Deletion of C-reactive protein ameliorates experimental cerebral malaria?

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Background: C-reactive protein (CRP) level correlates with parasitemia and severity of malaria, but whether this reflects causality remains unknown.

Methods: Using CRP-transgenic and CRP-deficient mice we compared the onset and severity of experimental cerebral malaria (ECM) induced by *Plasmodium berghei* ANKA (PbA).

Results: CRP-deficient mice were most resistant to ECM.

Conclusions: CRP might contribute to the development of cerebral malaria, rather than protect against it.

Keywords: Acute phase protein, Cerebral malaria, C-reactive protein, Parasitemia

Introduction

Cerebral malaria (CM), the severest form of malaria, is commonly seen in young children and travelers to endemic areas. CM has a high fatality rate and often causes long-term neurological consequences in survivors. The severity of CM is thought to be the consequence of a strong host immune and inflammatory response to the invading *Plasmodium* spp.¹ Blood levels of C-reactive protein (CRP) are positively associated with parasite burden in malaria^{2–5} and CRP levels are higher in CM compared to non-CM or mild malaria.^{6–8} CRP is an acute phase protein that can activate complement, bind Fc receptors and stimulate cytokine production,⁹ thus, the protein's elevation in blood might be expected to exacerbate CM. Yet paradoxically, in patients from malaria-endemic regions, high parasitemia does not always induce CRP elevation and a CRP genetic variation linked to heightened blood CRP¹⁰ associates with increased susceptibility to malaria infection.^{11,12} To investigate the possible role of CRP in malaria we investigated the influence of CRP excess versus CRP deficiency on the outcome of experimental cerebral malaria (ECM) in mutant mice.

Materials and methods

We compared the outcomes of *Plasmodium berghei* ANKA (PbA)-induced ECM in wild type C57BL/6 mice (WT) versus their human CRP-transgenic (CRPtg) and mouse CRP-deficient (CRPko) counterparts. The CRPtg and CRPko mice have both been fully described elsewhere.^{13–16} Human CRP is present in CRPtg at blood concentrations that are physiologically relevant in humans

i.e., low levels under steady-state conditions (<1 to 30 µg/ml), and much higher levels during an acute phase response (100 to 500 µg/ml).^{13,14} In WT and CRPtg mice, mouse CRP is still expressed, but mouse CRP is not a major acute phase protein.¹⁵ In CRPko mice expression of mouse CRP is entirely eliminated by Cre-Lox recombination, as described elsewhere.¹⁶ Mice were infected with 5×10⁵ PbA-infected red blood cells (iRBCs) and clinical symptoms were monitored as previously described.¹⁷ Briefly, mice were monitored twice daily for clinical signs of neurologic disease, using the following scoring scale: 0. asymptomatic; 1. symptomatic (ruffled fur); 2. mild disease (slow righting); 3. moderate disease (difficulty righting); 4. severe disease (ataxia, seizures, coma) and 5. dead. Mice observed having seizures were given a score of 4 regardless of other clinical signs of disease. Moribund animals were scored 4.5 and humanely sacrificed. Mice were classified as having ECM if they displayed these symptoms between days 6 to 9 post-infection, had positive thin-blood smears and had a corresponding drop in external body temperature or succumbed to infection. A cumulative disease index (CDI) was calculated for each animal by plotting clinical scores between days 5 to 10 and calculating the area under the curve. Blood samples were collected via retro-orbital bleeding and were used for both estimation of parasitemia by Giemsa-stained blood smears and for measurement of human and mouse CRP by ELISA.

Results and Discussion

Survival curves for the three genotypes of mice differed significantly ($p=0.0235$; Mantel-Cox logrank test, Figure 1A) with

Figure 1. Survival is increased and clinical signs of experimental cerebral malaria are improved in mice lacking C-reactive protein (CRP). Wild type (WT), CRP knockout (CRPko) and human CRP transgenic (CRPtg) mice were injected intraperitoneally on day 0 with 5×10^5 PbA-infected mouse red blood cells (iRBCs) and monitored for 10 days. (A) Survival of CRPko mice was significantly greater than that of WT and CRPtg mice. The asterisks indicate $p < 0.025$ for χ^2 tests comparing survival of WT and CRPtg to that of CRPko. (B) CRPko mice had significantly reduced clinical scores compared to CRPtg and WT mice (asterisk indicates $p < 0.05$ for Student's t tests comparing CRPko to WT and CRPko to CRPtg). (C) The cumulative disease index (CDI) was significantly different among the 3 genotypes (single asterisk indicating $p < 0.01$ by ANOVA) and compared to WT mice the CDI was significantly lower in CRPko and significantly higher in CRPtg (double asterisks indicating $p < 0.05$ by Student's t tests). (D) Parasitemia (% of iRBCs) is unaffected by presence or absence of CRP and both human CRP (E) and mouse CRP (F) are significantly reduced by day 7 post-infection (asterisks indicate $p < 0.025$ for paired Student's t tests comparing day 0 and day 7 values). 'ltd' and the dashed line indicate the respective lower limits of detection for the human CRP and mouse CRP ELISAs employed. The data shown in panels (A-F) are pooled from three separate experiments.

significantly more CRPko surviving to day 10 (9 of 13 mice) compared to WT (3 of 12 mice) and CRPtg (2 of 13 mice) ($p = 0.0236$ and $p = 0.0077$, respectively, by χ^2 tests). For all three genotypes neurological symptoms first were apparent on day 6 post-infection (Figure 1B), but CRPko had significantly reduced clinical scores compared to either CRPtg ($p = 0.0166$; paired Student's t

test) or WT mice ($p = 0.037$; paired Student's t test). The CDI was also significantly different among the three genotypes ($p < 0.01$; ANOVA, Figure 1C), with the CDI of CRPko being significantly lower than that of CRPtg and WT ($p = 0.02$ and $p = 0.036$, respectively, for 1-tailed Student's t tests). Despite the significantly different severity of ECM and the significantly different

mortality rates in the three genotypes there was no remarkable difference in their levels of parasitemia (Figure 1D), indicating that CRP modifies the disease phenotype, but not infection rates. At the time points tested neither human nor mouse CRP blood levels were significantly elevated above baseline values (Figure 1E and 1F, respectively). Rather, the blood levels of both CRP species trended downwards after day 4, concomitant with increasing parasitemia (compare Figure 1D to Figure 1E and 1F). This decrease in blood human and mouse CRP levels achieved statistical significance on day 7 for CRPtg mice ($p < 0.0237$ and $p < 0.0198$ for human and mouse CRP, respectively, by paired Student's *t* tests). The mechanisms underlying the decline of CRP levels as ECM progressed are not fully clear but may be due, in part, to hepatic toxicity and reduced production associated with the rapidly accelerating acute phase response.

The combined data are consistent with the conclusion that both human and mouse CRP actively participate in ECM. To our surprise both CRP species promoted ECM rather than protected against it, i.e., CRPko mice had the least severe symptoms and highest survival rate, while CRPtg mice had the most severe symptoms and lowest survival rate. These disparate clinical outcomes were realized despite nearly equal blood parasite burdens in the three different CRP genotypes. This is the first study suggesting that CRP plays an active role in ECM, with increased CRP levels associated with worse disease outcome. The mechanism for the exacerbating effect of CRP is currently unknown but might involve CRP-mediated complement activation, Fc receptor signaling, or cytokine production.² One weakness of our study is that because of a lack of early blood samples (prior to day 4) we were unable to ascertain if ECM was accompanied by an early CRP acute phase response. Furthermore, although murine ECM has proven to be a powerful research tool for discovery of mechanisms that might contribute to the pathophysiology of malaria,¹⁸ ECM in mice is simply not identical to CM in humans. Consequently, to ascertain the full range of CRP responsiveness during ECM and to dissect the pathways involved in the exacerbating effect of CRP on ECM will require more animal studies. Finally, determining whether or not our observations have any clinical merit will ultimately require further studies of CRP genetics and biology in malaria-infected humans.

Authors' contributions: AJS, TNR and SRB conceived the study; TNR performed the biological analyses; all authors contributed to interpretation of the data, wrote the manuscript and critically revised it for intellectual content. All authors read and approved the final manuscript. SRB and AJS are guarantors of the paper.

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Competing interests: None declared.

Ethical approval: Animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Alabama, Birmingham.

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