Brief Definitive Report

Maintenance of the Human Malarial Parasite, *Plasmodium falciparum*, in *scid* Mice and Transmission of Gametocytes to Mosquitoes

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Summary

The study of human malaria has been hampered by the lack of small animal models for the human-infecting malarial parasites. To approach this problem, the erythrocytic stages of the human malarial parasite *Plasmodium falciparum* were adapted to in vitro growth in the presence of ascites fluid from mice homozygous for the severe-combined immunodeficiency (*scid*) mutation. Human red blood cells (hRBCs) infected with these adapted parasites were then injected i.p. into nonobese diabetic *scid/scid* (NOD/LtSz-*scid*) mice. With daily supplemental intraperitoneal boosts of uninfected hRBCs, parasites were detected in the peripheral circulation of these mice for an average of 7 d after injection. Splenectomy of NOD/LtSz-*scid* mice increased both the level and duration of parasitemia in the periphery, and it also promoted the circulation of mature sexual stage parasites (gametocytes). When Anopheline mosquitoes were allowed to feed on the splenectomized mice, the gametocytes were ingested by the mosquitoes and developed into oocysts in the mosquito midguts. To our knowledge, these results are the first demonstration of human malarial parasite propagation in mice and transmission of these parasites to the invertebrate vector.

Human malaria remains a serious health threat in many tropical regions of the world despite decades of research aimed at combating this prevalent infection. The spread of drug-resistant parasites has made prevention and control increasingly difficult, and no successful vaccine has been developed yet, partly because parasite–human host interactions, particularly the factors that mediate acquired resistance, are poorly understood. Various life cycle stages found in the vertebrate host and in the invertebrate vector are targets for the many malaria vaccines that are currently being developed (reviewed in reference 1). Ultimately, vaccines must specifically target antigenically distinct human malarial parasites, particularly *Plasmodium falciparum*, the most deadly of the four human Plasmodial species, and must be tested in human subjects. Likewise, newly developed drugs must undergo extensive animal and human trials before being released for general antimalarial use.

While animal models of malaria have been indispensable in expanding our knowledge of the genus *Plasmodium*, there are limits to which observations in these systems can be extrapolated to human malaria. The development of a murine model for falciparum malaria will greatly enhance our ability to study the pathophysiology of this infection and will facilitate the pretesting of candidate drugs and vaccines, thus reducing the need for costly and time-consuming human trials.

Materials and Methods

Parasites. *Plasmodium falciparum* isolate NF54 and 3D7, a clone of NF54, were maintained according to a modification of the method described by Trager and Jensen (2): standard culture medium contains 90% RPMI 1640 supplemented with 25 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (Ultrol grade; Calbiochem-Novabiochem Corp., La Jolla, CA), 0.37 mM hypoxanthine (Sigma Chemical Co., St. Louis, MO), 28.6 mM sodium bicarbonate, and 10% human serum or plasma. Over several passages, human plasma was gradually replaced with *scid* mouse serum, plasma, or ascites fluid. In general, the replacement was done in 1–1.5% increments, and parasites were acclimated to each increase of mouse fluid for a minimum of three subcultures. The parasites used in these studies were maintained in medium containing 5% human plasma and 5% ascites fluid (5+5 medium).

Ascites. C.B-17-*scid/scid* (hereafter-*scid*) and BALB/c-*scid* mice were primed with an intraperitoneal injection of pristane or IFA 7 d before inoculation with SP-2/0 myeloma cells. Ascites fluid was tapped three times 14–20 d after injection of cells and was collected in 5 mM EDTA.

Mice. Several strains of *scid* mice were used. C.B-17-*scid*, HRS/J (*hr/hr*)-scid, and w^{41}/w^{41}-*scid* mice were derived from stocks at The Jackson Laboratory (Bar Harbor, ME). Male NOD/LtSz-*scid* mice were generated at The Jackson Laboratory by L. D. Shultz and were used at 6–12 wk of age. They were housed in microisolator cages and were provided with sterilized food and water at all times.

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Water supplemented with 12 mg/ml Sulfamethoxazole and 2.4 mg/ml Trimethoprim (Gensia Laboratories, Ltd., Irvine, CA) was provided 3 d/wk.

The strains of mice named above were screened for suitability for this study by a single injection of 2 ml i.p. of washed human red blood cells (hRBCs) at 60% hematocrit (hct). The appearance of these cells in the peripheral circulation was monitored by examination of venous blood (see below) at 24-h intervals.

Parasite Injection. Cultures of parasites maintained in 5+5 medium were grown to a parasitemia of 3–5%. Fresh hRBCs were added to bring the parasitemia to 1%. Cells were washed twice in medium lacking ascites/plasma. 1.0 ml of hRBCs at 50% hct was injected i.p. into NOD/LtSz-*scid* mice on day 0. Mice were boosted daily with i.p. injections of the same volume of fresh uninfected hRBCs.

Monitoring hRBC Level and Parasitemia in Peripheral Circulation. Heparinized blood was taken daily by lateral tail vein puncture; the hRBC level in circulation was determined by staining with an FITC-conjugated, blood group H antigen–specific lectin from *Tetragonolobus purpureas* (Sigma Chemical Co.). The lectin was dissolved at 1 mg/ml in PBS and used at 6.7–33 μg/ml. 1 μl of cells was suspended in 150 μl of the diluted lectin (in PBS) and incubated at room temperature in the dark for 30 min. After two washes with PBS, 1,000 cells were counted by UV-illuminated fluorescence microscopy. At least 10,000 total RBCs per sample were counted on Giemsa-stained (Harleco Giemsa blood stain; EM Industries, Inc., Gibbstown, NJ) thin smears to determine total parasitemia. hRBC parasitemia was calculated using the following formula: hRBC parasitemia = total parasitemia/fraction of total cells that are hRBCs.

Mosquito Feedings. Male NOD/LtSz-*scid* mice infected with parasitized hRBCs 16–21 d previously were anesthetized by an injection of 1 mg i.p. of sodium pentobarbital in saline. *Anopheles stephensi* and *Anopheles freeborni*, starved for 3–4 h, were allowed to feed on the mice through a nylon mesh for 20–30 min. Blood-fed mosquitoes were maintained in an insectory (26° C, 70–80% relative humidity) and were dissected 7–10 d later. Midguts were examined microscopically to score for oocysts.

Reinvasion. Mice were injected with type O^+ parasitized hRBCs as described above and were boosted i.p. daily with uninfected type A^+ hRBCs. A control animal was boosted with type O^+ hRBCs. All mice were killed on day 4, and cells were recovered from their peritoneal cavities. The vital dye hydroethidine (Polysciences, Inc., Warrington, PA) was used to detect viable parasites (3). Briefly, hydroethidine was dissolved at 10 mg/ml in DMSO (Sigma Chemical Co.) and diluted 1:200 in PBS. 1 μl of cells were mixed with 50 μl of this solution and incubated at 37°C in the dark for 20 min. After two washes with PBS, cells were stained (as described above) using an FITC-conjugated, blood group A antigen–specific lectin from *Helix aspersa* (Sigma Chemical Co.). Parasitized type A^+ hRBCs were identified by viewing under UV-illuminated fluorescence microscopy using the appropriate filters.

Results and Discussion

A primary requirement for the growth of blood stage malarial parasites is RBCs from a specific host species. Thus, in the development of a mouse model for *P. falciparum* infection, the mouse strain that is used must be able to maintain hRBCs for an extended period of time. Normal immunocompetent mice cannot serve as suitable hosts, since they have naturally occurring cross-reactive anti-hRBC antibodies that will cause immediate complement-mediated lysis of introduced hRBCs. On the other hand, *scid* mice completely lack antibodies and thus do not present this obstacle.

To introduce hRBCs into the circulation gradually rather than instantaneously, we chose the i.p. over the i.v. route for hRBC administration. We have observed that hRBCs injected i.p. into intact C.B-17-*scid* mice appear rapidly in peripheral circulation, presumably transported via lymphatic drainage (4, 5). However, once these cells enter the vascular space, they are cleared within 24 h (6). There are several possible explanations for this phenomenon. hRBCs have been shown to be coated nonspecifically with normal human serum immunoglobulins (7–9), and macrophages, as part of the reticuloendothelial system (RES), remove such opsonized particles through Fc receptor–mediated endocytosis. In addition, it is possible that NK cells, which are intact in C.B-17-*scid* mice (10), might mediate their elimination. Finally, the mean corpuscular volume of normal adult hRBCs is 80–100 μm^3 (11), whereas that of normal adult murine RBCs is 41–51 μm^3 (12). Thus, in mice, hRBCs may be damaged during passage through undersized capillary beds and subsequently cleared by the RES.

To investigate which of these factors account for hRBC clearance in immunodeficient mice, several genetic stocks of mice homozygous for the *scid* mutation were administered a single i.p. dose of washed hRBCs. Since we had already determined that intact C.B-17-*scid* mice are unable to support hRBCs in the peripheral circulation, splenectomized C.B-17-*scid* mice were used to determine the role of the spleen. Hairless HRS/J (*hr/hr*)-*scid* mice, which have a presumptive macrophage defect (13), were included to determine the magnitude of hRBC clearance caused by the RES. To address the issue of volume difference between mouse RBCs and hRBCs, w^{41}/w^{41} mice, which have a macrocytic anemia (14) and thus are naturally tolerant of larger cells in circulation, were examined for their ability to allow circulation of hRBCs. Finally, NOD/Lt mice, carriers of a combination of defects that might contribute to the persistence of hRBCs, were also tested. An FITC-conjugated lectin specific for the human blood group H antigen was used to differentiate between mouse and human RBCs obtained daily from venous circulation. hRBCs could not be detected in most of the mice tested past the 3rd d after injection. NOD/LtSz-*scid* mice, however, supported a high level of circulating hRBCs and maintained these cells for 5–6 d (Fig. 1). The latter strain manifests at least four defects that might contribute to this phenomenon. The first is the result of a mutation in the gene that encodes the high affinity Fc receptor for immunoglobulin G, FcγRI, which might reduce Fc receptor–mediated endocytosis in macrophages (15). These mice also exhibit abnormally low NK cell activity (16) and have a mild macrocytic anemia (17). Finally, as a result of a mutation in the gene that encodes the fifth component of complement (C5), these mice are deficient in hemolytic activity (17, 18). Based on these data, we concluded that NOD/LtSz-*scid* mice may be appropriate for maintaining *P. falciparum*–infected hRBCs.

In our initial attempts to condition *P. falciparum* for injection into mice, we partially replaced the human plasma in

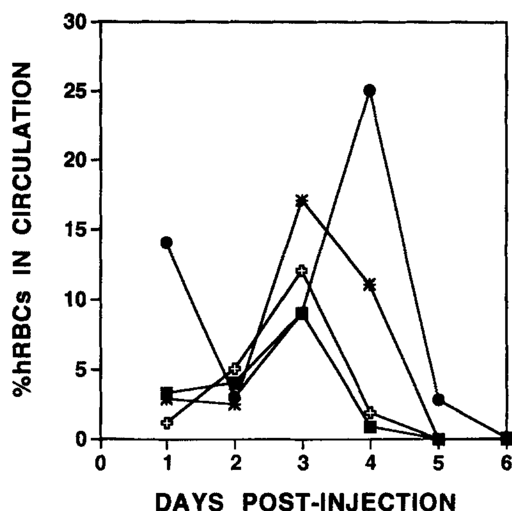
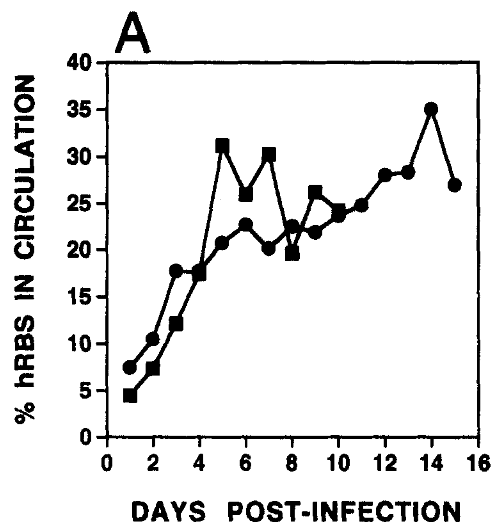


Figure 1. Appearance of hRBCs in the peripheral circulation of NOD/LtSz-*scid* mice after a single i.p. injection.



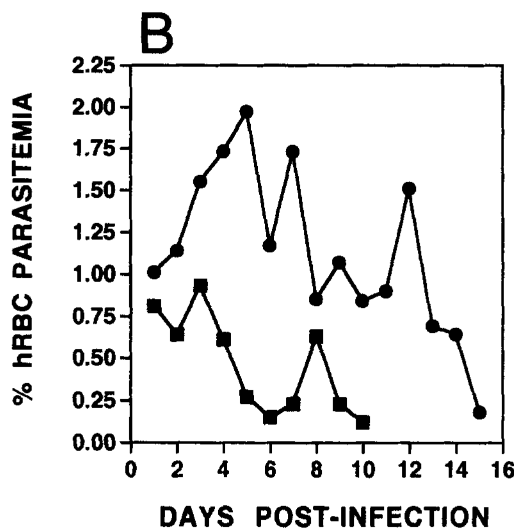


Figure 2. Comparison of hRBCs in circulation and parasitemia between intact and splenectomized NOD/LtSz-*scid* mice after i.p. injection of parasitized hRBCs. (*A*) Geometric means of circulating hRBC levels in intact (*squares*) and splenectomized (*circles*) mice. (*B*) Geometric means of hRBC parasitemias in both groups. Note the difference in lengths of time that intact and splenectomized mice supported parasites in circulation. For intact mice, n = 7 (days 1–5); n = 5 (days 6 and 7); n = 1 (days 8–10). Intact mice were removed from study when parasitemia fell below 0.1%. For splenectomized mice, n = 6 (days 1–6); n = 5 (days 7–10); n = 4 (days 11 and 13); n = 1 (days 12, 14, and 15). Splenectomized mice were removed from study when parasitemia fell below 0.2% (n = 3) or for use in gametocyte transmission experiments (n = 3, day 14) (see Table 1). For clarity, we have not shown the data for each individual mouse. Data are derived from three independent experiments.

in vitro culture medium with *scid* mouse serum or plasma. We found that parasites were rendered nonviable at a concentration of mouse serum or plasma ≥5% of the total medium volume. This observation indicated to us that the murine environment is potentially inhospitable for parasites, and it supported our notion that adaptation of parasites before introduction into the mice would be necessary. Since we intended to inject the parasites i.p., we also tested the ability of ascites fluid from *scid* mice to support growth in vitro. In contrast to mouse serum or plasma, murine ascites was well tolerated up to 7 or 8% of the total culture volume. Thus, ascites-adapted parasites were used in all of the experiments described below.

Infection of intact male NOD/LtSz-*scid* mice was initiated with a single i.p. injection of 1.0 ml hRBCs at 1% parasitemia. The mice were boosted daily with i.p. injections of the same volume of fresh, uninfected hRBCs because as much as 90% of the injected volume is transported out of the peritoneal cavity into the vascular space within 24 h. Cells infected with all asexual parasite forms (rings, trophozoites, and, to a lesser extent, schizonts) were detected in the peripheral circulation up to 10 d after injection (Fig. 2 *B*, *squares*).

Although splenectomy did not result in increased persistence of hRBCs in the circulation of C.B-17-*scid* mice, it did have a profound effect in NOD/LtSz-*scid* mice that had been injected with parasite-infected cells. While not significantly altering the ratio of human/mouse RBCs in circulation (Fig. 2 *A*), splenectomy resulted in markedly increased levels of parasitized hRBCs in circulation and a longer time period during which these cells could be detected (Fig. 2 *B*, *circles*). There was considerable variability in levels of parasitemia between individual mice in each group, and in a few cases, intact mice revealed values within the range observed for the splenectomized group. Nevertheless, the data in Fig. 2 represent a clear trend toward improved support of parasitized hRBCs in splenectomized versus intact NOD/LtSz-*scid* mice.

Parasites isolated from the peripheral circulation of both intact and splenectomized NOD/LtSz-*scid* mice were identical to those found in in vitro cultures (Fig. 3, *A*–*C*). Occasional parasites appeared unhealthy (Fig. 3 *D*), perhaps damaged during injection or by inflammatory factors in the peritoneal cavity. For the most part, however, parasites that were recovered from both the peritoneal cavity and peripheral circulation of infected mice were viable, displaying normal growth kinetics when reintroduced into culture.

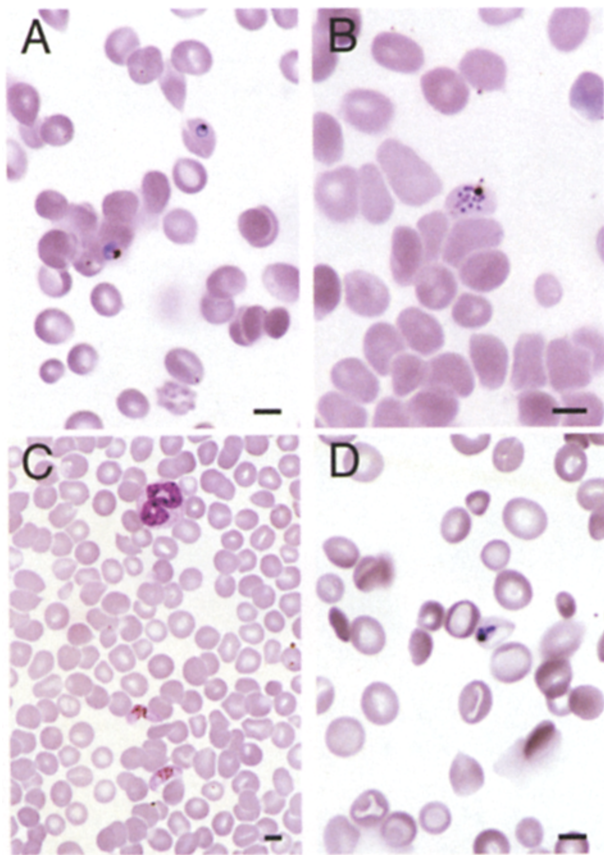


Figure 3. Light micrographs of various parasite stages isolated from the peripheral circulation of *P. falciparum*-infected NOD/LtSz-*scid* mice. (*A*) Early trophozoites, (*B*) a schizont, (*C*) mature gametocytes and a murine neutrophil, and (*D*) an unhealthy schizont with few segmenters. Bar, 5 μm.

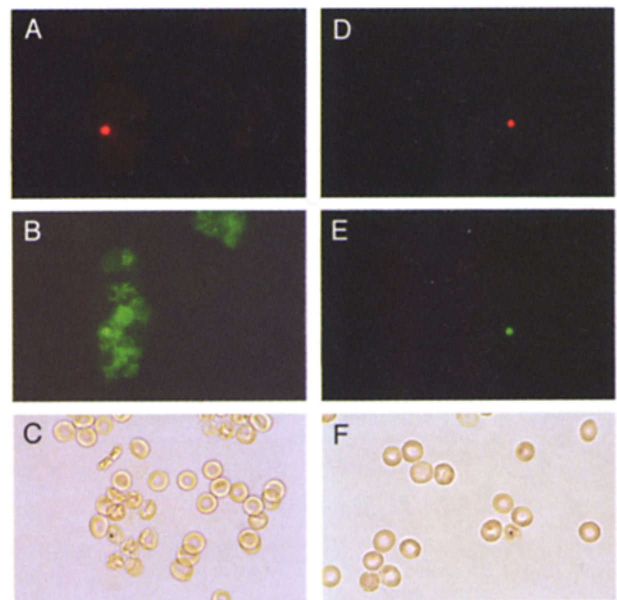


Figure 4. Representative micrographs of parasitized hRBCs recovered from the peritoneal cavities of NOD/LtSz-*scid* mice that were injected with *P. falciparum*-infected type O hRBCs. (*A–C*) hRBCs derived from a mouse boosted with type A hRBCs. (*D–F*) hRBCs derived from a control animal that received only type O hRBCs. (*A* and *D*) Hydroethidine staining of parasite RNA and DNA. (*B* and *E*) Blood group A-specific, FITC-conjugated lectin staining. Note that because of the wide emission spectrum of ethidium, the parasites can also be seen using the filters for FITC emission (*A* vs *B*) and (*D* vs *E*). (*C* and *F*) Light microscopy of same fields shown in *A* and *B* and *D* and *E*, respectively. Note that type A (FITC-labeled) but not type O (unlabeled) cells are agglutinated by lectin. The hemozoin crystal in both cases is visible (100×).

To formally prove that reinvasion of fresh uninfected hRBCs occurs in the mice, infection was initiated with human blood group O (type O) erythrocytes, and it was followed with i.p. boosts of human blood group A (type A) cells. A blood group antigen A–specific lectin was used to distinguish type O hRBCs from type A hRBCs, and parasites were stained with the vital dye hydroethidine. Examination of cells recovered from the peritoneal cavities of NOD/LtSz-*scid* mice 4 d after initiation of infection with parasitized type O hRBCs revealed parasites in type A cells (Fig. 4 *A–C*).

In addition to acting as suitable reservoirs for asexual stage parasites, splenectomized mice supported the maturation and circulation of gametocytes. The relative proportions of sexual to asexual forms (Fig. 5) and the total numbers of gametocytes (see below) increased over time, and by ∼16 d after injection, gametocytes were the predominant parasite forms in circulation. Fully mature male and female gametocytes were generally detected beginning ∼13 d after injection and were morphologically indistinguishable from those seen in in vitro cultures (Fig. 3 *C*).

Because the inoculating parasites contained low levels of gametocytes (0–3%), it could be argued that the increase in gametocytemia over time (Fig. 5) represents preferential survival of sexual versus asexual parasites. However, additional

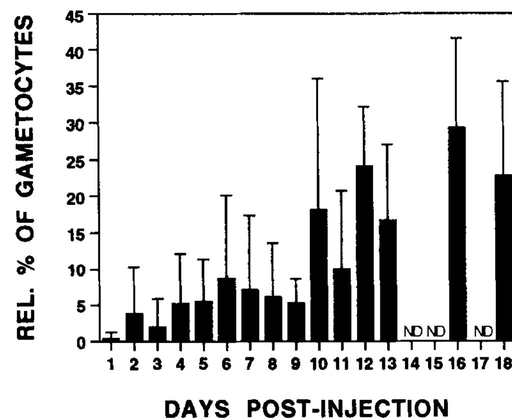


Figure 5. Fraction of sexual parasite forms as a percentage of all parasites found in circulation of splenectomized NOD/LtSz-*scid* mice after injection of infected hRBCs. Experiments performed with *P. falciparum* clone 3D7. n = 12 (days 1–5); n = 11 (day 6); n = 10 (days 7 and 8); n = 8 (days 9 and 10); n = 7 (day 11); n = 3 (day 12); n = 6 (day 13); and n = 4 (days 16 and 18). Data represent means ± SD of four separate experiments. Paired t test analysis shows that the value for day 16 is significantly higher than those for days 1–4 and 7 (P <0.025) and day 9 (P <0.03). Likewise, the value for day 12 is significantly higher than those for day 1 (P <0.04) and days 3, 7, and 8 (P <0.02).

Table 1. *Transmission of Gametocytes from Mice to Mosquitoes via Blood Feeding*

Mouse	Days P.I.§	Oocysts/midgut‖	Positive/total
849*	16	1,0,0,0,0,0,0,0,1,0,0,0,1,0,0,1,0,0,0	4:19
850*	16	1,3,0,0,0,2,0,0,0,0,0,0,1,1	5:14
851*	16	1,0,1,0,0,0,0,0,0,0,2,0,0,0,0,0	3:16
			Total = 12:49
859*	17	0,0,0,0,0,0,0,0,1,1,0,0,0,0,0,0	2:16
860*	17	0,0,0,0,0,1,0,0,0,0,0	1:11
			Total = 3:27
892‡	16	2,0,0,0,0,1,0,0,0,0,2,0,1,0,0,0,3,0	5:18
893‡	16	1,0,0,0,1,1,1,1,0,0,0,1,2,0	8:15
892‡	17	3,0,2,0,1,1,1,0,0,0,0,1,4,2,0,1,1,0,4,0	11:20
893‡	17	0,0,1,0,0,1,0,0,0,0,0	2:11
892‡	21	4,0,0,0,0,1,3,0,2,1,2,0,0,4,0,2,0,0,0,0,0,1,1,0,0,1,1,3	13:28
893‡	21	1,0,0,0,0,2,1,1,0,0,0,0,0,0,2,0,0,0,1,0,0,0,0,0,0,1,0,0,0	7:29
			Total = 46:121

Infectiousness of circulating gametocytes from splenectomized NOD/LtSz-*scid* mice to *A. stephensi* and *A. freeborni*. Mosquitoes, starved for 3–4 h, were allowed to feed through a nylon mesh on anesthetized mice that had been infected with parasitized hRBCs (*clone 3D7 or isolate ‡NF54) 16–21 d previously. Blood fed mosquitoes were dissected 7–10 d later, and their midguts were examined microscopically to score for oocysts. Data represent three independent experiments.

§ Days post-injection.

‖ *A. freeborni* was used with mice 859 and 860; *A. stephensi* was used with all others.

observations suggest that this increase resulted from in situ generation, and not from preferential persistence, of sexual forms. First, the appearance of gametocytes varied from day to day, i.e., a mouse with many gametocytes (at various maturational stages) on one day did not necessarily display the same amount or the same stages on the next day. Second, by our calculations, the absolute number of gametocytes in mouse circulation late in infection was as much as 20-fold greater than the input (inoculum) number.

Finally, in humans, the gametocyte generation time is 10–12 d (19), after which mature parasites have a half-life of 2.5 d in circulation (20); thus, sexual parasites present in the inocula would have matured and begun to disappear no later than on days 14–15, before the date on which we observed peak gametocytemia (Fig. 5, day 16) and performed mosquito blood feeds (see below, and Table 1, d 16–21).

To determine whether the gametocytes found in NOD/LtSz-*scid* mice were infectious, *A. stephensi* and *A. freeborni* mosquitoes were allowed to feed on mice that had been infected at least 16 d previously. In three independent experiments, oocysts were detected in the mosquito midguts 7–10 d after feeding, demonstrating transmission (and infectiousness) of *P. falciparum* from NOD/LtSz-*scid* mice to Anopheline mosquitoes (Table 1). A strain (NF54) known to produce high numbers of gametocytes in vitro was used in one experiment. Importantly, in our culture conditions, these parasites did not generate levels of gametocytes that were statistically significantly different from those of clone 3D7. In addition, the generation of gametocytemia in NF54-infected NOD/LtSz-*scid* mice was similar to that seen with 3D7-infected mice (Fig. 5). Nonetheless, mosquito blood feeds on the former mice resulted in markedly improved transmission (Table 1). It is noteworthy that these transmission rates are comparable with those reported in human infections (21).

In conclusion, we have successfully maintained both the sexual and asexual stages of *P. falciparum* in NOD/LtSz-*scid* mice for extended periods of time and have shown that the sexual parasites are transmittable to mosquitoes. The usefulness of this model in the study of human malaria should be far reaching. With improved levels of asexual parasites, it should be possible to test antimalarial drugs. Perhaps most significantly, NOD/LtSz-*scid* mice are exceptionally suited to reconstitution with human immune cells (22). It should then be possible to reconstitute these mice, immunize them with candidate antigens, and infect them to study the human immune response to *P. falciparum*.

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