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 days 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. 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. 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. 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. 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. 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.

Our aim in this study was to determine whether the percentages of peripheral blood Th1, Th2, and Th2 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 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

<table>
<thead>
<tr>
<th></th>
<th>IFN-β1a (Avo)</th>
<th>IFN-β1a (Reb)</th>
<th>GA</th>
<th>6-MP</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of subjects</td>
<td>16</td>
<td>16</td>
<td>15</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Male/female</td>
<td>6/10</td>
<td>6/10</td>
<td>4/11</td>
<td>2/7</td>
<td>4/6</td>
</tr>
<tr>
<td>Age (years) [mean (SD)]</td>
<td>30 (7)</td>
<td>30 (5)</td>
<td>33 (7)</td>
<td>33 (6)</td>
<td>32 (8)</td>
</tr>
<tr>
<td>Range (years)</td>
<td>21 to 44</td>
<td>21 to 40</td>
<td>24 to 42</td>
<td>28 to 42</td>
<td>22 to 48</td>
</tr>
<tr>
<td>EDSS (mean [SD])</td>
<td>1.5 (1.5)</td>
<td>1.5 (2.0)</td>
<td>2.0 (1.5)</td>
<td>5.0 (1.5)</td>
<td>na</td>
</tr>
<tr>
<td>Range</td>
<td>1.0 to 3.5</td>
<td>1.0 to 4.0</td>
<td>1.5 to 4.0</td>
<td>2.7 to 7.5</td>
<td>na</td>
</tr>
<tr>
<td>Duration of disease (years) [mean (SD)]</td>
<td>3 (4)</td>
<td>3 (5)</td>
<td>4 (5)</td>
<td>9 (8)</td>
<td>na</td>
</tr>
<tr>
<td>Range (years)</td>
<td>2 to 7</td>
<td>1 to 7</td>
<td>2 to 8</td>
<td>2 to 25</td>
<td>na</td>
</tr>
</tbody>
</table>

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, with modifications. Monoclonal antibodies, their isotype controls, and Cytofix/Cytoperm™ and Perm/Wash™ solutions were purchased from PharMingen (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 (Roswell 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% CO₂, cells were incubated with an erythrocyte lysis solution for 10 minutes. After washing with Dulbecco’s phosphate buffered saline (PBS) without Ca²⁺ or Mg²⁺ 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 isotype matched 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-4⁻ T cells invariably comprised less than 1% of the cell population, the following subpopulations were detected: IFN γ producing T cells (CD3⁺IFN γ⁺), Th1 cells (CD3⁺CD4⁻IFN γ⁺), Th1 cells (CD3⁺CD8⁻IFN γ⁺), and Th2 cells (CD3⁺CD4⁺IL-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 28.3 (4.8); Th1, 18.0 (4.0) v 19.1 (4.0); Th1, 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 x 10⁶/µl and Th2, 64.0 x 10⁶/µl (pre-treatment), and Th1, 66.0 x 10⁶/µl and Th2, 33.0 x 10⁶/µl (post-treatment), whereas a patient in the glatiramer acetate group had Th1, 230.5 x 10⁶/µl and Th2, 29.2 x 10⁶/µl (pre-treatment), and Th1, 251.2 x 10⁶/µl and Th2, 25.6 x 10⁶/µl (post-treatment). The 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...
DISCUSSION

T cells produce mediators that are thought to be responsible for oligodendrogial and neuronal damage in multiple sclerosis. 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, we did not detect CD2 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.

IFNβ 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. We found that Avonex and Rebif, which represent two different pharmaceutical preparations of IFNβ1a, 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 decreases in Th1, Tc1, and Th2 cells after six months of treatment. Downmodulating effects on IFNγ-producing T cells in multiple sclerosis induced by IFNβ have been reported by some groups, but denied by others. IFNβ treatment also accounts for decreases in IL-4-producing cells, 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.). Our data indicate that IFNβ1a 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. However, the findings of a multiple sclerosis trial (reported by Hohlfeld and Wiendl) 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, without important cytokine mediated systemic effects on peripheral Th1, Tc1, and Th2 cells. In contrast, Miller et al found that glatiramer acetate treatment upregulated TGFβ and IL-4 messenger mRNA in peripheral blood leukocytes, 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 IFNβ could not be more effective than single treatments. Indeed, if it is true that the former upregulates specific Th2-like regulatory cells, then an IFNβ 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, and others have confirmed it. It can be interpreted as an activated T cell transmigration into the CNS. Corticosteroids induce leukocytosis, characterised by granulocytosis and lymphocytopenia. We found that short term effects of 6-methylprednisolone treatment did not modify the baseline low percentages of IFNγ 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. 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 IFNγ and IL-4 producing T cell downmodulation could be among the many actions possibly responsible for the beneficial effect of IFNβ 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.

ACKNOWLEDGMENTS

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REFERENCES