Short-term sequential analysis of sex hormones and helper T cells type 1 (Th1) and helper T cells type 2 (Th2) cytokines during and after multiple sclerosis relapse

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ABSTRACT. Multiple sclerosis (MS) is an immune-mediated disease with a clear sex-bias that may be attributed to sex hormones, sex' linked genes or both. Here we sought to determine the evolution pattern of cortisol and sex hormones at MS relapse and 2-months later in 7 male patients with relapsing remitting MS, and whether there was a correlation with a specific Th1 and Th2 cytokine pattern. Our findings indicate the activation of the hypothalamic-pituitary-adrenal axis and the concomitant upregulation of pro- and anti-inflammatory cytokines during relapse. The further increase of sex hormones, in particular estradiol in our male MS patients suggest their possible implication in the physiopathology of the illness and a putative anti-inflammatory and neuroreparatory effect.

Keywords: cytokines; hypothalamo-pituitary-adrenal axis; multiple sclerosis

INTRODUCTION

Compelling evidence supports the notion that multiple sclerosis (MS) is a cell-mediated, autoimmune disease. The prevailing hypothesis postulates that autoreactive T-lymphocytes of the $CD4^+$ helper type-1 (Th1) subset orchestrate the pathogenic process in MS. These lymphocytes recognise antigens presented by antigen-presenting cells and subsequently secrete proinflammatory cytokines, such as interleukin (IL)-1, interferon (IFN)-γ, and tumour necrosis factor (TNF)-á, which induce the immunopathological cascade resulting in damage to the myelin, oligodendrocytes and axons within the central nervous system (CNS). The recovery phase is believed to be mediated by Th2 lymphocytes, which secrete anti-inflammatory cytokines, such as IL-4 and IL-10.

The fact that there is a peak of incidence of MS during the reproductive age, a higher preponderance in women, a reduction in MS activity (clinical and magnetic resonance imaging, MRI) during pregnancy, and a resumption of activity in the early postpartum, support the notion that MS could be influenced by the endocrine system. The precise reasons for this sex bias are unclear, although genes and hormones are powerful putative factors. Genes linked to sex or the sex hormones could be responsible for susceptibility to MS [1-3].

Th1 and Th2 lymphocytes are said to be mutually inhibitory. In patients with relapsing-remitting MS (RR-MS), there is an increase in the levels of Th1 proinflammatory cytokines concomitant with clinical relapse [4], whereas an increase in Th2 anti-inflammatory cytokines during MS remission have been reported. On the other hand, lymphocytes have glucocorticoids and sexual steroid receptors, which can directly affect their function, and influence in the Th1/Th2 balance. The anti-inflammatory [5, 6] and neuroprotective effects of sex hormones have gained increasing attention during recent years [7]. In experimental autoimmune encephalomyelitis (EAE) that serves as an animal model of MS, sex hormones (oestrogen, testosterone, progesterone) have been reported to act as modulators of the immune response towards Th2 phenotype, and decrease the severity of EAE [8, 9].

Several experimental studies have highlighted trophic effects of sex hormones on normal neuronal activity [10]. Androgens affect the morphology and survival of the spinal and brainstem motoneurons [11], and may prevent the neurotoxicity mediated by glutamate [12]. The main goal of the present study was to evaluate in patients with relapsing remitting (RR) MS, whether the cortisol and sex hormone at relapse and two months later correlate with a specific Th1/Th2 cytokine pattern.

Patients and control subjects

Twenty patients (13 women and 7 men) with RR MS who were consecutively admitted to our centre because of MS relapse were evaluated. With the purpose of avoiding the hormonal oscillations related to the ovarian cycle, only seven male and six age-adjusted healthy control men were studied. The control subjects were recruited from healthy blood donor volunteers during the study period. We analysed serum cortisol, sex hormones (estradiol, testosterone and progesterone), and the cytokines TNF-α and IL-10 at relapse and two months later. Relapse was defined as the emergence of new symptoms or worsening of existing symptoms lasting more that 24 hours after a period of 30 days of improvement or stability in the absence of infection or fever. All patients were diagnosed as having clinically defined RR MS, except one who had laboratory – supported, probable MS according to Poser *et al.* [13] criteria. In all the patients, an MRI and laboratory analyses (IgG index, and/or oligoclonal IgG bands (OB) were performed as part of routine assessment. Clinical disability was measured using Kurtzke's Expanded Disability Status Scale (EDSS) [14].

Patients with kidney, liver, endocrine, immunological, inflammatory or infectious disorders were excluded by history, physical examination, and laboratory evaluations. None of the patients had received any anti-inflammatory, immunosuppressive, steroid or endocrine treatment for at least three months prior to this study-point. Informed consent was obtained from each subject. The Ethical Committee of the hospital also gave consent. Venous blood samples were collected in prechilled, ethylenediamine tetra-acetic acid (EDTA) tubes, obtained between 8:30 and 10:30 am, centrifuged at 1 500 rpm for 10 min, and stored at – 70 ^{0}C, until analysis of hormones and cytokines was performed. The patients were subsequently treated with intravenous methylprednisolone 1 g/day for five days plus 0.5 g/day for three days according to the severity of relapse. None of the patients was treated with any of the beta-interferons. All seven patients were re-evaluated clinically at two months and blood was extracted similarly to the first analysis.

METHODES

Hormones and cytokines quantification

Serum cortisol was quantified by competitive immunoassay (IMMULITE 2000, Diagnostic Products Corporation, LA, USA). Serum testosterone, estradiol and progesterone were determined using a chemiluminescent immunoassay (Immuno I, Bayer, Germany). Serum TNF- α and IL-10 were quantified by specific ELISAs as described by the manufacturer (R&D Systems, Minneapolis, MN, USA) at MS relapse and two months later in parallel. There were no detectable levels of IL-10 in blood healthy donors. The average level of human TNF-alpha in plasma samples was 1.4 pg/mL, range, 0-4.9.

Statistical analysis

Data are presented as mean ± standard deviation, and median and interquartile range. Comparisons between patient and control variables were performed using the non-parametric Mann-Whitney U-test because of the small sample size. For comparison of paired data before and after relapse, we used the Wilcoxon test. Cox regression analysis was used to estimate the correlation among different variables. Correlations between different variables were performed using the Spearman correlation analysis.

RESULTS

The clinical and demographic characteristics of the seven male MS patients at baseline are illustrated in *Table 1*. The mean age at diagnosis was 32.2 ± 8.9 years (median, 30 years, range, 20-48). The mean duration of the illness was 4 ± 5.1 years (median, 2, range, 0-14). The mean number of relapses from the beginning of the illness was 2.4 ± 1.4 (median, 3, range, 0-5) and the degree of disability measured by the mean EDSS at relapse was 2.8 ± 0.6 (median, 2.5, range, 1.5-3.5). The number of days' evolution of the relapse on extraction of blood for the analysis of hormones and cytokines was 21.4 ± 13.8 days (median, 15, range, 5-40). All but one of the seven patients (patient #6) had the symptoms resolve and thus MS activity was finished by the two month study.

Interestingly, male MS patients exhibited a comparable hormonal and cytokine profile when examined individually *(Figure 1)*. The levels of all hormones (cortisol, progesterone, testosterone and estradiol) were elevated in the patients at the two different time-points (relapse and two months later), and with respect to healthy controls *(Figure 1)*. All hormones, TNF-α and IL-10 were significantly higher at MS relapse than in controls *(Table 2)*. This elevation was as statistically significant during the relapse as at the two month point with respect to control levels

Table 1
Clinical and demographical characteristics of the MS patients

Pt. ID	Age (years)	MS Type	No. of relapses	Duration of MS (years)	Duration of relapse (days) at baseline	EDSS at relapse	EDSS at 2-mo	IgG index	OB
Patient #1	30	RR	5	14	5	2.5	0	0.56	yes
Patient #2	39	RR	4	1	10	1.5	0	0.61	yes
Patient #3	30	RR	4	2.5	35	3.5	1,5	1.09	yes
Patient #4	27	RR	3	1.5	15	2.5	1	1.54	yes
Patient #5	48	RR	2	2	40	3	1	1.22	yes
Patient #6	32	RR	2	9	25	3	1	2.48	yes
Patient #7	20	P	1	0	21	2	0	0.80	not

RR: Relapsing-remitting; P: Probable; EDSS (Expanded Disability Status Scale); IgG index (normal, < 0.70); OB: presence of oligoclonal bands in CSF.

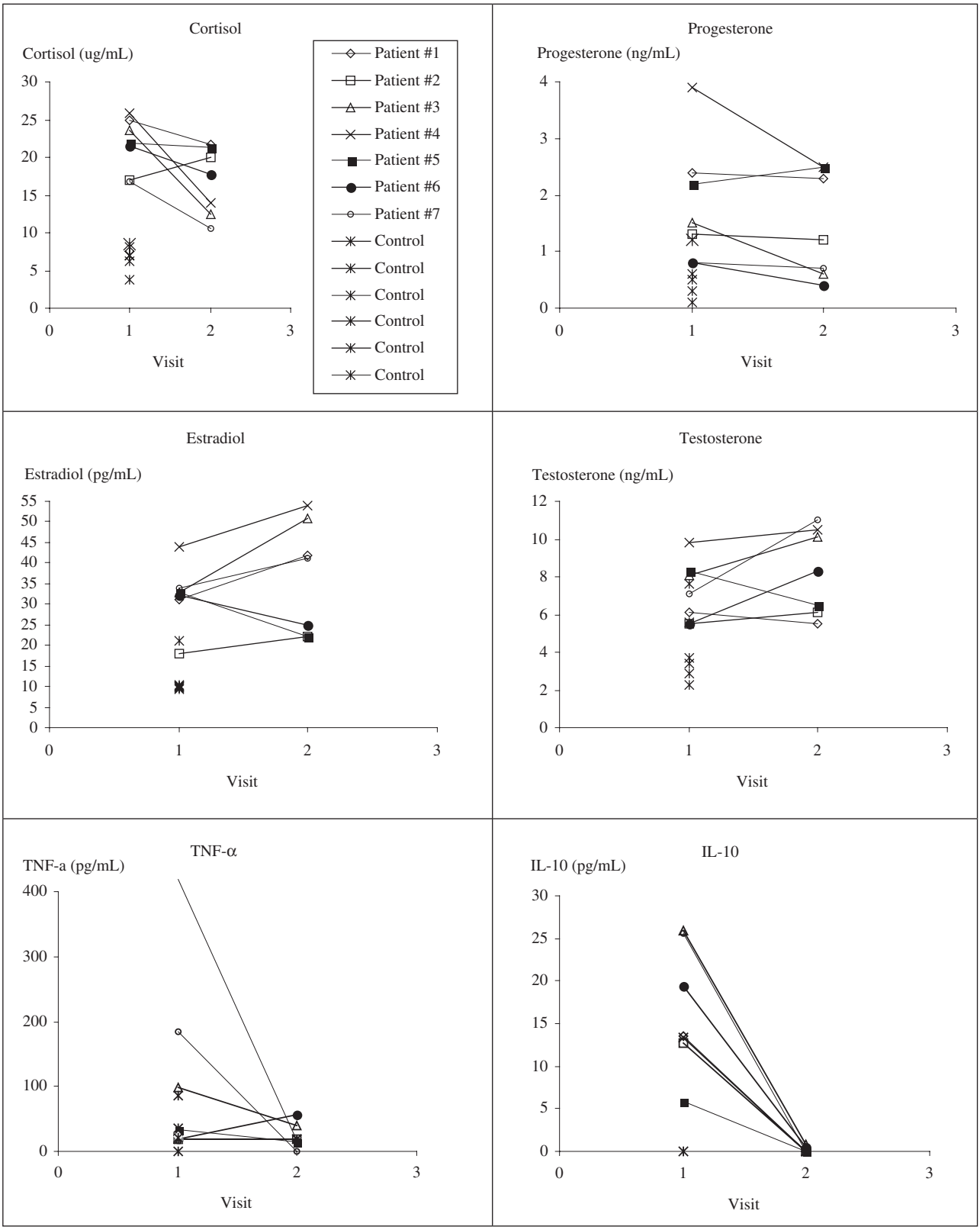

Figure 1
Serum hormones and cytokines levels at two different time-points studied in individual MS patients with respect to controls.

(Table 2). Patient #5 showed a slightly different pattern as illustrated in *Figure 1*. He had a longer time-course evolution from relapse, and was older than the other patients. Only the cortisol was significantly higher at relapse than after two months ($P < 0.01$) *(Table 2)*. Therefore, a clear dissociation was observed in the curves of the different steroid hormones studied: cortisol levels fell by 38% two months after relapse ($P = 0.04$), progesterone decreased by 21%, whereas estradiol increased by 14%, and testosterone increased by 15% *(Table 2)*. Only the fall in the cortisol levels was statistically significant with respect to the relapse.

Table 2
Serum hormones and cytokine levels in the study group at two different time-points and in the control group

Characteristic	At MS relapse	Two months after MS relapse	Control group
Cortisol	21.9 ± 3.6**§§	17.8 ± 4.4**	7.0 ± 1.6
Estradiol	33.0 ± 7.6**	41.0 ± 13.0**	10.0 ± 4.4
Progesterone	1.5 ± 1.0**	1.2 ± 0.9	0.5 ± 0.3
Testosterone	7.1 ± 1.6*	8.3 ± 2.2*	3.5 ± 1.9
IL-10	13.6 ± 3.3**§§	UD	UD
TNF-alpha	147.8 ± 18.6*	17.2 ± 15.2	21.3 ± 8.0

Median ± s.d. UD = undetectable; Statistical significance of *$p < 0.05$; **$p < 0.01$ with respect to control group, and § $p < 0.05$; §§ $p < 0.01$ values at relapse with respect to two months later.

With regard to cytokines, TNF-α and IL-10 levels were significantly higher in MS patients than controls *(Table 2)*. By two months from relapse, TNF-α levels clearly decreased and IL-10 had decreased to undetectable levels. Indeed, IL-10 significantly decreased from relapse to the two month follow-up *(Table 2)*.

No significant correlation was observed among hormone levels and cytokines either during relapse or two month later according to the Spearman correlation analysis (data not shown). When we analysed any correlation between the estradiol/progesterone ratio and both cytokines, again we found no correlation.

DISCUSSION

The endocrine system, the nervous system and the immune system have important interactions with reciprocal effects. Neuroendocrine host responses are known to occur during certain infectious, inflammatory, autoimmune and neoplastic processes. Lymphocytes contain receptors for glucocorticoids, prolactin, estradiol, testosterone, beta-adrenergic agents and endorphins. Alternatively, receptors of lymphocyte products have been identified in the endocrine glands, and cytokine receptors have been observed in the pituitary, adrenal and thyroid glands, testicles and ovaries [15]. It has been demonstrated that the observed effects of hormones on the immune system depend on the dose, route and timing of administration [16].

The increase in serum cortisol levels during relapse observed in our patients agrees with previous studies, since in the context of an excessive immune stimulus, the hypothalamic-pituitary-adrenal (HPA) axis is activated as a protective mechanism and releases anti-inflammatory corticosteroids [17]. Local or systemic proinflammatory cytokines (such as TNF-α, IL-1 and IFN-γ) are thought to stimulate the activation of the HPA axis. This activation plays a crucial role for resolution of inflammation, since ACTH output by the pituitary gland increases the production of glucocorticosteroids by adrenal cells. Indeed, corticosteroids are the most potent endogenous inhibitors of the immune and inflammatory process known. Adrenal androgens are usually secreted concomitantly with cortisol by the adrenal cortex, and are also thought to exert primarily anti-inflammatory effects [18]. Schumann *et al.* explored the interrelationship between the findings on MRI and the activity of the HPA axis, using dexamethasone-corticotropin-releasing hormone. They observed that the poor responders showed more gadolinium (Gd)-enhanced lesions than those that were hyper-responders, suggesting that the activity of the HPA was higher in those with less active MRI lesions [19]. This agrees with the increase in cortisol in our data.

During relapse, we observed that the levels of the sex hormones (progesterone, estradiol, testosterone) were significantly higher in our patients with respect to controls. The finding that testosterone levels were elevated as compared with healthy controls are not in agreement with data presented by Wei [17], although only two out of the 25 male patients studied had RR MS. The specific mechanism of glucocorticoid and sex steroid interaction in cytokine production is still unknown. However, anti-inflammatory/trophic effects of sex steroids alone are well documented in experimental data [20-22]. The influence of estrogens on the neuroregenerative process may be mediated by up-regulation of many molecules, including cytoskeletal components, which participate in the process of axonal sprouting and target recognition [23, 24]. One of the most accepted explanations of the neuroprotective effects of estrogen is related to the endogenous antioxidant role of the estradiol molecule [25, 26], but its antioxidant activity is observed at higher concentrations than physiological ones. Also, it is widely accepted that glucocorticoids may delay the onset and slow the progression of neurodegenerative disorders [27]. On the other hand, estrogens may also influence the production of proinflammatory cytokines by reactive glial cells, which may be toxic for neurons [5].

Recent studies in a series of patients with RR-MS in remission, have examined whether there is a correlation between sex hormones and disease activity measured by Gd-enhanced MRI lesions, showing that those patients with high estradiol and low progesterone levels [28], and those with high progesterone/17-beta-estradiol ratio [29], had a significantly greater number of Gd-enhanced lesions than those with low levels of both these hormones. In addition, Sicotte [30] demonstrated that treatment with high doses of oral estriol significantly diminishes the number of Gd-enhanced lesions and the volume of lesions in cerebral MRI. These observations support the hypothesis that absolute or relatively high levels of sex steroids hormones could exert an anti-inflammatory role on the course of the illness or that decreased levels of sex and adrenal steroids could mediate the increased susceptibility to autoimmune disorders, as occurs within the postpartum period.

Our data are in agreement with those of other authors that demonstrate the parallel upregulation of both Th1 and Th2 cytokines during MS relapse compared to age and sex-matched healthy controls [31, 32], in the context of what Link referred to as the cytokine storm in MS [33]. More-

over, a parallel downregulation of both Th1 and Th2 cytokines was observed during the resolution phase of relapse. These observations may indicate that not one single cytokine is responsible for driving the pathogenic process in MS. The simultaneous liberation of Th1 (TNF-α) and Th2 (IL-10) cytokines could be reflecting a compensatory mechanism to control the inflammation by Th2 subsets during relapse. It is clear that neither cytokine are independent effector molecules, but instead take part within the complex network of counteracting cytokines. It seems reasonable that the outcome of an immune response in MS is determined by the imbalance between pro- and anti-inflammatory cytokines. Neither during relapse nor later did we observe any correlation between cytokines and hormones levels. We do not know whether the moderate, persistent elevation of steroid hormones with respect to controls up to the two months following relapse may contribute to the clinical improvement through primary anti-inflammatory effects, or whether it represents a premorbid risk status of susceptibility for developing MS. During MS relapse, the activation of lymphocytes and macrophages account for the increased levels of cytokines, both Th1 and Th2 in what Link has defined as a cytokine storm. The decrease of Il-10 and TNF-alpha two months after relapse may occur spontaneously with the resolution of the inflammatory response or/and due to the direct effects of methylprednisolone therapy. Indeed, an important mechanism for the antiinflammatory effect of corticosteroids in MS results from a suppression of the activation of the peripheral immune compartment through inhibition of cytokine production [34].

We are aware of the limitations of our data given the small sample size, but we would like to use them to propose the following hypotheses: a first hypothesis would be that persistence of subclinical autoimmune inflammation within the CNS, which would induce the compensatory neuroendocrine changes as part of an integrated protective response, and the increased hormonal levels contribute to the clinical stability. Arguments substantiating the first hypothesis include various observations suggesting a persistent activation of teh HPA axis [35], and that MS lesions activate the HPA system. Proinflammatory cytokines that are produced in the MS lesions, such as IL-1 and TNF-α, are powerful activators of CRH neurons [36]. Therefore, the absence of any correlation between TNF-α and cortisol could be explained more by local production of TNF-alpha within the CNS than by systemic cytokines. A second hypothesis would be a dysfunction of the feedback mechanisms of the HPA axis in these patients, as has been suggested by other authors [15, 19].

In summary, our findings confirm the activation of the HPA axis and the elevation of pro- and anti-inflammatory cytokines during relapse. We also found higher levels of sex hormones during the relapse although this did not correlate with cytokine levels. The increase in sex hormones, particularly of estradiol in our MS patients, indicates the possible implication of the sex hormones in the physiopathology of the illness and the suggestion that hormones could exert both anti-inflammatory and neuroreparatory effects. Our data indicate that cortisol and sex hormones are upregulated in MS which may be part of the problem and the solution. Longitudinal studies of larger samples will be required to clarify whether the increase in sex hormones is a consequence of the illness or whether, on the contrary, these endocrine alterations have relevance in the physiopathology of the illness. Sex hormones hold promise in the treatment of neurological disease, although many questions remain to be answered.

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