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Activity profile in multiple sclerosis: an integrative approach A preliminary report

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In order to define an activity profile in patients with multiple sclerosis (MS), T-cell subpopulations and proliferative responses to myelin basic protein (MBP) associated with anti-MBP antibodies, nitrotyrosine levels in serum and cerebrospinal fluid (CSF), and serum CD40L (sCD154) were simultaneously assessed in 29 consecutive and untreated MS patients. When compared to controls, patients in secondary progressive stable (SP/I), or in full remission (RR/I) stages, individuals with secondary progressive active disease (SP/A) or in acute relapse (RR/A) showed a significant decrease of CD4/CD45RA$^+$ T cells associated with an increase of absolute numbers of CD4/45RO$^+$ T cells ($p<0.001$). In addition, in vitro-specific T-cell proliferative responses against MBP (SP/A, RR/A, SP/I: $p<0.001$ versus controls) in association with augmented sCD154 serum levels (SP/A, RR/A, versus controls $p<0.001$) and a significant increase of both CSF and serum levels of anti-MBP antibodies and nitrotyrosine levels ($p<0.001$) were also found. Thus, the simultaneous evaluation of antibody and cell-mediated immunopathological parameters, along with the effector mediators of inflammation such as the nitric oxide products, offers a new integrative approach to characterize markers of clinical activity in MS patients, which may be used at the moment of the initial diagnosis and during an apparent recurrences of the disease to monitor therapeutic protocols and to determine whether immune-based nerve destruction mechanisms are still operating in patients with few clinical findings.
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Key words: *cerebrospinal fluid; multiple sclerosis; myelin basic protein; nitric oxide; sCD40L*

Introduction

Multiple sclerosis (MS) is a demyelinating inflammatory disease of the central nervous system (CNS) frequently found among young adults.[1–3] Even though the etiology of MS remains elusive, both experimental and immunopathological data suggest a genetically coded susceptibility to develop an organ-specific autoimmune process, which leads to the progressive destruction of the myelin-furnished structures of the CNS.[4–9] The immunoregulatory defect seen in MS is characterized at the cellular level by a rapid migration of activated T lymphocytes from the peripheral blood to the CNS, inducing acute demyelination with a focal predominance of macrophages and CD4/CD45RO$^+$ lymphocytes and to a lesser degree CD4/CD45RA$^+$ cells.[10–13] In the fluid phase, an increase of IgG, IgA, IgM, kappa, and lambda chains synthesis in cerebrospinal fluid (CSF) has been associated with typical oligoclonal bands (OB).[14] In periods of clinical activity or during recurrence or flare of the disease, conflicting data have reported diminished values of peripheral blood and CSF CD4/CD45RA$^+$ lymphocytes[15–20] and increased levels of antibodies against myelin basic protein (MBP), proteolipid protein (PLP), and myelin-associated glycoprotein (MOG) in both CSF and serum.[21–27] This, in turn, may allow autoantibodies and complement access to the CNS to initiate demyelination.[28] More recent findings suggest a hyperactive response of CD4/CD45R0$^+$ memory T cells to myelin components.[29,30] At the effector inflammatory level, nitric oxide (NO) and its metabolic products have been implicated as mediators of the final phase of the myelin damage seen in both MS and experimental acute encephalomyelitis (EAE).[31–34] In addition, the CD40–CD40L (CD154) ligand pair has been shown to be important in EAE and MS induction. Blockade of this interaction using anti-CD154 antibody may inhibit the early events of EAE.[35–39] In a prospective research protocol, we have simultaneously evaluated antibody and cell-mediated MBP-specific immune responses, NO, and soluble CD40L (sCD40L) values with the purpose of integrating markers, allowing us to define an immunoclinical activity profile applicable in the assessment of the different MS clinical stages.

Materials and methods

Patients and controls

Twenty-nine untreated MS patients were evaluated in whom the diagnosis was established according to the Poser criteria.[40] Eighteen women and 11 men with age range 15–55 years (mean 36 years), visiting the Neurological Departments of Luis Razetti and José Mara Vargas Schools of Medicine, were selected for the research protocol with the previous approval of the Ethics Committee of the Institute of Immunology. Magnetic resonance imaging (MRI) of the brain was performed in all patients. CSF and sera

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were evaluated to detect OB using the isoelectric focusing (IEF) method with silver dye.[41] Activity of the disease was classified following Lublin and Reingold.[42] Eight secondary progressive patients were active (SPA), 10 patients of the remission/relapse group were in acute relapse (RR/A) – defined by the appearance of new neurological manifestations within the previous 4 weeks – four secondary progressive individuals were stable (SP/I) and seven patients were in full remission (RR/I). The control group consisted of 20 blood donors (12 men and 8 women) from the Blood Bank of the Central University Hospital (Caracas) with average ages of 33±11 years. CF samples were obtained from 14 MS patients and from patients (non-MS group) with brain injury (n=3), retinoblastomas (n=2), meningitis (n=3), idiopathic polyneuropathy (n=3) and acute lymphocytic leukemia (n=4).

All patients were negative for HTLV-I (ELISA; Abbott) and HIV (Virinostika, the Netherlands) antibodies.

Antigens

MBP was obtained following the technique of Deibler *et al*[43] with minor modifications. Briefly, human brain tissue, kindly donated by the Neuropathology Section of the Pathology Institute, was obtained from individuals less than 6 h after death. The specimens were minced as previously described.[43] A second purification process was done through a separation technique in a Mini-Prep-Cell apparatus (Bio-Rad Life Science Products, CA). The purity was confirmed by polyacrylamide gel electrophoresis using sodium dodecyl sulfate (SDS-PAGE) at 12.5% and Western blot analysis employing rabbit anti-human MBP monoclonal antibody from Dako (CA) (revealed by autoradiography with luminol, using an anti-rabbit peroxidase antibody from Sigma, St. Louis, MO). A single 18.5-kDa band corresponding to human MBP was demonstrated. *Candida albicans* (CA) and tetanus toxoid (TT) used in lymphocyte proliferation assays as recall antigens were kindly donated by the Biomedicine Institute and by the Venezuelan National Institute of Health. Phytohaemagglutinin (PHA) was purchased from Welcome Diagnostic (Darford, England, UK).

Anti-MBP antibodies

Anti-MBP levels were determined by a solid phase radioimmunoassay (RIA) as described by Warren *et al*.[26] Isolated MBP was adsorbed to RIA tubes (Falcon; Becton Dickinson, CA) using 0.5 μg/ml in carbonate–bicarbonate buffer, pH 9.6, incubated at 37°C for 1 h and overnight at 4°C. The plates were washed with phosphate-buffered saline (PSB)–Tween 20, 0.05%, pH 7.2, blocked with bovine serum albumin (BSA) at 1% in PBS for 1 h at 37°C and rinsed six times with PBS–Tween 20. One hundred microliters of patient and control (rabbit antibody against human MBP; Dako) serum diluted at 1/500 in PBS was added and incubated at 37°C during 1 h followed by six rinses with adequate buffer. One hundred micrograms of conjugate [^{125}I] goat anti-human IgG (1/500) (New England Nuclear®; Life Science Products, Boston, MA) was added to each well and incubated at 37°C for 1 h; after being rinsed with PBS–Tween 20, the tubes were counted in a Compugamma 1282 scintillation counter (LKB, Wallac, Finland). The results were calculated as: (sample counts–target counts)/(total counts–target counts) and expressed in radioactivity units.

Nitrotyrosine detection

The total amount of nitrotyrosine was determined by a standard sandwich ELISA assay as described by Ye *et al*.[44] Mouse IgG monoclonal antibodies for capturing the modified amino acid, polyclonal IgG against nitrotyrosine, and polyclonal goat anti-rabbit IgG peroxidase were obtained from Upstate Biotechnology (Lake Placid, NY). As previously described by Zabaleta *et al*[45] from our laboratory, nitrotyrosine was quantified using a standard curve with known nitrotyrosine concentrations from chemically modified and BSA. The inter- and intratest variation coefficients were 8% and 11%, respectively. Results are expressed as nanograms per milliliter.

Cellular preparations

Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors and MS patients.[46] Cells were isolated from heparinized venous blood samples by Ficoll-Hypaque gradients (Pharmacia, Uppsala, Sweden). The mononuclear cells fraction was washed three times in PBS, pH 7.4, and resuspended in RPMI complete medium 1640 (Gibco, Grand Island, NY) supplement with 2 mmol/ml L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% of fetal bovine serum (FBS).

Soluble CD40 ligand (sCD154)

sCD154 levels were assessed by a two-step quantitative ELISA as indicated by the manufacturer (Chemicon International, CA). Briefly, microwells were sensitized with mouse anti-human sCD154; sCD40L in serum or standard was added to the plates allowing binding to the anti-CD40L. Finally, an HRP-conjugated monoclonal anti-sCD40L antibody was used as a secondary antibody. A standard curve with less than 5% variation coefficient was set using recombinant CD154. The results are expressed in nanograms per milliliter.

Immunophenotypic analysis

Patient and control PBMC were separated by Ficoll-Hypaque gradients and diluted to a concentration of 1×10^6 cells/ml. Then, the PBMC were mixed with different surface markers for one/two color flow cytometry (Epics Elite; Coulter Electronics, Hialech). The monoclonal antibodies panel included: CD3, CD4, CD8, CD19, CD16, and CD56 (Coulter Electronics) and CD45RA and CD45RO (Dako, Denmark). After 45 min of incubation at 4°C, three washes were done with PBS–azide 0.1%, pH 7.2, for 10 min and resuspended in 0.5 ml of PBS for reading on the flow cytometer. The results were expressed in cell counts×mm^3.

Proliferation assays

T-cell *in vitro* proliferative responses were performed as previously reported from our laboratory.[47] Briefly, cells were precultured in complete medium in 200-μl volumes at concentration of 1×10^6 cells/ml in flat-bottomed 96-well microculture plates (Falcon 3872; Becton Dickinson) and incubated for 18 h at 37°C and 5% CO_2 atmosphere. Then,

Table 1 1) RR/A: relapsing–remitting active; 2) SP/A: secondary progressive active; 3) SP/I: secondary progressive inactive; 4) RR/I: relapsing–remitting inactive

Patients	*Age (years)*	*Sex*	*Dx (years)*	*Phase*	*OB*	*MRI*
MS1	30	M	2	RR/A	+	D
MS2	40	F	2	RR/A	+	D
MS3	39	M	11	RR/A	+	D
MS4	41	F	10	RR/A	+	D
MS5	31	F	4	RR/A	+	D
MS6	41	F	7	RR/A	+	D
MS7	16	F	6	RR/A	+	D
MS8	38	F	12	RR/A	+	D
MS9	14	F	2	RR/A	+	D
MS10	15	F	2	RR/A	+	D
MS11	55	M	18	SP/A	+	D
MS12	42	F	8	SP/A	+	D
MS13	51	M	9	SP/A	+	D
MS14	32	M	5	SP/A	+	D
MS15	53	M	10	SP/A	+	D
MS16	49	F	6	SP/A	+	D
MS17	40	M	8	SP/A	+	D
MS18	28	M	9	SP/A	+	D
MS19	29	F	14	SP/I	+	D
MS20	50	M	20	SP/I	+	D
MS21	49	M	15	SP/I	+	D
MS22	38	F	11	SP/I	+	D
MS23	41	F	5	RR/I	+	D
MS24	41	M	4	RR/I	+	D
MS25	32	F	6	RR/I	+	D
MS26	25	F	5	RR/I	+	D
MS27	25	M	5	RR/I	+	D
MS28	20	F	6	RR/I	+	D
MS29	40	F	8	RR/I	+	D

OB: CSF oligoclonal band; MRI: magnetic resonance imaging, MS: multiple sclerosis, D: demyelination.

the precultured cells were centrifuged at 1800 rpm, the supernatant was eliminated, and fresh medium was added. Predetermined optimal doses of antigens, MBP (10 μg/ml), TT (0.625 μg/ml), CA (85 μg/ml), and PHA (1 μg/ml) were established. For each combination, all cultures were performed in triplicate and incubated at 37°C in a CO_2 atmosphere for 7 days. PHA was used for cultures of 72 h.

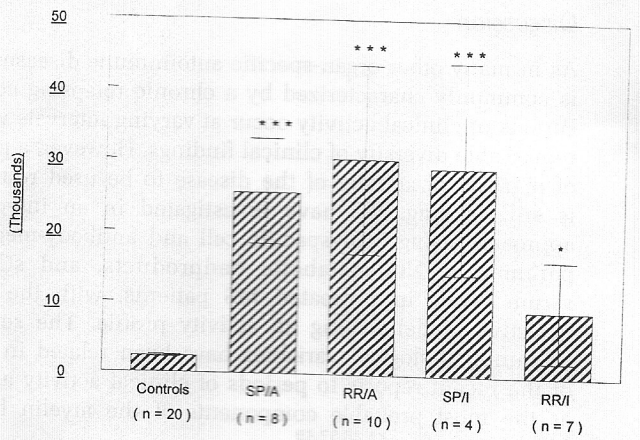

Figure 1 *Serum anti-MBP antibodies in MS patients and control sera. Data are presented as mean±SD. Radioactivity units (ru). ***p<0.0001, *p<0.01 (ANOVA)*

Cultures were pulsed with 1 μCi of [^{3}H]thymidine (Amersham Life Sciences, IL) 18 h before the end of incubation. Cells were harvested on glass fiber filter and thymidine incorporation was measured in a liquid scintillation counter (1205 Betaplate; LKB, Wallac, Finland). The results were expressed using a Stimulation Index (SI=cpm in the well with antigen/cpm of wells without antigen). Responses were scored positive when SI values were above 2 (higher than 1000 cpm).

Statistical analysis

The results are expressed as the mean (X)±standard deviation (SD); the intergroup statistical comparisons were done through the ANOVA test. Statistical significance of data was determined using the unpaired two-tailed Student's t-test. p values <0.05 were considered statistically significant. The frequencies of anti-MBP versus serum nitrotyrosine levels between individual patients in clinical activity were correlated using Pearson's lineal regression analysis.

Results

Clinical staging

The clinical staging of the MS patients is depicted in Table 1. Most of the patients were in either in RR/A or SP/A stage. The duration of the disease was greater in patients with a progressive pattern (11±5 years) than those showing relapse/remission course (6±3 years). OB and altered MRI were observed in all patients.

Serum and CSF anti-MBP antibodies

Significantly elevated serum anti-MBP antibodies levels were found in the four groups of MS patients when compared to controls (SP/A: 25 552±7238; RR/A: 30 291±13 260; SP/I: 29 169±14 843; RR/A: 9257±7142; controls: 2292±160, p<0.001) (Figure 1). In addition, SP/A, RR/A, and SP/I levels were also statistically significantly different to those of RR/I patients (Figure 1). In relation to CSF anti-MBP

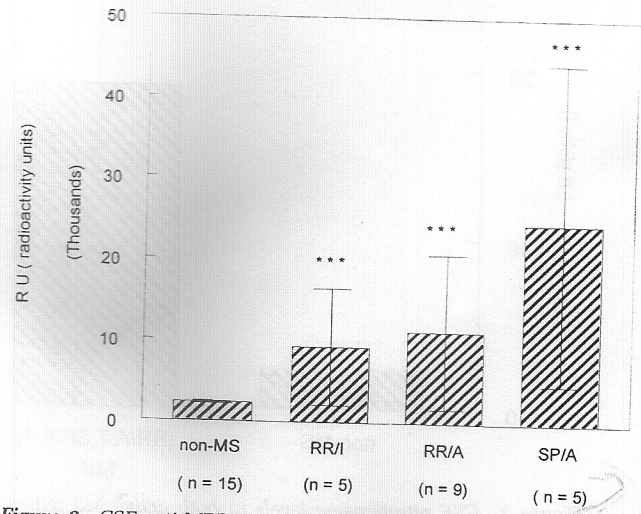

Figure 2 *CSF anti-MBP antibodies in both MS and non-MS groups. The values are presented as radioactivity units (cpm sample–cpm blank)/(cpm total–cpm blank). ***p<0.0001*

antibodies, significantly elevated levels were only demonstrated in SP/A and RR/A individuals when compared to RR/I and non-MS group (SP/A: 24586±19767 versus non-MS group: 3974±2422, $p<0.0003$; RR/A: 11285±9508 versus non-MS group, $p<0.01$) (Figure 2).

Serum and CSF nitrotyrosine levels
All MS patients exhibited significantly elevated nitrotyrosine serum levels when compared to controls (RR/A: 123.3±55.7; SP/A: 155.6±18.3; SP/I: 117±92; RR/I: 91.7±25.7 versus controls: 20±8 ng/ml; $p<0.001$). The difference between SP/A and RR/A versus RR/I patients was also significant ($p<0.001$). Furthermore, nitrotyrosine in CSF of clinically active MS patients (RR/A: 7 and SP/A: 1) showed increased levels when compared to the non-MS group (Figure 3). A positive correlation between serum anti-MBP antibodies and nitrotyrosine levels was also found in patients with active disease (SP/A and RR/A; $n=12$, $r=0.71$, $p<0.05$) (Figure 4).

T-lymphocyte subpopulations As depicted in Table 2, $CD4^+/CD45RA^+$ naive T cells showed a significant decrease in SP/A (332±293, $p=0.007$), RR/A (231±200, $p=0.0006$), and SP/I (353±227, $p=0.04$) patients when compared to controls (759426), RR/I (782±393) versus RR/A, $p<0.0001$; RR/I versus SP/A, $p=0.02$. In contrast, $CD4^+/CD45RO^+$ memory T cells showed a significant increase in SP/A (1182±78, $p<0.001$) and RR/A (1096±52, $p<0.001$) in MS patients when compared to controls (998±37).

CD154 (sCD40L) serum levels As demonstrated in Figure 5, serum sCD154 levels ($n=15$) were significantly augmented in SP/A (3.69±2.02, $p<0.001$) and RR/A (5.65±2.87, $p<0.001$) when compared to SP/I (0.76±0.41), RR/I (0.64±0.30), or controls (0.14±0.12 ng/ml).

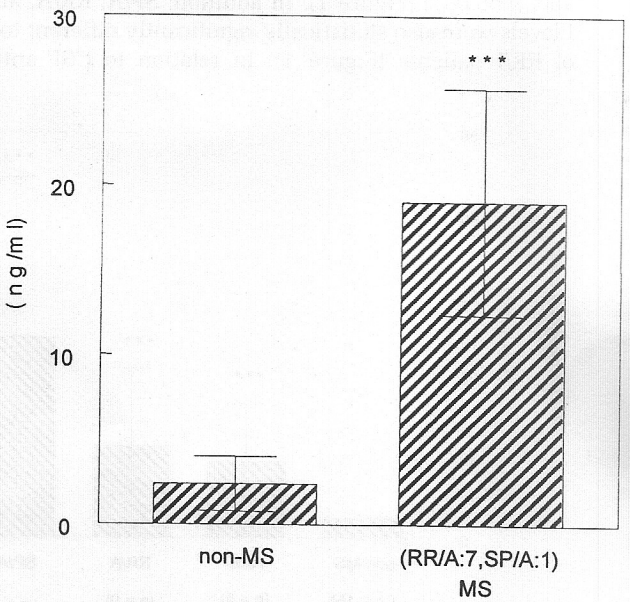

Figure 3 *CSF nitrotyrosine levels in both active MS patients and non-MS individuals. Data are presented as nanograms per milliliter. ***$p<0.0001$ (ANOVA)*

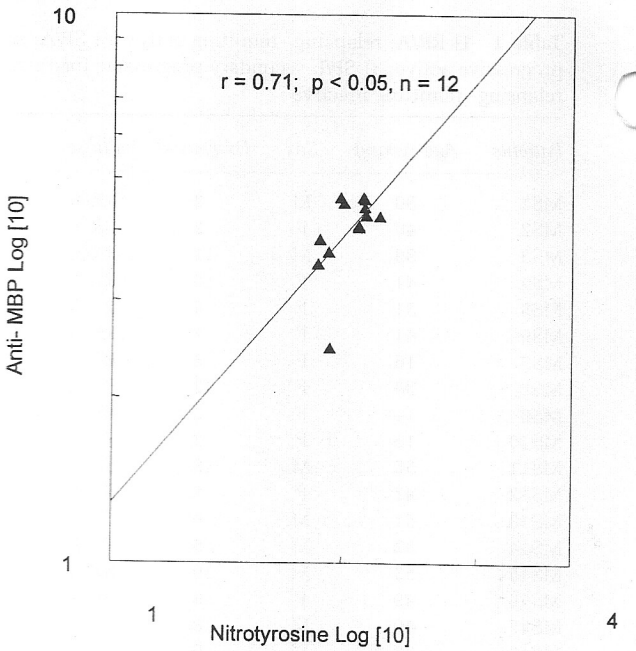

Figure 4 *Positive correlation (linear regression analysis) between the logarithm of anti-MBP antibodies versus the logarithm of nitrotyrosine serum levels among individuals with clinically active MS*

MBP T-cells proliferative responses
A hyperactive specific T-cell proliferative response to isolated human MBP was encountered in the MS groups as observed in Figure 6. T cells from RR/A patients exhibited the highest proliferation against MBP when compared no only to controls but also to RR/I patients (RR/A: 83.24±2.4, $p<0.0001$; PS/A: 27±5, $p<0.05$; PS/I: 29±2.5, $p<0.05$; RR/I: 16.3±3.3, $p<0.001$ versus controls: 10.3±1.73). The hyperactive T cells *in vitro* proliferation when tested against recall antigens were also remarkable in RR/A MS patients when compared to controls (TT: 89.08±7.4 versus 19.3±8.7, $p<0.001$; CA: 153±11.6 versus 33.08±7.3, $p<0.001$). Finally, the same significant difference was encountered upon PHA polyclonal stimulation (RR/A: 281.9±145.05 versus controls: 42.23±19, $p<0.05$).

Discussion

As in many other organ-specific autoimmune diseases, MS is commonly characterized by a chronic relapsing course. Periods of clinical activity occur at varying intervals with a remarkable diversity of clinical findings. However, a profile of markers of activity of the disease to be used routinely is still lacking. We have investigated in an integrated approach, using MBP-specific cell and antibody-mediated parameters, NO metabolic endproducts, and sCD154 serum levels in untreated MS patients, with the main objective of delineating an activity profile. The selected immunopathological variables have been related in some of the recent reports to periods of clinical activity as well as the most probable components of the myelin lesion-inducing factors.[25,28,37,48]

Thus, in our group of MS patients, the significant and simultaneous demonstration that increased hyperactive

Table 2 T-cell subpopulations

Subset	*SP/A (n=8)* *Cell×mm^{3a}*	%	*RR/A (n=10)* *Cell×mm^{3a}*	%	*SP/I (n=4)* *Cell×mm^{3a}*	%	*RR/I (n=7)* *Cell×mm^{3a}*	%	*Controls (n=20)* *Cell×mm^{3a}*	%
$CD3^+$	2791±1507	(74±7)	2076±97	(70±13)	1127±541	(80±7)	1879±799	(68±13)	2237±612	(72±7)
$CD3^+/CD4^+$	1469±0	(42±12)	1363±620	(44±7)	716±43	(44±6)	1349±84	(39±14)	1335±50	(43±8)
$CD3^+/CD8^+$	713±367	(27±12)	882±443	(27±8)	750±165	(32±16)	962±474	(25±7)	899±326	(29±8)
$CD4^+/CD45RO^+$	1324±78	(32±18)	1098±52	(35±9)	860±365	(23±19)	813±302	(18±5)	998±37	(31±9)
$CD4^+/CD45RA^+$	332±293	(11±9)	231±200	(7±6)	353±227	(15±4)	782±393	(19±7)	759±426	(25±11)
$CD16^+/CD56^+$	373±385	(9±7)	274±290	(7±7)	717±984	(6±5)	487±285	(3±9)	400±250	(10±14)
$CD19^+$	343±219	(11±5)	248±215	(12±4)	159±22	(8±5)	593±444	(14±6)	207±400	(0.2±0.4)

[a]Mean±SD and percentages (%).

MBP-specific memory $CD4/CD45RO^+$ T cells was associated with diminished $CD4/CD45RA^+$ absolute values and, with significantly elevated levels of both CSF/serum anti-MBP antibodies, nitrotyrosine, and serum CD40L (sCD154), allowed us to distinguish patients with active disease or else during flare-up episodes from those individuals in full clinical remission. Remarkably, the data obtained in our four SP/I patients revealed that in spite of the presence of very few clinical findings, the underlying immunopathological autoimmune process may still be actively operating.

As previously stated, the components of the suggested activity profile seem to be intimately related to demyelination, axonal degeneration, and the progressive impaired nerve function (recently reviewed in Ref. [3]). MBP-specific Th1 memory cells and demyelinating autoantibodies are two of the most powerful effector mechanisms (direct and/or macrophage-mediated T-cell cytotoxicity, lysis due to complement activation, and antibody-dependent cell cytotoxicity) mediating the CNS dysfunction typically seen in MS patients.

In addition, NO metabolic products may act at the inflammatory end of demyelination since their myelotoxic potential has been reported.[31–34,49] Furthermore, a temporary blockade of axonal conduction has also been attributed to NO. In a preliminary report from our laboratory, serum nitrotyrosine levels in MS active patients were sixfold higher in comparison to controls.[46] This increased production suggests that oxygen radicals such as superoxide may also be involved in nerve damage. Thus, the NO metabolic endproduct effect on nerve function may partly underlie the symptoms found in SP/A and RR/A MS patients.

Moreover, we report for the first time the striking correlation between serum anti-MBP-specific antibodies and nitrotyrosine levels, which further supports the concept of the integration of both specific and inflammatory effector mechanisms not only in the genesis of nerve lesions but in expression of activity of the disease.

Recently, $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ lymphocytes bearing CD40–CD40L have been found in active MS and EAE lesions.[37–39] Furthermore, in the animal model, treatment with monoclonals against CD40L effectively blocks not only the development of EAE but IL-12 production, while CD40L-deficient mice expressing MBP-specific TCR seem unable to develop EAE.

In addition, Balashov *et al*[37] showed increased expression of CD40L in activated $CD4^+$ cells in progressive MS, and more recently, Huang *et al*[39] reported elevated mRNA expression levels of both CD40/CD40L in non-stimulated MS PBMCs. In our MS patients, only those clinically active patients showed significantly elevated sCD154 levels, adding new evidence of the possible role of CD40 and its ligand in MS immunopathology and their usefulness as an activity marker. From the technical and standardization standpoint, we anticipate no difficulties in the different procedures involved in measuring antibody and cell-mediated

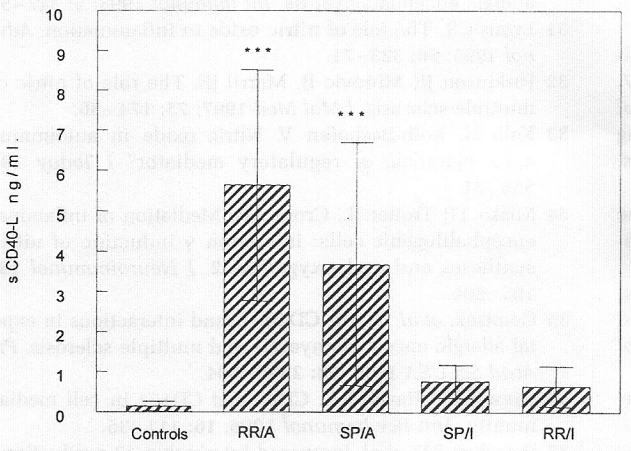

Figure 5 *Serum sCD40L in patients and controls. Data are presented as nanograms per milliliter in RR/A (n=4), SP/A (n=4), SP/I (n=3), RR/I (n=4), and control (n=20) groups. ***p<0.0001 (ANOVA)*

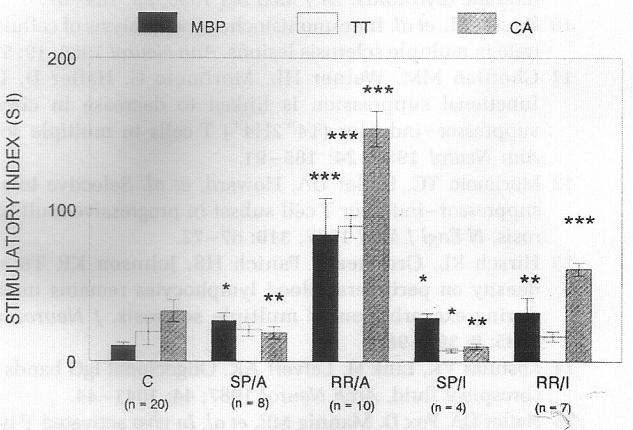

Figure 6 *MBP, recall antigens T-cell proliferative responses in both MS and controls. Results are presented in Stimulatory Index. *p<0.05, **p<0.00, ***p<0.0001 (ANOVA)*

anti-MBP responses or in the assessment of nitrotyrosine and sCD154 levels.

Thus, we suggest that the simultaneous evaluation of these four parameters would allow one to clearly determine the presence of clinical activity at the moment of initial diagnosis or during a recurrence of the disease and also help in the follow-up of patients under therapy. In addition, the findings in SP/I patients suggest not only an ongoing immunopathological process but also the need to reevaluate whether therapy should be kept in place until full immunoclinical remission is achieved.

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