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Endothelin and nitric oxide levels in cerebrospinal fluid of patients with multiple sclerosis

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In order to investigate the potential role of endothelins (ETs) and nitric oxide (NO) in the pathogenesis of multiple sclerosis (MS) we evaluated the levels of these vasoactive mediators in cerebrospinal fluid (CSF) of relapsing remitting MS patients and in a group of subjects with other neurological diseases (OND) and in a control group of subjects without neurological disease. Eighty patients affected from clinically diagnosed MS were selected, 44 of them were studied during an acute clinical attack and 36 in a stable phase. The OND group included 21 subjects affected by degenerative non inflammatory (*n*=9) and inflammatory (*n*=12) neurological disease while the control group included 22 subjects with cancer of the prostate (*n*=11) and with bladder disease (*n*=11). ET levels were significantly increased in CSF of relapsing remitting MS patients with an acute clinical attack in comparison with those in a stable phase, the OND group and the control group. Moreover significant differences were observed among the four groups with regard to the NO levels: MS patients in a stable and acute phase like OND group have high levels of NO compared to the control group. Since the blood-brain barrier index values did not differ significantly between the three groups, the data of this study suggest an important role for NO and ET in cerebral microcirculation in MS patients. *Journal of NeuroVirology* (2000) **6,** S62–S66.

Keywords: endothelins; nitric oxide; multiple sclerosis; cerebrospinal fluid

Introduction

In multiple sclerosis (MS), demyelination is the result of a series of pathological events which include local oedema, perivascular infiltration and production of cytokines and neurotoxic substances. It has been recently suggested that various substances may be involved within the central nervous system (CNS) and among the other endothelins (ETs) (Levin, 1995; Rubaniy, 1992) and nitric oxide (NO) (Merril *et al*, 1993; Hooper *et al*, 1995) are a group of vasoactive mediators suspected of playing a leading role. Endothelins are produced by endothelial and microglial cells and astrocytes in CNS as neuro-transmitters (Harland *et al*, 1995) and are involved in CNS cerebrovascular disorders (Fujimori *et al*, 1990; Suzuki *et al*, 1990; Ehrenreich *et al*, 1992). Some reports indicate that Endothelin 1 probably acts as modulator of inflammatory status in CNS up-regulating the local expression of intercellular and vascular adhesion molecules in microvascular endothelial brain, implicating the peptide in the recruitment of blood cells at site of inflammation (Barone *et al*, 1995). ET also contribute to mitogenesis and proliferation of the glia and astrocytes (Ross and Snyder, 1990; Harland *et al*, 1995) and can participate in tissue regeneration (Yamada *et al*, 1995).

Also NO can influence cerebrovascular resistance and the permeability of the blood-brain barrier under pathological condition and is over produced by microglial cells during inflammation in the brain (Wong *et al*, 1996) becoming responsible for demyelinating process (Merril *et al*, 1993) death and loss of oligodendrocytes (Ikeda *et al*, 1995). Moreover low levels of NO lead to a decrease in myelin production from oligodendrocytes (Merril *et al*, 1993).

In this study the potential role of ETs and NO in MS pathogenesis has been investigated by measuring their level in cerebrospinal fluid (CSF) collected from relapsing remitting MS patients and from a group of patients with other neurological diseases (OND) and in a control group without neurological diseases. A significant

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increase of ETs and NO levels have been observed in the CSF of MS patients.

Results

The results of the measurement of ET levels in the CSF of the four groups of patients is exposed in the Figure 1A. ET levels (mean=11.0; s.d.=12.67 pg/ml) in CSF of acute relapsing remitting MS patients were significantly higher ($P<0.05$) than those observed in stable relapsing remitting MS patients (mean=5.1; s.d.=9.13 pg/ml) and the OND group (mean=2.83; s.d.=4.3 pg/ml ($P<0.05$) and the control group (mean=1.5; s.d.=2.3) (Figure 1). The data obtained by measuring the NO levels in the CSF of the same patients are reported in Figure 1B. Significant differences in total nitrate/nitrite have been observed between acute relapsing remitting MS (mean=54.52; s.d.=23.9) and stable relapsing remitting MS (mean=56.53; s.d.=27.6) *versus* the control group (mean=28.7; s.d.=8.1) and in the OND patients (mean=44.29; s.d.=11.0) *versus* the control group. No differences were observed in the two groups of MS patients.

In order to verify if the presence in the CSF of ET and/or NO could be due to damage of a BBB, the IgG intrathecal synthesis index and the Link IgG index have been evaluated in all the patients studied. The results obtained are shown in Table 1 in which it is possible to see that the value of BBB integrity does not differ among the three groups of the MS or OND patients while as expected the IgG index values are increased in MS patients but are within normal range in the OND cases.

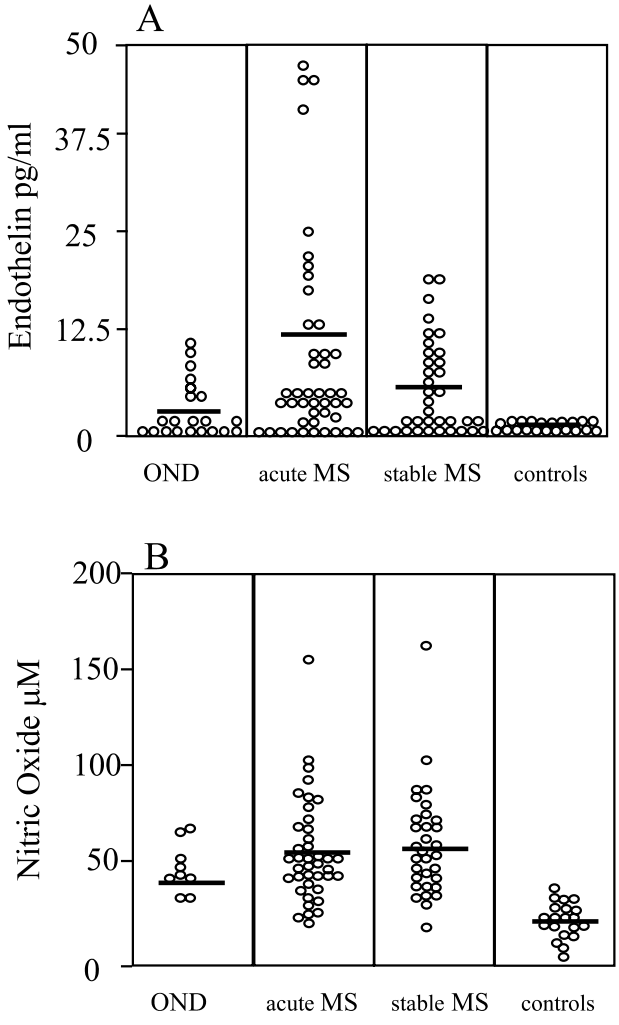

Figure 1 Distribution of Endothelin (**A**) and Nitric Oxide (**B**) levels in CSF from OND patients, acute and stable MS patients and not neurological controls.

Discussion

ETs have been suggested to act as neuropeptides in the CNS and to modulate neuronal activity (Giaid *et al*, 1991). In normal brain glial cells, including astrocytes and endothelial capillary cells, do not express endothelin-like immunoreactivity while the production of ETs is increased in presence of CNS diseases and injuries (Lee *et al*, 1990; Jiang *et al*, 1993). For instance reactive human astrocytes and macrophages express ET mRNA during viral CNS diseases, such as progressive multifocal leukoencephalopathy (PML) and subacute sclerosing panencephalitis (SSPE) (Ma *et al*, 1994). In the present study we report the presence of enhanced levels of ETs in CSF of acute relapsing remitting MS patients in comparison to those in a stable disease phase and to the OND patients. Moreover the elevated levels of nitric oxide in CSF of patients with MS provides further evidence for NO in immunopathogenesis of MS according to already published data (Giovannoni, 1998). However nitrite (NO_2^-) and nitrate (NO_3^-) as charged anions have difficulty to cross the blood-brain barrier and this suggests that a greater quantity of intrathecal NO is produced in MS patients (Felgenhauer *et al*, 1982).

Moreover we did not observe a higher frequency of BBB damage in acute MS patients, the increased ETs levels found in the CSF of these patients are probably due to a local synthesis by glia, neurons, astrocytes or infiltrating monocytes. Although the role of this peptide family is still not clearly defined

Table 1 IgG synthesis index and blood-brain barrier levels in CSF of patients with acute MS, stable MS and OND reported as mean value and standard deviation.

	OND	*Acute MS*	*Stable MS*
Link IgG synthesis index	0.668±0.86	0.811±0.46	0.701±0.22
Blood-brain barrier integrity	5.16±4.08	5.034±1.92	5.191±2.25

it has been suggested that the increased synthesis of ET during CNS injury promotes the mitogenesis of glioma cells and astrocytes and may contribute to the reduction of the local blood flow (O'Brien *et al*, 1992; Bonte *et al*, 1993), and that, on the other hand, it could play a role in the extravasation and migration of peripheral blood lympho-monocytes through the BBB (MacCumber *et al*, 1990; Yamada *et al*, 1995; Kohzuki *et al*, 1995). It has also been suggested that ETs may participate in tissue regeneration since they are largely expressed from astrocytes in the areas surrounding necrotic lesions or demyelinating plaques in PML and SSPE (Yamada *et al*, 1995). Moreover these peptides have been shown to be capable of promoting the activation of astrocytes and microglial cells (Holzwarth *et al*, 1992; Lin and Chuang, 1992; Jiang *et al*, 1993; Stanimirovic *et al*, 1995) and therefore to induce the production of Transforming growth factor beta (TGFβ) (Lindholm *et al*, 1992; Merril *et al*, 1993; Yamada *et al*, 1995), a cytokine that induces differentiation of the precursor of oligodendrocytes into myelin-producing cells *in vitro* (Eddleston *et al*, 1993; Gard *et al*, 1995).

Increased levels of NO have been observed in Parkinson, Alzheimer and in HIV-1-induced CNS diseases suggesting a neurotoxic activity of this neuro-transmitter molecule (Qureshi *et al*, 1995; Goodwin *et al*, 1995). NO causes injury to oligodendrocytes also if the adjacent astrocytes and microglial cells are not damaged or killed *in vivo* and *in vitro* (Prineas, 1985; Merril *et al*, 1993). Since the oligodendrocytes seem to be the target in MS pathogenesis it is possible that a lower concentration of NO lead to the damage of oligodendrocytes by alteration of myelin biochemical turnover (Merril and Benveniste, 1996). Our data show the presence of high levels of NO in CSF of MS and OND *versus* control group. The presence of NO in CSF of MS patients contribute to the hypothesis that the low levels of NO can mediate the blood-brain-barrier breakdown that occur in CNS inflammation.

On the whole the results of our study suggest that the higher ET levels observed in the CSF of the acute MS patients may be indicative of its potential as a marker of disease activity, while the presence of NO levels may be considered as an indicator of BBB dysfunction in CNS inflammation. The role of ETs and NO in the inflammatory process leading to MS pathogenesis requires further investigation and more evidence is needed to establish whether ETs play a role in triggering local reduction of blood flow in the CNS or if they are produced as consequence to damage to endothelial cells.

Materials and methods

Patients and controls

The CSF samples analysed in this study were collected from 80 MS patients (51 females and 29 males, mean age 38 years) with clinically definite relapsing remitting MS diagnosed according to the criteria of Poser *et al* (1993). MS patients with a new clinical acute attack and with a cerebral gadolinium-enhancing area evidenced by magnetic resonance imaging (MRI), performed on a 0.5 Tesla operating unit (General Electric MR MAX), were defined as acute, while those without a clinical relapse in the previous 6 months and without gadolinium-enhancing areas were classified as stable MS. According to the clinical observation and to the MRI results of the 80 RRMS patients 44 were in the acute phase whereas 36 were in the stable phase of disease.

The mean period of disease is 6.7 years for the RRMS in stable phase and 6 years for the patients in acute phase. None of the patients had received ACTH, corticosteroid or any other relevant pharmaceutical agent for at least 3 months, or any immunosuppressive agent for at least 6 months before CSF collection. Atypical dietary habit rich in nitrates was investigated and if present used as exclusion criteria.

A group of 21 patients suffering from other neurological diseases (OND) was studied as control. Ten of these were females and 11 males with a mean age of 35 years and a mean disease duration of 1.6 years. This group included nine patients with degenerative non inflammatory neurological disease (seven amyotrophic lateral sclerosis (ALS), one labyrinthine syndrome, one spastic paresis) and 12 with inflammatory neurological diseases (four encephalomyelitis, five pyramidal syndrome, 2 Beçhet, one cerebellar ataxia).

The non neurological control group included 22 subjects (21 males, one female) suffering from cancer of the prostate (n=11) and of bladder (n=11).

CSF routine laboratory analysis

For all the MS patients and the OND controls, routine analyses were performed on the CSF samples obtained by sterile lumbar puncture. Blood-brain barrier (BBB) integrity, the IgG intrathecal synthesis indexes and the presence of IgG oligoclonal bands (OB) were evaluated using standard procedures. The Link IgG index, (Lefvert and Link, 1985) the Tourtellotte IgG synthesis index (Tourtellotte *et al*, 1985) and BBB integrity value were calculated by measuring serum and CSF IgG and albumin levels, using the nephelometer system APS Beckman (Beckman Instruments, Inc., Galway, Ireland). Normal values were established as $\leqslant$0.7 mg/ml/day for the Link IgG index, $\leqslant$3.3 mg/dl/day for the Tourtellotte IgG synthesis and $\leqslant$5.5 for the BBB integrity index.

The presence of CSF IgG OB was evaluated by sodium dodecylsulfate (SDS) acrylamide gel electrophoresis with silver staining, using the Phast System apparatus (Pharmacia Biotech, Uppsala, Sweden).

Endothelin and nitric oxide measurement
Endothelin levels in the CSF were assayed using the Endothelin Immunoassay Kit (Cayman Chemical Company, Ann Arbor, MI, USA) adopting the supported protocol. After ultrafiltration on 10 000 molecular weight cut off filter (Sartorius AG, Göttingen, Germany) to eliminate protein, nitrate and nitrite was assayed using NO_2^-/NO_3^- assay (R & D System, Inc, Minneapolis, USA). This assay determines total nitric oxide based on the enzymatic conversion of nitrite by nitrate reductase. The reaction is followed by a colorimetric detection of nitrite as an azo dye product of the Griess reaction (Ding *et al*, 1988). Separated procedure for nitrite and nitrate assays have been performed. Finally the concentration of endogenous nitrite present in each sample was added to each nitrate concentration and the results have been expressed as μmol/l of NO_2^- plus NO_3^-. The sensitivity of the nitrite assay was less than 0.22 μmol/l while the sensitivity of the nitrate assay was less than 0.54 μmol/l.

Statistical analysis
The statistical evaluation was performed by Kruskal-Wallis test and Mann Whitney test and the values have been considered significant for $P \leqslant 0.05$.

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