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Targeted Inhibition of Complement Using Complement Receptor 2-Conjugated Inhibitors Attenuates EAE

Xianzhen Hu[a], **Stephen Tomlinson**[b], and **Scott R. Barnum**[a,c]

Stephen Tomlinson: tomlinss@musc.edu; Scott R. Barnum: sbarnum@uab.edu

[a]Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294, United States

[b]Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, SC 29425 and the Ralph H. Johnson Veterans Affairs Medical Center, Charleston, SC 29401, United States

[c]Department of Neurology, University of Alabama at Birmingham, Birmingham, AL 35294, United States

Abstract

Multiple sclerosis (MS) is the most common autoimmune demyelinating disease, affecting millions of individuals worldwide. In the last two decades, many therapeutic options for the treatment of MS have become available, however they are limited in terms of effectiveness and some remain plagued by safety issues. The currently available treatment options target relapsing remitting forms of MS and are not effective against the more progressive forms of the disease. These limitations highlight a significant unmet treatment need for MS. In experimental autoimmune encephalomyelitis (EAE) studies from our laboratory, we have previously shown, using a number of complement mutant and transgenic mice, that inhibition of the alternative complement pathway and the C3 convertase confers significant protection from disease. We report here that targeted inhibition of complement activation using complement receptor 2 (CR2)-conjugated inhibitors significantly attenuates EAE. Administration of CR2-Crry (blocks all complement pathways at C3 activation) and CR2-fH (specifically blocks the alternative pathway) just prior to and during the onset of EAE blocks progression of both acute and chronic disease. These data indicate that inhibition of complement may offer an effective therapeutic approach to treating both acute and chronic forms of demyelinating disease through blocking the alternative pathway or complement convertases.

Keywords

experimental autoimmune encephalomyelitis; neuroimmunology complement; immunology; autoimmune disease

Correspondence: Dr. Scott R Barnum, Department of Microbiology, University of Alabama at Birmingham, 845 19th St. S. BBRB/842, Birmingham, AL 35294, 205-934-4972 (office), sbarnum@uab.edu.

Introduction

Multiple sclerosis remains one of the most common demyelinating diseases worldwide affecting millions of individuals [10, 13]. Progress has been made in recent years with respect to the development of disease-modifying therapies for MS, including IFN-β, glatiramir acetate, natalizumab (Tysabri) and fingolimod with others in the developmental pipeline [7, 34, 41, 50]. Despite their widespread use, all of these MS immunotherapeutic reagents have limitations in terms of overall clinical effectiveness within the relapsing-remitting population or, serious safety concerns [18–21, 23, 38]. Although there are several MS therapeutics in development or clinical trials, none show greater efficacy than the currently available therapeutics for relapsing remitting MS and few of these target progressive MS, which currently has no therapeutic options [7, 41, 50]. Thus despite the current clinical successes with disease-modifying MS drugs, there are still significant unmet needs for progressive forms of disease [15, 39].

Complement has been implicated in the pathology of MS and experimental autoimmune encephalomyelitis (EAE) for nearly forty years (reviewed in [4, 24, 26, 31, 32]). In early studies, animals treated with cobra venom factor (CVF), which transiently depletes serum complement activity, had reduced disease severity and CNS infiltration and inflammation [24, 25, 27, 31, 32, 35] demonstrating for the first time the potential therapeutic benefit of inhibiting complement activation in demyelinating disease. In subsequent studies, a soluble version of the complement receptor type 1 (sCR1) was used to treat EAE. sCR1 inactivates the C3 and C5 convertases and effectively blocks complement activation through all three activation pathways (reviewed in [22, 28]). sCR1 significantly reduced both inflammation and demyelination, but did not completely suppress disease [36]. Attempts to use sCR1 in human clinical studies were abandoned due in part to difficulty in properly expressing a large protein (>150kDa) with over 40 disulfide bridges. These initial studies raised the questions: could a more targeted approach that inhibits only one complement pathway or effector molecule provide insight into potential complement therapeutics for MS and what is the best target to pursue? Through the use of complement mutant mice, our laboratory and others have demonstrated that EAE is significantly attenuated on deletion of factor B or C3, key alternative pathway components [30, 46]) or on deletion of the complement receptors for iC3b (CR3 and CR4) [8, 9], but not through other complement pathways or proteins [4]. The results of these animal studies set the stage for proof of concept studies to determine if alternative pathway inhibitors would be effective in attenuating EAE. For these studies, we employed the recently described complement therapeutic agents CR2-Crry or CR2-fH [17, 43]. These agents specifically target sites of complement activation through the amino-terminal domain encoding the iC3b/C3d binding site from the complement receptor type 2 (CR2) and inhibit complement convertase activity through a carboxy-terminal domain containing either the complement receptor-1 related gene/protein y (Crry) or the amino-terminal domains of mouse factor H. CR2-Crry inhibits the C3 and C5 convertases generated by any of the complement activation pathways, while CR2-fH specifically inhibits the alternative pathway. We report here that both CR2-Crry and CR2-fH significantly inhibit MOG-induced EAE, particularly in the chronic phase of disease. These results suggest that inhibiting complement activation through the alternative pathway may be therapeutically useful for progressive forms of demyelinating disease.

Materials and Methods

Mice

Inbred C57BL/6 mice, originally from The Jackson Laboratory (Bar Harbor, ME), were from our own colony. All studies were performed with approval from the UAB IACUC.

CR2-Crry and CR2-fH preparation

The recombinant fusion proteins CR2-Crry and CR2-fH were produced and purified as previously described [2, 17]. Protein purity was assessed by SDS gel electrophoresis and complement inhibitory activity was evaluated by zymosan assay [17].

Induction of active and adoptive transferred EAE and CR2-Crry and CR2-fH treatment

For active EAE, mice were immunized as previously described [47] on day -1 with 250 ng of PT (i.p.) and on day 0 with CFA emulsion containing 1 mg heat- inactivated *Mycobacterium tuberculosis* and 250 μg MOG peptide$_{35–55}$ (Biosynthesis, Inc., Lewisville, TX). On day 1 mice received a second PT injection and progression of EAE clinical signs were monitored daily for 30 days using a clinical scale ranging from 0 to 6 as follows: 0, asymptomatic; 1, loss of tail tone; 2, flaccid tail; 3, incomplete paralysis of one or two hind limbs; 4, complete hind limb paralysis; 5, moribund; 6, dead. Only mice with a score of at least 2 (flaccid tail) observed for 2 or more consecutive days were judged to have onset of EAE. A cumulative disease index (CDI) was calculated from the sum of the daily clinical scores observed between day 7 and day 30. **All mice regardless of disease status were included in the CDI calculations**. For transferred EAE, spleens of control donors were removed two to three weeks following induction of active EAE, and prepared as previously described [47]. Adoptive transfer EAE was induced by injecting ~5×10^6 purified T cells (i.p.) into wild type recipient mice and scored as described above. At various time points after induction of either active or transferred EAE, mice were injected i.p. with PBS (control group), CR2-Crry or CR2-fH as delineated in the Results section.

Statistics

Statistical significance between PBS, CR2-Crry and CR2-fH-treated mice for EAE onset, incidence and severity was calculated using the Student's t-test (Prism 5, GraphPad Software, Inc.).

Results

Treatment with CR2-Crry or CR2-fH delays and attenuates EAE

In preliminary EAE studies using CR2-Crry, we examined several dosing regimens and determined that two injections (500 μgs each injection) on days 7 and 12 were sufficient to attenuate EAE compared to PBS-treated controls. Disease severity was significantly reduced throughout the acute and chronic phases of disease (Fig. 1A, Table 1, days 12–30, p=0.01, Student's t-test). The cumulative disease index in CR2-Crry-treated mice was reduced 35% compared to PBS-treated mice (CDI: 60 vs. 39). Treatment with CR2-Crry also delayed the onset of EAE (16 days vs. 13 days, p=0.021, Student's t-test). The course of disease in CR2-Crry-treated mice is similar to what we reported for sCrry/GFAP mice in MOG-induced EAE in which a soluble form of Crry is produced in the CNS under the control of an astrocyte-specific promoter [11].

We also performed EAE studies using CR2-fH, which specifically targets alternative pathway activity [3, 17]. Preliminary studies to determine the optimal dosing regimen demonstrated that more frequent administration of CR2-fH was required to delay and attenuate EAE compared to CR2-Crry treatment. We found that mice injected with 400μg of CR2-fH on days 7, 9, 11 and 13 post-induction developed significantly less severe EAE compared to PBS-treated controls (Fig. 1B, Table 1). Disease onset in CR2-fH treated mice was markedly delayed (20 days vs. 14 days, p=0.005, Student's t-test). Similar to CR2-Crry-treated mice, we observed that disease severity was reduced throughout the acute and chronic phases of EAE (days 12–30, p=0.01, Student's t-test) and the cumulative disease

index was reduced over 50% (CDI: 28 vs. 61). Interestingly, shifting the treatment regimen earlier two days (days 5, 7, 9 and 11) resulted delayed disease, but little protection during the chronic phase of disease (data not shown). These data demonstrate that inhibition of complement by two different complement-targeted inhibitors can significantly attenuate an inflammatory and chronic form of EAE that is arguably reminiscent of progressive MS in humans [14, 44, 45].

Treatment with CR2-Crry prior to or after onset delays and attenuates transferred EAE

We extended our EAE studies with CR2-Crry to examine for protection in transferred EAE, which more closely mimics human demyelinating disease. For these studies we induced disease by transferring encephalitogenic T cells to wild type mice as previously described [46] after which they received either PBS or CR2-Crry (500 μgs/injection) on days 3, 5, 7 and 9 post-transfer. Similar to what we observed for active EAE, CR2-Crry-treated mice presented with delayed (13 days vs. 8 days) and attenuated EAE (CDI: 12 vs. 28.8) compared to PBS-treated mice (Fig. 2A, Table 2). Furthermore, the incidence of EAE was reduced by over 30% (100% vs. 67%). In a paired experiment, we also treated mice with CR2-Crry once they reached a clinical score of 1. In this treatment regimen, onset of EAE was not different compared to PBS-treated mice, however chronic disease was attenuated (Fig. 2B, Table 2; CDI 28.8 vs. 21).

Discussion

The data we present here provides proof of concept that inhibition of complement using CR2-based inhibitors offers a viable therapeutic approach to the treatment of demyelinating disease such as MS. This concept is supported by numerous studies using complement mutant mice and a variety of complement inhibitor molecules [4, 16, 29]. Complement receptor 2-based inhibitors have also proven to be effective in a number of inflammatory disease settings including several in the CNS such as spinal cord injury [37], stroke [12] and macular degeneration [40]. Both CR2-Crry and CR2-fH function by binding to covalently attached C3d fragments within 30–60 minutes of administration and subsequently prevent continued local complement activation [17]. Both proteins have relatively short fluid-phase half-lives (8–9 hrs.) [1, 17], but the half-life of C3d-bound, CR2-based inhibitors in the CNS is currently unknown. Our data suggest that both inhibitors are functional for extended periods of time since clinical EAE scores remained attenuated for at least 30 days post disease induction (Fig. 1). The clinical course EAE we show here with CR2-Crry and CR2-fH treatment is remarkably similar to that reported for factor B- and C3-deficient mice during EAE [30, 46]. In both cases, disease is significantly attenuated in the acute phase and fails to progress to the severity levels seen in control animals. At present it is not known if attenuated disease severity during the chronic phase of EAE is due to increased inhibitor half-life while bound to C3d on the vascular endothelium and/or brain parenchymal surfaces. From a mechanistic point of view, limiting complement-mediated inflammation and tissue damage may prevent or lessen epitope spreading [49], thereby by reducing overall disease severity, and possibly contribute to complement-mediated tolerance mechanisms [33, 48].

Currently there are no disease-modifying drugs approved for clinical use that block complement alternative pathway activity. Although it seems counterintuitive to inhibit the alternative pathway as a therapeutic approach because of the subsequent increased risk of infectious disease, numerous animal model studies have shown significantly better outcome using this approach (reviewed in [16]). The use of CR2-targeted complement inhibitors allows increased anti-inflammatory efficacy without altering the host response to infectious agents [2, 42]. Additional support for a complement inhibitory approach comes from the clinical success using Eculizumab, a human monoclonal antibody directed to C5 that is used

to treat paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome. This FDA-approved treatment has proven highly effective and very safe with respect to infection rates [5, 6] with the caveat that Eculizumab systemically inhibits complement but targets the terminal complement pathway. Taken together, these studies suggest that long-term use of targeted complement inhibitors, including alternative pathway inhibitors, in chronic inflammatory diseases such as MS, where treatment may be required for decades, are feasible and warrant further development.

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Abbreviations

CR2	complement receptor 2
Crry	complement receptor related protein y
fH	factor H

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Highlights

- Therapeutic approaches to treating demyelinating disease have unmet needs
- Based on EAE studies with complement-deficient mice, complement represents a therapeutic target
- CR2-based inhibitors are efficacious in blocking complement-mediated inflammation and damage
- We show that treatment with CR2-based inhibitors is protective in murine demyelinating disease

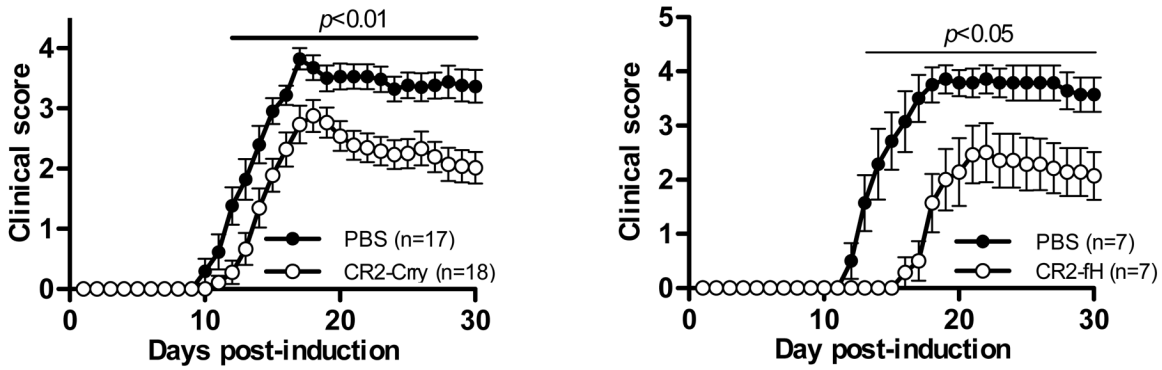

Figure 1. Clinical course of MOG-induced EAE in mice treated with CR2-Crry or CR2-fH

A. Wild type mice were either treated with saline (n=17; black circles) or with CR2-Crry (n=18; open circles) after induction of EAE and the course of disease was monitored for 30 days. Mice were injected with 500 μgs of CR2-Crry on days 7 and 12-post immunization. Disease severity was significantly attenuated in antibody treated mice (day 12 to 30, $p<0.01$, Student's t-test). Results shown are the mean of four experiments. **B**. Same as A except mice received 400μg of CR2-fH on days 7, 9, 11 and 13 (n=7; open circles) or PBS (n=7, black circles). Disease severity was significantly attenuated in CR2-Crry treated mice (day 13 to 30, $p=0.05$, Student's t-test). Results shown are the mean of two experiments.

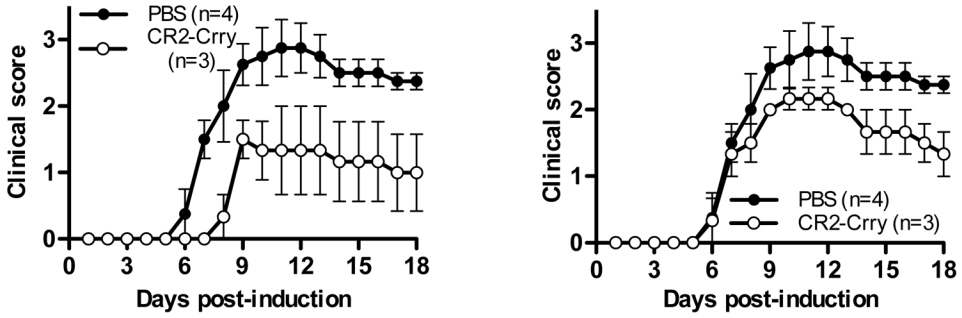

Figure 2. Treatment with CR2-Crry prior to or after disease onset attenuates transferred EAE

A. Transferred EAE was induced and monitored as in (A) but mice were either treated with PBS (n=4; black circles) or injected with 500 μgs of CR2-Crry on days 3, 5, 7 and 9-post immunization (n=3; open circles). **B.** In the same set of experiments as in A, a second group of mice were injected with 500 μgs of CR2-Crry on reaching a clinical score of 1 (n=3; open circles) (days 7, 9, 11, 13). Disease severity was significantly attenuated in CR2-Crry treated mice (p=0.002, Wilcoxon rank sign test).

Table 1

Active EAE phenotypes on treatment with CR2-Crry or CR2-fH.

Inhibitor Treatment	CDI[A]	Disease Onset[B]	Disease Incidence[C]
PBS (n=17)	60	13d	100%
CR2-Crry (n=18)	39*	16d	89%
PBS (n=7)	61	14d	100%
CR2-fH (n=7)	28*	20d	86%

[A] Cumulative Disease Index (CDI) - mean of the sum of daily clinical scores observed between days 7 and 30.

[B] Disease onset is defined as the first day of two consecutive days with a clinical score of 2 or greater.

[C] Disease incidence is the percent of mice that displayed any clinical signs of disease.

* $p<0.05$, control vs. inhibitor treated mice

Table 2

Transferred EAE phenotypes on treatment with CR2-Crry before and after disease onset.

Inhibitor Treatment	CDI[A]	Disease Onset[B]	Disease Incidence[C]
Saline control (n=4)	28.8	8d	100%
CR2-Crry (n=3) pre-onset	12.2	13d	67%
CR2-Crry (n=3) post-onset	21	8d	100%

[A] Cumulative Disease Index (CDI) - mean of the sum of daily clinical scores observed between days 0 and 30.

[B] Disease onset is defined as the first day of two consecutive days with a clinical score of 2 or greater.

[C] Disease incidence is the percent of mice that displayed any clinical signs of disease.