Medical Science Research 1998, 26:407–408

Serum nitrotyrosine levels in patients with multiple sclerosis: relationship with clinical activity

Mercedes E. Zabaleta, Nicolas E. Bianco and Juan B. De Sanctis

Instituto de Immunología, Facultad de Medicina, Universidad Central de Venezuela, Caracas, Venezuela

Requests for reprints to Dr Juan B. De Sanctis, Instituto de Immunología, Universidad Central de Venezuela, Aerocav #1216, PO Box Miami, Fl, PO Box 02-5304, Miami, Fl 33102-5304, USA. Tel: (58) (2) 605-3429; fax: (58) (2) 693-2734. E-mail: inmuno@cantv.net

Received 20 March 1998; accepted 25 March 1998

Abstract: We have assayed levels of nitrotyrosine, one of the products of peroxynitrate ($ONOO^-$), in the sera of 20 controls and 14 patients with multiple sclerosis (MS). These were in different stages of clinical activity: seven with clinical remission (RR) (clinically inactive) and seven with chronic progressive (CP) disease (four active and three inactive). The levels of nitrotyrosine were significantly higher ($P < 0.0001$) in MS patients (112.79 ± 33.24 ng/ml) than in the controls (20.0 ± 8.0 ng/ml). The highest increase (155.6 ± 18.3 ng/ml) was in active chronic progressors (PC A) and these values were significantly higher than in RR patients (91.7 ± 25.7 ng/ml, $P < 0.0001$) and inactive CP (117.5 ± 9.2 ng/ml, $P < 0.05$) patients. Serum nitrotyrosine levels are raised considerably in MS patients and they may vary with the stage and activity of the disease. Future studies should ascertain the importance of serum peroxynitrate formation in MS.

Med Sci Res 26:407–408

Keywords: nitrotyrosine, multiple sclerosis, peroxynitrate

Introduction: nitric oxide (NO), initially identified as an endothelial factor responsible for the relaxation of smooth muscle cells, is generated through the enzymatic conversion (by nitric oxide synthase) of arginine to citruline using NADPH as a cofactor. The end product is quickly transformed into a radical which, in the presence of superoxide radical ($O_2^{\cdot-}$), can be transformed into peroxynitrate ($ONOO^-$) [1,2].

Peroxynitrate reacts with CO_2 to produce various noxious products that deplete antioxidants, and oxidize and nitrate proteins, lipids and DNA [1,2]. Peroxynitrate has been suggested to be involved in the pathology of different diseases, as assessed by the detection of 3-nitrotyrosine in injured tissues either by antibody immunostaining, or HPLC-based and gas mass chromatography techniques [2].

In vivo, there is increasing evidence that NO plays an important role in central nervous system inflammation [3]. Raised levels of NO metabolites have been found in the cerebrospinal fluid and serum samples of multiple sclerosis (MS) patients [3]. However, there was no relationship between the clinical activity observed in the patients and NO metabolites.

We have now studied the possible importance of nitrotyrosine in different clinical stages of MS.

Patients and methods: 14 patients (seven female and seven male, age 40 ± 11 years old) with a diagnosis of MS supported by clinical and laboratory findings [4] were enrolled from the department of neurology of the Vargas Hospital (Caracas, Venezuela) or the Clinical University Hospital (Caracas, Venezuela). Written consent was obtained from each patient. The study was approved by the hospital's Ethical Committee.

None of the patients had received any immunosuppressor for 3 months prior to the study, nor any other drug for a period of no less than 72 h. Clinically, four patients were classified as having disease activity and 10 were stable or inactive. The patients were divided into three groups: relapsing remitting (RR), which were clinically inactive, and chronic progressive (CP), either clinically inactive (CP I) or active (CP A).

The controls were 20 normal blood donors.

Blood samples were taken after the patients had fasted for no less than 4 h and no longer than 24 h. Their habitual food intake was similar. All patients and controls received a diet with low contents of nitrite and nitrate, following guidelines described elsewhere [5].

The amount of total nitrotyrosine was determined by a standard sandwich ELISA assay as described by Ye and coworkers [6]. Both antibodies, the mouse IgG monoclonal for capturing the modified amino acid, the polyclonal against nitrotyrosine and the polyclonal goat anti-rabbit IgG-peroxidase were obtained from Upstate Biotechnology (Lake Placid, New York, USA). Nitrotyrosine was quantified using a standard curve with known concentrations of nitrotyrosine from chemically modified bovine serum albumin as described previously [6]. The inter- and intra-assay coefficients of variation were 8% and 11% respectively.

Results are expressed as means ± SD, and statistical comparisons were made by ANOVA.

Results: Table 1 illustrates the characteristics of the patients and their serum levels of nitrotyrosine. These levels were significantly higher in MS patients than in the controls ($P < 0.001$). However, if the patients were divided according to the classification specified in Table 1, marked differences became apparent. There were significant differences between the age and the years of the clinical onset of the disease in the different groups. There was, however, no correlation between age or between the time of the onset of the disease and nitrotyrosine levels.

As compared to controls, patients classified as RR,

0269-8951

Table 1. Characteristics of the different groups of MS patients and their serum nitrotyrosine levels (means ± SD)

	Controls	RR	CP I	CP A
Number	20	7	3	4
Sex (F:M)	1:1	5:2	1:2	2:2
Age (yr)	38 ± 8	33 ± 9	50 ± 1	46 ± 6
Disease onset (yr)		6 ± 3	17 ± 3#	9 ± 2
Nitrotyrosine (ng/ml)	20.0 ± 8.0	91.7 ± 25.7*	117.5 ± 9.2*	155.6 ± 18.3*

**As compared to controls, P < 0.0001 (ANOVA).*
#As compared with RR and CP A patients, P = 0.0003 (ANOVA).

inactive CP (CP I) and active CP (CP A) had significantly higher ($P < 0.0001$) levels of nitrotyrosine. However, there were marked differences among groups in the levels of nitrotyrosine. CP A patients had the highest values of nitrotyrosine and these levels were significantly different from those in the RR ($P < 0.0001$) and CP I groups ($P < 0.05$). In addition, patients defined as inactive CP had significantly higher levels of nitrotyrosine ($P < 0.05$) as compared to RR patients.

Discussion: In a previous report [7], we found that patients with MS had significantly lower levels of nitrites and nitrates as compared to controls, suggesting that there could be an increased formation of other toxic NO radicals such as peroxynitrate. In order to investigate this possibility, we assessed the serum levels of 3-nitrotyrosine in MS patients and compared them to those in controls.

MS patients had serum levels of nitrotyrosine more than six-fold higher in comparison to controls. These raised levels contrast with the lower values for nitrites and nitrates reported previously [7], especially in the RR and CP I groups. Furthermore, the highest nitrotyrosine values observed in the CP A group, contrast with the normal (as compared to controls) [7] values for nitrite and nitrate. In CP A patients, NO production, evaluated as the sum of nitrites, nitrates and nitrotyrosine, appeared to be higher than in the other groups. Thus nitrotyrosine levels may be an important marker in a sustained inflammatory response and could be related to clinical activity in MS patients.

The increased production of nitrotyrosine in MS patients suggests that oxygen radicals such as superoxide may also be involved in the generalized inflammatory response observed in this disease. Moreover, high NO formation, along with that of superoxide radicals, seem to be related to the clinical activity observed in CP A patients.

Elevated levels of NO as reported in the cerebrospinal fluid of MS patients and animal models of this disease (allergic encephalomyelitis) have confirmed the importance of NO in the lesions [3]. However, we believe this is the first study to have assessed the presence of nitrotyrosine in MS patients and suggested a clear relationship between this peroxynitrate product and disease activity. We propose, therefore, that serum nitrotyrosine levels may be more reliable markers than nitrites and nitrates in the assessment of nitric oxide in inflammatory disease. Future studies should ascertain the role of superoxide in MS.

Acknowledgements: This research was supported by Inmunobiologia A.C. and the Genic program.

1. Kiechle FL, Malinski T. Nitric oxide. Biochemistry, pathophysiology and detection. *Am J Clin Pathol* 1993; 100:567–575.
2. Halliwell B. What nitrates tyrosine? Is nitrotyrosine specific as a biomarker of peroxynitrate formation in vivo? *FEBS Lett* 1997; 411:157–160.
3. Parkinson JF, Mitrovic B, Merril JE. The role of nitric oxide in multiple sclerosis. *J Mol Med* 1997; 75:174–186.
4. Poser CM, Paty DW, Scheiberg L, McDonald WI, Davis FA, Ebners GC, *et al*. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. *Ann Neurol* 1983; 13:227–231.
5. Garmendia JV, Gutiérrez Y, Blanca I, Bianco NE, De Sanctis JB. Nitric oxide in different types of hypertension during pregnancy. *Clin Sci* 1997; 93:413–421.
6. Ye YZ, Strog M, Huang Z-Q, Beckman JS. Antibodies that recognize nitrotyrosine. *Meth Enzymol* 1996; 269:201–209.
7. Zabaleta M, Bianco NE, De Sanctis JB. Serum nitric oxide products in patients with multiple sclerosis: relationship with clinical activity. *Med Sci Res* 1998 submitted