Journal of NeuroVirology (2000) 6, Suppl 2, S109–S114

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Molecular evidences for a role of HSV-1 in multiple sclerosis clinical acute attack

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To verify the possible role of human herpesviruses as triggering or aggravating factors in relapsing-remitting multiple sclerosis (RRMS) clinical acute attack, we studied the prevalence of some herpesviruses in the peripheral blood mononuclear cells (PBMCs) collected from 22 MS patients during an MS relapse and in a stable phase and from 18 healthy controls (HC). DNA belonging to Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), Human cytomegalovirus (HCMV), Epstein-Barr virus (EBV) and Human Herpes virus 6 (HHV-6) has been searched by specific nested polymerase chain reaction (n-PCR). EBV and HHV6 DNA has been detected with high frequency in acute and stable MS and in healthy controls without significant differences. HCMV DNA was observed both in acute and stable MS but not in HC, and, more interestingly, HSV-1 DNA was only found in 13% of acute MS, while both stable MS and healthy controls were negative. On the basis of these results we focused on HSV-1, and to confirm them and to demonstrate that HSV-1 is actively replicating in MS patients during clinical relapse, we searched both messenger RNA (mRNA) and DNA of HSV-1 in the PBMCs of 15 acute MS patients and 15 healthy controls. We found HSV-1 mRNA and DNA in a significant number of acute MS patients but not in the control group. On the whole these data indicate that HSV-1 reactivate in the peripheral blood of MS patients during clinical acute attack and probably play a role in the triggering of MS relapses. *Journal of NeuroVirology* (2000) **6,** S109–S114.

Keywords: multiple sclerosis; herpesviruses; herpes simplex type 1 and 2; DNA; messenger RNA; clinical relapse

Introduction

Multiple sclerosis (MS) is a chronic human demyelinating disease of the central nervous system characterised from an autoimmune pathogenic process in genetically predisposed individuals (Poser, 1993). The mechanism that triggers the autoimmune disorder is still not clear, however several evidences suggest that environmental factors and in particular one or more viruses can be implicated in it (Sibley *et al*, 1985; Kurtzke, 1993; Ferrante and Mancuso, 1996; Kirk and Zhou, 1996; Dalgleish, 1997; Perron *et al*, 1997; Monteyne *et al*, 1998; Ross *et al*, 1999). Despite all the efforts and the numerous reports of viral isolation and the abundance of serological and molecular data, direct evidences in favour of the viral aetiology of MS are still lacking (Kurtzke, 1993; Dalgleish, 1997).

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Recently, different authors suggested that, besides their possible aetiologic role, viruses could trigger the exacerbation in the relapsing remitting form (Andersen *et al*, 1993; Panitch, 1994; Gran *et al*, 1999). In some of these surveys an association between clinically relevant viral-like infections, mostly characterised by fever and upper respiratory tract symptoms, and MS relapses has been evidenced. Moreover, serological evidences of asymptomatic viral infections or reactivations concurrent with an acute clinical attack have been obtained (Sibley *et al*, 1985; Andersen *et al*, 1993; Panitch, 1994). Furthermore, current long-term therapy with antiviral drugs like interferon, can be seen as a support to the idea of viral action in the evolution of MS (Bergstrom, 1999).

Since not all the acute MS phase are associated to a clinically evident viral infections, it must be hypothesised that also asymptomatic viral infections or reactivations could act as triggering factors

of the MS relapses. Many viral families are well known for their capability of establishing persistent infections in humans. Among these human herpesviruses seems of particular interest for their neurotropism and different members of this family have repeatedly suggested as possible aetiologic agent in MS (Martin, 1981; Bray and Culp, 1992; Bray and Luka, 1992; Sanders *et al*, 1996; Soldan *et al*, 1997).

In order to verify the possible relation between human herpesviruses and MS acute phase, we designed a longitudinal study of 26 relapsing remitting MS and on 20 healthy subjects to assess the prevalence of DNA belonging to five human herpesviruses by nested Polymerase Chain Reaction (PCR).

Then, since herpes simplex type 1 (HSV-1) DNA was found only in acute but not in stable MS patients nor in controls, we performed a second study to verify the active replication of HSV-1 by searching HSV-1 messenger RNA (mRNA) in the peripheral blood of 15 relapsing remitting MS patients during a clinical relapse and of 15 healthy controls.

Here we report the data obtained showing the possible involvement of HSV-1 in clinical acute attack of relapsing remitting MS.

Results

The results of the search of DNA belonging to the five different herpesviruses considered in the PBMCs collected from acute and stable relapsing-remitting MS patients and from healthy controls are reported in Table 1. As it is possible to note, EBV DNA detection was significantly more frequent in both acute and stable MS patients than in healthy controls only in the samples collected on the first day of enrolment in the study, while no difference among the three subjects categories have been found during the follow-up period. Likewise, no difference in the frequency of HHV-6 DNA detection has been found between the MS patients and controls.

The data obtained searching HCMV DNA are more interesting since it has been detected only in the PBMC samples collected from acute and stable RRMS patients (with a prevalence of 7.7% and 5.5% respectively) but not in those from the healthy controls.

Of particular relevance are the results on the HSV-1 and HSV-2 DNA that has been detected only in the acute relapsing remitting MS patients already on the first day of the acute attack (15.3%) and, with a variable frequency, during the follow-up. All the samples collected from the MS patients during the remission and from the healthy controls were negative. It is interesting to note that out of the seventeen HSV amplified DNA 16 belonged to HSV-1 and only one to HSV-2. This HSV-2 strain was detected from an MS patients that was positive only in one occasion on the third day of follow-up during a clinical relapse.

A general overview of the frequency of detection of DNA for the various viruses considered is exposed in Figure 1, in which the weighted mean prevalence for the acute MS patients and healthy controls and the absolute mean value for the stable MS cases are reported. Also from this type of analysis it is possible to see that the frequency of detection of EBV DNA is very high in all the three studied group, with 49.9% of positivity in acute MS, 50% in stable MS and 39.2% in healthy controls. Also for the HHV-6 a very high frequency of detection has been observed in acute MS (41.6%) and healthy controls (45.9%), and to a lesser degree, also in stable MS patients (22.2%), without any significant difference between the three groups. More interesting are the data about the HCMV DNA that has been detected with a low frequency only in acute (7.2%) and stable MS (5.5%) but not in the healthy subjects. Finally HSV-1 or HSV-2 DNA was evidenced only in the acute MS patients, with a mean prevalence of 12.1%, and not in the stable MS and healthy individuals.

The results of the first part of the study, induced us to perform a second set of experiments in order to verify the presence of HSV-1 DNA and to assess if this virus was latent or replicating in the PBMCs of MS patients. The data obtained on the 15 relapsing remitting MS cases and healthy subjects

Table 1 Distribution of viral DNA (expressed as positive samples/total samples and percentage) in the PBMC samples of relapsing-remitting MS patients and healthy controls (HC) at the various days of follow-up during clinical relapse and in a stable phase

		Acute attack days						
Virus	*Subject*	*0*	*3*	*7*	*10*	*14*	*17*	*Remission*
EBV	MS	14/26 (53.8)	12/26 (46.1)	12/26 (46.1)	14/26 (53.8)	10/23 (43.4)	7/11 (63.6)	9/18 (50)
	HC	4/18 (22.2)	8/18 (44.4)	8/18 (44.4)	6/17 (35.2)	6/13 (46.1)	6/13 (46.1)	–
HHV-6	MS	13/26 (50)	11/24 (45.8)	9/24 (37.5)	9/24 (43.8)	10/23 (43.8)	2/9 (22.2)	4/18 (22.2)
	HC	6/16 (40)	9/16 (56.2)	7/17 (41.1)	7/16 (43.7)	6/11 (54.5)	5/11 (45.4)	–
HCMV	MS	2/26 (7.7)	3/26 (11.5)	3/26 (11.5)	0/26 (0)	2/23 (8.6)	0/11 (0)	1/18 (5.5)
	HC	0/18 (0)	0/18 (0)	0.18 (0)	0/17 (0)	0/13 (0)	0/13 (0)	–
HSV-1/2	MS	4/26 (15.3)	2/26 (7.7)	3/26 (11.5)	5/26 (19.2)	1/23 (4.3)	2/11 (18.2)	0/18 (0)
	HC	0/18 (0)	0/18 (0)	0/18 (0)	0/17 (0)	0.13 (0)	0/13 (0)	–

included in the second part of the study are shown in Figure 2, in which it is possible to see that HSV-1 DNA was present in 36% of the patients already on the first day of acute phase (T0), in 35% on the tenth day (T10) and in 20% after 20 days (T20). HSV-1 DNA was also found in one of the two healthy controls that, at the moment of blood collection, were suffering from herpes labialis. The search of mRNA resulted positive in 45% of the MS patients at T0, in 28% at T10 and 20% at T20, with a slight decrease similar to that observed for DNA. We were unable to detect HSV-1 mRNA in the healthy controls including the two that had herpes labialis.

Discussion

Many epidemiological and laboratory evidences have suggested the possible role of viral infections in the triggering of the acute attack in RRMS patients (Andersen *et al*, 1993; Panitch, 1994), moreover many authors have already suggested the idea that herpesviruses could be involved in the complex aetiopathogenic processes that induce MS and characterise its clinical course. The study we performed is one of the few devoted to find possible relationships between asymptomatic herpes reactivation and the triggering of clinical acute attack in MS, and we believe that the large amount of data obtained lead us to draw some conclusions about the role of these viruses in the relapse of relapsing-remitting MS.

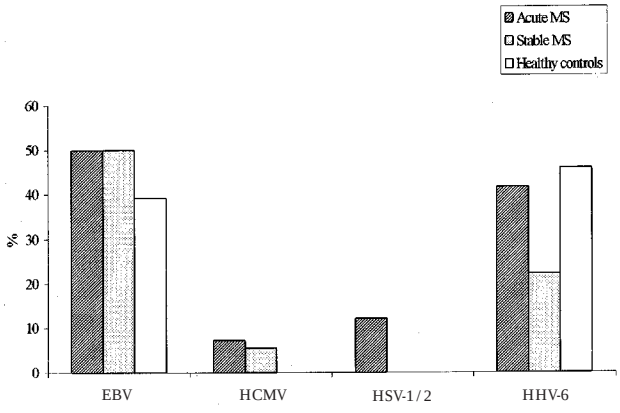

Figure 1 Percentages of prevalence of EBV, HCMV, HSV-1/HSV-2 and HHV-6 DNA in PBMCs of relapsing-remitting acute and stable MS patients and of healthy controls. The value observed in acute MS cases and in healthy subjects are expressed as weighted mean prevalence.

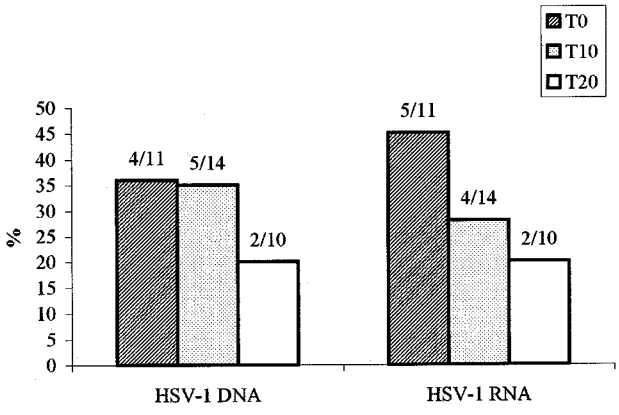

Figure 2 HSV-1 DNA and mRNA detection in the PBMC samples collected from MS patients during a clinical acute attack.

A first general conclusion that can be made is that a large number of PBMC samples from acute and stable MS patients and from healthy subjects resulted positive for EBV and HHV-6 DNA thus indicating that these viruses are widely circulating, while, on the contrary, the finding of HCMV and HSV-1 and-2 is very rare.

EBV has been already suspected of being involved in MS as a potential aetiopathogenic factor (Haahr *et al*, 1992,1995, Lindeberg *et al*., 1991, Munch *et al*, 1998) and in a preliminary study including a limited number of acute relapsing remitting MS cases (but not stable patients and HC) we have supposed a possible association of this virus with the acute attack to (Ferrante *et al*, 1997).

The data obtained in the present study, and in particular the observation of an high frequency of EBV DNA in PBMCs of acute and stable RRMS and of healthy controls without statistically significant differences, do not suggest a possible active role of this virus in the acute clinical attack of MS. It should be added that because EBV establishes a persistent infection in B-lymphocytes of the large majority of the general population, the high prevalence we observed in MS patients and healthy controls is not an unexpected result. HHV-6 can induce neurological diseases (Ishiguro *et al*, 1990; Huang *et al*, 1991) and it has recently been indicated as an aetiological agent of MS (Soldan *et al*, 1997). In our study, we did not observe significant differences in the frequency of HHV-6 DNA. Taken together these data don't allow us to totally exclude a role of these viruses in the triggering of relapse in relapsing-remitting MS. Further investigations, like the search of EBV and HHV-6 DNA and mRNA in the serum, should be used to verify if evident differences are present in the expression of these viruses between acute, stable MS patients and healthy controls.

The most interesting data obtained in our study are those regarding the distribution of HCMV and HSV-1 or HSV-2 DNA that has been detected only in MS patients but not in healthy controls. However it should be pointed out that some of the biological differences between these two viruses can give a different value to the results that at a first look seem to be very similar. It must

be underlined that our finding of HCMV DNA in the peripheral blood, although less common than that observed for EBV and HHV-6, is, in some way, not completely unexpected, since this virus is known for its capability to establish latency in peripheral blood leukocytes of a large majority of normal population. In an attempt to explain the finding of HCMV DNA only in MS patients, it should be underlined that MS is a disease in which several immunological abnormalities have been observed and thus it could be hypothesised that an imbalance in the leukocyte population in MS patients can favour the finding of HCMV DNA in the PBMCs. On the contrary it is well known that HSV-1 and HSV-2 establish the latency in sensorial neuronal ganglia and that during reactivation viral presence is restricted to the nervous system and to the innervated mucosal regions (Whitley, 1996). Thus the finding of HSV-1 in MS patients, and in one case of HSV-2, is of particular relevance. It should be pointed out that HSV-1 DNA has also been detected in the PBMCs collected on the first day of clinical acute attack, when the patients were still free from any therapy.

The value of this observation is greatly enforced from the results of the viral expression study, in which HSV-1 mRNA has been detected only in a relevant percentage of MS cases, but not in healthy controls. It should also be noted that one of the two healthy individuals that at the moment of blood collection were suffering of cold sores had HSV-1 DNA, but not mRNA, in his PBMCs.

In conclusion the results obtained in the present study indicate, as already suggested (Bergstrom *et al*, 1989; Lycke *et al*, 1996), that HSV-1 should be taken into consideration as a possible risk factor in the triggering and in the maintaining of acute clinical attack, and suggest the possible employment of specific antiviral drugs in pharmacological treatment of MS patients, and in particular of those with HSV-1 or HSV-2 reactivation, in order to reduce the frequency and gravity of relapses.

Materials and methods

Viral DNA prevalence study

Patients A total of 26 relapsing-remitting MS patients (17 females and nine males) diagnosed according to the criteria of Poser *et al* (1983), and followed at the Don C. Gnocchi Multiple Sclerosis Unit in Milan, have been considered. The mean of age at the moment of the enrolment was 31 years (range 20–46 years). As controls 18 healthy subjects, (12 female, six males) with a mean age of 29 years (range 19–36) have been studied. None of the relapsing-remitting MS patients or the healthy subjects was affected from immunological disorders and none of them had clinical symptoms suggestive of viral infection.

A sample of PBMCs was collected from all the MS patients at the moment of the admission at the MS Unit within 2 days from the onset of a clinical acute attack, confirmed by magnetic resonance imaging with gadolinium showing demyelinating areas of enhancement, and, twice a week for the following 3 weeks. A further PBMC sample was collected from 18 of these MS patients some months later (mean 8 months, range 6.5–9) when they were in a stable phase, documented from the absence of areas of enhancement at magnetic resonance imaging with gadolinium. PBMC samples were also collected from the healthy controls with the same timing applied to the RRMS cases.

Sample preparation for PCR PBMC pellet was obtained from heparinated peripheral blood centrifuged on a Ficoll solution (Amersham Pharmacia Biotech Italia, Cologno Monzese, Italy), stored at -20°C and lysates (lysis buffer: 10 mM Tris-HCl pH 8.3, 1 mM EDTA, 0.5% NP-40, 0.5% Tween-20 and 120 μg Proteinase-K). To perform PCR assay, 20 μl of PBMCs lysate (corresponding to 2.5×10^5 cells) were utilised.

PCR analysis To detect viral DNA, regions belonging to the DNA Polymerase gene of HSV1/2 and HCMV, and to the EBNA1 gene of EBV have been respectively searched by using specific nested-PCR methods whose primers and protocols have been previously described in detail (Ferrante *et al*, 1997). The major capsid protein gene of HHV6 has been searched using the primers and PCR protocol previously described by Secchiero *et al* (1995). In order to differentiate HSV-1 from HSV-2, the n-PCR product was subjected to enzymatic restriction by *Bam*HI (Roche Diagnostics GmbH, Mannheim, Germany), which specifically cleaves the HSV-2 n-PCR products.

HSV-1 mRNA study

Patients To evaluate the presence of HSV-1 replication, a second group of 15 acute MS patients (11 females and four males), diagnosed according to the criteria of Poser *et al* (1983) have been considered. The mean age was 36 years (range: 21–58). As control, we studied a group of 15 healthy subjects (13 females, two males), with a mean age of 31 (range 22–42). PBMCs were collected from RRMS patients at the first day of acute phase, before any pharmacological treatment, and at 10 and 20 days later, while for the healthy controls one sample of PBMCs was considered. It is important to underline that the healthy controls included two subjects suffering from cold sores.

RNA preparation RNA from peripheral blood were isolated using the RNAeasy Blood Mini (QIAGEN GmbH, Hilden, Germany) purification procedure. Briefly, erythrocytes were selectively

lysed and leukocytes were recovered by centrifugation. The leukocytes were then lysed using highly denaturation which immediately inactivated RNases to allow the isolation of intact RNA; the sample was applied to a spin column provided with a silicagel-based membrane; total RNA bound to this membrane, was washed away from contaminants, eluted in RNase free water and stored at -80°C. Prior to RT–PCR amplification, the elimination of any possible DNA contamination was performed treating all the samples with Deoxyribonuclease I (DNaseI, GIBCO BRL- Life Technologies Italia Srl, S. Giuliano Milanese, Milan, Italy) according to the protocols supplied by the producer.

Reverse transcriptase nested PCR Oligonucleotide primers specific for HSV-1 DNA polymerase gene were selected, to detect active replicating virus (Table 2). RT–PCR was performed on 0.5 μg RNA with the Titan One Tube RT–PCR System (Roche Diagnostics GmbH, Mannheim, Germany); to exclude possible HSV-1 DNA contamination, all the samples were tested for the presence of β-globin gene (Colombo *et al*, 1995). RNA from VERO cells infected with HSV-1 was included as positive control and to verify the sensibility of method. Likewise RNA from V0801 infected respectively with HCMV and VZV was utilized to evaluate the specificity of RT–PCR.

Table 2 Nucleotide sequence of the primers employed for HSV-1 reverse transcriptase nested PCR. The reported nucleotide position on HSV-1 genome is referred to the complete DNA sequence of the long unique region in the genome of HSV-1 (McGeoch *et al*, 1988)

Primer	*Nucleotide position*	*Sequence*
FHEX	62990–63011	5′-ACCCAGCGCCATACGTACTATA-3′
RHEX	63315–63334	5′-TGTGATGGCGTCCATAAACC-3′
FHINT	63021–63041	5′-ATGAATTTCGATTCATCGCCC-3′
RHINT	63302–63321	5′-ATAAACCGCGCGTGGAACTG-3′

Statistical analysis
The data obtained in the different groups were evaluated and compared using the Fisher's exact Test for small population.

Acknowledgements

This work was partially supported from a grant of the Fondazione Cassa di Risparmio delle Provincie Lombarde (CARIPLO), and from a grant Ricerca Corrente 1998, Ministero della Sanità, of the Don C. Gnocchi Foundation, IRCCS, Italy.

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