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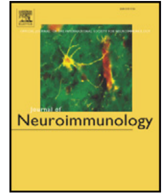

Additive effects of combination treatment with anti-inflammatory and neuroprotective agents in experimental autoimmune encephalomyelitis

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ABSTRACT

We studied the effects of combination treatment with an anti-inflammatory agent, interferon (IFN)-β, and a putative neuroprotective agent, an estrogen receptor (ER)-β ligand, during EAE. Combination treatment significantly attenuated EAE disease severity, preserved axonal densities in spinal cord, and reduced CNS inflammation. Combining ERβ treatment with IFNβ reduced IL-17, while it abrogated IFNβ-mediated increases in Th1 and Th2 cytokines from splenocytes. Additionally, combination treatment reduced VLA-4 expression on $CD4^+$ T cells, while it abrogated IFNβ-mediated decreases in MMP-9. Our data demonstrate that combination treatments can result in complex effects that could not have been predicted based on monotherapy data alone.

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1. Introduction

MS is a putative autoimmune demyelinating disease with a neurodegenerative component. Currently all of the approved MS treatments are targeted at the inflammation, and while this may confer some indirect neuroprotection, there are no directly neuroprotective drugs available. Often patients have progressive disability accumulation despite robust immunosuppression, thereby suggesting the need for treatment approaches which involve using an anti-inflammatory agent in combination with a neuroprotective agent.

Type I interferons (IFN-α and -β) have been widely established as effective anti-inflammatory agents in modifying the course of several inflammatory diseases, including collagen-induced arthritis (CIA), viral inflammation, and EAE (Axtell and Steinman, 2008; Billiau, 2006). IFNβ is one of several widely approved treatment choices for relapse remitting MS patients. Despite its widespread use, some patients are not responsive to therapy (Arnason, 1999). Further, IFNβ treatment often has only modest effects in slowing permanent disability accumulation even in those who initially are considered responsive based on a reduction in relapses. While relatively higher doses of IFNβ therapy may be more effective, such higher doses may be less well tolerated (Clanet et al., 2002, 2004).

IFNβ receptors are endogenously expressed in a variety of tissue and cell types; therefore, it has the potential to exert influence on many aspects of MS pathogenesis. For example, IFNβ therapy in MS patients has been shown to reduce pro-inflammatory Th1 and Th17 cytokines, increase T regulatory cells, downregulate VLA-4 expression on effector T cells, and decrease MMP-9 to limit immune cell trafficking to the CNS (Comabella et al., 2009; Martin-Saavedra et al., 2007, 2008b; Muraro et al., 2000; Shinohara et al., 2008). Though the IFNβ receptor is expressed in neurons and glia, there is currently no evidence for a direct effect of IFNβ on cells outside of the immune system (Prinz et al., 2008). Thus, it is possible that IFNβ treatment could be combined with a directly neuroprotective treatment to increase efficacy.

Estrogens are good candidates to combine with IFNβ therapy since there is abundant evidence using *in vitro* systems and non-inflammatory neurodegenerative *in vivo* models that estrogen treatment can be directly neuroprotective (Honda et al., 2000; Jansson et al., 1994; Morale et al., 2003; Wu et al., 2005). Estrogens act primarily through nuclear ER subtypes, ERα and ERβ (Garidou et al., 2004; Liu et al., 2003; Morales et al., 2006; Polanczyk et al., 2003; Tiwari-Woodruff et al., 2007), while more rapid membrane effects have also been described (Wang et al., 2009b). In EAE and other inflammatory diseases, the role of ERα ligand treatment has been shown to be anti-inflammatory by decreasing Th1 cytokines, altering chemokines, and increasing T regulatory cells (Douin-Echinard et al., 2008; Elloso et al., 2005; Jansson and Holmdahl, 2001; Liu et al., 2003; Offner, 2004; Polanczyk et al., 2003). Together this results in less CNS inflammation. While ERα ligand treatment also preserves myelin and prevents axonal loss, it is unknown whether this is due to merely blocking CNS inflammation as opposed to providing direct neuroprotection to neurons, oligodendrocytes, and astrocytes since these CNS cell types all express ERα and ERβ (Maret et al., 2003; Mitra, 2003). In contrast, recent evidence suggests that ERβ ligand treatment is directly neuroprotective in

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EAE since it preserves myelin and prevents axonal loss without altering peripheral cytokine production or reducing CNS inflammation (Tiwari-Woodruff et al., 2007). Clinically, ERα ligand treatment is effective early during EAE (Liu et al., 2003), while ERβ ligand treatment has significant effects only in the chronic stage of disease (Tiwari-Woodruff et al., 2007). Regarding combination treatment in MS, ERβ ligand treatment provides intriguing possibilities since side effects of high dose estrogen treatment, including breast and uterine cancer, are mediated by ERα, not ERβ (Ali and Coombes, 2000).

Here, we examined combination treatment of chronic EAE using IFNβ, a primarily anti-inflammatory agent, with an ERβ ligand as the neuroprotective agent. While additive effects on clinical and neuropathologic outcomes were found, both additive and antagonistic immune interactions were observed, thereby underscoring the complexity of such combination treatments.

2. Material and methods

2.1. Animals

Female B6.Cg-Tg (Thy1-YFP) 16Jrs/J (Thy1-YFP) mice 8–10 weeks old were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were maintained under environmentally controlled conditions in a 12 hour light/dark cycle with access to food and water *ad libitum*. All procedures involving animals were carried out in accordance to the NIH guidelines for the care and use of laboratory animals and approved by the UCLA Chancellor's Animal Research Committee and Division of Laboratory Animals Medicine.

2.2. Reagents

The ERβ ligand diarylpropionitrile (DPN) was purchased from Tocris Biosciences (Ellisville, MO) and dissolved with molecular-grade ethanol purchased from EM Sciences (Hatfield, PA). Miglylol 812N liquid oil was Sasol North America (Houston, TX). Recombinant mouse interferon-beta (IFNβ) was purchased from PBL InterferonSource (Piscataway, NJ). All reagents were prepared and stored according to manufacturer's instructions.

2.3. EAE induction and treatments

Animals were injected subcutaneously with Myelin Oligodendrocyte Glycoprotein (MOG), amino acids 35–55 (200 µg/animal, American Peptides), emulsified in complete Freund's adjuvant (CFA) and supplemented with *Mycobacterium Tuberculosis H37ra* (50 µg/animal, Difco Laboratories), over four draining inguinal and axillary lymph node sites in a volume of 0.1 ml/mouse. Seven days prior to immunization, animals received treatment that continued to the endpoint of the experiment with DPN (8 mg/kg/day, s.c. injections) dissolved in 10% molecular-grade ethanol and diluted with 90% Miglylol 812N liquid oil, rmIFNβ (20 KU, i.p. injections) diluted with injection grade PBS and 0.1% FBS carrier protein, vehicle consisting of 1:9 molecular-grade ethanol/ Miglylol 812N, or a combination of DPN and IFNβ. Animals were monitored daily for EAE signs based on a standard EAE 0–5 scale scoring system: 0—healthy, 1—complete loss of tail tonicity, 2—loss of righting reflex, 3—partial paralysis, 4—complete paralysis of one or both hind limbs, and 5—moribund.

2.4. Histological preparation

Mice were deeply anesthetized in isoflurane and perfused transcardially with ice-cold 1× PBS for 20–30 min, followed by 10% formalin. Spinal cords were dissected and submerged in 10% formalin overnight at 4 °C, followed by 30% sucrose in PBS for 24 h. Spinal cords were cut in thirds and embedded in 75% gelatin/15% sucrose solution. 40 µm thick free-floating spinal cord cross-sections were obtained with a microtome cryostat (model HM505E) at −20 °C. Tissues were collected serially and stored in 1× PBS with 1% sodium azide in 4 °C until immunohistochemistry.

2.5. Immunohistochemistry

40 µm thick free-floating sections were thoroughly washed with 1× PBS to dilute residual sodium azide. In the case of anti-MBP labeling, tissue sections undergo an additional 2 h incubation with 5% glacial acetic acid in 100-proof ethanol at room temperature (RT), followed by 30 min incubation in 3% hydrogen peroxide in PBS. All tissue sections were permeabilized with 0.3% Triton X-100 in 1× PBS and 2% normal goat serum (NGS) for 30 min RT, and blocked with 10% NGS in 1× PBS, except in the case of MBP labeling, which was blocked with 10% normal sheep serum (NSS), for 2 h or overnight at 4 °C. The following primary antibodies (Abs) were used: anti-sheep MBP (1:1000), anti-CD45 (1:500), anti-CD3 (1:500), anti-Mac3 (1:500) (Chemicon), and anti-neurofilament-NF200 (1:750, Sigma). Tissues labeled with anti-sheep MBP continue with second Ab labeling step consisting of 1 h incubation with biotinylated anti-sheep IgG Ab (1:1000, Vector Labs), followed by 1.5 h incubation with streptavidin Ab conjugated to Alexa 647 fluorochrome (Chemicon). All other tissues followed with second Abs conjugated to TRITC (1:1000) or Cy5 (1:750) (Vector labs and Chemicon) for 1.5 h. To assess the number of cells, a nuclear stain DAPI (2 ng/ml, Molecular Probes) was added 10 min prior to final washes after secondary Ab incubation. Sections were mounted on slides, allowed to semi-dry, and cover slipped in fluoromount G (Fisher Scientific). IgG-control experiments were performed for all primary Abs, and only non-immunoreactive tissues under these conditions were qualified for analysis.

2.6. Microscopy

Stained sections were examined and photographed using a confocal microscope (Leica TCS-SP, Mannheim, Germany) or a fluorescence microscope (BX51WI; Olympus, Tokyo, Japan) equipped with Plan Fluor objectives connected to a camera (DP70, Olympus). Digital images were collected and analyzed using Leica confocal and DP70 camera software. Images were assembled using Adobe Photoshop (Adobe Systems, San Jose, CA).

2.7. Quantification

To quantify immunohistochemical staining results, three consecutive spinal cord cross-sections at the T1–T5 level from four mice per group for a total of twelve sections per group were captured under microscope at 10× magnification for YFP/CD45 labeled sections, or 40× magnification for YFP/MBP labeled sections using the DP70 Image software and a DP70 camera (both from Olympus). All images in each experimental set were captured under the same light intensity and exposure limits. Analysis was performed on images using ImageJ Software v1.30, downloaded from the NIH website: http://rsb.info.nih.gov/ij. Inflammatory infiltrates were quantified by measuring the intensity of CD45 staining in the lateral funiculus and posterior column in captured 10× images. Axons were identified by YFP expression in the lateral funiculus and posterior column in captured 40× images and quantified with the measure function in the ImageJ software. All quantifications were performed by a blinded observer.

2.8. Splenocyte culture

Splenocytes were cultured in 24-well plates at the concentration of 4×10^6 cells/ml of complete RPMI medium containing 5% heat-inactivated fetal calf serum (FCS), 1 mM sodium pyruvate, L-glutamine, 2ME, NEAA, Pen-strep, and 25 mM Hepes Buffer. Cells were stimulated with 25 µg/ml MOG, amino acids 35–55, and 20 ng/ml IL-12 (BD Biosciences) for 72 h at

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