Increased Survival in B-Cell-Deficient Mice during Experimental Cerebral Malaria Suggests a Role for Circulating Immune Complexes

Rosane B. de Oliveira, Jennifer P. Wang, Sanjay Ram, Ricardo T. Gazzinelli, Robert W. Finberg, Douglas T. Golenbock

R.W.F. and D.T.G. contributed equally to this article.

ABSTRACT The pathogenesis of malaria, an insect-borne disease that takes millions of lives every year, is still not fully understood. Complement receptor 1 (CR1) has been described as a receptor for Plasmodium falciparum, which causes cerebral malaria in humans. We investigated the role of CR1 in an experimental model of cerebral malaria. Transgenic mice expressing human CR1 (hCR1+) on erythrocytes were infected with Plasmodium berghei ANKA and developed cerebral malaria. No difference in survival was observed in hCR1+ mice compared to wild-type mice following infection with P. berghei ANKA; however, hCR1 detection was significantly diminished on erythrocytes between days 7 and 10 postinfection. hCR1 levels returned to baseline by day 17 postinfection in surviving animals. Immunoblot assays revealed that total erythrocyte hCR1 levels were diminished, confirming that immune complexes in association with erythrocyte hCR1 were likely removed from erythrocytes in vivo by clearance following immune adherence. Decreases in hCR1 were completely dependent on C3 expression, as mice treated with cobra venom factor (which consumes and depletes C3) retained hCR1 on erythrocytes during C3 depletion through day 7; erythrocyte hCR1 decreases were observed only when C3 levels recovered on day 9. B-cell-deficient mice exhibit a marked increase in survival following infection with P. berghei ANKA, which suggests that immune complexes play a central role in the pathogenesis of experimental cerebral malaria. Together, our findings highlight the importance of complement and immune complexes in experimental cerebral malaria.

IMPORTANCE Cerebral malaria is a deadly complication of infection with Plasmodium falciparum. Despite its high prevalence, relatively little is understood about its pathogenesis. We have determined that immune complexes are generated and deposited on erythrocytes specifically expressing human complement receptor 1 in a mouse model of cerebral malaria. We also provide evidence demonstrating the importance of immunoglobulins in the pathogenesis of cerebral malaria in mice. These findings may have important implications in human cerebral malaria.
The role of the complement system in the pathogenesis of several diseases has been increasingly recognized (20–24). Complement proteins or receptors may modulate the course of malaria in distinct ways. C5^−/− mice have a slight survival advantage in cerebral malaria (25), while others found that C3^−/− mice have no survival advantage (26). Hematin has been shown to activate the alternative pathway on erythrocytes (27). Human complement receptor 1 (hCR1) has been reported to serve as a receptor for Plasmodium falciparum invasion via direct binding of the parasite ligand (28). Erythrocyte CR1 is also involved in the rosette formation of uninfected erythrocytes with P. falciparum-infected erythrocytes (29). CR1 plays an important role in the control of complement activation and also serves as the “immune adherence receptor” to facilitate clearance of immune complexes (ICs), as IC-coated erythrocytes traverse macrophage-lined liver and spleen sinusoids. ICs activate complement, which results in the deposition of C3 and C4 fragments on the ICs. By virtue of its ability to bind to C3 and C4 fragments, erythrocyte CR1 binds to complement-coated ICs, and CR1 and complement-coated ICs together are cleared from the circulation as erythrocytes traverse the liver and spleen. Following removal of complement-coated ICs and CR1, erythrocytes return to the circulation. ATP release by the IC-coated erythrocyte may promote CR1 clustering to increase avidity of binding of complement-coated ICs with CR1 and also facilitate the phagocytosis of immune-adherent ICs (30).

We investigated whether murine models of Plasmodium infection could be used to address the roles of complement, ICs, and erythrocyte CR1 during malaria. Because normal murine erythrocytes do not express CR1, we employed transgenic mice that express hCR1 on their erythrocytes (31) to elucidate the role of human erythrocyte CR1 and circulating immune complexes (CICs) during experimental cerebral malaria. We found that infecting either wild-type or human CR1 transgenic mice with P. berghei ANKA results in equal rates of lethal cerebral malaria. Strikingly, a transient but reproducible reduction in erythrocyte CR1 levels is observed following infection. We sought to determine the mechanism by which this decrease in erythrocyte CR1 occurs.

**RESULTS**

**The presence of erythrocyte CR1 does not influence the disease course in murine malaria.** Infections with P. berghei ANKA are typically established by an intraperitoneal injection of 10^4 to 10^5 infected erythrocytes simultaneously exhibiting all of the parasite developmental stages in the blood. Experimental cerebral malaria (ECM) develops in susceptible mice between 6 and 8 days postinfection and is a major cause of mortality. Following infection with 10^5 infected erythrocytes, the following signs of cerebral malaria were used to score disease severity in wild-type C57BL/6 and hCR1 transgenic (hCR1^+/−) mice: ruffled fur, abnormal posture, disturbances in balance, limb paralysis, convulsion, coma, and death. No significant differences were observed in either morbidity or survival in hCR1^+/− mice versus wild-type mice (Fig. 1) or...
disease severity (data not shown). In addition, parasitemia levels were similar between hCR1/H11001 and wild-type mice (Fig. 1B). Erythrocytes were monitored by expression of the TER-119 antigen, a 52-kDa glycophorin A-associated protein that is expressed from the early proerythroblast stage to mature erythrocytes (32), and parasites were stained with ethidium bromide according to previously published methods (33).

hCR1 on erythrocytes decreases after mice are infected with *P. berghei* ANKA. Flow cytometric analysis of hCR1 levels during the course of *P. berghei* ANKA infection revealed an apparent loss of CR1 expression on erythrocytes that is greatest between 7 and 10 days postinfection, though some decreases can be detected as early as day 5 postinfection (Fig. 2A). Detection of hCR1 was restored by day 17 postinfection in surviving mice. By day 17, the presence of a second high-CR1-staining peak suggested that new erythrocytes entered the circulation during infection, likely in response to infection-related anemia (Fig. 2A). The average values of hCR1 detected in mice through day 25 following infection are plotted in Fig. 2B. As expected, some animals succumbed to disease over time.

To ensure that the diminished erythrocyte CR1 levels observed by flow cytometry were not the result of ICs binding to CR1, thereby restricting access of the anti-CR1 antibody to its epitope on CR1, we performed immunoblotting assays on erythrocytes from hCR1/H11001 mice infected with *P. berghei* ANKA. Decreases in hCR1 were observed at day 9 postinfection compared to baseline (day 0) (Fig. 3). hCR1 levels rose significantly by day 15 compared to baseline. A second independent experiment revealed decreases in erythrocyte CR1 in three mice at day 7 compared to baseline (day 0). (B) Densitometry ratios of CR1 to TER-119, relative to day 0 baseline values.

**FIG 2** Infection of hCR1+ mice with *P. berghei* ANKA results in diminished detection of hCR1 on erythrocytes at 7 to 10 days postinfection. (A) Flow cytometry histograms from an experiment with one representative wild-type and two representative hCR1+ mice that survived 25 days following *P. berghei* ANKA infection are shown over a time course. Freshly isolated erythrocytes from infected or uninfected mice were analyzed on days 0, 5, 7, 9, 10, 17, and 25. The expression of cell surface hCR1 was analyzed. The histograms show the fluorescence intensity of erythrocyte CR1 detected (the x axis indicates FITC or CR1, and the y axis represents the percentage of the maximum); 1 × 10^6 cells were analyzed in each event. Percent parasitemia of each mouse, also measured by flow cytometry (data not shown), is indicated with asterisks. (B) Average values of CR1 detection for each time point for available mice are shown. Error bars indicate the standard errors of the means.

**FIG 3** hCR1+ mice had decreased protein levels of erythrocyte hCR1 at day 7 after infection with *P. berghei* ANKA. (A) Representative immunoblots of CR1 and TER-119 in erythrocytes from hCR1+ mice infected with *P. berghei* ANKA. CR1 bands (200 kDa) were quantified by densitometry and normalized to TER-119 (52 kDa). Decreases in hCR1 normalized to TER-119 were observed at day 9 postinfection relative to baseline (day 0). CR1 levels rose significantly by day 15 compared to baseline. A second independent experiment revealed decreases in erythrocyte CR1 in three mice at day 7 compared to baseline (day 0). (B) Densitometry ratios of CR1 to TER-119, relative to day 0 baseline values.

**FIG 4** Changes in disease severity (data not shown). In addition, parasitemia levels were similar between hCR1+ and wild-type mice (Fig. 1B). Erythrocytes were monitored by expression of the TER-119 antigen, a 52-kDa glycophorin A-associated protein that is expressed from the early proerythroblast stage to mature erythrocytes (32), and parasites were stained with ethidium bromide according to previously published methods (33).

Decreases in hCR1 following *P. berghei* ANKA infection are dependent on C3. hCR1 binds to C3b via its long homologous repeat B (LHR-B) and LHR-C, which facilitate the clearance of complement-coated immune complexes (“immune adherence”-mediated clearance). Thus, we sought to establish if the apparent decrease in hCR1 during *P. berghei* ANKA infection would occur in the absence of C3. Treatment of mice with cobra venom factor (CoVF) leads to a decrease in **Cerebral Malaria, Immune Complex, and CR1**
with the knowledge that CoVF is highly immunogenic and antibodies elicited after a week will block CoVF activity (34). We found that control hCR1⁺ mice not treated with CoVF showed the typical decline in erythrocyte hCR1 levels at day 7 after infection with P. berghei ANKA, whereas hCR1⁺ mice treated with CoVF did not show decreased erythrocyte hCR1 levels until day 9 postinfection. Average hCR1 levels are plotted in Fig. 4; individual histograms are shown in Fig. S1 in the supplemental material.

Circulating immune complexes are detected during the course of infection of P. berghei ANKA. We next confirmed the presence of ICs in serum during the course of P. berghei ANKA infection. Serum samples from wild-type and hCR1⁺ mice were collected at days 8 and 10 postinfection. In both wild-type and transgenic mice, CICs peaked at day 8 and began to decline at day 10 (Fig. 5). This was consistent with the data above and suggested that CICs bind to erythrocyte CR1 between days 7 and 10 postinfection. At day 10, the IgG and IgM levels were significantly lower in erythrocyte hCR1 detection, but the decrease was delayed until day 9 postinfection, which was consistent with the kinetics of CoVF activity. Average values of CR1 detection for each time point for mice are shown. Error bars indicate the standard errors of the means. **, P < 0.01.

B-cell-deficient mice had significantly increased survival, with a median survival of 12.5 days compared to 10 days in wild-type mice (P < 0.0001; \( \chi^2 = 47.18 \), log rank test). Multiple cohorts did not result in any confounding effect, as unadjusted and adjusted hazard ratios from the Cox model were similar. Only 2% of wild-type mice (1 of 44) escaped cerebral malaria, versus 50% of B-cell-deficient mice (21 of 42). Histopathologic analysis of brains of moribund, infected B-cell-deficient mice revealed distinct microvascular lesions (Fig. 6B). The most striking observation was that blood vessels of B-cell-deficient mice had significant leukocyte plugging and cellular infiltration with minimal hemorrhage. In contrast, wild-type mice consistently had moderate hemorrhage with minimal leukocyte plugging of the microvasculature. Vascular lesions were similar in number and distribution between wild-type and B-cell-deficient mice. Infected red blood cells (iRBCs) were present in both groups without significant differences in parasitemia at day 11 postinfection; levels in wild-type mice were 25% ± 2% (n = 16) and levels in B-cell-deficient mice were 23% ± 1% (n = 16). Of note, B-cell-deficient mice exhibited distinct signs of illness at terminal stages of disease. These mice did not have the convulsions typically seen in wild-type mice with ECM. B-cell-deficient mice presented with hind limb paralysis during end-
stage disease, whereas wild-type animals had unilateral hemiplegia.

DISCUSSION

We have made several important findings with *P. berghei* ANKA ECM in transgenic mice expressing human CR1 on erythrocytes. We have identified the generation of ICs during *P. berghei* infection, which is accompanied by decreases in erythrocyte hCR1 levels between days 7 and 10 following infection. However, erythrocyte CR1 did not appear to affect parasitemia, disease course, or survival. The transient decrease in CR1 was detected by flow cytometry and validated by immunoblot assay. In mice that survived the infection, erythrocyte CR1 levels eventually recovered and surpassed baseline levels both by flow cytometry (manifested as a second peak) and by immunoblot assay, and that recovery probably is the result of introduction of new erythrocytes into the circulation. The decreased CR1 levels noted by flow cytometry and by immunoblotting confirm true loss of CR1 from the erythrocyte, rather than “masking” of CR1 epitopes for the detecting antibody by ICs. The decreases in erythrocyte CR1 during ECM are dependent on C3, as demonstrated by the delay in kinetics following treatment of mice with CoVF. A schematic of erythrocyte CR1 clearance following immune adherence is shown in Fig. 7.

The role of ICs in the pathogenesis and severity of autoimmune diseases such as systemic lupus erythematosus is well established. ICs also contribute to the pathogenesis of several infectious diseases. Examples include IC-mediated vasculitis and renal damage in streptococcal infections, hepatitis B virus, HIV-1 infection, bacterial endocarditis, and cryoglobulinemia associated with hepatitis C virus (37). The interplay between erythrocyte CR1 and ICs could contribute to the complications of malaria. In humans, polymorphisms can dictate the size (length) and the expression levels of CR1 on erythrocytes. Four different size variants have been described (CR1*1 through 4) that result from deletions or duplications of LHRs during unequal crossover events (38, 39). The level of CR1 expressed is regulated by the H (high-expression) and L (low-expression) codominant alleles: LL, HL, and HH genotypes give rise to low, intermediate, and high CR1 expression, respectively (40). In humans, CR1 levels also vary with age—levels are high at birth, followed by low levels between 6 and 24 months of age (41), a period at which children are most susceptible to malaria.

Complement activation triggered by ICs may precipitate severe malarial anemia (42), and in this instance, individuals with low CR1 levels (and therefore with decreased ability to degrade C3b and C4b to their hemolytically inactive fragments) could conceivably be at a greater risk of developing severe malarial anemia (43–45). It is not clear whether the low CR1 levels observed in these instances are the result of removal of the CR1 from erythrocytes.
(as we described here in our model) or the result of genetically determined low CR1 levels. Evidence supporting the former hypothesis stems from a study which showed normalization of CR1 levels following resolution of infection (44). Other studies have not shown a correlation between severe disease and CR1 levels (46).

Rosetting, a phenomenon where a specific variant PfEMP1 expressed by parasitized erythrocytes adheres to CR1 on uninfected erythrocytes to form clumps, is believed to play a central role in the pathophysiology of *P. falciparum* cerebral malaria (29). Rosetting was also demonstrated in 14 of 15 clones of *Plasmodium chabaudi* tested in a murine model using wild-type C57BL/6J mice (47), suggesting that this phenomenon may occur independently of erythrocyte CR1 in mice. One would expect CR1 polymorphisms that are associated with reduced ability to form rosettes (e.g., SL2 and SL2/2) to be associated with a lower incidence of cerebral malaria, but studies addressing this issue have yielded conflicting results (48, 49).

ICs have proinflammatory properties, and indeed, studies currently being carried out by our group have shown that human peripheral blood mononuclear cells stimulated with ICs isolated from the serum of individuals with *Plasmodium vivax* malaria secrete interleukin-6, tumor necrosis factor, and IL-1β (D. Golenbock and R. Gazzinelli, unpublished observations). The loss of CR1 that we have observed coincides with a rise in IgG- and IgM-containing ICs and may represent an attempt to clear these proinflammatory complexes from the circulation.

Fernandez-Arias et al. recently reported diminished levels of surface CR1 on monocytes/macrophages both in a rodent malaria model and in patients infected with either *P. falciparum* or *P. vivax* (50). These data together illustrate the importance of IC deposition and clearance during malaria infection.

Our data led us to investigate the role of immunoglobulins in the pathogenesis of ECM. B-cell-deficient mice, devoid of immunoglobulins, exhibited increased survival and delayed onset of disease. Histopathology revealed striking differences, with a lower degree of microvascular hemorrhage in the B-cell-deficient mice. To our knowledge, only one other study reports data with *P. berghei* ANKA ECM using B-cell-deficient mice having the targeted deletion in the μ region of the IgM locus (also known as B-cell-null, BKO, μMT, or Igh-6null mice). Yáñez et al. reported that 3 of 4 μMT mice and 8 of 8 wild-type mice developed cerebral malaria in a single experiment; the limited numbers presumably precluded achieving statistical significance (51). Infections of IγD mice, which have a targeted deletion of the Iγ region of the IgM locus, were more suggestive of a role of B cells in disease; in three experiments, only 8 of 31 IγD mice developed cerebral malaria, whereas 24 of 27 wild-type mice developed cerebral malaria (51). In our studies, the pathological findings of decreased hemorrhage and increased leukocyte plugging in the microvasculature of brains of the B-cell-deficient mice with ECM were striking. In ECM, *P. berghei* ANKA parasites do not infect the brain parenchyma; rather, a fraction of infected erythrocytes accumulates intravascularly, including in the brain (52). CD4+ and CD8+ T cells have both been implicated in the development of ECM (51, 53–55). Mice deficient in T cells are ECM resistant and lack microvascular lesions, endothelial cell death, and mononuclear cell infiltration. It would be interesting to determine if the decreased hemorrhage in the microvasculature is directly associated with a lack of IC formation, as this could have important implications for the pathogenesis of cerebral malaria.

Of note, we did not observe any decreases in hCR1 when hCR1+ transgenic mice were challenged with either *P. chabaudi*, which results in parasite clearance, or *P. berghei* NK65, which causes severe anemia (data not shown). Disease was comparable between transgenic and wild-type mice in both infection models in terms of parasitemia, anemia, and clinical signs. Thus, the decreases in erythrocyte CR1 appear to be specific for *P. berghei* ANKA infection and/or ECM. Altogether, we propose that CIC plays a major role in the pathogenesis of ECM and that immunoglobulin deficiency alters the disease outcome following *P. berghei* ANKA infection. Future studies to define the antigenic content of malarial IC and to determine how ICs may specifically contribute to microvascular damage and hemorrhage are merited.

**MATERIALS AND METHODS**

**Ethics.** All experiments involving animals were in accordance with guidelines set by the American Association for Laboratory Animal Science (AAALAS). All protocols related to this work were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Massachusetts Medical School.

**Mice.** All mice were bred and maintained under specific-pathogen-free conditions in the animal facilities at the University of Massachusetts Medical School in accordance with the University of Massachusetts Medical School’s IACUC. B-cell-deficient mice (IγD−/−) on the C57BL/6 background as well as control wild-type C57BL/6 mice were originally purchased from Jackson Laboratories. Transgenic mice expressing human CR1 on erythrocytes (hCR1+/) were generated on the C57BL/6 background as previously described (31). Age- and sex-matched groups of mice were used in all experiments.

**Plasmodium infection.** Mice (5 to 10 weeks old) were infected with the frozen stock of *Plasmodium berghei* ANKA (gift of A. Luster, Massachusetts General Hospital, Boston, MA). Parasitemia of infected RBCs (iRBCs) was assessed every 2 or 3 days by microscopy of Giemsa-stained thin blood smears, and 7 to 8 days later, when the parasitemia showed mostly ring stages and the mice suffered from cerebral malaria symptoms, blood was drawn and used for infection studies.

Wild-type, transgenic, and knockout mice were each infected with 10⁵ iRBCs in 200 μl phosphate-buffered saline (PBS) by intraperitoneal injection. Survival and signs of disease were monitored daily. Animals that showed neurological signs, such as convulsions, ataxia, and paralysis, followed by death, between 7 and 12 days after infection were considered to have cerebral malaria (56). Whole blood was collected periodically in hirulog for assessment of CR1.

**Cobra venom factor treatment.** For some experiments, animals received either four doses of cobra venom factor (CompTech, Tyler, TX), each dose at 200 μg/kg of body weight, administered intraperitoneally prior to infection and on days 3, 5, and 7 postinfection to deplete complement, or sterile normal saline (control animals).

**Immunoblot assays.** Red blood cell lysates in NuPAGE LDS sample buffer (4×) were separated and then transferred to an Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore) (57). Membranes were blocked with PBS-1% milk and probed with a polyclonal rabbit IgG, anti-CR1 affinity-purified antibody that was provided by J. P. Atkinson (Washington University, St. Louis, MO). CR1-reactive bands were detected with goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma-Aldrich) followed by the addition of 5-bromo-4-chloro-3-indolyolphosphate and nitroblue tetrazolium (BCIP/NBT)-Purple Liquid substrate (Promega, Madison, WI). Rat anti-mouse TER-119 antibody was obtained from eBioscience, and TER-119 bands were detected with goat anti-rabbit IgG conjugated to alkaline phosphatase (Promega).
Flow cytometry. The relative expression of mouse erythrocyte CR1 was determined using the anti-CR1 monoclonal antibody 7G9 (provided by Ron Taylor, University of Virginia School of Medicine). Either 7G9 was directly labeled with Alexa Fluor 647 according to the manufacturer’s directions (Molecular Probes, Eugene, OR, USA), or a fluorescein isothiocyanate (FITC)-labeled secondary antibody was used for detection. The erythrocyte population was identified by staining with FITC-conjugated anti-TER-119 antibody. Subsequently, the cells were washed twice, and 100,000 cells were analyzed using an LSRII flow cytometer (BD Bioscience). Data were acquired with DIVA software (BD Bioscience) and analyzed with Flowjo (Tree Star).

Immune complex ELISA. Plates were coated with purified human C1q at 5 μg/ml in PBS overnight at 4°C and then blocked with 1% bovine serum albumin (BSA) in PBS for 1 h. Serum from infected and uninfected animals was diluted 1:100 and added for 1 h at 37°C. Captured immune complexes were detected with anti-mouse IgG and anti-mouse IgM alkaline phosphatase (1:1,000 dilution in PBS-0.05% Tween 20).

Complexes were detected with anti-mouse IgG and anti-mouse IgM alkaline phosphatase (1:1,000 dilution in PBS-0.05% Tween 20). Tissue sections were prepared and stained with hematoxylin and eosin as described elsewhere (9). Slides were reviewed independently by a veterinarian pathologist.

Histopathology. Eight days following P. berghei ANKA infection, brains were carefully removed and fixed in formaldehyde solution (4%, vol/vol). Tissue sections were prepared and stained with hematoxylin and eosin as described elsewhere (9). Slides were reviewed independently by a veterinarian pathologist.

Statistical analysis. All data were analyzed using GraphPad InStat 4.0 software. Unless stated otherwise, all comparisons were performed using a two-tailed Student t test. Mann-Whitney U testing was used for non-parametric analysis when data did not fit a Gaussian distribution. A P value of ≤0.05 was considered to be statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00949-14/-/DCSupplemental.

ACKNOWLEDGMENTS

We thank Ronald Taylor for his generous donation of antibodies and other reagents used in these studies and especially for his invaluable intellectual input. We thank Anna Cerny for animal husbandry, Nancy Nowak for assistance with immunoblotting assays, and Melanie Trombley with graphics.

This work was supported by Public Health Service grants NIH R01 AI072923 from the National Institute of Allergy and Infectious Diseases to D.T.G., NIH R01 AI054544 to S.R., and NIH R01 AI092105 to J.P.W.

REFERENCES


