Published in final edited form as:

Mol Immunol. 2013 July ; 54(3-4): 302–308. doi:10.1016/j.molimm.2012.12.018.

Therapeutic Inhibition of the Alternative Complement Pathway Attenuates Chronic EAE

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Abstract

Previous studies from our laboratory using complement-mutant mice demonstrated that the alternative pathway is the dominant activation pathway responsible for complement-mediated pathology in demyelinating disease. Using a well-characterized inhibitory monoclonal antibody (mAb 1379) directed against mouse factor B, we assessed the therapeutic value of inhibiting the alternative complement pathway in experimental autoimmune encephalomyelitis (EAE), the animal model for multiple sclerosis. Administration of anti-factor B antibody to mice prior to the onset of clinical signs of active EAE had no effect on the onset or acute phase of disease, but significantly attenuated the chronic phase of disease resulting in reduced cellular infiltration, inflammation and demyelination in antibody-treated mice. Attenuation of the chronic phase of disease was long lasting even though antibody administration was terminated shortly after disease onset. Chronic disease was also attenuated in transferred EAE when anti-factor B antibody was administered before or after disease onset. Similar levels of disease attenuation were observed in transferred EAE using MOG-specific encephalitogenic T cells. These studies demonstrate the therapeutic potential for inhibition of factor B in the chronic phase of demyelinating disease, where treatment options are limited.

Keywords

Complement; Alternative pathway; Factor B; Demyelinating disease

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Disclosure: V.M.H. is co-inventor on patent 7,964,705 titled "Humaneered anti-factor B antibody", and V.M.H and J.M.T. serve as consultants to Alexion Pharmaceuticals, Inc.

The other authors declare they have no competing interests.

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1. INTRODUCTION

Demyelinating diseases including multiple sclerosis (MS), chronic inflammatory demyelinating polyneuropathy (CIDP), Guillain-Barre syndrome (GBS) and multifocal motor neuropathy (MMN) together affect millions of individuals worldwide (1–6). These diseases are characterized by a loss of central or peripheral nervous system myelination due to autoimmune responses, complement-mediated destruction of oligodendrocytes or Schwann cells and culminate in axonal degeneration. Progress in clinical treatment of these diseases has been variable, with the greatest success seen for relapsing-remitting MS, where a number of disease-modifying therapies are in use. Treatment for the remaining diseases is limited to corticosteroids, high-dose intravenous immunoglobulin, or plasmapheresis. The commonality of complement-mediated damage in these demyelinating diseases suggests that inhibition of complement activation may provide a broad spectrum therapeutic option, particularly for progressive forms of MS where there are currently no treatment options (7–9).

The role of complement in the pathology of MS and other demyelinating diseases has been known for several decades based primarily on studies treating animals with cobra venom factor (CVF) which transiently depletes complement and delays and/or reduces disease severity (reviewed in (10, 11)). However, because CVF activates rather than inhibits complement, it is difficult to interpret such studies due to the release of the inflammatory complement anaphylatoxins, non-specific opsonization of multiple cell types and the potential for membrane attack complex (MAC)-mediated tissue damage (12). The question then is, what is the best therapeutic target in a complex cascade with multiple activation pathways? Using a systematic approach our laboratory has examined the course of experimental autoimmune encephalomyelitis (EAE), the animal model for MS, in mice deficient in complement components or receptors required for the activation pathways or effector functions of complement (reviewed in (10)). Not surprisingly, deletion of C3 markedly delayed EAE onset and reduced disease severity (13, 14) since absence of this central complement component effectively cripples the autoimmune contributions of the complement system. However, C3 is a poor therapeutic target for a chronic autoimmune disease such as MS because continuous complement immunosuppression can lead to a high risk of infectious disease. Of the numerous complement mutant mice we examined, only factor B-deficient mice presented with significant protection in EAE, suggesting that the alternative pathway is a key complement protein in the development of EAE/MS (13).

Complement activation through the alternative pathway generates the amplification loop C3 convertase resulting in additional C3 activation and rapid amplification of complement activation (15). This mechanism is important for pathogen neutralization and removal but can be damaging to host tissues when complement is inappropriately targeted. Nevertheless, the alternative pathway has, in recent years, proven to be a valuable therapeutic target in a wide range of animal autoimmune and injury models including rheumatoid arthritis, lupus, ischemia-reperfusion injury and brain and spinal cord injury (16–20). We report here proof of concept studies using anti-factor B antibody (mAb 1379) to treat mice both before and after disease onset in a chronic EAE model. We found that limited dosing with mAb 1379 significantly attenuated disease severity and provided long-lasting protection. We observed similar protection in transferred EAE and, importantly, EAE was significantly attenuated when mAb 1379 was administered after disease onset. These data suggest that inhibition of the alternative pathway may be a viable therapeutic approach for progressive MS.

2. Materials and Methods

2.1 Mice

Inbred C57BL/6 mice, originally from The Jackson Laboratory (Bar Harbor, ME), were from our own colony. The factor B^{-/-} mice have no serum factor B, cannot form the alternative pathway C3 convertase and have no alternative pathway activity (21). All studies were performed with approval from the UAB IACUC.

2.2. Induction of active and adoptive transfer EAE and anti-factor B treatment

For active EAE, mice were immunized as previously described (22) on day -1 with 250 ng of pertussis toxin (i.p.) and on day 0 with CFA emulsion containing 1 mg heat-inactivated *Mycobacterium tuberculosis* and 250 µg MOG peptide₃₅₋₅₅ (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. Disease onset was defined as the first day of two consecutive days with a clinical score of two or more. Disease incidence was defined as the percent of mice that displayed any clinical signs of disease. For transferred EAE, spleens of control donors were removed two to three weeks following induction of active EAE, and prepared as previously described (22). Adoptive transfer EAE was induced by injecting ~5 × 10⁶ purified T cells (i.p.) into wild type recipient mice and scored for 15–20 days as described above. At various time points after induction of either active or transferred EAE, mice were injected i.p. with either PBS (control group) or anti-factor B monoclonal antibody (clone 1379, 1 mg in 400 µl PBS, (23)).

2.3. Histopathology

Mice from both treatment groups with actively induced EAE were sacrificed at 30 days p.i. by CO₂ inhalation, and spinal columns were removed, fixed in 10% buffered-formalin and paraffin embedded. Sections (5 µm thick) from the cervical, thoracic and lumbar spinal cord were cut and either stained with hematoxylin and eosin for overall lesion evaluation and characterization of inflammatory responses or with Luxol fast blue for evaluation of demyelination. The extent of inflammation and demyelination was scored based on lesion size (0–4) and lesions were evaluated for lymphocyte accumulation, neutrophil infiltration, demyelination, axonal degeneration and gliosis (0–4). Tissues were evaluated without identification as to experimental group. Severity scores were calculated as the mean over all segments of the products of the intensity scores multiplied by the extent scores for each lesion characteristic (inflammation, axonal degeneration, gliosis, and demyelination). The means of the individual lesion characteristic severity scores were summed to give the overall severity score.

2.4. T cell proliferation and infiltration

Antigen-specific T cell proliferation assays were performed as previously described (22). Single cell suspensions from spleens of wild type and factor B^{-/-} mice obtained 14 days after EAE induction were cultured in triplicate in 96-well plates at 5 × 10⁵ cells/well with increasing concentrations of MOG₃₅₋₅₅ peptide (0.125 – 2 µg/ml). After 48 h, cultures were pulsed with ³H-thymidine for 18 h and incorporation of thymidine was measured. A stimulation index was calculated based on the fold-increase in CPM uptake between unstimulated and MOG-stimulated cultures.

To examine T cell infiltration spinal cords were removed from PBS and anti-factor B-treated mice with active EAE (day 13) after perfusion with PBS, ground through a cell strainer, washed in PBS, resuspended in 40% Percoll and layered on 70% Percoll. After centrifugation at 2000 rpm (RT, 25 min.), cells at the interface were removed and washed in PBS. Cells were incubated with anti-CD16/32 (24G2, FcR block) to prevent non-specific staining. Spinal cord leukocytes were stained with anti-CD4-FITC (GK1_(CR1).5), antiCD8-PE (53–6.7), anti-CD45-FITC (30F11), and anti-IFN- γ -FITC (XMG1.2), all from eBiosciences, San Diego, CA. Stained cells and forward scatter were analyzed using a FACSCalibur and the data analyzed using FlowJo software (Tree Star).

2.5. Statistics

Statistical significance between the clinical scores of PBS- or mAb 1379-treated mice in active and transferred EAE experiments was calculated using the Wilcoxon signed rank test. Statistical significance between disease onset and incidence and T cell proliferation (in control versus factor B^{-/-} mice) was calculated using the student's t-test. Results of evaluations for inflammation and demyelination were analyzed using analysis of variance for main effects and Tukey's test for pair-wise mean comparisons.

3. Results

3.1. Attenuation of chronic active EAE on treatment with anti-factor B mAb 1379

In initial experiments we determined the optimal dosing regimen of mAb 1379 in order to inhibit the course of MOG_{35–55} peptide-induced EAE. For these studies we induced EAE and then treated mice with either PBS or mAb 1379 and followed the course of EAE for 30 days as described in the Materials and Methods. We found that mice treated on days 5, 7, 9 and 11 had significantly attenuated EAE in the chronic phase of disease (days 15 through 30, $p < 0.05$, Wilcoxon ranked sign test) as reflected in reduced CDI for antibody-treated mice (57.7 vs. 41.6, PBS-treated vs. mAb 1379-treated, respectively) (Fig. 1A, Table 1). There was no significant difference in disease onset (13.1d vs. 15.4d, PBS-treated vs. mAb 1379-treated, respectively) or the initial rate of disease development (Fig. 1A, Table 1). Alternative treatment regimens did not provide significant protection or provided no greater protection than the optimal dosing regimen (data not shown).

Interestingly, the severity of EAE was reduced throughout the chronic phase of disease even though final administration of mAb 1379 was on day 11 post-induction. Alternative pathway activity returns to normal levels within 48hrs after mAb 1379 treatment (23). Because protection in the chronic phase of EAE continued in the presence of a functional alternative pathway, we repeated our initial experiment, following the course of EAE for over 50 days to determine if protection waned over time. Similar to the results shown in Fig. 1A, we observed that chronic disease remained significantly attenuated, (days 11 through 52, $p < 0.05$, Wilcoxon ranked sign test, CDI 102 vs. 179, PBS-treated vs. mAb 1379-treated, respectively) (Fig. 1B, Table 1). These data demonstrate that inhibition of the alternative pathway early in disease development leads to long-term protection, but does not prevent or delay disease onset.

3.2. Reduced cellular infiltration and demyelination in mAb 1379-treated mice with EAE

Histological evaluation of spinal cord sections taken at 30 days post-immunization (active EAE) revealed an overall decrease in the severity of lesions in mAb 1379-treated mice compared to PBS-treated mice. Luxol fast blue and periodic acid-Schiff staining (Figure 2A) and H&E stained sections (Figure 2C) from PBS-treated mice showed a pattern of demyelination and leukomyelitis, predominantly affecting the outer ventrolateral white matter tracts. The inflammatory cell response was mixed and included neutrophils,

macrophages, and lesser numbers of lymphocytes, as well as perivascular cuffing, primarily by lymphocytes. In multiple areas, myelin sheaths were dilated and appeared empty (“moth-eaten” appearance) with some containing swollen homogeneous eosinophilic (degenerating) axons. In contrast, demyelination, inflammation and infiltration were significantly reduced in mAb 1379-treated mice (demyelination, $p=0.018$; infiltration and inflammation, $p=0.029$) (Figure 2B and D). These results are consistent with extended attenuation during chronic disease as shown in Figure 1.

We also analyzed for leukocyte infiltration during the acute phase of the disease (day 13 post-induction) and observed that CD4⁺ and CD8⁺ T cell infiltration into the spinal cords of mAb 1379-treated mice was substantially reduced (62% and 33%, respectively) compared to PBS-treated mice (Table 3). Although T cell infiltration was reduced in mAb 1379-treated mice, absence of factor B does not alter the capacity of T cells to proliferate in antigen-specific restimulation assays using MOG peptide as shown in Figure 2E. IFN- γ -production by infiltrating CD4⁺ T cells was similar between mAb 1379- and PBS-treated mice. In contrast the percentage of FoxP3⁺CD25⁺CD4⁺ T cells was elevated in mAb 1379-treated mice compared to PBS-treated control mice (Table 2), suggesting that Treg cell infiltration may account in part for the reduced disease in mAb 1379-treated mice.

3.3. Attenuation of transferred EAE on treatment with anti-factor B mAb 1379

We next examined the efficacy of mAb 1379 treatment in transferred EAE, as this model is more relevant to human disease than adjuvant-induced EAE. Similar to what we observed in active EAE studies, mAb 1379 treatment prior to disease onset (on days 2, 4, 6 and 8) significantly attenuated chronic disease compared to PBS-treated mice (days 7 through 16), as reflected in reduced CDI for antibody-treated mice (28.8 vs. 10.8, PBS-treated vs. mAb 1379-treated, respectively, $p<0.05$, Wilcoxon ranked sign test) (Figure 3A Table 3). There was also a significant difference in disease onset (9d vs. 15.4d, PBS-treated vs. anti-factor mAb-treated, respectively, $p=0.021$, Wilcoxon ranked sign test) and the initial rate of disease development in mAb 1379-treated mice (Figure 3A, Table 3). We also examined for the ability of mAb 1379 to attenuate transferred EAE after the onset of clinical signs of disease. For these studies, we treated mice with mAb 1379 once they developed early signs of clinical disease (clinical score of 1–2). We found mAb 1379 treatment after disease onset on days 9, 11, 13 and 15 significantly attenuated chronic disease compared to PBS-treated mice (days 10 through 20, $p<0.05$, Wilcoxon ranked sign test) as reflected in reduced CDI for antibody-treated mice (44.2 vs. 26.7, PBS-treated vs. anti-factor mAb-treated, respectively) (Figure 3B, Table 3). There was no significant difference in disease onset (8.3d vs. 9.4d, PBS-treated vs. anti-factor mAb-treated, respectively) or the initial rate of disease (Figure 3B, Table 3).

4. DISCUSSION

The complement system represents a significant challenge with respect to therapeutic targeting due the number of activation pathways leading to C3 cleavage and its integration with the coagulation, fibrinolytic, bradykinin and kallikrein systems (24, 25). Clinical inhibition of complement activity is complicated by the risk of infection, especially in chronic disease settings (e.g. rheumatoid arthritis or MS) where patients will be treated for extended periods of time. Inhibition at the level of the complement terminal pathway to prevent formation of the membrane attack complex through the use of an anti-C5 antibody (eculizumab) has proven clinically successful in paroxysmal nocturnal hemoglobinuria with few complications (26–28). This therapeutic approach has recently been extended to other diseases where eculizumab treatment may provide significant protection against central nervous system and kidney damage due to Shiga-toxin-mediated complement activation (29, 30) and does ameliorate the loss of renal function and hemolysis in atypical hemolytic

uremic syndrome (31, 32). The clinical success of eculizumab in several disease settings provides a strong rationale for exploring complement inhibition in other pathways in the system.

In animal models inhibition of the alternative pathway has proven effective in acute injury including traumatic brain and spinal cord injury (19, 20, 33) and ischemia-reperfusion in multiple tissues (34–36). In humans, transient inhibition of the alternative pathway, to manage complement-mediated immunopathology occurring during an acute crisis, likely presents a low clinical risk with respect to infection because treatment duration would presumably be short-lived. In animal models of chronic inflammatory disease such as rheumatoid arthritis, lupus, asthma and multiple sclerosis, inhibition of the alternative pathway (through gene deletion or inhibitors) also provides significant protection from disease development or reduces disease severity (13, 37–41). Long-term inhibition of the alternative pathway in chronic disease may however present a greater clinical challenge since partial suppression of complement activation over extended periods of time could increase the risk of infection.

Our studies yielded two interesting results that may have clinical implications for alternative pathway inhibition at the level of factor B. First, and most importantly, treatment with mAb 1379 delayed and attenuated EAE after the development of clinical signs of disease. This suggests that alternative pathway inhibition, whatever the clinical risks, could be of therapeutic value in the face of ongoing MS. Secondly, we observed that treatment with mAb 1379 provided significant long-term reduction in EAE severity - almost 40 days after the last antibody injection. Previous animal studies have documented that alternative pathway inhibition by mAb 1379 is limited to two to three days after the last dose is administered (23). Thus in our hands mAb 1379 was able to attenuate EAE well after treatment was terminated. This finding suggests that alternative pathway inhibition prior to disease onset not only interferes with complement-mediated mechanisms required for full disease development, but that these mechanisms are disabled for extended periods – possibly permanently. The implication of these findings are significant because they suggest that continuous inhibition of the alternative pathway may not be required to achieve long-term, significant clinical protection. A limited treatment paradigm would have the advantage of reducing treatment side effects, especially the risk of immunosuppression.

Presently there are a number of complement inhibitors in clinical use or in development that might be efficacious in demyelinating disease (42–44). We have recently shown that targeted inhibition using complement receptor 2 (CR2) conjugated inhibitors significantly attenuates EAE (45). In these studies, treatment with CR2-Crry (which inhibits all complement activation pathways at the level of C3) (46) and CR2-fH (which inhibits the alternative pathway) (47) EAE was attenuated, however there were interesting differences in disease course when using the two inhibitors. CR2-Crry treatment had little effect on disease onset and led to limited attenuation of chronic active EAE. In contrast, CR2-fH delayed disease onset by almost a week and reduced overall disease severity by over 50% compared to PBS-treated controls in active EAE (45). Although mAb 1379 treatment did not markedly alter active EAE onset, it was as effective as CR2-fH treatment in attenuating disease and, in transferred EAE, it both delayed onset and attenuated disease (Fig. 3). Taken together our results indicate that inhibition of the alternative pathway may represent a powerful new approach in treating demyelinating disease.

Acknowledgments

Support: NIH grants NS069365 (to S.R.B.), DK076690 (to J.M.T.), AR51749 (to V.M.H.) and NS077811 (to T.N.R.),

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Highlights

The value of inhibiting the alternative complement pathway in EAE was assessed.

Anti-factor B antibody significantly attenuated the chronic phase of EAE.

Attenuation of the chronic phase of EAE was long lasting with short-term treatment.

Anti-factor B attenuated transferred EAE if administered before/after disease onset.

Inhibition of factor B may offer a therapeutic avenue in multiple sclerosis.

Figure 1.

Treatment with anti-factor B mAb 1379 administered before the onset of disease reduces the severity of the chronic phase of active EAE. Active EAE was induced with MOG₃₅₋₅₅ peptide and clinical signs scored for 30 days as described in Materials and Methods. **(A)** Mice were injected i.p. with either PBS or mAb 1379 (1 mg) on days 5, 7, 9 and 11 post disease-induction. EAE was significantly attenuated in mAb 1379-treated mice from day 16 through 30 post-induction ($p < 0.05$, Wilcoxon ranked sign test). **(B)** Mice were injected i.p. with either PBS or mAb 1379 (1 mg) on days 5, 7, 9 and 11 post disease-induction and the course of disease followed for 52 days. EAE was significantly attenuated in mAb 1379-treated mice from day 10 through 52 post-induction ($p < 0.05$, Wilcoxon ranked sign test). Arrowheads indicate injection with PBS or mAb 1379.

Fig. 2.

Leukocyte infiltration and demyelination in PBS- and anti-factor B-treated mice during EAE. Spinal cords from wild type mice treated with PBS or anti-factor B mAb (n=4 for each group) were obtained at 30 days post-immunization, fixed in 10% buffered formalin and paraffin-embedded. Sections (5µm) were stained with Luxol fast blue (**A and B**) or H&E (**C and D**) and scored as described in the Materials and Methods. Representative sections from PBS-treated (**A**) and mAb 1379-treated (**B**) mice stained with Luxol fast blue are shown. In PBS-treated mice there was significantly more widespread demyelination ($p=0.018$) compared to antibody-treated mice. Representative sections from PBS-treated (**C**) and mAb 1379-treated (**D**) mice stained with H&E are also shown. In PBS-treated mice there was significantly more infiltration and inflammation ($p=0.029$) compared to antibody-treated mice. Original magnification 4×. (**E**) Factor B^{-/-} T cells proliferate comparably compared to wild type T cells. Encephalitogenic T cells enriched by nylon-wool adherence from the spleens of wild type and Factor B^{-/-} mice (n=4, both groups) undergoing active EAE, or T cells from healthy controls (naïve cells), were co-cultured with irradiated splenic APCs plus MOG peptide (0.125 – 4 µg/ml). The cells were pulsed with ³H-thymidine and harvested at 18 h for determination of radioisotope incorporation. The results shown are expressed as the mean + SEM of fold-induction of wild type and Factor B^{-/-} T cell proliferation relative to background proliferation.

Fig. 3.

Treatment with anti-factor B mAb 1379 administered before or after the onset of disease reduces the severity of the chronic phase of transferred EAE. **(A)** Transferred EAE was induced in wild-type mice by injecting encephalitogenic T cells ($\sim 5 \times 10^6$) derived from wild type mice with active EAE. Mice were then injected i.p. with either PBS or mAb 1379 (1 mg) on days 2, 4, 6 and 8 post-transfer and scored for clinical signs of disease for 16 days. Transferred EAE was significantly attenuated in mAb 1379-treated mice from day 7 through 16 post-induction ($p < 0.05$, Wilcoxon ranked sign test). **(B)** Transferred EAE was induced in wild-type mice as in (C). Mice were then injected i.p. with either PBS or mAb 1379 (1 mg) on days 9, 11, 13 and 15 post-transfer and scored for clinical signs of disease for 20 days. Transferred EAE was significantly attenuated in mAb 1379-treated mice from day 9 through 20 post-induction ($p < 0.05$, Wilcoxon ranked sign test). Results shown are the mean $\pm$ SE for clinical score from two to three independent experiments. Arrowheads indicate injection with PBS or mAb 1379.

Table 1

EAE clinical signs in wild-type mice treated with PBS or mAb 1379.

		CDI	Disease Onset	Disease Incidence
30 Days PI ^D	PBS-treated n=9	57.7	13.1d	100
	mAb 1379-treated n=10	41.6 *	15.4d	80
52 Days PI ^D	PBS-treated n=8	179	9.1d	100
	mAb 1379-treated n=9	102 *	13.8d	89

CDI = cumulative disease index, PI = post-induction of EAE.

* Significantly different than PBS-treated controls, $p < 0.05$.

Table 2

CNS T cell infiltration in mice in mice with active EAE treated with PBS or mAb 1379.

	%CD4 ⁺	%CD8 ⁺	%CD4 ⁺ /IFN- γ producing	%FoxP3 ⁺ expressing CD25 ⁺ CD4 ⁺
PBS-treated (n=3)	7.14	4.05	95	70
mAb 1379-treated (n=3)	2.74	2.7	96	79

Table 3

Transferred EAE clinical signs in mice treated with PBS or Mab 1379 before or after disease onset.

		CDI	Disease Onset	Disease Incidence
Treatment before disease onset	PBS-treated n=4	28.8	9d	100
	mAb 1379-treated n=7	10.8 *	15.4d *	86
Treatment after disease onset	PBS-treated n=4	44.2	8.3d	100
	mAb 1379-treated n=9	26.7 *	9.4d	100

CDI = cumulative disease index, PI = post-induction of EAE.

* Significantly different than PBS-treated controls, $p < 0.05$.