

In Vivo Sequestration of *Plasmodium falciparum*-infected Human Erythrocytes: A Severe Combined Immunodeficiency Mouse Model for Cerebral Malaria

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Summary

Cerebral malaria is a fatal complication of infection by *Plasmodium falciparum* in man. The neurological symptoms that characterize this form of malarial disease are accompanied by the adhesion of infected erythrocytes to the vasculature of the brain. To study this phenomenon in vivo, an acute phase severe combined immunodeficiency (SCID) mouse model was developed in which sequestration of *P. falciparum*-infected human erythrocytes took place. During acute cerebral malaria in humans, the expression of intercellular adhesion molecule-1 (ICAM-1) is induced in vascular endothelium by inflammatory reactions. Acute phase ICAM-1 expression can also be obtained in SCID mice. The endothelium of the midbrain region was the most responsive to such inflammatory stimulus. It is noteworthy that the reticular formation in the midbrain controls the level of consciousness, and loss of consciousness is a symptom of cerebral malaria. We found that infected human erythrocytes were retained 24 times more than normal erythrocytes in ICAM-1-positive mouse brain. Sequestration to the brain was reduced by anti-ICAM-1 antibodies. These in vivo results were confirmed by the binding of *P. falciparum*-infected erythrocytes to the ICAM-1-positive endothelium in tissue sections of mouse brain. We conclude that the SCID mouse serves as a versatile in vivo model that allows the study of *P. falciparum*-infected erythrocyte adhesion as it occurs in human cerebral malaria. Upregulation of ICAM-1 expression in the region of the midbrain correlates with increased retention of malaria-infected erythrocytes and with the symptoms of cerebral malaria.

Plasmodium falciparum, one of the four plasmodium species that infect man, produces a fulminant disease that can lead to severe cerebral symptoms. Every year, this parasite causes about 250 million clinical cases of malaria (1). Approximately 2 million individuals, mainly children under 5 years of age, die from the main complications, which are cerebral malaria and severe anemia. A special feature of *P. falciparum* pathology is the absence of mature intraerythrocytic stages of the parasite in the circulating blood. Mature forms are found attached to the lining of microvessels in peripheral organs. This adhesion of infected erythrocytes to the vascular endothelium is called sequestration. Clinically severe neurological symptoms correlate with a much higher frequency of sequestered parasitized erythrocytes in the brain vasculature compared to other organs (2).

Sequestration is thought to depend on differences between *P. falciparum* strains leading to the expression of different erythrocyte surface molecules mediating adhesion to endothelia, as well as variation in the production of endotoxins eliciting

a TNF- α response by host macrophages (3). Thus, sequestration depends also on the susceptibility of the host to endotoxins, the subsequent regulation of adhesion molecules, and the host's level of immunity (4). A key role is played by TNF. Its plasma concentration levels are elevated in patients with fatal cerebral malaria (5), and it has been shown that homozygotes for the *TNF2* allele, which leads to a high production of TNF, carry an increased risk of developing cerebral malaria (6). The inflammatory cytokine TNF regulates the expression of the adhesion molecules intercellular adhesion molecule-1 (ICAM-1),¹ vascular adhesion molecule-1 (VCAM-1), and E- and P-selectin by vascular endothelium.

¹ Abbreviations used in this paper: APAAP, alkaline phosphatase/anti-alkaline phosphatase; ICAM, intercellular adhesion molecule-1; IRBC, *Plasmodium falciparum*-infected human erythrocytes; rhICAM-1, recombinant human ICAM-1; nmICAM-1, recombinant mouse ICAM-1 construct; SCID, severe combined immunodeficiency; VCAM-1, vascular cell adhesion molecule-1.

ICAM-1 has been identified *in vitro* as a receptor for *P. falciparum*-infected erythrocytes (IRBCs) (7). The expression of ICAM-1 is high during inflammation and mediates adhesion of leukocytes under physiological conditions.

The experimental study of *P. falciparum* sequestration is hampered by the fact that the characteristic pathology develops only in humans and in squirrel monkeys (8). Therefore, the analysis of the interaction of the parasitized erythrocytes with host endothelium is practically restricted to the examination of postmortem samples. Rodent malaria models are useful for the study of many aspects of the disease, but the pathology of murine plasmodium infections is different from that of human cerebral malaria, and sequestration of parasitized erythrocytes to the brain vasculature has not been observed in the models used so far, except for *Plasmodium yoelii* XL, where infection was not associated with inflammatory reactions in the brain (9, 10). An alternative is to investigate the adhesion of isolated *P. falciparum*-infected erythrocytes to cultured cell lines *in vitro*. The present paper describes a newly developed rodent model that allows the sequestration of *P. falciparum*-infected human erythrocytes to the brain *in vivo*. It involves the severe combined immunodeficient (SCID) mouse as a host for IRBC. For the acute phase of malaria, we present a humanized animal model in which the expression of adhesion molecules by vascular endothelium have been induced by an inflammatory stimulus. It can be used for quantitative evaluation, permitting identification of adhesion molecules involved in cerebral malaria.

Materials and Methods

Reagents

All culture media and supplements including BSA and AlbuMAX™ were obtained from Gibco Ltd. Glasgow (Paisley, Scotland). The rat antimurine ICAM-1 antibodies YN 1/1.7.4 (11, 12) were prepared from the hybridoma cell line CRL 1878 (American Type Culture Collection, Rockville, MD). The IgG fraction was purified by affinity chromatography using protein G-Sepharose Fast 4 (Pharmacia LKB, Uppsala, Sweden). Normal rat IgG was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Anti-rat antibodies (Z 494) and rat alkaline phosphatase/anti-alkaline phosphatase (APAAP) complex (Z 488) were obtained from Dakopatts (Glostrup, Denmark). LPS from *Salmonella typhosa* was obtained from Difco Laboratories (Detroit, MI).

Parasites

The *P. falciparum* strain ItG2.F6 was cultured *in vitro* according to the method of Trager and Jensen (13), but using serum-free medium instead. RPMI 1640 was supplemented with 25 mM Hepes (BDH Laboratories Supplies, Poole, England), 50 µg/ml hypoxanthine, 0.21% (wt/vol) NaHCO₃, 50 µg/ml neomycin sulfate, and 0.5% lipid-rich BSA (AlbuMAX™ I) as a substrate for human serum (14). Erythrocytes for passages were obtained from the Swiss Red Cross (Berne, Switzerland).

Mice were infected with the *Plasmodium berghei* ANKA strain.

Transfected Cells

Murine L cells, stably transfected with human ICAM-1, were a gift from Carl C. Figdor (Division of Immunology, The Nether-

lands Cancer Institute, Amsterdam, The Netherlands). These L ICAM-1 cells were cultured in IMDM supplemented with 1 mg/ml Geneticin (G-418 sulphate) and 5% FCS as described (15). Untransfected control L cells were maintained without Geneticin.

Animals

Female 6–8-wk-old BALB/c mice were supplied by Charles River Ltd. (Sulzfeld, Germany). Female C.B-17 SCID mice were bred to the age of 6–12 wk under specific pathogen-free breeding conditions at the Basel Institute for Immunology (Basel, Switzerland).

Experimental Infections and LPS Treatment

BALB/c mice were infected by exposure to parasitized *Anopheles stephensi* mosquitoes at the facilities of the Swiss Tropical Institute. These mice were held under normal breeding conditions during the infection and killed shortly before they died. Healthy BALB/c mice served as controls. SCID mice were injected intravenously with 10 µg LPS in 100 µl PBS 16 h before further experiments. An infrared lamp provided additional warmth during LPS treatment.

Immunoenzymatic Staining Technique for the Detection of ICAM-1

Samples. Frozen tissue sections were prepared from LPS-treated SCID mice or control mice receiving PBS (Fig. 1), and from *P. berghei* ANKA-infected and normal BALB/c mice (Fig. 2).

Brains were collected immediately after death and frozen in TissueTek (Miles Inc., Elkhart, IN). 7-µm sections were air dried and fixed in acetone at room temperature for 10 min, then incubated for 30 min with primary antibodies YN1/1.7.4 or control rat IgG (10 µg/ml in PBS containing 1% BSA). All incubations were carried out at room temperature in a humid chamber and followed by repeated washes in PBS or TBS. After the application of the secondary anti-rat IgG antibodies (1:100 in TBS/1% BSA), the sections were incubated with the rat APAAP complex (1:50 in TBS/1% BSA) and exposed to the substrate solution consisting of 0.2 mg/ml naphthol-AS-BI-phosphate (Fluka AG, Buchs, Switzerland), 0.4 mg/ml FastRed-TR salt (practical grade; Sigma Immunochemicals, St. Louis, MO) and 0.2 mg/ml Levamisol (Sigma) in Michaelis buffer (0.14 M sodium acetate [Merck, Darmstadt, Germany], 0.14 M 5,5-diethyl-barbituric acid sodium salt [Merck], pH 9.4). 30–40 min after color development, tissue sections were counterstained with Mayer's hematoxylin (Fluka) and mounted with Mowiol (16). Antibody-labeled tissue zones appeared red. Staining with normal rat IgG as the primary antibody showed a negative reaction.

Quantification. Several microscopical photographs in the regions of cerebellum, midbrain, and corpus striatum were taken. They were analyzed by two observers. Positively stained vessels seen in three randomly selected independent areas of 0.5 mm² per region were counted.

Adhesion of *P. falciparum*-infected Human Erythrocytes on L ICAM-1 Cells

Cytoadherence assays were performed as previously described (17, 18). Briefly, cultured IRBCs were diluted with binding medium (RPMI 1640, 25 mM Hepes, 0.5% Albu MAX™ adjusted to pH 7) to 5% hematocrit and 6–8% parasitemia. They were allowed to adhere for 1 h at 37°C on monolayers of L ICAM-1 cells, previously cultured for 48 h on glass slides (Semadeni, Ostermundigen, Switzerland). The slides were gently washed 4× with binding medium, air dried, fixed with methanol, stained with 5% Giemsa solution (Fluka) and mounted in Eukitt (Merck). Adherent IRBC

were counted by light microscopy in 4-mm² areas of six different cell monolayers.

Adhesion of P. falciparum-infected Human Erythrocytes on Coated Soluble Molecules

The generation of the recombinant mouse ICAM-1 construct (rmICAM-1, domains 1 and 2 coupled to mouse Ig κ constant region) is described elsewhere (19). Briefly, total RNA was isolated from endothelioma cells and reverse transcribed into cDNA by standard methods. The primers used for the amplification of the coding region for the extracellular domain were 5'-ATTAGAGCTCATGGCTTCAACCCGTGCCAAGCC-3' (5' primer) and 5'-TATACTTACCTTCGAAAGTCCGGAGGCTCTGGC-3' (3' primer). The amplified product was purified and inserted into the pHT4 vector containing the mouse Ig κ constant region as a fusion partner, cloned into competent *Escherichia coli* K803, and transfected into J558L myeloma cells by protoplast fusion. Supernatants were purified by affinity chromatography with goat anti-mouse κ mAbs (Southern Biotechnology Associates, Birmingham, AL). The purified molecules were equilibrated with PBS and frozen at -70°C. The recombinant human CD4 construct (rhCD4) used as control is a CD4-anti-CD3- κ Janusin molecule composed of the two first Ig-like domains of human CD4 linked to an Fv fragment specific for the human CD3 molecule and coupled to mouse κ (20). This molecule was a kind gift from A. Traunecker (Basel Institute for Immunology). The recombinant human ICAM-1 molecule (rhICAM-1) contains the five extracellular Ig-like domains and was a kind gift from J. E. Merritt (Roche Products Ltd., Welwyn Garden City, Hertfordshire, England). Drops of 15 μ l of soluble rhICAM-1, rmICAM-1, and rhCD4 (100 μ g/ml in PBS) were placed in bacterial plastic petri dishes (Greiner, O. Kleiner AG, Wohlen, Switzerland) and incubated in a humid chamber at 4°C overnight, washed with PBS and blocked with 1% BSA in PBS for 30 min at room temperature. The petri dishes were flooded with 10 ml of cultured IRBCs at 5% parasitemia, diluted to 0.5% hematocrit with culture medium. IRBCs were allowed to adhere for 1 h at 37°C in an atmosphere of 3% O₂, 4% CO₂, 93% N₂. The dishes were gently washed with culture medium (6 \times), air dried, fixed with methanol, and stained with 5% Giemsa solution (Fluka). IRBCs adhering to each coated area were counted by light microscopy.

Adhesion of IRBCs on Frozen Sections

In situ binding of IRBCs was performed in analogy to a leukocyte binding assay described previously (21). Freshly prepared frozen sections (brain and lung) of LPS-primed and control SCID mice were preincubated with binding medium and placed in a humid chamber, before IRBCs were allowed to adhere for 1 h at room temperature with gentle agitation. IRBCs from suspension culture were used at 0.1% hematocrit at a parasitemia of 10%. The medium was decanted from the slides and the sections were immersed in 4% paraformaldehyde (Merck) in Dulbecco's PBS which fixed adherent cells. They were then stained with 5% Giemsa solution, air dried, and mounted with Eukitt for microscopic evaluation.

Analysis of Brain Sections. Bound pRBCs were counted by light microscopy. At least four different areas of 60 fields (at a magnification of 100) were counted in the regions of cerebellum and mid-brain on brain sections.

Analysis of Lung Sections. A minimum of 150 IRBCs were counted in each section. Each experiment was carried out using three separate sections from an organ.

Inhibition experiments with the antibody YN1/1.7.4 were per-

formed with brain sections from LPS-primed mice. IRBC suspension was used at a parasitaemia of 6%. Both IRBC and brain and lung sections were preincubated using PBS containing 10 μ g/ml antibody (IRBCs, 30 min on ice; sections, 30 min at room temperature). All other experimental conditions were as described above. Each experiment was carried out using five different sections.

In Vivo Sequestration Experiments

Radioactively labeled mature forms of IRBCs were prepared in vitro. IRBC cultures of 5% hematocrit were expanded and synchronized by sorbitol treatment (22) so that cultures contained mainly young ring form parasites 24 h before the injection into mice. Subsequent labeling of parasite-containing erythrocytes with radioactive [³H]hypoxanthine (2 μ Ci/ml culture; Amersham International, Buckinghamshire, England) was carried out overnight during the maturation of the parasites to the late trophozoite stage in hypoxanthine-free culture medium. The medium of labeled cultures was exchanged several times with medium containing cold hypoxanthine to remove free radioactivity. Uninfected human RBC, which are normally used for IRBC culture, were labeled with ⁵¹Cr by a 30-min incubation of packed RBCs with 300 μ Ci of sodium chromate/ml (Amersham) of cells at 37°C. The cells were washed with ice-cold medium and stored for 30 min at room temperature before the experiment. Labeled IRBCs and RBCs were resuspended in PBS at 5% hematocrit shortly before the injection into the SCID mice.

Sequestration Experiment. Normal and LPS-treated mice were injected intravenously with 200 μ l of the erythrocyte suspension. Labeled infected and normal human erythrocytes were allowed to circulate for 3 h before the mice were killed by exposure to CO₂. The organs were excised and washed in PBS. They were lysed in 2 ml Soluene 350 (Packard Instruments Inc., Meriden, CT) during 12 h at 40°C and resuspended in 18 ml Picofluor (Packard) for β -scintillation counting of ³H incorporation, or they were counted as intact organs for γ -radiation of ⁵¹Cr. The organ retention of adherent normal RBCs and IRBCs was calculated using weight and radioactivity of each organ. The recovery of radioactivity in the dissected organs represented 42–45% ³H and 53–57% ⁵¹Cr of the injected dose.

The antibody YN1/1.7.4 was administered intravenously along with the IRBC suspension (0.2 mg per mouse).

Statistical Analysis

Significance analysis was performed using the Student's *t* test for two unpaired samples with equal variance.

Results

To study acute-phase sequestration of IRBCs in vivo, we developed an animal model using the SCID mouse. Since this mouse has a hampered immune system, it tolerates the presence of human and malaria antigens.

The acute phase includes the production of inflammatory cytokines, which in turn induce expression of adhesion molecules by vascular endothelium of the brain that are essential for sequestration of circulating IRBCs. The inducible IRBC receptor ICAM-1 is an adhesion molecule expressed by cytokine-activated endothelial cells (23). It was found to bind IRBC in vitro (7). During acute-phase malaria, ICAM-1 is expressed by the vascular endothelium of the brain (24–27). In the SCID mice used as Plasmodium hosts in these experi-

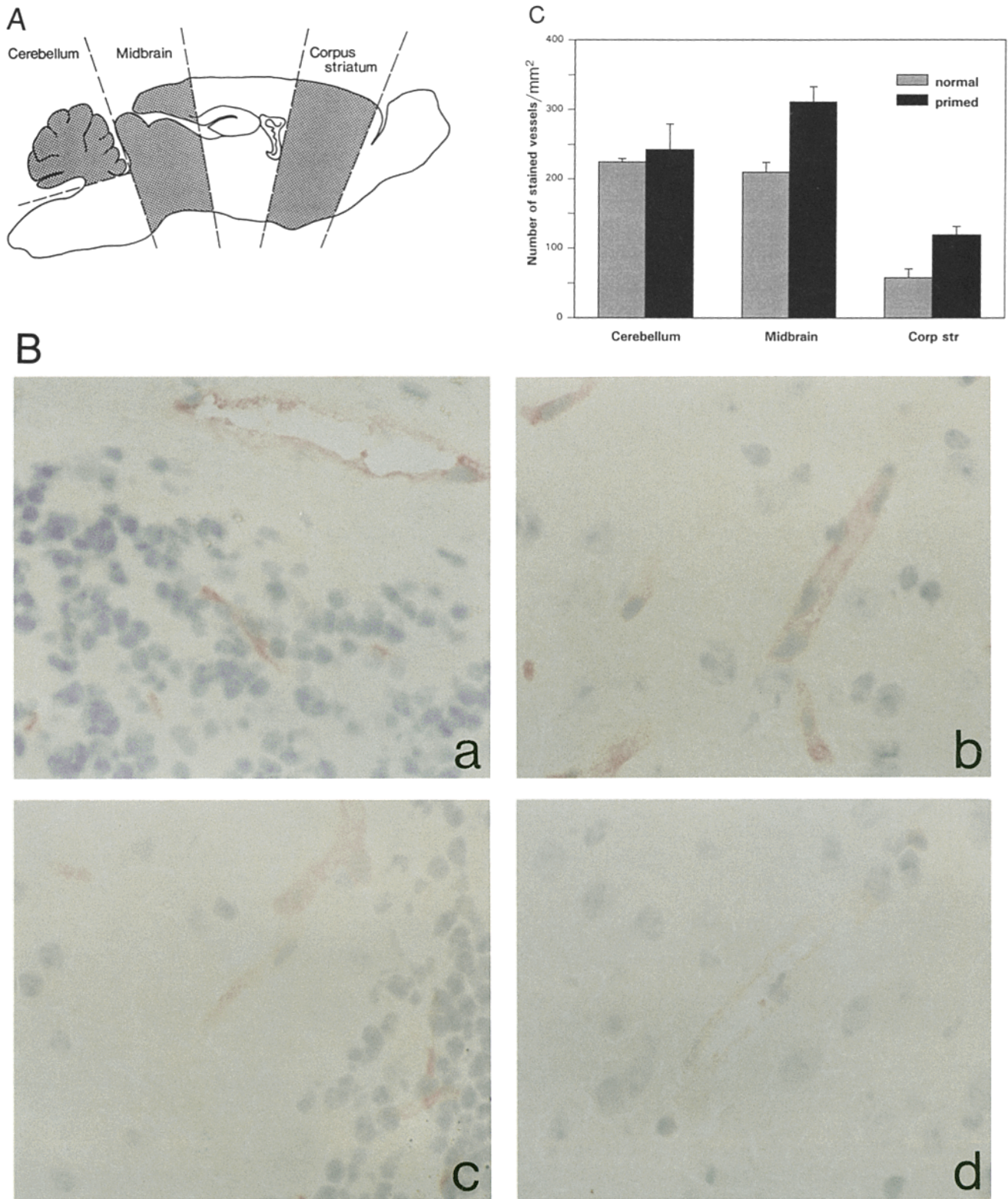


Figure 1. Effect of LPS priming on the expression of ICAM-1 in the brains of SCID mice. SCID mice were treated with LPS (10 $\mu\text{g}/\text{mouse}$) during 16 h. Brain tissue was embedded and sagittal sections were stained with anti-ICAM-1 antibody (YN 1/1.7.4) using the APAAP technique; cell structures were counterstained with hematoxylin. (A) Map of sagittal section of a mouse brain. Indicated regions were of interest for analysis since they showed differential regulation of ICAM-1 expression. (B) Photographs represent regions of cerebellum (a) and midbrain (b) of LPS-primed mice, as well as the corresponding regions (c and d) of normal control mice. They display longitudinal and cross-sections of several venules, whose endothelial cell nuclei are clearly evident. Positively stained endothelial cells appear red ($\times 680$). (C) Quantification of ICAM-1 expression in the brain of SCID mice. Photographs of APAAP-stained brain sections of LPS-primed and normal SCID mice were analyzed. Positive blood vessels in 0.5-mm² areas were counted. Three independent areas per indicated region in the brain were examined. LPS treatment induced ICAM-1 expression by endothelial cells in the region of midbrain and corpus striatum.

ments, acute-phase ICAM-1 expression was induced by treatment with LPS. The expression of ICAM-1 was analyzed in different regions of the brain and compared with that of untreated animals. ICAM-1 expression was visualized using the APAAP technique. LPS treatment resulted in upregulated ICAM-1 expression on the endothelial surface of small and large vessels in midbrain and corpus striatum (Fig. 1, B and C). The number of positive vessels found in the cerebellum was not influenced by LPS induction, but the level of expression was increased. Normal SCID mice injected with PBS only, showed a small number of vessels expressing ICAM-1 in midbrain and corpus striatum; additionally, the expression level was extremely low (Fig. 1, B and C).

Upregulation of ICAM-1 expression by brain endothelium of SCID mice was further compared with that found in mice suffering from acute-phase murine malaria resulting from infection with *P. berghei* ANKA. The expression of ICAM-1 in terminally ill animals was identical to that of LPS-primed SCID mice, suggesting that the SCID mouse can indeed be used as a model for the acute-stage disease (Fig. 2). In contrast to human malaria, erythrocytes infected with the murine plasmodium species do not adhere to ICAM-1 expression vascular endothelium (9). In murine *P. berghei* malaria, ICAM-1 expression is known to be responsible for massive infiltration of leucocytes causing a different cerebral pathology. Although the *P. berghei* mouse model is useful for the investigation of other aspects of malaria disease, sequestration to the brain may only be studied with IRBCs.

The organ retention of normal and *P. falciparum*-infected human RBCs in SCID mice was determined for brain, lung,

spleen, kidney, liver, and heart, 3 h after the injection of labeled infected [^3H]hypoxanthine) and normal human erythrocytes (^{51}Cr). The most striking difference between infected and normal human erythrocytes in the LPS-induced, ICAM-1-positive SCID mouse was found in the brain. Retention of parasitized human erythrocytes was 24 times higher than that of uninfected ones, which indicated that there was specific adhesion of infected erythrocytes to brain endothelium (Table 1 A). In comparison to the control SCID mouse, in which ICAM-1 had not been induced, the number of infected erythrocytes sequestered in the brain was significantly increased in the ICAM-1-positive mouse (Table 1 B). The amount of parasitized erythrocytes sequestered to the brain, lung, and heart was elevated, though there were fewer in the spleen. In contrast, control mice accumulated infected erythrocytes in the spleen.

The involvement of ICAM-1 in adhesion of IRBCs to the brain of ICAM-1-positive mice was investigated by the injection of anti-ICAM-1 antibodies along with IRBCs (Table 2). The antibody inhibited adhesion of IRBCs in the brain by 34%, and this reduction was highly significant. The antibody treatment had no inhibitory effect on the adhesion of IRBCs in other organs; IRBC retention in the lung was actually increased in antibody-treated animals; however, this aspect was not further pursued. The number of IRBCs remaining in the blood circulation of antibody treated mice increased by 100% compared to control animals (Table 2 legend).

The determination of the adhesion of IRBC to ICAM-1 in vitro showed that mature forms bound with high efficiency

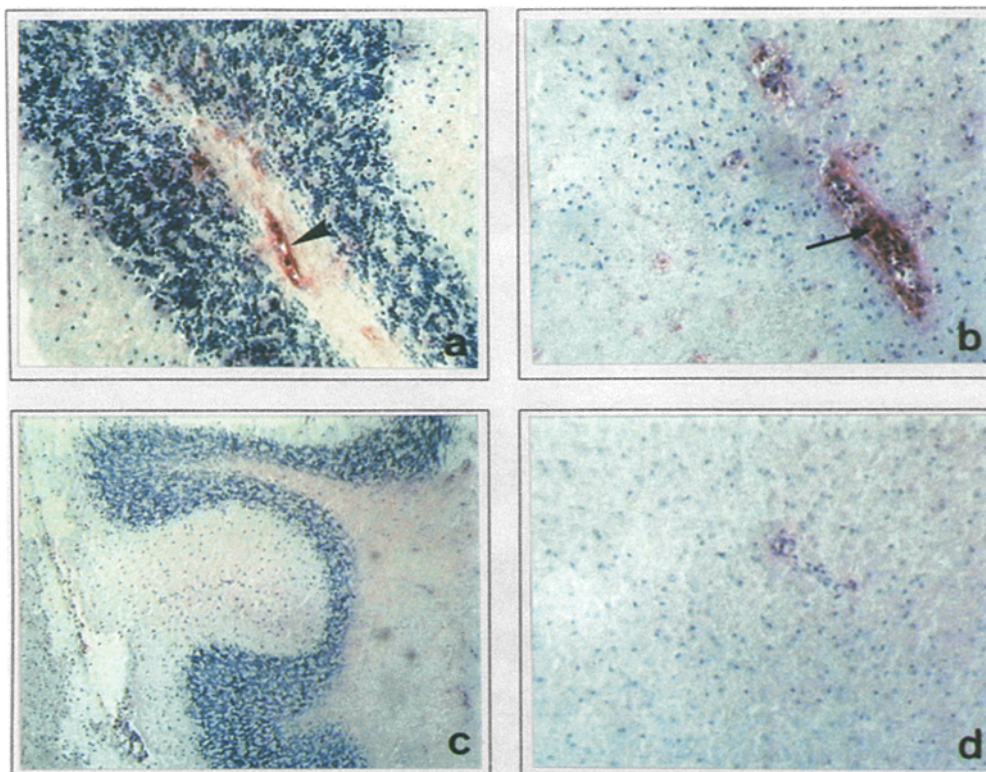


Figure 2. Effect of *Plasmodium berghei*-infection on the expression of ICAM-1 by brain endothelium. BALB/c mice were infected with *P. berghei* ANKA. Brain tissue of mice that were terminally ill were embedded, and sagittal sections were incubated with anti-ICAM-1 antibody (YN1/1.7.4) using the APAAP technique. Antibody-labeled tissue zones appear red and other cell structures are counterstained with hematoxylin. Photographs show a region of cerebellum (a) and midbrain (b) of infected mice, and corresponding regions (c and d) of noninfected control mice. ICAM-1 expression by endothelial cells lining the blood vessels (arrowhead) was strongly induced after infection. ICAM-1 was also expressed on activated leukocytes (arrow). ($\times 80$ in a, b, and d or $\times 40$ in c).

Table 1. Frequency of Normal Human and IRBCs in Organs of SCID Mice

Organ	IRBC	RBC	Ratio
A.			
Brain	5.8% (415.677 ± 48.642)	0.24% (2.300 ± 720)	24
Lung	7.1% (507.998 ± 97.226)	4.0% (38.315 ± 9.634)	1.8
Spleen	8.0% (576.806 ± 209.015)	10.0% (95.062 ± 47.348)	0.8
Liver	71.6% (5.146.360 ± 777.436)	79.0% (749.263 ± 47.398)	0.9
Kidney	4.8% (343.987 ± 55.790)	5.2% (49.668 ± 28.884)	0.9
Heart	2.8% (201.063 ± 28.208)	1.5% (14.572 ± 4.145)	1.9
B.			
Brain	4.6% (308.100 ± 48.102)	0.37% (3.764 ± 1.182)	12
Lung	5.4% (360.865 ± 88.010)	4.0% (40.867 ± 11.201)	1.4
Spleen	13.3% (887.034 ± 299.285)	6.8% (69.820 ± 31.108)	2.0
Liver	69.2% (4.627.482 ± 583.432)	81.9% (842.906 ± 82.663)	0.8
Kidney	4.5% (303.676 ± 41.342)	4.1% (42.537 ± 17.295)	1.1
Heart	3.0% (202.879 ± 42.069)	2.9% (29.699 ± 9.366)	1.0

The retention of IRBCs (strain ItG2.F6) was compared with the retention of normal human RBCs in LPS-primed (A) and control (B) SCID mice. Cultured IRBCs labeled with [³H]hypoxanthine and normal human RBCs labeled with ⁵¹Cr were injected intravenously into SCID mice. LPS (10 μg) was injected 16 h before the injection of erythrocytes. 3 h after the injection of erythrocytes, the mice were killed and dissected. The organs were washed in PBS, weighed, lysed, and the radioactivity was counted. Recovery of counts in analyzed organs was 42–45% for IRBCs and 53–57% for RBCs respectively. The number of counts for each organ was calculated using the average organ weights and disintegrations per minute per gram of tissue. The total counts of all recovered organs was taken as 100%. When compared with normal human RBCs, IRBC retention in brain was increased by a factor of 24 in LPS-primed and by a factor of 12 in control mice. LPS priming increased the adhesion of IRBCs significantly in brain ($P = 0.001$) and lung ($P = 0.007$), and decreased it in the spleen ($P = 0.031$). $n = 8$ for IRBC, $n = 4$ for RBC per group. Numbers in brackets represent counts per minute per organ.

to murine L cells transfected with human ICAM-1, while infected erythrocytes did not adhere to nontransfected control L cells (data not shown). Furthermore, IRBC adhered to recombinant mouse and human ICAM-1 coated to the

Table 2. Effect of the Anti-ICAM-1 Antibody on the Retention of IRBCs in LPS-primed SCID Mice

Organ	Control	YN 1/1.7.4	P
Brain	4.2% (130.983 ± 10.374)	2.6% (86.536 ± 7.869)	0.001
Lung	6.9% (214.697 ± 34.439)	15.9% (536.394 ± 113.207)	0.013
Spleen	8.3% (260.217 ± 115.087)	7.6% (256.731 ± 158.733)	0.979
Liver	74.5% (2.290.155 ± 281.050)	68.1% (2.302.590)	*
Kidney	4.0% (125.126 ± 3.317)	3.4% (115.561 ± 15.719)	0.455
Heart	2.2% (67.581 ± 7.303)	2.5% (83.670)	*

The effect of the anti-ICAM-1 antibody YN 1/1.7.4 on the retention of IRBC in several organs of LPS-primed SCID mice. Experimental conditions were as described in Table 1. The antibody (0.2 mg IgG per mouse) was injected along with IRBC. The anti-ICAM-1 antibody reduced adhesion of IRBC in the brain by 34%; the blood of these mice contained significantly more IRBC than control mice (YN 1/1.7.4, 906.592 ± 178.565 cpm/g; control, 455.308 ± 139.251 cpm/g; $P = 0.002$). $n =$ five per group.

* Liver and heart were taken from one animal. Numbers in brackets represent counts per minute per organ.

surface of culture dishes (Table 3). rhCD4 and BSA served as control and showed marginal IRBC binding, i.e., adhesion to mouse and human ICAM-1 was significant. Human ICAM-1 was three times more efficient in IRBC binding than mouse ICAM-1. This may partially result from the composition difference of the constructs; mouse ICAM-1 contains only the first two Ig-like domains, whereas human ICAM-1 is composed of all five extracellular domains.

The interaction of IRBCs with vascular endothelium in the brain was examined by an adhesion assay using frozen sections of normal and ICAM-1-positive SCID mice (Fig. 3 A). The IRBCs had been allowed to adhere to the sections of brain and lung tissue. Brain regions shown on the map of Fig. 2 A were evaluated separately. Infected erythrocytes adhering to the endothelial lining of the venules of the mid-brain region and the cerebellum of an ICAM-1-positive mouse are shown in Fig. 3 A, a and b. Note the clearly visible mature *P. falciparum* trophozoites located in the cytoplasm of the erythrocytes in contact with the endothelium. LPS induction caused a significant increase in IRBC adhesion to the midbrain. The IRBC binding to cerebellum was the same in control and LPS-induced mice, and it correlated with the constitutive ICAM-1 expression found in this region of the brain (Fig. 3 B). Anti-ICAM-1 antibodies inhibited IRBC adhesion in the region of the midbrain and the cerebellum by 63 and 40% respectively (Fig. 4). The lung vasculature of both normal and ICAM-1-induced mice showed a high

Table 3. Adhesion of IRBCs to Recombinant ICAM-1

Coated molecule	Adhering IRBC per area (n = 4)
BSA	11.1 ± 1.8
rhCD4	10.7 ± 3.0
rmICAM-1	61.8 ± 2.3
rhICAM-1	206.0 ± 9.2

rmICAM-1, rhICAM-1, and the controls, rhCD4 and BSA, were coated to plastic culture dishes as 15- μ l drops at a concentration of 100 μ g/ml at 4°C overnight. Cultured IRBC were allowed to adhere to the coated surface for 1 h at 37°C. Subsequently, the dishes were washed, air dried, and stained with Giemsa solution. Bound IRBC per coated area were counted by light microscopy.

level of ICAM-1 expression (data not shown). In this tissue, IRBCs adhered to venules and arterioles (Fig. 3 A, c). Binding efficiency to lung tissue was generally higher than binding to brain. This may result from the fact that the lung endothelium of animals kept under normal breeding conditions expressed ICAM-1 irrespective of induction by LPS, and the lungs contain more endothelial surfaces than the brain (Fig. 3 B).

In conclusion, adhesion of IRBCs was more efficient in tissue with high ICAM-1 expression and was partially inhibited by anti-ICAM-1 antibodies. This indicates that IRBCs use ICAM-1 as adhesion receptors on vascular endothelium.

Discussion

Sequestration of infected erythrocytes is an important feature of human acute phase malaria. In this report, we present a mouse model for the acute phase of malaria, in which the sequestration of IRBCs in internal organs can be studied in vivo. This humanized mouse model allowed quantification of IRBC adhesion to vascular endothelium, and we examined the distribution of sequestered IRBC in different organs, including the brain. We were able to show that binding was related to the presence of ICAM-1 by demonstrating for the first time that anti-ICAM-1 antibodies are able to reduce cytoadherence of malaria-infected erythrocytes to the microvasculature of the brain. The in vivo findings were verified by binding of IRBC to brain tissue sections, on which they adhered predominantly to endothelium displaying elevated ICAM-1 expression.

Upregulation of ICAM-1 expression in the vascular endothelium is reported for human (24), simian (25), and murine (26, 27) severe malaria. It has been demonstrated that ICAM-1 is coexpressed with CD36, thrombospondin, VCAM-1, and E-selectin on the brain microvasculature of postmortem samples of patients who had died of cerebral malaria. None of these adhesion molecules were detected in samples of patients who had died of other causes (24). Al-

though their presence in the brain of malaria patients was demonstrated, neither the expression level nor the precise distribution was described. ICAM-1 expression in the brain can also be induced by LPS treatment. Its expression pattern was similar in LPS-treated animals and in terminally ill *P. berghei*-infected mice. VCAM-1 expression was also investigated and showed the same results (Willimann, K., and B. A. Imhof, unpublished observation). The careful analysis of ICAM-1 and VCAM-1 expression in the brain of SCID mice showed that ICAM-1 was induced in midbrain and corpus striatum on the surface of small and large vessels, whereas VCAM-1 was restricted to the macrovasculature (data not shown). This explained the 10-fold higher number of ICAM-1-positive vessels as compared to VCAM-1. Since small vessels are predominant in cerebral tissue, ICAM-1 may play a major role in cerebral sequestration. Furthermore, it could be speculated that constitutive basal expression of ICAM-1 in small vessels might lead to primary adhesion of a few IRBCs, which in turn lead to the production of ICAM-1-inducing cytokines (28–30). This could result in focal clustering of large numbers of erythrocytes and local elevated concentrations of endotoxins initiating vascular pathology (31, 32). Our acute phase mouse model represents the final stage of such an activation cascade.

In a study of IRBC binding to ICAM-1, it was established by mAb domain assignment that a specific binding site for malaria-infected erythrocytes that were repeatedly selected for ICAM-1 binding is located in domain 1 of human ICAM-1 (33, 34). Molecular modeling defined a malaria binding site distinct from the one for lymphocyte functional antigen-1 (LFA-1), the physiological ligand of ICAM-1. Erythrocytes infected with the *P. falciparum* strain selected for strong binding to human ICAM-1 bound to recombinant mouse ICAM-1 with low efficiency. These data were obtained by static in vitro binding assays and may not represent physiological conditions. In our in vivo assays, we used nonselected malaria strains representing a heterogeneous population. These strains bound to several adhesion molecules including ICAM-1, but with low affinity (Table 3, reference 35). Certain malaria strains selected for ICAM-1 binding displayed a reduced ability to interact with other adhesion molecules, such as CD36 (35). This may suggest that high and low affinity binding sites exist on ICAM-1. In contrast, another set of experiments showed that IRBCs selected for their adhesion to endothelial cells acquired the capacity of ICAM-1 binding while they maintained the ability to bind to CD36 (36). The antibody YN1/1.7.4 blocked the interaction of mouse LFA-1 with mouse ICAM-1 in vitro (11, 12), and it interfered with the adhesion of human IRBCs in the mouse brain in vivo. This locates the low affinity malaria binding site close to the LFA-1 binding domain, which clearly separates it from the well-defined high affinity binding site. Since normal malaria infection is never caused by cloned parasites and anti-ICAM-1 antibodies reduced the number of IRBC that were sequestered in the brain in our experiments, it is conceivable that the human-mouse model represents the physiological condition of sequestration as a result of low affinity binding of IRBC. The adhesion of human malaria-infected erythrocytes to rodent tissue was also demonstrated by Raventos-Suarez et

A

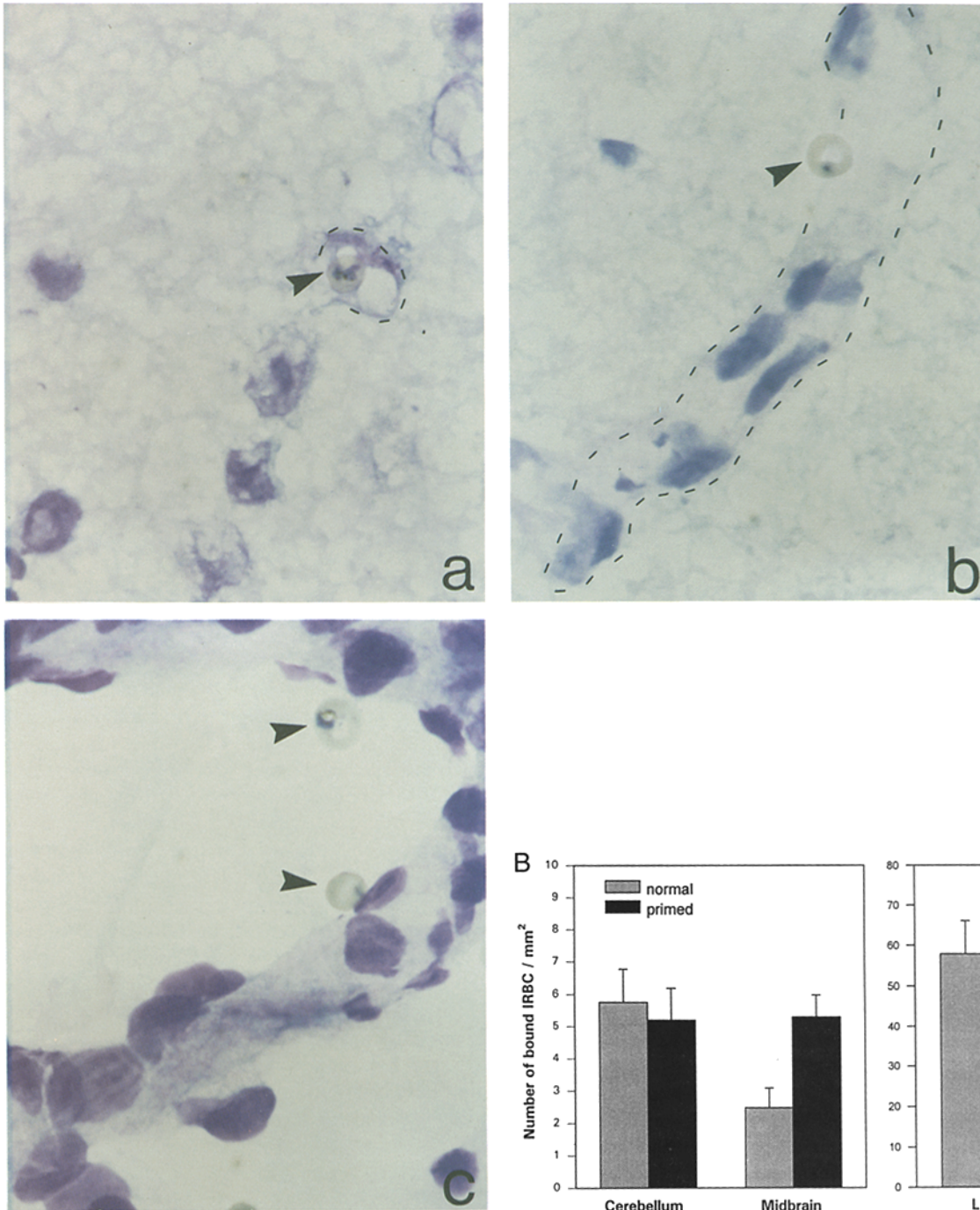


Figure 3. Binding of parasitized RBC to brain and lung tissue sections of LPS-primed and normal SCID mice. IRBC (strain ItG2.F6) from suspension culture were allowed to adhere for 1 h at room temperature to freshly prepared frozen sections of LPS-primed and normal SCID mice. Adhering cells were fixed with paraformaldehyde and stained with Giemsa solution. (A) Photographs showing IRBCs adhering to brain (a and b) and lung (c) endothelium of an LPS-primed mouse; arrowheads point to adherent IRBCs ($\times 720$). (B) Quantification of IRBCs adhering to frozen sections of lung and brain tissue. Two different observers counted at least four different areas of 60 fields (magnification of the microscope objective was 100) in the region of the cerebellum and midbrain, and a minimum of 600 IRBCs in the lung. Each experiment was carried out in triplicate. LPS induction caused increased adhesion of IRBCs in the midbrain ($P = 0.005$). In the lung, where expression of ICAM-1 by vascular endothelium was constitutively high, the IRBC binding was high, irrespective of LPS treatment.

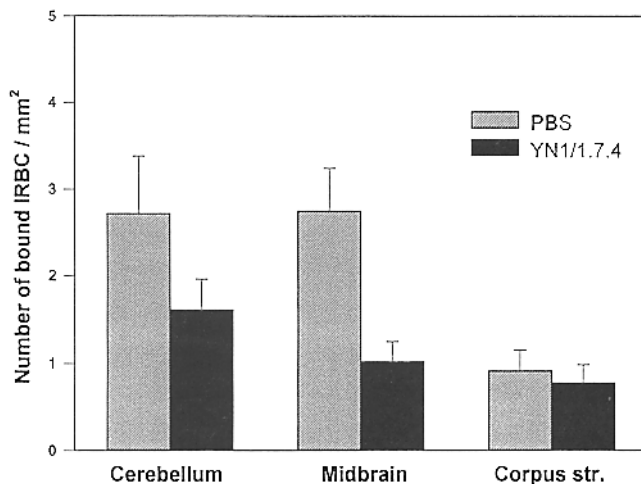


Figure 4. Inhibition of IRBC binding to brain tissue of LPS-primed SCID mice by anti-ICAM-1 antibodies. The effect of the anti-ICAM-1 antibody YN1/1.7.4 on the binding of IRBCs in different regions of the brains of LPS-primed SCID mice. Quantification and experimental conditions were as described in Fig. 3 B, but sections and parasites were preincubated with 10 μ g/ml antibody in PBS or PBS only. Each experiment was carried out with five different sections. The anti-ICAM-1 antibody reduced adhesion of IRBC in the cerebellum by 40% and in the midbrain by 63%.

al. They studied microvascular obstruction by *P. falciparum*-infected human erythrocytes *ex vivo* in the isolated perfused rat mesocaecum (37). The addition of IRBC to the perfusate was shown to slow down or block *in vitro* blood flow, as a result of cytoadherence to the vascular endothelium. These data demonstrate that adhesion experiments performed *in vivo* or under flow conditions can provide valuable additional information on malaria sequestration. It was previously shown that anti-mouse ICAM-1 antibodies were ineffective against cerebral pathology (26, 27) when injected into mice bearing acute *P. berghei* malaria. This is not astonishing since murine erythrocytes infected with *P. berghei* do not sequester. Their presence leads to cytokine release and subsequent accumulation of activated monocytes in the brain vasculature of susceptible mice. In the *P. falciparum*-SCID mouse model, the antibodies interfered specifically with the adhesion of human IRBC in the brain, reducing their retention by one third. This may indicate that the expression of adhesion ligands by the infected red cell is plasmodium species specific and that binding to ICAM-1 is restricted to *P. falciparum*. Such a concept is supported by the fact that humans infected with *Plasmodium vivax* have high TNF levels in blood circulation that are able to induce ICAM-1 expression, but sequestration of infected erythrocytes has not been documented (38). The limited number of infected erythrocytes that can be transferred into a SCID mouse makes it difficult to quantify *in vivo* sequestration by light microscopy. Although occasional parasitized erythrocytes were seen in histological specimens, their number was too small to allow statistical evaluation. The use of radioactively labeled infected erythrocytes facilitated quantification and permitted the examination of whole-organ

samples. Control injection of labeled normal human erythrocytes clearly distinguished the retention of IRBC from the organ distribution of uninfected erythrocytes in the SCID mouse. Finally, IRBC sequestration was determined by the ratio of parasitized to uninfected erythrocyte retained in an organ. In the acute phase mouse model, the IRBC sequestration occurred in various organs, being most pronounced in the brain and most minor in lung and heart. Thus, the pattern in the primed SCID mouse resembles the pattern reported for human cerebral malaria (2, 39).

The clinical picture of severe malaria disease in humans reveals involvement of vital organs. The most severe manifestation of an organ dysfunction is found with cerebral malaria. Cerebral malaria begins fulminantly with a generalized convulsion followed by loss of consciousness. Further characteristic symptoms in comatose patients are opisthotonus and disconjugate gaze (38). So far, the precise mechanism for the development of these clinical symptoms remains unclear; however, adhesion molecule-mediated sequestration of *P. falciparum*-infected erythrocytes seems to be the main pathologic event. In this study, we obtained evidence for the differential upregulation of ICAM-1 expression in distinct brain areas, suggesting a correlation of cerebral symptoms with disturbances in specific regions. If high ICAM-1 expression is taken as an indicator for the inflammation of the endothelium, the midbrain region was shown to be most sensitive to the inflammatory stimulus. LPS stimulation increased ICAM-1 expression in the midbrain dramatically, whereas the constitutive production of ICAM-1 in the endothelium of the cerebellum was affected minorly. The location of the reticular formation, the "activating system" of the central nervous system, in the midbrain region of the brainstem may have implications for the clinical symptoms produced. The reticular formation controls muscle tone, respiration, blood flow, and general activity of the cerebral cortex, which determines the level of consciousness. Pronounced reduction of the activity of the reticular formation is associated with unconsciousness (40). Furthermore, ascending connections to the cerebellum are involved in the control of quite specific motor tasks such as the coordination of eye movement. It is therefore probable that pronounced inflammation in this area, leading to the local production of toxic mediators, could cause the specific neurological dysfunctions observed in cerebral malaria. Additionally, it is likely that impaired flow and metabolic disturbances resulting from the active metabolism of adherent, parasitized cells could also contribute to local toxicity, enhancing the derangement of neuronal function (38).

We conclude that the SCID mouse model is a versatile *in vivo* model for the investigation of acute phase sequestration of human malaria. It allowed the localization of a highly responsive zone in the brain that reacts to the inflammatory stimulus caused by malaria infection. Increased ICAM-1-mediated adhesion of IRBCs was observed in the midbrain region of the brainstem, a zone responsible for the level of consciousness. Thus, the humanized mouse model offers an experimental platform for the investigation of the molecular basis of *P. falciparum* sequestration. Further problems may be addressed under conditions that closely resemble the human disease.

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