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Regular Article

Neuroprotective dimethyl fumarate synergizes with immunomodulatory interferon beta to provide enhanced axon protection in autoimmune neuroinflammation

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ABSTRACT

Introduction: Despite recent advances in development of treatments for multiple sclerosis, there is still an unmet need for more effective and also safe therapies. Based on the modes of action of interferon-beta (IFN-β) and dimethyl fumarate (DMF), we hypothesized that anti-inflammatory and neuroprotective effects may synergize in experimental autoimmune encephalomyelitis (EAE).
Methods: EAE was induced in C57BL/6 mice by immunization with $MOG_{35–55}$-peptide. Murine IFN-β was injected s.c. every other day at 10.000 IU, and DMF was provided at 15 mg/kg by oral gavage twice daily. Control mice received PBS injections and were treated by oral gavage with the vehicle methylcellulose. Mice were scored daily by blinded observers and histological, FACS and cytokine studies were performed to further elucidate the underlying mechanism of action.
Results: Combination therapy significantly ameliorated EAE disease course in comparison to controls and monotherapy with IFN-β. Histological analyses showed a significant effect on axon preservation with almost twice as much axons present in inflamed lesions as compared to control. Remarkably, the effect on axonal preservation was more pronounced under combination therapy than with both monotherapies. Neither monotherapy nor combination therapy demonstrated modulation of cytokines and frequency of antigen presenting cells.
Discussion: Combination of IFN-β and DMF resulted in greater beneficial effects with improved tissue protection as compared to the respective monotherapies. Further combination studies of these safe therapies in human disease are warranted.

Introduction

Multiple sclerosis (MS) is a chronic neurological disease of the central nervous system (CNS) of presumed autoimmune origin that is characterized by inflammation-driven demyelination, and subsequent axonal damage and progressive neurological deficits. Studies in MS and experimental autoimmune encephalomyelitis (EAE), an established animal model mimicking several pathophysiological features of MS, demonstrated that demyelinating lesions are accompanied by T cell and macrophage infiltration (Barnabe-Heider and Miller, 2003; Gold et al., 2006; Weiner, 2009). Subsequent to T cell and macrophage activation and infiltration, these cells release pro-inflammatory cytokines, interact with antigen presenting cells (APCs) and are involved in destruction of the myelin sheet, which is associated with chronic axonal damage (Herrero-Herranz et al., 2008).

For as long as 20 years interferon-beta (IFN-β) has been an established immunomodulatory agent in patients with relapsing remitting MS (RRMS). In phase III studies IFN-β showed beneficial effects on relapse rate, disease progression and development of new CNS lesions as shown by magnetic resonance imaging (MRI) (Burks, 2005; Jacobs, 1996). So far, different mechanisms of action have been described for IFN-β with a focus on downregulation of macrophage and microglia activity (Mendes and Sa, 2011).

Dimethyl fumarate (DMF; study name BG-12) has shown beneficial effects in both EAE and in models of neurodegenerative diseases as well as in two phase III studies in patients with RRMS (Ellrichmann et al., 2011; Ghoreschi et al., 2011; Linker et al., 2011). So far, different mechanisms of action, including neuroprotection, have been described. DMF

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was shown to mediate neuroprotection by inducing the nuclear factor E2-related factor 2 (Nrf2) pathway and thereby addressing toxic-oxidative stress (Ghoreschi et al., 2011; Johnson et al., 2008; Williamson et al., 2012). In APCs, DMF induced glutathione depletion and thus stimulated type II dendritic cells (DCs) with an impaired secretion of interleukin- (IL) 12 and IL-23. Simultaneously, DMF induced both IL-4 producing T helper (Th) 2 cells and generated type II dendritic cells (DCs) that produce IL-10 (Ghoreschi et al., 2011).

Based on their different modes of action we postulated that IFN-β and DMF synergize in early and early chronic EAE. We show that the combination of IFN-β and DMF resulted in a synergistic effect at the clinical level, as reflected by decreased disease severity of EAE. This effect was confirmed histologically by reduced inflammation and in particular preservation of axonal density in the spinal cord of EAE mice. The beneficial effects of combination therapy with IFN-β and DMF may result from effects on different pathophysiological pathways in EAE.

Material and methods

Animal models

8–10 week old female C57BL6/J mice were obtained from the Harlan Laboratories (Borchen, Germany, nowadays Rossdorf, Germany) and kept under standardized, pathogen free conditions at the local animal facility, Ruhr-University, Bochum, Germany. Food and water were given ad libitum to all animals. Experiments were approved by the local authorities for animal experimentation (approval no. 8.87-50.10.32.08.032).

EAE induction and treatment

An emulsion consisting of myelin oligodendrocyte glycoprotein 35–55 (MOG_{35-55}) in phosphate buffered saline (100 μg) and an equal volume of complete Freund's adjuvant containing 100 μg of Mycobacterium tuberculosis (Difco, BD Bioscience) was prepared and subcutaneously injected at the tail base of 8–10 week old female C57BL6/J mice. In addition to immunization with MOG_{35-55} emulsion, on days 0 and 2, 100 ng pertussis toxin (List, Quadratec) was administered i.p. DMF-treated animals received 15 mg/kg body weight (bw) DMF dissolved in 0.08% methylcellulose twice a day per oral gavage. Mice treated with murine IFN-β received an s.c. injection of 10.000 units every other day. IFN-β was dissolved in endotoxin free recombinant mouse serum albumin (MSA) 1%. Preventive treatment started at the day of immunization. Animals were weighed and clinically monitored daily. The blinded observer used a 10-scale score (Linker et al., 2002) for clinical signs. Mice were sacrificed on day 10 for ELISA and FACS and on day 18 for histological analysis. For ELISA and FACS spleens and lymph nodes (superficial cervical lymph nodes, deep cervical lymph nodes, lumbar lymph nodes) were removed to prepare a single cell suspension. For histological studies, mice were deeply anesthetized with ketamine and were transcardially perfused with 4% paraformaldehyde.

Immunohistochemistry

Immunohistochemistry was performed on 5 μm paraffin embedded spinal cord cross sections (lumbar, thoracal and cervical part). If necessary, antigen unmasking was performed via boiling of sections in citric acid buffer. After inhibition of nonspecific binding with 10% bovine serum albumin (BSA), sections were incubated overnight at 4 °C with the appropriate primary antibody in 1% BSA. After blocking of endogenous peroxidase with 0.3% H_2O_2, the peroxidase-based ABC detection system (Vectastain, Vector Laboratories, via Linaris, Wertheim, Germany) was employed with diaminobenzidine as the chromogenic substrate. Specificity of staining was confirmed by omitting the primary antibody as a negative control. T cells were labeled by rat anti-CD3 (1:200; Serotec, Düsseldorf, Germany), macrophages by rat anti-mouse Mac-3 (1:200; BD Pharmingen, Heidelberg, Germany). Bielschowsky silver impregnation was employed to assess axonal pathology and Luxol Fast Blue staining was used to assess demyelination as described previously (Linker et al., 2011). Axonal damage was additionally visualized by double staining with CNPase and the neurofilament antibody SMI31 (1:1000; Invitrogen, Karlsruhe, Germany) with anti-mouse Alexa 488- and Cy3-conjugated secondary antibodies. The sections double labeled for neurofilaments (SMI31) and CNPase using immunofluorescence were analyzed on a fluorescence microscope (Olympus BX51).

To calculate the inflammatory index, spinal cord cross sections were stained with haemalaun. Histological and immunohistochemical quantifications were performed by a blinded observer as described earlier (Linker et al., 2011).

Splenocyte and lymphocyte culture and ELISA experiments

Splenocytes were cultured in 24-well plates at a concentration of 5×10^6 cells/ml of complete GluMax medium (GIBCO®, Invitrogen, Germany), containing 10% heat inactivated fetal calf serum (FCS), 1% sodium pyruvate, 1% L-glutamine, 1% MEM-NEAA and 1% penicillin/streptomycin. Cells were stimulated with 10 μg/ml MOG or 1.25 μg Concanavalin A (ConA) for 24, 48 and 72 h at 37 °C, 5% CO_2. At the indicated time points, supernatants were collected and centrifuged to eliminate cellular debris. Cell culture supernatants were stored at −20 °C and brought to room temperature immediately before the measurement of different cytokines, including IL-6 (BD OptEIA 555240, Pharmingen, Germany), IL-10 (R&D Systems DY417, Heidelberg, Germany), IL-17 (R&D Systems DY421, Heidelberg, Germany) and IFN-γ (R&D Systems DY485, Heidelberg, Germany) according to manufacturers' protocols. Results shown are the mean of triplicates ± SEM.

Flow cytometry (FACS) experiments

FACS analyses were performed using a FACS Canto II and CellQuest software (BD). Splenic single cell suspensions and lymphocyte from lymph nodes were incubated for 20 min at 4 °C and washed with FACS buffer, centrifuged and resuspended in FACS buffer. Monoclonal antibodies purchased from BD (Heidelberg, Germany) and Miltenyi Biotech (Gladbach, Germany) were used to detect CD11b (clone M1/70) CD11c (clone HL3), CD80 (clone 16-10A1), CD86 (clone GL1) and MHC-II (clone 2G9) and CD25CD4 in lymph nodes.

Statistical analysis

All histological analyses were performed completely blinded with respect to treatment. Data are presented as mean ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) or Kruskal–Wallis test (all analyses done by Graph Pad Prism 6, San Diego, CA, USA). As post-hoc tests Bonferroni's Multiple Comparison Tests were performed. A probability level (p-value) of $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ was considered to be statistically significant for all tests. All error bars represent SEM.

Results

Combination of DMF and IFN-β improves the clinical disease course in EAE mice

To compare monotherapy of DMF and IFN-β with a combination of both compounds, we analyzed clinical symptoms and disease severity of MOG-EAE in C57BL/6 mice until the early chronic phase of the disease. The incidence of EAE was 100% in the DMF-group (25/25) and in the IFN-β-group (24/24) versus 22/24 in the combination-group (92%) and 19/23 in the sham treated control group (83%). The difference was statistically not significant. There were no unexpected adverse events upon daily inspection of mice with no differences in mortality