Recombinant Human Erythropoietin Prevents the Death of Mice during Cerebral Malaria

Karine Kaiser,¹ Anthony Texier,¹ Josette Ferrandiz,¹ Alain Buguet,² Anne Meiller,³ Christine Latour,¹ François Peyron,¹ Raymond Cespuglio,² and Stéphane Picot¹

¹Équipe d’Accueil 37-32, Parasitologie et Médecine Tropicale, ²Équipe d’Accueil 37-34, Neurobiologie des États de Vigilance, and ³Plateforme de Physiologie, Faculté de Médecine, University Claude Bernard Lyon 1, Lyon, France

Cerebral involvement during malaria is a complication that leads to seizure, coma, and death. The effect of new neuroprotective therapies has not yet been investigated, although cerebral malaria shares some features with neurological stroke. Erythropoietin (EPO) is one of the more promising drugs in this area. We measured the effect of EPO on the survival of mice infected with Plasmodium berghei ANKA and demonstrated that inoculations of recombinant human EPO at the beginning of the clinical manifestations of cerebral malaria protect >90% of mice from death. This drug has no effect on the course of parasitemia. The effect of EPO was not related to either the inhibition of apoptosis in the brain or the regulation of the increase and decrease of nitric oxide production in the brain and blood, respectively. Tumor necrosis factor–α and interferon–γ mRNA overexpression was inhibited by EPO, and treated mice had fewer brain hemorrhages. EPO has been used in patients with chronic diseases for years, and more recently it has been used to treat acute ischemic stroke. The data presented here provide the first evidence indicating that this cytokine could be useful for the symptomatic prevention of mortality during the acute stage of cerebral malaria.

Malaria remains one of the most common life-threatening illnesses in the tropics, taking a dramatic toll of >1 million deaths each year. The majority of malaria cases are uncomplicated, and only a few evolve to severe malaria, which results from the combination of parasite-specific virulence factors and host inflammatory responses [1]. Severe falciparum malaria is now considered to be a complex syndrome that affects many organs and aspects of metabolism [2]. Cerebral malaria, which leads to seizure and coma, is associated with severe intracranial hypertension caused by brain swelling [3]. Recent imaging and postmortem findings in adults with cerebral malaria have confirmed the presence of diffuse cerebral edema with thalamic and cerebellar white-matter hypoattenuation, diffuse petechial hemorrhages, and symmetric ischemic changes involving the thalamus and cerebellum [4]. Nonetheless, the nature of the pathogenetic processes that lead to cerebral malaria is incompletely understood, but mechanisms that link cytokines with the activation of endothelial cells in the cerebral microvasculature have been recently stressed [5]. The effect of new neuroprotective therapies has not yet been investigated, although the manifestations of cerebral malaria partly share features with neurological stroke and acute nonspecific neurological disorders. The hormone erythropoietin (EPO) is probably one of the more promising drugs in this area. Thus, to explore the cytoprotective effect of EPO during cerebral malaria, we measured its effect on the survival of CBA/J mice infected with Plasmodium berghei ANKA, the most commonly used model of cerebral malaria [6].

EPO has been recognized recently as a member of
the type I cytokine superfamily with multiple functions, including playing a prominent role in erythropoiesis and neuroprotection. Systematically administered EPO crosses the blood-brain barrier via the abundant expression of EPO receptors (EPORs) in brain capillaries and acts as an antiapoptotic and cytoprotective cytokine [7]. Moreover, EPO prevents inflammation by inhibiting the expression of proinflammatory cytokines, including tumor necrosis factor (TNF)-α by preserving the integrity of endothelial cells; and by preventing the permeability of the blood-brain barrier [8]. Recent pharmacological analyses have demonstrated that human EPO (HuEPO) and murine EPO have a number of similarities and that recombinant HuEPO (rHuEPO) crosses the blood-brain barrier in mice and is present in the brain parenchymal space at levels in the range seen for agents that are centrally active [9]. Thus, rHuEPO has been widely used in murine models to explore the effect of EPO on the course of various neurological diseases [10, 11].

MATERIALS AND METHODS

Animal model. Animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee. Female CBA/J mice (6–8 weeks old and weighing 20–22 g) were infected with the malarial parasite Plasmodium berghei ANKA (gift from G. Grau, Université de la Méditerranée, Marseille, France) by intraperitoneal (ip) inoculation of 1 × 10⁶ parasitized red blood cells (RBCs).

Thirty infected mice were used for the survival experiments. On days 4, 5, and 6 after infection, 15 mice received saline solution alone ip, and 15 mice received rHuEPO (NeoRecormon; Roche) ip at a dose of 50 μg/kg (~120 IU/mouse). Considering that the total volume of blood in a 20-g mouse is ~1.7 mL, it can be calculated that the maximum EPO blood level is <85 IU/mL. Data on parasitemia, RBC counts, body weight, and clinical presentation were collected every day during the study.

For histological analysis, a group of infected mice inoculated with either saline solution alone or rHuEPO in the same fashion with the malarial parasite Plasmodium berghei ANKA was examined. Apoptotic cells were detected by the TUNEL method, according to the manufacturer’s instructions.

Gene expression. To analyze gene expression, another group of infected mice inoculated with either saline solution alone or rHuEPO were killed days 3–7; their brains were immediately removed, postfixed in formalin for 72 h, and embedded in paraffin; serial 4-μm-thick horizontal sections were then cut. Hematoxylin–phloxin–safran–stained preparations were examined. Apoptotic cells were detected by the TUNEL method, using the In Situ Cell Death Detection Kit (Roche), in accordance with the manufacturer’s instructions.

Real-time PCR was done using the LightCycler System and the Fast-Start DNA Master SYBR Green I Kit (Roche Applied Science). PCRs were set up in a total volume of 20 μL containing 2 μL of SYBR Green Fast-Start reaction mix and enzyme, 3.5 mmol/L MgCl₂, 1 μmol/L each primer, and 5 μL of cDNA. Thermocycling profiles were as follows: 95°C for 10 min, 40 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 10 s, and extension at 72°C for 20 s. Melting-curve analysis was done in the range of 65°C–95°C, to confirm the specificity of the PCR products.

Gene-expression levels were assessed by the comparative-threshold method, using expression of the mouse glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) gene as a reference [12]. Cycle-threshold (Ct) values were defined as the PCR cycle at which amplified product was first detected. The corrected value, ΔCt, was defined as the difference between the Ct values derived for the specific gene being assayed and for the GAPDH control. The ΔCt value for each gene was then used to determine ΔΔCt values, which represented the difference between paired tissue samples and was calculated as the ΔCt value for a normal brain minus the ΔCt value for the tested brain. Because each ΔΔCt unit represents a 2-fold difference in the amount of product, the n-fold differential expression of a specific gene in a sample, compared with that of a normal counterpart, was expressed as 2^ΔΔCt.

Brain and blood NO measurements. Twenty-four mice were infected for these experiments. On days 4, 5, and 6 after infection, 12 mice each received saline solution alone or rHuEPO, as described above. An additional 12 uninfected mice were used to calibrate the NO sensors at the beginning and end of NO quantification. Direct NO-specific voltammetric measurements were taken using a specific, controlled sensor in the brain on day 6 after infection [13, 14]. After mice were anesthetized, the sensor was inserted stereotactically into the sensorimotor cerebral cortex through a trepanation hole. Reliable cortical readings were obtained in 8–20 min, and blood was collected via heart puncture and transferred to an airtight, 400-μL blown-glass chamber equipped with a voltammetric sensor. All mice died of the heart puncture, and none of them were permitted to recover from the anaesthetizing throughout the whole procedure. Cortical and blood NO measurements were taken every 2 min by scanning applied voltages in a 400–1400-mV range; the voltage at which NO was oxidized averaged 650 mV.

Statistical analysis. The unpaired Student’s t test was used to compare continuous data. Cumulative, long-term survival rates were calculated according to the Kaplan-Meier method, and groups were compared using the log-rank test via SPSS (version 11.5). All of the measured parameters were considered for the survival analysis; survival time was the dependant variable. P < .05 was considered to be significant. The paired Student’s t test was used to compare NO voltammetric values (ob-
Figure 1. Cumulative survival analysis. A, Protection of Plasmodium berghei ANKA–infected CBA/J mice from fatal cerebral malaria by recombinant human erythropoietin (rHuEPO) treatment. Survival data are shown by use of a Kaplan-Meier curve. On days 4, 5, and 6 after infection, mice received either rHuEPO at a dose of 50 µg/kg (~120 IU/mouse) or saline solution alone (n = 15 mice/group). The log-rank test revealed that treatment had a significant effect on survival (P = .0016). B, Univariate analysis of survival for tested parameters. Cumulative Kaplan-Meier survival curves are shown for mice grouped on the basis of the parameters; median values for parasitemia increase (4.2-fold), body-weight loss (6.5%), and red blood cell (RBC) loss (12.5%) were used to define the groups and compare survival rates. The log-rank test revealed that none of these parameters had a significant effect on survival (P = .39, P = .76, and P = .74, respectively).

RESULTS

As was expected, the 15 mice that received saline solution alone showed severe symptoms of malaria (such as reduced locomotion, ataxia, and convulsions) on days 4–6 after infection and then developed fatal cerebral malaria; 14 (93.3%) died on day 7 or 8, and 1 (7.1%) survived until day 19. The 15 mice that received 3 consecutive daily inoculations of rHuEPO on the days (4–6) when clinical signs began to appear showed symptoms similar to those of the untreated mice, but only 6 (40.0%) died on day 7 or 8. The mice that survived beyond this time point recovered from their symptoms during the second week of follow-up; however, their RBC counts progressively decreased to dramatically low levels from day 8 to their death. Eight (53.3%) survived until day 19, and 2 (13.3%) were still alive on day 30. Kaplan-Meier analysis of cumulative survival clearly demonstrated an effect of the 3-day course of rHuEPO treatment during cerebral malaria (P = .0016, log-rank test) (figure 1A). Similar data were observed in 3 different experiments.

Because both body-weight loss during the course of disease and high parasitemia could be involved in poor outcome, these parameters were measured every day and were compared be-
Figure 2. A, Presence of perivascular hemorrhages in infected mice, as revealed by hematoxylin-phloxin-safran staining of brain sections. Shown are areas within the cerebellum (a and b) and cortex (c) in an infected, untreated mouse on day 7 after infection. These hemorrhages were not observed in infected mice that received treatment with recombinant human erythropoietin (rHuEPO); the normal vessels of these mice are shown (e, f, and g). Sections of brains from infected mice were also analyzed by TUNEL staining; TUNEL-positive neuronal cells were detected in several areas of brain sections from untreated mice (d), which contrasts with the very limited but intense binding in focal areas of DNA fragmentation in treated mice (h). The scale bar indicates 200 μm. B, Frequency of brain hemorrhages in infected mice that received either rHuEPO treatment or saline solution (control). Frequencies were determined by light-microscopic examination of brain sections. Data are means ± SEs of values for 5–8 mice per time point; *P < .02 for the comparison between the treated and untreated mice.

To deeply investigate the potential antimalarial role of EPO, in vitro IC_{50} against Plasmodium falciparum was measured and found to be 145 ± 10 IU/mL of blood. This level is higher than that in infected mice.

Because EPO was initially described as a hematopoietic factor, and because malarial parasites infect RBCs, we wondered...
whether rHuEPO treatment would have an impact on RBC counts during the study. Interestingly, there was no difference in RBC count between the treated and the untreated mice \((P > .4, \text{ Student’s } t \text{ test})\), with mean values on day 6 of \(83.3 \times 10^8\) RBCs/mL and \(83.7 \times 10^8\) RBCs/mL, respectively. As was expected, the 3-day treatment with rHuEPO had no impact on RBC count.

Figure 1B summarizes the results of the univariate analysis of survival for each of the parameters tested during follow-up. The median values for body-weight loss (6.5%), RBC loss (12.5%), and parasitemia increase (4.2-fold) were used to define groups of mice (i.e., those with lower values and those with higher values), which were then compared on the basis of survival rate. None of these parameters were found to be associated with outcome; the only factor that was found to be strongly associated with outcome was receipt of the rHuEPO treatment \((P = .0016)\).

To better characterize the neuroprotective effect of EPO, brain sections obtained on days 5–10 were analyzed by histological staining (figure 2A). Increasing numbers of ring hemorrhages were observed in both the treated and the untreated mice when clinical signs of severe malaria appeared on days 5 and 6. A huge increase in focal hemorrhages in various levels of brain sections was evident on day 7 for the untreated mice, which contrasted with the low number observed in brain sections from the treated mice (figure 2B). Therefore, it is possible that the highly significant reduction in the mortality of treated mice on day 7 was related to vascular protection and limited brain hemorrhaging.

These findings call into question whether EPO treatment leads to the inhibition of apoptosis in neuronal cells, as has been previously described for stroke, or to a decrease in the inflammatory response to cerebral malaria. To explore the role played by EPO in the inhibition of apoptosis, brain sections obtained during follow-up were subjected to TUNEL staining. Surprisingly, few TUNEL-positive neuronal cells were observed.

**Figure 3.** Relative expression of erythropoietin (EPO), EPO receptor (EPOR), caspase 3, and caspase 8 mRNA on days 4 and 7 after infection in mice that received treatment with recombinant human EPO (black bars) and in mice that did not receive treatment (gray bars). Expression is shown as a fold increase, compared with expression in the uninfected mice (which were ascribed a value of 1, as indicated by the vertical line). Data are means \(\pm\) SEs of values from 3 independent reverse-transcription polymerase chain reactions performed for each time point.
in the treated and the untreated mice when they presented clinical evidence of cerebral malaria (figure 2A). A slight and diffuse neuronal-cell DNA fragmentation was observed in several areas of the brain sections from the untreated mice, which contrasted with the very limited but intense binding observed in focal areas of DNA fragmentation in brain sections from the treated mice.

To control for the effect of rHuEPO treatment on the expression of EPO and EPORs in the brain, total RNA was isolated from the brains of mice killed on days 3–7. Real-time quantitative RT-PCR revealed a significant increase in EPO mRNA expression (P < .05) and a significant decrease in EPOR mRNA expression (P < .01) during the course of disease in the infected mice, compared with that in the uninfected mice (figure 3). However, there was no difference in the level of EPO mRNA expression between the treated and the untreated mice on days 4 and 7. The decrease in EPOR mRNA expression was more significant in the treated mice (P < .02) than in the untreated mice (P < .09).

To further investigate the relative role played by the inhibition of apoptosis and inflammatory processes by EPO treatment, the levels of caspase 3 and caspase 8 mRNA expression as well as the levels of expression of mRNA for the proinflammatory cytokines TNF-α and interferon (IFN)–γ and their receptors (TNF-αR1, TNF-αR2, and IFN-γR) were measured under the same conditions. Surprisingly, caspase 3 and caspase 8 mRNA was not overexpressed in the brain during cerebral malaria, and, thus, no significant effect could be observed in the mice treated with rHuEPO (figure 3). From day 5 to 6, we observed a dramatic increase in TNF-α and IFN-γ mRNA expression in the untreated mice (figure 4); for both cytokines, this effect was delayed 24 h in the mice treated with rHuEPO. TNF-αR1, TNF-αR2, and IFN-γR mRNA expression progressively increased during the acute stage of the disease, but the patterns were quite similar in the treated and the untreated mice (data not shown).

A rapid increase in inducible NO synthase (iNOS) mRNA expression was observed as early as day 4 in the treated and the untreated mice, but this effect was controlled by rHuEPO until day 7. The gaseous NO production in the cortex was lower in the uninfected mice (used for calibration) than in the infected mice that were untreated (P < .05). No difference was observed between the uninfected mice and the infected mice treated with rHuEPO or between the infected mice that received treatment and the infected mice that did not. With respect to the blood, mean NO levels were decreased in the infected mice that received treatment, compared with those in the uninfected mice (P = .01) (data not shown). A similar trend was seen in the infected mice that did not receive treatment, but it did not reach significance (6 df instead of 7 df). Accordingly, NO levels did not differ between the infected mice that received treatment and those that did not.

**DISCUSSION**

Systemic injections of rHuEPO (~120 IU/mouse) at the time when the symptoms of cerebral malaria began to appear protected a majority of *P. berghei* ANKA–infected mice from death. rHuEPO had no effect on the course of parasitemia, and mice treated with only 3 inoculations survived acute cerebral malaria and died of severe anemia 20–30 days after infection. The timing of EPO administration during the course of infection is crucial, as it has been demonstrated that early administration is associated with fatal outcome [15]. Because critical symptoms may occur <24 h after infection in human cerebral malaria, we decided to administer rHuEPO at the appearance of clinical signs. We did not observe any clear clinical improvement until day 6 in the mice treated with rHuEPO, and most of the untreated mice died. These observations demonstrate that treatment with EPO during the acute stage of severe malaria protects against death without affecting parasitemia. The therapeutic
window we used is of interest for future potential clinical studies, because it allows a standard follow-up of patients presenting with severe malaria without interfering with the clinical presentation of the disease and without inducing the adverse effects that have been reported to result from long-term EPO treatment. rHuEPO shares main features with murine EPO and crosses the blood-brain barrier with high efficacy [8]; it has been widely used in a murine model of cancer (reviewed in [16]). Moreover, in a rodent model of multiple sclerosis, rHuEPO has been demonstrated to modulate 3 distinct intracellular pathways (including Akt phosphorylation) after binding to EPOR, confirming the reliability of its use [17].

Preclinical studies are promptly needed to explore the effect of a single inoculation of EPO on the occurrence of symptoms of severe malaria and the potential interactions between this cytokine and specific antimalarial drugs. The potential hazard of EPO administration in such cases is probably minor, considering the potential benefits of avoiding premature death caused by an acute disease that can be effectively treated by antimalarial drugs.

Given the multiple protective effects of EPO in patients experiencing severe traumatic or infectious diseases, it could be speculated that inhibition of cell apoptosis, decreases in the production of proinflammatory cytokines, and protection of the microvasculature integrity could be involved in survival from cerebral malaria. Many studies have demonstrated that EPO protects cells from cytotoxic events by inhibition of apoptosis in neuronal structures (reviewed in [18]). It has been recently shown that Fas-deficient mutant mice are less susceptible to cerebral malaria [19]. Surprisingly, neuronal-cell DNA fragmentation, as assessed by TUNEL staining, was not abolished by EPO treatment. On days 6 and 7 after infection, there was no difference in the level of apoptosis of neuronal cells between the mice treated with rHuEPO and the untreated mice. Moreover, no difference in caspase 3 and caspase 8 mRNA expression was observed during the course of disease between the treated and the untreated mice. Thus, the potential link between protection against cerebral malaria and the antiapoptotic effect of EPO could not be demonstrated here.

In a recent review, Coleman and Brines stated that the tissue response to stress is characterized by a rapid and marked increase in the local EPOR level, followed by an increase in local EPO production within the penumbra of injury [20]. Analyzing mRNA expression in the whole brain of infected mice, we observed a significant increase in EPO mRNA expression during the course of infection. Except for a significant inhibition of EPOR mRNA expression, rHuEPO treatment did not affect EPO mRNA expression. Because cerebral malaria could not be related to topographically defined cerebral lesions, it might be suspected that modification of mRNA expression in limited focus was masked by nondamaged tissue. In light of these data and the low sensitivity of the available rHuEPO ELISA, it is not surprising that the direct measurement of this cytokine in the brains of mice has not yet been done [21]. Nevertheless, because malarial parasites have been shown to induce EPO production during the course of infection and because cross-reactions could be observed between murine EPO and HuEPO assays, it appears that this measurement would be useless in our experiments.

However, considering the favorable outcome of cerebral malaria in the rHuEPO-treated mice, we could speculate that this cytokine can act via a means beyond the inhibition of apoptosis. EPO has been shown to influence extrinsic-cell homeostasis through the control of cytokine release [18]. In our model, we observed that TNF-α and IFN-γ mRNA was overexpressed in untreated mice on day 6, compared with that in EPO-treated mice. Thus, the inhibition of TNF-α and IFN-γ mRNA overexpression could be one of mechanisms of the protective effect of EPO. The role that these proinflammatory cytokines play in the pathogenesis of cerebral malaria has been widely explored for decades [22–24]. Grau et al. have clearly demonstrated that neutralization of IFN-γ is capable of preventing TNF-α overproduction and the development of cerebral malaria in mice [25]. A clinical trial of a monoclonal antibody in children with cerebral malaria failed to demonstrate an improvement in survival [26], probably because the pathogenic mechanisms involved in this disease are more sophisticated than overexpression of a single cytokine. It is not clear whether the beneficial effect of EPO on the outcome of murine cerebral malaria observed here is related to a global anti-inflammatory effect or to a more specific action. For example, we were unable to differentiate the role played by TNF-αR1 and TNF-αR2 between the mice treated with rHuEPO and the untreated mice. However, EPO is known to exert an anti-inflammatory effect on the central nervous system by reducing TNF levels during experimental autoimmune encephalomyelitis and cerebral ischemia [27, 28]. It could be speculated that the progression from cerebral malaria to death, induced by proinflammatory cytokine production leading to vascular damage and perivascular hemorrhages, could be counteracted by EPO treatment administered at the onset of the clinical stage of the disease. This is in accordance with our observation that the rHuEPO-treated mice had fewer brain hemorrhages than did the untreated mice on day 7, when the peak of mortality occurs, and with the reported observations that EPO can prevent the permeability of the blood-brain barrier, maintain the establishment of cell-to-cell junctions, and protect cerebral endothelial cells during injury [29, 30].

Excess production of NO has been implicated in the pathogenetic mechanisms of cerebral malaria [31] and in various types of cell death [32]. As has been previously described in P. berghei-infected Swiss mice [14], we observed a significant increase in
NO production in the brain and a decrease in the blood during the critical stage of the disease, but no effect of rHuEPO treatment. This could be explained by the fact that EPO offers cellular protection against NO exposure without interacting with its production; however, it most likely explained by an impaired induction of a secondary cellular pathway, such as GAPDH s-nitrosylation [33].

In a model of staurosporine-induced neuronal-cell apoptosis, it has been also demonstrated that NO production inhibits caspase activity by nitrosylation of a critical cysteine residue in the active site of caspases, rendering the protease inactive [34]. However, this effect was not associated with reduced cell death by nonapoptotic mechanisms. The direct impact of iNOS overexpression and NO production on caspase activation and neuronal-cell apoptosis during the acute stage of the disease has to be taken into account in the analysis of this complex phenomenon.

We have demonstrated the protective effect of EPO on survival during cerebral malaria, with more evidence for an immunomodulatory effect than for an antiapoptotic effect. EPO has been used for years in patients with anemia and chronic kidney diseases, and more recently it has been used in patients with acute ischemic stroke and cardiac disorders [35]. Most of the adverse effects of EPO are associated with long-term administration (including vascular thrombosis and upper respiratory tract infections), except for uncontrolled hypertension, which is a contraindication. For these reasons, administration of EPO for symptomatic treatment of acute cerebral malaria is not likely to be harmful. The data presented here provide, for the first time, an efficacious way to improve the outcome of severe malaria.

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