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Pattern of cytokine secretion by peripheral blood cells of patients with multiple sclerosis in Brazil

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Autoimmune T cells play a key role as regulators and effectors of organ-specific autoimmune disease. In multiple sclerosis (MS), activated T cells specific for myelin components produce a plethora of inflammatory cytokines and mediators that contribute to myelin damage. The production of proinflammatory and regulatory cytokines by peripheral blood cells from patients with active and stable MS and healthy controls were examined. The results show that TNFα production was somewhat elevated in active MS with no significant increase in the level IFNγ, whereas in the chronic phase the anti-inflammatory cytokines IL-10 and TGFβ increased, accompanied by a reduction in IFNγ when stimulated by myelin basic protein.
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Introduction

Multiple sclerosis (MS) is the most important demyelinating disease that affects man. One feature which distinguishes MS from most other conditions is its geographic distribution. The geographic pattern based on prevalence surveys suggests that rates are high in southern Canada and the northern part of the United States, but appear to be low in Mexico and Central America. Few reliable data from Latin America are available, however, and there is a need for information about South America in particular. Although little information is available about the prevalence of MS in Brazil, specialists are concerned with its increase in southern parts of the country, where the population consists mainly of descendents of European immigrants.

Multiple sclerosis is a chronic inflammatory disease characterized by lymphocyte infiltration and demyelination of the central nervous system.[1] Autoreactive T cells which exist in an activated state in the peripheral blood and accumulate in the cerebrospinal fluid (CSF) of patients with MS[2] are thought to recognize myelin components such as myelin basic protein (MBP) and thus contribute to the pathogenesis of the disease.

Cytokines play a particularly important role in cell immune mechanisms. The investigation of the role of cytokines in autoimmune diseases has expanded rapidly over the past few years, and there is now considerable evidence that cytokines such as TNFα and IFNγ contribute to the pathogenesis of MS. The inflammatory responses caused by Th1 cytokines may be inhibited by the emergence of a Th2 type response, resulting in the down regulation of the Th1-mediated immune response. Significantly higher levels of TNFα were found in the CSF of patients with chronic progressive MS than in that of patients with a stable form of the disease.[3] The level of TNFα also correlated with the degree of disability in patients with progressive forms of the disease,[4] and it has been suggested that the increased production of TNFα precedes the clinical manifestations of MS relapse.[5]

A pathogenetic role for IFNγ has also been demonstrated in a clinical trial which revealed its enhancement of exacerbation frequency in MS patients, possibly associated with the activation of monocytes, as evidenced by up-regulation of MHC class II antigen expression on these cells.[6] IFNγ also stimulates the expression of adhesion molecules on endothelial cells, promoting T cell homing and activating blood-derived macrophages and astrocytes to produce proinflammatory mediators such as TNFα.[7,8]

It has become apparent that in inflammatory diseases involving the increased production of cytokines with proinflammatory potential, there is a concomitant increase in production of cytokines with immunoregulatory properties such as ILH 10 and TGFβ.[9–11] IL-10 was initially identified as a factor produced by T helper 2 inhibiting the cytokine synthesis of Th1, an effect attributed to the inhibition of the accessory function, including downregulation of MHC class II expression, and leading to impaired antigen presentation to reactive T cells.[12–14]

Several studies indicate that TGFβ is a potent immunosuppressive factor, affecting the proliferation,

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activation and differentiation of the cells that participate in immunity. Treatment of rats or mice with TGFβ ameliorates Experimental Autoimmune Encephalomyelitis (EAE),[10] and the appearance of TGFβ in the CNS is related to recovery.[15]

In the present study, pro-inflammatory and anti-inflammatory cytokines from the peripheral blood cells of patients with active MS and from those in remission were quantified.

Materials and methods

Patients

Three groups of patients were studied: those with active MS, those with MS in remission and normal controls. Patients were evaluated in this study according to the criteria of Poser *et al*,[16] with patients free of clinical manifestation for more than 6 months being considered to have a stable form of the disease (remission) or with active disease in the last 6 months (relapses). Sixty-three individuals were studied: 31 (26 women and 5 men) with stable MS; 15 (11 women and 4 men) with active MS and 17 (13 women and 4 men) normal subjects. The EDSS was 2.6 ± 1.2 (mean $\pm$ s.d.). The individuals had a mean age of 31 ± 2 years and all subjects came from the same geographic region and same mixed ethnic origin. With the exception of two subjects (who had received immunosuppressive drugs and corticosteroid treatment 2 months prior to the blood donation), none of the subjects had received any treatment prior to donating blood for the study.

Antigen, antibody and recombinant cytokines

Polyclonal chicken anti-TGFβ1 antibody was purchased from R&D Systems (Minneapolis, MN, USA); purified bovine TGF-β1 and monoclonal mouse anti-TGFβ1 were provided by Genzyme, MA, USA; and anti mouse IgG peroxidase was also obtained (Vector Burlingame, CA, USA). The following reagents were purchased from PharMingen (San Diego, CA, USA), IL-10 (clones JES3-9D7; JES3-12G8) mAb, IFN-γ (clones NIB42;4S.B3) mAb, TNFα (clones Mab1;Mab11) mAb and recombinant TNFα, IL-10 and IFN. Human Myelin Basic Protein was obtained according to Deibler *et al*.[17]

Determination of cytokines synthesis

Lymphocytes were cultured in 2 ml RPMI 1640 medium supplemented with 2% FCS, at a cell density of 2×10^6 ml. The cells were then stimulated with either MBP (25 μg/ml) or PHA (10 μg/l): 40 h for IL-10 production, 60 h for IFNγ and 72 h for TGFβ. For TNFα production, the whole blood cells were stimulated with either LPS (100 ng/ml) for 2 h or PHA (10 μg/ml) for 24 h.

Capture ELISA for quantitation of cytokines

TGFβ was measured using a capture ELISA developed in the laboratory.[18] TGFβ Antibody (polyclonal antibody obtained from R&D, MN, USA) (1 μg/ml in PBS, pH 7.4) was added to 96 well microtiter plates (Immulon I, Nunc, Roskilde, Denmark). After overnight incubation at 4°C, the plates were washed three times with ELISA wash buffer (PBS containing 0.05% Tween 20, 0.001% Thimerosal) and blocked for 1 h with ELISA diluent (PBS containing 0.05% Tween 20, 1% BSA). The plates were washed three times with wash buffer, and 100 μl of standard, control, or sample blood was added to duplicate wells for overnight incubation at 4°C. The plates were washed three times with wash buffer and incubated for 1 h at room temperature with TGFβ mAb (Genzyme MA, USA) 1 μg/ml in ELISA buffer. The plates were then washed three times with ELISA wash buffer and incubated an additional hour with biotinylated anti-mouse IgG (Vector, Burlingame, CA, USA) 1 : 2000. Avidin-peroxidase complex and the substrate were then added. Orthophenylene diamine (Sigma Chem. USA) prepared at 5 mg/ml, and 0.05 M hydrogen peroxide was added and left 30 min at room temperature and the plates read at 492 nm.

For quantification of TNFα, IFNγ and IL10 three different kinds of human cytokine-specific mAb were used (PharMingen, San Diego, CA, USA). Briefly, the wells of 96-well microtiter plates (Immulon I, Nunc, Roskilde, Denmark) were coated with $1-2$ μg/ml of the capture mAb of each cytokine in 0.1 M $NaHCO_3$ (pH=8.5) and incubated overnight at 4°C. Following blocking with 3% dry milk in PBS at room temperature for 2 h, samples and standard recombinant IFNγ, TNFα and IL-10 were added and incubated overnight at 4°C. Then, $0.5-2.0$ μg/ml of biotinylated mAb for the detection of human IFNγ, TNFα and IL10 were added followed by 1/400 avidin-peroxidase (Sigma Chem. USA). Finally, the peroxidase substrate and the stop solution were used to obtain OD determined at 492 nm.

Statistical analysis

Results are expressed as the mean $\pm$ 1s.d. of the mean, and statistical significance ($P<0.05$) was analyzed by the Mann-Whitney *U*-test.

Results

TNFα production from peripheral blood cells

Whole blood cells of patients with active and stable MS and healthy controls were stimulated with LPS and PHA *in vitro*, and the TNFα in the plasma was quantified using an ELISA assay. The TNFα was measured in samples of blood from 31 patients with clinically stable MS, 15 with the active disease, and 17 healthy control subjects. When the cells were stimulated with LPS, no significant differences between means of TNFα were observed for the stable and healthy controls (243 ± 201 pg/ml *versus* 132 ± 154 pg/ml respectively; $P>0.05$; patients with active MS produce significantly more TNFα than the healthy controls (355 ± 368 pg/ml *versus* 132 ± 154 pg/ml respectively; $P<0.05$ (Figure 1a). When the cells were stimulated *in vitro* with PHA, the mean TNFα was 356 ± 418 pg/ml and 180 ± 200 pg/ml for stable and healthy controls, with 162 ± 98 and 98 ± 72, without PHA, with no significant difference found ($P>0.05$).

For patients with active disease, the mean was 433±470 pg/ml stimulated with PHA and 128±64 pg/ml without PHA, again with no significant difference from the control ($P<0.05$) (Figure 1b).

IFNγ production from peripheral blood cell

Mononuclear cells from patients with active and stable MS and healthy controls were stimulated for 60 h with PHA (10 μg/ml) and MBP (25 μg/ml) and IFNγ production was quantified by a capture ELISA assay. For this experiment, 11 patients with active MS, 15 with stabilized MS, and 12 healthy controls were used. When the cells were stimulated *in vitro* with PHA, the mean IFNγ was 1205±1092 pg/ml and 1315±1060 pg/ml for stable and healthy controls, with 182±128 pg/ml and 498±390 pg/ml, without PHA, with no significant difference found ($P>0.05$). For patients with active disease, the mean was 2393±2148 pg/ml stimulated with PHA and 634±465 pg/ml without PHA, again with no significant difference from the control ($P>0.05$) (Figure 2a).

Stimulation with MBP resulted in a somewhat lowered IFNγ production for the stable patients in relation to the healthy controls (223±430 pg/ml stimulated with MBP and 182±128 without MBP *versus* 642±668 pg/ml and 489±390 pg/ml) respectively $P<0.03$; no differences in presence in the supernatants was found for patients with an active form of the disease and the healthy controls. For patients with stable disease the mean was (702±607 pg/ml stimulated with MBP and 634±465 pg/ml without MBP *versus* 642±668 pg/ml and 489±390 pg/ml, respectively; $P>0.05$ (Figure 2b).

IL-10 production by peripheral blood cells

Mononuclear cells from patients with active and stable MS and healthy controls were stimulated for 40 h with PHA or MBP, and IL-10 was quantified by a capture ELISA assay. For this part of the study, eight patients with active MS, 15 in remission, and 17 healthy controls were used. When the cells were stimulated by PHA *in vitro*, the mean IL-10 production was 1725±720 pg/ml *versus* 1701±950 pg/ml and 469±398 pg/ml *versus* 402±396 pg/ml without PHA for stable patients and healthy controls. There was no significant difference between the two groups ($P>0.05$). Mean IL-10 production by mononuclear cells from patients with active disease however, was 548±419 when stimulated with PHA (398±324 without stimulation), representing a significant decrease of the production of IL-10 in relation to healthy controls ($P=0.003$) (Figure 3a).

In vitro MBP stimulation of mononuclear cells led to a significant increase in mean IL-10 production

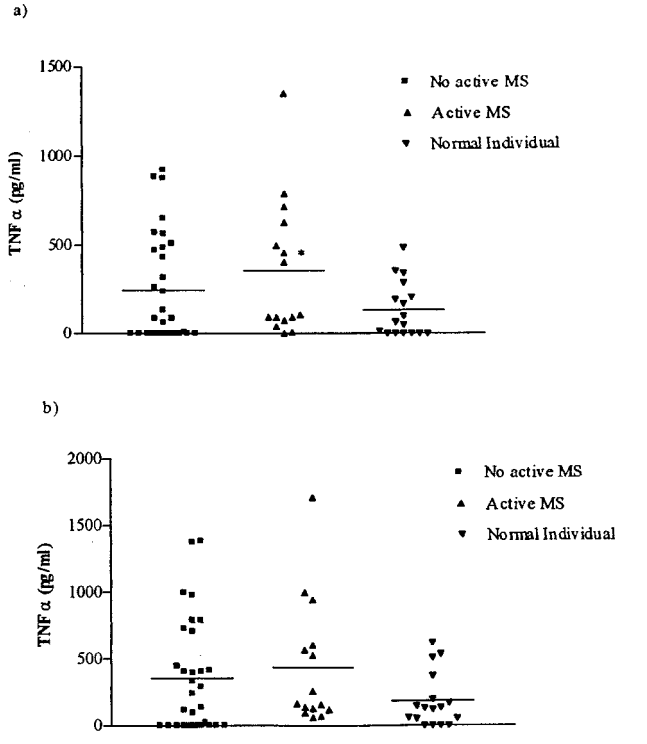

Figure 1 *Production of TNFα* by MS patients with active ▲ *or stable disease* ■ *and healthy controls* ▼. *After whole peripheral blood cell stimulation with LPS (100 ng/ml) for 2h* (***a***) *and PHA for 24 h (10 μg/ml)* (***b***), *TNFα concentration were detected by ELISA assay. Results corresponding to results from stimulated cells minus results for unstimulated cells. *(*$P<0.03$*)*

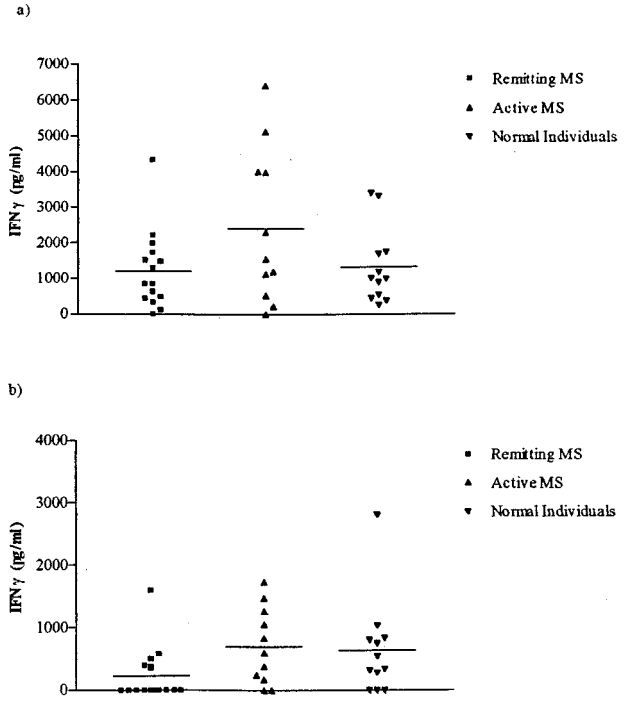

Figure 2 *Production of IFNγ by MS patients with active* ▲ *or stable disease* ■ *and healthy controls* ▼. *After lymphocyte stimulation* in vitro *with or PHA (10 μg/ml)* (***a***) *or MBP (25 μg/ml),* (***b***) *IFNγ were detected by ELISA in supernatants from 60 h culture. Results corresponding to results from stimulated cells. (*$P<0.05$*)*

1430 ± 627 pg/ml when stimulated with MBP and 469 ± 398 without MBP *versus* 385 ± 254 pg/ml and 402 ± 396 for stable MS and healthy controls, respectively; (*$P < 0.01$). No significant differences in the mononuclear cells subjected to *in vitro* MBP activation from active and the healthy controls were observed (482 ± 350 pg/ml stimulated with MBP and 398 ± 324 ng/ml without MBP *versus* 385 ± 254 and 402 ± 396 pg/ml, without MBP) respectively; ($P > 0.05$) (Figure 3b).

Production of TGFβ from mononuclear cells

Blood from seven patients with active MS, 15 with stable MS and 15 healthy controls were studied. *In vitro* PHA and MBP stimulation of cells for 72 h was followed by TGFβ quantification. The mean TGFβ production by mononuclear cells from patients with a stable form of the disease stimulated with PHA was 921 ± 1067 pg/ml (632 ± 543 pg/ml without PHA) *versus* 983 ± 967 pg/ml (253 ± 198 pg/ml without PHA), for the healthy control group. The production in the active disease group 699 ± 487 pg/ml (296 ± 298 pg/ml without PHA) *versus* 983 ± 967 pg/ml (253 ± 198 pg/ml without PHA) was somewhat lower, although the difference did not reach significant levels in statistical terms ($P > 0.05$) (Figure 4a).

Peripheral blood cells from stable patients stimulated with MBP produced significantly greater amounts of TGFβ (1700 ± 1461 pg/ml stimulated and 632 ± 543 pg/ml (unstimulated) *versus* 254 ± 264 pg/ml (stimulated) and 253 ± 198 pg/ml (unstimulated) (*$P < 0.002$) for stabilized and healthy controls, respectively. No significant differences in mononuclear cells subjected to *in vitro* MBP activation from active and the healthy controls were observed (201 ± 169 pg/ml (296 ± 298 without MBP) *versus* 254 ± 264 pg/ml (253 ± 198 pg/ml without MBP) respectively; $P > 0.05$ (Figure 4b).

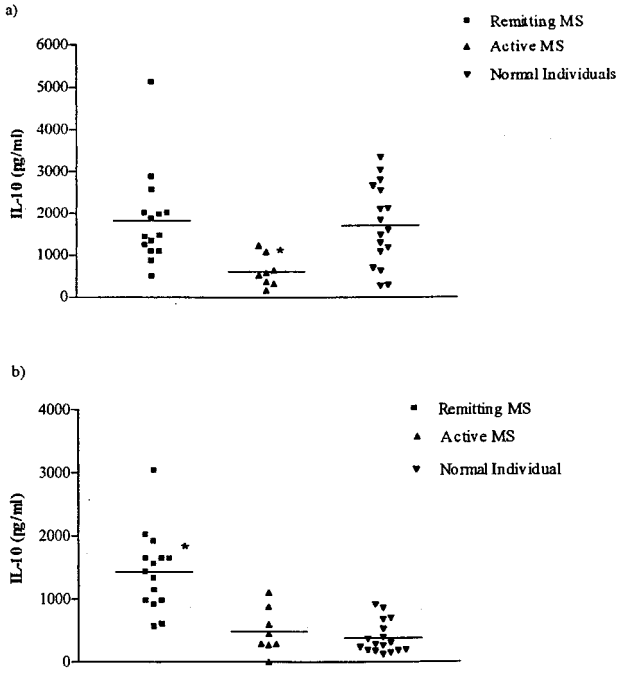

Figure 3 *Production of IL-10 by MS patients with active ▲ or stable disease ■ and healthy controls ▼. After lymphocyte stimulation with PHA (10 μg/ml (**a**) or MBP (25 μg/ml) (**b**), IL-10 levels were detected by ELISA in supernatants from 40 h culture. Results corresponding to results from stimulated cells. *(*P* < 0.05)*

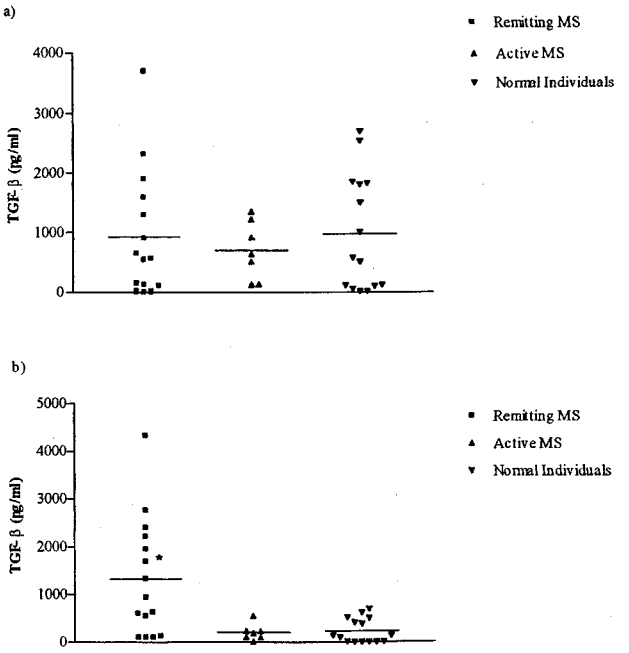

Figure 4 *Production of TGFβ by MS patients with active ▲ or stable disease ■ and healthy controls ▼. After lymphocyte stimulation with PHA (10 μg/ml (**a**) or MBP (25 μg/ml (**b**), TGFβ were detected by ELISA in supernatants from 72 h culture. Results corresponding to results from stimulated cells. *(*P* < 0.05)*

Discussion

These data show that changes in cytokine secretion patterns do occur in the peripheral blood cells of patients with active and stable MS. Whole leukocytes were used because, although Th1 and Th2 cells are the major sources of their respective cytokines, many other cells both within and outside of the immune system also produce such cytokines. Since various cell types can contribute to the overall Th1 and Th2 cytokine pattern, it has been suggested that these responses should possibly be described as type 1 and type 2 cell response. For example: subsets of $\gamma\delta$ T cells, CD4 and CD8 can secrete Th1 and Th2-like cytokine patterns. NK cells also produce IFNγ and TNFα and contribute to Th1-like responses,[19] moreover, IL-4 and possibly other Th2 cytokines are synthesized by mast cells, B cells, basophils and NK 1.1 cells.[20] Furthermore, IL-10 is produced by macrophages and B cells.[21]

Although many interesting type 1 or type 2 responses have been observed in autoimmune diseases, a careful consideration of anatomical location and the kinetics of a response can provide more definitive data. Moreover, many immune responses may remain localized so that sampling of peripheral blood lymphocytes may provide information about potential responses, whereas sampling of CSF or brain tissue represents an ongoing response. Peripheral blood was selected for investigation of the cytokines patterns here since MS involves both a localized immune response in the CNS and various immune abnormalities correlated with disease activity in the peripheral immune compartment.[22]

Proinflammatory cytokines such as TNFα and IFNγ are associated with disease activity in both MS patients and the experimental model of Experimental Autoimmune Encephalomyelitis (EAE).[6,7] Since TNFα is a major inducer of endothelial adhesion molecules and chemokines, it can be predicted that it will have a major effect on the recruitment of lymphocytes by the central nervous system.[23] A recent study describes the spontaneous development of a chronic inflammatory demyelinating disease in transgenic mice that constitutively have a dysregulate murine TNFα gene. Transgenic expression was restricted to the central nervous system, and the direct involvement of TNFα in the pathogenesis of the disease was confirmed by peripheral administration of neutralizing murine TNFα antibodies.[24] The present study showed that *in vitro* LPS stimulation of peripheral blood cells from patients with active MS led to a moderate increase in TNFα production; this is in agreement with several authors who revealed an increase in TNFα in patients with active MS.[23,24] The stimulation of lymphocytes using PHA, however, did not induce a significant difference in TNFα production for the individuals in the groups studied.

No increase in IFNγ production in the active form of the disease was induced by MBP stimulation nor by polyclonal activation with PHA. These results are in contrast to previous studies,[25,26] where the authors found an increase in the levels of this cytokine. On the other hand, the data found here are in agreement with previous observations which found no significant increase in IFNγ levels during the active phase of disease. There was, indeed, a significant reduction in level of IFNγ observed in patients with a stable form of the disease when the cells were stimulated with MBP. Until recently, observations pointed to a critical role for IFNγ in the pathogenesis of MS and in the EAE model, although recent data using a variety of systemic and CNS-localized manipulations of IFNγ production have led to the questioning of the overall importance of this cytokine in disease initiation and progression. A number of investigations have shown that systemic administration of neutralizing IFNγ antibodies leads to exacerbation of the disease in both susceptible and resistant strains of mice, rather than the amelioration of clinical signs.[27,28] In addition, the *in vivo* administration of IFNγ has been found to decrease the severity of EAE in murines. Further support for this protective role of IFNγ during the pathogenesis of EAE has come from studies showing that mice with disrupted IFNγ gene are susceptible to the induction of the disease.[29]

The characteristic cytokine products of Th1 and Th2 are mutually inhibitory for the differentiation and effector function of the reciprocal phenotype. IFNγ has been shown to prevent Th2 cell proliferation, whereas IL-10 profoundly inhibits the synthesis of Th1 cytokines.[30–32] TGFβ has been described as a Th3 cytokine[33] and its effects on suppressing EAE have also been demonstrated.[10] The present study has shown that in the active phase of the disease there is a decrease in the level of IL-10 when cells are stimulated with PHA, whereas no differences in TGFβ levels were found, despite previous suggestions to the contrary published in the literature. Patients with stable forms of the disease revealed no differences in the production of IL-10 and TGFβ when stimulated under similar conditions. Patients with stable forms of the disease, however, were found to reveal increases in the production of IL-10 and TGFβ when stimulated with MBP.[34]

Myelin basic protein seems to have stimulated the production of anti-inflammatory cytokines in the cells from patients with stable MS; the production of anti-inflammatory cytokines by T cell clones from MS patients sensitized to PLP has also been observed[34,35] The mechanisms for stimulation of the increased production of anti-inflammatory cytokines while inhibiting that of pro-inflammatory ones by neuroantigens such as MBP are not well understood. In the Lewis rat model, administration of myelin basic protein has been shown to be an effective means of suppressing EAE, an effect mediated by the TGFβ, secreted by T-cells from orally tolerized animals after being triggered by the oral tolerogen. We previously demonstrated that suppressor determinants exist on the MBP molecule, since T cells from animals fed with 21–40 peptide secreted TGFβ, whereas no TGFβ was released in animals fed with MBP encephalitogenic determinant (71–90).[36] It is possible that suppressive determinants present on the MBP molecule stimulate the production of anti-inflammatory cytokines (IL-10 and TGFβ) by MBP-specific T cells from patients with stable MS. The increased levels of anti-inflammatory cytokines, on the other hand, may inhibit the production of pro-inflammatory cytokines such as IFNγ, as seen in this study.

The results of stimulation of the cells of patients with the active form of the disease suggest a reduction in clones producing anti-inflammatory cytokines in the peripheral blood since even stimulation with polyclonal mitogens such as PHA was not capable of simulating the production of significant levels of these cytokines.

Thus, in autoimmune diseases, type 1 and 2 cytokines may polarize forms of the specific immune response that result from the combined action of genetic and environmental conditions. Consequently, inhibiting mechanisms such as the production of anti-

inflammatory cytokines can be used to interrupt the progression of events leading to chronic inflammation thus, seems to be a promising way of treating organ-specific autoimmune diseases such as MS.

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