Acta Neurol. Belg., 2009, **109**, 277-282

Original articles

Broad screening for human herpesviridae DNA in multiple sclerosis cerebrospinal fluid and serum

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Abstract

Members of the human herpesviridae family are candidates for representing the macroenvironmental factors associated with multiple sclerosis (MS) pathogenesis. Real-time PCR was used to search for DNA of herpes simplex virus type-1/-2, varicella-zoster virus, cytomegalovirus, Epstein-Barr virus (EBV), human herpesvirus 6 type A/B in paired cerebrospinal fluid (CSF) and serum samples from 54 patients with MS, 34 of whom with active disease, 10 patients with other non-infectious neurological diseases, and 15 healthy individuals. All the CSF and serum samples were negative for the examined herpesviruses DNA, except one CSF sample from an MS patient, which was positive for EBV DNA. These findings do not support a role for the here-studied herpesviruses replication, whether in systemic or in the intrathecal compartment, as co-pathogenetic factors, nor as inducers of relapses, in MS.

Key words: Cerebrospinal fluid; Epstein-Barr virus; multiple sclerosis; real-time PCR; varicella-zoster virus.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the CNS, in which genetic and environmental factors could play a pathogenetic role. The latter factors are macroenvironmental, as they act at population level, and include infectious agents (Dyment *et al.*, 2006).

Biological characteristics and experimental observations make at least some human herpesviruses suitable candidate pathogenetic factors in MS. Indeed, i) herpes simplex virus type-1 (HSV-1)/-2 and varicella-zoster virus (VZV) latently infect ganglionic neuronal cells, with periodic reactivations that mimic the relapsing-remitting course of MS; ii) some herpesviruses induce demyelination in animals and in humans; iii) intrathecal antibody production to HSV, VZV, human herpesvirus 6 (HHV-6), and Epstein-Barr virus (EBV) characterizes MS (Sindic *et al.*, 1994; Simmons, 2001; Serafini *et al.*, 2007). Recent studies have focused the attention on EBV and VZV. EBV proteins were identified as targets of humoral and cellular immune response in MS patients (Cepok *et al.*, 2005) and EBV-infected B cells were found to be enriched in intrameningeal follicles in MS brains (Serafini *et al.*, 2007), whereas Sotelo and co-workers reported the presence of VZV in the CSF of all the studied MS patients with active disease (Sotelo *et al.*, 2008). Moreover, Herpesviruses active infection/reactivations could play a role in triggering MS relapses (Wingerchuk and Lucchinetti, 2007).

We aimed to analyse cerebrospinal fluid (CSF) and serum samples from MS patients and controls for the presence of herpesvirus DNA, and to assess whether viral replication in intrathecal/systemic compartment associates with MS, acute MS phases, or both.

Material and methods

CSF and serum samples from 54 consecutive patients with definite relapsing-remitting MS (McDonald *et al.*, 2001), 10 patients with other non-infectious neurological diseases, namely Alzheimer disease (n = 7) and amyotrophic lateral sclerosis (n = 3), and 15 healthy individuals were studied for the presence of HSV-1, HSV-2, VZV, cytomegalovirus (CMV), EBV, HHV-6 type A/B DNA. Table 1 shows the demographic and clinico-laboratory characteristics of patients and controls. All MS

Table 1

Demographic, clinical and laboratory characteristics of patients with multiple sclerosis (MS), patients with other non infectious neurological diseases (ONIND), and healthy controls (HC)

	MS patients (n = 54)	ONIND patients (n = 10)	HC (n = 15)
Sex, F/M	34/20	3/7	3/12
Mean age ± SD, yrs (range)	32 ± 7 (20-52)	71 ± 5 (58-80)	43 ± 7 (26-66)
Mean disease duration ± SD, yrs (range)	9 ± 5 (0.5-18)	4 ± 5 (1-6)	NA
Mean EDSS ± SD, yrs (range)	2.5 ± 1.5 (0.0-6.0)	NA	NA
Disease activity, active/stable	34/20	NA	NA
CSF OCBs, positive/negative (%)	52/2 (96)	0/10 (0)	NA
Moderate/severe blood-CSF barrier damage, positive/negative (%)	0/54 (0)	1/9 (11)	NA

EDSS, expanded disability status scale (Kurtzke, 1983); NA, not applicable; CSF, cerebrospinal fluid; OCBs, oligoclonal IgG bands.

patients were immuno-modulatory/-suppressive drug-free for at least 3 months prior to sampling, and studied within two weeks of onset of clinical symptoms (acute MS, n = 34), or at least 6 months after the end of relapses (stable MS, n = 20). In 12 of 34 acute MS patients, sampling coincided with the onset of the disease. CSF was centrifuged at 400 g for 10 min. CSF supernatants were analysed for routine examinations, including the assessment of oligoclonal IgG bands and aliquots stored at -80°C until analysis, together with corresponding paired sera. The samples were taken from patients for diagnostic purposes, or from healthy volunteers for studies on markers in HIV infection (Cinque *et al.*, 2005). Patients and controls gave informed consent to sample collection and storage, according to protocols approved by local ethics committees.

DNA was extracted from 200 mL CSF or serum using QIAamp Blood Kit (QIAGEN Inc., Chatswoth, CA, USA), according to the manufacturer's instructions, and eluted in 100 mL of water. Table 2 shows primers and probes used to detect DNA of the examined herpesviruses by real-time PCR. For each assay, a standard curve was prepared using serial dilutions of plasmidic DNA (for HSV-1, HSV-2, VZV, CMV, HHV-6A/B), or of two copies of EBV genomes, each of which contained in Namalwa cells (for EBV). Standard curves were constructed, in duplicate, using 10^6, 10^5, 10^4 and 10^3 copies/mL. CSF and serum samples were analyzed in triplicate. To exclude the presence of inhibitory substances in the samples, 10^5 copies/mL of standard was added into one of the three aliquots. Amplification and detection were performed using an ABI Prism 7900 Sequence Detection System (PE-Applied Biosystems, Cheshire, UK). To each well, 20 mL PCR mixture, consisting of 12.5 mL Universal Mastermix (PE-Applied Biosystem), 900 nM each primer, and 175 nM probe, was added to 5 mL sample. Cycling parameters were 50°C for 2 minutes, 95°C for 10 minutes, 50 cycles of 95°C for 15 seconds, and 60°C for 1 minute. A threshold cycle value was calculated for each sample by determining the point at which the fluorescence exceeds the threshold limit chosen for the specific plate, and plotted against each standard to create standard curves. Table 2 shows real-time PCR analytical sensitivities.

Results

No CSF or serum sample contained DNA of the examined herpesviruses, but one CSF sample from an active MS patient, which was positive for EBV DNA (2573 copies/mL). MS features of this patient showed no peculiarities, except high median relapse rate (3/year; disease duration, 12 years), and poor response to immunomodulatory therapies.

Discussion

In MS, while intrathecal, antiviral humoral immune response might result from non-specific B-cell activation (Franciotta and Lolli, 2005; Meinl *et al.*, 2006), the detection of viral genomes in the brain/CSF could provide direct evidence of intrathecal viral replication, thus supporting a viral role in MS pathogenesis.

We found evidence of the here-studied herpesvirus DNA in CSF and serum samples of none but one MS patient, and of none of neurological and healthy controls. These findings, which derive from a large screening within herpesviridae family, settle in a split literature on the topic. Whereas some members of the Herpesviridae family, such as HSV-1, HSV-2, CMV, HHV-7, and HHV-8, have

Table 2

Real-time PCR protocols for viral DNA detection

Virus	Genome region	Primers and probe	Primers and probe (5'-3')	Amplicon length (bp)	Detection limit (c/mL)	Ref.
Epstein-Barr virus	LMP1	LMP1 F	AAG GTC AAA GAA CAA GGC CAA G	64	100	§
		LMP1 R	GCA TCG GAG TCG GTG GG			
		LMP1 probe	6-FAM-AGG AGC GTG TCC CCG TGG AGG-TAMRA			
Herpes simplex virus-1	gD	HSV-1 Q1 F	GGC CTG GCT ATC CGG AGA	63	1000	
		HSV-1 Q2 R	GCG CAG AGA CAT CGC GA			
		HSV-1 probe	6-FAM-CAG CAC ACG ACT TGG CGT TCT GTG T-TAMRA			
Herpes simplex virus-2	US4-US5	HSV-2 Q1 F	AGA TAT CCT CTT TAT CAT CAG CAC CA	73	1000	
		HSV-2 Q2 R	TTG TGC TGG CAA GGC GA			
		HSV-2 probe	6-FAM-CAG ACA AAC GAA CGC CGC CG-TAMRA			
Varicella-zoster virus	ORF 4	Orf 4 F	ATG GCG TAC CGA GTC AAT GG	86	100	
		Orf 4 R	TAC GGG CCG TGC TAT TGA AG			
		Orf 4 probe	6-FAM-CAC GCT GGC TCC CGC GGT-TAMRA			
Human herpesvirus-6 A/B	US67	HHV-6 F	ATG CTG CCA GGT ACA AAG AGC	87 (A/B)	100	*
		HHV-6 R	AAA TGA CAA GYG CAC YGA G			
		HHV-6 probe A	6-FAM-CAG CCA TAT TTC CGG TAT ATG ACC TTC GTA AGC T-TAMRA			
		HHV-6 probe B	6-FAM-CAG CGA TAT TTC CGG TAT ATG ACC TTC GTA AGC T-TAMRA			
Cytomegalovirus	US17	US17 F	GCG TGC TTT TTA GCC TCT GCA	151	100	
		US17 R	AAA AGT TTG TGC CCC AAC GGT A			
		US 17 probe	6-FAM-TGA TCG GCG TTA TCG CGT TCT TGA TC-3-TAMRA			

Ref., references; § (Bossolasco *et al.*, 2002); * (Persson *et al.*, 2003).

only occasionally been associated with MS, a stronger association has been invoked for HHV-6, VZV and, EBV (reviewed in Simmons, 2001).

HHV-6 is a lymphotropic virus, but can infect various cell types, including CNS glial cells, and increased frequency of anti-HHV-6 antibodies and the periplaque presence of the virus have been reported in MS (reviewed in Ascherio and Munger, 2007). Several groups also detected HHV-6 DNA in MS CSF with frequencies ranging from 10 to 22% of the cases (Wilborn *et al.,* 1994; Rotola *et al.*, 2004; Mancuso *et al*., 2007; Alvarez-Lafuente *et al.*, 2008). However, we and others were unable to confirm these findings (Martin *et al.*, 1997; Mirandola *et al.*, 1999; Taus *et al.*, 2000; Beck *et al.*, 2003; Ahram *et al*., 2009; Yao *et al.*, 2009), despite the same methodology enabled to detect HHV-6 in the CSF of patients with encephalitis (Yao *et al.*, 2009; Bossolasco *et al*., 1999 and our unpublished results).

VZV is a neurotropic virus that latently infects the host. Recently, Sotelo and co-workers found high copy numbers of VZV DNA in the CSF of all the tested patients with MS (Sotelo *et al.*, 2008). The highest DNA copy numbers were detected at MS relapses, but high numbers were present even at remissions, months after the relapses (Sotelo *et al.*, 2008). This scenario of long-lasting VZV replication could support the efficacy of anti-herpetic drugs in MS, but MS clinical trials with these drugs proved they did not work (reviewed in Simmons, 2001), whereas high-dose corticosteroids, which could boost VZV replication (Torigo *et al.*, 2000), are the current therapy for MS relapses. Eventually, Burgoon and Colleagues, using the same technologies used by Sotelo and Colleagues (2008), found no herpesvirions or VZV DNA in MS CSF, or in acute MS plaques (Burgoon *et al.*, 2009).

EBV is a lymphotropic virus, whose strong association with MS is based on the increased frequency of late infectious mononucleosis, high titers of anti-EBNA IgG, and the almost 100% prevalence of latent EBV infection in MS patients (reviewed in Ascherio and Munger, 2007). Serafini and Colleagues, using in situ hybridization and immunohistochemistry techniques, found evidence of a preminently latent EBV infection in 21 of 22 autopsy MS cases (Serafini *et al.*, 2007), whereas Willis and Colleagues, using similar techniques, were not able to confirm the data (Willis et al., 2009). Serafini and Colleagues' series also included 16 autopsy CSF samples of MS patients, two of which were EBV DNA positive at a low copy number, whereas they were DNA negative for the other investigated viruses, namely HSV-1, HSV-2, VZV, CMV, HHV-6 and JC virus (Serafini *et al.*, 2007).

To give robustness to the present study, a) we included MS patients with a wide spectrum of disease duration and disability, and samples were collected during relapses and remissions; b) we used sensitive and specific real-time PCR assays currently employed for diagnostics, whose proficiency was regularly evaluated through international quality control programmes (e.g., our EBV DNA assay was 87% specific and 80% sensitive for the diagnosis of primary CNS lymphoma (Bossolasco *et al.*, 2002), and our CMV DNA assay was 92% specific and 87% sensitive for the diagnosis of CMV encephalitis (unpublished observation); c) actual analytical sensitivity values of our real-time PCR assays fall below those reported in Table 2 (e.g., analytical sensitivity values for HSV-1/2 range from approximately 200 to 1000 c/mL); and d) we tested, for the first time, healthy individuals to have a reference group of 'firm' negative controls and to enhance specificity.

Many methodological variables affect the rate of detection of viral genomes in CSF, including selection of patients and controls, stratification into pertinent subgroups, accuracy of sample collection and treatment, storage conditions, DNA purification, primer design and amplification procedures (Linde *et al.*, 1997). Still, there is concern about the variability of results obtained by different studies. Biological features of some herpesviruses, such as EBV and HHV-6, which can infect and persist in circulating B cells, imply that they might be detected in CSF due to the presence of CNS-patrolling lymphocytes, but also to traumatic lumbar puncture and/or inadequate cell centrifugation that make blood-derived cells be present in CSF specimens. Such possibilities, which could lead to misinterpreting latent intracellular infections as evidence of viral reactivations, were minimized in the present study, in which cell-free CSF samples were analysed. We did not store, and thus could not examine, cell pellets, and therefore we were not able to evaluate the presence of VZV DNA in the CSF mononuclear cells. On the other hand, other studies failed to demonstrate an association between higher numbers of CSF lymphomononuclear cells and the presence of viral DNA (Wilborn *et al.,* 1994; Rotola *et al.*, 2004; Mancuso *et al*., 2007; Alvarez-Lafuente *et al.*, 2008; Sotelo *et al.*, 2008).

We also studied serum samples since, in principle, peripheral reactivation of herpesviruses could trigger MS flares without need of intrathecal replication. Our negative results are consonant with the lack of efficacy of acyclovir/valacyclovir on clinical and radiological outcomes in MS (reviewed in Simmons, 2001).

In conclusion, our data do not support an association between the intrathecal or systemic herpesvirus replication and MS primary manifestations, or clinical relapses. This study also highlights the need for systematic use of methodological standards for molecular assays, in diagnostics and research settings.

Acknowledgements

This study was supported by grant RC2008 from the "Ministero della Salute", Italy. The authors thank Dr. Roberto Bergamaschi, head of the 'Mondino MS Center'.

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