Attenuating the Progression and Preventing the Onset of Experimental Autoimmune Encephalomyelitis by Transient pH Disturbances

Wei-ping Zeng^{1,*}

¹ Therazwimm Incorporation, 3128 Ferguson Road, Huntington, WV 25705

*Correspondence: Wei-ping Zeng, Therazwimm Incorporation, 3128 Ferguson Road, Huntington, WV 25705. ORCID iD: 0000-0002-8268-3583

E-mail: weipingzengny@gmail.com

Summary

There is currently a lack of effective treatment of progressive multiple sclerosis (MS). In a mouse model of MS, slightly lowering pH in the animals at early stages stops disease progression. When the animals are treated before symptoms appear, disease onset is prevented or delayed.

Abstract

Remitting-relapsing multiple sclerosis is now considered a treatable disease thanks to disease modifying treatments with good efficacy of reducing relapses. In contrast, effective treatment of progressive multiple sclerosis remains elusive. Using a murine experimental autoimmune encephalomyelitis (EAE) model, this study showed that transiently lowering the pH in the mice at early stages of EAE attenuated disability progression to advanced stages. When the treatments were initiated prior to the appearance of clinical signs, onset of EAE was prevented or delayed. Histological analysis showed that the treatments cleared inflammation in the spinal cords.

Introduction

Multiple sclerosis (MS) is a neurodegenerative inflammatory disease that affects the central nervous system (CNS). Worldwide it affects approximately 2.5 million people. The disease typically starts between 20 and 40 years of ages, and is the leading cause of non-traumatic disability among young adults. (Lozano et al., 2012; Tullman, 2013). MS is a heterogeneous disease. About 85% of MS cases are relapsing-remitting MS (RRMS) characterized by episodes of acute worsening of neurologic functioning with total or partial recovery and no apparent progression of disability. (Klineova and Lublin, 2018). At an older age, about two thirds of RRMS transition to secondary progressive MS (SPMS) characterized by steady progression of disability with or without relapse. (Klineova and Lublin, 2018; Tutuncu et al., 2013). The third subtype is the primary progressive MS (PPMS), which account for about 10-15% of MS cases and is characterized by steady progression of disability from onset without initial relapses or remission. (Klineova and Lublin, 2018; Miller and Leary, 2007). Characterizing MS into these three different phenotypic subtypes is important for the development of treatments of MS. While there is currently no cure for MS, a number of disease-modifying treatments (DMTs) are available for treating RRMS. (Krajnc et al., 2021; Vaughn et al., 2019). While the current DMTs have proven to be effective for the reduction of relapses, they have shown only modest or no efficacy for progressive MS (PMS). (Vaughn et al., 2019).

The pathological hallmark of MS is focal demyelination in the CNS accompanied by inflammation, axonal damage, and astrogliosis. (Popescu et al., 2013). Inflammation

is the driving force for demyelination and neurodegeneration. Although inflammation subsides in old patients with long-standing disease, it is closely associated with active demyelination and axonal damage at all stages of MS. (Frischer et al., 2009). While the etiology of MS is not fully understood, evidence points to immune response to an unidentified myelin antigen as the primary cause of MS. Autoreactive lymphocytes, mostly T cells but also B cells, and/or monocytes infiltrate the CNS, first accumulate in the perivascular areas, eventually also penetrate the parenchyma where the monocytes may further differentiate into macrophages. (Frischer et al., 2009; Prineas and Parratt, 2021). The autoreactive T cells activate macrophages and microglial cells. The activated macrophages and microglial cells, as well as other inflammatory cells, induce apoptosis of oligodendrocytes by oxidative stress. (Mendiola et al., 2020). The inflammatory milieu of the demyelinating lesion also activates astrocytes leading to astrocytic scar, which impedes remyelination of the damaged nerves. (Sofroniew, 2009)

Given the pathogenic role of inflammation in MS, novel ways of depleting inflammatory cells in the CNS could prove to be effective treatment of MS including PMS. The author previously found that low intracellular pH correlates with apoptosis, and pH modifiers such as small amounts of acids can effectively lower intracellular pH and induce apoptosis in lymphocytes and other inflammatory cells while preserving normal tissue cells. (Manuscripts submitted). Therefore, lowering pH could potentially be a new therapy of MS, effective even for the currently hard-to-treat PMS. This study was designed as a preclinical test of this hypothesis using a murine experimental autoimmune encephalomyelitis (EAE) model.

EAE is a widely used animal model for research and drug development of MS that reproduces most of the hallmark pathological features of MS. (Palumbo and Pellegrini, 2017). The disease phenotypes of the murine EAE models are in large part determined by the genetic background of the mice and the encephalitogenic antigen used for EAE induction. Immunization of C56BL/6 mice with the myelin oligodendrocyte glycoprotein peptide MOG₃₅₋₅₅ induces chronic progressive disease, therefore is particularly useful for developing treatments for PMS. (Palumbo and Pellegrini, 2017). Here, this model was used to show that transient mild acid treatments attenuated EAE progression to advanced stages, prevented and delayed the onset of the disease.

Results and Discussion

Acid treatments attenuated EAE progression

In a separate study with a murine allergic asthma model, it was found that treatments with pH modifiers such as acids effectively depleted pulmonary inflammation without affecting the structural integrity of the lungs. (Manuscript submitted). Like allergic diseases, autoimmune diseases are caused by overzealous immune and inflammatory responses. Therefore, acid treatments may have similar therapeutic and preventive effects on autoimmune diseases. To test this hypothesis, C57BL/6 mice were immunized with MOG₃₅₋₅₅ peptide to induce EAE. Mice at early stages of EAE with clinical scores of 1.5 to 2.5 were treated with i.p. injection of saline or saline containing HCl or HOAc. The treatments were repeated every other day for a total of 4 treatments. In the meantime, the mice were monitored daily for disease progression for 6 or 7 days after the initiation of the treatments (some mice reached clinical score of 5 or higher after 6 days, therefore had to be sacrificed). Clinical scores of all control saline-treated mice

continued to increase after the initiation of the treatment, and peaked before day 4 after treatment initiation, after which the disease entered remission. Within the observation period, 10 of the 11 control saline-treated mice reached clinical scores of 4 or above. (Figure 1a). In contrast, none of the 5 mice treated with HCl progressed to clinical score of 4 or above. By day 4 after the initiation of treatment, all of the HCl-treated mice had clinical scores lower than their scores at the time of the treatment initiation. (Figure 1a). The results of the HOAc-treated mice were similar to those of the HCl-treated mice. Only 1 of the 9 HOAc-treated mice reached clinical score of 4 or above. By day 4, the clinical scores of 6 of the mice were lower than their scores at the time of initial treatment, and 2 remained the same. (Figure 1a). At day 4 the turning point for remission for most mice, the average clinical score of the control saline-treated mice was 3.46, whereas the average score of the HCl-treated mice was 0.9, and that of the HOAc-treated mice was 1.78. (Figure 1b). Histology analyses showed that at day 4, the spinal cords of the control saline-treated mice showed heavy inflammatory infiltration. In contrast, no obvious inflammation was observed in either the HCl- or HOAc-treated mice. (Figure 2).

Acid treatment reduced and delayed the onset of EAE

On day 11 after immunization with MOG₃₅₋₅₅, at which time no clinical sign of EAE had developed in this study, the mice received i.p. injections with saline or saline containing HOAc. The treatments were repeated every other day for two times. As shown in Figure 3, clinical signs started to appear 2 days after the initiation of treatments. In the control saline-treated mice, 50% of them developed EAE on day 2 after the initiation of treatment, the rate of disease onset quickly peaked at 80% on day 3. In contrast, in the HOAc-treated mice, the rate of disease onset on day 2 after the initiation

of treatment was 30%, and it did not reach the maximum rate of 60% until 5 days after the initiation of treatment. (Figure 3).

In summary, in this study, transient mild acid treatments were found to both attenuate the progression of EAE from early to advanced stages, and prevent and/or delay the onset of diseases in the immunized mice. These findings have important clinical implications for MS. RRMS is now considered a treatable disease thanks to the many available DMTs. In contrast, only few treatments, most notably mitoxantrone, ocrelizumab, and siponimod, are available for PPMS and/or SPMS. (Ciotti and Cross, 2018; Hartung et al., 2002; Kappos et al., 2018; Vaughn et al., 2019; Wolinsky et al., 2020). However, these treatments provide only modest improvement over placebo. In addition, adverse side effects are of serious concerns. In fact, mitoxantrone is not frequently used because serious risks of malignancies, cardiac and infectious diseases. (Buttmann et al., 2016; Ciotti and Cross, 2018). Therefore, effective treatment of PMS remains an elusive goal. (Metz and Liu, 2018; Ontaneda et al., 2017). Although the EAE model is not exact equivalent of MS, the strong effect of acid treatments on EAE progression to advanced stages in the current study suggests that pH modifiers such as acids could be powerful therapeutic agents for PMS.

In this regard, it is worth noting that mice recovered from EAE after the acid treatments appeared to be in excellent health condition as judged by their gaits and shiny coats (data not shown), which indicated low risk of long-term adverse side effects. This is perhaps not completely surprising because humans can experience transient severe acidosis without serious pathological consequences in the aftermath. (Bangsbo et al., 1993; Hopkins and Sharma, 2019; Orringer et al., 1977). Therefore, it can be expected

that treatments with pH modifiers such as acids would be acceptable options for therapy and prevention of MS.

Given that there is no cure for MS, prevention is a particularly attractive alternative. The current study shows that initiation of acid treatments of MOG₃₅₋₅₅ - immunized mice at a time prior to disease onset can prevent or delay the onset of EAE. For application to MS, similar time points for preventive acid treatments could be at the time of diagnosis of radiologically isolated syndrome (RIS) or clinically isolated syndrome (CIS). RIS has 34% conversion rate to clinically diagnosed MS (CDMS) within 5 years, (Okuda et al., 2014), whereas the conversion rates of CIS to CDMS range from 60 – 85%, (Klineova and Lublin, 2018). In addition, individuals with other risk factors such as genetic predisposition or family history could also be candidates for the preventive treatments.

There is a general consensus that MS relapse is caused by inflammation. Given that acid treatments in this study cleared inflammation in the CNS, they could be effective treatments for all forms of MS including the RRMS. In contrast, the role of inflammation in PMS, especially in old patients, is less conclusive. The view that PMS may be independent of inflammation is supported by the fact that the effective DMTs for RRMS but not PMS are anti-inflammatory. However, inflammation, albeit less prominent, is present in the active demyelinating lesions in PMS, suggesting that inflammation plays a role in PMS at least in relatively young patients. Indeed, the DMTs that do have modest but significant efficacy on PMS also target inflammation. (Ciotti and Cross, 2018; Hartung et al., 2002; Kappos et al., 2018; Vaughn et al., 2019; Wolinsky et al., 2020). Two theories could explain the seemingly contradictory

evidence. First, inflammation may play a role in the initiation of the pathologic process of demyelination, which once initiated may develop autonomously. In such a scenario, if anti-inflammatory treatment starts early enough, it could potentially stop the transition from RRMS to SPMS and/or prevent PPMS and SPMS from progressing further by limiting the development of new demyelinating lesions. Under this theory, acid treatment could be effective for PMS because early treatments did indeed attenuate disability progression in EAE. The second theory is that low-grade inflammation plays a continuous role in PMS. In other words, the inflammation required for PMS does not have to be as strong as that for relapse. The current DMTs are not effective for PMS because they are not powerful enough to eliminate the low-grade inflammation. Since inflammation was essentially eliminated in the current study, the anti-inflammatory effect of the acid treatments could be powerful enough to make them effective for PMS.

While only acids were used in the current study, other pH modifiers can be used for the same purpose. Such other pH modifiers could be pH buffering agents, ionophores, or inhibitors and stimulators of the expression or activity of enzymes (e.g., carbonic anhydrases), membrane transporters (e.g., monocarboxylate transporters, Na^+/H^+ exchangers) and ion channels that facilitate the production and cross-membrane movement of the contents of intra- and extra-cellular fluids such as protons, bicarbonates and monocarboxylates that are responsible for the maintenance and change of pH in and around a cell. Thus, this study demonstrated that pH modifiers are a class of broad-spectrum anti-inflammatory agents with potential medical applications.

Materials and Methods

Mice. Female C57BL/6 mice of 9 weeks of age were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The mice were housed in the animal facility of Charles River Accelerator and Development Lab (CRADL) (Cambridge, MA, USA). The mice were acclimated at CRDAL for 1 week before use. Animal studies were performed according to the protocols approved by the CRADL Institutional Animal Care and Use Committee.

EAE model. Induction of EAE was carried out as previously described. (HookeLaboratories, 2017; Zeng et al., 2009). Antigen emulsion was prepared by mixing MOG₃₅₋₅₅ (G. L. Biochem, Shanghai, China), desiccated *M. tuberculosis* H37Ra (Difco Laboratories, Detroit, MI, USA) and incomplete Freund's adjuvant (IFA) (Difco Laboratories) in PBS. On day 0, mice were immunized by s.c. injection of the antigen emulsion (containing 1.5mg/ml of MOG₃₅₋₅₅) at 4 evenly distributed sites in the dorsal area (25µl/site). Two hours later, the mice were i.p. injected with Pertussis Toxin (List Biological laboratories, Campbell, CA, USA) freshly prepared in ice-cold PBS (200ng in 100µl PBS/mouse). On day 1, the mice received a second dose of Pertussis Toxin. Starting on day 10, at which time no disease onset was observed, EAE clinical scores of the mice were recorded using the scoring system described in Table 1. Mice with clinical score of 3 or above were provided wet chows and hydrogels.

Acid treatments. Treatments of the mice were carried out by i.p. injection of 200µl of saline or saline containing 87.5mM HCl or HOAc at specific times in the clinical course of EAE as described in each experiments.

Analysis of inflammatory infiltration. Spinal cords were harvested and fixed in 10% neural formalin (Fisher Scientific, Kalamazoo, MI, USA). The fixed spinal cords

were paraffin embedded and sectioned on a Microtome (Leica Biosystem, Buffalo Grove, IL, USA). The tissue sections were stained with hematoxylin and eosin (H&E). The tissue slides were examined under a light microscope with mounted camera, and images were taken and processed with Motic Image Plus 2.0 software (Swift Optical Instruments, San Antonio, TX, USA).

Declaration of Conflict of Interest

WZ owns shares of Therazwimm.

Author contribution

WZ designed and carried out the study, and wrote the manuscript.

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Figure Legends

Figure 1. Attenuation of EAE progression by acid treatments. When mice had EAE clinical scores of 1.5 to 2.5, i.p. treatments with saline, or saline containing HCl or HOAc were initiated. The treatments were repeated every other day for a total of 4 treatments. (a) EAE clinical scores of each treated mice from the initiation (day 0) of the treatments up to 7 days after the initiation of the treatments. (b) Graphic presentation of the averages of the EAE clinical scores of the treated mice on day 4 after the initiation of the treatments. Statistical significance of the differences between the saline-treated mice and the HCl- or HOAc-treated mice was determined by Student t test; ** $p < 0.01$. Data were pooled from 3 experiments.

Figure 2. Clearance of inflammatory infiltration in the spinal cords by acid treatments. Tissue sections of spinal cords of mice of the EAE model were subject to H&E staining. (a) Histological views of the spinal cords of mice treated with saline or saline containing HOAc. (b) Histological views of the spinal cords of mice treated with saline or saline containing HCl. Arrows indicate inflammatory infiltration.

Figure 3. Prevention and delay of the onset of EAE by acid treatments. On day 11 after immunization with MOG₃₅₋₅₅ when there is no onset of disease (EAE clinical score = 0), mice received i.p. injection of either saline or saline containing HOAc. The percentages of mice that developed EAE on different days after the initiation of treatments are shown.

Table 1. Definition of EAE clinical scores

Score	Clinical phenotype
0	Tail is normal with full strength
0.5	Mild tail weakness
1	Whole tail weakness; when laid on a flat surface on the back, mouse can flip up very well
1.5	When laid on a flat surface on the back, mouse can't flip up very well
2	When laid on a flat surface on the back, mouse can hardly flip up; can stand on a metal grid very well; can climb into the cage from the grid very well (within 3 seconds)
2.5	Can stand on the metal grid, but occasionally hind legs fall from the grid; has difficulty climbing into the cage from the grid (needs 3-10 seconds)
3	Can hardly stand on the grid, most of the time the legs fall through the grid; can hardly climb into the cage from the grid (needs more than 10 seconds); hind legs are weak but still movable
3.5	One hind leg is paralyzed; front legs are fine; ability to ambulate not affected
4	Both hind legs are paralyzed, but front legs are fine; the whole body can move well
4.5	Hind legs are paralyzed, front legs are weak; whole body ambulates with difficulty
5	Hind legs are paralyzed, front legs are very weak; mouse rarely moves
6	Death

Figure 1

Figure 2

Figure 3