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Interferon beta treatment: Bioavailability and antiviral activity in multiple sclerosis patients

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Viral infections have been appointed as the main component of environmental susceptibility to multiple sclerosis (MS). Interferon beta is an immunomudulatory treatment that is able to modify the natural course of the disease; nonetheless, its mechanism of action in not well established yet. The objectives of the present study were (1) to evaluate the bioavailability of interferon beta through the measurement of the expression of myxovirus resistance protein (MxA), metalloproteinase 9 (MMP-9), and its inhibitor (TIMP-1); (2) to analyze its antiviral efficiency through the measurement of human herpesvirus-6 (HHV-6) prevalence; and (3) to correlate both parameters (bioavailability and antiviral efficiency) with the relapse rate in multiple sclerosis (MS) patients treated with interferon beta. Pairs of blood and serum samples were collected from 54 MS patients during five visits in 1 year: one before the start of the treatment and four during interferon beta treatment. Expression of MxA, MMP-9, and TIMP-1 was analyzed by quatitative real-time polymerase chain reaction (qRT-PCR) and HHV-6 genomes were detected by qPCR. The results showed a correlation between MxA and relapse rate ($P = .014$). MMP-9/TIMP-1 ratio was increased among the patients with relapses, and decreased among the relapse-free patients, although differences were not statistically significant. Furthermore, our results suggest a possible role for HHV-6 in MS, because 42.8% of patients with viral reactivations experienced at least one relapse versus 22.5% of patients without viral reactivations. Lastly, regarding the antiviral effectiveness of the interferon beta, the HHV-6 prevalence decreased from 58% to 36% in PBMCs and from 18.5% to 12.2% in sera; furthermore, a good correlation with the bioavailability of interferon beta was found, because patients with a decrease in HHV-6 prevalence had higher levels of MxA ($P = .046$, at the third month). *Journal of NeuroVirology* (2007) **13,** 504–512.

Keywords: HHV-6; interferon beta; MxA; MMP-9; multiple sclerosis; TIMP-1

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease whose etiology remains unknown. Nevertheless, genetic and environmental factors are thought to be involved in its origin. Viral infec-
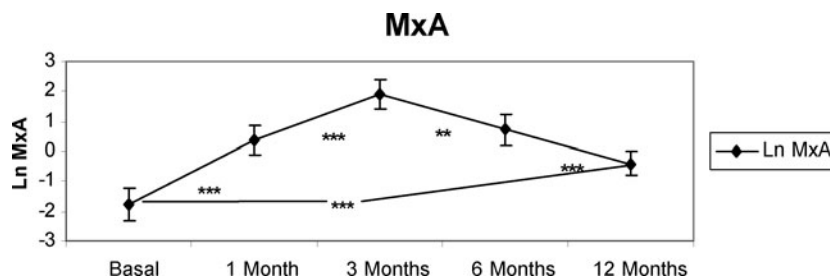
tions have been appointed as the main component of environmental susceptibility. Human herpesvirus-6 (HHV-6) is one of most studied virus in MS, although there are no definitive evidences of its implication until this moment. It has been shown that MS patients have increased levels of HHV-6 DNA and immunoglobulin M (IgM) against the virus in blood and serum samples (Alvarez-Lafuente *et al*, 2002a, 2002b); HHV-6 has been directly detected in demyelinating lesions (Challoner *et al,* 1995; Cermelli *et al,* 2003); there are some evidences of molecular mimicry between HHV-6 and myelin basic protein (MBP) (Tejada-Simon *et al*, 2003); and there are *in vitro* evidences of HHV-6 active infection of oligodendrocytes (Ahlqvist *et al*, 2005).

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Figure 1 Evolution of mean levels of MxA in all patients (with and without relapses) along the 1-year follow-up study. Data are expressed as mean of Ln MxA ± SEM. *Significant differences between visits. $**P < .01;***P < .001$.

Interferon beta is an immunomodulatory treatment that is able to modify the natural course of the disease reducing the number and severity of relapses and decreasing the accumulation of expanded disability status scale (EDSS)-estimated disability. Mechanism of action of interferon beta is not well established yet, but it is known that this molecule is bound to membrane-specific receptors, starting a cascade of intracellular signals that finish in the secretion of different proteins called interferon-induced genetic products with antiviral, antiproliferative, and immunomodulatory effects (Suhayl *et al*, 2002). However, one problem related to this therapy is the development of neutralizing antibodies (NAbs) against interferon beta. Data from pivotal trials suggest that the frequency of NAbs ranges from ~5% to 42%, depending on a variety of factors that appear to include interferon beta type, dosage/dosage frequency, and route of administration (Sorensen *et al*, 2003). NAbs are associated to a decrease in treatment efficacy (both in clinical parameters and in magnetic resonance imaging [MRI]) (The IFNB MS Study Group, 1996; Ross *et al*, 2000; Rudick *et al,* 1998), because they interfere the interaction of interferon beta with its receptor and the subsequent transduction of the signal through interferon beta–induced genes. Therefore, it is very important to know as soon as possible if patients are positive for NAbs in order to recommend another alternative treatment. Of the interferon beta–induced products, the myxovirus resistance protein (MxA) is highly specific for type I interferons and it is strongly stimulated by interferon beta binding to its receptor. Thus, it has been shown that is possible to measure interferon beta bioavailability by measuring mRNA or protein levels of MxA (Pachner *et al*, 2003, 2005; Deisenhammer *et al*, 1999; Gilli *et al*, 2002). Nevertheless, in a recent study (Gilli *et al*, 2004), it is suggested that the detection of MxA would not be a useful tool for the detection of interferon beta bioavailability in MS, and the authors proposed the measurement of the matrix metalloproteinase 9 (MMP-9). MMP-9 is regulated by the tissue inhibitor of metalloproteinases 1 (TIMP-1), and it has been demonstrated that MS patients present an unbalanced MMP-9/TIMP-1 ratio and that an increase in the ratio could predict new enhancing lesions (Waubant *et al,* 2003; Lee *et al,* 1999).

The purposes of this study are to evaluate interferon beta antiviral efficiency through the measurement of the evolution of the prevalence and viral load of HHV-6; to evaluate interferon beta bioavailability by analyzing the evolution of the expression of MxA and MMP-9/TIMP-1 ratio; and to correlate both parameters with the clinical evolution (relapse rate) in MS patients treated with interferon beta along 1-year follow-up.

Results

Evolution of MxA expression during the treatment
The results are shown in Figures 1 and 2. During the 1-year follow-up study, MxA increased during the first 3 months and then it fell, although the levels at the end of the study were higher than at the basal visit ($P < .001$). Comparisons of expression levels between successive visits were always statistically significant ($P < .001$). Also, it is possible to see in Figure 2 that patients with relapses had lower expression of MxA than relapse-free patients, mainly at the third month ($P = .014$).

Evolution of MMP-9/TIMP-1 ratio during the treatment
The results are shown in Figures 3 and 4. As it could be expected, a decrease in the MMP-9/TIMP-1 ratio was observed from the pretreatment visit to the second one. From 1 to 6 months, the ratio tended to rise again, but the maximum level that it reached was not statistically different from the pretreatment point. From 6 month to the last visit, a new decrease occurred. At the end of the follow-up, no statistically significant differences were found. When patients with relapses were compared with relapse-free patients (Figure 4), different trends were shown: whereas in the first group the ratio was increased from the basal visit (mean = −2.62) to the 12th one (mean= −1.69), in the second group the ratio tended to decrease (from −1.01 to −1.21); nevertheless, none of the differences reached a statistical significance ($P = .48$ and $P = .54$, respectively).

Comparison of MxA evolution between patients with relapses and without relapses
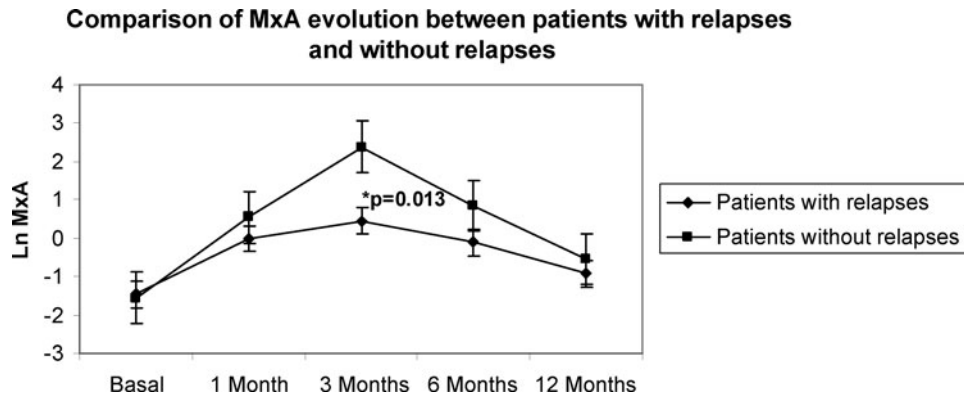
Figure 2 Evolution of MxA expression in patients that had experienced at least one relapse during the year of interferon beta treatment (patients with relapses) compared with those patients that did not suffer any relapse during the interferon beta treatment (patients without relapses). Data are expressed as mean of Ln MxA $\pm$ SEM. At the third month there is a statistically significant difference between groups (*$P = .013$).

Evolution of HHV-6 prevalence along the interferon beta treatment

The results are shown in Figure 5. There are statistically significant differences in HHV-6 prevalence in PBMCs between pretreatment visit and last visit: 58% and 36%, respectively ($P = .03$). When we analyzed the HHV-6 active infection, we found again a difference between the HHV-6 prevalence in serum samples at the basal visit (18.5%) and 1 year later (12.2%), but it did not reach a statistical significance ($P = .17$). HHV-6 prevalence in samples of HBD was only measured once, to have a reference, and the results we obtained were that 22.1% of HBD were positive for HHV-6 in PBMCs (P value $= .29$ in comparison with patients at 12th month visit), whereas we did not find any HHV-6 sequence among their serum samples (P value $= .011$ in comparison with patients at 12th month visit).

Lastly, it is important to note that in the group of patients that had some HHV-6–positive serum sample along the follow-up the percentage of individuals with relapses was 42.8%, whereas in the group of patients that had never presented HHV-6 reactivation the percentage of individuals with relapses was 22.5% ($P = .13$; OR $= 2.58$).
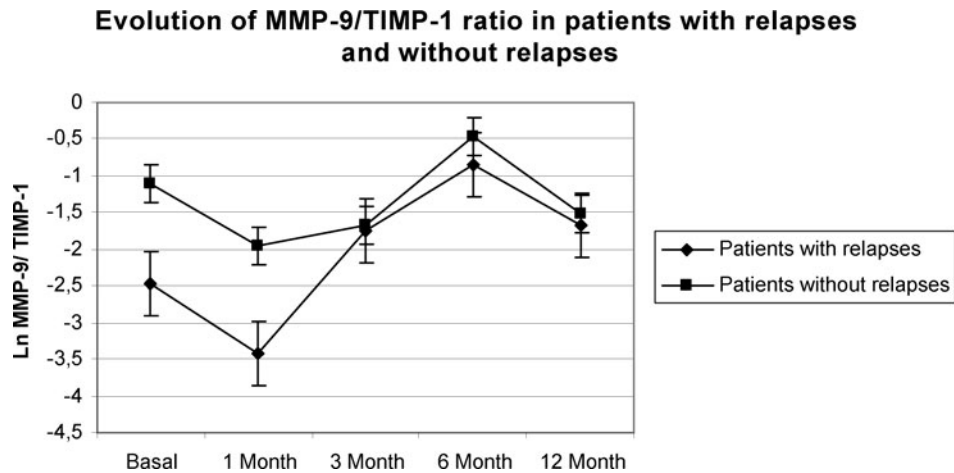
Comparison of evolution of MxA and MMP-9/TIMP-1 ratio according HHV-6 prevalence

The results are shown in Figures 6 and 7. Evolution of MxA was analyzed according to HHV-6 prevalence and it was possible to realize that those patients who were positive for HHV-6 in peripheral blood mononuclear cells (PBMCs) at the basal visit (before the onset of the interferon beta treatment) and were negative at the end of the follow-up, namely "patients with a decrease in HHV-6 prevalence," had higher levels of MxA than the rest of the patients (patients who were negative for HHV-6 in PBMCs at the basal visit and positive at the 12th month of the treatment, namely "patients with an increase in HHV-6 prevalence" or patients that were positive before the onset of the treatment and still at the end of the follow-up, namely "patients with no changes in HHV-6 prevalence").

As it could be seen in Figure 7, when we compared the patients who had had at least one positive serum sample for HHV-6 during the year of treatment with those patients who had never presented HHV-6 reactivation, it was found that whereas in the first group the MMP-9/TIMP-1 ratio increased from the first visit to the 12th one ($P = .02$), in the second group the ratio tended to decrease ($P = .1$).
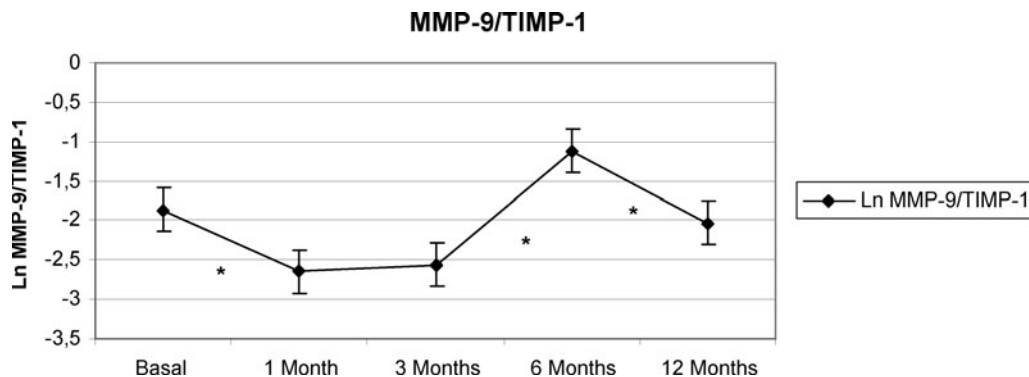
MMP-9/TIMP-1

Figure 3 Evolution of the mean of the ratio MMP-9/TIMP-1 in all patients (with and without relapses) along the 1-year follow-up study. Data are expressed as mean of Ln MMP-9/TIMP-1 $\pm$ S.E.M. *Significant differences between visits (*$P < .05$).

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## Evolution of MMP-9/TIMP-1 ratio in patients with relapses and without relapses

Figure 4 Evolution of MMP-9/TIMP-1 ratio along the 1-year follow-up study in patients that had suffered at least one relapse during the follow-up (patients with relapses) compared with patients that had not experienced any relapse during the year of treatment (patients without relapses). Data are expressed as mean of ln ratio ± SEM.
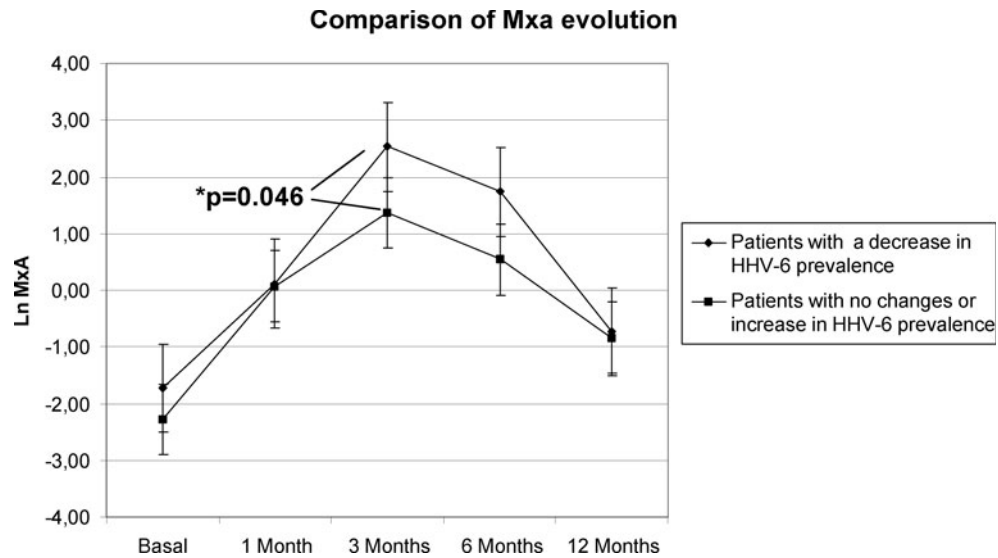
Discussion

There is a great controversy about the best method to measure interferon beta bioactivity in MS patients. This is a very interesting topic because many studies have shown that a high percentage of patients treated with interferon beta develop NAbs, and they inhibit or neutralize biological activity of interferon beta. Although type I interferons have many biological activities, only two assays are commonly used to measure NAbs: the cytopathic effect (CPE) assay and the MxA assay. Although more sensitive, CPE is complex and more expensive than MxA assay. Among all the interferon beta products, MxA is the most specific because it does not respond to activation to other receptors (Pachner *et al*, 2003). Also, MxA present a quite high dose-dependent specificity (Myhr *et al*, 2003; Deisenhammer *et al*, 1999), compared with other biological markers; hence, its expression has long been thought to be a sensitive measure of interferon beta bioactiv-

ity and reduced bioavailability following NAb formation (Gilli *et al*, 2004). In a recent study, Pachner *et al* (2003) showed that mRNA level of MxA is a good biomarker of interferon beta activity because it decreases by the presence of NAbs. As it is shown in Figure 1, in our study, MxA expression progressively increased from the beginning of interferon beta treatment until the third month and then it decreased. However, mean levels after a year of treatment were higher than basal levels. This result could mean that bioavailability of interferon beta reaches a maximum and then it falls, which is in agreement with studies that show that 42% to 98% of patients develop antibodies against interferon beta-1b administration (Giovannoni *et al*, 2002; Ross *et al*, 2000; Panitch *et al*, 2002). The time of appearance of NAbs depends on the product and dose regimen, but in general, it is possible to detect them between 3 and 18 months after the start of the treatment (The IFNB Multiple Sclerosis Study Group, 1995; Rice *et al*, 2001;

HHV-6 prevalence in PBMCs and serum samples
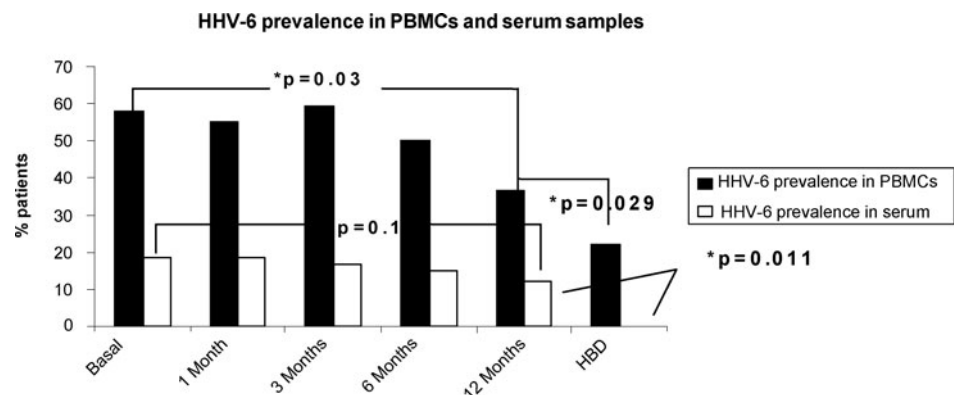
Figure 5 Evolution of HHV-6 prevalence in PBMCs and serum samples of patients treated with interferon beta along the five visits and HHV-6 prevalence of HBD. There are significant differences between basal visit and 12th month visit in PBMCs samples of interferon beta treated patients ($P = .03$). Moreover, HHV-6 prevalence in PBMCs and serum samples of HBD was significantly lower than in interferon beta treated patients at any visit ($P = .029$ and $P = .011$, respectively).

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Comparison of Mxa evolution

Figure 6 Comparison of evolution of MxA levels between two groups: (1) patients who were positive for HHV-6 in PBMCs at the basal visit and became negative at the end of the follow-up (patients with a decrease in HHV-6 prevalence) and (2) patients who were negative for HHV-6 in PBMCs at the basal visit and positive at the 12th month of the treatment (patients with an increase in HHV-6 prevalence) or patients who were positive for HHV-6 in PBMCs at basal visit and remain without changes along the 1-year follow-up (patients with no changes in HHV-6 prevalence). There is a statistically significant difference at third month ($^*P = .046$).

European Study Group, 1998). Interferon beta-1b provokes sooner apparition of NAbs, with most of patients being positive 6 months after starting treatment, compared with interferon beta-1a, in which it takes 9 to 15 months for the NAb-positive rate to reach a plateau (Ross *et al*, 2000). As it could be seen in Figure 2, patients who suffered at least one relapse in the year of observation presented low levels of MxA expression, compared with relapse-free patients, mainly at third month. This result coincides with earlier studies showing that the presence of NAbs decreases clinical effectiveness of interferon beta both in relapse rate and in number of gadolinium-enhancing lesions (Rudick *et al*, 1998;

Ross *et al*, 2000; The IFNB Multiple Sclerosis Study Group, 1995).

Although NAb formation has been associated with reduced MxA concentrations, novel data suggest that MxA may not be operative in the therapeutic effect of interferon beta in MS (Gilli *et al*, 2004). Recently, some studies have shown that matrix metalloproteinases (MMPs) may function as effectors in specific stages of MS pathogenesis. MS is an inflammatory disease characterized by blood-brain barrier (BBB) breakthrough, perivascular inflammation, and demyelination. Activation of systemic T cells and their transmigration throughout BBB are crucial events in MS pathogenesis (Raine *et al*, 1990; Hickey *et al*,

Comparison of Ratio evolution
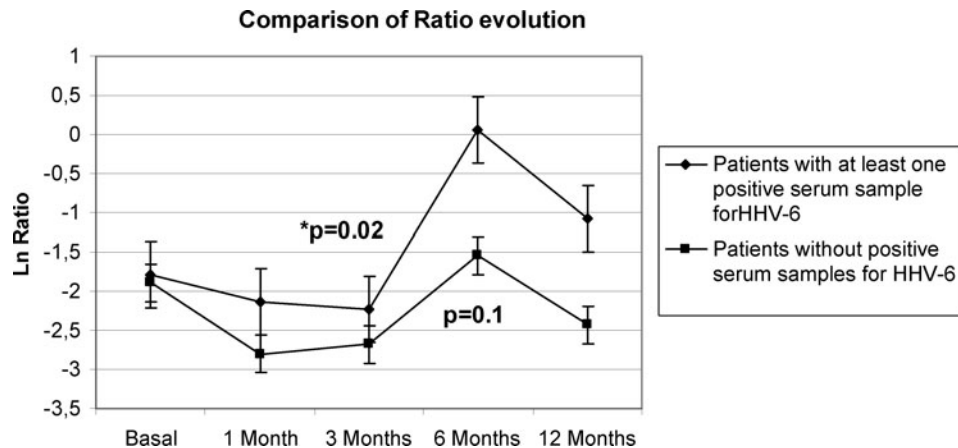
Figure 7 Comparison of MMP-9/TIMP-1 ratio between interferon beta–treated patients that had had at least one positive serum sample for HHV-6 during the follow-up and patients that had never presented virus reactivation. In the first group, the ratio was increased ($^*P = .02$) and in the second group, the ratio tended to decrease ($P = .10$). Comparison of two groups at last visit was statistically significant ($P = .03$).

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1991). Local secretion of MMPs may help T lymphocytes to cross the BBB and enter the central nervous system (CNS) (Opdenakker *et al*, 2001). Also, MMPs could be involved in the generation of autoantigenic epitopes through proteolysis of myelin proteins (Lepert *et al*, 2001). To prevent the destruction of tissues, MMPs activity must be hardly controlled by tissue inhibitors of metalloproteinases (TIMPs). The inhibitor of MMP-9, one of the most active MMPs in MS, is TIMP-1, and it acts by forming a heterodimeric noncovalent complex with MMP-9 (Yong *et al*, 1998). Recently, many works have suggested that the beneficial effect of interferon beta could be explained, at least in part, by its action on these two components of BBB integrity (Avolio *et al*, 2005; Waubant *et al*, 2003). In the present study, we have found a decrease in the MMP-9/TIMP-1 ratio (Figure 3) after 1 month of treatment, although there is hardly any change after 1 year of treatment. However, when patients with relapses were compared with relapse-free patients (Figure 4), different trends were shown: whereas in the first group the ratio was increased from the first visit to the fifth one, in the second group the ratio tended to decrease. These results are in agreement with previous studies probing that MMP-9/TIMP-1 ratio is a good marker of disease activity measured by new gadolinium-enhancing lesions (Avolio *et al*, 2005; Waubant *et al*, 2003), both in relapsing-remitting MS (RRMS) and in secondary progressive MS (SPMS).

Finally, the results seem support the hypothesis that interferon beta, as well as through its immunomodulatory effect, may be exerting its beneficial effect on MS through its antiviral activity. As it could be seen in Figure 5, HHV-6 prevalence decreased both in PBMCs and serum samples after 1 year of treatment. These results are in agreement with the study of Hong *et al* (2002), which shows that the prevalence and the amount of immunoglobulin M (IgM) against HHV-6 were lower in the serum samples of a group of patients treated with interferon beta compared to an untreated one. Furthermore, our results suggest that HHV-6 active infection could be involved in the pathophysiology of MS, because the percentage of individuals who had experienced at least one relapse during the study was lower for the group of patients who had not any positive serum samples for HHV-6. Likewise, in a work published by Chapenko *et al* (2003), the authors found a correlation between HHV-6 active infection and disease activity measured by MRI, both in RRMS and in SPMS; the researchers also found that the risk of exacerbation was higher in patients with active infection than in patients with latent infection. Also, it is important to note that MxA levels, which are the best correlated with disease activity, were correlated to HHV-6 prevalence (Figure 6) as well, because patients who experienced a decrease in HHV-6 prevalence had higher levels of MxA than patients with no effect of interferon over HHV-6 prevalence. Moreover, in the group of patients who had no HHV-6 re-

activations along the follow-up, the MMP-9/TIMP-1 ratio tended to decrease, whereas in the group of patients with viral reactivations, the ratio was increased (Figure 7).

In conclusion, these results suggest that MxA expression and MMP-9/TIMP-1 ratio correlate with interferon beta clinical effectiveness and, therefore, both parameters could be markers of bioavailability, although in our study MxA showed a better correlation than MMP-9/TIMP-1 ratio. Moreover, our results seem to indicate a possible role of HHV-6 in MS pathogenesis, because interferon beta appears to diminish HHV-6 prevalence and it seems to correlate with disease activity, because patients with viral reactivations had a higher relapse rate. Lastly, MxA and MMP-9/TIMP-1 ratio appear to correlate with HHV-6 prevalence, showing a good correlation between bioavailability and antiviral effectiveness. All in all, in order to confirm these results, new studies with more patients and longer follow-up should be performed.

Materials and methods

Patients and controls

Fifty four patients with definite relapsing-remitting MS (RRMS) were included in the study. None of them has been treated with immunosuppressive drugs, including steroids, or immunomodulatory agents (e.g., interferon beta or glatiramer acetate), or antiviral medications, for at least 6 months before the enrollment in the study. All the patients were treated with interferon beta-1b. Fifty healthy blood donors (HBDs) were recruited as controls. All the subjects were attended at Hospital Clinico San Carlos of Madrid. RRMS patients and controls were of similar age and sex distribution (Table 1). The cohorts were established with the approval of the local Ethics Committee of the centre, and all the participants signed informed consent after explaining the experimental procedures.

Samples

During a period of 1 year, pairs of blood and serum samples were collected along five scheduled visits: the first one at the basal visit (1 week before starting

Table 1 Clinical and demographic characteristics of MS patients and controls

	Patients	Controls	*P* value
Subjects	50	50	n.s
Males	17	17	n.s
Females	33	33	n.s
Age at recruitment (mean ± SD)	35.6 ± 6.9	31.4 ± 5.7	n.s
Age at onset (mean ± SD)	27.3 ± 7.1	—	—
EDSS (mean ± SD)	2.8 ± 1.3	—	—

interferon beta treatment), and four during the treatment (1, 3, 6, and 12 months after the beginning of the treatment). Moreover, a pair of blood and serum sample was collected when a patient suffered a relapse. A relapse was diagnosed when a patient experienced new symptoms or worsening of previous ones, at least 30 days after the onset of the latest attack. To be considered as a relapse, these symptoms must persist at least 24 h in the absence of fever or infectious process. Objective neurological signs must be present on examination with an increase in the EDSS and/or multiple sclerosis functional composite (MSFC) scores. Fatigue, sexual disturbances, or sphincter dysfunction cannot be considered as a relapse when they appear isolate.

At the time of visit, 10 ml of peripheral blood were drawn by vein puncture into sterile tubes with EDTA and directly used for DNA extraction. Furthermore, 2 ml of serum were isolated in serum separator tubes with another 10 ml of peripheral blood by centrifugation, they were separate into aliquots of 1 ml, and finally stored at –80°C for 3 months (median; range 2 to 5 months) until use.

DNA extraction
After collection, total DNA was extracted from PBMCs and serum. The QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) was used for the extraction of total DNA from PBMCs according to the manufacturer's protocol. DNA was resuspended in 200 μl of buffer. DNA concentration was determined by reading the optical density at 260 nm. Two blank reactions (only reagents without sample) were extracted together with each set of 10 samples in order to evaluate a possible cross-reaction.

Serum DNA was isolated by DNA spin column technique of QIAamp Ultrasens Virus Kit (Qiagen), from 1 ml of serum, according to the manufacturer's instructions. DNA was finally eluted in 70 μl of elution buffer. Two negative controls, consisting of reagents only, were processed with each set of 10 samples.

RNA extraction and reverse transcription (RT)
The total RNA was extracted from peripheral blood using the QIAamp RNA Blood Mini Kit (Qiagen). Recommendations of the manufacturer were followed. Total RNA was eluted in 60 μl of Rnase-free water. Blank reactions (extraction mixture alone) were interspersed within experimental samples. Purified RNA was quantified by spectrophotometry. Prior to RT, all the samples were digested with RNase-free DNase (Sigma, St. Louis, MO), at 70°C for 15 min, to ensure the removal of any possible DNA contamination.

RT was carried out with the Transcriptor first-strand cDNA synthesis kit (Roche Diagnostics, Barcelona, Spain). A previous 25°C incubation was followed by one cycle of 50°C for 1 h and 1 cycle of 85°C for 5 min to inactivate the reaction, and the cDNA was stored at –80°C until used for quantitative real-time polymerase chain reaction (PCR).

Table 2 qPCR primers and probes

HHV-6	FP: 5′-cgaaacgcctacacagaat-3′ RP: 5′ -caaagccaaattatccagagcg-3′ PROBE: 5′-MGB-DQ-cgtcacacccgaaggaat-FAM-3′
MxA	FP: 5′-aagctgatccgcctccactt-3′ RP: 5′-tgcaatgcacccctgtatacc-3′ PROBE: 5′-FAM-ccagatggaacagattgtctactgccag-TAMRA-3′
MMP-9	FP: 5′-ccctggagacctgagaacca-3′ RP: 5′-aaccatagcggtacaggtattcct-3′ PROBE: 5′-FAMtctcaccgacaggcagctggca-TAMRA-3′
TIMP-1	FP: 5′-ctgcggatacttccacaggtc-3′ RP: 5′-gcaagagtccatcctgcagtt-3′ PROBE: 5′-FAM-cacaaccgcagcgaggagtttctc-TAMRA-3′
18s RNA	FP: 5′-gcccgaaagcgtttactttga-3′ RP: 5′-tccattattcctagctgcggtatc-3′ PROBE: 5′-FAM-aaagcaggcccgagccgcc-TAMRA-3′

HHV-6 quantitative real-time PCR for DNA extracted from blood and serum
For the common strain detection of both variants (A and B) of HHV-6, quantitative real-time PCR was carried out with a specific set of two primers and probe (Table 2) (Hymas *et al, 2005*). A beta-globin PCR was carried out as internal control to ensure the quality of the extracted DNA from PBMCs of each sample and its suitability for PCR.

The reaction mixture for each PCR test contained Master Mix (1×) (Bioron, Germany), HHV-6 primers 400 μM (Bonsai Technologies, Madrid, Spain), HHV-6 probe 100 μM (Bonsai Tech), 8.4 μl of extracted DNA, and water to achieve a final reaction volume of 20 μl. Each sample was analyzed in duplicate.

The PCR was performed in a Rotor-Gene 3000 real-time cycler (Corbett Research, Sydney, Australia). After a preincubation at 95°C for 15 min to activate the Taq DNA polymerase, two-step thermocycling was performed for 45 cycles at 95°C for 15 s and 58°C for 60 s. To exclude the possibility of contamination during the PCR, one negative control was amplified for every five samples in each experiment, consisting of all reagents except sample DNA. We obtained a C_T value for each sample. For the final estimation of the DNA copy number, standard curves of known amounts of DNA from HHV-6B (Z-29) virions and from HHV-6A (U1102) (ABI Advanced Biotechnologies, Columbia, MO) were made. In every PCR assay, we made standard curves with a known copy number of HHV-6 (A and B) genomes (10000, 1000, 100, 10, and 1 copies). Each point of the curve was triplicate and C_T values of the samples in the assay were interpolated in the standard curve to obtain the exact HHV-6 viral load.

Quantitative real-time PCR for cDNA of MxA, MMP-9, and TIMP-1
To measure MxA, MMP-9, and TIMP-1 expression, quantitative real-time PCR was performed with primers and probes published elsewhere (Table 1) (Medeiros *et al*, 2003; Pachner *et al*, 2005; Higashikata *et al*, 2004; Jordan *et al*, 2004). Reaction

mixture for each PCR test contained Master Mix ($1\times$) (Bioron), 400 μM of primers (Bonsai Tech), 100 μM of probe (Bonsai Tech), 5 μl of cDNA, and water to achieve a final reaction volume of 20 μl. Each sample was analyzed in duplicate. The reaction conditions were 95°C for 15 min, and then two-step thermocycling (denaturation and annealing/extension) was performed for 50 cycles at 59°C.

As MxA, MMP-9, and TIMP-1 transcriptional expression were expressed in a relative manner, the results were normalized using the housekeeping gene rRNA18S as reference to avoid differences due to possible RNA degradation/contamination or different reverse transcription efficiency. As it has been previously described (Pachner *et al*, 2003), for the final relative quantification of the expression, in each assay, each RNA sample was assigned a ΔC_T value, calculated as C_T (for MxA or MMP-9 or TIMP-1) minus C_T (rRNA18S). Results were then normalized to normalization standard samples of RNA (healthy individuals), run at the same time as the RNA of the experimental points. These controls were assigned the normalization ratio of 1, and the normalization ratio of each experimental sample was calculated according to the following formula: $NR_{exp} = 2^{-\Delta\Delta Ct}$,

where $-\Delta\Delta Ct$ was the difference between ΔC_T for the experimental sample and the ΔC_T of the normalization standard. Total RNA of 25 healthy individuals was extracted and a pool was made to be used as the normalization control in each real-time run.

Statistical analysis
Data did not present a normal distribution (Kolmogorov-Smirnov test), therefore a logarithmical transformation was made. Data are expressed as mean $\pm$ standard error mean (SEM). To compare data from different visits (basal, 1 month, 3 months, 6 months, and 12 months), a paired-sample t test was undertaken. To compare data from two different groups of patients (patients with relapses versus relapses-free, patients with viral reactivations *vs* patients without reactivations, etc.), an independent-sample t test was conducted. The chi-square or two-tailed Fisher's exact test was used to test differences in categorical variables. Statistically significant differences were considered when $P < .05$.

GenBank accession numbers
MxA: M30817; MMP-9: AX011001; TIMP-1: AY932824; 18SRNA: X03205; HHV-6: NC001664.

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