Diminished Myelin-Specific T Cell Activation Associated with Increase in CTLA4 and Fas Molecules in Multiple Sclerosis Patients Treated with IFN-β

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ABSTRACT

Multiple sclerosis (MS) is a chronic inflammatory disease of the white matter of the central nervous system (CNS) characterized by focal areas of demyelination. Interferon-β (IFN-β) provides an effective treatment that lessens the frequency and severity of exacerbations in relapsing-remitting multiple sclerosis (RRMS), but the mechanisms by which IFN-β is efficient remain uncertain. The data presented here demonstrate that IFN-β impairs the proliferative response to myelin basic protein (MBP) and myelin, as well as increasing the expression of the CTLA4 intracellular molecule. Moreover, this treatment increases the expression of surface Fas molecules and of the soluble form of these molecules. Our hypothesis is that the increase in Fas and CTLA4 molecules in MS patients may lead to lymphocyte apoptosis, which suggests possible mechanisms underlying the therapeutic response to IFN-β.

INTRODUCTION

TREATMENT OF RELAPSING-REMITTING multiple sclerosis (RRMS) with interferon-β (IFN-β) reduces the frequency and severity of clinical exacerbations and has a beneficial effect on the progression of the disease.[1] The pathogenesis of MS assumes that autoreactive T lymphocytes and macrophages, after crossing the blood-brain barrier (BBB), produce central nervous system (CNS) demyelination.[2] Therefore, regulation though the induction of anergy or the elimination of autoreactive T cells may be a possibility for preventing MS lesions.

Two signals are needed for T cell activation. Binding of the T cell receptor (TCR) to a peptide-MHC complex provides the first, and the second is provided by cytokines, such as interleukin-2 (IL-2), and costimulatory proteins, such as CD80 and CD86, expressed on antigen-presenting cells (APCs) or CD28 and CTLA4 molecules expressed on lymphocytes. These CD28 and CTLA4 proteins share amino acid sequences but appear to serve different functions: crosslinking with the CD28 receptor enhances T cell activation, whereas that with the CTLA4 receptor inhibits it.[3,4] One previously suggested possibility is that

signaling through CTLA4 may induce apoptosis in activated cells. It has been suggested that CTLA4 may downregulate the proliferative response of lymphocytes when the cell surface of the CTLA4 peaks, correlating with a decrease in survival factors involved in the mechanisms of apoptosis.[5,6]

Apoptosis is a physiologic process that plays a critical role in the elimination of autoreactive T cells and, thus, immune regulation. Apoptosis of the cells that express the Fas molecules (also known as CD95 or APO-1) results from the crosslinking of the Fas molecule with the Fas ligand (FasL).[7,8] Mice strains carrying mutations in the Fas *(lpr)* and FasL *(gld)* genes exhibit abnormal lymphocyte proliferation and autoimmune syndromes.[9,10] Cell death in MS, as well as in its animal model, experimental autoimmune encephalomyelitis (EAE), has been demonstrated to be an essential mechanism in the regulation of the inflammatory reaction, and infiltrating autoreactive lymphocytes seem to be eliminated *in situ* through the apoptotic process.[11–15] The involvement of the Fas system in MS and in other neurologic disorders has been reported, and it may play a role in modulation of apoptosis.[16]

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In the present study, the association between the activation of myelin-specific T lymphocytes in MS patients treated or not with IFN-β was investigated, as was the expression of CTLA4 and Fas molecules in these individuals.

MATERIALS AND METHODS

Patients

The patients in this study were identified using the criteria of Poser et al.[17] to define MS. A total of 47 patients with stable RRMS, secondary progressive MS, and primary progressive MS, 55 patients with RRMS in treatment with IFN-β1b, and 30 normal subjects were studied (Table 1). The control group was recruited from the local community and had no family history of neurologic or psychiatric illness. Patients were chosen, and Expanded Disability Status Scale (EDSS) scores were assessed in sequential visits. None of the patients in any of the groups had received corticosteroids or other immunosuppressive drugs during a period of at least 6 months prior to donating blood for the study. The patients in the treated group had been receiving IFN-β treatment in standard doses for 18–24 months. This study was approved by the Ethics Committee of the Universidade Estadual de Campinas—UNICAMP, and the volunteers gave written informed consent for participation in the study.

Human myelin basic protein

Human myelin basic protein (MBP) was obtained according to Deibler et al.[18]

Isolation of human myelin

Humans brains were removed from patients who had died from nonneurologic causes, with removal occurring an average of 2 h after death. The white matter was removed, and myelin was isolated by overlaying the homogenate in isotonic (0.32 M) sucrose on a denser sucrose (0.85 M) gradient, allowing the myelin to migrate down to the interface.[19,20] The myelin was then isolated by centrifugation, dialyzed against water at 4°C, and lyophilized.

Purification of mononuclear cells

Blood samples (15 mL) were collected under sterile conditions. The cells were separated on a Ficoll-Hypaque gradient (1.077 density), and the cell concentration was adjusted to 2×10^6 cells/mL.

Proliferation assay

Peripheral mononuclear blood cells (PBMCs) were purified using a Ficoll-Hypaque gradient. The cells were suspended in Hank's balanced salt solution (HBSS) and washed before the addition of RPMI 1640 medium supplemented with 5×10^{-5}M 2-mercaptoethanol, 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin-streptomycin, 12.5 mM HEPES buffer, pH 7.4, 0.2% NaHCO$_3$, and 10% AB$^+$ human serum. The cells were cultured in 96-well flat-bottom culture plates, 10^5 per well, in the presence of 25 μg/mL MBP or 10 μg/mL human myelin as well as phytohemagglutinin (PHA) (5 μg/mL). Cells were incubated for 72 h for the nonspecific mitogen and for 144 h for the antigen in a humidified, 5% CO$_2$ atmosphere at 37°C; the plates were pulsed with 1.0 μCi of ^{3}H-thymidine per well and harvested 18 h later. The incorporation of ^{3}H-thymidine was assessed by standard liquid scintillation techniques. The results were expressed as stimulation index (SI), which is the mean count per minute (cpm) of stimulated cells/cpm of unstimulated cells.

Quantification of surface Fas and CTLA4 molecules

Five microliters of biotin anti-Fas antibody or anti-CTLA4 molecules, as well as the isotype controls (PharMingen, San Diego, CA) were added to the cells (5×10^6 cells/mL). After 30 min of incubation on ice and two washes with HBSS, avidin-FITC antibody (5 μg/mL) was added. After 30 more min of incubation and further washing, the presence of the Fas or CTLA4 molecules was determined by flow cytometry.

TABLE 1. SUMMARY OF CLINICAL CHARACTERISTICS OF MS PATIENTS

Parameter	IFN-β-treated MS[a]	Untreated MS[b]	Healthy controls
n	55	47	30
Female/male	37/16	29/18	19/11
Age, years	30	46	29
Mean $\pm$ SD	$\pm$9.6	$\pm$11.2	$\pm$4.0
Range	16–46	18–57	18–52
Duration of disease, years	10	17	
Mean $\pm$ SD	$\pm$5.8	$\pm$6.9	
Range	3–24	11–24	
EDSS[c]	4.3 $\pm$ 2.0	5.9 $\pm$ 1.8	
Mean $\pm$ SD			
Range	1.0–8.0	4.0–8.0	

[a]The treated MS group comprises relapsing-remitting multiple sclerosis.
[b]The untreated group comprises stable relapsing-remitting multiple sclerosis; secondary progressive multiple sclerosis; primary progressive multiple sclerosis.
[c]EDSS, Expanded Disability Status Scale.

Quantification of surface Fas molecules on CD4$^+$ and CD8$^+$ cells

Anti-Fas antibodies (10 μL) conjugated with FITC (PharMingen) and 5 μL anti-CD4 or anti-CD8 antibodies (or both) conjugated with phycoerythrin (PE) (PharMingen) were added to the cells (2 $\times$ 10^6 cell/mL). After 30 min of incubation and appropriate washes, the percentage of Fas molecules was determined using a Becton Dickinson FACScan (Becton Dickinson, Mountain View, CA).

Cytometric analysis of intracellular CTLA molecules

Lymphocytes (10^6 cells/mL) were stimulated with 5.0 μg/mL PHA for 20 h, the last 4 h in the presence of 10 μg/mL monensin. After stimulation, cells were washed twice with HBSS (pH 7.2), fixed 15 min with formaldehyde (2% in phosphate-buffered saline [PBS], pH 7.2), permeabilized with PBS (pH 7.2) containing 0.5% bovine serum albumin (BSA) and 0.5% saponin, and then incubated for 15 min at ambient temperature with the specific monoclonal antibody (mAb) to the CTLA4 molecule. Cells were then washed and analyzed on a Becton Dickinson FACScan.

Quantification of soluble Fas molecules

To measure the levels of soluble Fas molecules (sFas) in sera, an ELISA kit with specific mAbs was used (Opteia-Human Fas kit, PharMingen). Briefly, an mAb specific for human Fas was coated on a 96-well plate. Standards and samples were added to the wells so that any sFas present would bind to the immobilized antibody. The wells were washed, and a mixture of biotinylated antihuman Fas antibody with horseradish peroxidase (HRP)-conjugated streptavidin was added, producing an antibody-antigen sandwich. The wells were again washed, and a substrate solution that produces a blue color with intensity in direct proportion to the amount of sFas present in the initial sample was added. A stop solution was used to change the color from blue to yellow, and the wells were read at 450 nm. All samples were assayed in duplicate, and the differences between replicate wells were uniformly <5%.

Statistical analysis

The statistical significance of the results was determined by a Wilcoxon test, a Kruskal Wallis test, and a Spearman Rank Correlation test. A *p* value <0.05 was considered to be significant.

RESULTS

Proliferative response

The proliferative response to PHA was evaluated for the three groups (untreated MS, treated MS, and healthy donors), and the results, expressed as SI, are shown in Figure 1A. The healthy donor group demonstrated an extensive proliferative response to PHA (SI = 96 ± 48, *n* = 20), which was not significantly different (*p* > 0.05) from that of the untreated patients
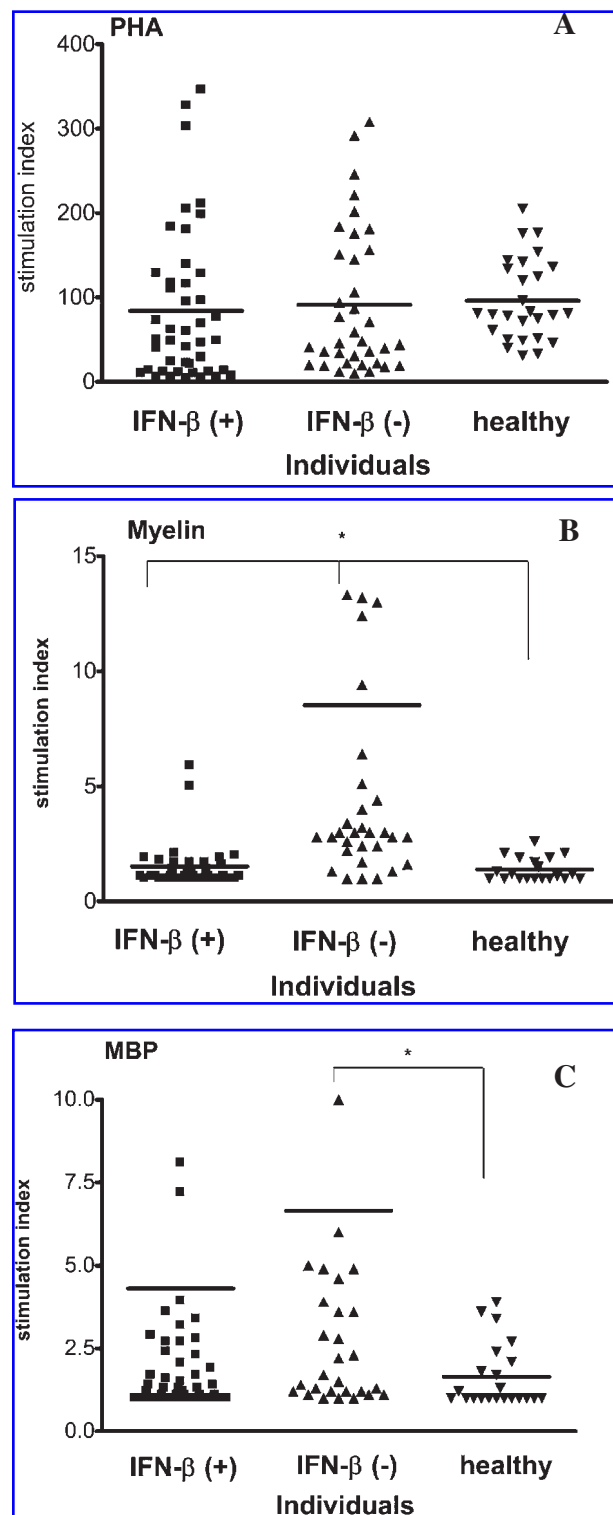
FIG. 1. Proliferative response of lymphocytes from patients with multiple sclerosis, both with and without treatment with IFN-β, as well as healthy individuals, after stimulation with (**A**) PHA, (**B**) myelin, and (**C**) MBP. Treatment with IFN-β significantly reduced (*p = 0.003) the proliferative response of lymphocytes to neuroantigens (myelin and MBP). Results are expressed as stimulation index.

(SI = 91 ± 85, n = 36) or of the treated MS patients (SI = 84.4 ± 89, n = 27).

The lymphocyte proliferative response to myelin was evaluated for the three groups. The results shown in Figure 1B demonstrate that the lymphocyte blastogenic response increased significantly (p = 0.003) for the untreated MS patients (SI = 8.2 ± 1.1, n = 30) in relation to that of the healthy individuals (SI = 1.3 ± 0.4, n = 20). There was no significant difference ($p > 0.05$) between the treated patients (SI = 1.5 ± 1.0, n = 39) and the healthy individuals (SI = 1.3 ± 0.4, n = 20).

The lymphocyte proliferative response to MBP was also evaluated. The results in Figure 1C show a significantly higher response (p = 0.003) level for the untreated MS patients (SI = 6.6 ± 4.1, n = 28) than for the healthy group (SI = 1.6 ± 0.9, n = 20). There was no significant difference ($p > 0.05$) between the treated patient group (SI = 4.3 ± 1.2, n = 44) and the healthy group (SI = 1.6 ± 0.9, n = 20).

Quantification of CTLA4 molecules

The presence of CTLA4 molecules was quantified by flow cytometry on the lymphocyte surface and intracellularly in the two groups of MS patients, both untreated and those treated with IFN-β, as well as in the healthy donor controls. When analyzed on the surface of *ex vivo* lymphocytes or even after 24 h in culture, no significant changes were observed in the expression of CTLA4 molecules for the three groups (1.7 ± 0.8%, 1.5 ± 0.2%, 1.4 ± 0.6% for treated patients, untreated patients, and healthy controls, respectively). Marked changes were observed, however, in the intracellular CTLA4 molecules. The mean expression of CTLA4 intracellular molecules was 15.8 ± 2.1% for treated MS patients (n = 19) vs. 3.4 ± 0.7% for healthy controls (n = 15), revealing a significant difference between the two groups (p = 0.0001). This expression for the untreated patients was statistically equivalent to that for the healthy subjects (6.2 ± 1.6% and 3.4 ± 0.7%, respectively, n = 9) ($p > 0.05$) (Fig. 2).

Association between lymphocyte proliferative response to myelin antigens (myelin and MBP) and expression of intracellular CTLA4 molecules

As CTLA4 molecules are known to inhibit the activation of T lymphocytes, the increase in these molecules was associated with the lymphocyte proliferative response to myelin antigens. The results showed that there is a negative correlation between intracellular CTLA4 molecules and the lymphocyte proliferative response to myelin (R^2 = −0.57775, p = 0.00757) and to MBP (R^2 = −0.5215, p = 0.0230) for IFN-β-treated patients. No correlations were found for untreated patients and healthy controls. The lymphocyte proliferative response was presented as delta cpm, which is the mean cpm of stimulated cells minus the cpm of unstimulated cells (Fig. 3).

Quantification of surface Fas molecule

The surface expression of Fas molecule was studied in 8 MS patients, both before IFN-β therapy and for 6 months after its initiation. A significantly greater expression (p = 0.001) of Fas surface molecules was observed after treatment (an increase from 8.5 ± 1.6% to 26.8 ± 1.5%) (Fig. 4).

Quantification of surface Fas on CD4$^+$ and CD8$^+$ cells

Because CD4 T lymphocytes seem to be involved in the genesis of MS lesions, the expression of surface Fas mole-
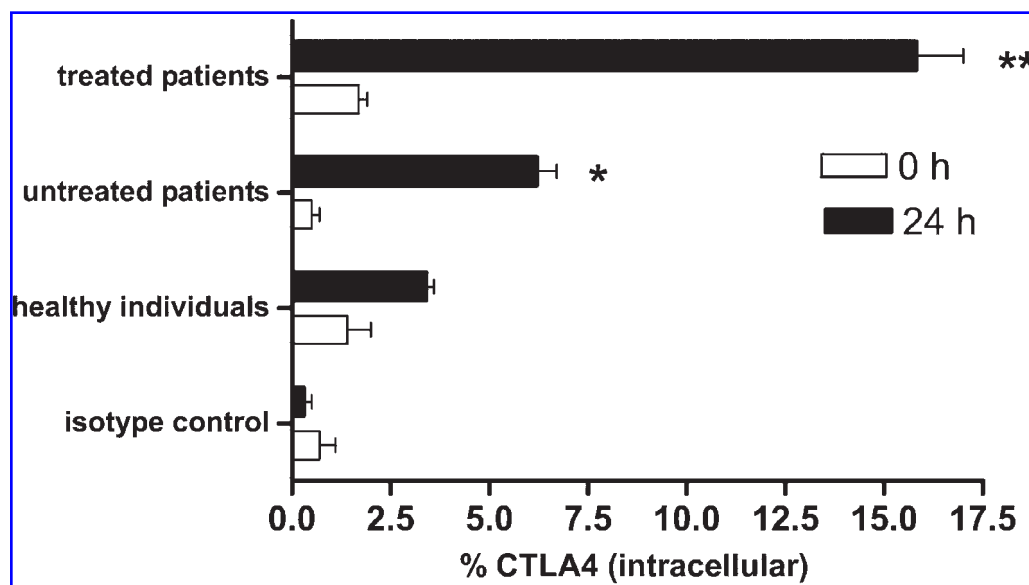

FIG. 2. Percentage of surface (0 h) and intracellular CTLA4 molecules (20 h in culture) in patients with multiple sclerosis, both with and without treatment with IFN-β, as well as healthy individuals. Treatment with IFN-β significantly increased (*p = 0.001; **p = 0.0001) the intracellular expression of CTLA4 molecules in lymphocytes stimulated with PHA for 20 h in culture. CTLA4 molecules were quantified by flow cytometry.
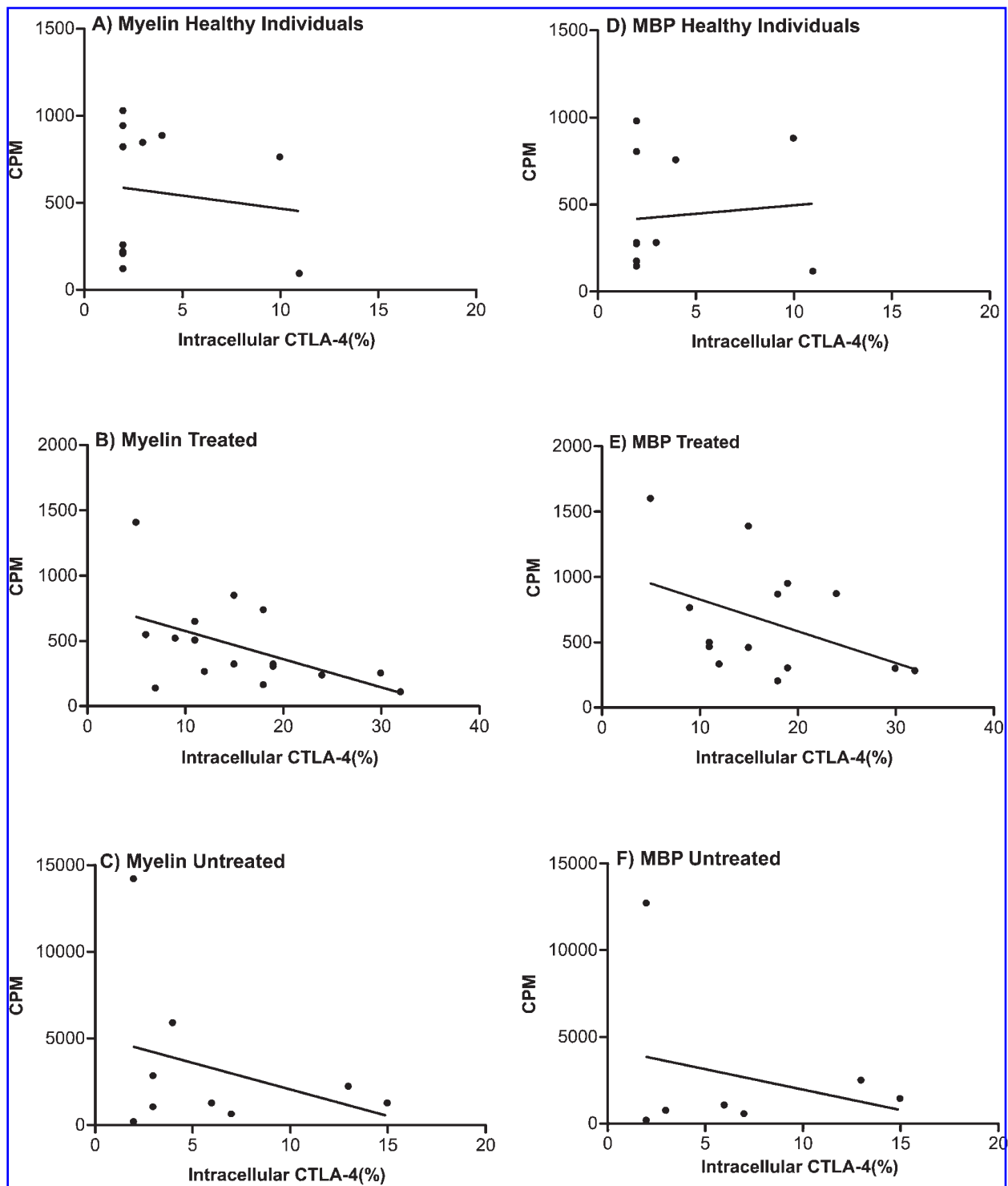
FIG. 3. Correlation between intracellular CTLA4 molecule expression and lymphocyte proliferative response to MBP and myelin. (**A**) $R^2 = -0.35091$ ($p = 0.1317$). (**B**) $R^2 = -0.57775$ ($p = 0.00757$). (**C**) $R^2 = -0.11765$ ($p = 0.3815$). (**D**) $R^2 = -0.24997$ ($p = 0.2166$). (**E**) $R^2 = -0.5215$ ($p = 0.0230$). (**F**) $R^2 = 0.21622$ ($p = 0.3207$).

cule was determined by two-color flow cytometry. The results showed an increase ($p = 0.001$) in the percentage of CD4$^+$Fas$^+$ in the group of treated patients ($24.3 \pm 1.2\%$, $n = 27$) in comparison with the other two groups: $19.2 \pm$ 0.7% and $22.2 \pm 0.8\%$ for healthy individuals ($n = 17$) and those with untreated MS ($n = 16$), respectively (Fig. 5A). There was no significant difference in the percentage of CD8$^+$Fas$^+$, with $11.7 \pm 1.0\%$, $12.6 \pm 1.2\%$, and $13.6 \pm 2\%$
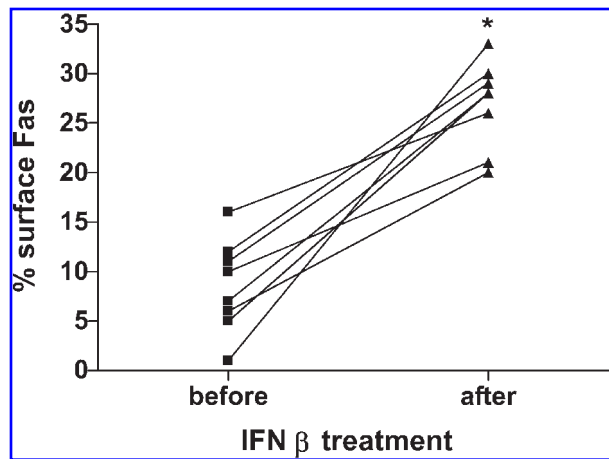
FIG. 4. Percentage of surface Fas molecules on mononuclear cell surfaces in patients with multiple sclerosis, before and after treatment with IFN-β. A significant (*$p = 0.001$) increase in the percentage of Fas molecules was observed in the treated MS patients. Fas molecules were quantified by flow cytometry.

for healthy individuals ($n = 16$), and those with treated ($n = 26$) and untreated MS ($n = 15$) (Fig. 5B).

Quantification of sFas in sera

The administration of IFN-β caused the presence of significantly more sFas molecules in both treated MS patients (227.8 ± 18.4 pg/mL, $n = 38$) ($p = 0.003$) and untreated ones (180.1 ± 14.0 pg/mL, $n = 37$) ($p = 0.004$) in comparison with healthy donors (114.4 ± 10.3 pg/mL, $n = 20$) (Fig. 6).

DISCUSSION

In the present study, the T cell response to myelin antigens and a nonspecific mitogen was investigated, as well as the expression of CTLA4 and Fas molecules in the peripheral blood cells of patients with MS, whether or not being treated with IFN-β.

We have shown that untreated patients evidence a greater lymphocyte proliferative response to myelin and MPB than do the normal healthy controls. These results match the findings of others, which have demonstrated the presence of activated T cells specifically recognizing the myelin antigen in the peripheral blood cells in MS patients, although the antigenic target was confined to the CNS.[21,22] These data suggest that the immune mechanism that maintains the autoreactive lymphocytes under control is impaired in MS patients. We were able to show that the proliferative response to myelin antigens was significantly reduced in the group of MS patients treated with IFN-β. These results are in agreement with those previously described[23,24] Thus, such approaches as immunotherapy with IFN-β, which downregulates the activation of T cells, are useful in minimizing the damage of autoreactions.

The mechanisms by which IFN-β limits the expansion of myelin-reactive T cells, however, deserve additional studies.

One possibility that has been suggested is that IFN-β acts mainly by reducing the antigen-presenting capacity of APCs, which in turn inhibits the effectors functions of autoreactive T cells[25] or by inducing tolerogenic dendritic cells (DCs) in MS patients.[26] Another possibility is the induction of apoptosis in the T cells[27] or modification of the expression of costimulatory molecules, such as CTLA4.

The importance of the CTLA4 molecule in demyelination has been demonstrated in the experimental model of EAE for studying MS. Blocking of CTLA4 accelerates the onset of EAE and is associated with an increased frequency of inflammatory lesions in the CNS, enhanced secretion of proinflammatory cytokines, and increased proliferative responses to *in vitro* antigen stimulation.[28,29] In the present study, the number of intracellular CTLA4 molecules increased in MS patients treated with IFN-β. This increase correlated with a decrease in the proliferative response of the T cells to myelin antigens. The intracellular portion of the CTLA4 tends to be highly conserved for various species, which suggests that control of intracellular trafficking is an important part of its function. This importance is
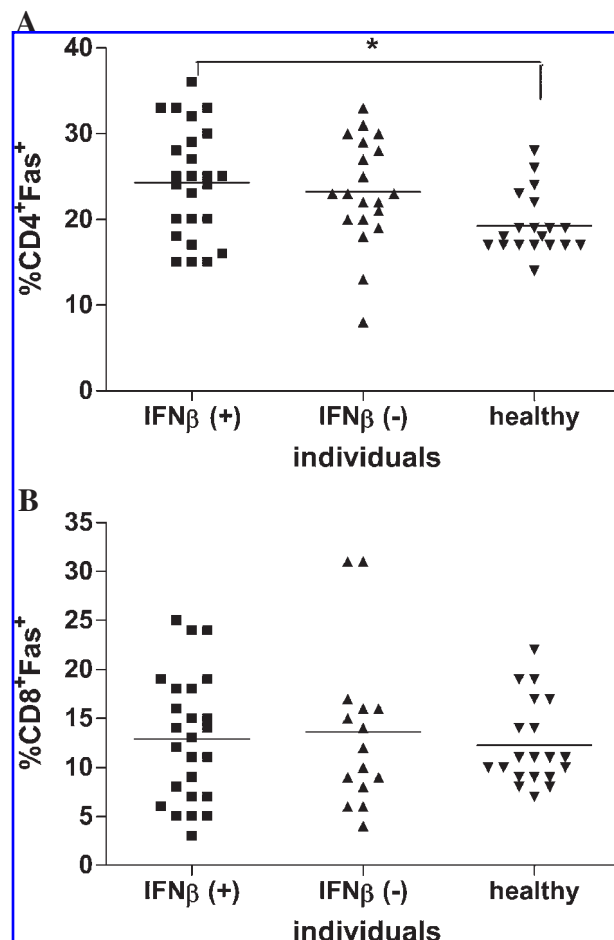
FIG. 5. Percentage of surface Fas molecules on (**A**) CD4 and (**B**) CD8 lymphocytes in patients with multiple sclerosis, both with and without treatment with IFN-β, as well as healthy individuals. A significant (*$p = 0.002$) increase in the percentage of CD4$^+$Fas$^+$ was observed in the treated MS group. Fas molecules were quantified by flow cytometry.
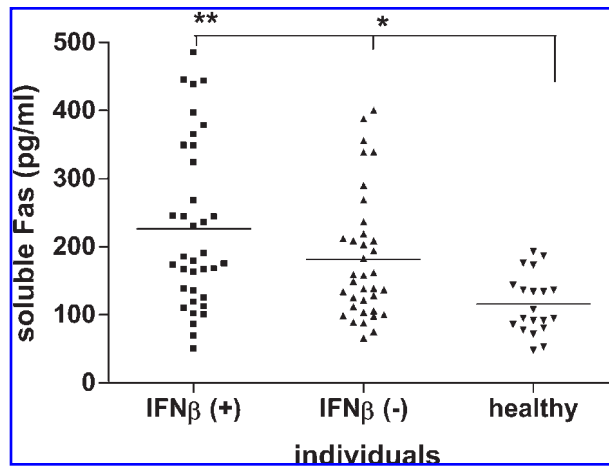
FIG. 6. Quantification of sFas molecules in patients with multiple sclerosis, both with and without treatment with IFN-β, as well as healthy individuals. A significant increase in sFas was observed in both treated and untreated groups of MS patients (*$p = 0.003$). The levels of sFas were higher (**$p = 0.004$) for the treated group. Fas molecules were quantified by an ELISA assay.

emphasized by the fact that the intracellular CTLA4 molecule is polarized toward those sites facing T cell contact.[30,31] As CTLA4 is known to inhibit the activation of T lymphocytes, inhibition of autoreactive T cells may be, at least in part, responsible for the beneficial effects of IFN-β treatment.

Despite considerable efforts, the mechanisms by which CTLA4 molecules exert their suppressive effect on T activation remain poorly understood. The CTLA4 molecules may function at least in part by competing with CD28 for CD80/86 ligands, thus serving as an indirect attenuator of costimulatory signals.[32] The crosslinking of CTLA4 molecules may inhibit IL-2 production and consequent T cell activation,[33] and there is also the possibility of an indirect mode of action of CTLA4, as its engagement costimulates the secretion of inhibitory cytokines, such as transforming growth factor-β (TGF-β). A CTLA4 molecule facilitates TGF-β-mediated suppression by intensifying the TGF-β signal at the point of suppressor cell-target cell interaction.[34] Moreover, in recent studies, the involvement of the CTLA4 molecule in the induction of CD4$^+$CD25$^+$ T cells expressing FOXp3 through TGF-β signaling has also been reported.[35,36] CTLA4 crosslinking may induce T cell apoptosis, as it has been shown that CTLA4 crosslinking on the surface of prestimulated murine T lymphocytes leads to the death of those T lymphocytes.[37]

There is considerable evidence that apoptosis of the myelin autoreactive T lymphocytes is deregulated in MS patients. It has been demonstrated that the activation-induced cell death triggered by the Fas receptor is impaired in MS patients[38–40] and that IFN-β treatment reduces the expression of the inhibitors of apoptosis and increases the apoptosis levels of T lymphocytes of MS patients.[41] In the present study, no direct association between the increased expression of CTLA4 molecules and apoptosis was studied, although an increase in the expression of intracellular CTLA4 molecules was associ-

ated with a simultaneous increase in Fas molecules, both on the cell surface and in soluble form, after treatment with IFN-β. The results obtained here show significant increase in the expression of Fas molecules on the surface of leukocytes in a small group of MS patients after IFN-β treatment. Moreover, a moderate increase in the expression of Fas molecules on the surface of CD4 T lymphocytes in relation to untreated or normal control groups reinforces the role of IFN-β in the induction of apoptosis of autoreactive T cells. The expression of Fas molecules on CD4 Th1 lymphocytes may be linked to the apoptosis of these cells, which may be involved in the development of MS. Thus, the absence or decrease in the expression of the Fas molecule may be indicative of worsening of the disease.

Fas molecules also occur in a soluble form (sFas), which lacks a transmembrane region and is present in normal human sera. As has been shown here, both groups of treated and untreated MS patients show an increase in sFas molecules, although treatment significantly reduced the level. Previous findings have demonstrated that an increase in sFas molecules is associated with disease activity.[42] Thus, reduction in these molecules may be beneficial for MS patients. The results obtained here may be a reflection of this, but it could also be that in some way, the presence of sFas may prevent cells from undergoing Fas-induced apoptosis,[43,44] thus playing a role in modulation of the process.

Taken together, the results presented here provide evidence of the complexity of the mechanisms that control T cell activation in MS patients. IFN-β treatment reduces the proliferative response of lymphocytes to myelin antigens as well as inducing the expression of intracellular CTLA4 molecules and surface Fas molecule on CD4 T lymphocytes. This increase in surface Fas molecules should be favorable to the induction of apoptosis; although sFas molecules released during treatment may result in the survival of some of the T cells. Thus, the beneficial effects of IFN-β treatment, that is, reduction of myelin-specific T cell activation and reduction in clinical signs, may occur because the mechanisms of apoptosis have prevailed, thus reducing the inflammatory response despite the presence of sFas.

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