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Deferiprone modulates in vitro responses by peripheral blood T cells from control and relapsing–remitting multiple sclerosis subjects

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ABSTRACT

T cells are important mediators of autoimmune inflammation in relapsing–remitting multiple sclerosis (RRMS). Previous studies found that deferiprone, an iron chelator, suppressed disease activity in a mouse model of multiple sclerosis, and inhibition of T cell proliferation was implicated as a putative mechanism. The objective of the present study was to examine the effects of deferiprone on suppressing in vitro responses of T cells from control and RRMS subjects. Peripheral blood T cells were co-stimulated with anti-CD3+anti-CD28 and cultured with or without interleukin 2 (IL-2). Proliferating CD4+ T cells from control and RRMS subjects, cultured with or without IL-2, decreased in response to 75 μM deferiprone, although the extent of decreased proliferation of CD4+ T cells from RRMS subjects was less than for control subjects. Proliferating CD8+ T cells from control subjects, cultured with or without IL-2, also decreased in response to 75 μM deferiprone, and this decrease was seen in proliferating CD8+ T cells from RRMS cultured with IL-2. CD4+CD25+ and CD8+CD25+ cells from control subjects, cultured with or without IL-2, declined in 75 μM deferiprone, but the decrease was smaller than for the CD4+ and CD8+ proliferative responses. CD4+CD25+ and CD8+CD25+ cells from RRMS subjects showed more variability than for control subjects, but CD4+CD25+ cultured with IL-2 and CD8+CD25+ cells cultured without IL-2 significantly declined in 75 μM deferiprone. CD4+FoxP3+ and CD4+CD25+FoxP3+ cells tended to remain constant or increase. In summary, deferiprone induced declines in proliferative responses at a dosage that is within peak serum pharmacological concentrations.

1. Introduction

There is a scarcity of efficacious treatment options for disease exacerbations during relapsing–remitting multiple sclerosis (RRMS). The most common intervention is a steroid, e.g., methylprednisolone delivered via the oral or intravenous route. Corticosteroids hasten recovery from a relapse [1], but do not lower the rate of new exacerbations or lessen the progression of long-term disability [2]. Side effects from corticosteroid therapy include hyperglycemia, hypokalemia, ankle swelling, facial flushing, insomnia, psychiatric changes, bone loss and gastric disturbances [1,3–5].

For patients that do not respond or who cannot tolerate corticosteroids, other interventions can be considered such as plasma exchange or intravenous immunoglobulin [5,6]. However, these treatments are very expensive and time consuming, and have limited effectiveness [1].

The lack of efficacious secondary treatment options, the occurrence of side effects attributable to corticosteroids, and the failure of corticosteroids to lessen subsequent relapses or alter the disease progression, establish a need for new interventions for disease exacerbations. The animal model experimental autoimmune encephalomyelitis (EAE) is commonly used to test compounds for possible usefulness in MS. This strategy has resulted in the development of several compounds that are now standard treatments for RRMS [7]. EAE studies often utilize an experimental design that entails the administration of a test compound prior to the onset of EAE clinical signs. However, this design is not optimal for testing a compound for use during disease exacerbation since early administration of the compound may interfere with the development of disease rather than suppressing ongoing clinical signs [8]. When the drug deferiprone was tested in the SJL mouse model of EAE, it was administered after the onset of clinical signs [9]. This drug suppressed disease activity in a pronounced manner at a time when clinical signs are normally rapidly advancing. Further tests showed that deferiprone suppressed the proliferation of activated T cells [9], which are key mediators of pathogenesis in EAE and RRMS [10,11].

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Deferiprone is an orally available iron chelator that causes immunomodulation in patients experiencing immunostimulation due to transfusion iron overload [12]. In this population, levels of CD8 lymphocytes, TNFα, and IL-2 were elevated prior to treatment, and returned to normal levels following deferiprone administration. The level of the anti-inflammatory cytokine, IL-10, increased after treatment but remained within the normal range. Furthermore, deferiprone was also found to suppress oxidative damage in this patient population [13]. The action of deferiprone on immune cells from MS subjects is unknown. The objective of the present studies was to examine the effects of deferiprone on T lymphocytes from RRMS and healthy control subjects.

2. Materials and methods

2.1. Human subjects

This study was approved by the Human Subjects Committee of the University of Kansas Medical Center. Patients and control subjects were recruited at the University of Kansas Medical Center Multiple Sclerosis Clinic, and informed consent was obtained. Patients had a definite diagnosis of MS, as defined by the revised McDonald Criteria [14], and were classified as having RRMS according to the Lublin–Rheingold classification [15]. T cells from seven female patients with RRMS (age range 38–57; mean age 49.0 +/− 2.7 standard error of the mean) and four female and one male healthy controls (age range 24–51; mean age 39.6 +/− 5.1) were evaluated. All RRMS patients but one were in remission at the time of blood collection, and four of seven were on a MS disease modifying therapy, i.e., interferon beta-1a or 1b, but none was receiving a corticosteroid.

2.2. Antibodies and reagents

PE-Cy™5 mouse anti-human CD4, PE-Cy™7 mouse anti-human CD8, APC-Cy™7 mouse anti-human CD25 and Alexa Fluor 647 mouse anti-human FoxP3 were obtained from BD BioSciences, San Jose, CA. Anti-human CD3 was obtained from BD BioSciences and eBioscience, San Diego, CA. Anti-human CD28 was obtained from Ancell, Bayport, MN.

Live/dead fixable violet stain and 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) were obtained from Invitrogen, Carlsbad, CA. Deferiprone was kindly supplied by ApoPharma, Inc., Toronto, ON. EasySep® T cell Enrichment Cocktail Magnetic Nanoparticle separation system was obtained from Stemcell Technologies, Vancouver, BC. Fetal bovine serum (FBS), interleukin 2 (IL-2), L-glutamate, Penicillin–Streptomycin, and RPMI 1640 medium were obtained from Sigma-Aldrich, St. Louis, MO. Ficoll-Paque PLUS™ was obtained from Amersham Biosciences, Uppsala, Sweden.

2.3. Blood collection, leukocyte separation, and cell plating

Blood was collected in green top tubes containing heparin, stored at room temperature, and processed within ~24 h of collection. Leukocytes were separated from the whole blood by Ficoll-Paque PLUS™ density centrifugation followed by negative T-cell selection using an EasySep® T cell Enrichment Cocktail Magnetic Nanoparticle separation system. After separation, cells were washed with phosphate buffered saline (PBS), resuspended in PBS with 0.1% FBS, incubated in 1.5 μM CFSE in dimmed lighting for 8 min at room temperature, diluted with an equal volume of FBS, incubated for an additional 10 min at 37 °C in dimmed lighting, washed with PBS + 2% FBS and resuspended in RPMI 1640 medium, 10% FBS, 1% Penicillin–Streptomycin, and 1% L-glutamate.

Cells were plated at $1.5–2\times10^5$ cells/well in 96 well Falcon microtiter plates that were precoated with 1 μg/mL anti-human CD3 and 5 μg/mL anti-human CD28 antibodies in PBS. Cells were cultured for 4 days with 0–200 μM deferiprone in the presence or absence of 100 IU IL-2/well.

2.4. Cell staining and flow cytometry

Following 4 days of culture, cells were stained with PE-Cy5 mouse anti-human CD4, PE-Cy7 mouse anti-human CD8, APC-Cy7 mouse anti-human CD25, and live/dead fixable violet stain followed by Alexa Fluor 647 mouse anti-human FoxP3 and fixation with 1% paraformaldehyde in PBS. Approximately 1.5×10^4 cells/well were analyzed with a BD LSRII Flow Cytometer, using FACSDiva software.

2.5. Data analysis and statistics

Dot plots and histograms representing the number of mitoses per gated cells were prepared using FlowJo software (Ashland, OR). Ratios were prepared for data obtained for deferiprone divided by data obtained for vehicle, which was the complete culture media. Measurements of area under the curve were calculated from ratio data collected over a range of 25–100 μM deferiprone concentrations for each subject (controls, n = 5; RRMS, n = 7, one subject's values utilized an extrapolated line for area calculation). Comparisons of ratios between responses at 75 μM deferiprone and vehicle within a subject group utilized the one-tailed Wilcoxon matched-pairs signed-rank test (controls, n = 5; RRMS, n = 6 as one RRMS subject didn't receive 75 μM deferiprone). Comparisons of ratio values at 75 μM deferiprone between subject groups utilized a one-tailed Wilcoxon two sample test. Analysis of areas under the curve utilized a one-tailed Student *t*-test. Significance was set at $p \leq 0.05$ for all tests.

3. Results

3.1. Proliferative responses of CD4+ and CD8+ T cells, in the presence of T cell receptor (TCR) co-stimulation, evaluated over a range of deferiprone concentrations

Absolute raw data values displayed variable results between human subjects as expected. Due to this inherent variability, each subject served as their own control to normalize the data so that comparative analyses between populations, i.e., control subjects vs. RRMS subjects, could be made. Thus, data obtained in the presence of deferiprone were divided by data obtained in the absence of deferiprone (vehicle control) where a value below 1 represented a decreased response relative to vehicle.

Proliferating CD4+ and CD8+ T cells from control subjects, cultured in the presence or absence of IL-2, had reached values of <0.14 (response to deferiprone divided by response in vehicle) at 75 μM deferiprone ($p = 0.031$ with or without IL-2) and the ratios were generally unchanged with increasing deferiprone concentrations up to 150 μM (Fig. 1A). In contrast, the ratios of proliferating CD4+ cells from RRMS subjects had a ratio of >0.4 at 75 μM deferiprone in either the presence ($p = 0.016$) or absence ($p = 0.031$) of IL-2, and at higher concentrations of deferiprone up to 150 μM the ratios typically continued to drop. Proliferating CD8+ cells from RRMS subjects (Fig. 1B) showed more variability than proliferating CD4+ cells, but significant decreases were observed at 75 μM for RRMS subjects with IL-2 ($p = 0.031$). At 75 μM, significant differences were observed between RRMS and control subjects for proliferating CD4 cells cultured with ($p = 0.041$) or without ($p = 0.009$) IL-2, but did not reach significance for proliferating CD8 cells ($p = 0.063$ with or without IL-2).

For proliferating CD4+ or CD8+ T cells cultured with or without IL-2, the area under a line connecting the data points at 25, 50, 75 and 100 μM did not show a dependency on the subject's age. Although the areas under the curve were greater for RRMS subjects compared to control subjects (Fig. 1C and D), the differences failed to reach statistical significance. T cells from RRMS patients receiving a disease modifying therapy of interferon beta-1a or 1b did not show greater