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Treatment of multiple sclerosis with Copaxone (COP)

Elispot assay detects COP-induced interleukin-4 and interferon-γ response in blood cells

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Summary

Copolymer-1 (Copaxone or COP) inhibits experimental allergic encephalomyelitis and has beneficial effects in multiple sclerosis. There is presently no practical *in vitro* assay for monitoring the immunological effects of COP. We used an automated, computer-assisted enzyme-linked immunoadsorbent spot assay for detecting COP-induced interferon-γ (IFN-γ)- and interleukin-4 (IL-4)-producing cells and a standard proliferation assay to assess the immunological response to COP in peripheral blood mononuclear cells from 20 healthy donors, 20 untreated multiple sclerosis patients and 20 COP-treated multiple sclerosis patients. Compared with untreated and healthy controls, COP-treated patients showed (i) a significant reduction of COP-induced proliferation; (ii) a positive IL-4 Elispot response mediated predominantly by CD4 cells after stimulation with a wide range of COP concentrations; and (iii) an elevated IFN-γ response partially mediated by CD8 cells after stimulation with high COP concentrations. All three effects were COP-specific as they were not observed with the control antigens, tuberculin-purified protein or tetanus toxoid. The COP-induced changes were consistent over time and allowed correct identification of COP-treated and untreated donors in most cases. We propose that these criteria may be helpful to monitor the immunological response to COP in future clinical trials.

Keywords: multiple sclerosis; immunotherapy; copaxone; autoimmune T cells; cytokine response

Abbreviations: Ab = antibody; CD = cluster of differentiation; COP = copolymer-1; Elispot = enzyme-linked immunoadsorbent spot (assay); FACS = fluorescence-activated cell sorter; IFN = interferon; IL = interleukin; PBMC = peripheral blood mononuclear cells; PPD = tuberculin purified protein; SEB = staphylococcal enterotoxin B; SI = stimulation index; TH = T helper; TT = tetanus toxoid

Introduction

Copolymer-1 (Cop-1, Copaxone, glatiramer acetate; COP) is a mixture of synthetic polypeptides composed of the four amino acids, L-alanine, L-glutamic acid, L-lysine and L-tyrosine in a defined molar ratio of 0.43 : 0.14 : 0.33 : 0.1 and with an average molecular weight of 4700 to 11 000. COP is one of the currently approved agents for immunomodulatory therapy of multiple sclerosis (Wolinsky, 1995; Arnon, 1996; Noseworthy *et al.*, 1999). In contrast to interferon (IFN)-β, COP is believed to selectively downmodulate the immune response to myelin autoantigens, especially to myelin basic protein (Sela *et al.*, 1990; Arnon, 1996). In previous clinical trials, the efficacy of COP was demonstrated by monitoring clinical changes (Bornstein *et al.*, 1991; Johnson *et al.*, 1995, 1998) and more recently, disease activity by means of brain MRI (Mancardi *et al.*, 1998; Ge *et al.*, 2000; Comi *et al.*, 2001). Thus far, however, there are no practical laboratory techniques available to assess the immunological effects of COP treatment.

We have demonstrated recently that COP-reactive T-cell

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Table 1 *Characteristics of the subjects investigated in this study*

Healthy donors

Name	Gender	Age (years)	HLA–DR
W.H.	M	31	2, 11
F.T.B.	M	34	n.d.
R.Gu.	M	24	6, 11
S.Ka.	F	37	1, 8
H.P.	M	47	n.d.
M.Ke.	M	28	4, 6
F.S.	M	30	7, 11
E.M.	M	37	3
A.Sc.	F	28	11
T.M.	M	28	1, 4
M.Kl.	M	33	3, 13
N.G.	M	38	13, 14
R.H.	M	46	3, 7
C.H-S.	F	30	2, 4
E.Al.	F	56	1, 11
H.N.	M	35	7, 13
S.P.	F	32	7, 11
M.H.	F	27	2
C.L.	M	45	4, 8
I.E.	F	49	1, 7

Untreated multiple sclerosis patients

Name	Gender	Age (years)	HLA–DR	Multiple sclerosis	EDSS
K.G.	F	60	7, 13	SP	4.5
T.W.	M	29	2	RR	4
M.W.H.	F	40	1, 7	SP	4
R.G.	M	57	1, 2	SP	6.5
M.W.	M	47	1, 8	PP	4
A.V.	M	55	4, 11	SP	7.5
T.K.	M	34	3, 12	PP	7.5
M.E.	M	25	2, 8	RR	2
H.Se.	F	56	2, 3	SP	6.5
S.V.	M	45	2	SP	6.5
R.Mo.	M	32	2, 13	RR	1.5
L.N.	F	36	1, 2	RR	2.5
K.W.	M	26	2, 4	RR	1
S.S.	M	30	2, 11	RR	2.5
B.M.	F	29	13, 14	RR	1
S.D.	F	47	2, 11	RR	3
A.Fr.	M	48	1, 4	PP	2
B.L.	F	51	2, 13	SP	3.5
H.K.	M	36	3, 12	RR	1
S.W.	F	41	2, 8	RR	3.5

COP-treated multiple sclerosis patients

Name	Gender	Age (years)	HLA–DR	Multiple sclerosis	EDSS	COP treatment (months)*
H.S.	F	41	2, 4	RR	3.5	44
N.K-M	M	47	2, 8	RR	6.5	42
B.G.	F	33	4, 7	RR	1.5	39
M.S.	F	25	7, 8	RR	2	45
B.B.	F	27	2, 11	RR	2	38
E.A.	F	43	2, 8	RR	6	43
T.S.	M	33	2, 3	RR	2	42
A.G.	F	39	2, 3	RR	2	40
M.R.	F	31	2, 11	RR	2	43
R.S.	F	47	2, 7	RR	5.5	39
S.K.	M	22	8, 11	RR	1	25
A.Se.	F	29	2, 4	RR	2.5	12
R.R.	M	29	2, 11	RR	4	12
C.S.	F	42	4, 8	RR	1	7
S.Z.	F	34	2, 7	RR	2.5	1
L.N.	F	36	1, 2	RR	2.5	1
W.Ha.	F	43	2, 11	RR	2.5	3
H.C.	F	32	11, 12	RR	2.5	2
G.J.	F	31	13, 14	RR	1	2
S.H.	F	31	3	RR	0.5	12

n.d. = not done; n.a. = not applicable; SP = secondary progressive multiple sclerosis; RR = relapsing–remitting multiple sclerosis; PP = primary progres
EDSS = expanded disability status scale. *At the time of the first sampling.

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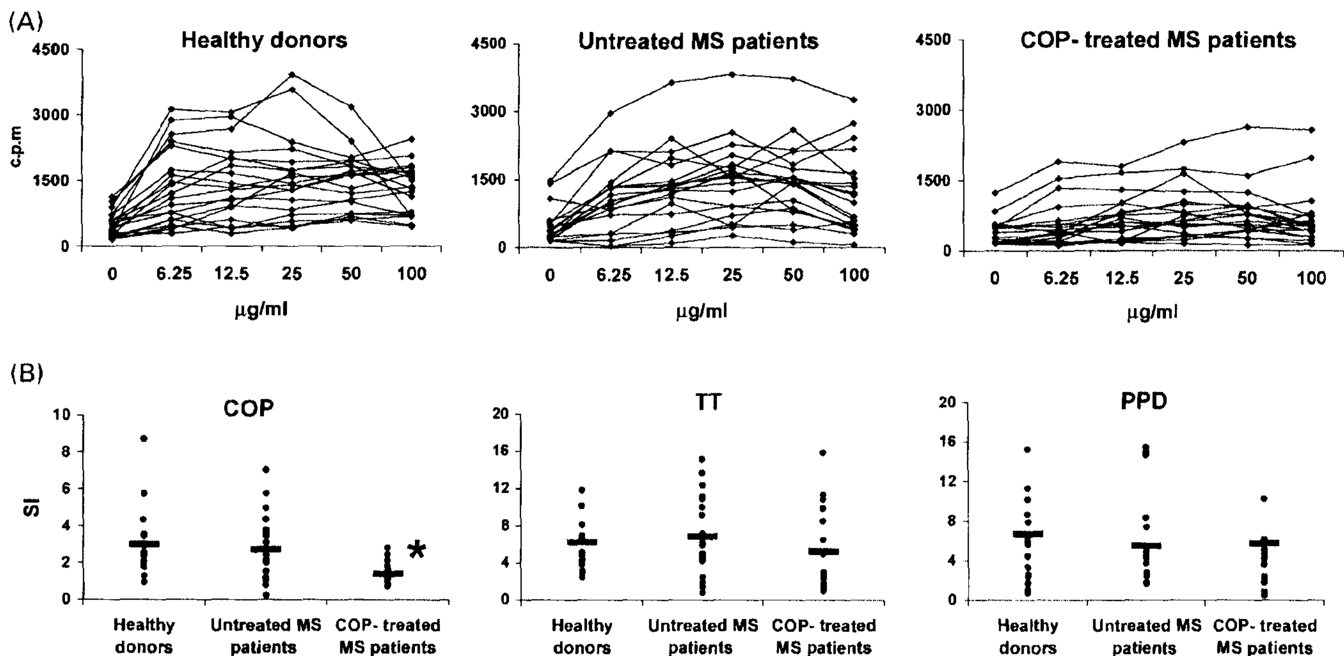

Fig. 1 Reduction of COP-induced proliferation in COP-treated patients. (**A**) Proliferative response of PBMC expressed as counts per min (c.p.m.) after stimulation with different COP concentrations. Each group [healthy donors, untreated multiple sclerosis (MS) patients, and COP-treated multiple sclerosis patients) comprised 20 subjects (black curves). All experiments were performed in triplicate. All standard deviations were below 20% of the mean (not shown). (**B**) Comparison between the proliferative response to COP (6.25 µg/ml) and to recall antigens TT (4 µg/ml) and PPD (20 µg/ml). Horizontal bars indicate group mean values. Statistically significant differences are marked with an asterisk.

lines from COP-treated patients are preferentially T helper (TH) type-2, whereas COP-specific T-cell lines from untreated patients and healthy controls are predominantly TH1 (Neuhaus *et al.*, 2000). However, the isolation of COP-specific T-cell lines is technically demanding and time-consuming, and hence inappropriate for routine monitoring of treatment effects during clinical trials. In the present study we used a simple, automated enzyme-linked immunoadsorbent spot (Elispot) assay to detect interleukin-4 (IL-4) and IFN-γ in short-term (18 h) cultures of freshly isolated peripheral blood mononuclear cells (PBMC).

Methods

Patients and control subjects

Blood was drawn after informed consent from 20 healthy donors, 20 untreated multiple sclerosis patients, and 20 COP-treated multiple sclerosis patients (Table 1). The untreated multiple sclerosis patients had received no immunosuppressive or immunomodulatory treatment for at least 3 months preceding the study. The group of untreated patients included 10 relapsing–remitting, seven secondary progressive, and three primary progressive patients (Table 1). The group of COP-treated multiple sclerosis patients (20 mg/day COP subcutaneously) consisted of 20 relapsing–remitting patients (Table 1). All patients were clinically stable at the time of sampling. All donors were HLA (human leukocyte antigen)-typed (Dr E. Albert, Department of Immunogenetics, University of Munich, Germany). The proportion of DR2-positive patients was similar in the untreated (12 out of 20) and COP-treated (13 out of 20) multiple sclerosis patients.

Antigen stimulation and proliferation assay

PBMC were isolated on a discontinuous density gradient (Lymphoprep, Nycomed, Oslo, Norway). Viable cells were counted with Trypan Blue (Sigma-Aldrich, Deisenhofen, Germany) and resuspended in culture medium (RPMI 1640 supplemented with 5% foetal calf serum, 1% glutamine and 1% penicillin/streptomycin; Gibco, Karlsruhe, Germany). One batch of foetal calf serum was used throughout the study.

PBMC (1×10^5 cells/well) were cultured in 96-well microtitre plates for 5 days in the presence of one of the following antigens: COP (6.25, 12.5, 25, 50 and 100 µg/ml; batch 242992899; Teva Pharmaceutical Industries, Petah Tiqva, Israel), tetanus toxoid (TT, 4 µg/ml; Chiron-Behring, Marburg, Germany), tuberculin purified protein (PPD, 20 µg/ml; Statens Serum Institut, Copenhagen, Denmark) and staphylococcal enterotoxin B (SEB, 1 µg/ml; Toxin Technology, Sarasota, Fla., USA) as a positive control. [^{3}H]Thymidine (0.2 µCi/well) was added during

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the last 18 h of culture. Cells were harvested and [^{3}H]thymidine incorporation was measured using a direct β-counter (Matrix 9600, Packard, Frankfurt, Germany). All experiments were performed in triplicate. Standard deviations were below 20% of the mean. The results were expressed as absolute counts per minute and as stimulation index (SI = ratio of the counts obtained in the presence of antigen and the counts in the absence of antigen). In all experiments, the counts in the presence of SEB were >5000.

Elispot assays

The Elispot assays were performed in parallel with the proliferation tests and analysed with an automated imaging system and appropriate computer software (KS ELISPOT automated image analysis system; Zeiss, Jena, Germany). Briefly, 96-well polyvinylidene difluoride plates (Millipore, Eschborn, Germany) were coated at 4°C overnight with 10 μg/ml capture antibody [anti-IFN-γ antibody (Ab) clone 1-D1K; Mabtech, Nacka, Sweden; or with anti-IL-4 Ab clone MP4–25D2, Pharmingen, Hamburg, Germany]. The plates were then washed and blocked with culture medium for 1 h at 37°C. PBMC (2×10^5 cells/well for the IFN-γ and 4×10^5 for the IL-4 Elispot assay) were cultured for 18 h at 37°C and 5% CO_2. For each subject, quadruplicate wells were exposed to the same antigens used in the proliferation assay. After culture, the plates were washed and incubated first with 1 μg/ml biotinylated detector Ab (anti-IFN-γ Ab clone 7-B6–1 or anti-IL-4 Ab clone 12–1; Mabtech), then with

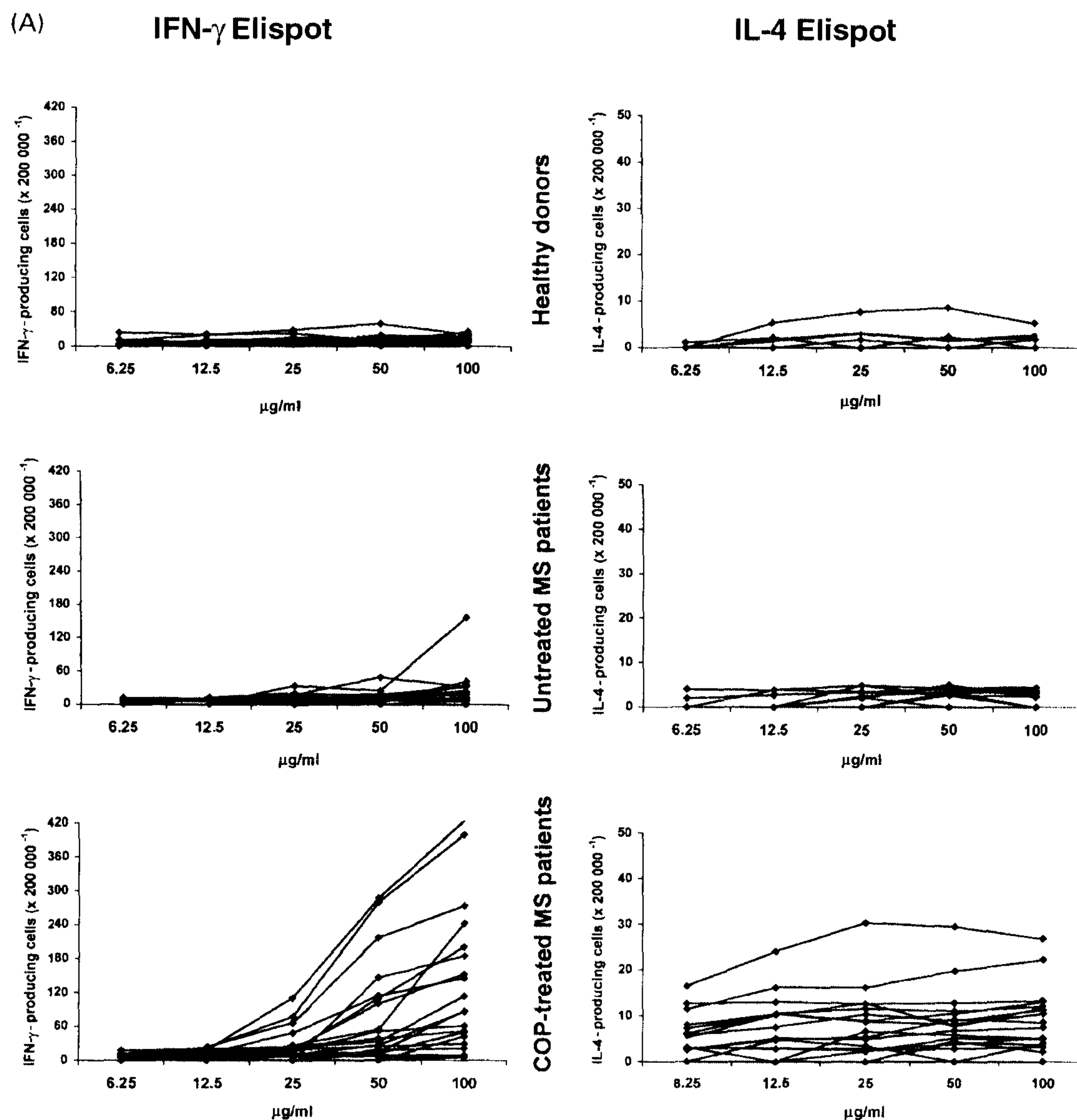

Fig. 2

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1 : 1000 streptavidin-alkaline phosphatase (Mabtech), and finally with BCIP/NBT (bromo-chloro-indolyl phosphate/ nitroblue tetrazolium; Sigma-Aldrich). The frequency of cytokine-producing, antigen-reactive cells was expressed as the difference between the mean number of spots after antigen stimulation and the mean background for each experiment. Overall, the mean absolute spot background was ~10 spots (9.4 ± 2.3) for the IFN-γ assay and ~5 spots (5.3 ± 2) for the IL-4 assay. A value equal to zero was assigned to spot frequencies smaller than the mean background of the individual assay plus 2 SD. All standard deviations were below 20% of the mean. In all experiments, stimulation with SEB induced more than one out of 200 IFN-γ- or IL-4-producing cells.

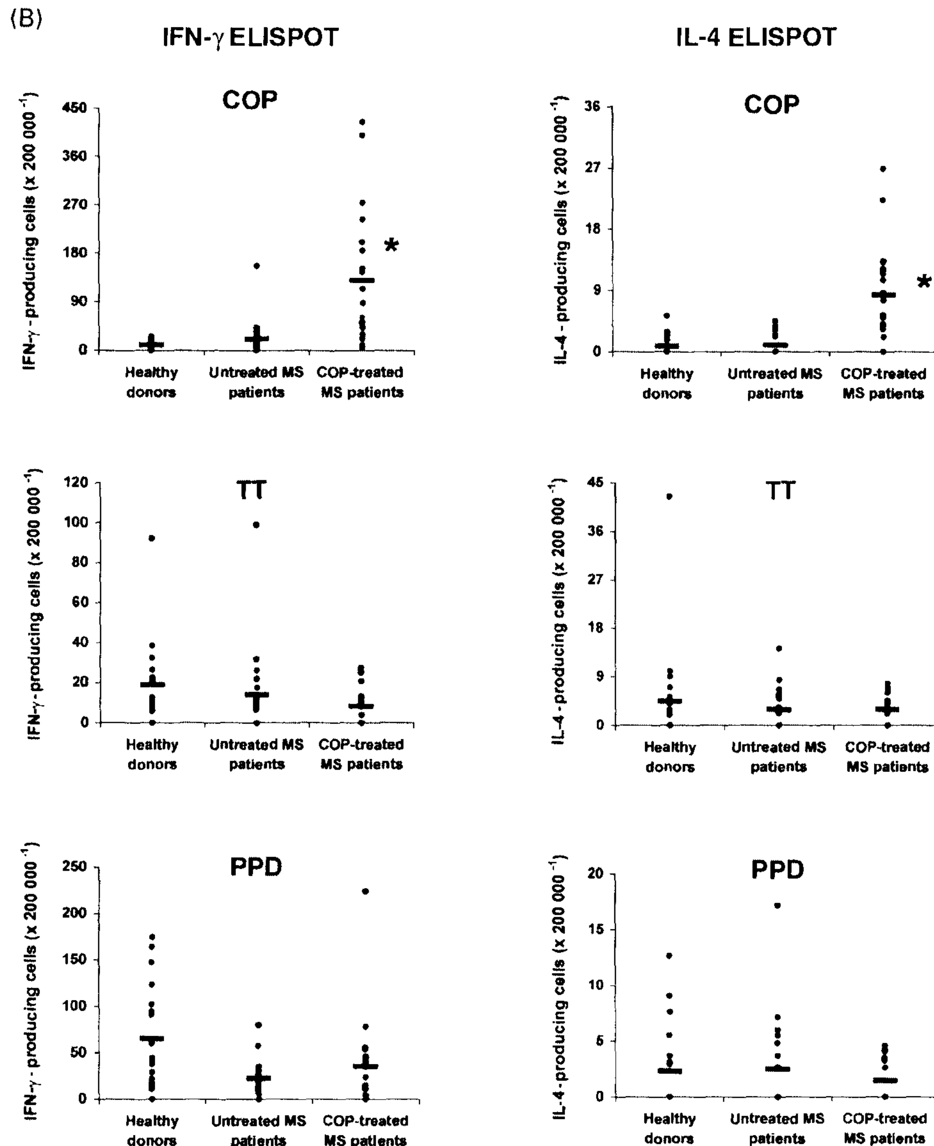

Fig. 2 Differential IFN-γ and IL-4 Elispot response in COP-treated patients. (**A**) Frequency of IFN-γ and IL-4-producing cells (given as spots per 2 x 10^5 PBMC) in response to different COP concentrations in 20 healthy donors, 20 untreated multiple sclerosis (MS) patients, and 20 COP-treated multiple sclerosis patients. Black curves represent individual subjects. In the treated patients, COP induces a strong IFN-γ response at high concentrations (lower left panel), and a positive IL-4 response over the whole range of concentrations (lower right). All assays were done in quadruplicate. SD were below 20% of the mean (not shown). Spots were counted using the KS ELISPOT automated image analysis system (Zeiss, Jena, Germany). (**B**) Comparison between the Elispot response to COP (100 μg/ml) and to the recall antigens TT (4 μg/ml) and PPD (20 μg/ml). Horizontal bars indicate group mean values. Statistically significant differences are marked with an asterisk. The elevated IFN-γ and IL-4 response in COP-treated patients (upper panels) is specific for COP, as it is not observed with TT (middle) or PPD (bottom).

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