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Journal of Neuroimmunology 92 (1998) 113–121

Journal of
Neuroimmunology

Treatment of multiple sclerosis with Copolymer-1 (Copaxone®): implicating mechanisms of Th1 to Th2/Th3 immune-deviation

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Received 19 May 1998; revised 28 July 1998; accepted 28 July 1998

Abstract

The synthetic polypeptide copolymer-1 (Cop-1; Copaxone®; Glatiramer Acetate) has been recently approved as an effective treatment in relapsing multiple sclerosis (MS). A large body of evidence demonstrates that Cop-1 induces active suppression of CNS-inflammatory disease in animal models. However, Cop-1-mediated suppressor mechanisms have not yet been elucidated in humans. A 12-month open study following clinical and immunological parameters of ten relapsing MS patients treated with Cop-1 is presented. Relapse rates and disability scores (EDSS) were evaluated prior to and after 12 months of treatment. The immunological parameters assessed prior to and at 3 months' interval during treatment included serum levels of soluble IL-2 receptor (sIL-2R) and IL-10 as well as leukocyte cytokine mRNA expression of TNFα, IL-4 and TGF-β. Copaxone treatment was found to lead to a significant reduction in the mean annual relapse rate (from 1.4 prior to treatment to 0.6 during treatment) and stabilization of disability in 90% of the patients. The treatment was accompanied by an elevation of serum IL-10 levels, suppression of the pro-inflammatory cytokine TNFα mRNA, and an elevation of the anti-inflammatory cytokines TGF-β and IL-4 mRNAs in PBLs. These results suggest that the beneficial clinical effects of Copaxone in MS patients may be attributed to changes in activation of T cell subsets and a shift from Th1 to Th2/Th3 cytokine profile, probably leading to Cop-1-driven mechanisms of bystander suppression.

Keywords: Multiple sclerosis; Immunomodulation; Copolymer-1; Th1/Th2; Cytokines; Bystander suppression

1. Introduction

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) of suspected autoimmune origin. Studies in MS and its animal model, experimental autoimmune encephalomyelitis (EAE), suggest that MS results from immune-dysregulation and aberrant activation, whereby CNS myelin proteins serve as autoantigens leading to a T cell-driven inflammatory and demyelinating process (Martin et al., 1992; Steinman et al., 1994; Hafler and Weiner, 1995). The immune-dysregulation in MS involves both cellular and humoral arms of the immune response and can be identified in the peripheral blood, cerebrospinal fluid (CSF) and CNS. These include: defective immune-suppressor responses (Antel et al., 1986); elevated T cell reactivity against various myelin antigens, such as myelin basic protein (MBP), proteolipid protein (PLP) or myelin oligodendrocyte glycoprotein (MOG) (Steinman et al., 1995); increased expression of MHC class II molecules on antigen presenting cells (APCs), monocytes, endothelial as well as glial cells; elevated levels of circulating memory T cells ($CD4^+$/IL-$2R^+$/$CD45RO^+$) (Martin et al., 1992; Steinman et al., 1994; Hafler and Weiner, 1995; Inobe et al., 1996); raised

Abbreviations: CNS, central nervous system; Cop-1, Copolymer-1; EAE, experimental autoimmune encephalomyelitis; IL, interleukin; MS, multiple sclerosis; MBP, myelin basic protein; PBL, peripheral blood leukocytes; sIL-2R, soluble IL-2 receptor; TGF, transforming growth factor; Th, T helper cells; TNF, tumor necrosis factor

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0165-5728/98/$ - see front matter
PII: S0165-5728(98)00191-X

levels of cell surface adhesion molecules on T cells and macrophages (Antel and Owens, 1993; Cannella and Raine, 1995; Weller et al., 1996). The elevated levels of adhesion molecules correlate with the degree of blood brain barrier (BBB) eruption (Sharief et al., 1993; Rieckmann et al., 1994). MS is also characterized by elevated levels of pro-inflammatory cytokines such as tumor necrosis factor-α (TNFα), interleukin (IL)-1, IL-2 and interferon (IFN)-γ, in the peripheral blood (Sharief and Hentges, 1991; Trotter et al., 1991; Hartung et al., 1995), CSF (Hauser et al., 1990; Rudick and Ransohoff, 1992) and in brain lesions (Hofmann et al., 1980). The association of these inflammatory cytokines with disease activity (Hauser et al., 1990; Sharief and Hentges, 1991; Rudick and Ransohoff, 1992; Hartung et al., 1995) implies that $CD4^+$ T cells of the T helper type 1 (Th1) (Voshuhl et al., 1993; Windhagen et al., 1996) and macrophages (Cua et al., 1995; van der Laan et al., 1996), play a pivotal role in the immunopathogenesis of the CNS demyelinating disease. Moreover, the relative low levels and defective production of IL-10, IL-4 and TGF-β in patients with active multiple sclerosis (Mokhtarian et al., 1994), and their protective role in EAE (Johns et al., 1991; Kuruvilla et al., 1991; Racke et al., 1991; Kennedy et al., 1992; Van der Veen and Stohlman, 1993; Fabry et al., 1995; Falcone and Bloom, 1997), suggest that T cells of the Th2 and Th3 phenotypes, and their characteristic cytokine products may be involved in induction of remission and in suppression of the disease process.

Increased understanding of the animal model and the human demyelinating disease has recently led to the implementation of a number of immunomodulatory strategies in the treatment of MS. These include: IFN-β-1b (IFNβ multiple sclerosis study group, 1993; Paty, 1993), IFN-β-1a (Jacobs et al., 1994; Pozzilli et al., 1996), as well as the synthetic polypeptide copolymer-1 (Cop-1; Copaxone®; Glatiramer Acetate), originally synthesized with the aim of mimicking the myelin antigen MBP. Cop-1 treatment suppresses EAE induced in a variety of species (Teitelbaum et al., 1997), and has been demonstrated to reduce clinical disease activity in humans with relapsing–remitting MS (Abramsky et al., 1977; Bornstein et al., 1987; Johnson et al., 1995). The mechanism(s) of Cop-1 action are not fully elucidated. It has been suggested that following binding to MHC class II molecules on antigen presenting cells (APCs), Cop-1 competes with myelin antigens for presentation by APC, thus preventing myelin-specific T cell activation (Racke et al., 1992; Teitelbaum et al., 1992; Fridkis-Hareli et al., 1994; Teitelbaum et al., 1997). An additional mechanism suggested to be involved in Cop-1 immunomodulatory activity is the induction of specific suppressor cells (Teitelbaum et al., 1997). However, Cop-1-driven suppressor mechanisms have not yet been clarified in patients. To further evaluate Cop-1 immunomodulatory activities, we conducted a clinical and immunological study in relapsing remitting MS patients treated with Copaxone® for 12 months in an open trial.

2. Materials and methods

2.1. Patients and treatment

Ten patients (nine female and one male), age range 21–58 years (mean, 37.5 ± 11.94 years), with clinically definite and laboratory supported relapsing remitting MS according to the criteria of Poser et al. (Poser et al., 1983) were followed at the MS Center, Lady Davis Carmel Hospital, Haifa, Israel, in an open trial. Mean disease duration was 9.4 years. Inclusion criteria included a history of at least two clearly identified and documented relapses in the 2 years prior to study entry; all patients were ambulatory, defined by an expanded disability status scale (EDSS) (Kurtzke, 1993) of 0 through 5.0; patients had not been corticosteroid treated for at least 3 months prior to trial. Patients were excluded if they had ever received immunosuppressive therapy with cytotoxic activity (azathiorine, cyclophosphamide or cyclosporine) or lymphoid irradiation. Additional exclusion criteria included pregnancy or lactation, and all women were required to use an adequate contraceptive method. Signed informed consent was obtained from patients and the study was approved by the Ethical Committee, Lady Davis Carmel Medical Center. Age and sex matched controls ($n = 6$) were included in all immunological assays. Copaxone®, 20 mg (supplied by Teva Pharmaceutical Industries, Petach Tiqva, Israel), was daily self administered, subcutaneously, under a protocol approved by the Israeli Health Administration.

Clinical outcome measures were: annual relapse rate, the absolute changes in EDSS score and the proportion of patients with 'improved', 'stable' or 'worsened' clinical disability, by the end of the treatment. Relapse was defined as the appearance or reappearance of one or more neurological abnormalities that persisted for at least 24 h, and had been preceded by a stable or improving neurological state of at least 30 days.

2.2. Serum levels of cytokines

The IFN-γ, IL-2 (Endogen, MA, USA), sIL-2R, IL-4 and IL-10 (high sensitivity, R&D Systems, CA) serum levels were determined by commercial ELISA kits. Data was calculated from duplicate samples on the basis of titration curves obtained from standards supplied by the manufactures.

2.3. Peripheral blood leukocytes

Peripheral blood (10 ml) was collected in EDTA and immediately placed in ice. Red blood cells (RBC) were spun down at $100 \times g$, 4°C and leukocytes (PBL) were then pelleted from the plasma by centrifugation at $650 \times g$,

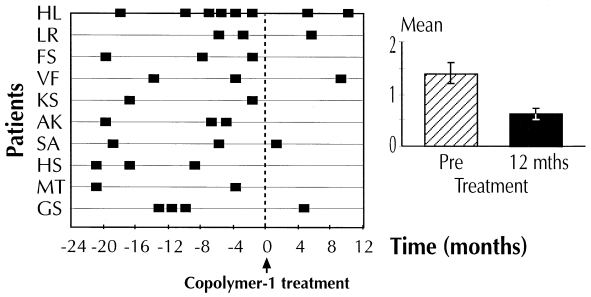

Fig. 1. Copolymer-1 treatment leads to reduced annual relapse rate in MS patients. The number of relapses for each patient during the 2 years prior (pre) to treatment and during 1 year of treatment are presented (figure on left; ↑ designates start of treatment). Figure on the right shows mean annual relapse rate prior to and after 1 year of treatment ($p = 0.001$).

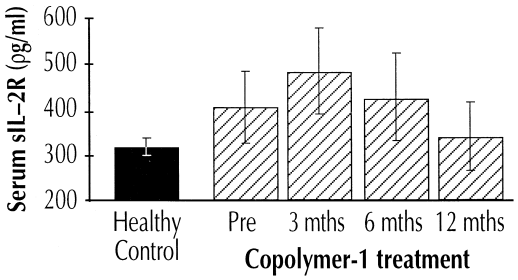

Fig. 3. Copolymer-1 treatment is associated with reduction in serum IL-2 soluble receptor (sIL-2R). Serum sIL-2R levels were determined by ELISA in MS patients, prior to and during Cop-1 treatment, and in healthy controls. The values presented were calculated using a standard titration curve and represent mean values for each group ± standard error of mean.

4°C, for 10 min. Residual RBC were lysed with TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8.0). RNA was extracted from either fresh or liquid nitrogen frozen PBL.

2.4. RT-PCR

Total cellular RNA was isolated from PBL with Tri-Reagent (Medical Research Center, OH) according to the manufacturer's instructions. RNA samples were washed twice in 75% ethanol, air dried and suspended in 10 μl RNase free TE buffer. RNA quantity and quality were determined by spectrophotometric absorbance at 260/280 nm. RNA samples were stored at −70°C. Complementary DNA (cDNA) was prepared by reverse transcription at 37°C for 60 min in 50 μl reaction mixture containing 2 μg RNA, 400 U Moloney Murine leukemia virus reverse transcriptase (Amersham, OH) in the presence of RNA-guard (Pharmacia, Germany) and oligo-hexamers (Pharmacia, NJ). The enzyme was than inactivated and the integrity of the RNA was assessed by amplifying 1 μl cDNA using G3PDH specific primers (Clontech, CA). The polymerase chain reaction (PCR) mix (25 μl volume) included reaction buffer (10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100) 0.2 mM each dNTP (Sigma, MO), 0.4 μM 5′ and 3′ primers and 1U DNA Taq polymerase (Appligene, France). Thirty-five cycles of PCR amplification were performed, each consisting of denaturation at 94°C, annealing at 68°C, and extension at 72°C in a thermal cycler (MJ Research, MA, USA). PCR products were visualized by UV following electrophoresis of products in ethidium bromide stained 3% agarose gels (Sigma). Standard molecular size markers, negative controls (PCR mix without sample cDNA) and positive controls (standard cDNA supplied by manufacturer, Clontech) were run with each PCR assay.

2.5. Semi-quantitation of cytokine mRNA

Semi-quantitative assessment of cytokine (IL-4, TNFα, and TGF-β) mRNA was performed by parallel PCR using cytokine specific primers and G3PDH primers (Clontech). To ensure that amplification was being performed at linear phase, three different quantities of cDNA (2, 1, and 0.5 μl cDNA for G3PDH amplification; 4, 2, and 1 μl cDNA for TNFα and TGF-β amplification; 6, 4, and 2 μl for IL-4 amplification, determined in preliminary experiments) were utilized in all assays. PCR was performed as above using

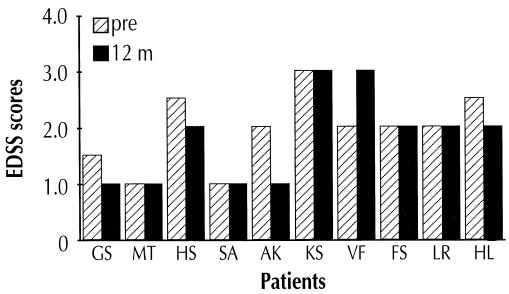

Fig. 2. Copolymer-1 treatment leads to stabilization of clinical disability. Disability of patients, assessed and graded according to Kurtzke's extended disability status scale (EDSS scores), were evaluated prior to treatment (pre) and after 1 year of Cop-1 treatment (12 m).

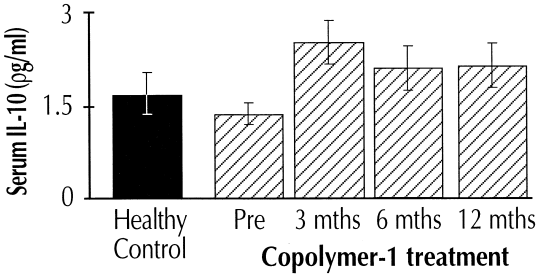

Fig. 4. Copolymer-1 treatment leads to elevation in serum IL-10. Serum IL-10 levels were determined in MS patients, prior to and during Cop-1 treatment, and in healthy controls by ELISA. The values presented were calculated using a standard titration curve and represent mean values for each group ± standard error of mean.

appropriate annealing temperatures (60–65°C) for each cytokine. Resulting PCR products were visualized by UV following electrophoresis, intensity of bands was measured by video densitometry (Bio Imaging Systems, Applitec, Israel) and the ratio between sample to G3PDH products was calculated.

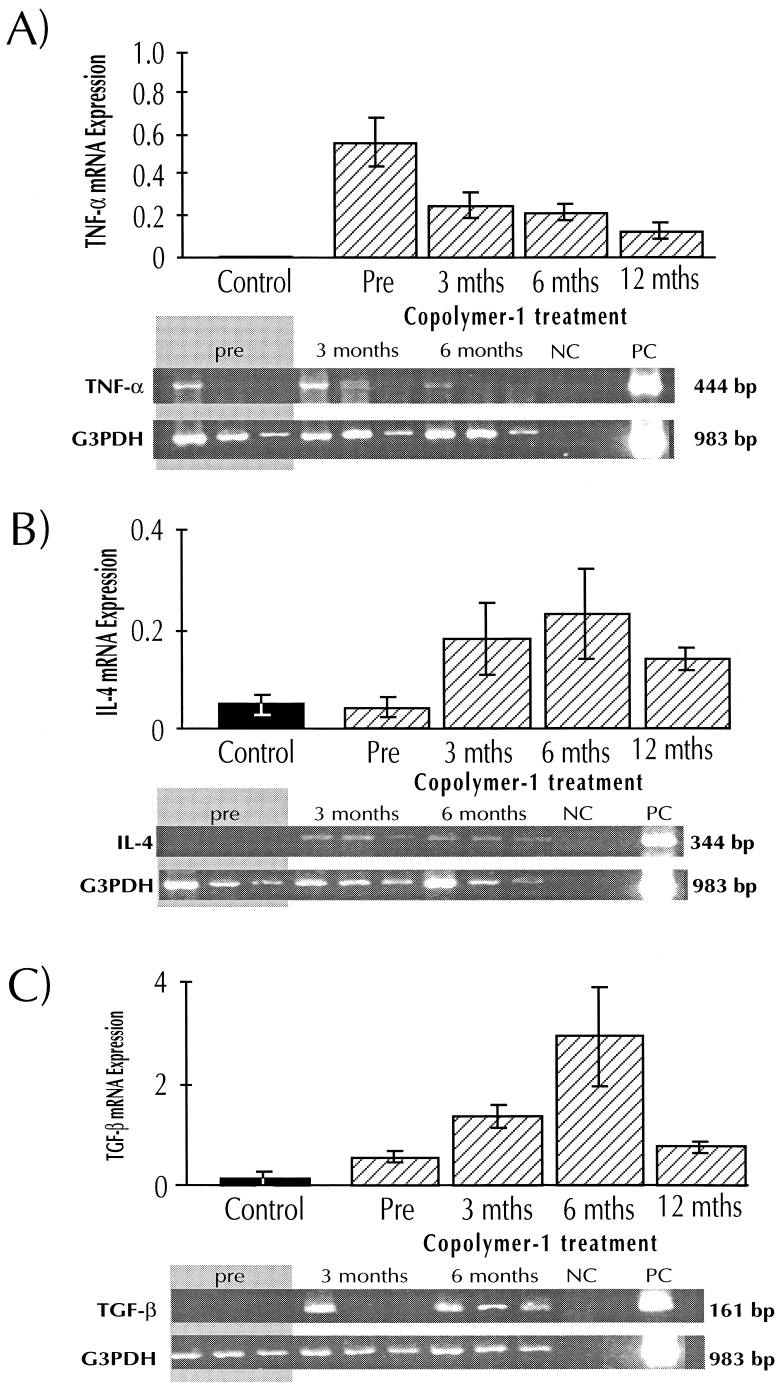

Fig. 5. Copolymer-1 treatment leads to modulation of cytokine mRNA expression in peripheral blood leukocytes. Semi-quantitative assessment of cytokine (TNFα, A; IL-4, B; TGF-β, C) mRNA from PBL was performed by RT-PCR using three concentrations of cDNA (for each time period) as described in Section 2. Housekeeping gene, *G3PDH*, was amplified in parallel. Histogram (upper panel) shows mean ratio (±standard error) between densitometric intensity of specific cytokine to G3PDH PCR products for 10 treated MS patients at each time period examined and for four controls. Results of representative samples obtained at three different times (pre = prior to treatment; 3 and 6 months; NC = negative and PC = positive controls for PCR amplification) of Cop-1 treatment, are shown in bottom panel of figures.

2.6. Statistical evaluation

Mean relapse rate and changes from baseline according to EDSS, were assessed using repeated-measures ANOVA. Statistical significance of data was determined using Student's *t*-test for paired analysis of parameters with normal distribution when comparing a single parameter at different times and Ranked Signed Wilcoxon for comparison of groups. P values of < 0.05 were considered significant.

3. Results

3.1. Copolymer-1 treatment leads to reduced annual relapse rate

During the 1-year follow-up the Cop-1 treated patients had six confirmed relapses (Fig. 1). The mean annual relapse rates was 0.6 during the treatment as compared to 1.4 prior to Cop-1 therapy, a 57% reduction, which was statistically significant ($p = 0.001$). Five (50%) MS patients receiving Cop-1 were relapse-free throughout the trial.

3.2. Stabilization of neurological disability by Copolymer-1 treatment

Among the 10 patients receiving Cop-1, neurological disability improved in four, was stable in five, and worsened in only one patient (Fig. 2). Thus, 90% of the patients treated with Cop-1 were free of disease progression. No clinically significant adverse event was noted. The most commonly recognized adverse event during treatment was a localized injection-site reaction consisting of mild erythemia and induration, which sometimes persisted for several days.

3.3. Modulation of serum sIL-2R and IL-10

The levels of sIL-2R in the sera were higher (though not of statistical significance, $p = 0.7$) in pretreated MS patients compared to healthy controls (Fig. 3). Slight changes, including first a significant elevation at 3 months ($p = 0.04$) and eventually a slight decrease in the mean value of this receptor at 12 months of treatment ($p = 0.43$; pre vs. 12 months) were observed. At all times a wide variability in the level of sIL-2R was found amongst the patients.

The mean level of serum IL-10 was slightly lower, though not significant, in pre-treated MS patients, compared to healthy controls ($p = 0.4$) (Fig. 4). A 2-fold significant ($p = 0.04$) increase in the mean level of this cytokine was observed at 3 months of treatment, as compared to baseline levels. However, the increment was attenuated at 6 and 12 months of treatment ($p = 0.16$). It should be noted that no detectable levels of IFN-γ, IL-2 and IL-4 were observed in these serum samples.

3.4. Cytokine mRNA expression of TNFα, IL-4 and TGF-β in PBL

Profound differences in baseline cytokine mRNAs expression were observed in PBL derived from MS patients as compared to controls and marked changes were identified in patients during Cop-1 treatment (Fig. 5). Elevated mRNA expression levels of the pro-inflammatory cytokine TNFα were observed in all MS patients prior to treatment, whereas no detectable levels were observed in healthy controls. The TNFα levels decreased in nine of the 10 MS patients following Cop-1 treatment and after 12 months of treatment there was a 4-fold, statistically significant ($p < 0.004$), decrease in the mean mRNA level of this cytokine compared to the mean level prior to treatment (Fig. 5A).

The changes in TNFα mRNA were accompanied by enhanced mRNA expression of the anti-inflammatory cytokines IL-4 and TGF-β (Fig. 5B–C). In 70% of patients no mRNA expression for IL-4 was observed prior to Cop-1 administration. After 12 months of treatment, seven of the 10 patients showed IL-4 mRNA expression in PBL (Fig. 5B). Following 3 and 6 months of treatment, the expression of this cytokine was significantly elevated in comparison to pre-treatment levels ($p = 0.05$ and $p = 0.03$, respectively). Although still higher than baseline or control levels, after 12 months of treatment the levels of IL-4 mRNA were more attenuated. TGF-β mRNA expression was observed in 80% of MS patients prior to Cop-1 treatment (Fig. 5C). However a 5-fold, statistically significant ($p = 0.03$) increase in the mean level of this cytokine was observed after 6 months of treatment. At 12 months of treatment, the differences between MS treated patients and healthy controls were still statistically significant ($p = 0.02$) though they were not significant ($p = 0.38$) compared to pre-treatment values.

4. Discussion

The immunological follow-up carried out in the present study adds additional insight into the mechanisms of Cop-1 immunomodulatory activities in MS patients and is, to the best of our knowledge, the first report of regulatory mechanisms of Cop-1 in humans. Although only 10 patients were studied in an open trial, our results demonstrating that Cop-1 treatment leads to a significant reduction of the mean relapse rate as well as stabilization of disability, confirm previous published clinical studies (Abramsky et al., 1977; Bornstein et al., 1987; Johnson et al., 1995). The association of the clinical reduction of disease activity with the shift of cytokine profile, observed in the present work, may contribute to our understanding of the immunomodulatory effects mediated by Cop-1.

It has been proposed that the predominant mechanism underlying the immunomodulatory activities of Cop-1 re-

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