

## **Visualization of *Plasmodium falciparum*–Endothelium Interactions in Human Microvasculature: Mimicry of Leukocyte Recruitment<sup>Ⓞ</sup>**

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### **Abstract**

*Plasmodium falciparum*–infected erythrocytes roll on and/or adhere to CD36, intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and P-selectin under shear conditions in vitro. However, the lack of an adequate animal model has made it difficult to determine whether infected erythrocytes do indeed interact in vivo in microvessels. Therefore, we made use of an established model of human skin grafted onto severe combined immunodeficient (SCID) mice to directly visualize the human microvasculature by epifluorescence intravital microscopy. In all grafts examined, infected erythrocytes were observed to roll and/or adhere in not just postcapillary venules but also in arterioles. In contrast, occlusion of capillaries by infected erythrocytes was noted only in approximately half of the experiments. Administration of an anti-CD36 antibody resulted in a rapid reduction of rolling and adhesion. More importantly, already adherent cells quickly detached. The residual rolling after anti-CD36 treatment was largely inhibited by an anti-ICAM-1 antibody. Anti-ICAM-1 alone reduced the ability of infected erythrocytes to sustain rolling and subsequent adhesion. These findings provide conclusive evidence that infected erythrocytes interact within the human microvasculature in vivo by a multistep adhesive cascade that mimics the process of leukocyte recruitment.

Key words: cytoadherence • adhesion molecules • erythrocytes • malaria • pathogenesis

### **Introduction**

Malaria is by far the most important parasitic disease worldwide and is second only to tuberculosis as an infectious cause of death, particularly in rural Africa, where resources to combat the infection are limited (1). Of the four species of *Plasmodium* that infect humans, *P. falciparum* accounts for the majority of the infections and is also the most lethal. Approximately two million individuals, mostly children, die annually, partly from complications associated with underperfusion of vital organs as a result of the sequestration of infected erythrocytes in the microcirculation (2). Although infected erythrocytes may be passively trapped in microvessels due to a loss of deformability (3), it has also

been widely postulated that parasites actively adhere in postcapillary venules to evade splenic clearance (4).

In vitro experiments would suggest that infected erythrocytes can interact with various host proteins under both static and flow conditions (for review see reference 5). For example, application of hydrodynamic shear to infected erythrocytes permits them to roll on CD36, intercellular adhesion molecule (ICAM)-1, P-selectin, and vascular cell adhesion molecule (VCAM)-1 (6–9). Subsequent adhesion to CD36 has also been noted. Although these observations imply that *P. falciparum* can induce the infected erythrocytes to roll and adhere, the experiments to date by our laboratory and others have been designed to optimize the conditions for cellular interactions. Shear forces are used at levels at which interactions of infected erythrocytes with adhesion molecules can be observed. In fact, the highest shear stress used in vitro is thought to be at the very low end of the shear forces in vivo (10). Moreover, adhesion molecules are abundantly supplied in the form of immobi-

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lized receptor protein or expressed on transfected cells. The lack of a suitable animal model for cytoadherence has meant that the hypothesis that infected erythrocytes can actively adhere in the microcirculation to allow for propagation of the infection has never been tested directly.

In this study, we made use of a well established model of human skin grafted onto SCID mice but extended the model to directly visualize the human microvasculature using intravital microscopy. We provide direct evidence that there is sufficient constitutive expression of CD36 and ICAM-1 to allow infected erythrocytes under physiologic shear to roll on and adhere to not just human postcapillary venules but also to human arteriolar vasculature. Moreover, in some of the human skin grafts, capillary plugging was not observed, yet active CD36-dependent adhesion was evident, suggesting that in at least some cases active adhesion may be the predominant mechanism that enables the parasite to complete its life cycle without causing the demise of the host. Most importantly, an anti-CD36 antibody was able to reverse the firm adhesion of infected erythrocytes, raising the possibility that antiadhesive therapy could be employed in severe falciparum malaria.

## Materials and Methods

**Parasite Culture.** Three clinical isolates of *P. falciparum* obtained from acutely infected patients admitted to the Hospital for Tropical Diseases, Bangkok, Thailand were cultured as described (7). The collection of patient specimens was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Thailand. Cryopreserved parasites were thawed and cultured for 24–36 h in RPMI medium (Life Technologies) supplemented with 25 mM HEPES (Sigma-Aldrich), 100 U/ml penicillin, and 100 µg/ml streptomycin (GIBCO BRL), 2 mM glutamine (Sigma-Aldrich), and 10% AB serum from a normal Thai donor. Infected erythrocytes were harvested for experiments when they were at the late trophozoite/early schizont stage. The parasitemias of the three isolates studied were between 5 and 7%. In a previous study (7), these parasite isolates were shown to roll on and adhere to CD36 transfectants, whereas the interaction with ICAM-1 transfectants was mainly one of rolling, with <5% of the rolling cells eventually becoming adherent. These cytoadherent characteristics are well documented for infected erythrocytes obtained from the peripheral blood of infected patients (5).

**Preparation of Human Skin Graft in SCID Mice.** CB-17 SCID/beige mice (Harlan Canada) were grafted with split thickness human skin as described previously (11) under a protocol approved by the Health Sciences Animal Welfare Committee and the Health Research Ethics Board of the University of Alberta. In brief, split thickness grafts from a single donor were prepared from discarded human skin using a 0.1-inch dermatome. Recipient mice were anesthetized using halothane. A 0.5 × 0.5-cm defect was excised from the posterior thorax and covered with human skin anchored using skin staples (US Surgical). The graft was allowed to heal for 3 wk before the animal was used in the intravital microscopy experiments.

**Intravital Microscopy.** Animals were anesthetized with ketamine and xylazine, and body temperature was maintained at physiological levels using a heating pad and rectal thermometer as previously described (12). The procedure was approved by the

Animal Care Committee, University of Calgary. The jugular vein was cannulated for administration of additional anesthetic, boluses of infected erythrocytes, and antibodies. A midline dorsal incision was made from the neck to the lower back without disrupting the lateral dermal blood supply. The area of mouse skin supporting the human graft was carefully separated from the underlying tissue, with care taken not to disrupt the neovasculature that supplied the base of the graft. The skin was reflected onto a pedestal and secured with suture. The microvasculature within the graft was exposed by gently dissecting the connective tissue supporting the graft, causing minimal disruption to the graft blood supply within this tissue. The graft was then moistened with saline and covered with a coverslip. The preparation was examined using an upright microscope (Optiphot-2; Nikon) with a 20× water immersion objective.

To identify human vessels, 100 µg of FITC-labeled *Ulex europaeus* (Sigma-Aldrich) was injected intravenously immediately before microscopic visualization. FITC-derived fluorescence was visualized by epiillumination at 450–490 nm using a 520 nm emission filter. Infected erythrocytes were labeled with rhodamine 6G (1:10 of 0.05%, 5 min), and unbound rhodamine 6G was removed by three washes in PBS. Rhodamine-labeled infected erythrocytes were visualized by excitation at 510–560 nm using a 590 nm emission filter. Pilot studies revealed that neither *U. europaeus* nor rhodamine 6G at the doses used affected the ability of infected erythrocytes to interact with endothelial cell monolayers under flow conditions in vitro (Ho, M., unpublished observations). The procedure did not label uninfected erythrocytes that were devoid of nuclear material. Each 200-µl bolus contained infected erythrocytes at ~50% hematocrit and 5–7% parasitemia. Images of the labeled infected erythrocytes and human microvessels were visualized using a silicon-intensified CCD camera (C-2400-08; Hamamatsu Photonics) and recorded with a VCR for playback analysis. The numbers of rolling and adherent infected erythrocytes were determined off-line. Rolling of infected erythrocytes was expressed as percentage flux fraction, determined by counting the number of interacting infected erythrocytes in an individual vessel and expressing this relative to the total number of infected erythrocytes passing through the vessel over the same period (determined by frame-by-frame analysis). Infected erythrocytes that remained stationary on the vascular wall for at least 30 s were defined as adherent.

In experiments involving inhibitory mAbs, 20–40 µg (µg/ml) was injected as a bolus in a total volume of 100 µl of PBS after baseline interactions had been recorded. The anti-human CD36 (OKM5; gift of Ortho Diagnostic, Raritan, NJ) and anti-human ICAM-1 (84H10; R&D Systems) antibodies were found to be effective at these concentrations under in vitro conditions (7–9). The antibodies were allowed to circulate for 2–3 min before a second bolus of infected erythrocytes was injected. The videotapes of the antibody experiments were analyzed without knowledge of the effect of the antibodies on cytoadherence.

**Immunohistochemical Staining.** At the time of intravital microscopy studies, skin was harvested from a representative animal in the series and snap frozen in OCT. Sections were stained with the antihuman mAbs anti-PECAM-1 (Becton Dickinson), OKM5 (Ortho), 84H10 (R&D Systems), or irrelevant control IgG (Cappel Laboratories) for expression of CD31, CD36, and CD54, respectively. The biotin-conjugated secondary sheep anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc.) was developed using the ABC kit (Vector Laboratories) and chromagen diaminobenzadine (Pierce Chemical Co.). Images were obtained using a CCD digital camera (Nikon).

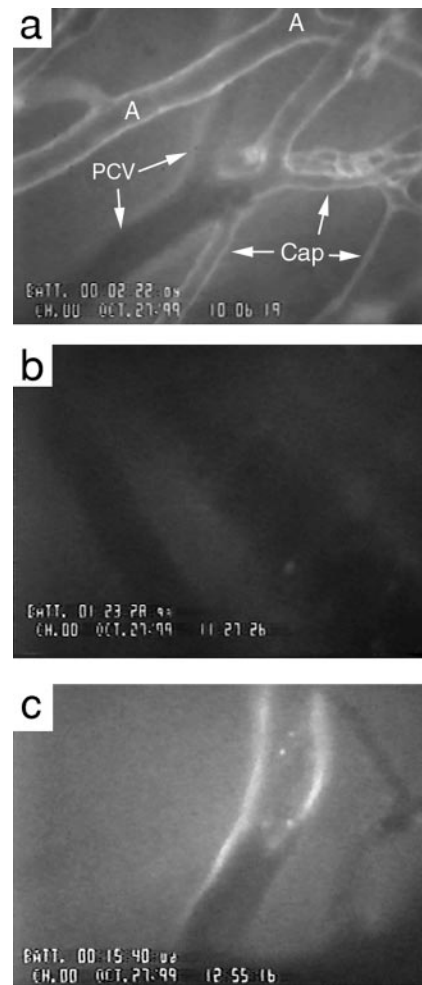
**Online Supplemental Material.** The online supplemental data comprises two video sequences taken from an experiment examining the interactions of *P. falciparum*-infected erythrocytes in the microvasculature of a human skin graft on a SCID mouse.

The video illustrates a vascular field within the human skin graft, containing at least three separate microvessels that were labeled with FITC-*U. europaeus*. Infected erythrocytes were labeled with rhodamine 6G, and unbound stain was removed by multiple washes. Uninfected erythrocytes were unlabeled. A bolus of infected erythrocytes was injected into the circulation of the mouse, and interactions with endothelial cells were examined in the graft microvasculature over the following 10 min. Video 1 illustrates interactions of infected erythrocytes in an untreated human microvasculature. In several vessels, adherent infected erythrocytes were apparent as cells stationary on the vascular wall. In addition, infected erythrocytes can be observed rolling along the walls of two vessels. Blood flow was maintained throughout this area of the microvasculature. In Video 2, the graft microvasculature was pretreated with mAbs against the human adhesion molecules CD36 and ICAM-1. Despite many infected erythrocytes flowing through the graft microvasculature (observed as streaks in the centerline of blood flow), very few of them interacted with the endothelium. These observations conclusively demonstrate a role for CD36 and ICAM-1 in mediating the cytoadherence of *P. falciparum*-infected erythrocytes in a human microvasculature in vivo. Videos are available at <http://www.jem.org/cgi/content/full/192/8/1205/DC1>.

## Results

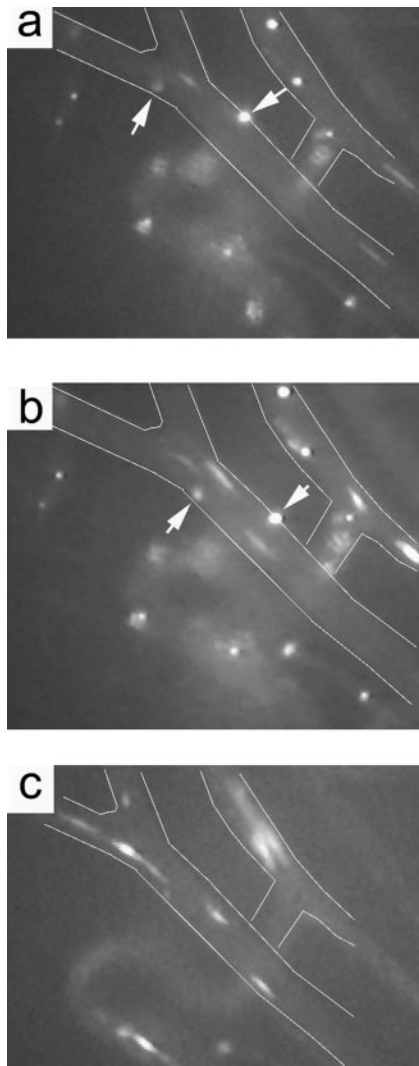
The adhesive interactions between infected erythrocytes from clinical parasite isolates and a human microvasculature were examined by epifluorescence intravital microscopy of human skin grafted onto SCID mice. The skin graft retained the human microvascular bed of the superficial dermis, and the graft blood supply was restored by spontaneous anastomosis of the mouse and human microvessels at the base of the graft (11). By fluorescently labeling the microvessels in vivo with the lectin *U. europaeus*, which specifically recognizes human endothelial cells of vessels of all sizes (13), we were able to distinguish the species origin of the microvessels in the fields of observation. Fig. 1 a demonstrates that endothelial cells lining microvessels in the grafts were indeed of human origin, as FITC-conjugated *U. europaeus* clearly labeled the graft vessels but not adjacent murine skin (Fig. 1 b). However, the intensity of labeling did differ and was in the order of arterioles > capillaries > postcapillary venules. At the periphery of the graft, some chimeric vessels were observed, lined by a mixture of labeled (human) and unlabeled (murine) endothelium (Fig. 1 c).

Rhodamine-labeled infected erythrocytes containing trophozoites and schizonts were injected intravenously into the animals. Infected erythrocytes continued to circulate in significant numbers for ~10–15 min, after which parasite accumulation was apparent in the spleen. In all eight grafts examined, infected erythrocytes were observed to roll and/or adhere in postcapillary venules of the human skin graft (Fig. 2). Surprisingly, infected erythrocytes also interacted with arterioles in 75% of grafts examined. The percentages



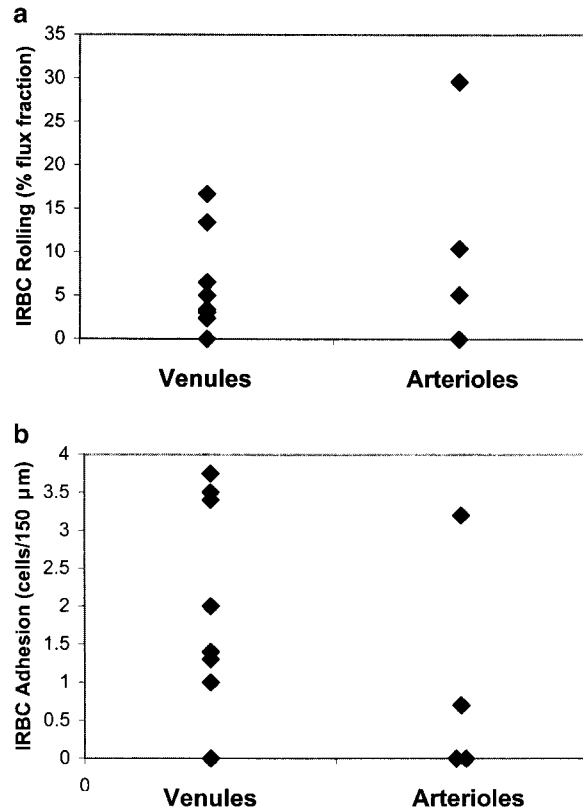
**Figure 1.** Identification of microvessels in vivo in human/SCID mouse chimeras. (a) FITC-*U. europaeus*-labeled microvessels are detected in the centre of the skin graft, indicating the presence of human endothelium. A, arteriole; Cap, capillary; PCV, postcapillary venule. (b) Absence of *U. europaeus* labeling in the microvasculature of adjacent murine skin. (c) A chimeric microvessel lined by human (labeled) and murine (unlabeled) endothelium at the periphery of the skin graft.

of infected erythrocytes that undergo rolling on and adhesion to both sides of the microvasculature are shown in Fig. 3. In postcapillary venules, ~6% of the total number of passing infected erythrocytes underwent rolling interactions, whereas 10% rolled in arterioles. Adhesion of infected erythrocytes was also observed. Significant adhesion of infected erythrocytes was observed in postcapillary venules in seven of eight grafts and in arterioles in one of four grafts. Approximately two-thirds of the adherent infected erythrocytes rolled for various distances before becoming arrested, while the rest appeared to bypass the rolling event and adhered immediately after tethering. In addition, occlusion of some capillaries by infected erythrocytes was observed after parasite injection in five of eight grafts. Infected erythrocytes did not interact with murine blood vessels in the surrounding skin, consistent with the species specificity of the infection.



**Figure 2.** Interactions of infected erythrocytes and human endothelial cells in the human skin graft model. Panels a–c illustrate the same vascular field within the human skin graft. To aid identification, the vascular walls are indicated by lines. Adherent or slowly moving (rolling) infected erythrocytes are visible as discrete circular objects, while noninteracting infected erythrocytes are observed as streaks in the centerline of blood flow. Uninfected erythrocytes are unlabeled. Panels a and b are separated by ~3 s. Arrows indicate infected erythrocytes undergoing rolling interactions with the endothelial surface of a postcapillary venule within the graft. Over the 3-s time course, the rolling infected erythrocytes have moved slowly along the vascular wall. At the same time, the patency of the microcirculation is apparent as rapidly-moving, noninteracting infected erythrocytes are observed throughout this period. Panel c illustrates the same area of microvasculature after dual treatment with mAbs against human CD36 and ICAM-1. Very few interacting infected erythrocytes are observed despite many cells continuing to pass through the graft microvasculature. Videos illustrating the above points are explained in the online supplemental material section and are available at <http://www.jem.org/cgi/content/full/192/8/1205/DC1>.

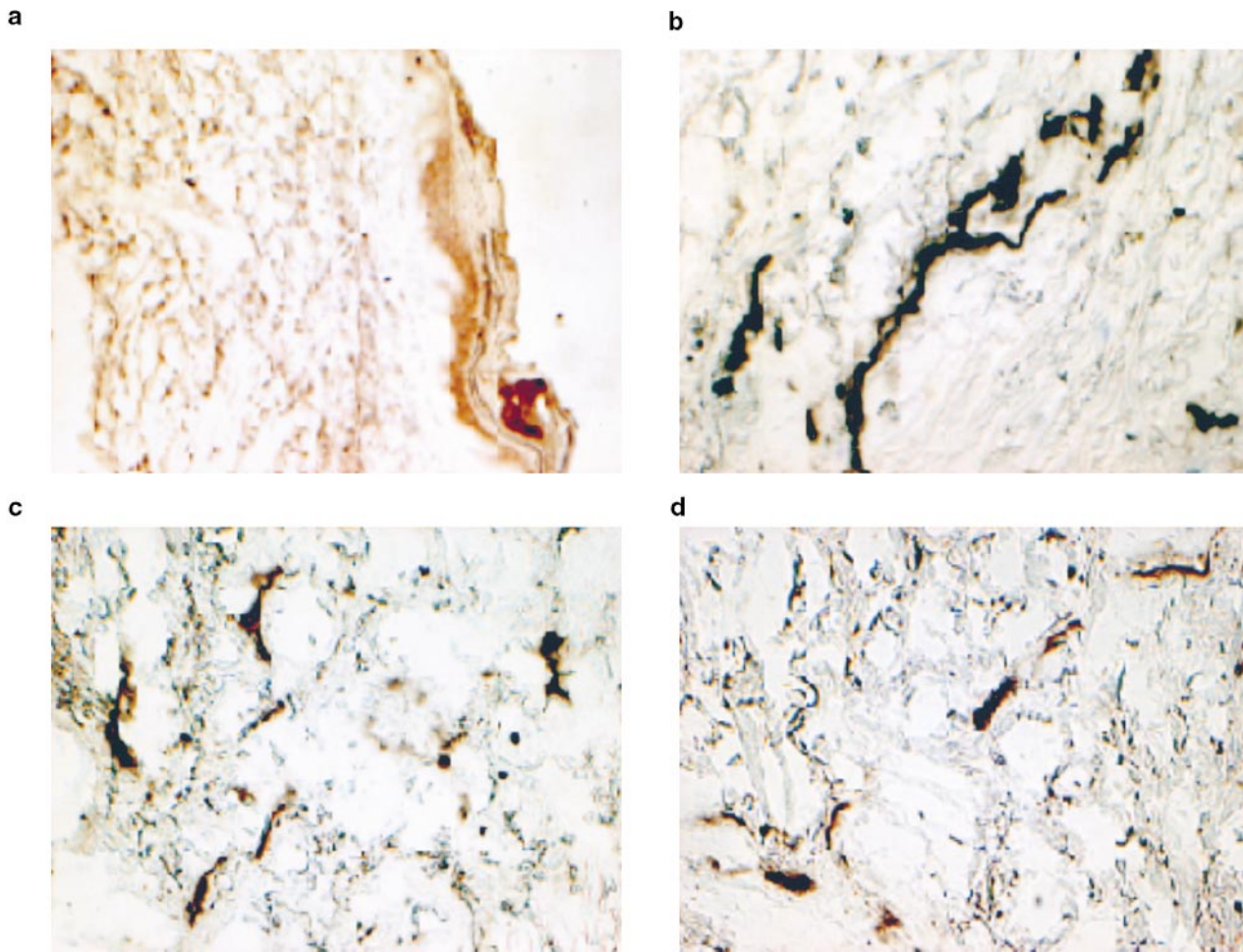
In the next series of experiments, the molecular mechanisms by which infected erythrocytes interacted with the human microvessels were identified. Human dermal microvascular endothelial cells have been shown to constitutively



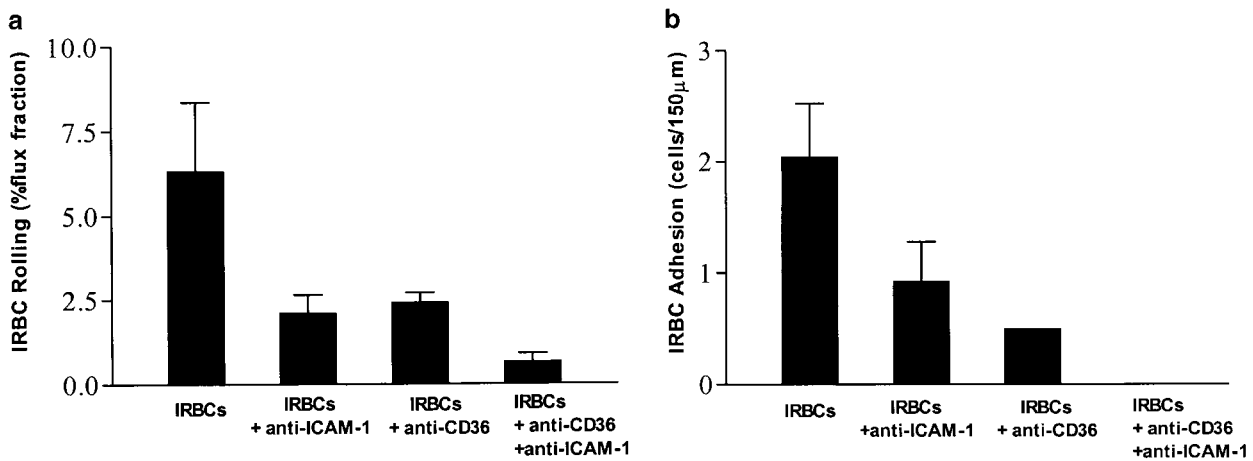
**Figure 3.** Rates of rolling (a) and adhesion (b) of infected erythrocytes in postcapillary venules and arterioles in human skin grafts. Data shown are mean data from each graft, generated by averaging observations from multiple vessels (four to seven venules, two to seven arterioles) in each graft. IRBC, infected erythrocytes.

tively express CD36 and ICAM-1 (14), the two major molecules implicated by in vitro studies as mediating cytoadherence (15, 16). We showed that the microvasculature in the human skin grafts retained expression of these molecules as well as human platelet–endothelial cell adhesion molecule (PECAM)-1 (Fig. 4, a–d). Intravital microscopy experiments showed that the addition of an anti-CD36 mAb rapidly reduced rolling and adhesion of infected erythrocytes in postcapillary venules (Fig. 5). Moreover, already adherent infected erythrocytes quickly detached after antibody administration. The residual rolling of infected erythrocytes after anti-CD36 treatment was largely inhibitable by anti-ICAM-1 treatment, resulting in further reduction in the number of adherent cells. Treatment of mice with anti-ICAM-1 alone also reduced rolling and adhesion, although the parasite isolates tested were not known to adhere to ICAM-1 under flow conditions in vitro.

The effect of inhibitory antibodies on interactions of infected erythrocytes in arterioles was also studied. Anti-ICAM-1 reduced arteriolar rolling by 70%, but no effect on adhesion was apparent (data not shown). Arteriolar interactions were eliminated after treatment with a combination of anti-CD36 and anti-ICAM-1.



**Figure 4.** Immunohistochemical staining of sections from human skin grafts with mAbs specific to human endothelial antigens. (a) Control IgG; (b) Anti-human CD31 (PECAM-1); (c) Anti-human CD36; (d) Anti-human CD54 (ICAM-1).



**Figure 5.** Effect of antibodies against human CD36 and ICAM-1 on rolling (a) and adhesion (b) of infected erythrocytes in postcapillary venules in human skin grafts. Data are shown as the mean  $\pm$  SEM of multiple observations before and after antibody administration in at least two skin grafts. IRBCs, infected erythrocytes.

## Discussion

It is now well established that leukocytes are initially captured from the mainstream of blood flow by selectins and roll along the endothelial surface. This rolling process serves as a prerequisite for the subsequent arrest (adhesion) of leukocytes on endothelial ICAM-1 (17). Despite the hundreds of *in vivo* studies that repeatedly demonstrate the multistep cascade of events for leukocytes in animal models, our data demonstrate for the first time that *P. falciparum*-infected erythrocytes undergo similar interactions under the unique physiological shear conditions and configurations of adhesion molecule expression present in a human microvasculature *in vivo*. Infected erythrocytes also rolled and adhered but via a very different series of adhesive mechanisms.

First, CD36, the scavenger receptor, was the dominant adhesive molecule for rolling and adhesion. This molecule does not support interactions with leukocytes (our unpublished observations). Second, ICAM-1 supported infected erythrocyte interactions with blood vessels under flow conditions, but the interactions were of a rolling phenotype. As leukocytes adhere but do not roll on ICAM-1 (10), *P. falciparum* appears to have evolved the novel ability to roll on a molecule designed to support human leukocyte adhesion. Furthermore, ICAM-1 and CD36 acted synergistically in the recruitment of infected erythrocytes, as inhibition of rolling on ICAM-1 resulted in a reduction in the number of adherent cells. Third, rolling and active adhesion of infected erythrocytes occurred on both the arteriolar and venular sides of the circulation, whereas leukocytes adhered predominantly in venules and not at all in uninflamed microvessels. This finding suggests that adherent infected erythrocytes can withstand very high shear rates. As two very different cell systems, leukocytes and an intraerythrocytic pathogen, appear to make use of the rolling to adhesion cascade, from an evolutionary standpoint it suggests that the stepwise mechanism is likely the optimal strategy for cellular recruitment under flow in blood vessels many times the cell diameter.

The observation that infected erythrocytes interacted with arteriolar endothelium was unexpected and was in apparent contrast to all known *in vivo* evidence for cytoadherence from the histological examination of post mortem tissues in which infected erythrocytes (but not uninfected erythrocytes) are found in close apposition to venular endothelium via electron-dense protrusions (18). It should be stressed that the predominant adhesive interaction of infected erythrocytes with arteriolar endothelium observed in this study was rolling, which would not have been captured in tissue sections. Nevertheless, significant adhesion to arterioles was observed in one of four skin grafts. Whether this reflects lower shear stress in the murine compared with human microcirculation remains to be determined.

The experimental model in this study likely reflects a noninflammatory state. Indeed, our own work has previously revealed that the human/SCID mouse chimera has

no evidence of inflammation 21 d after transplant inasmuch as no mouse leukocytes were noted in and around the human microvasculature (11). Our data suggest that CD36 and ICAM-1 by themselves are sufficient to support rolling and adhesion of infected erythrocytes on noninflamed microvessels. However, it is well documented that TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IFN- $\gamma$  are all elevated during severe falciparum malaria (19–21), and it is likely that these cytokines would induce the expression of adhesion molecules such as P-selectin and VCAM-1 that have been shown to enhance the adhesion of infected erythrocytes on human microvascular endothelial cells *in vitro* (9).

In summary, the use of the human/SCID mouse model has enabled us to directly visualize cell–cell interactions in a human microvasculature. The results indicate that infected erythrocytes from clinical parasite isolates roll and adhere to both venules and arterioles *in vivo*, where the hydrodynamic shear stress is significantly higher than that applied to flow chambers *in vitro*. The human/SCID mouse chimera thus provides an excellent model to further elucidate the role of adhesion molecules in the pathogenesis of *P. falciparum* malaria and to evaluate potential therapeutic interventions that would disrupt the adhesive interactions of infected erythrocytes with vascular endothelium *in vivo*. Ultimately, this experimental model could be used to establish the importance of adhesion molecules in other disease states involving red blood cells, such as sickle cell anemia, and leukocyte recruitment in a variety of inflammatory processes.

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