Cerebral malaria refers to the severe complications of falciparum malaria in which vascular plugging of Plasmodium falciparum–infected red blood cells (RBCs) in the brain can lead to coma and death, often in children.1,2 Cytoadherence of parasitized RBCs to endothelial cells, via ligands present on the erythrocyte membrane knobs, is thought to be responsible for this phenomenon.3 However, exceptions to the requirement for knobs have been observed.4 Others have postulated an important role for cytokines and nitric oxide in the pathogenesis of cerebral malaria.5

The molecular mechanisms underlying the cytoadherence of P. falciparum-infected RBCs to the endothelium is under intensive investigation. Most laboratory-maintained and field isolates of infected RBCs appear to bind to CD36. However, binding to intercellular adhesion molecule-1 (ICAM-1) has also been observed.4 Importantly, variation in specificity for endothelial ligands is common among different isolates.6 Vascular cell adhesion molecule-1 (VCAM-1), a member of the same immunoglobulin-like superfamily as ICAM-1, and E-selectin have also been found to be receptors for P. falciparum-infected RBCs in vitro.7 This binding is dependent on activation of human umbilical vein endothelial cells with tumor necrosis factor-α (TNF-α). Thus, the relative importance of the various endothelial cytoadherence receptors for P. falciparum–infected RBCs in the etiology of cerebral malaria may depend on both isolate specificity and cytokine effects.

The endothelial cell is one of the major targets of cytokine function. The inflammatory cytokines TNF-α and interleukin-1, for example, induce or increase expression of two of the structural groups of adhesion molecules on endothelium, members of the selectin family (P-selectin or E-selectin) and the immunoglobulin gene superfamily (ICAM-1 and VCAM-1).8 Levels of TNF-α are elevated during severe falciparum malaria;9 however, there is no direct evidence for its role in human cerebral malaria.

To study cerebral malaria experimentally, it was necessary to have an animal model of the syndrome, preferably in rodents, where reagents including mutant mouse strains and monoclonal antibodies (MAbs) are available. For this reason, we have focused our attention on the model of cerebral malaria induced by P. yoelii 17XL, initially described by Yoel and Hargreaves.10 This lethal strain of P. yoelii arose as a spontaneous mutation from the nonlethal strain P. yoelii 17XNL. We confirmed the plugging of capillaries with parasitized erythrocytes in the brains of mice infected with P. yoelii 17XL that occurred without significant inflammation. Important, studies in vivo and ex vivo demonstrated cytoadherence of infected RBC in the postcapillary venules, decreased red blood cell velocities, and increased shear stress.11 This model is different than the P. berghei ANKA model in which the cerebral malaria seems to have an inflammatory pathogenesis,12 unlike human cerebral malaria.2

To understand further the pathogenesis of cerebral malaria in the P. yoelii model, we studied the expression of two ligands, ICAM-1 and VCAM-1, thought to be involved in adhesion of infected red blood cells. Our results suggest that up-regulation of ICAM-1 is a distinctive feature of P. yoelii cerebral malaria. Since TNF-α is thought to play an important role in cerebral malaria, we also assayed serum levels of TNF-α in mice infected with either this parasite or the nonlethal parent strain. Levels of TNF-α increased more rap-
idly in lethal compared with non-lethal infection. To test the contribution of TNF-α to the lethal phenotype, we followed the course of infection in mice with a targeted disruption of the TNF-α gene. The TNF-α-deficient mice (TNF-α−/−) mice were still susceptible to death from the lethal parasite. We suggest that this model may be useful in studying therapeutic approaches to this often fatal syndrome.

MATERIALS AND METHODS

Animals and infection with malaria parasites. SW mice (female, 6–8 weeks old) were obtained from Taconic Farms (Germantown, NY). Animals were housed and cared for in an American Association of Accredited Laboratory Animal Care (AAALAC)-accredited facility at Montefiore Hospital (Bronx, NY). The TNF-α−/− mice on a C57BL/6 × 129 background were generated as recently described and maintained in accordance with protocols approved by the Memorial Sloan-Kettering Cancer Center Institutional Care and Use Committee. Both male and female mice were used and no difference was noted between them. Differences in parasitemia between TNF-α−/+ and TNF-α−/− mice were compared with an unpaired t-test (two-tailed).

Plasmodium yoelii 17XL (lethal) and P. yoelii 17XNL (nonlethal), stored in liquid nitrogen, were used to infect source mice. Experimental mice were infected by intraperitoneal inoculation of 1 × 10⁸ or 1 × 10⁹ infected RBCs obtained from a source mouse. At various intervals after infection, parasitemia was determined, mice were exsanguinated, and the brains were removed. Parasitemia was monitored by making smears of blood obtained from the tail vein and counting 50 Giemsa-stained fields or at least 300 RBCs under oil immersion (1,200×). Serum was stored at −70°C. This experiment was repeated three times with two infected and two control mice killed per time point.

Immunohistochemistry. Brains were fixed overnight in 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1M sodium phosphate buffer, pH 7.2, containing 0.005% CaCl₂ and stored in phosphate buffer. Sagittal sections, 40 μm thick, were cut on a Vibratome 1000 (Technical Products International, St. Louis, MO) and stained using the avidin-biotin-peroxidase technique, as follows. Sections were first incubated in 100% methanol containing 0.8% H₂O₂ (Sigma, St. Louis, MO) to remove endogenous peroxidase activity. Sections were washed twice in 0.1 M Tris-buffered saline (TBS, pH 7.2), blocked for 60 min in a solution of 5% fetal calf serum and counted 50 Giemsa-stained fields or at least 300 RBCs obtained from a source mouse. At various intervals after infection, parasitemia were similar (1% on day 4 of 17XNL). No staining of vessels appeared when an unrelated MAb was used as the primary antibody or the primary antibody was not used. Infected erythrocytes were more easily visualized when an unrelated MAb was used (Figure 1H). This experiment was repeated two more times and showed the same results.

RESULTS

Increased expression of ICAM-1 on brain endothelium during P. yoelii infection. Since previous studies have shown that ICAM-1 may play a role in the vaso-occlusive pathology of human cerebral malaria, we examined its role in the P. yoelii 17XL model. Brains from mice infected with the lethal or nonlethal strains of P. yoelii at several time points were examined histologically using anti-ICAM-1 MAb. The entire section was viewed by light microscopy. Areas of cortex and cerebellum were positive in infected mice. Figure 1 shows the results for mice infected with P. yoelii 17XL for four, five, and eight days (Figure 1A, B, and C) and P. yoelii 17XNL for five, seven, and 21 (recovered) days (Figure 1D, E, and F). Controls included brain tissue from a normal mouse stained with anti-ICAM-1 (normal control, Figure 1G), sections stained with an isotype control MAb (negative control, Figure 1H), and sections stained without primary antibody. There was greater expression of ICAM-1 in capillaries and small venules of mice infected with either parasite than in the normal mice. Furthermore, in both infections, the level of expression of ICAM-1 increased with time after infection, corresponding to the increase in parasitemia. However, the expression of ICAM-1 was clearly greater in mice infected with P. yoelii 17XL compared with P. yoelii 17XNL. This was not strictly determined by parasitemia since early in infection, levels of parasitemia were similar (1% on day 4 of P. yoelii 17XL and 2% on day 5 of P. yoelii 17XNL). No staining of vessels was observed when an unrelated MAb was used as the primary antibody or the primary antibody was not used. Infected erythrocytes were more easily visualized when an unrelated MAb was used (Figure 1H). This experiment was repeated two more times and showed the same results.

To quantify these results, we used video microscopy. Immunostained brain sections were viewed under a Nikon diaphot microscope and the intensity of staining was measured. The results of this analysis confirmed the immunochromatographic staining. In the lethal infection, there is a clear shift in pixel intensity, a measure of darkness, with time after infection from day four to five to eight. In the nonlethal infection, there is also a shift to stronger staining on day 7 compared with day 5, but then a reduction in intensity on day 9 and low levels in mice that had recovered from infection (day 21).
FIGURES 1 and 2. (Top), increased expression of intercellular adhesion molecule-1 (ICAM-1) in the brain vasculature of mice infected with *Plasmodium yoelii* 17XL (A, C) and 17XNL (G, H). Vibratome sections of brains of mice infected with *P. yoelii* 17XL for four (A), five (B), or eight (C) days or *P. yoelii* 17XNL for five (D), seven (E), or 21 (F) days were stained with anti-mouse ICAM-1. G is a section of brain from a normal mouse stained with anti-ICAM and H is a section of brain from a mouse infected with the lethal strain for eight days and stained with an unrelated monoclonal antibody of the same isotype. Note the increase in staining in the *P. yoelii*-infected mice compared with normal mice, the increase in staining in the *P. yoelii* 17XL-infected mice (A–C) compared with the nonlethal infection (D–F), and the increase with days after infection (A–C and D–E) and the decrease upon recovery from the nonlethal infection (F). Large arrows indicate stained vessels and small arrows indicate parasitized erythrocytes and pigment. (Magnification × 400.) (Bottom), increased expression of vascular cell adhesion molecule-1 (VCAM-1) in the brain vasculature of mice infected with *P. yoelii* 17XL and NL. Vibratome sections of
Increased expression of VCAM-1 on brain endothelium during *P. yoelii* infection. The expression of VCAM-1, another candidate adhesion molecule of human cerebral malaria, was measured in the same mice as for ICAM-1. Staining with anti-VCAM-1 MAb was greater in lethal (Figure 2A, B, and C) compared with nonlethal infection (Figure 2D, E, F) and increased with time after infection. In the nonlethal infection, VCAM-1 expression returned to control levels upon recovery (Figure 2F). Interestingly, anti-VCAM-1 MAb stained larger vessels than did anti-ICAM-1 MAb. These appeared to be mainly small veins and venules. The intensity of staining with anti-VCAM-1 MAb was determined as for anti-ICAM-1. Although there was an increase in staining as the lethal infection progressed, the intensity of staining was not as great as for ICAM-1. This was also true in the nonlethal infection.

**Serum TNF-α levels during *P. yoelii* 17X infection.** Levels of serum TNF-α were determined at different stages of lethal and nonlethal infection. The typical course of *P. yoelii* 17XL and NL are shown in Figures 3A and B. In the lethal infection, the concentration of TNF-α increased very rapidly and reached 200 pg/ml by day 4 of infection (Figure 3A). In the nonlethal infection (Figure 3B), TNF-α levels increased more gradually, reached a peak of approximately 230 pg/ml by day 10, and then decreased when the mice recovered. On day 4, TNF-α levels were 4–5 times higher in mice with lethal infections compared with those with nonlethal infections (*P < 0.01*).

**Course of *P. yoelii* in mice lacking TNF-α.** Since TNF-α levels are elevated in both lethal and nonlethal infections, it was of interest to determine the course of these infections in the absence of this cytokine. Animals with a targeted disruption of the TNF-α gene were infected with *P. yoelii* 17XL and NL and parasitemia and mortality were analyzed. In mice infected with $1 \times 10^5$ infected RBCs of the lethal strain (which did not kill the mice), parasitemias were not significantly different in TNF-α+/+ and TNF-α−/− mice. However, by day 25, TNF-α+/+ mice had recovered and some TNF-α−/− still had high levels of parasites (Figure 4A). The TNF-α−/− mice infected with the nonlethal strain had significantly higher levels of parasitemia than the wild type litter mates on days 7 and 12 (*P < 0.02*), and wild-type mice recovered 10 days earlier than TNF-α−/− mice (Figure 4B). When mice were infected with 10-fold more *P. yoelii* 17XL infected RBCs ($1 \times 10^6$), both groups succumbed to infection (Figure 4C). This study was repeated and showed the same results.

**DISCUSSION**

Cerebral malaria remains a major cause of death associated with severe *P. falciparum* infection. Experimental models of cerebral malaria may facilitate an understanding of the pathogenesis of this syndrome and lead to better therapeutic intervention and reduced mortality associated with human cerebral malaria. Unfortunately, there are few experimental models of this syndrome. Infection of Rhesus monkeys with *P. coatneyi* induces knobs on the surface of the infected RBC; however, it does not cause the cerebral symptoms associated with human cerebral malaria. Squirrel monkeys infected with *P. falciparum* do display symptoms of cerebral malaria; however, a rodent model would allow a dissection of the immune mechanisms involved in this syndrome and provide a host that is suitable for larger experiments of treatment protocols.

In an attempt to study cerebral malaria in a rodent model, Grau and others made use of the *P. berghei* ANKA infection of certain strains of mice, a model originally described...
by Wright. Serum levels of TNF-α were elevated in mice displaying symptoms of cerebral malaria but not in mice without such symptoms. Furthermore, injection of antibodies to TNF-α protected mice from cerebral malaria without affecting parasitemia. However, the lesions in the brain are characterized by the presence of large mononuclear cells and endothelial damage. This is in contrast to the histopathology of human cerebral malaria, which usually does not present with an inflammatory pathogenesis. However, an increase in adhesion molecules could occur in P. berghei ANKA due to local inflammatory cells. Thus, the pathogenesis of P. berghei ANKA cerebral malaria may be fundamentally different from that of human cerebral malaria.

The P. yoelii 17XL model of cerebral malaria shares significant features with human cerebral malaria. There is an accumulation of infected RBCs in the brain, in the absence of an influx of mononuclear cells. Importantly, in the P. yoelii 17XL model, we observed cytoadherence in vivo, mainly of infected RBCs, and reduced blood flow. Although P. yoelii-infected RBCs do not display surface knobs, close adherence of infected RBCs to brain endothelium was observed by electron microscopy. Like P. falciparum, P. yoelii 17XL can invade both mature and immature RBCs. Since the overwhelming majority of RBCs in circulation are mature, parasites are mainly in mature RBCs. The nonlethal parent strain, P. yoelii 17XNL, and P. berghei preferentially invade reticulocytes. Mice infected with P. yoelii 17XL often develop hind limb paralysis but rarely become comatos. In a study of comatose Malawian children, 34% exhibited signs of decerebration or decortication (arms or legs flexed or extended).

To compare further the P. yoelii model of cerebral malaria with that of P. falciparum, in which the increased expression of ICAM-1 and VCAM-1 may play a key role, we stained brain sections with MAbs specific for mouse ICAM-1 and VCAM-1. We found a striking increase in ICAM-1 expression that was associated with the lethal P. yoelii infection and with time after infection. In addition, staining was confined to capillaries and small venules, which are the sites of the vaso-occlusive pathology. In contrast, expression of VCAM-1 was not increased as much as ICAM-1. In addition, VCAM-1 staining was restricted to larger vessels, mainly small venules and small veins. Although the MAb against VCAM-1 that was used may have been of a lower affinity than the ICAM-1 MAb, this is a less likely explanation in view of other findings. For example, experiments using an in vitro flow chamber also demonstrated that VCAM-1 was not an important cytoadherence ligand for P. falciparum. Importantly, in our recent studies, we have found that anti-ICAM MAb can reverse cytoadherence in vivo and release schizonts into the circulation. Thus, ICAM-1, and not VCAM-1, is a likely cytoadherence molecule in this model.

Since most strains and isolates of P. falciparum bind to CD36, a constitutively expressed protein, but only a small fraction of individuals with P. falciparum malaria develop cerebral malaria, there must be additional factors that cause this syndrome. For example, as with leukocytes, several ligands may have to act cooperatively to withstand blood shear stress. In support of this, it has been shown that P. falciparum-infected RBCs adhere more strongly to cells expressing both CD36 and ICAM-1. Interestingly, a recent post-mortem study indicated that CD36 is not elevated in the brains of patients who died of cerebral malaria but ICAM-1 and E-selectin expression were significantly elevated. In addition, the specific ligand that a P. falciparum isolate binds to does not necessarily correlate with severity of infection. Therefore, some aspect of the host response may also play a role in cerebral malaria.

Studies indicate that high TNF-α levels are closely associated with severe malaria in humans. In the present study, however, high levels of TNF-α were observed in both lethal and nonlethal infections. Although TNF-α levels are often higher in mice infected with lethal compared with nonlethal P. yoelii, in the experiment shown in Figure 3, peak TNF-α levels were not significantly different in the two infections. However, the kinetics of TNF-α synthesis was very much accelerated in the mice infected with the lethal infection. This may partially explain the difference in ICAM-1 expression that we observed.

Other evidence suggests that the presence of high levels of TNF-α is not solely responsible for cerebral malaria. Treatment of patients with an MAb against TNF-α did not reduce the incidence of cerebral malaria. In addition, pentoxifylline, an inhibitor of TNF-α, did not affect TNF-α levels or improve outcome in controlled study of P. falciparum. However, a different study did find reduction of both TNF-α and duration of coma in cerebral malaria in children. Interestingly, in P. vivax infection at the time of cri-
sis, serum levels of TNF-α may be as high as in Plasmodium falciparum, with no cerebral symptoms in P. vivax. An increase in the frequency of the TNF2 allele in humans reported from the Gambia, particularly in children with cerebral malaria, suggests that TNF-α plays a dual role in malaria, perhaps by increasing the risk of cerebral malaria, but also by protecting against blood stages. Taken together, our data and data in the literature suggest that other factors, in addition to TNF-α, may be required for cerebral malaria.

In nonlethal malaria, TNF-α−/− mice demonstrated much higher levels of blood-stage parasitemia during infection with P. yoelii 17XNL (Figure 4B). Nevertheless, the high level of TNF-α was not sufficient to control parasitemia in wild type mice infected with the lethal strain. The TNF-α−/− mice infected with 1 × 10^6 infected RBCs died at the same rate as wild type mice (Figure 4C). However, it appears that mice of this background are not as susceptible to cerebral mice as SW mice. Similarly, studies with ICAM-1-deficient mice and wild-type controls indicated that these animals did not die of cerebral malaria (Wanidworanun C, Shear, HL unpublished data). Studies of ICAM-1 expression in TNF-α−/− mice are in progress.

Our results indicate that as in human malaria, increased expression of ICAM-1, not VCAM-1, is an important correlate of cerebral malaria. In addition, similar to human disease, TNF-α levels are elevated in rodent malaria. However, here we show that elevated TNF-α levels are not necessary for the lethal outcome. Thus, the P. yoelii 17XL mouse model of cerebral malaria is relevant to human disease and may elucidate some aspects of its etiology. Furthermore, this model may be useful in testing experimental treatments of this syndrome that are urgently needed.

Acknowledgments: We are grateful to Bijal Das for expert technical assistance and the Analytical Imaging Facility, Albert Einstein College of Medicine for assistance with the video microscopy.

Financial support: This work was supported by grant AI-34064 from the National Institutes of Health.

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