Cutting Edge: The Membrane Attack Complex of Complement Is Required for the Development of Murine Experimental Cerebral Malaria

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Cerebral malaria is the most severe complication of Plasmodium falciparum infection and accounts for a large number of malaria fatalities worldwide. Recent studies demonstrated that C5 deficient mice are resistant to experimental cerebral malaria (ECM) and suggested that protection was due to loss of C5a-induced inflammation. Surprisingly, we observed that C5aR knockout mice were fully susceptible to disease, indicating that C5a is not required for ECM. C3aR knockout and C3aR knockout mice were equally susceptible to ECM as were wild-type mice, indicating that neither complement anaphylatoxin receptor is critical for ECM development. In contrast, C9 deposition in the brains of mice with ECM suggested an important role for the terminal complement pathway. Treatment with anti-C9 Ab significantly increased survival time and reduced mortality in ECM. Our data indicate that protection from ECM in C5 deficient mice is mediated through inhibition of membrane attack complex formation and not through C5a-induced inflammation. The Journal of Immunology, 2011, 186: 6657–6660.

Malaria remains one of the most deadly infectious diseases throughout the developing world (1). Attempts at immunological control of this parasite through vaccine development have not been successful after decades of effort. This failure is due, in part, to our poor understanding of the host immune response to the parasite at the level of both innate and adaptive immunity (2). Cerebral malaria (CM) is one of the most severe clinical complications of Plasmodium falciparum malaria, with a fatality rate of 15–30%: >10% of CM survivors have permanent neurologic sequelae. Despite the impact of CM, our understanding of the molecular mechanisms leading to pathology is limited, particularly as it relates to the innate immune response.

The role of complement in the development and progression of malaria and CM has been explored for several decades. Malaria infection activates the classical and alternative pathways, leading to elevated serum levels of many complement activation fragments (reviewed in Ref. 3). These observations have provided little insight into the contribution of complement to malaria pathogenesis. Recent studies, seeking to determine the susceptibility of several congenic mouse strains to experimental cerebral malaria (ECM), demonstrated that mice naturally deficient in C5 were resistant to ECM development. Furthermore, treatment of wild-type mice with anti-C5aR or anti-C5a Ab protected against the development of ECM, and anti-C5aR Ab significantly inhibited monocyte-derived cytokine production (4, 5). These data suggest that C5, through the biological activities of C5a, is an important contributor to ECM pathology. Although these data are compelling, ECM development and progression were never assessed in C5aR knockout mice, nor was the possibility examined that ECM could be dependent on C5b and the membrane attack complex (MAC).

The present study demonstrated that neither complement anaphylatoxin receptor is crucial for disease development. Instead, the critical role of C5 in ECM is mediated at the level of C5b and MAC formation. Targeting the terminal complement pathway may offer a therapeutic approach to lessen the severity of CM.

Materials and Methods
Mice, malaria parasites, and ECM
C5-deficient DBA/2 mice were backcrossed to C57BL/6 mice for eight generations. C5aR−/− and C3aR−/− mice were described previously (6, 7). C3aR−/−/C5aR−/− mice were generated by crossing C3aR−/− and C5aR−/− mice. Male and female mice between the ages of 8 and 12 wk were used for all experiments. All studies were performed with approval from the University of Alabama at Birmingham Institutional Animal Care and Use Committee. Plasmodium berghei ANKA (PbA) was maintained by passage in BALB/c mice, as previously described (8). ECM was induced by injecting mice i.p. with 5 × 106 PbA-infected RBCs. Parasitemia was monitored on day 6 postinfection by Giemsa-stained, thin-blood smears. Mice were monitored for survival. The online version of this article contains supplemental material.

Abbreviations used in this article: CM, cerebral malaria; ECM, experimental cerebral malaria; MAC, membrane attack complex; PbA, Plasmodium berghei ANKA.

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twice daily for clinical signs of neurologic disease, in a blinded fashion, using the following scoring scale: 0, asymptomatic; 1, symptomatic (ruffled fur); 2, mild disease (slow righting); 3, moderate disease (difficult 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 5–9 post-infection and had a corresponding decrease in external body temperature or succumbed to infection.

CNS cytokine serum protein levels and analysis of leukocytes from brains

Whole blood was collected via retro-orbital bleed on day 6 post-ECM induction. Samples were assayed for TNF-α and IL-6 by ELISA (Invitrogen), which was performed according to the manufacturer’s instructions. Mice were transcardially perfused with PBS for 2 min, and brains were processed for flow cytometry, as previously described (9).

Immunofluorescence for C9 deposition in the brains of wild-type mice with ECM

On day 6 postinfection, brains from wild-type PbA-infected mice were removed and snap-frozen in Tissue-Tek OCT embedding medium. Sections (10 μm) were cut, fixed in acetone, air dried, and blocked overnight with an anti-mouse CD16/32 Ab (eBioscience, San Diego, CA). Sections were immunostained with rabbit polyclonal anti-mouse C9 (100 μg/ml) generated using a mouse C9 peptide consisting of amino acid sequence 21–40 coupled to keyhole limpet hemocyanin (Biosynthesis, Lewisville, TX). This Ab detects a single band ~72 kDa on Western blot analysis of normal mouse (but not rat) serum, which corresponds to the correct molecular mass of mouse C9 (Supplemental Fig. 2). The Ab was visualized with a goat anti-rabbit, Alexa Fluor 555-conjugated Ab (Molecular Probes). Blood vessels were stained using a biotin-conjugated anti-CD31 Ab and visualized using streptavidin-488 (Biolgend). Nuclei were stained using DAPI Fluoromount G (Southern Biotech).

Inhibition of C9 in ECM with anti-C9 Ab treatment

Wild-type mice were infected as described above. Starting on either day 3 or 4 postinfection, mice were treated with 400 μg rabbit anti-mouse C9 Ab, 400 μg rabbit IgG isotype control, or PBS. Mice were injected daily until the onset of symptoms (day 6 postinfection). This dose was determined to be optimal based on dose-response experiments.

Statistical analysis

Statistical significance of ECM survival was calculated using the log-rank test using Prism 4 (GraphPad Software). Student t test (one way), Mann–Whitney test, or Wilcoxon rank-sum tests were used to determine significance for parametric and nonparametric data. Data are shown as mean ± SEM. A p value < 0.05 was considered statistically significant.

Results and Discussion

C5−/− mice are highly resistant, whereas C5aR−/− and C3aR−/− mice are fully susceptible to ECM

All strains of mice examined to date succumb to ECM within 8–10 d postinfection, with the exception of AKR/J (naturally C5-deficient) and BALB/c (C5-sufficient) (4, 10–12). This prompted us to examine for differences in ECM onset and progression between C5−/− and wild-type C57BL/6 mice. We observed that C5−/− mice were remarkably resistant to ECM, with >90% surviving past day 10 postinfection, whereas all wild-type mice succumbed to ECM by day 9 postinfection (Fig. 1A; p = 0.0001, log-rank test). Based on these observations, C5−/− mice on the C57BL/6 background are one of the most ECM-resistant strains examined to date. In addition, C5−/− mice had a corresponding reduction in clinical disease scores, proinflammatory cytokine production (TNF-α and IL-6), and leukocyte infiltration (CD8+ T cells) into the CNS compared with wild-type mice (Supplemental Fig. 1A–C). Together, these results suggested that C5−/− mice have a reduced inflammatory environment in the cerebral microvasculature and, therefore, a correspondingly lower propensity to develop ECM.

Based on previous reports (4, 5), it was reasonable to predict that C5aR−/− mice would have a higher survival rate than wild-type mice in PbA-induced ECM because of an inability to respond to C5a-mediated inflammation. To verify this possibility, we induced ECM in C5aR−/− mice. Surprisingly, we found that C5aR−/− and wild-type mice were equally susceptible to ECM (p = 0.14, log-rank test) (Fig. 1B) and that overall disease severity mimicked that of wild-type mice (Supplemental Fig. 1D). One possible explanation for these results is that C3aR substitutes for C5aR in the development of ECM, in keeping with the known significant functional overlap between these two anaphylatoxin receptors (13). We induced ECM in C3aR−/− mice and observed results similar to those obtained for the C5aR−/− mice: C3aR−/− and wild-type mice were equally susceptible to ECM (p = 0.9, log-rank test; data not shown). Furthermore, C3aR−/−/C5aR−/− mice were equally susceptible to ECM (p = 0.20, log-rank test; data not shown). In contrast to previously reported studies using Ab to block C5a-mediated effects (4), our genetic-based

FIGURE 1. C5−/− mice are resistant to the development of ECM, whereas C5aR−/− mice are fully susceptible. Wild-type, C5−/−, and C5aR−/− mice were injected i.p. with 5 × 107 PbA-infected RBCs, and clinical scores and survival were monitored twice daily for 10 d, as described in Materials and Methods. A, C5−/− mice (n = 16) were significantly resistant to disease-induced mortality (p = 0.0001, log-rank test; >90% survival past day 10) compared with wild-type mice (n = 19). B, C5aR−/− (n = 17) and wild-type mice (n = 17) had identical disease-induced mortality.
findings clearly indicated that the complement anaphylatoxins receptors are not critical to ECM susceptibility.

If C5 is not acting through the C3aR and C5aR anaphylatoxin receptors, it is likely that C5b and the terminal complement components leading to the formation of the MAC are responsible for complement-mediated disease susceptibility in ECM. To determine whether C9 deposits in the brain during ECM, we performed immunofluorescence studies on frozen brain sections, obtained from wild-type mice 6 d postinfection using a newly generated rabbit anti-mouse C9 Ab. This Ab recognizes mouse C9, but not rat C9, as determined by Western blot analysis of normal mouse and rat serum (Supplemental Fig. 2). C9 staining was readily detected throughout the cortex of infected mice (Fig. 2A, shown in red), whereas no staining was observed using rabbit IgG control Ab (Fig. 2C). C9 staining frequently, but not always, colocalized with blood vessels, as determined by staining simultaneously for PECAM (CD31) as a marker for blood vessels (Fig. 2B, arrows). In general, C9 staining was punctate; however, staining in some regions was cellular in appearance (Fig. 2B, arrowheads).

Attenuated ECM in C5aR−/−, but not C5aR−/− mice, combined with C9 deposition in the brains of mice with ECM suggested that ECM may be dependent, in part, on MAC formation. To address this possibility, we treated mice with rabbit anti-C9 Ab after induction of ECM. Injections of anti-C9 Ab on days 3–6 postinduction significantly delayed the time to death (p = 0.0001, log-rank test) and reduced clinical scores compared with PBS control-treated mice (p < 0.001, days 6–9, Wilcoxon rank-sum test) (Fig. 2C, 2D).

Table I shows that the cumulative disease index was significantly reduced and that the time to 50% survival was significantly longer in anti-C9–treated mice compared with either PBS- or isotype control-treated mice (6.5 versus 8.4 d). These are, to our knowledge, the first data to demonstrate that inhibition of C9 and, by extension, prevention of MAC formation, is protective in ECM. It is unclear whether the anti-C9 Ab crosses the blood–brain barrier; however, the majority of the inflammation and pathophysiology of ECM occurs in the cerebral microvasculature, precluding the need for the Ab to reach the brain parenchyma.

Our results contradict recent studies suggesting that C5a is a primary inflammatory mediator in ECM (4, 5). Although C5a may contribute to inflammation in CM, our data demonstrating that C5aR−/− mice are highly susceptible to ECM indicated that C5a does not drive development of cerebral disease. The protective effect of anti-C5aR Ab reported in the study by Patel et al. (4) remains unexplained in the absence of additional supporting data. The specificity of C5aR Ab used in those studies (14) was not documented by Western blot analysis or other methodologies. Assessing the effectiveness of

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<th>Cumulative Disease Indexa</th>
<th>Days to 50% Survivalb</th>
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<tr>
<td>Isotype control (n = 14)</td>
<td>19.6</td>
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<tr>
<td>Anti-C9 treatment (n = 14)</td>
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a Mean of the sum of daily clinical scores assigned between days 1 and 10.
b The day at which 50% of the mice have survived postinfection with infected RBCs.

*p = 0.0001, compared with control; unpaired Student t test.

FIGURE 2. C9 deposits in the CNS during ECM, and inhibition of C9 delays development of ECM. Wild-type mice were injected with 5 × 10⁸ PbA-infected RBCs, as described in the text. Mice were sacrificed at day 6, and brains were harvested and embedded in OCT. A, Sections (10 μm) were treated with Fc block, stained with rabbit anti-C9 sera (100 μg/ml), and visualized with goat anti-rabbit Alexa Fluor 555 (original magnification ×10). B, Boxed area in A magnified to highlight colocalization of C9 and CD31 staining (arrows) and punctate and cellular C9 staining patterns (arrowheads) (original magnification ×16). C, Staining as in A, except that preimmune rabbit sera (100 μg/ml) was used (original magnification ×16). All sections were mounted in DAPI Fluoromount. Anti-C9 Ab–treated mice (D) were significantly protected from ECM (p = 0.0001, log-rank test) and had reduced clinical scores compared with mice treated with isotype-control Ab (E) (p < 0.05 on days 6–9, Wilcoxon rank-sum test).
C5a inhibition in ECM, as reported in the same study, is not possible because the data were not shown. A potential confounding factor could be the use of different mouse strains between the study by Patel et al. (4) and our study (B10.D2/NsnJ versus C57BL/6J). This seems unlikely because disease susceptibility between the strains seems linked to C5 and uncoupled to the MHC (4). All other experimental parameters between the previous study and what we report in this article were essentially the same. C5L2, a second C5aR (15), may also participate in the development of disease pathogenesis in malaria, but its contribution alone, or together with the canonical C5aR, in ECM has yet to be reported. C5L2 functions are broadly defined, ranging from a nonsignaling decay receptor to glucose and lipid metabolism. The consensus of numerous studies indicates that C5L2 is uncoupled from G proteins and intracellular signaling events (13, 16–18). This suggests that any role C5L2 has in malaria may be distinct from that traditionally associated with complement-mediated inflammation.

The protective effect that we observed with C5−/− mice, or upon treatment with anti-C9 Ab, demonstrated an important role for the terminal complement pathway in ECM. Intuitively, MAC-mediated parasite lysis would logically provide a critical protective function. Surprisingly, protection from ECM in C5−/− mice seems to be independent of parasite load, because both wild-type and C5−/− mice had similar parasite levels at the peak of disease (data not shown). This suggests that complement-mediated clearance of parasites by terminal-pathway mechanisms is minimal and that this pathway enhances disease severity rather than provides protection through parasite elimination. There is growing evidence of platelet/complement interactions, whereby platelets directly activate the complement system and, in turn, undergo complement-mediated lysis (19–22). Platelets release numerous inflammatory mediators and cytokines that contribute to disease pathology in CM (23, 24). This inflammatory dynamic may be blocked in C5−/− mice and partially abrogated on treatment with anti-C9 Ab.

Our data indicated that the complement terminal pathway is an important contributor to the pathogenesis of ECM and suggest that studies to address this possibility in human CM are warranted. It is now important to develop experimental-intervention protocols in a murine model that combines inhibition of the terminal complement pathway with antimalarial treatment and postdiagnosis of ECM to mimic more closely the challenges of treating human CM in the clinic. In this regard, examining for elevated serum levels of MAC or deficiencies or polymorphisms in terminal-pathway components in patients with CM compared with those with severe malaria may prove useful. Should human studies in these areas provide support for the results that we reported in this article, it would pave the way for testing the usefulness of eculizumab, a U.S. Food and Drug Administration-approved anti-C5 therapeutic in use for treating paroxysmal nocturnal hemoglobinuria (25, 26), in human malaria.

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Disclosures
The authors have no financial conflicts of interest.

References