Peripheral blood leukocyte NO production in MS patients with a benign vs progressive course

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Abstract—*Background:* Nitric oxide (NO) may play a role in tissue destruction and axonal degeneration in multiple sclerosis (MS). *Objective:* To investigate NO production by peripheral blood leukocytes (PBL) in patients with a benign and progressive course of MS. *Methods:* PBL were isolated from 25 patients with a benign course of MS (BMS), 33 with secondary progressive MS (SPMS), 21 with primary progressive MS (PPMS), and 29 healthy individuals. Leukocyte supernatants were assayed for nitrite concentration, which is an index of NO generation, using the Griess reaction. Serum levels of tumor necrosis factor (TNF)α and interleukin (IL)-12 were measured using ELISA. *Results:* Compared to healthy controls, nitrite concentrations were higher in patients with BMS ($p < 0.001$), SPMS ($p < 0.001$), and PPMS ($p < 0.05$). There were no significant differences among the three clinical subgroups of MS. There was a correlation between nitrite concentrations and serum levels of IL-12 ($p = 0.04$), but not of TNFα. *Conclusion:* Increased NO production by PBL in patients with MS is independent of the disease course.

NEUROLOGY 2004;62:239–242

Nitric oxide (NO) has been implicated in the pathogenesis of immune-mediated inflammation of the CNS in multiple sclerosis (MS).[1,2] Reactive astrocytes and microglia in MS plaques express high levels of inducible NO synthase (iNOS).[3-5] NO is thought to play a role in demyelination, oligodendrocyte destruction, and the functional and structural injury of axons.[1,2] NO is an unstable molecule and rapidly converted in vivo into its metabolites nitrite and nitrate. Levels of nitrate and nitrite can been used as an index of NO generation. NO metabolite concentrations are increased in the CSF of patients with MS, and seem to correlate with disease activity.[6-10]

Increased levels of NO metabolites have also been measured in serum of patients with MS.[6,11-14] It is believed that elevated concentrations of NO metabolites in serum are derived from the CNS.[2] However, two studies involving a small number of patients found increased NO production by peripheral blood mononuclear cells[15] and peripheral blood leukocytes (PBL) in patients with MS.[16] This suggests that the increased concentrations of NO metabolites in serum of patients with MS may be derived from PBL rather than from cells in the CNS. The significance of the NO production by PBL in patients with MS is unclear. Because axonal pathology is not limited to demyelinated lesions, but also extends into normal-appearing white matter (NAWM),[17,18] the questions arises whether NO produced by PBL might contribute to the widespread axonal degeneration that characterizes disease progression in MS.

The aim of the current study was to investigate PBL NO production in patients with a benign course of MS (BMS) lacking progression, secondary progressive MS (SPMS), and primary progressive MS (PPMS). We used PBL because both mononuclear cells and neutrophils can produce NO.[15,19] We also determined serum levels of interleukin (IL)-12 and tumor necrosis factor alfa (TNFα), which can trigger the transcription of iNOS mRNA.[20,21]

Methods. *Patients.* The study was approved by the medical ethics committee of the University Hospital Groningen. All patients gave their informed consent before inclusion in the study. Venous blood was obtained from 29 healthy controls, 25 patients with BMS, 33 with SPMS, and 21 with PPMS. BMS was defined as an Expanded Disability Status Scale (EDSS) score of 3 or less, despite at least 10 years of disease duration,[22] and without disease progression. Patients with SPMS had switched from a relapsing-remitting disorder to a progressive downhill course that could still be accompanied by some overlapping relapses. The term PPMS was used for patients in whom the disease was characterized by a progressive course from the onset, without superimposed relapses. Exclusion criteria were the presence of infections, fever, or a relapse, and the use of corticosteroids within the past 3 months. MRI with gadolinium administration to exclude subclinical active disease was not performed. Fifteen patients were using interferon β. No other immunomodulatory or immunosuppressive drugs were used by any of the patients. Demographic data are given in the table.

Leukocyte NO production. Samples of blood were drawn into 10-mL heparinized tubes through an IV cannula in the forearm. Red cells were allowed to sediment in 1 mL dextran 5% (Pharma-

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Supported by Multiple Sclerosis International (MSI), Amsterdam (the Netherlands).

Received August 4, 2003. Accepted in final form September 24, 2003.

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Table *Subject characteristics*

Characteristics	BMS, n = 25	SPMS, n = 33	PPMS, n = 21	Healthy controls, n = 29
Sex, F/M	16/9	21/12	14/7	15/14
Age, y, mean ± SD	49.7 ± 9.7	49.4 ± 9.3	51.0 ± 8.5	44.1 ± 10.2
Disease duration, y, mean ± SD	22.0 ± 8.1	19.6 ± 8.3	12.2 ± 5.1	—
EDSS, median (range)	2.0 (0.0–3.0)	7.0 (4.0–8.5)	6.0 (4.0–8.5)	—
Nitrite, nmol/mg protein, mean ± SEM	12.48 ± 1.18*	12.17 ± 0.92*	10.23 ± 1.06†	5.94 ± 0.58
IL-12, pg/mL, mean ± SEM	17.20 ± 2.06	17.62 ± 1.76	20.54 ± 2.88	16.78 ± 1.38
TNFα, pg/mL, mean ± SEM	2.60 ± 0.16‡	3.03 ± 0.21	3.00 ± 0.22	3.41 ± 0.19

* $p < 0.001$, † $p < 0.01$, ‡ $p < 0.05$ vs healthy controls.

BMS = benign multiple sclerosis; SPMS = secondary progressive multiple sclerosis; PPMS = primary progressive multiple sclerosis; EDSS = Expanded Disability Status Scale score; IL = interleukin; TNF = tumor necrosis factor.

cia, Uppsala, Sweden) for 90 minutes at room temperature. The leukocyte-rich upper layer was removed and leukocytes were isolated according to the method of Percy and Brady.[23] The final suspension of leukocytes was mixed with 2 mL of aqua bidest, and the lysated leukocytes were kept at −20 °C until used. Protein content was measured by the method of Lowry et al.[24] Nitrite levels in leukocyte supernatants were quantified by a spectrophotometric assay based on the Griess reaction.[25] In this system, nitrite ions react with 1% sulfanilamide in 5% orthophosphoric acid/0.1% N-1 naphthylethylenediamine dihydrochloride to yield an azochromophore. Absorbance was measured at 540 nm.

Serum TNFα and IL-12. Serum specimens were assayed for concentrations of TNFα and IL-12 using ELISA kits according to the manufacturer's instructions (R&D Systems Inc., Minneapolis, MN).

Statistical methods. The significance of between-group differences was assessed using the Kruskal-Wallis test. Only when these results were significant comparisons between specific groups were done using Dunn's multiple comparisons test. The Mann-Whitney *U*-test was used for pairwise comparisons. Spearman correlation analysis test was performed for correlation studies. All statistical tests were interpreted at the 5% two-tailed significance level.

Results. There were significant differences in mean PBL NO production among the four groups (healthy controls, BMS, SPMS, or PPMS; $p < 0.0001$). Mean (± SEM) nitrite level in controls was 5.94 (±0.58) nmol/mg protein. Compared with controls, nitrite levels were significantly higher in patients with BMS ($p < 0.001$), SPMS ($p < 0.001$), and PPMS ($p < 0.01$). There were no significant differences among the three subgroups of MS (figure 1; see the table). There was no correlation between PBL NO production and age, sex, disease duration, or EDSS (not shown).

Serum levels of TNFα and IL-12 did not differ significantly among patients with BMS, SPMS, and PPMS (see the table). The levels were not different from those in healthy controls, except for lower levels of TNFα in patients with BMS ($p < 0.05$). There was a significant correlation between PBL nitrite concentrations and serum IL-12 levels, but not TNFα levels (figure 2). Treatment with interferon β did not influence PBL NO production. Mean nitrite PBL levels were 12.48 (±0.82) nmol/mg protein in patients using interferon β, and 11.58 (±0.96) nmol/mg protein in patients not using interferon β ($p = 0.63$).

Discussion. Compared to healthy controls, NO production by PBL was significantly elevated in patients with MS irrespective of a benign or progressive disease course. None of the patients had a clinical relapse. This suggests an ongoing activation of iNOS in PBL in patients with MS.

Expression of iNOS can be stimulated by proinflammatory cytokines, including IL-12 and TNFα.[20,21] Serum levels of IL-12 and TNFα were not significantly different between the three clinical subtypes of patients with MS and controls, except for lower serum levels of TNFα in patients with BMS compared to controls. We found a significant correlation between PBL nitrite concentrations and serum levels of IL-12, which is important for the development of T helper 1 (Th1) cell responses.[26] Our findings do not necessarily imply that IL-12 is involved in the NO production by PBL, and other mechanisms might be involved. Interestingly, enhanced NO production by PBL has also been observed in Alzheimer disease (AD) and chronic congestive heart failure.[27,28] In pa-

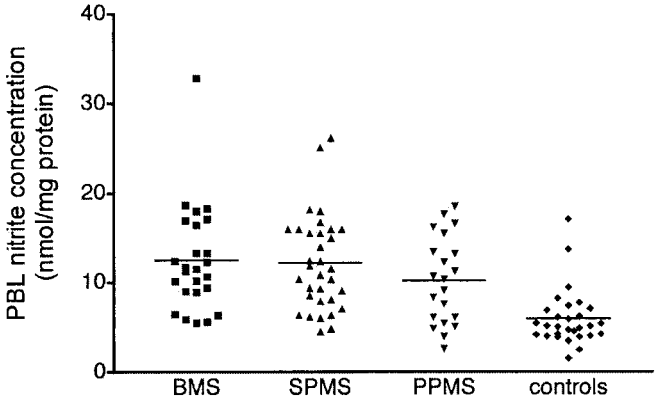

Figure 1. Peripheral blood leukocyte (PBL) nitrite levels in patients with a benign course of multiple sclerosis (BMS), secondary progressive MS (SPMS), primary progressive MS (PPMS), and healthy controls. The transverse solid lines represent the mean value. There were significant differences (p < 0.0001; Dunn's multiple comparisons test) between controls and each of the three clinical subgroups of MS.

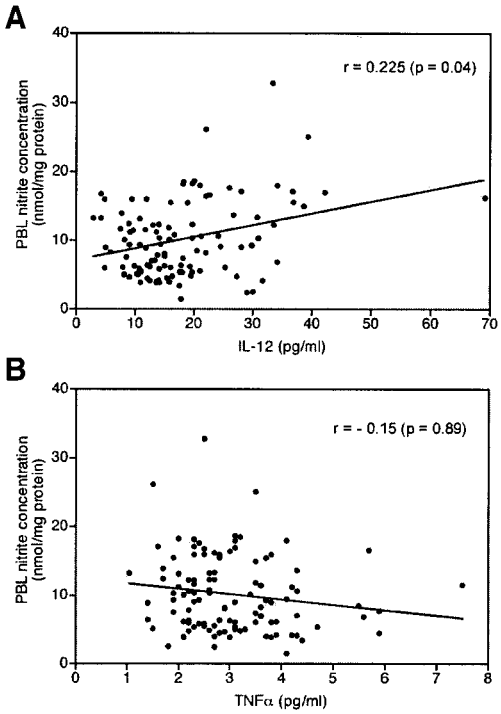

Figure 2. Correlation between peripheral blood leukocyte (PBL) nitrite levels and (A) serum levels of interleukin (IL)-12 and (B) serum levels of tumor necrosis factor (TNF)α.

tients with chronic heart failure, there was a relationship between iNOS activity in PBL and, among other parameters, serum levels of norepinephrine, suggesting that a stimulation of the sympathetic nerve system might play a role. Sympathoadrenergic activity is also increased in patients with AD and patients with MS.[29,30] It would be of interest to further investigate a possible relationship between sympathoadrenergic activity in MS and PBL NO production.

The clinical significance of an enhanced NO production by PBL in MS is unclear. In contrast to CSF,[6-10] serum NO metabolite levels do not seem to be related to clinical relapses or MRI evidence of lesion activity.[6,7,14] Our findings support the idea that nitrite levels in serum are mainly derived from NO produced by PBL rather than by cells in the CNS. NO is a diffusible gas that can readily pass the blood–brain barrier and enter the CNS. In a study investigating the effects of body cooling in patients with MS with heat-sensitive symptoms, improvement of symptoms was associated with a decrease in PBL NO production.[16] Because NO can block conduction in demyelinated axons in vitro,[31,32] this finding suggests that NO produced by PBL may be involved in fluctuations of MS symptoms.

Progressive disability in MS is believed to result from a widespread axonal degeneration. Studies using proton magnetic resonance spectroscopy to measure levels of *N*-acetylaspartate (NAA), an amino acid that is primarily localized to neurons, and histopathologic studies have shown diffuse axonal dysfunction/loss in NAWM in patients with MS.[17,18] NAA levels in NAWM were preserved in patients with BMS.[33] We found no difference in PBL NO production between patients with a benign and progressive course of MS, indicating that peripherally produced NO is unlikely to be a major contributor to axonal degeneration.

Interestingly, there is experimental evidence indicating that an enhanced PBL production of NO might represent an immunosuppressive effect. NO inhibits Th1 $CD4^+$ T cell proliferation,[34] and the expression of adhesion molecules and proinflammatory cytokines.[35] Rats recovering from experimental allergic encephalitis, which is an animal model of MS, had significantly increased serum NO production, and this protected against a second episode of disease.[36] Oral treatment with N-methyl-l-arginine, an iNOS inhibitor, reduced serum NO levels and led to more severe disease,[37] or spontaneous relapse.[36]

Acknowledgment

The authors thank Natasha Maurits for help in the statistical analyses, and Reint Hartman, Koos Roggeveld, Magreet Schaaf, Tatyana I Oparina, and Michael G. Stepanov for help in the laboratory measurements.

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