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Research on the exoerythrocytic (EE) stages of human malaria parasites has been hindered because of the lackof an easily available suitable animalmode. 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 macrozoites 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 malaria 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.

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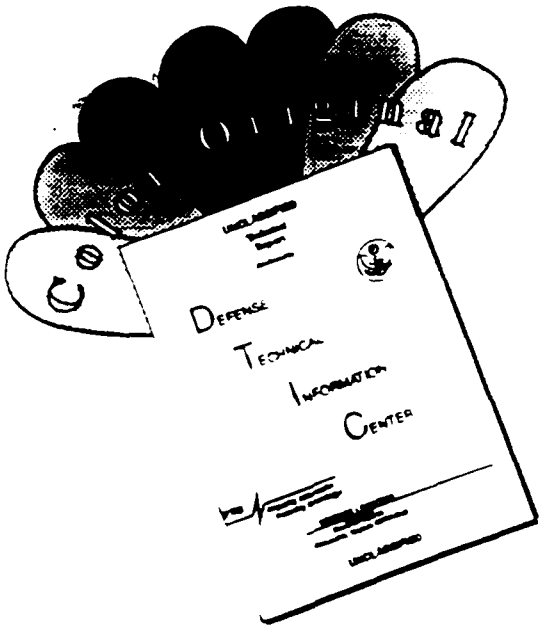

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Mouse model for exoerythrocytic stages of *Plasmodium falciparum* malaria parasite

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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 malaria 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/Icr *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 Ca^{2+} or Mg^{2+} 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* (isolate 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

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Abbreviations: EE, exoerythrocytic; mAb, monoclonal antibody; CS, circumsporozoite; IFA, indirect immunofluorescent assay; MSA-1, merozoite surface antigen 1; LSA-1, liver schizont antigen 1.

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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 1.8×10^4 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

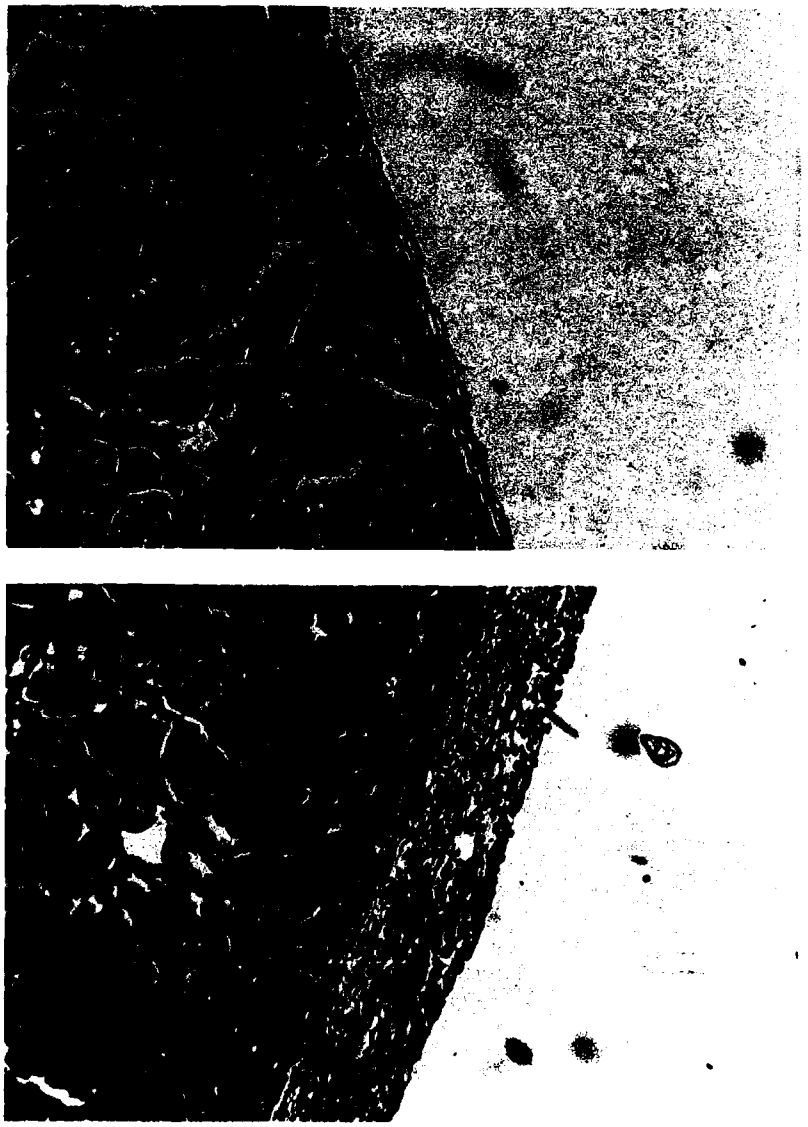

FIG. 1. Hematoxylin and eosin stain of normal *scid* kidney (*Upper*) and *scid* hu-hep kidney (*Lower*). Kidneys from nontransplanted and transplanted mice were removed and fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The capsule area is substantially thickened in the *scid* hu-hep mouse kidney 1 week after transplantation of 2×10^6 viable human hepatocytes. Capillary buds (some containing erythrocytes) are evident throughout the transplant cell mass and little necrosis is present. (×170.)

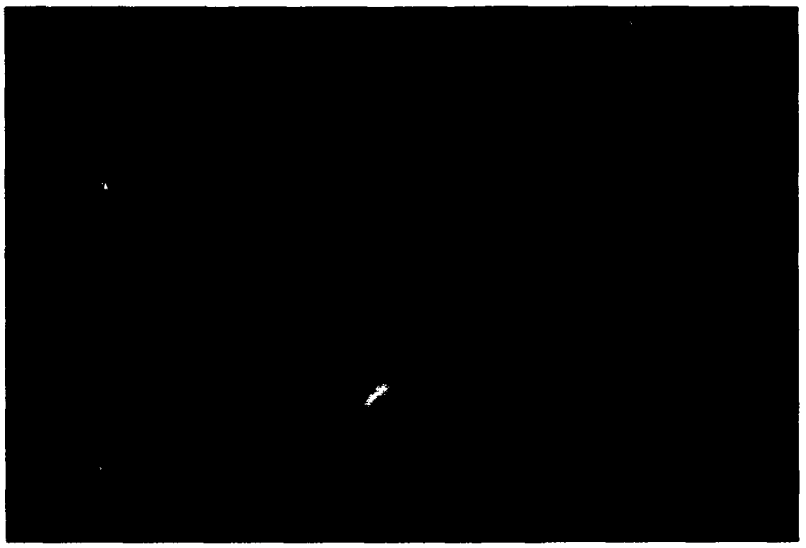

FIG. 2. Fluorescence photomicrograph of a kidney frozen section from a *scid* hu-hep mouse infected with *P. falciparum* sporozoites. Mice received 5×10^4 sporozoites i.v. 7 days after transfer of 2×10^6 viable human hepatocytes under the right kidney capsule. Twenty-four hours postinfection, the mouse was sacrificed and the kidney was frozen for cryosectioning. Sections were fixed in absolute methanol for 2 min, probed with fluorescein isothiocyanate-labeled mAb 2A10, and counterstained with Evans blue. In each section, numerous cells (arrowheads) containing brightly fluorescing parasites are observed. Positively stained parasites were confined to the area under the kidney capsule containing the transplanted hepatocytes.

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 bergei* 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

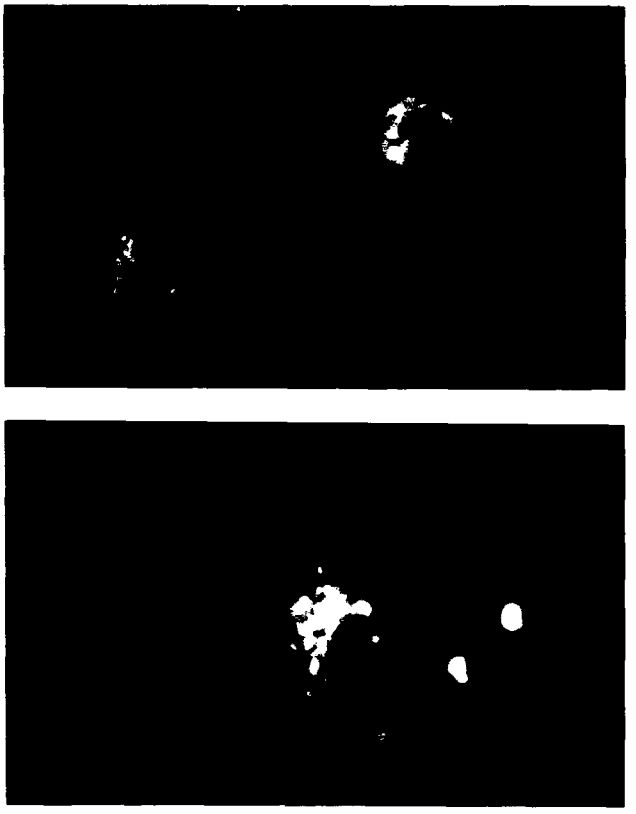

FIG. 3. Indirect immunofluorescent micrograph of *scid* hu-hep kidney, 8 days after infection with *P. falciparum*, stained with mAb 7H10 or anti-LSA-1 antiserum. Mice received 5×10^4 sporozoites i.v. 4 months after transfer of 2×10^6 viable human hepatocytes under the right kidney. (*Upper*) Eight days after infection, the right kidney was processed as described in Fig. 2 for frozen sections, probed first with mAb 7H10 and then with goat anti-mouse fluorescein conjugated antiserum. Positively staining merozoites are clearly visible filling both of these infected hepatocytes. (*Lower*) A section stained with anti-LSA-1 rabbit antiserum and goat anti-rabbit fluorescein-conjugated antibodies demonstrated positive fluorescence for late-stage EE merozoites. (×260.)

Table 1. Indirect immunofluorescent reactivity of antibodies to liver schizonts

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

*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).

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).

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

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.)

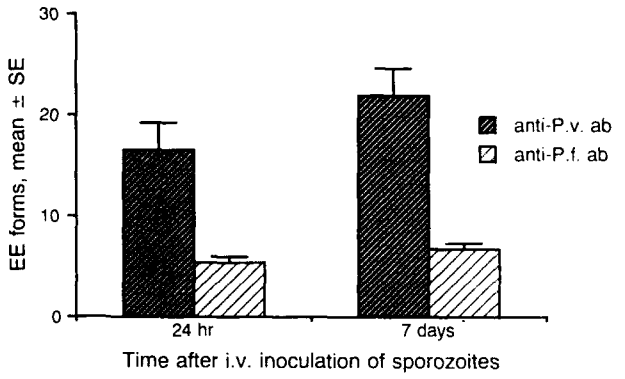

FIG. 5. Passive transfer of anti-CS mAb into *scid* hu-hep mice and subsequent infection with *P. falciparum* sporozoites. Six weeks after hepatocyte transplants, *scid* hu-hep mice received 2.5 mg of anti-*P. falciparum* CS mAb (Pf 49 1B2.2) ($n = 5$) or anti-*P. vivax* CS mAb (PvNVS 3) ($n = 4$) i.p. Six hours later, each mouse received 1.8×10^4 salivary gland sporozoites i.v. via the tail vein. At 24 hr postinfection, three *P. falciparum* and two *P. vivax* mAb-treated mice were sacrificed and their transplanted kidneys were prepared for cyrosectioning. Similarly, 7 days postinfection the remaining two *P. falciparum* and two *P. vivax* mAb transferred mice were sacrificed. The number of EE-stage schizonts in five Giemsa-stained frozen sections (5 μm thick) from each animal, separated by 100 μm, was quantified. The mean number of schizonts (±SE) per section is represented in the histogram. Paired t test determined that the differences between the anti-*P. falciparum* (anti-P.f. ab) and anti-*P. vivax* (anti-P.v. ab) mAb-treated mice, at both 24 hr and 7 days, were significant at $P < 0.001$.

routinely ≥20 per section (Fig. 5, day 7). The size of the mature EE schizonts varies between 20 and 67 μm in the *scid* mouse model at 7 days, larger than *in vitro* infected human hepatocytes (2) but consistent with other *in vivo* systems (3, 24–26).

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-hep 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-hep 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 vivo* model to study the effect of activated immune cells and soluble factors on liver-stage parasites.

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