particular chimpanzees, have provided in vivo experimental models for the human malaria EE stage (3), their use has been difficult because of economic/ethical considerations and a limited supply. Development of a more convenient in vivo model would eliminate some of the difficulties associated with current in vitro and in vivo models.

The characterization of severe combined immunodeficient (scid) mice has provided investigators with a model suitable for the study of many human diseases (4, 5). The scid mouse lacks functional B and T cells; therefore, xenografts of human tissues can be successfully transplanted and maintained without rejection (6, 7). We report here the successful transplantation of human hepatocytes into scid mice (designated scid-hu-hep), their subsequent i.v. infection with *P. falciparum* (NF54) sporozoites, and development of mature liver-stage merozoites.

**MATERIALS AND METHODS**

Mice. C.B-17/1cr scid/scid mice, originally obtained from Leonard Schultz (The Jackson Laboratories, Bar Harbor, ME), were bred at the University of Maryland at Baltimore animal facility and housed in microisolator cages. The animals were cared for and used strictly in accordance with the Public Health Service guidelines (8).

*Human Hepatocytes.* Hepatocytes were prepared by collagenase digestion of human liver obtained from surgical resections or immediate autopsies (9). Briefly, liver tissue was minced, washed with phosphate-buffered saline/EGTA repeatedly, and digested with collagenase (0.05%). Isolated hepatocytes were washed in Williams' medium containing 10% fetal calf serum, and their viability was determined by trypan blue exclusion. In some cases, cells were maintained overnight in Hanks' balanced salt solution containing a high glucose concentration without Ca2+ or Mg2+ at 4°C (10) or were cultured on collagen-coated 35-mm Petri dishes before transplantation. Cultured hepatocytes were released from the collagen substratum by trypsin/collagenase treatment (11). Both cold (4°C) stored cells and cultured cells were centrifuged over a Nycodenz cushion to remove dead cells (12). The viability of the cells in both groups, as determined by trypan blue exclusion, was ~90% after Nycodenz separation.

*Hepatocyte Transplantation.* For surgical implantation of human hepatocytes, mice were anesthetized by i.p. injection with Nembutal (sodium pentobarbital; 0.05 mg per g of body wt), supplemented by Metofane (methoxyflurane) inhalation. A small laparotomy was performed on each scid mouse. The right kidney was isolated using a Demarres chalazion forceps and 2 × 10^6 isolated hepatocytes in 50 μl were injected under the kidney capsule with a 25-gauge needle. The laparotomy was then closed with 5-0 chromic (muscle layer) and 5-0 silk (skin) suture. All surgery and animal handling procedures were done using a strict aseptic technique in a laminar flow hood.

*Parasites.* Viable *P. falciparum* sporozoites were collected from *Anopheles stephensi* salivary glands by trituration in a tissue grinder with Williams' medium containing 10% heat-inactivated fetal calf serum. The mosquitoes were infected by membrane feeding with blood containing *P. falciparum* (iso- late NF54) gametocytes.

*Hepatocyte Infection and Detection.* One week posttransplantation, and up to 4 months thereafter, mice were injected via the tail vein with 5 × 10^4 salivary gland sporozoites. Infected mice were sacrificed 24, 48, and 72 hr and 7, 8, and 11 days postinfection. *P. falciparum* merozoites, as evidenced by immunofluorescence microscopy, were seen in infected mice.

Abbreviations: EE, exoerythrocytic; mAb, monoclonal antibody; CS, circumsporozoite; IFA, indirect immunofluorescent assay; MSA-1, merozoite surface antigen 1; LSA-1, liver schizont antigen 1.†To whom reprint requests should be addressed.

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9 days after injection with sporozoites. Kidney, liver, and spleen tissue were removed and processed for routine histology or cryosectioning. Frozen sections were examined with a panel of monoclonal and polyclonal antibodies specific for sporozoite or merozoite antigens. Direct fluorescent assay was undertaken with a monoclonal antibody (mAb 2A10, directly conjugated to fluorescein isothiocyanate), specific for the *P. falciparum* circumsporozoite (CS) protein (13). Indirect immunofluorescent assays (IFAs) were done with mAbs 7H10, 7B2, and 3B10 (specific for merozoite surface antigen 1; MSA-1) (14), polyclonal antiserum against liver schizont antigen 1 (LSA-1) (15), or human anti-liver schizonts antibody (16). Several other mAbs (8B7, 3D5, and 5E3) (17, 18) with specificities for blood-stage antigens were also used in IFAs against the collected tissues. Giemsa staining for the presence of infected hepatocytes was also done on some sections without prior immunofluorescent staining. In addition, sections were stained for the presence of human albumin with an anti-human albumin mAb and the avidin–biotin complex method (19).

**Passive mAb Transfer.** To demonstrate one practical application of the *scid* hu-hep mouse, a passive transfer experiment using anti-CS mAb was undertaken. Six weeks after human hepatocyte transplant, five mice received 2.5 mg of anti-*P. falciparum* CS mAb (Pf 49 1B2.2) (13) and four mice received 2.5 mg of anti-*P. vivax* CS mAb (PvNVS 3) (20) i.p.

Six hours later, each mouse was injected in the tail vein with $1.8 \times 10^4$ viable *P. falciparum* salivary gland sporozoites. Mice were sacrificed from both antibody-treated groups 24 hr and 7 days postinfection. The tissues were cryosectioned and stained by IFA and with Giemsa as detailed above to visualize infected hepatocytes. The number of EE schizonts in a minimum of five nonserial tissue sections (separated from each other by 100 μm) from each animal were counted. The mean number of schizonts per section was determined and significance was assessed by paired *t* test.

**RESULTS AND DISCUSSION**

Transplantation of human hepatocytes under the kidney capsule produced a well-defined mass of cells (when viewed microscopically) that was easily sampled and very distinct, as compared to the kidney tissue, when examined using routine histological stains (Fig. 1). In general, the hepatocytes distributed circumferentially under the capsule at the site of injection. In addition, immunohistochemical staining of the transplanted hepatocytes, with an anti-human albumin mAb, demonstrated albumin production in the hepatocytes for at least 3 weeks posttransplantation (data not shown), suggesting normal hepatocyte function (21). Frozen sections of kidneys taken from mice 24 hr after infection that were probed by direct fluorescent assay with mAb 2A10 displayed...
many cells containing fluorescing parasites (Fig. 2). Infected hepatocytes were limited to the area under the capsule containing the transplanted cells, with no parasites being found in the cortex or medulla of the mouse kidney. Other tissues—e.g., nontransplanted kidney, spleen, and liver—were negative when stained for parasites using mAb 2A10, suggesting efficient recognition and invasion of the implanted human hepatocytes by the inoculated sporozoites. Sections probed with an unrelated mAb (anti-Plasmodium berghei CS) or scid hu-hep kidney sections from mice that received the contents of uninfected mosquito salivary glands served as negative controls and did not contain any positively fluorescing cells (data not shown).

The intensity of the staining with mAb 2A10 decreased after 48 hr and was much less intense 72 hr after infection. This finding is consistent with earlier reports about the fate of CS protein in older EE schizonts (16, 22). The mAb used was specific for CS protein and therefore was not a suitable probe for the later stages of the liver schizonts. Several other reagents [mAbs 7H10, 7B2, and 3B10 (generous gifts from Jeffrey Lyon) and polyclonal antiserum against LSA-1 (gift of Michael Hollingsdale)] were used to stain frozen sections collected 7, 8, and 9 days postinfection. mAbs 7H10, 7B2, and 3B10 react with an epitope on a polymorphic 195-kDa glycoprotein (MSA-1) found on both liver and blood-stage merozoites (23). Polyclonal antisera to LSA-1 reacts very strongly with EE-stage parasites. An IFA using mAb 7H10 demonstrated the presence of MSA-1 on the parasites within the infected hepatocytes at 7, 8 (Fig. 3 Upper), and 9 days postinfection, although there was no apparent difference in the intensity of the staining among these time points. Similar results were obtained when the antisera to LSA-1 was used for IFA (Fig. 3 Lower). As demonstrated by Szarfman et al. (23), liver-stage schizonts contain some antigens that cross-react with antibodies made against blood-stage parasites, but not all blood-stage antigens are expressed during EE development. A summary of the fluorescent antibody staining results is presented in Table 1.

Subsequent Giemsa staining of antibody-probed frozen sections, and others not stained for IFA, revealed schizonts containing well-developed mature merozoites in the infected hepatocytes (Fig. 4).

Separate experiments were performed to determine whether overnight storage or short-term culture affected posttransplant hepatocyte viability or subsequent infection with sporozoites. No differences were noted in the survival of grafts or in the numbers of parasitized cells when hepatocytes were stored or cultured. In addition, scid hu-hep mice have been found to be permissive to infection with P. falciparum for up to 4 months after hepatocyte transplantation. These results indicate that hepatocytes from one source can be transplanted over a period of several days and then maintained in the mouse, optimizing the use of human liver
The results of the passive transfer experiment demonstrated the capacity of mAb Pf 1B2.2 to decrease the number of infected hepatocytes as compared to the anti-Plasmodium vivax CS control. The number of EE schizonts in the anti-P. falciparum CS mAb-treated mice was significantly (P < 0.001) lower than the controls treated with the anti-P. vivax CS mAb (Fig. 5). In addition, preliminary morphometric analysis of the sections suggests a significant difference in the size of the schizonts in the two groups, with the anti-P. falciparum group having the smaller EE-stage schizonts (data not shown).

This in vivo model for the EE stage of the human malaria parasite is being further evaluated with regard to quantitation of sporozoite rate of infection and how it relates to current chimpanzee in vivo and human in vitro models. Nonetheless, it does demonstrate that infection of transplanted human hepatocytes by P. falciparum sporozoites and their subsequent schizogony can be achieved in a small animal model. Based on our studies to date, the scid hu-hep model is comparable to the results recently published (3), using the chimpanzee model, regarding the level of infection, maturity of EE forms, and production of infectious merozoites (data not shown). Although the lack of quantitative data makes it difficult to assess overall infection rates, the number of schizonts in P. falciparum-infected scid hu-hep kidney was as it becomes available. To date, of the 47 scid hu-hep mice that have survived the transplant surgery (>95%), successful infections with P. falciparum (as determined by immunological or Giemsa staining) have been achieved in all attempts (n = 34).

Table 1. Indirect immunofluorescent reactivity of antibodies to liver schizonts

<table>
<thead>
<tr>
<th>Antibodies against liver schizonts and asexual blood stages</th>
<th>kDa</th>
<th>7 day EE stages</th>
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<tbody>
<tr>
<td>7B2 (IgG1), 7H10 (IgG2), 3B10 (IgM)*</td>
<td>195</td>
<td>+++</td>
</tr>
<tr>
<td>8B7 (IgG1)†</td>
<td>300</td>
<td>+</td>
</tr>
<tr>
<td>3D5 (IgG1) specific for ABRA‡</td>
<td>101</td>
<td>+</td>
</tr>
<tr>
<td>5E3 (IgG2a)‡</td>
<td>113</td>
<td>+</td>
</tr>
<tr>
<td>Human anti-liver schizonts§</td>
<td></td>
<td></td>
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<tr>
<td>Rabbit anti-LSA-1§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2A10 (IgG)§</td>
<td></td>
<td>±</td>
</tr>
<tr>
<td>PvNVS 3 (IgG3)**</td>
<td></td>
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</tbody>
</table>

* Lyon et al. (14).
† Howard et al. (17).
‡ Chulay et al. (18).
§ Druilhe et al. (16).
∥ Hollingdale et al. (15).
¶ Wirtz et al. (13).
** Charoenvit et al. (20).

Fig. 4. Giemsa-stained frozen section from scid hu-hep kidney 8 days after infection with P. falciparum sporozoites. Several infected hepatocytes are visible (arrows) in each low-power field with numerous merozoites filling the cytoplasm of the infected cells. (Upper, ×170; Lower, ×850.)
Nonhuman primate models currently being used have been valuable in the study of the malaria parasite, but these animals are increasingly rare and expensive to use in biomedical research (27). The scid hu-hap has the potential for decreasing the need for nonhuman primates for the assessment of anti-EE-stage chemotherapeutic agents and candidate vaccines and making the use of human liver feasible. The scid hu-hap mouse represents a model with a well-defined inbred genetic background. Groups of mice with transplanted hepatocytes would be genetically identical and as such would not be prone to the confounding effects of genetic variability. Furthermore, the model could be used to evaluate the potential for reagents developed for malaria prevention or control, for immunologic and biochemical characterization of liver-stage antigens, and as an in vitro model to study the effect of activated immune cells and soluble factors on liver-stage parasites.

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