Whole-Body Imaging of Sequestration of 
Plasmodium falciparum in the Rat

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The occlusion of vessels by packed Plasmodium falciparum-infected (iRBC) and uninfected erythrocytes is a characteristic postmortem finding in the microvasculature of patients with severe malaria. Here we have employed immunocompetent Sprague-Dawley rats to establish sequestration in vivo. Human iRBC cultivated in vitro and purified in a single step over a magnet were labeled with 99mTc, injected into the tail vein of the rat, and monitored dynamically for adhesion in the microvasculature using whole-body imaging or imaging of the lungs subsequent to surgical removal. iRBC of different lines and clones sequester avidly in vivo while uninfected erythrocytes did not. Histological examination revealed that a multiadhesive parasite adhered in the larger microvasculature, inducing extensive intravascular changes while CD36- and chondroitin sulfate A-specific parasites predominately sequester in capillaries, inducing no or minor pathology. Removal of the adhesive ligand Plasmodium falciparum erythrocyte membrane protein 1 (PIEMP1), preincubation of the iRBC with sera to PfEMP1 or preincubation with soluble PIEMP1-receptors prior to injection significantly reduced the sequestration. The specificity of iRBC binding to the heterologous murine receptors was confirmed in vitro, using primary rat lung endothelial cells and rat lung cryosections. In offering flow dynamics, nonmanipulated endothelial cells, and an intact immune system, we believe this syngeneic animal model to be an important complement to existing in vitro systems for the screening of vaccines and adjunct therapies aiming at the prevention and treatment of severe malaria.

Cerebral malaria, respiratory distress, and anemia, or combinations thereof, are the major clinical syndromes associated with severe Plasmodium falciparum malaria. These disease states are in part attributable to the blockage of the microvasculature (9) with a reduction of the blood flow and an indiction of inflammatory processes in the surrounding tissues. This is generally accepted to depend on the unique ability of the P. falciparum-infected erythrocyte (iRBC) to sequester away from the peripheral circulation during the intraerythrocytic cycle (23), confirmed by iRBC found adherent to the endothelial lining and to RBC in autopsy material (20, 25, 26, 34). The sequestration is in part attributable to the relatively high rigidity of the iRBC (35), but also to expression of the adhesive ligand Plasmodium falciparum erythrocyte membrane protein 1 (PIEMP1) at the iRBC surface. PIEMP1 interacts with receptors on host cells, mediating adhesion to the endothelial lining of the microvasculature (cytoadhesion) and to uninfected erythrocytes (RBC) and to iRBC (rosetting and autoagglutination, respectively) (1, 8, 19, 37).

While iRBC of patients with uncomplicated malaria predominantly adhere to CD36 at the endothelial cell surface (24), iRBC of children with severe malaria are frequently found to also bind to other endothelial receptors (multiadhesive) and to form rosettes and autoagglutinates (2, 15, 30, 32).

Sequestration of iRBC is commonly accepted to participate in the generation of severe disease, but whether the pathology is a direct consequence of hypoxia or if it is mediated by the release of proinflammatory cytokines remains unclear. Thus, while it is generally agreed that sequestration is a key event in the development of severe P. falciparum malaria, the finer details of the molecular pathogenesis remain unresolved, in part due to the lack of a reasonable animal model.

The models so far developed to study sequestration all depend on surgically adapted or immunodeficient rodents or on delicate and rare primates. Infection of Aotus and Saimiri monkeys from the New World is important as it to some extent mimics the course of natural infections. However, the monkeys are often resistant to reinfection and difficult to acquire (6, 7, 14, 22). The more recent use of SCID mice combined with transplanted human tissue has verified important mechanisms in real-time interaction between iRBC and the endothelium (16). The drawback of this model, apart from being technically complicated, is the lack of a complete immune system, limiting its applicability in vaccine screening. The sequestration of human iRBC in the rat microvasculature has previously been elegantly demonstrated in an ex vivo model (17, 28) but the model requires advanced surgery and does not provide an intact immune system. Although important for our understanding of malaria pathology, none of these models is the tool required for the screening of antisequestration drugs and vaccines.

Here, we present a model employing human RBC and im-
munocompetent Sprague-Dawley rats to study the sequestration in vivo. By injecting radioactively labeled human iRBC into the tail vein and tracing the cells in a gamma camera we show strain- and clone-specific sequestration in the pulmonary circulation, and the adhesion is demonstrated to depend on parasite-derived proteins at the surface of the iRBC. The applicability of the model to drug and vaccine development is shown as sequestration is inhibited by soluble receptors CD36 and heparan sulfate (A. M. Vogt et al., unpublished data), by vaccination of the animals with the adhesive ligand FcEPI (5) or by preincubation of iRBC with sera from immunized animals.

Although undoubtedly artificial we believe this new robust and inexpensive animal model will provide a needed tool for the study of sequestration and for the development of vaccines and drugs aimed at preventing sequestration in the clinical setting.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (B&K, Sweden) were kept either in the animal facility of the Swedish Institute for Infectious Disease Control or in the animal facility of the Microbiology and Tumorbiology Centre (MTC) at Karolinska Institutet and the experiments were conducted at 3 to 6 months of age. All animal experiments were performed with the permission of the Swedish Ethical Committees (permission no. 177/01, 178/01, and 176/03).

Animal immunization. The rats were immunized as described elsewhere (5). In brief the rats were immunized subcutaneously three times (on days 0, 21, and 42) with recombinant Semliki forest virus particles expressing the DBL1a domain of FCR3S1.2 FcEPI. On day 63, they were boosted with Escherichia coli-expressed recombinant FCR3S1.2 DBL1a protein (200 μg/rat) in incomplete Freund's adjuvant and blood was collected 3 weeks later for preparation of sera.

Parasites. The highly rosetting and multiadhesive parasite FCR3S1.2 originated from the clone FCR3S1 generated by the selection of a rosetting iRBC by means of one drop of acridine orange (10 μg/ml) and 2% bovine serum albumin (BSA). When present, rosettes were disrupted with 1640 and resuspended in 5 to 10 ml of phosphate-buffered saline (PBS) with 2% BSA after removing the column from the magnet, spun down at 500 g for 7 min, and resuspended in 10 ml 150 mM NaCl and further diluted 3/20 in 150 mM NaCl, and pH was adjusted to 7.4. The samples were incubated for 5 min in 1 ml of the solution at 37°C, resuspended in 1 ml of 99mTc–150 mM NaCl solution (~1,000 MBq/ml), incubated 20 min at 37°C, and finally washed thrice and resuspended in RPMI 1640.

In vivo sequestration assay. The rats were sedated by a subcutaneous injection of a mixture of Dormicium (Roche, Basel, Switzerland)–Hypnorm (Janssen Pharma Centica, Beers, Belgium)–distilled water (1:1:2) and placed on a heat pad (37°C) located in a triple-headed gamma camera (TRIAD XLT; Triniux Research Lab, Twinsburg, OH). 99mTc-labeled iRBC or RBC in RPMI 1640 (0.3 to 0.5 ml, 2 × 10^10 to 5 × 10^10 cells) were injected into the tail vein, and dynamic whole-body images where acquired in a 256 by 256 matrix during 30 min (30 1-min acquisitions). The sedated animals were euthanized by injecting pentobarbital sodium (60 mg/ml; Apoteksbolaget, Sweden) into the heart. Acquired images were analyzed in HERMES analysis software (Nuclear Diagnostics AB, Stockholm, Sweden) by placing separate regions of interest (ROI) over each lung and over the whole animal. The proportion of injected material retained in the lungs was determined by the count rates of these ROI. In a smaller set of animals the lungs were removed and 1-min images where acquired separately in the gamma camera.

Inhibition of in vivo binding with immune sera and soluble 99mTc-labeled human FCR3S1.2 iRBC (2 × 10^10) were incubated (37°C/65 min) in human sera from nonimmunized rats, FCR3S1.2 iRBC and/or recombinant human FcEPI (50 μg/ml) or recombinant human FcEPI (50 μg/ml), washed twice in RPMI 1640, and then incubated with sera from immunized rats prior to injection. As controls FCR3S1.2 iRBC were incubated accordingly in naïve sera from nonimmunized rats. For blocking with CD36 the same number of FCR3S1.2 iRBC were incubated in 0.5 ml of RPMI 1640 supplemented with 25 μg of human recombinant CD36 (R&D Systems Europe, Abingdon, United Kingdom), washed once in RPMI 1640, and then incubated with sera from rats prior to injection. As controls iRBC were accordingly incubated in RPMI without the addition of CD36. The samples were inspected in a Nikon Optiphot UV microscope (Tokyo, Japan), using a 10× ocular and 40× lens, after addition of one drop of acridine orange (10 μg/ml) to exclude lysis.

Histological analysis of lungs. Sedated rats were injected with 2.2 × 10^10 to 3 × 10^10 99mTc-labeled human iRBC at a parasitemia of ~75%. The lungs were surgically removed 30 min after the injection and placed in 4% paraformaldehyde in PBS, and the count rate was determined in the gamma camera. Ten 4-μm cuts from the central part of the lower left lung lobes, each separated by 200 μm from the previous, were selected for analysis. The sections were stained with hematoxylin-eosin and examined in a Nikon Optiphot (Tokyo, Japan) light microscope.

Isolation and cultivation of RLEC. The isolation of primary endothelial cells was done according to a modified protocol previously published by Miller et al. (21). The lungs were aseptically removed and placed in PBS supplemented with heparin (14 U/ml), penicillin (100 U/ml), streptomycin (0.1 mg/ml) and amphotericin B (0.25 μg/ml). The tissue was minced into 2- to 3-mm pieces and digested with 50 mg collagenase (Type I; Sigma) and 50 U elastase (Type I; Sigma) in 50 ml of Hank’s balanced salt solution (HBSS; Sigma) at 37°C for 30 min. The suspension was passed through 100-μm and 40-μm nets, centrifuged (250 × g, 10 min) into a 10-ml bed of fetal calf serum (FCS), and resuspended in 1% FCS in HBSS. The cells were subsequently incubated with a mouse monoclonal antibody (MAB) to rat CD31/platelet endothelial cell adhesion molecule 1 (PECAM-1, MCA1334G; Serotec Inc.) at 10 μg/ml washed thrice in 1% FCS in HBSS and incubated with 4 × 10^9 goat anti-mouse immunoglobulin G (IgG)-coated Dynabeads (Dynal M-450, Dynal Biotech). Incubations were performed in bidirectional rotation for 30 min at 4°C. Dynabeads with bound endothelial cells were washed five times using a Dynal magnetic particle concentrator (Dynal Biotech) before resuspension in 5 ml of rat lung endothelial cell (RLEC) complete medium (low-glucose DMEM supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 90 μg/ml heparin, and 60 μg/ml endothelial cell growth supplement; all from Sigma) and plated on 60- by 15-mm plates (Primaria plates, BD Falcon). A second round of sorting was carried out when cells were confluent. The cells were further cultivated either on Primaria plates or in 25-cm² cell culture flasks, and subculture was conducted at 70 to 90% confluence. The origin of the cells was confirmed by morphology and by incubation with either a MAB against rat endothelial cell antibody (CDL40; Cederlane) or a mouse MAB to rat CD31/PECAM-1 antibody (BD 558025; BD Bio-
sciences) at 10 μg/ml for 30 min at room temperature (RT), following three washes in 1% FCS in PBS and incubation with a fluorescent secondary antibody (Alexa Fluor goat anti-mouse IgG; Molecular Probes) at 4 μg/ml for 30 min at RT. The surface fluorescence was evaluated by UV microscopy (Nikon Optiphot, Tokyo, Japan).

**Binding of iRBC to primary RLEC.** RLEC were harvested, plated onto coverslips (Thermanox, Nunc, Labassco, Sweden) and grown in RLEC complete medium in 24-well plates (Nunc) for 24 to 48 h. MACS-enriched iRBC were washed thrice in RPMI 1640 and resuspended in binding medium (RPMI 1640, 25 mM HEPES, 1% FBS at a 0.5% hematocrit. The RLEC were washed once with PBS before the addition of 400 μl of iRBC suspension to each well and incubation for 30 min at 37°C. Unbound iRBC were removed by washing the coverslips three times in the binding medium and the cells were fixed for 30 min in 1% glutaraldehyde (Sigma) in PBS at RT before staining with 1% Giemsa for 30 min. The number of iRBC bound per 100 target cells was determined by light microscopy (Nikon Optiphot, Tokyo, Japan). The inhibitory capacity of the anti-rat CD31/PECAM-1 or the anti-human CD36 MAb on the iRBC adhesion was studied by the addition of the reagents to the binding medium at given concentrations. Alternatively, the RLEC were pretreated with heparinase III (Sigma) at 0.2 IU/ml in PBS for 30 min at 37°C and rinsed in PBS before the binding assays.

**Binding of iRBC to cryosections of rat lung in vitro.** Rat lungs were cut into pieces, snap-frozen in Tissue-Tek (Miles) on dry ice, and stored at −70°C. The frozen pieces were cut into 10 μm sections, mounted on three-well glass slides (Novakemi, Sweden), and stored at −20°C. The slides were equilibrated in a humidity chamber for 30 min at 37°C before the binding assays. MACS (150 μl)-enriched iRBC mixed with binding medium to a final hematocrit of 0.2% were added to each well and incubated in a humidity chamber for 30 min at 37°C Unbound iRBC were removed by submerging the slides thrice in RPMI 1640. The sections were fixed 30 min in 1% glutaraldehyde (Sigma) in PBS at RT, stained with 1% Giemsa stain, and examined by light microscopy (Nikon Optiphot, Tokyo, Japan). Four parallel lanes from the top to the bottom and four parallel lanes from the left to the right were counted, and the number of iRBC bound per mm² was calculated. Inhibition of binding was studied by adding antibodies (anti-CD31/PECAM-1, anti-CD36) to the binding medium prior to the addition of iRBC. Alternatively the sections were pretreated with heparinase III or chondroitinase ABC (Sigma), both at 0.2 IU/ml, in a humidity chamber for 30 min at 37°C and washed in PBS before the binding assays. Uninfected human RBC were added to untreated cryosections and processed in the exact same way as controls.

**Data analysis.** The data were stored and formatted in Microsoft Excel (Microsoft Corp.) and statistical analysis was performed in StatView 4.5 (Abacus Concepts, Inc.) using the Mann-Whitney U test.

**RESULTS**

**In vivo sequestration in the lung.** *Plasmodium falciparum*-infected and noninfected human RBC were radioactively labeled and injected into the tail vein of Sprague-Dawley rats in order to develop an in vivo model genuinely reflecting the events occurring in the human microvasculature during *P. falciparum* infection (Fig. 1A and B). Whole-body images were acquired in a gamma camera during 30 min (Fig. 1C and D) and the proportion of the injected material localized in different organs was determined by the count rate in different areas of the acquired images (ROI analysis) (Fig. 1E and F) or by surgical removal of organs and separate measurement of their count rates in the gamma camera. While the vast majority of the injected human RBC, regardless of being infected or not, were nonspecifically absorbed in liver, spleen, and kidneys, significant differences regarding the amount of retained material were noted in the lungs, both between iRBC and RBC and between the different strains and clones of malaria parasites used. In about 50% of the animals some (1 to 5%) of the total activity ended up in the urinary bladder, most likely a consequence of free 99mTc from the sample injected and from catabolism of labeled cells (11).

iRBC of the highly rosetting clone FCR3S1.2 were initially used to investigate their possible sequestration. Neither rosettes nor autoagglutinates were present in the samples injected, as rosettes were disrupted mechanically prior to enrichment. A total of 21 rats injected with 99mTc-labeled human FC3S1.2 iRBC showed accumulation of iRBC in the lungs as
The proportion of the material injected localized in the lungs reached a maximum of ~25 to 30% at the start of the experiment (time zero, not shown) and then decreased during the 30 min (Fig. 2A). The mean proportion of the injected material found in the lungs of the 21 animals during the last 10 min was in average 6.6% (4.6% to 11.9%, standard deviation [SD] of ±1.6%) (Fig. 2C). The mean proportion of injected material located in the lungs during the last 10 min was subsequently calculated for each animal and used for comparing results in and between the groups (Fig. 2A, B, and D, marked area).

In the 18 control animals injected with 99mTc-labeled uninfected human RBC, the proportion of injected material in the lungs was at no time significantly different from that of animals injected with nontreated iRBC, nor was the average proportion of injected material found in the lungs during the last 10 min (6.3% compared to 6.7%, P = 0.3). In contrast, trypsin-treated iRBC demonstrated a lower proportion of injected material in the lungs at all times (Fig. 2A), and a significantly lower average proportion of injected material present in the lungs during the last 10 min was 2.7% (1.9% to 3.8%, SD of ±0.7%), significantly lower as compared to FCR3S1.2 (P < 0.01) (Fig. 2C).

To verify the dependency of the binding on parasite expressed proteins at the cell surface, enriched FCR3S1.2 iRBC were trypsin treated or mock treated before labeling and injection. In the four animals injected with mock-treated iRBC, the proportion of injected material was at no time significantly different from that of animals injected with nontreated iRBC, nor was the average proportion of injected material found in the lungs during the last 10 min (6.3% compared to 6.7%, P = 0.3). In contrast, trypsin-treated iRBC demonstrated a lower proportion of injected material in the lungs at all times (Fig. 2A), and a significantly lower average proportion of injected material present in the lungs at different time points. The sequestration of iRBC of the multiahesive clone FCR3S1.2 is compared to background trapping of uninfected human RBC and the sequestration is reduced as the iRBC are trypsin treated prior to injection (A). iRBC of the non-PfEMP1-expressing clone FCR3S1.6 sequester significantly less as compared to iRBC of FCR3S1.2 (B). Error bars show ±1 standard deviation. (C) Box plot summarizing the different proportions of injected material sequestered in the rats when using iRBC of different parasites, or trypsin-treated iRBC, as compared to uninfected RBC. Numbers are based on noninvasive ROI analysis (Fig. 1F) and represent the average of the mean of the last 10 min (shaded areas in panels A, B, and D) of four experiments or more. All the differences are significant (P < 0.05) except that between FCR3S1.6 and trypsin-treated FCR3S1.2 (P = 0.378) and between FCR3S1.6 and FCR3CSA (P = 0.055). (D) The average proportion of injected material present in the rat lungs at different time points when injecting IRBC of the CD36-specific binding clone 3D7AH1S2 or IRBC of the CSA-specific clone FCR3CSA, compared to uninfected human RBC. Error bars show ±1 standard deviation. (E) Detailed analysis of the dynamics of sequestration during the first 10 min showing the difference between clones FCR3S1.2/3D7AH1S2 and uninfected human RBC. All levels were adjusted to 100% at time zero. K values indicate the derivative slope of the curve at 3 min.
The parasites FCR3S1.6, FCR3CSA and 3D7AH1S2 were subsequently used to investigate the sequestration of iRBC of different adhesive specificities. Six rats injected with FCR3S1.6 iRBC showed retention in the lungs comparable to that of animals injected with trypsin-treated FCR3S1.2 iRBC (Fig. 2B). The mean proportion of injected material present in the lungs during the last 10 min was on average 4.1% (3.4% to 5.1%, SD of ±0.64%), significantly lower than FCR3S1.2 iRBC (P < 0.01), but still higher than uninfected RBC (P < 0.01) (Fig. 2C). Injecting six rats with FCR3CSA iRBC resulted in a slightly lower proportion of injected material in the lungs as compared to FCR3S1.2 iRBC (Fig. 2D). On average, 5.2% of the injected material was localized in the lungs during the last 10 min of the experiment (4.2% to 6.4%, SD of ±0.8%) (Fig. 2C), significantly higher than in rats injected with uninfected RBC (P < 0.01) but slightly lower than in rats injected with iRBC of FCR3S1.2 (P = 0.02). In four rats injected with 3D7AH1S2 iRBC, the proportion of material present in the lungs was at all times well above that of rats injected with iRBC of any of the other parasites (Fig. 2D). The mean proportion of injected material in the lungs during the last 10 min was in average 12.7% (9.0% to 16.5%, SD of ±3.2%), significantly higher than with iRBC of the other clones tested (P < 0.012). A more detailed analysis of the amounts of material in the lungs during the first 10 min after injection of 3D7AH1S2 iRBC, FCR3S1.2 iRBC, and noninfected RBC revealed significant differences in the dynamics.

While almost 60% of the uninfected cells were lost during the first 5 min, 40% of the FCR3S1.2 iRBC and 20% of the 3D7AH1S2 iRBC were removed during the same time (Fig. 2E).

In a smaller set of 14 animals, injected with iRBC of clone 3D7AH1S2, FCR3S1.2 or FCR3CSA or with uninfected human RBC, the lungs were removed and analyzed separately in the gamma camera. The results confirmed the previous ROI-generated results as only 0.9% of the noninfected RBC injected were retained in the lungs compared to 5.1% and 10.0% in rats injected with iRBC of clones FCR3S1.2 or 3D7AH1S2 respectively (Fig. 3). FCR3CSA bound at 4.2%, replicating the results previously seen with ROI with a slightly lower binding compared to FCR3S1.2, but this time the difference between the two parasites was nonsignificant (P = 0.07). All other differences were highly significant (P < 0.015). A summary of the in vivo results obtained is given in Table 1.

**Inhibition of in vivo sequestration.** To further investigate the role of PfEMP1 and to verify the role of homologue receptors in the rat, inhibition of binding in vivo was performed by incubation of FCR3S1.2 iRBC with sera from animals immunized against the DBL1α domain of the FCR3S1.2 PfEMP1 or with soluble CD36 prior to injection. In three separate experiments, a total of six animals were injected with iRBC incubated in immune sera diluted 1:5. The inhibition of sequestration ranged from 32% to 63% resulting in an average, after removal of the background binding of noninfected human RBC, of 54% inhibition of sequestration as compared to animals injected with FCR3S1.2 iRBC incubated with GST control sera. To confirm these results, FCR3S1.2 iRBC were incubated in serum concentration from 1:10 to 1:2.5 before injection resulting in a concentration-dependent inhibition of binding (Fig. 4A). In three animals, FCR3S1.2 iRBC were pretreated with soluble CD36 at 50 μg/ml resulting in an inhibition of 25% as compared to rats injected with mock-treated iRBC (Fig. 4B).

**Histological analysis of rat lungs.** iRBC (identified by the presence of pigment) of FCR3S1.2 were found in small- to medium-sized venules and small veins of, on average, ~45 μm (20 to 300 μm) in diameter and more rarely in capillaries. An average of 1,608 iRBC, localized in 7 to 16 sites containing 10 to 550 iRBC, were identified in each of the 10 sections examined, resulting in a total of 1.6 × 10^4 iRBC counted (Fig. 5A). This correlated well with the 1.9 × 10^4 cells predicted with the gamma camera by dividing the count rate of the analyzed lungs...
sections with the count rate per injected iRBC. Plenty of the iRBC were localized in the vicinity of the endothelium, some adhering to endothelial cells but many looking as if previously attached but now separated from the endothelium by a small gap (Fig. 5B). The majority of the iRBC were found in central parts of the vessels, aggregating with other iRBC and with rat RBC (Fig. 5C). Differently sized areas of pink degenerate material, with parasitic pigment incorporated, were commonly found in the affected vessels (Fig. 5D). In total, >50% of the iRBC were in different stages of degradation, ranging from empty looking cells with thin membranes to extracellular parasites.

Lung sections from rats injected with FCR3CSA or 3D7AH1S2 iRBC were also examined. More than 80% of the FCR3CSA iRBC were located one by one in capillaries (5 to 10 μm; Fig. 5E) and degenerated parasites and degenerate material were very rarely seen (Fig. 5F). In the lung sections from the rat injected with 3D7AH1S2 virtually all iRBC were found to be located one by one in capillaries, and there were no signs of degeneration of the iRBC or any presence of degenerate material (Fig. 5F and G). A summary of the histological findings can be found in Table 2.

**Binding and inhibition of binding of iRBC to endothelial cells and lung sections in vitro**. Primary RLEC were isolated from lungs of male Sprague-Dawley rats and used for subsequent adhesion assays in vitro. Confirming the origin of the cells with anti-rat endothelium MAb gave a strong even fluorescence on 96/100 cells counted, while using anti-rat CD31 MAb gave a more uneven and dotty staining of 89/100 cells counted. Using only the secondary antibody gave a very weak background on 5/100 cells counted. FCR3S1.2 iRBC were found to bind at an average of 165 iRBC per 100 RLEC (mean of 21 readings) (Fig. 6A and B) while the 3D7AH1S2 iRBC bound at an average of 315 iRBC per 100 RLEC (mean of six readings) (Fig. 6A and B). Anti-human CD36 antibodies, cross-reacting with rat CD36, almost completely blocked the binding of iRBC of 3D7AH1S2 to RLEC at 10 μg/ml (mean of six readings) while anti-rat CD31/PECAM-1 antibodies at the same concentration had no impact, confirming the selective CD36-binding phenotype of this clone (Fig. 7A). The adhesion of FCR3S1.2 iRBC to RLEC was reduced by both antibodies, with anti-human CD36 blocking 25% of the binding and anti-rat CD31/PECAM-1 blocking 57% of the binding, confirming the capacity of the parasite to use both CD31/PECAM-1 and CD36 as receptors (Fig. 7A and B). Combining the two antibodies at 10 μg/ml each resulted in 65% reduction of the binding (Fig. 7B) and pretreatment of the RLEC with heparinase III resulted in a 37% reduction of binding. Combining heparinase III treatment with the anti-rat CD31/PECAM-1 antibody alone, or with a combination of the antibodies, resulted in an inhibition of the binding by 86% and 97% respectively (Fig. 7B).

Cryosections of snap-frozen rat lung were used to confirm the results generated with the RLEC and to investigate the specificity of the binding of FCR3CSA. The higher binding of 3D7AH1S2 iRBC (333 iRBC/mm²) as compared to FCR3S1.2 iRBC (160 iRBC/mm²) was verified using the lung sections (Fig. 6A and C) while the controls using uninfected RBC showed low binding (0.3 RBC/mm²). A difference between the two parasites was also seen regarding the size of the vessels to which the parasites bound, with FCR3S1.2 iRBC adhering in somewhat larger venules of up to 50 μm in diameter whereas 3D7AH1S2 iRBC were typically seen in vessels of <20 μm in diameter. Using the CD36 and CD31/PECAM-1 antibodies at 10 μg/ml to block the binding of FCR3S1.2 iRBC resulted in the same pattern of inhibition as when using RLEC (Fig. 7A) as the binding was reduced by 22% and 56%, respectively (Fig. 7B). Combining anti-CD36 and anti-CD31/PECAM-1 antibodies at 10 μg/ml each resulted in 71% reduction of the binding, and pretreatment of the sections with heparinase III reduced the binding by 73% (Fig. 7B). Heparinase III treatment combined with anti-CD31/PECAM-1 alone, or with a combination of the antibodies, at 10 μg/ml resulted in 94% and 96% blockage of binding, respectively (Fig. 7B). The inhibitory effect of the antibodies on the binding of the iRBC to cryosection was titrated as can be seen from Fig. 7C. iRBC of FCR3CSA were similarly incubated on lung cryosections and found to bind at 309 iRBC/mm². Pretreatment with chondroitinase ABC at 0.2 U/ml for 30 min inhibited this binding by ~90% (35 iRBC/mm²).

**DISCUSSION**

Due to the inability of *P. falciparum* to infect any but human RBC (and those of a few primates) there is to date no handy, robust animal model established and in general use to study *P. falciparum* sequestration. To overcome this we have here developed a small-animal model to investigate the acute phase of sequestration using immunocompetent Sprague-Dawley rats. The three *P. falciparum* clones employed in this investigation (FCR3S1.2, 3D7AH1S2, FCR3CSA), covering binding phenotypes previously found associated with different forms of mild or severe malaria (10, 15, 24), were found to sequestrate in a strain- and clone-specific manner and to induce distinct pathological changes.

Several independent approaches were utilized in order to verify that the binding of iRBC in the rat lungs truly reflects parasite-specific receptor-ligand interactions. First we used...
mild trypsin digestion to remove parasite derived adhesive polypeptides from the surface of iRBC prior to their injection and thereby reduced the sequestration by more than 70% (Fig. 2A and C). This treatment has previously been shown to remove the major adhesive ligand PfEMP1, but simultaneous removal of other trypsin-sensitive proteins from the surface of the iRBC cannot be excluded (13). Secondly we used the weakly PfEMP1-expressing clone FCR3S1.6 (13) and found that this parasite accumulated in the lungs at a level comparable to that of the trypsin treated iRBC of FCR3S1.2 (Fig. 2B and C). The remaining accumulation in these first two experiments may be due to residual PfEMP1, passive trapping of the iRBC, or the presence of additional, trypsin-resistant proteins such as RIFINS or SURFINS (12, 18, 40). Thirdly, we incubated iRBC of FCR3S1.2 with sera from rats immunized with the DBL1/H9251 domain of PfEMP1 (4) prior to injection and thereby significantly reduced their binding in a concentration dependent manner (Fig. 4). The DBL1α-immunized rats have previously been found to be protected against iRBC sequestration using the model presented here, and the sera from the

FIG. 5. Histological examination of hematoxylin-eosin stained lung sections from rats injected with P. falciparum iRBC of different strains and clones. (A) Scanned image of a whole lung section from a rat injected with FCR3S1.2 iRBC. Sites and numbers of iRBC found at each site are indicated. (B) Magnification of iRBC of FCR3S1.2 interacting with the endothelial lining. (C) Picture from the central part of a vessel showing a plentitude of FCR3S1.2 iRBC interacting with other iRBC and with rat erythrocytes. (D) Medium sized vein from the central part of the lung of a rat injected with FCR3S1.2 iRBC. Dark spots are pigment present in iRBC, each spot representing one parasite. The iRBC are found along the endothelial lining as well as in the central part of the vessel. Note pink degenerate areas (arrows) with residual pigment. (E) Magnification of lung sections from a rat injected with iRBC of FCR3CSA showing typical location of iRBC in the capillaries (arrow). (F and G) Magnification of lung section from a rat injected with iRBC of 3D7AH1S2 showing typical location of the iRBC in the capillaries (arrows). Original magnifications: panel D, ×400; panels B, C, E, F, and G, ×1,000. Scale bars: panel D, 50 μm; panels B, C, E, F, and G, 10 μm.
immunized rats have been found to recognize the surface of FCR3S1.2 iRBC in vitro (5). Lastly, FCR3S1.2 iRBC were incubated with soluble CD36 prior to injection resulting in a 25% reduction of binding. This level of inhibition was indeed expected since CD36 is shown to contribute to about 25% of the binding of the iRBC of FCR3S1.2 to rat lung endothelial cells in vitro (Fig. 7). The remaining 75% of binding is mediated primarily by heparan sulfate but also by CD31/PECAM-1 (Fig. 7). Data showing significant reduction of binding when coinjecting the FCR3S1.2 iRBC with the soluble receptor heparan sulfate/heparin further support the specificity of the binding and demonstrate the applicability of the model for drug studies (A. M. Vogt, unpublished data). Taken together, these data demonstrate that the binding of iRBC in the rat lung reflecting sequestration, at least partly mediated by PfEMP1, and hence that the model is useful for the study of iRBC adhesion in vivo.

ROI analysis of whole-body imaging was used in the majority of the experiments to quantify sequestration resulting in significant differences regarding the level of sequestration between all tested parasites \( (P < 0.02) \) except for FCR3S1.6 that was not significantly separated from FCR3CSA \( (P = 0.055) \) or from trypsin-treated FCR3S1.2 \( (P = 0.38) \). In a subset of the animals the lungs were surgically removed and measured separately in the gamma camera. This gave less interexperimental variability and a substantial decrease in activity seen in the animals injected with uninfected material, most likely due to parts of the lungs being covered by the liver during ROI analysis and to a reduction of the background activity from nonlung tissue when measuring the radioactivity of excised lungs. Despite the small animal groups it therefore resulted in highly significant differences between all investigated parasites \( (P < 0.02) \) except between FCR3S1.2 and FCR3CSA \( (P = 0.06) \). Using excised lungs hence dramatically reduced the number of animals needed to obtain significance.

The ROI images revealed that human RBC, regardless of being infected or not, ended up in the liver, spleen and kidneys, an unspecific accumulation previously noted and suggested to depend on the phagocytosis of syngeneic RBC (11). Although the dynamics of the binding may superficially look similar between rats injected with uninfected RBC or with iRBC, a more thorough analysis of the first 5 min after injection shows that the rats injected with iRBC still maintained 60 to 80% of the initial binding while those injected with uninfected cells

<table>
<thead>
<tr>
<th>Material injected</th>
<th>No. of cells counted</th>
<th>Proportion of cells found in:</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Small vessels (^b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Without degenerate deposits</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Without degenerate deposits</td>
</tr>
<tr>
<td>FCR3S1.2 1.6 (\times) 10(^4)</td>
<td>31.3%</td>
<td>12.5%</td>
</tr>
<tr>
<td>FCR3CSA 1.0 (\times) 10(^3)</td>
<td>83.4%</td>
<td>14.5%</td>
</tr>
<tr>
<td>3D7AH1S2 1.0 (\times) 10(^3)</td>
<td>96.4%</td>
<td>0%</td>
</tr>
</tbody>
</table>

\(^a\) Total number of infected erythrocytes counted in 3 to 10 cryosections of rat lung.
\(^b\) Postcapillary venules with a diameter of \(<20 \mu m\).
\(^c\) Postcapillary and collecting venules with a diameter of \(>20 \mu m\).

![FIG. 6. In vitro binding of iRBC of different strains and clones of P. falciparum to primary RLEC and to cryosections of rat lung. (A) Binding of iRBC of FCR3S1.2 or 3D7AH1S2 to primary RLEC (number of iRBC per 100 target cells) and to cryosections of rat lung (number of iRBC per mm\(^2\)). Error bars show 1 standard deviation. (B) In vitro binding of iRBC of FCR3S1.2 (left) and iRBC of 3D7AH1S2 (right) to RLEC. (C) In vitro binding of iRBC of FCR3S1.2 (left) and iRBC of 3D7AH1S2 (right) to cryosections of snap-frozen rat lung; note different preferences regarding size of vessels between the two clones. Original magnification, \(\times1,000\). Scale bars, 30 \(\mu m\).](http://iai.asm.org/)
had lost more than 60% of the binding. As human erythrocytes previously have been shown to have a half-life of ~7 min in the rat (11), this is likely to result from a combination of continuous binding of circulating iRBC as well as higher stability of the specific binding of iRBC as compared to the passive trapping of noninfected cells. This also fits with previous observations that initial iRBC adherence is sometimes transitory, followed by progressive cell recruitment. These differences in dynamics are important as they demonstrate a time frame during which antisequestration measures may be studied in this model.

The results from the gamma camera were confirmed by histological analysis of lung tissue from rats injected with iRBC of parasite clones FCR3S1.2, 3D7AH1S2, and FCR3CSA. iRBC in numbers comparable to what was predicted by the gamma camera were found present in the lung sections of rats injected with FCR3S1.2 iRBC. These sections also revealed the FCR3S1.2 iRBC to be mainly located in somewhat larger venules and that a large number of the iRBC were in different stages of degradation combined with the presence of degenerate, fibrin-like material in the lumen of the affected vessels. In contrast, when lung sections from rats injected with either CD36-specific iRBC (3D7AH1S2) or CSA-specific iRBC (FCR3CSA) were analyzed, they revealed the vast majority of the iRBC to be located one by one in capillaries, with no, or minor, amounts of degenerate material being present. Interestingly, the preference of the FCR3S1.2 parasite to adhere in somewhat larger venules was reproducible in vitro when parasites where allowed to bind to cryosections of rat lung, although the binding was then restricted to vessels of less than 50 μm. This most likely represents an uneven distribution of receptors. The rare binding in larger vessels (up to 300 μm) was exclusively seen in conjunction with degenerate material and as it was never seen in vitro it might be a consequence of in vivo rosetting and fibrin deposition, possibly in areas of reduced blood flow downstream of clogged venules. Sequestration in these large vessels has not been reported in human histopathological studies and might therefore represent an artifact of the model. However, as it was only seen with FCR3S1.2 iRBC, it could also represent an important property of this parasite, and the fact that it has never been reported in human material is possibly due to most of the samples being nonlung tissue. Degradation of iRBC and presence of fibrin-like material have both been described in human autopsy specimens (20, 26) and may reflect a common trait in the development of severe disease (9). In a recent clinicopathological study by Pongponratn et al. it was noted that the vessels of deceased cerebral malaria patients were more commonly seen “blocked by a mass of fibrillar material that is surrounded by red blood cells . . . and iRBCs” as compared to deceased non-cerebral-malaria patients (26). This was true for all areas of the brain investigated, and if looking separately at the medulla this difference was significant ($P = 0.046$) (26). Further, “vessel . . . packed with degenerate material . . . pigment were present . . . with no intact parasites present . . .” (26). Although these findings seem to bear a resemblance to our findings in the rats

FIG. 7. Inhibition of binding of *P. falciparum* iRBC to primary RLEC or to cryosections of rat lung. (A) The effect of a MAb anti-rat CD31/PECAM-1 or a MAb anti-human CD36 (cross-reactive with rat CD36) at 10 μg/ml on the binding of iRBC of FCR3S1.2 or iRBC of 3D7AH1S2 to RLEC. Numbers are given as percentages of controls (mean of three experiments). (B) Effect of MAb anti-rat CD31/PECAM-1, MAb anti-human CD36 or heparinase treatment on the binding of iRBC of FCR3S1.2 to primary RLEC or to cryosections of rat lung. Antibodies were used at 10 μg/ml. Cells and cryosections were pretreated with heparinase at 0.2 IU for 30 min prior to the experiment (bars shown on the far right), with or without the addition of antibodies at 10 μg/ml as indicated. Numbers are given as percentages of controls (mean of three experiments). (C) The effect of MAb anti-human CD36 and MAb anti-rat CD31/PECAM-1 on the binding of FCR3S1.2 iRBC to cryosections of rat lung at the concentrations given in the graph. Binding is given as a percentage of the control (mean of three experiments). Error bars in all three graphs show 1 standard deviation.

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injected with iRBC of FCR3S1.2, there are several important differences to keep in mind. First, while not known, the histopathological changes seen in human tissue are generally thought to be the result of a long lasting infection while the reaction in the rat is indeed very rapid. Secondly, naturally occurring anti-human antibodies have previously been demonstrated in rodents and may very well contribute substantially to the changes observed. Thirdly, sequestration in such large vessels as occasionally seen with FCR3S1.2 iRBC in the rat has not been reported in human specimens and may, as discussed above, represent an artifact of the model. Lastly, but not least, we are in the model limited to studying the lungs while most of the findings in the quoted articles are from brain tissue. However, the extensive histopathology induced by the FCR3S1.2 iRBC as compared to the lesser changes induced by the even more sequestrating 3D7AH1S2 iRBC to us indicate the presence of a certain level of parasite specific immunological reaction. If allowed to speculate one may hypothesize that the rapid induction of pathology in the rat might be triggered by acute local hypoxia in combination with tissue factor release causing polymerization of fibrinogen at the iRBC surface, as iRBC of FCR3S1 previously has been shown to bind to fibrinogen (36). Cerebral malaria in humans has often a quite sudden onset with meningitis-like symptoms which might indicate a rapid progression of pathology, possibly following a phenotypic change of the parasite. The potential relevance of the pathological changes seen in the lungs of the rat to the pathology of the brain and other organs in humans with severe malaria remains to be elucidated.

In vitro binding of iRBC to cryosections and primary RLEC was used to further verify the in vivo results and to investigate the specificity of the binding of iRBC. The binding of iRBC of 3D7AH1S2 to RLEC was approximately twice that of iRBC of FCR3S1.2, reproducing the relationship in the level of sequestration in vivo between the two parasites. Further, the specificities in binding of the iRBC to the RLEC and to human endothelial cells were shown to be comparable, as concluded from inhibition experiments using anti-human CD36 antibodies and/or anti-rat CD31/PECAM-1 antibodies. These results authenticate the dependency on different endothelial receptors in the two different strains, as known from other in vitro binding experiments (13). Previous findings have revealed heparan sulfate and CSA to be receptors for iRBC on the human endothelium and placental syncytiotrophoblasts (10, 29, 31, 39), and we demonstrate this to be true also on the rat lung endothelium. Thus, taken together these results show levels of receptor expression to be similar, if not identical, between human and rat endothelial cells.

Sequestration of iRBC occurs in asymptotic immune individuals as well as in nonimmune individuals with malaria. Led by the results in the rat we suggest FCR3S1.2, 3D7AH1S2, and FCR3CSA all represent strains sequestering at different levels by the results in the rat we suggest FCR3S1.2, 3D7AH1S2, and FCR3S1.2, but not with 3D7AH1S2, may reflect an important mechanism in the development of severe malaria disease.

In conclusion, we here describe the establishment of a new robust animal model for the study of sequestration of P. falciparum. We demonstrate the level of sequestration in vivo to be in keeping with in vitro binding using endothelial cells as well as cryosections of rat lung, and to be dependent on PIEMP1. Furthermore, we show that the sequestration can be specifically inhibited in vivo and that the induced histopathology carries possible resemblances with findings in patients who succumb to P. falciparum malaria.

Compared to in vitro systems the present model provides the full complexity of the microvasculature with a physiological blood flow, an unmodified endothelial cell lining and an intact immune system. In contrast to the animal models currently used in malaria research this system offers a cheap and straightforward way of studying unmodified parasites in non-adapted and immunocompetent animals.

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REFERENCES


33. Reference deleted.


