SHORT REPORT

Interferon γ and interleukin 4 producing T cells in peripheral blood of multiple sclerosis patients undergoing immunomodulatory treatment

D Franciotta, E Zardini, R Bergamaschi, L Andreoni, V Cosi

J Neurol Neurosurg Psychiatry 2003;**74**:123–126

Intracellular cytokine flow cytometry was used to analyse the percentages of interferon (IFN) γ and interleukin (IL)-4 producing T cells in the peripheral blood of multiple sclerosis patients, before and after immunomodulatory treatment, and of healthy controls. After six months of treatment, different doses of IFN β1a (Avonex or Rebif) decreased $CD4^+$ (Th1, Th2) and $CD8^+$ (Tc1) cells to a similar extent, without affecting the Th1/Th2 ratio. These T cell subsets were unmodified after nine months of glatiramer acetate (Copaxone) treatment, and after six day courses of high dose 6-methylprednisolone. The data suggest that IFN β1a produces sustained downmodulation of IFN γ and IL-4 producing T cells in vivo, which may contribute to its therapeutic efficacy; that glatiramer acetate possibly acts without altering non-specific cellular immunity; and that glucocorticoid induced lymphocytopenia does not affect the percentages of Th1, Th2, and Tc1 cells; at least in the periphery, none of the treatments caused a Th1 to Th2 shift that could account for their respective therapeutic effects.

The rationale for immunomodulatory treatment in multiple sclerosis derives from current knowledge about its pathogenesis. B cells, T cells, and macrophages account for the inflammatory response that leads to demyelination, which is mainly promoted by Th1 circuits.[1] Th1 cells secrete interleukin (IL)-2, interferon (IFN) γ, and tumour necrosis factor α/β, whereas Th2 cells produce IL-4, IL-5, and IL-10. Cytokines of Th1 subsets are inhibitory towards Th2 cells and vice versa. Accordingly, boosting Th2 response is considered to be beneficial in multiple sclerosis.

IFN β1a/b and glatiramer acetate are currently used for the chronic treatment of multiple sclerosis. IFN β could act by antagonising proinflammatory cytokines, and—in accordance with recent hypotheses—by modifying the IL-12/IL-10 axis.[2 3] Glatiramer acetate resembles myelin in structure. It appears to inhibit T cell reactivity towards some myelin antigens and to generate glatiramer acetate reactive Th2-like regulatory cells.[4 5] Glucocorticosteroids are administered in acute attacks of multiple sclerosis because of their immunosuppressive and anti-inflammatory activity. At high concentrations, their effects could exploit caspase dependent apoptosis in lymphocytes.[6]

The mechanisms of these drugs are only partially known, and could at least in part be mediated by cytokines. As it is possible to stain both extracellular epitopes and intracellular cytokines on the same cell, flow cytometry is a powerful technique for the analysis of cytokine expression in defined cell populations.[7] $CD4^+$ T lymphocytes act as helper/cytotoxic cells, whereas $CD8^+$ T lymphocytes mediate cytotoxicity and suppression. IFN γ/IL-12 drives $CD4^+$ T cells to Th1 and Tc1, and IL-4 $CD8^+$ T cells to Th2 and Tc2 polarisation.[8]

Our aim in this study was to determine whether the percentages of peripheral blood Th1, Tc1, Th2, and Tc2 subsets changes in multiple sclerosis patients after treatment with IFN β1a (two different products), glatiramer acetate, and 6-methylprednisolone.

METHODS

Patients and controls

We enrolled 56 cases of definite relapsing–remitting multiple sclerosis[9] and 10 healthy controls. Table 1 shows their demographic characteristics. The patients were divided into subgroups according to treatment. On sampling, their disease had been clinically stationary for at least the preceding three months, and none of the patients had used immunosuppressive agents during this period. The patients who underwent 6-methylprednisolone treatment were suffering from exacerbations of their disease and had never taken IFN β1a/b or glatiramer acetate. The healthy subjects were tested at baseline (time 0) and again at six months.

The local ethics committee approved the protocol.

Table 1 Demographic characteristics of multiple sclerosis patients and healthy controls

	IFN-β1a (Avo)	IFN-β1a (Reb)	GA	6-MP	Controls
No of subjects	16	16	15	9	10
Male/female	6/10	6/10	4/11	2/7	4/6
Age (years) (mean (SD))	30 (7)	30 (5)	33 (7)	33 (6)	32 (8)
Range (years)	21 to 44	21 to 40	24 to 42	25 to 42	22 to 48
EDSS (mean (SD))	1.5 (1.5)	1.5 (2.0)	2.0 (1.5)	5.0 (1.5)	na
Range	1.0 to 3.5	1.0 to 4.0	1.5 to 4.0	2.7 to 7.5	na
Duration of disease (years) (mean (SD)	3 (4)	3 (5)	4 (5)	9 (8)	na
Range (years)	2 to 7	1 to 7	2 to 8	2 to 25	na

EDSS, expanded disability status scale; GA, glatiramer acetate (Copaxone®); IFN-β1a (Avo), Avonex™; IFN-β1a (Reb), Rebif®; 6-MP, 6-methylprednisolone; na, not applicable.

IFN β1a (Avo), Avonex™, was given in a dose of 30 μg by weekly intramuscular injection, while IFN β1a (Reb), Rebif®, was given as a subcutaneous dose of 44 μg three times weekly. Blood was collected at baseline and after six months of continuous treatment; post-treatment collection was at three days (in the case of IFN β1a (Avo)) or two days (in the case of IFN β1a (Reb)) after injection, to avoid detecting the drugs' short term effects.

Glatiramer acetate, Copaxone®, was given subcutaneously in a dose of 20 mg daily. Blood was collected at baseline and after nine months of continuous treatment.

The patients with acute multiple sclerosis were treated with intravenous 6-methylprednisolone, 1 g/day for six days. Blood was collected before treatment (day 0) and at the end of the treatment (day 7).

Procedures

Intracellular cytokine staining was done by a standard technique,[10] with modifications.[11] Monoclonal antibodies, their isotype controls, and Cytofix/Cytoperm™ and Perm/Wash™ solutions were purchased from PharMigen (San Diego, California, USA). Briefly, within one hour of its collection in lithium heparin, 500 μl of whole peripheral blood was diluted 1:1 in RPMI (Rosswell Park Memorial Institute medium) and supplemented with 50 ng/ml phorbol 12-myristate 13-acetate (PMA), 1 μg/ml ionomycin, and 2 μM monensin (all from Sigma, Milan, Italy). After a four hour incubation at 37°C in 5% CO_2, cells were incubated with an erythrocyte lysing solution for 10 minutes. After washing with Dulbecco's phosphate buffered saline (PBS) without Ca^{2+} or Mg^{2+} and 1% fetal calf serum (staining buffer), cells were incubated for 15 minutes at 4°C in the dark with CyChrome® conjugated anti-CD4/CD8/CD69, as well as fluorescein isothiocyanate (FITC) or phycoerythrin conjugated anti-CD3 antibodies, and their related isotype controls. Anti-CD69 antibody was used to confirm T cell activation. Anti-isotype matched antibodies of irrelevant specificities were used as negative staining controls.

After washing with staining buffer, cells were fixed with Cytofix/Cytoperm for 20 minutes, and then with Perm/Wash for 10 minutes. Cells were incubated with FITC conjugated anti-IFN γ, phycoerythrin conjugated anti-IL-4 antibodies, and their isotype matched antibodies for 30 minutes. After washing with Perm/Wash, cells were suspended in staining buffer and analysed with a FACScan flow cytometer (Epics – Profile II, Coulter Electronics, Miami, Florida, USA). Lymphocytes were tightly gated by means of both forward and side scatter channels. Given that $CD3^+CD8^+IL\text{-}4^+$ T cells invariably comprised less than 1% of the cell population, the following four subpopulations were detected: IFN γ producing T cells ($CD3^+IFN\ \gamma^+$), Th1 cells ($CD3^+CD4^+IFN\ \gamma^+$), Tc1 cells ($CD3^+CD8^+IFN\ \gamma^+$), and Th2 cells ($CD3^+CD4^+IL\text{-}4^+$).

Statistical analysis

Data are reported as percentage of the median (95% confidence interval). The Mann–Whitney test was employed for data comparison in patients *v* controls and the Wilcoxon test for serial measurements. A probability (p) value of < 0.05 was considered significant.

RESULTS

The vast majority of the stimulated T cells were highly positive for the activation antigen CD69. This indicates that the lack of cytokine production by a given cell was not caused by incomplete activation.

In the healthy controls, the baseline *v* six months median percentages for the T cell subpopulations did not differ: IFN γ producing T cells, 30.3 (5.9) *v* 28.5 (4.8); Th1, 18.0 (4.0) *v* 19.1 (4.0); Tc1, 9.6 (2.4) *v* 9.8 (1.8); Th2, 2.3 (0.7) *v* 2.5 (0.6).

Figure 1 shows the percentages of both IFN γ and IL-4 producing T cells in the four patient subgroups before and after treatment.

The small group of patients in acute relapse before 6-methylprednisolone treatment had lower values for the subpopulations considered than the clinically stable patients and the healthy controls. Such values (except for Tc1 cells) tended to be even lower after 6-methylprednisolone treatment, but the difference was not significant.

In patients on IFN β1a, irrespective of the manufacturer, the percentages of both IFN γ and IL-4 producing T cells decreased significantly after the treatment. The pre- and post-glatiramer acetate treatment percentages of these cells were unchanged. For example, a patient of the IFN β1a group had Th1, $240.0 \times 10^3/\mu l$ and Th2, $64.0 \times 10^3/\mu l$ (pre-treatment), and Th1, $66.0 \times 10^3/\mu l$ and Th2, $33.0 \times 10^3/\mu l$ (post-treatment), whereas a patient in the glatiramer acetate group had Th1, $230.5 \times 10^3/\mu l$ and Th2, $29.2 \times 10^3/\mu l$ (pre-treatment), and Th1, $251.2 \times 10^3/\mu l$ and Th2, $25.6 \times 10^3/\mu l$ (post-treatment). The Th1/Tc1 and Th1/Th2 ratios did not differ significantly throughout the case series (data not shown).

Taken as a whole, the clinically stable multiple sclerosis patients showed a trend towards lower percentages for the

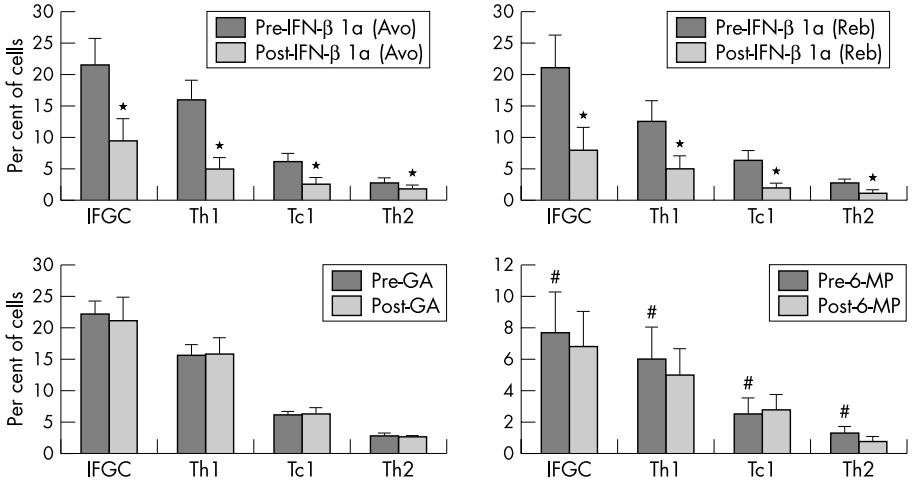

Figure 1 Percentages of IFN γ and IL-4 producing T cells in $CD3^+$ T cells from the peripheral blood of multiple sclerosis patients before and after immunomodulatory treatments. GA, glatiramer acetate (Copaxone); IFGC, IFN-γ producing T cells; IFN-β1a (Avo), Avonex; IFN-β1a (Reb), Rebif; 6-MP, 6-methylprednisolone. Values are medians, error bars = 95% confidence interval. *p < 0.05, pre- *v* post-treatment; #p< 0.05, clinically stable patients before chronic treatments (cumulative value for the three groups) *v* acute patients before 6-methylprednisolone treatment.

four subsets than those found in the healthy controls, though the difference was only significant for IFN γ producing T cells (21.4 (2.4) *v* 30.3 (5.9), or *v* 28.5 (4.8)).

DISCUSSION

T cells produce mediators that are thought to be responsible for oligodendroglial and neuronal damage in multiple sclerosis.[1] We analysed the percentages of $CD3^+$, $CD4^+$, and $CD8^+$ T cells—which differentiated into IFN γ producing (Th1, Tc1) or IL-4 producing (Th2) subpopulations after short term stimulation—in peripheral blood samples from multiple sclerosis patients. In accordance with our previous results,[11] we detected no Tc2 cells either in patients or in controls. In healthy controls, the subpopulation percentages were unchanged at time 0 and at six months. This indicates that biological variability does not affect the measurements of these cells.

IFN β is a pleiotropic cytokine with a confusing array of both pro- and anti-inflammatory activities; such activities even include that of driving IFN γ production.[2] We found that Avonex and Rebif, which represent two different pharmaceutical preparations of IFN β1a, induced equally profound decreases in Th1, Tc1, and Th2 cells after six months of treatment. The extent of decrease was such that it could contribute to the therapeutic efficacy of IFN β1a in multiple sclerosis; at the same time, it would be unlikely to allow differentiation between responders and non-responders (the present short term study is inherently uninformative about responsiveness to treatment). Downmodulating effects on IFN γ producing T cells in multiple sclerosis induced by IFN β have been reported by some groups,[12–14] but denied by others.[15] IFN β treatment also accounts for decreases in IL-4 producing cells,[13 14] although other studies emphasise the molecule's therapeutic role in specifically boosting the Th2 as opposed to the Th1 immune response (reviewed by Karp *et al* [2]). Our data indicate that IFN β1a does not affect Th1/Th2 balance, but rather downmodulates both subpopulations. The data do not exclude a regulatory role for IL-10 secreting cells as mediators of IFN β mechanisms of action.[2] However, the findings of a multiple sclerosis trial (reported by Hohlfeld and Wiendl[16]) indicate that recombinant IL-10 was not effective, and possibly induced exacerbations.

Our data on glatiramer acetate agree with the hypothesis that, once generated in the periphery, glatiramer acetate specific Th2 cells act within the central nervous system,[5] without important cytokine mediated systemic effects on peripheral Th1, Tc1, and Th2 cells. In contrast, Miller *et al* found that glatiramer acetate treatment upregulated TGF β and IL-4 messanger mRNA in peripheral blood leucocytes,[17] and proposed that a shift from Th1 to Th2/Th3 subsets could contribute to the efficacy of this agent.

Taken as a whole, our results on chronic treatment for multiple sclerosis suggest that a combination of glatiramer acetate and IFN β could not be more effective than single treatments. Indeed, if it is true that the former upregulates specific Th2-like regulatory cells,[4 5] then an IFN β induced profound Th2 cell downregulation could interfere with the generation of glatiramer acetate stimulated specific Th2 cells.

Before undergoing 6-methylprednisolone treatment, our patients with clinically active multiple sclerosis showed a decrease in the percentages of Th1, Tc1, and Th2 cells. We have previously described the same phenomenon,[11] and others have confirmed it.[18] It can be interpreted as an activated T cell transmigration into the CNS. Corticosteroids induce leucocytosis, characterised by granulocytosis and lymphocytopenia. We found that short term effects of 6-methylprednisolone treatment did not modify the baseline low percentages of IFN γ and IL-4 producing T cells. Thus the lymphocytopenia that we detected (data not shown) did not affect the percentages of these cells in the periphery. We cannot exclude the possibility that downmodulation of Th1, Th2, and Tc1 cells induced by 6-methylprednisolone could occur within the central nervous system, where activated T cells could be overrepresented before treatment. Ex vivo evidence has shown that high dose 6-methylprednisolone is proapoptotic for $CD4^+$ lymphocytes.[19] Our data suggest that this "non-genomic" effect does not reduce the Th1 and Tc1 percentages. Differences in experimental conditions may explain the discrepancy.

Conclusions

Substantial IFN γ and IL-4 producing T cell downmodulation could be among the many actions possibly responsible for the beneficial effect of IFN β in multiple sclerosis. At least in the periphery, none of the treatments reported here causes a Th1 to Th2 shift that could account for their respective therapeutic effects.

ACKNOWLEDGEMENTS

This work was supported by a grant from the "Ministero della Sanità" (RC 1999).

Authors' affiliations
D Franciotta, E Zardini, L Andreoni, Laboratory of Neuroimmunology, Foundation "Neurological Institute C Mondino," University of Pavia, Pavia, Italy
R Bergamaschi, V Cosi, Division B, IRCCS, Foundation "Neurological Institute C Mondino"

Competing interests: Two of the coauthors (RB and VC) have participated in clinical trials supported by the manufacturers of drugs mentioned in this paper.

Correspondence to: Dr Diego Franciotta, Foundation "Neurological Institute C Mondino", via Palestro 3, I-27100 Pavia, Italy; diego.franciotta@mondino.it

Received 7 May 2002
In revised form 10 September 2002
Accepted 12 September 2002

REFERENCES

1 **Martino G**, Hartung HP. Immunopathogenesis of multiple sclerosis: the role of T cells. *Curr Opin Neurol* 1999;**12**:309–21.
2 **Karp CL**, van Boxel-Dezaire AHH, Byrnes AA, *et al*. Interferon-β in multiple sclerosis: altering the balance of interleukin-12 and interleukin-10? *Curr Opin Neurol* 2001;**14**:361–8.
3 **Byrnes AA**, McArthur JC, Karp CL. Interferon-β therapy for multiple sclerosis induces reciprocal changes in interleukin-12 and interleukin-10 production. *Ann Neurol* 2002;**51**:165–74.
4 **Fridkis-Hareli M**, Teitelbaum D, Gurevich E, *et al*. Direct binding of myelin basic protein and synthetic copolymer 1 to class II major histocompatibility complex molecules on living antigen-presenting cells – specificity and promiscuity. *Proc Natl Acad Sci USA* 1994;**91**:4872–6.
5 **Neuhaus O**, Farina C, Wekerle H, *et al*. Mechanisms of action of glatiramer acetate in multiple sclerosis. *Neurology* 2001;**56**:702–8.
6 **Gold R**, Buttgereit F, Toyka KV. Mechanism of action of glucocorticosteroid hormones: possible implications for therapy of neuroimmunological disorders. *J Neuroimmunol* 2001;**117**:1–8.
7 **Franciotta D**, Zardini E, Bergamaschi R, *et al*. The relevance of cytokines within the immuno-inflammatory response: are they reliably measurable in biological fluids? *Funct Neurol* 2001;**16**(suppl):271–6.
8 **Sad S**, Marcotte R, Mosmann TS. Cytokine-induced differentiation of precursor mouse CD8+ T cells into cytotoxic CD8+ T cells secreting Th1 or Th2 cytokines. *Immunity* 1995;**2**:271–9.
9 **Poser CM**, Paty DW, Scheinberg L, *et al*. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. *Ann Neurol* 1983;**13**:227–31.
10 **Prussin C**, Metcalfe D. Detection of intracytoplasmic cytokine using flow cytometry and directly conjugated anti-cytokine antibodies. *J Immunol Methods* 1995;**188**:117–28.
11 **Franciotta D**, Zardini E, Bergamaschi R, *et al*. Interferon γ- and interleukin-4-producing T cells in peripheral blood of multiple sclerosis patients. *Eur Cytokine Netw* 2000;**11**:677–81.
12 **Becher B**, Giacomini PS, Pelletier D, *et al*. Interferon-γ secretion by peripheral blood T-cell subsets in multiple sclerosis: correlation with disease phase and interferon-β therapy. *Ann Neurol* 1999;**45**:247–50.
13 **Rep MH**, Schrijver HM, van Lopik T, *et al*. Interferon (IFN)-beta treatment enhances CD95 and interleukin-10 expression but reduces interferon-gamma producing T cells in MS patients. *J Neuroimmunol* 1999;**96**:92–100.

14 **Furlan R**, Bergami A, Lang R, *et al*. Interferon-β treatment in multiple sclerosis patients decreases the number of circulating T cells producing interferon-γ and interleukin-4. *J Neuroimmunol* 2000;**111**:86–92.
15 **Dayal AS**, Jensen MA, Lledo A, *et al*. Interferon-gamma-secreting cells in multiple sclerosis patients treated with interferon beta-1b. *Neurology* 1995;**45**:2173–7.
16 **Hohlfeld R**, Wiendl H. The ups and downs of multiple sclerosis therapeutics. *Ann Neurol* 2001;**49**:281–4.
17 **Miller A**, Shapiro S, Gershtein R, *et al*. Treatment of multiple sclerosis with Copolymer-1 (Copaxone®): implicating mechanisms of Th1 to Th2/Th3 immune-deviation. *J Neuroimmunol* 1998;**92**:113–21.
18 **Killestein J**, Rep MHG, Barkhof F, *et al*. Active MRI lesion appearance in MS patients is preceded by fluctuations in circulating T-helper 1 and 2 cells. *J Neuroimmunol* 2001;**118**:286–94.
19 **Leussink VI**, Jung S, Merschdorf U, *et al*. High-dose methylprednisolone therapy in multiple sclerosis induces apoptosis in peripheral blood leukocytes. *Arch Neurol* 2001;**58**:91–7.