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Dimethyl fumarate influences innate and adaptive immunity in multiple sclerosis

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ABSTRACT

Introduction: The mode of action of dimethyl fumarate (DMF), an immunomodulatory treatment for relapsing-remitting multiple sclerosis (RRMS), has not yet been fully elucidated. While *in-vitro* experiments and animal studies suggest effects on immune cell survival, proliferation, migration and oxidative stress response, corresponding observations from human studies are lacking. This study aims to characterize *ex-vivo* and *in-vivo* effects in a cohort of DMF treated RRMS patients.

Methods: Blood samples were collected from twenty well-characterized RRMS patients at baseline and after 3, 6 and 12 months of DMF treatment and an age- and gender-matched cohort of 20 healthy individuals at 0 and 3 months. Leukocyte subpopulations, immunoglobulin levels and cytokine secretion were measured. T cells were assessed for their levels of reactive oxygen species (ROS), metabolic status and their proliferative capacity. Levels of antioxidants were determined in serum by mass spectrometry. Responses of monocyte activation markers as well as NFkB and MAPK pathways to DMF were analysed.

Results: Upon DMF treatment, all lymphocyte subpopulations dropped significantly over the course of 12 months with cytotoxic and effector T cells being affected most significantly. DMF induced cell death and inhibited proliferation of T cells in-vitro. Interestingly, this anti-proliferative effect decreased under treatment. In-vivo DMF treatment led to decreased T cell glycolysis and higher turn-over of antioxidants. In line with these results a significant increase of cytosolic ROS levels after 3 months treatment was detected in T cells. In-vitro DMF treatment reduced NFkB (p65) translocation to the nucleus and MAPK (p38) levels decreased upon stimulation with monomethyl fumarate (MMF) in-vitro and ex-vivo. Consequently, the expression of co-stimulatory molecules like CD40 and CD150 was decreased in antigen presenting cells both in-vitro and ex-vivo.

Conclusion: This study translates knowledge from in-vitro and animal studies on DMF into the clinical setting. Our data suggest that DMF not only alters lymphocyte composition, but also has profound effects on proliferation and induces oxidative stress in T cells. It also acts on innate immunity by reducing the activation status of antigen presenting cells (APCs) via NFkB and MAPK inactivation.

1. Introduction

Relapsing-remitting multiple sclerosis (RRMS) [1,2] and psoriasis [3], two conditions of proposed autoimmune pathophysiology, can both efficiently be treated with oral dimethyl fumarate (DMF). Unlike most other recently approved medications for these disorders [4,5], DMF development was not specifically targeted to certain immune functions, but was established strictly empirically [6]. Treated patients show a substantial shift in the lymphocyte composition [7–10] and treatment leads to a reduced inflammatory disease activity [1–3]. An immunomodulatory mechanism of DMF hence seems probable, but has not been elucidated satisfactorily. Insight into its mode of action might therefore not only facilitate predicting treatment response and targeting its use in autoimmunity [11], but also uncover neglected aspects of the diseases treated.

DMF is a diester of the Krebs cycle intermediate fumaric acid. It

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is largely hydrolysed by esterases to monomethyl fumarate (MMF) in the gastrointestinal tract [12,13] suggesting it might exert local effects directly in the gut or systemic effects through its metabolite MMF in the blood (peak concentration in circulation 1.43 μg/ml = 10 μM [14]). A wide range of effects of these fumaric acid esters (FAE) has been observed in animal and cell culture models.

The suggested modes of action include specific T cell apoptosis [15] and altered cytokine secretion [16], inhibited lymphocyte migration [17], changes in the antioxidant profile [18] and polarization on antigen presenting cells (APCs) [13,19]. Underlying pathways as diverse as NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) [20], MAPK (mitogen-activated protein kinase) [20,21], Nrf2 (Nuclear factor (erythroid-derived 2)-like 2) [22] and HCA2 (hydroxycarboxylic acid receptor 2, also GPR109A) [23] have been debated. Whether these "laboratory" effects are present and relevant in the clinical setting, however, is unknown.

This study was designed to bridge this research gap in a prospective, longitudinal observation study of people with RRMS under DMF treatment. To potentially translate previously discussed effects from laboratory to clinic we characterized the immune phenotypes and immune responses of our cohort extensively. Identified in-vivo or ex-vivo effects were then assessed for their correlation with clinical outcome.

2. Materials and methods

2.1. Patients and healthy controls

From 20 individuals with RRMS diagnosed according to the 2010 revised criteria of McDonald et al. [24] that were started on DMF treatment (delayed release formulation, 240 mg twice daily) extensive clinical data (including Expanded Disability Status Scale (EDSS) and longitudinal magnetic resonance imaging) and blood samples were collected at baseline and after 3, 6 and 12 months. Only FAE treatment-naïve adult patients fulfilling the prescription criteria for DMF (as approved by Swissmedic) were enrolled. All eligible individuals with intention to DMF-treatment visiting the multiple sclerosis (MS) centre of the University Hospital of Basel were assessed for study participation and consecutively recruited. Patient recruitment for an extension phase of this study is being continued to date. Blood samples from an age- and gender-matched cohort of 20 healthy individuals were drawn at an interval of 3 months. Additional sampling after 6 and 12 months in controls was considered redundant. Informed consent was obtained from all participants. The study was approved by the local ethics committee for northwest and central Switzerland (EKNZ) and performed in accordance with the Declaration of Helsinki.

At enrolment patients had a mean age of 41 years (range 26–60; healthy controls: mean 40, range 26–61) and a gender distribution of 11:9 (female:male; healthy controls: 12:8). They presented with a mean EDSS of 2.25 (range: 1.0–4.0), a mean disease duration of 5.9 years from first symptoms (range: 0.3–16) and 3.1 years from diagnosis of RRMS (range: 0.1–12.8), and had had 1.1 relapses (range: 0–3) within the two previous years. 10 patients (50%) had no history of immunomodulatory or immunosuppressive treatment. Among patients with previous immune modulation, most patients (n = 7) had received one treatment. Preceding treatments comprised: interferon beta-1b (n = 4), interferon beta-1a (n = 3), glatiramer acetate (n = 3), fingolimod (n = 2), teriflunomide (n = 2), daclizumab (n = 1) and mitoxantrone (n = 1). All of these treatment regimens were paused for at least 3 weeks or until lymphocyte counts had normalized before first intake of DMF.

To assess treatment response, patients were grouped into responding (Rio-Score = 0) and non-responding individuals (Rio-Score > 0) as a function of their clinical evolution and imaging results at 12 months follow-up by an established score previously used in the outcome classification of immunomodulatory treatments in RRMS [25].

2.2. Sample processing

At each time point patient blood samples were freshly characterized for general lymphocyte subpopulations using antibodies against CD3, CD4, CD8, CD16, CD19 and CD56 by automated flow cytometry (BD FACSCanto II, BD Bioscience, San Jose, USA). Serum immunoglobulin levels were determined on an automated analyzer (Cobas 8000, modul c502, Roche Diagnostics, Basel, Switzerland). Peripheral blood mononuclear cells (PBMC) were separated from patients' and controls' EDTA blood by Ficoll gradient centrifugation (Lymphoprep, AXIS-Shield, Oslo, Norway). They were stored in liquid N_2 in a freezing medium consisting of 10% dimethyl sulfoxide (Sigma Aldrich, St. Louis, USA), 30% RPMI1640, 60% fetal calf serum (both Life Technologies, Carlsbad, USA) until analysed. Culture medium was composed of RPMI1640, 10% fetal calf serum and 1% Amphotericin B, Penicillin and Streptomycin (all Life Technologies, Carlsbad, USA).

2.3. Cell culture

Thawed PBMC were diluted in medium at a density of 1 million/ml and stimulated in volumes of 0.25, 0.5 or 1 ml in flat-bottomed 24-well plates or round-bottomed 96-well plates (TPP, Trasadingen, Switzerland) at 37 °C in 5% CO_2. The following reagents were used and diluted in cell culture medium: hydrogen peroxide (H_2O_2), DMF, MMF, lipopolysaccharides (LPS) (all Sigma Aldrich, St. Louis, USA) and Dynabeads T cell expander anti-human CD3/CD28 (Life Technologies, Carlsbad, USA). All cell counting was performed on an automated cell counter (ADAM MC, NanoEnTek, Seoul, Korea).

The following **protocols** were performed:

- Induction of oxidative stress response, adapted protocol [22]: incubation for 22 h with/without 10 μM and 30 μM DMF and subsequent incubation for 2 h with/without 100 μM H_2O_2. After washing 8 h rest before analysis.
- cytokine secretion: incubation for 24 h with/without 10 μM DMF and with/without 1:4 (bead:cell) CD3/CD28-stimulation.
- T cell proliferative capacity: incubation for 100 h with/without 10 μM, 30 μM and 100 μM DMF and with/without 1:4 CD3/CD28-stimulation.
- Activation of APCs: incubation for 8 h with/without 20 μM and 100 μM DMF and subsequent incubation with/without 100 pg/ml LPS for 16 h.
- determination of MAPK activation: incubation for 60 min with/without 20 μM DMF, 20 μM MMF or 100 pg/ml LPS.
- determination of NFkB activation: incubation for 4 h with/without 30 μM DMF or 30 μM MMF and subsequent incubation with/without 10 ng/ml LPS for 60 min.

2.4. Flow cytometry

The following antibodies and dyes were used: FITC anti-human Foxp3, PE anti-human CD25, PerCP/Cy5.5 anti-human CD127, PB anti-human CD4, BV605 anti-human CD3, Alexa Fluor 488 anti-GATA3, PB anti-T-bet, BV510 anti-human CD8a, PB anti-human CD56, (all BioLegend, San Diego, USA), live/dead violet fluorescent, live/dead aqua fluorescent, cell trace violet, CM-H_2DCFDA, MitoTracker green, MitoSOX red (all Thermo Fisher, Waltham, USA) PE anti-RORγt, PerCP/Cy5.5 anti-human CD4, FITC anti-human

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