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The effects of teriflunomide on lymphocyte subpopulations in human peripheral blood mononuclear cells in vitro

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ABSTRACT

Teriflunomide is an inhibitor of dihydro-orotate dehydrogenase (DHODH), and is hypothesized to ameliorate multiple sclerosis by reducing proliferation of stimulated lymphocytes. We investigated teriflunomide's effects on proliferation, activation, survival, and function of stimulated human peripheral blood mononuclear cell subsets in vitro. Teriflunomide had little/no impact on lymphocyte activation but exerted significant dose-dependent inhibition of T- and B-cell proliferation, which was uridine-reversible (DHODH-dependent). Viability analyses showed no teriflunomide-associated cytotoxicity. Teriflunomide significantly decreased release of several pro-inflammatory cytokines from activated monocytes in a DHODH-independent fashion. In conclusion, teriflunomide acts on multiple immune cell types and processes via DHODH-dependent and independent mechanisms.

1. Introduction

Teriflunomide is an oral, immunomodulatory disease-modifying therapy recently approved in several countries for the treatment of relapsing-remitting multiple sclerosis (MS), which has shown beneficial effects on both clinical and magnetic resonance imaging outcomes in patients with MS (O'Connor et al., 2011). Teriflunomide is a selective and reversible inhibitor of the mitochondrial enzyme dihydro-orotate dehydrogenase (DHODH) required for de novo pyrimidine synthesis in proliferating lymphocytes (Warnke et al., 2009; Palmer, 2010).

Pathologically, MS lesions are characterized by infiltrates of T-cells, B-cells, and macrophages. Cytokines released from activated immune cells contribute to this migration and infiltration of the central nervous system (CNS), ultimately leading to glial activation and neurodegeneration (Wingerchuk et al., 2001). Induction of experimental autoimmune encephalomyelitis (EAE) in the Dark Agouti (DA) rat leads to development of a relapsing–remitting disease course in these animals, which mimics many aspects of MS and is believed to model the chronic clinical disease closely (McFarland and Martin, 2007). In this experimental model, teriflunomide reduced infiltration of T-cells, B-cells and macrophages into the spinal cord (Petty et al., 2010) and was associated with reduced demyelination and axonal loss (Merrill et al., 2009). A goal of MS therapy is to limit abnormal peripheral immune expansion and CNS infiltration of activated immune cells targeting myelin.

Although the exact mechanism of teriflunomide in modulating MS is yet to be fully determined, it is hypothesized to block the proliferation of stimulated B and T lymphocytes, which require de novo synthesis of pyrimidine for their expansion and survival. This is supported by the observation that the anti-proliferative effect of teriflunomide can be overcome in vitro by the addition of exogenous uridine (Ruckemann et al., 1998). The effects of teriflunomide are thought to be limited to stimulated B and T lymphocytes, as resting lymphocytes and other immune cell lineages can meet their pyrimidine needs from the salvage pathway (Gold and Wolinsky, 2011; Claussen and Korn, 2012). In this study, we investigated the effect of teriflunomide on the activation, proliferation and survival of specific lymphocyte subsets from stimulated peripheral blood mononuclear cells (PBMCs) isolated from healthy human subjects. Additionally, cytokine/chemokine release by stimulated PBMCs was assessed in the presence and absence of teriflunomide.

2. Materials and methods

2.1. Teriflunomide and uridine

Teriflunomide was dissolved in dimethyl sulfoxide (DMSO) to give a stock solution of 100 mM, which was further diluted in complete cell culture medium to final test concentrations of 25, 50 or 100 μM (final DMSO concentration per well was 0.1% [v/v]).

To determine whether any inhibitory effect of teriflunomide could be attributed to de novo pyrimidine synthesis, exogenous uridine

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(Sigma-Aldrich, St. Louis, MO, USA) was added to appropriate wells at a final concentration of 50 μM.

2.2. PBMC isolation

Whole blood (containing sodium citrate as an anticoagulant) from a total of eight healthy donors was provided by the Sanofi-Aventis internal blood donor panel. Informed consent was obtained from each donor as per protocol BWIM08 approved by the MidLands Institutional Review Board. PBMCs were isolated from whole blood using Ficoll–Hypaque density gradient centrifugation. Briefly, whole blood was loaded into each Accuspin tube (Sigma-Aldrich) and centrifuged. The mononuclear cell layer was collected and washed three times with phosphate-buffered saline (PBS) containing 0.5% (w/v) bovine serum albumin and 2 mM ethylenediaminetetraacetic acid. PBMCs were re-suspended in complete culture medium (RPMI 1640 medium containing 10% [w/v] human AB serum [Invitrogen, Grand Island, NY, USA] plus penicillin/streptomycin [Invitrogen]).

2.3. Lymphocyte stimulation

To investigate polyclonal T-cell proliferation within a PBMC population, cells were treated with anti-CD3 antibody (10 ng/mL; BD Pharmingen, San Diego, CA, USA) and analyzed at day 5 post-stimulation. In separate experiments evaluating polyclonal T-cell activation, cells were treated with anti-CD3 antibody (100 ng/mL; BD Pharmingen) plus anti-CD28 antibody (1 μg/mL; BD Pharmingen) and analyzed at 24 h poststimulation for activation. For B-lymphocyte stimulation, CpG oligodeoxynucleotide (CpG) (sequence: 5′-TC(po) GTC(po)GTTTTGAC(po)GTTTTGTC(po)GTT-3′; TriLink BioTechnologies, San Diego, CA, USA; lot no. A25-0213-11) was used at a final concentration of 0.1 μM. Cells were analyzed for both activation and proliferation at day 5 poststimulation.

2.4. Flow cytometric analysis of stimulated PBMCs

Cell proliferation in PBMCs was measured using CellTrace™ Violet proliferation dye (Invitrogen), prepared according to the manufacturer's instructions. Briefly, PBMCs were re-suspended in PBS at a concentration of 5×10^6 cells/mL and then mixed with an equal volume of proliferation dye. After mixing, PBMCs were incubated for 3 min at 37 °C before being washed twice with centrifugation using complete culture medium. PBMCs were then re-suspended in complete culture medium, plated into 24-well plates at a density of 2×10^6 cells/well and treated with the appropriate T- or B-cell stimulant in the presence or absence of teriflunomide, plus or minus exogenous uridine.

After incubation with appropriate stimulant, PBMCs were stained with fluorescent-labeled cell-surface marker antibodies or corresponding isotype controls (for T-cells: anti-human CD3–PE-Cy7, anti-human CD4–APC-H7, anti-human CD8 Alexa Fluor 700, anti-human CXCR5 Alexa Fluor 488; for B-cells: anti-human CD19–PerCP-Cy5.5, anti-human CD80–PE [all BD Pharmingen]) for 20 min at room temperature in the dark. Cells were then washed twice, re-suspended in 100 μL/tube of LIVE/DEAD® viability dye (Invitrogen), and incubated for 30 min at room temperature to allow measurement of cell viability in each sample. After incubation with viability dye, cells were washed once and re-suspended in fluorescence-activated cell sorting (FACS) staining buffer (BD Pharmingen). Data were acquired on a BD LSRFortessa flow cytometer using BD FACSDiva software version 6.2 and analyzed using FlowJo 7.6.1 software (Tree Star Inc., Ashland, OR, USA).

2.5. Assessment of B-cell proliferation and activation

The effect of teriflunomide on CpG-stimulated $CD19^+$ B-cell proliferation within the PBMC population was expressed as percentage inhibition of maximum response (100%). The maximum response was defined as the percentage of divided $CD19^+$ B-cells generated from positive control (CpG alone) minus the percentage of divided $CD19^+$ B-cells generated from the unstimulated cell negative control.

B-cell activation, defined by expression of CD80, was measured in the same experiment as B-cell proliferation. The effect of teriflunomide on CpG-stimulated $CD19^+$ B-cell activation was analyzed by comparing the percentage of $CD19^+$ B-cells co-expressing $CD80^+$ from the unstimulated cell negative control with those in the stimulated cell positive control (with and without exogenous uridine).

2.6. Assessment of T-cell subset proliferation and activation

T-cell subsets were defined according to surface marker expression as $CD3^+CD4^+$, $CD3^+CD8^+$, and $CD3^+CXCR5^+$ cell subsets within the PBMC population. For each subset, the effect of teriflunomide on proliferation was expressed as percentage inhibition of maximum response (percentage of divided T-cells generated from the anti-CD3-stimulated positive control minus the percentage of divided T-cells generated from the unstimulated cell negative control). For analysis of T-cell activation, a separate set of samples was prepared from four additional donors. T-cell activation was induced by anti-CD3 plus anti-CD28 antibody stimulation of PBMCs for 24 h in the presence or absence of teriflunomide and exogenous uridine, as described above. After 24 h of incubation, cells were stained with anti-human CD3–PE-Cy7, anti-human CD4–APC-H7, anti-human CD25–V450, anti-human CD69–APC, and anti-human CD127–PerCP-Cy5.5 (with corresponding isotype controls), and prepared for flow cytometric analysis as above.

The effect of teriflunomide on anti-CD3 plus anti-CD28-stimulated $CD3^+CD4^+$ T-cell activation was determined by comparison of the percentage of T-cells expressing CD25, CD69, or CD127 from the unstimulated cell negative control with that from the stimulated cell positive control (with and without exogenous uridine).

2.7. Lymphocyte viability analysis

To investigate the possibility that any inhibition of activation or proliferation of PBMCs in the presence of teriflunomide could be due to cytotoxic effects, the viability of stimulated lymphocytes in the presence or absence of teriflunomide 25 and 100 μM was assessed based on staining with LIVE/DEAD® viability dye. Maximum viability (100%) was calculated based on staining in the stimulated cell positive control. Viability was measured in the same set of experimental samples prepared for proliferation studies.

2.8. Cytokine assay

PBMCs were re-suspended in complete cell culture medium and plated into 96-well plates at a density of 2×10^5 cells/well. Cells were treated with anti-CD3 antibody (10 ng/mL) plus teriflunomide at concentrations of 25 or 100 μM in the presence or absence of 50 μM exogenous uridine. Controls included stimulant alone (positive controls) or no stimulant (negative controls). After overnight incubation at 37 °C in a 5% CO_2 atmosphere, supernatants were harvested and stored at −80 °C until required for analysis. Monocytes were isolated from fresh PBMCs using the human monocyte negative isolation kit (StemCell Technologies, Vancouver, BC, Canada), according to the manufacturer's instructions. The enriched cells were determined to contain greater than 95% monocytes, based on flow cytometric analysis after staining with anti-CD14–PerCp-Cy5.5 antibody (BD Pharmingen). Monocytes were re-suspended in RPMI 1640 medium containing 10% (w/v) fetal bovine serum plus penicillin/streptomycin and plated into 96-well plates at a density of 1×10^4 cells/well. Cells were treated with 10 ng/mL lipopolysaccharide (LPS; Sigma-Aldrich) plus teriflunomide at various concentrations in the presence or absence of 50 μM exogenous uridine. Controls included LPS stimulant alone (positive controls) or no stimulant (negative controls). After overnight incubation at 37 °C in a