Brains and peripheral blood mononuclear cells of multiple sclerosis (MS) patients hyperexpress MS-associated retrovirus/HERV-W endogenous retrovirus, but not Human herpesvirus 6

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Multiple sclerosis (MS)-associated retrovirus (MSRV)/HERV-W (human endogenous retrovirus W) and Human herpesvirus 6 (HHV-6) are the two most studied (and discussed) viruses as environmental co-factors that trigger MS immunopathological phenomena. Autopsied brain tissues from MS patients and controls and peripheral blood mononuclear cells (PBMCs) were analysed. Quantitative RT-PCR and PCR with primers specific for MSRV/HERV-W env and pol and HHV-6 U94/rep and DNA-pol were used to determine virus copy numbers. Brain sections were immunostained with HERV-W env-specific monoclonal antibody to detect the viral protein. All brains expressed MSRV/HERV-W env and pol genes. Phylogenetic analysis indicated that cerebral MSRV/HERV-W-related env sequences, plasmatic MSRV, HERV-W and ERVWE1 (syncytin) are related closely. Accumulation of MSRV/HERV-W-specific RNAs was significantly greater in MS brains than in controls (P = 0.014 vs healthy controls; P = 0.006 vs pathological controls). By immunohistochemistry, no HERV-W env protein was detected in control brains, whereas it was upregulated within MS plaques and correlated with the extent of active demyelination and inflammation. No HHV-6-specific RNAs were detected in brains of MS patients; one healthy control had latent HHV-6 and one pathological control had replicating HHV-6. At the PBMC level, all MS patients expressed MSRV/HERV-W env at higher copy numbers than did controls (P = 0.00003). Similar HHV-6 presence was found in MS patients and healthy individuals; only one MS patient had replicating HHV-6. This report, the first to study both MSRV/HERV-W and HHV-6, indicates that MSRV/HERV-W is expressed actively in human brain and activated strongly in MS patients, whilst there are no significant differences between these MS patients and controls for HHV-6 presence/replication at the brain or PBMC level.

INTRODUCTION

The aetiopathogenesis of multiple sclerosis (MS) disease is complex and debated. Immunopathogenic phenomena are thought to be triggered by environmental (viral?) factors operating on a predisposing genetic background (Noseworthy et al., 2000). Among the viruses suggested as MS co-factors are ubiquitous members of the family Herpesviridae, Human herpesvirus 6 (HHV-6) (Alvarez-Lafuente et al., 2004; Moore & Wolfson, 2002) and Epstein–Barr virus (EBV) (Christensen, 2006), and a human endogenous retrovirus (HERV), the MS-associated retrovirus (MSRV) (Dolei, 2005; Perron et al., 1989), a member of the HERV-W multicopy family; links between HERVs and some human diseases have been observed increasingly (Dolei, 2006). HHV-6 can be neurotropic, can become latent and be reactivated, and has potential immunopathogenic properties. Meta-analyses indicate that the available reports provide some support for a link between HHV-6 and MS, but none shows causative relationships (Clark, 2004; Moore & Wolfson, 2002). The association of MS with EBV is based on the increased frequency of late infectious mononucleosis, higher titres of
anti-EBV EBV IgG and the almost absolute prevalence of latent EBV infection in MS patients (Delorenze et al., 2006; Hollsberg et al., 2005). MSRV/HERV-W produces extracellular virus particles with gliotoxic (apoptotic) (Menard et al., 1997), fusogenic (Perron et al., 1997a) and super-antigenic (Perron et al., 2001) properties; the virus causes a T cell-mediated neuropathology in vivo (Firozzi et al., 2003). Activities strikingly concordant with findings on MSRV and MS (Dolei et al., 2002; Firozzi et al., 2003; Lafon et al., 2002; Perron et al., 2005; Sotgiu et al., 2002b) were reported for syncytin (Antony et al., 2004), an env protein encoded by the ERVWE1 locus (Blond et al., 1999) and involved in embryo implantation during pregnancy (Mi et al., 2000; Muir et al., 2004); this element is a replication-incompetent endogenous retrovirus belonging to the HERV-W family, located on chromosome 7q21–22, in a region of candidate genetic susceptibility for MS (Blaise et al., 2002). Moreover, a complex interplay might occur between HHV-6 and MSRV/HERV-W, as MSRV expression is transactivated in vitro by herpesviruses (Lafon et al., 2002; Perron et al., 1993) and the simultaneous presence of HERV and herpesvirus antigens has synergistic effects on cell-mediated immune responses (Brudek et al., 2004).

To date, MSRV virions have been detected in the blood and cerebrospinal fluid (CSF) of MS patients by several groups, including ourselves (Dolei et al., 2002; Komurian-Pradel et al., 1999; Nowak et al., 2003; Perron et al., 1997b; Serra et al., 2001; Sotgiu et al., 2002b). The virus is also detectable in other neurological patients (Dolei et al., 2002; Karlsson et al., 2004; Nowak et al., 2003), at significantly lower frequencies, and in about 10% of healthy individuals (Dolei et al., 2002; Garson et al., 1998). In a population at high risk of MS, we detected MSRV particles in the plasma of 100% of patients with active MS, and the presence of MSRV particles in CSF was found to parallel the progression of the disease (Dolei et al., 2002). Notably, blind serial examinations revealed that patients with MSRV-free CSF had stable MS, whereas those with MSRV-positive CSF disclosed a more severe, treatment-requiring disease, suggesting that the presence of MSRV in CSF could be considered as a negative prognostic marker (Sotgiu et al., 2002b, 2007). Accordingly, MSRV presence in CSF of monosymptomatic optic neuritis patients is associated with increased conversion to definite MS (Sotgiu et al., 2006).

To provide some insight into the role of these two viruses in MS, we evaluated their expression in brain and blood cells of MS patients and controls, as the mere detection of their genomes per se would not imply viral activity. This is particularly important for HHV-6 studies, as the vast majority of reports have detected virus DNA, and the need for studies to examine antigen and virus mRNA expression in MS and control brains to delineate the relationship between latent and active virus and MS has recently been underlined (Fotheringham & Jacobson, 2005). We evaluated two different transcripts for each virus [MSRV/HERV-W env and pol, encoding envelope and reverse transcriptase (RT), respectively; HHV-6 U94/rep, which maintains the latent state, and DNA-pol, indicative of actively replicating virus]. To our knowledge, this is the first study to evaluate both MSRV/HERV-W and HHV-6 and the first to use a fully quantitative approach to determine MSRV/HERV-W load.

**METHODS**

**Human samples.** Early post-mortem (mean interval, 8.5 h; range, 4–12 h) brain tissue from five subjects (mean age, 48 years; range, 37–65 years) with a clinical diagnosis of chronic progressive MS was studied. In total, 14 blocks containing lesions and normal-appearing white matter were examined. Histopathologically, cases MS1–MS6 had predominantly chronic-active lesions with hypercellular margins, ongoing demyelination with macrophage and lymphocyte infiltration and a hypocellular demyelinated centre. In cases MS7 and MS8, the majority of lesions were chronic-silent (absence of inflammation and presence of demyelinated centres). Controls (Table 1) consisted of four normal brain controls (NBCs), autopsyed tissue from subjects with non-neurological diseases (mean age, 45 years; range, 37–60 years; post-mortem mean interval, 12.2 h; range, 8–29 h), six patients with other neurological diseases (OND), autopsyed tissue from cases OND1 and OND2, and OND3 (Alzheimer’s disease, age 66 years; cerebral infarct, age 65 years) and biopptic samples from four patients with grade IV astrocytoma (mean age, 45.5 years; range, 42–52 years), including neoplastic and normal peritumoral tissue. In all autopsyed cases, sampling provided cortical structures and white matter from frontal and parietal lobes. All samples were snap-frozen and stored at −80°C, and 15 μm thick cryostat sections underwent immunohistochemistry. Samples for RNA extraction were from chronic-active MS lesions and/or periplaque tissue: grey and white matter from NBCs, astrocytomas (OND1–OND2), peritumoral (OND3) and normal tissue (OND4) surrounding OND1 and fronto grey matter from OND2 and OND6. Integrity of brain samples was controlled by immunohistochemistry (see below). We could analyse only a small number of brain samples, as it is not easy to obtain freshly frozen brain samples. Brain samples employed in this study were already available [obtained by one of us (B.B.) for diagnostic purposes over the past 5 years]; therefore, ethical approval was not necessary.

Blood samples from 35 patients with active MS and 14 healthy donors (HDs; blood donors from a transfusion centre) were derived from a cohort described previously (Dolei et al., 2002).

**RNA extraction.** Peripheral blood mononuclear cell (PBMC) separation and RNA extraction were described previously (Dolei et al., 2002; Serra et al., 2003). Thawed brain fragments were washed with PBS to remove blood trails and then RNA extraction was performed as described above.

**RT-PCR for MSRV/HERV-W pol mRNA.** RNA-derived cDNA samples were exposed to nested qualitative and non-nested semi-quantitative RT-PCR using MSRV/HERV-W pol-specific primers, as described previously (Dolei et al., 2002; Perron et al., 1997b; Serra et al., 2003). Briefly, 100 ng aliquots of undiluted RNA sample or each of a series of serial dilutions underwent reverse transcription into DNA by using oligo-dT as primer and M-MLV (Moloney murine leukemia virus) RT (Gibco-BRL Life Technologies) as described previously (Dolei et al., 2002), followed by PCR amplification of DNA products in a Hybaid thermal cycler (Omnigene) utilizing primers specific for the MSRV/HERV-W pol gene (Dolei et al., 2002; Garson et al., 1998). Controls included PCR of RNAs not exposed to RT with primers specific for the β-globin gene (primer pair PC04/GH20; Synthetic Genetics) or with MSRV/HERV-W-specific primers (to ensure the absence of contaminating cellular DNA
sequences and of endogenous retroviral DNA sequences, respectively), PCR of cDNA samples without template (negative control) and samples of human cellular DNA (positive control). Cellular RNA from PBMCs of individuals shown previously to be negative for circulating MSRV (and whose PBMCs did not release or transcribe MSRV/HERV-W in culture; Serra et al., 2003) was also included. Presence/absence of MSRV/HERV-W was confirmed in repeated assays of the same sample. The specificity of the amplified products was confirmed by dideoxy sequencing (see below). Semi-quantitative data were expressed as the reciprocal of each end-point copied dideoxy sequencing in both directions, with the fluorescent

Quantitative real-time RT-PCR for MSRV/HERV-W env, HHV-6 DNA-pol and HHV-6 U94/rep RNAs and generation of recombinant DNA external calibration curves. Primers and TaqMan probes were designed by using Beacon Designer software (PREMIER Biosoft International) (see Supplementary Table S1, available in JGV Online). HHV-6 primers and probes recognize both HHV-6A and HHV-6B variants [GenBank accession numbers X83413 (HHV-6A) and AF157706 (HHV-6B)]. Samples were heated to 95 °C for 3 min and then subjected to 50 cycles of 94 °C for 15 s, 53 °C for 30 s and 60 °C for 30 s in an iCycler iQ PCR detection system (Bio-Rad). Fluorescent data were collected during the 60 °C step. Each sample was analysed at least twice and the specific content was obtained through external calibration curves; gene fragments of interest, generated by conventional PCR, were inserted into plasmids (pCR2.1-TOPO; Invitrogen) as standards for the production of recombinant DNA (rDNA) (Pfaffl & Hageleit, 2001). We used rDNA standards instead of RNAs for RNA quantification as they give better results in terms of sensitivity, quantification range, reproducibility and stability (Pfaffl & Hageleit, 2001) and DNA standards have also been used in a recent paper on the development of broadly targeted real-time PCRs for semi-quantification of HERV pol RNA expression (Forsman et al., 2005). To control for correct amplification and reverse transcription, a positive sample was also submitted to each RNA-extraction procedure and the resulting extract was amplified in triplicate. Parallel RNA samples were also exposed to PCR amplification without the RT step to detect contaminating DNA. Prevention measures against cross-contamination were employed (Kwok & Higuchi, 1989); in particular, sample processing and PCR amplification were carried out in separate laboratories, with different equipment.

Phylogenetic analysis of MSRV/HERV-W env sequences. Amplified products were obtained by RT-PCR amplification utilizing primers specific for MSRV/HERV-W env (see Supplementary Table S1, available in JGV Online). The size of amplified products covered almost one-third of the env RNA sequence, including the intracellular region. Amplified products were then exposed to automated dideoxy sequencing in both directions, with the fluorescent BigDye system (Perkin Elmer ABI PRISM 310 Genetic Analyzer). Sequence analysis was carried out by using CLUSTAL_X (Thompson et al., 1997) for multiple sequence alignment and UPGMA (unweighted pair-group method using averages) for phylogenetic analysis. Sequences from patients and controls were compared with those of other HERV env sequences [GenBank accession numbers

Table 1. MSRV/HERV-W and HHV-6 expression in human brains from MS patients and controls

<table>
<thead>
<tr>
<th>Group</th>
<th>Brain sample</th>
<th>MSRV/HERV-W</th>
<th>HHV-6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pol†</td>
<td>env‡</td>
</tr>
<tr>
<td>MS‡</td>
<td>MS1 Periphery</td>
<td>+</td>
<td>81 920</td>
</tr>
<tr>
<td></td>
<td>MS2 Periphery</td>
<td>+</td>
<td>71 570</td>
</tr>
<tr>
<td></td>
<td>MS3 Plaque</td>
<td>+</td>
<td>105 550</td>
</tr>
<tr>
<td>NBC§</td>
<td>NBC1 White matter</td>
<td>+</td>
<td>3 824</td>
</tr>
<tr>
<td></td>
<td>NBC2 Grey matter</td>
<td>+</td>
<td>6 144</td>
</tr>
<tr>
<td></td>
<td>NBC3 Grey matter</td>
<td>+</td>
<td>926</td>
</tr>
<tr>
<td></td>
<td>NBC4 White matter</td>
<td>+</td>
<td>8 016</td>
</tr>
<tr>
<td>OND‖</td>
<td>OND1 Astrocytoma</td>
<td>+</td>
<td>4 787</td>
</tr>
<tr>
<td></td>
<td>OND2 Astrocytoma</td>
<td>+</td>
<td>3 856</td>
</tr>
<tr>
<td></td>
<td>OND3 Astrocytoma</td>
<td>+</td>
<td>413</td>
</tr>
<tr>
<td></td>
<td>OND4 Astrocytoma</td>
<td>+</td>
<td>3 440</td>
</tr>
<tr>
<td></td>
<td>OND5 Peritumoral</td>
<td>+</td>
<td>3 454</td>
</tr>
<tr>
<td></td>
<td>OND6 Normal</td>
<td>+</td>
<td>460</td>
</tr>
<tr>
<td></td>
<td>OND7 Stroke</td>
<td>+</td>
<td>141</td>
</tr>
<tr>
<td></td>
<td>OND8 Alzheimer’s</td>
<td>+</td>
<td>22</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>2 072 ± 1 986</td>
<td>9.0 ± 12.3</td>
<td>1.2 ± 3.5</td>
</tr>
</tbody>
</table>

†Positivity in nested RT-PCR.
‡Copies per 100 ng RNA.
§MS vs NBCs, Kruskal–Wallis H = 0.014.
§NBCs vs OND, not significant.
‖MS vs OND, P = 0.006.
**Table 2.** MSRV/HERV-W and HHV-6 presence/activity in PBMCs from MS patients and healthy blood donors

<table>
<thead>
<tr>
<th></th>
<th>HD (Healthy blood donors)</th>
<th>MS (patients with active MS)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HD</strong></td>
<td>All</td>
<td>HHV-6&lt;sup&gt;+&lt;/sup&gt; high&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>No.</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>Age (years):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>32.3 ± 9.2</td>
<td>40.1 ± 8.1</td>
</tr>
<tr>
<td>Range</td>
<td>19–52</td>
<td>33–52</td>
</tr>
<tr>
<td>Median</td>
<td>32</td>
<td>37.5</td>
</tr>
<tr>
<td><strong>P value&lt;sup&gt;‡&lt;/sup&gt; vs HD</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. copies per 100 ng cell RNA:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>890.5 ± 2.791</td>
<td>352.5 ± 523.1</td>
</tr>
<tr>
<td>Range</td>
<td>0–10 520</td>
<td>0–953.5</td>
</tr>
<tr>
<td>Median</td>
<td>0</td>
<td>1540</td>
</tr>
<tr>
<td><strong>P value&lt;sup&gt;‡&lt;/sup&gt; vs HD</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>HHV-6 DNA-pol DNA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. positive/total</td>
<td>3/14</td>
<td>0</td>
</tr>
<tr>
<td><strong>P value&lt;sup&gt;§&lt;/sup&gt; vs HD</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. copies per 100 ng cell RNA:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>8.6 ± 17.4</td>
<td>40.4 ± 6.6</td>
</tr>
<tr>
<td>Range</td>
<td>0–47.4</td>
<td>34.2–47.4</td>
</tr>
<tr>
<td>Median</td>
<td>0</td>
<td>104.0</td>
</tr>
<tr>
<td><strong>P value&lt;sup&gt;‡&lt;/sup&gt; vs HD</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>HHV-6 DNA-pol RNA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. positive/total</td>
<td>0/14</td>
<td>–</td>
</tr>
<tr>
<td>No. copies per 100 ng cell RNA:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Range</td>
<td>0–0</td>
<td>0–664</td>
</tr>
<tr>
<td>Median</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>P value&lt;sup&gt;‡&lt;/sup&gt; vs HD</strong></td>
<td></td>
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</tr>
</tbody>
</table>

*High copy numbers of HHV-6 DNA-pol DNA, i.e. actively replicating HHV-6.
†Fewer than 400 HHV-6 DNA copies.
‡Kruskal–Wallis H <i>P</i> value.
§Two-tailed Fisher’s exact test.
||Fewer than 10 copies.

AF331500 (MSRV virionic genome), NM_014590 (ERVWE1-syncytin mRNA), AJ289710 (HERV-H), Y18890 (HERV-K) and NM_207582 (HERV-FRD/syncytin2)].

**Immunohistochemistry.** The anti-HERV-W env monoclonal antibody (mAb) 6A2B2 (Blond et al., 2000) was employed in both immunoperoxidase and double-immunofluorescence procedures to assess HERV-W reactivity in brain tissues on each MS block, on 12 blocks from NBCs (three blocks for each patient) and on each OND sample. Procedures were described in detail elsewhere (Bonetti et al., 2003; Lolli et al., 2005). Immunoperoxidase staining was performed in duplicate and reactivity was graded as follows: −, no staining; +, staining present on <20% of cells (four representative fields at 40 x magnification for each case); 2 +, staining on 20–40% of cells; 3 +, staining on 40–60% of cells; 4 +, staining present on >60% of cells. Slides were viewed under a Zeiss MC80 microscope. For double fluorescence, anti-HERV-W reactivity was detected with streptavidin–Texas red (Amersham Biosciences); sections were then incubated with the glial fibrillary acidic protein (GFAP) phenotypic marker (1:500; Dako) followed by fluorescein-conjugated anti-rabbit Ig or anti-HLA-DR (1:10; Dako). To assess the cellular distribution in brain sections, double immunofluorescence with the neural phenotypic markers myelin basic protein (MBP; 1:200) for mature oligodendrocytes, GFAP (1:500) for astrocytes, CD68 for microglia (1:100) (all from Dako) and NG2 (1:100; Chemicon) for oligodendrocyte precursors was performed as described previously (Lolli et al., 2005). The reaction was visualized with appropriate fluorescein-conjugated antibodies (Vector Laboratories).

**Statistical analysis.** Significance of the results was evaluated by means of the Epi Info database and statistics software program, version 6 (CDC/WHO, Atlanta, GA, USA).
**RESULTS**

**Detection of HERV-W RNA transcripts and interrelationships between the known HERV-W env sequences**

All brain samples tested contained HERV-W env and pol RNA transcripts, irrespective of the health/disease status (Table 1); MSRV/HERV-W env was detected in PBMCs from 35 of 35 MS patients and four of 14 HDs (Table 2), in keeping with data of extracellular MSRV (Dolei et al., 2002). Samples of MSRV/HERV-W env products, obtained by nested RT-PCR amplification of brain RNAs from MS patients, were sequenced in comparison with env sequences of extracellular virus from plasma of MS patients and HD individuals from a previously described cohort (Dolei et al., 2002) of the Italian island Sardinia (which has the second-highest MS prevalence worldwide) (Sotgiu et al., 2002a), as well as to other HERV-W sequences present in GenBank, such as that of extracellular genomic MSRV (Perron et al., 2001) and of ERVWE1 (syncytin) (Mi et al., 2000) mRNA, and to env sequences from other endogenous and exogenous human retroviruses. For the sequence under study, i.e. the env intracellular domain, MSRV and ERVWE1 env RNAs have >89 % identity, and the env consensus sequence of extracellular genomic MSRV/HERV-W detected in Sardinian bloods shares >95 and >93 % identity with MSRV and ERVWE1, respectively (if the whole env gene is compared, MSRV and ERVWE1 env RNAs have >93 % identity and the Sardinian env consensus sequence shares >95 % identity with both MSRV and ERVWE1; data not shown), thus indicating that all of these genes are homologous to each other and that, until now, no specific primers have been identified to discriminate env sequences of extracellular MSRV from those possibly transcribed from other HERV-W endogenous DNAs, such as the ERVWE1 locus. A computer-derived phylogenetic tree of the above sequences is shown in Fig. 1. As shown, all sequences are related closely and belong to the same branch. As for the extent of identity in the gene fragment of Fig. 1, the env sequences detected in brain tissues from the MS patients share up to 96 % identity with the MSRV, ERVWE1 and Sardinian MSRV/HERV-W consensus sequence. All of the env sequences detected in our human samples belong unequivocally to the HERV-W family and are distant from other HERV families (38–47 % identity with HERV-FRD/syncytin 2, HERV-H and HERV-K) and from exogenous human retroviruses, such as human T-lymphotropic virus 1 and human immunodeficiency virus (20–25 % identity; data not shown).

**MSRV/HERV-W env and pol genes are upregulated in MS patients**

Quantification of MSRV/HERV-W env and pol RNA transcripts in brain and PBMC samples was carried out by real-time RT-PCR and semi-quantitative RT-PCR, respectively. Data are reported in Tables 1 and 2 and Fig. 2. As shown, MSRV/HERV-W env transcripts accumulated similarly in brain samples from NBCs and OND controls, without significant differences between white and grey matter. In brain samples from MS patients, however, the accumulation of MSRV/HERV-W transcripts was increased by 20- to 25-fold. Notably, very similar increases were obtained in MS patients by testing two different MSRV/HERV-W genes and by using two different techniques (env, 21.4-fold by quantitative real-time RT-PCR; pol, 23.3-fold by semi-quantitative RT-PCR) and the difference between MS and controls was highly significant (Table 1; Fig. 2). In PBMCs, MSRV/HERV-W env RNA copy numbers were higher in MS patients than in controls ($P=0.00003$; Table 2), due to the increased frequency of MSRV/HERV-W positivity, as the difference in MSRV/HERV-W env RNA copy numbers between...
MSRV/HERV-W-positive MS and healthy individuals was not significant (data not shown).

**MSRV/HERV-W env protein is detectable in the brain of MS patients, but not in normal brain**

The cellular distribution of brain sections and the integrity of autopsied samples, in comparison with biopsied ones, were assessed through immunofluorescence detection of neural phenotypic markers, such as MBP for mature oligodendrocytes, GFAP for astrocytes, CD68 for microglia neural phenotypic markers, such as MBP for mature oligodendrocytes, GFAP for astrocytes, CD68 for microglia were assessed through immunofluorescence detection of autopsied samples, in comparison with biopsied ones, of autopsied samples, in comparison with biopsied ones, were assessed through immunofluorescence detection of normal-appearing white matter of MS (autopsied) brains with respect to either autopsied or biopsied normal-appearing or OND brains (data not shown).

No staining by the HERV-W env 6A2B2 mAb (Blond et al., 2000) was detected in either grey or white matter in brain from NBCs (Fig. 3a) or from Alzheimer’s disease patients, or in brain tissue surrounding malignant astrocytoma (Fig. 3b), whilst scattered glial cells within neoplastic lesions showed MSRV/HERV-W immunoreactivity (Fig. 3c). In MS lesions, upregulation of MSRV/HERV-W immunoreactivity was observed within plaques, correlated with the extent of active demyelination and inflammation. In fact, MSRV/HERV-W immunoreactivity was absent in normal-appearing white matter and in perilesional areas (not shown). In chronic-silent MS lesions, very faint MSRV/HERV-W staining, located at the lesion edge on a limited proportion of glial cells (Fig. 3e), was observed in three of seven plaques (1+); in the remaining silent lesions, no staining was detected throughout. In chronic-active MS lesions, MSRV/HERV-W immunoreactive cells were abundant (40–60 % of total glial cells, 3+) and present throughout the entire lesion. In terms of localization on glial-cell subpopulations, MSRV/HERV-W immunostaining was observed on cells resembling both astrocytes and microglia at the lesion edge (Fig. 3f), as described recently (Antony et al., 2004; Perron et al., 2005); this finding was confirmed by double immunofluorescence, where the MSRV/HERV-W signal co-localized either with GFAP- or HLA-DR-positive cells (data not shown). At variance with active lesion edges, in plaque centres, the MSRV/HERV-W signal was mostly localized on hypertrophic astrocytes (Fig. 3g and insert).

**Lack of expression of HHV-6 sequences in brain and PBMCs of MS patients**

Given the ubiquitous distribution of HHV-6 infection in humans, two different real-time RT-PCRs were performed to discriminate between actively replicating virus (expressing DNA-pol RNA transcripts) and latent virus, which it is known to express only the U94/rep gene to maintain latency (Yoshikawa et al., 2002). As reported in Table 1, brain samples from MS patients had levels of both HHV-6 transcripts that were below detection limits (similar to those of other reports: Griscelli et al., 2001; Pfaffl & Hageleit, 2001). One NBC had latent HHV-6 and one OND had replicating HHV-6; however, copy numbers were close to detection limits. This indicates that, at variance with studies from other populations, HHV-6 is expressed only by a small minority (if any) of brain samples from our country, none of them with MS.

As for PBMCs (Table 2), 21.4 % of HDs and 34.3 % of MS patients had HHV-6 DNA. Virus DNA copies were slightly more abundant in MS patients than in HDs, and one MS individual had replicating HHV-6, as judged by detection of DNA-pol transcripts. However, differences of neither percentage HHV-6 positivity nor DNA copy numbers reached statistical significance. In the MS cohort, mean MSRV/HERV-W copy numbers did not differ significantly in HHV-6-positive and HHV-6-negative patients ($P = 0.13$, Kruskal–Wallis test).

**DISCUSSION**

Virus involvement in MS pathogenesis is a highly debated issue. According to the literature, the most likely candidate viruses as MS co-factors are herpesviruses, such as HHV-6 (Alvarez-Lafuente et al., 2004; Moore & Wolfson, 2002) and EBV (Christensen, 2006; Delorenze et al., 2006; Hollsberg et al., 2004; Perron et al., 2005); this finding was confirmed by double immunofluorescence, where the MSRV/HERV-W signal co-localized either with GFAP- or...
et al., 2005), and endogenous retroviruses, such as MSRV/HERV-W (Dolei, 2005; Perron et al., 1989). Qualitative/semi-quantitative differences between patients and controls for presence/expression of either MSRV/HERV-W or HHV-6 have been reported (Christensen, 2005; Clark, 2004; Dolei et al., 2002; Garson et al., 1998; Komurian-Pradel et al., 1999; Moore & Wolfson, 2002; Nowak et al., 2003; Opsahl & Kennedy, 2005; Perron et al., 1997b; Yi et al., 2004). The present study shows, for the first time, data on the simultaneous expression of both viruses in PBMCs and brain from MS patients and controls; they are strengthened by quantification of the expression of two genes for each virus, because, given their ubiquity, genome detection per se would not necessarily imply viral activity.

The origin of MS-associated HERV-W-related transcripts is debated (Dolei, 2005; Garson et al., 2005): it could be expression of isolated genes, such as syncytin (Mi et al., 2000), extracellular MSRV endogenous retrovirus particles (Firouzi et al., 2003) or a new HERV-W exogenous member of the HERV-W family (Dolei, 2005; Serra et al., 2003). However, no primers or antibodies are available for discriminating virion-producing/pathogenic MSRV/HERV-W from RNA or proteins normally expressed by endogenous HERV-W proviruses (Perron et al., 2005), and all known HERV-W env sequences are homologous (Fig. 1), including sequences present in GenBank or detected experimentally by us in brain tissues (that could be either intracellular RNAs or extracellular genomic RNAs), as well as in plasma samples of patients and controls (extracellular, virionic genomes).

In all brain samples tested, we detected the presence of both MSRV/HERV-W env and pol RNAs, regardless of the health/disease status of the individual. However, a statistically significant increase in expression was observed in MS patients with respect to those with normal-appearing brains or with other neurological diseases (whose reciprocal

Fig. 3. Immunoreactivity for MSRV/HERV-W in control brains and MS lesions. Brain sections were processed as described previously (Bonetti et al., 2003) and stained with the anti-HERV-W env 6A2B2 mAb (Blond et al., 2000). No immunostaining was detected in normal white matter from normal brain controls (a) or in brain tissue surrounding malignant astrocytoma (b), whereas scattered glial cells within neoplastic lesions showed MSRV/HERV-W immunoreactivity (c). Immunostaining was absent in a serial section of the same neoplastic tissue as in (b), but stained with an isotype-matched unrelated antibody (d). In chronic-silent MS lesions, a very faint signal was present at the lesion edge (e). Intense signal was observed in chronic-active MS lesions (MS2), being localized on cells morphologically resembling microglia and astrocytes at the lesion edge (f), whereas in the plaque core, MSRV/HERV-W staining mainly decorated astrocytic profiles (g and insert). Magnification, × 240.
differences were minor and not significant); the increase was similar for both genes, despite their evaluation with different assays (env, 21.4-fold by quantitative real-time RT-PCR; pol, 23.3-fold by semi-quantitative RT-PCR). This suggests a coordinated accumulation of the two transcripts, as occurs for genes located close together. Quantitative real-time data suggest that brains from MS patients and controls have a viral load of approximately $10^5$ and $10^{1.3}$ MSRV/HERV-W env copies per 100 ng RNA, respectively.

When serial sections of the same brain sample were analysed at the protein level (Fig. 3), the MSRV/HERV-W env protein was clearly detected only in samples from MS patients, perhaps due to lower sensitivity of this assay compared with PCR methodology. Alternatively, one might assume that MSRV/HERV-W RNA expression in the healthy brain is not followed by protein synthesis (most HERVs are defective and/or lacking 3' untranslated-region sequences that are necessary to stabilize RNA translation in eukaryotic cells), whilst in MS lesions (and in scattered glial cells within the tumour), MSRV/HERV-W env has acquired features that allow the process of translation. Immunostaining was found only within MS lesions and its intensity correlated with active demyelination and inflammation, whereas normal-appearing white matter from the same patients was found to be negative. In chronic-silent lesions, very faint staining for MSRV/HERV env was present on a minority of glial cells, whilst in chronic-active MS plaques, immunoreactive cells were abundant throughout the entire lesion. Around 50 % of total glial cells contained the env protein. As for glial-cell subpopulations, at the active lesion edge, env immunoreactivity was observed on cells morphologically resembling both astrocytes and microglia; in plaque centres, instead, the MSRV/HERV-W signal was mostly localized on hypertrophic astrocytes. These findings are in keeping with studies of MS brains (Antony et al., 2004; Perron et al., 2005) showing relative accumulation of HERV-W env RNA and protein in brain from MS patients.

We also detected MSRV/HERV-W env and pol RNAs in normal brains, whilst MSRV/HERV-W env protein was absent in samples from normal brain controls and from Alzheimer’s disease patients, as well as normal peritumoral tissues, but present in scattered glial cells within the tumour, thus confirming in vivo HERV-W expression found in cancer cell lines (Yi et al., 2004). In the interpretation of data from various diseases, one should remember that we evidenced a positive-feedback loop on MSRV expression in blood cells from MSRV-positive individuals (Serra et al., 2003); these cells release MSRV spontaneously in culture, which can be upregulated by exposure to the MS detrimental cytokines gamma interferon (IFN-γ) and tumour necrosis factor alpha (TNF-α), whilst IFN-β, a therapeutic cytokine for MS, is a powerful inhibitor of MSRV release. In keeping with this, HERV-W RNA was detected in patients with Alzheimer’s disease only in the presence of TNF-α (Johnston et al., 2001).

With respect to HHV-6, the other MS co-factor candidate, we could not detect HHV-6 expression in brain tissues of MS patients (Table 1), despite the use of fully quantitative real-time RT-PCRs that allow detection of both HHV-6 A and B variants and discriminate between actively replicating and latent virus. One normal NBC had latent HHV-6 and one pathological brain control had replicating HHV-6, but in copy numbers close to detection limits (which are comparable to those of other real-time PCR studies) (Donati et al., 2003); similar viral loads were found in a study of various brain regions from English MS cases and controls, without differences in the distribution, variant type or quantity of HHV-6 in brains from patients with MS compared with controls (Tuke et al., 2004). In contrast, a higher prevalence in the MS brain was found for HHV-6 DNA in an American cohort (Cermelli et al., 2003) and for HHV-6 antigen in a Finnish study (Virtanen et al., 2005). Similarly, HHV-6 DNA was found in all early MS lesions from five patients, albeit with scarce production of virus antigens, if any (Goodman et al., 2003). It was also suggested that HHV-6 might be more relevant in early than in established MS disease (Rotola et al., 2004). A recent paper showed HHV-6 expression in all brain samples of seven MS patients and three controls of an English cohort, restricted to oligodendrocytes, whose percentage positivity was significantly higher in MS patients than in controls (Opsahl & Kennedy, 2005). Two meta-analyses have been performed on the association of HHV-6 and MS (Clark, 2004; Moore & Wolfson, 2002), including studies on brain, CSF, blood etc. Among their conclusions is that, as HHV-6 is detected in a high proportion of individuals without MS, HHV-6 PCR positivity in itself is not sufficient for its causality in the development of MS; the available reports provide some support for a relationship between HHV-6 and MS, but none are able to show a causative relationship, and studies of prevalence of HHV-6 infection do not provide conclusive evidence for HHV-6-MS association. A recent review of potential HHV-6-induced disease mechanisms in MS underlined the need for studies of antigen and virus mRNA expression in the brain, to delineate the relationship between latent and active virus and MS (Fotheringham & Jacobson, 2005). It has also been hypothesized that the dysregulated immune system of MS patients is unable to control periodic HHV-6 flare-ups, which possibly contribute to MS pathology, where HHV-6 infection might affect neural-cell function (Alvarez-Lafuente et al., 2004; Fotheringham & Jacobson, 2005; Opsahl & Kennedy, 2005). To our knowledge, only two reports have been published so far on HHV-6 RNA expression in MS, in the brain (Opsahl & Kennedy, 2005) and in the blood (Alvarez-Lafuente et al., 2004).

In our opinion, both HERV-W and HHV-6 have the potential for a role in MS. However, MSRV/HERV-W is/are thought to be present in all human cells, whereas HHV-6, although specific seroreactivity is acquired very early by humans, can be variably present in the nervous system, according to its circulation/reactivation in different populations and cohorts. This could explain the wide differences
observed in the various studies (Bonetti et al., 2003; Clark, 2004; Fotheringham & Jacobson, 2005; Moore & Wolfson, 2002). In addition, herpesviruses might activate HERV expression in patients, as MSRV expression can be transactivated in vitro by herpesviruses (Lafon et al., 2002; Perron et al., 1993) and the simultaneous presence of HERV and herpesvirus antigens has pronounced synergistic effects on cell-mediated immune responses (Brudek et al., 2004). Christensen (2005) recently reviewed the possible interactions between HERV and herpesvirus, proposing to synergize the herpesvirus and HERV findings, and presented several possible pathogenic mechanisms.

In conclusion, our study shows the co-ordinated expression of MSRV/HERV-W pol and env in all brain samples, which is increased by 1.4 logs in MS tissues, where the env protein is also detectable. The data exclude actual HHV-6 involvement in the MS lesions under study and provide very limited evidence of brain infection by HHV-6 in our controls. This suggests either that HHV-6 acts very early during MS or that its reportedly increased presence is an epiphenomenon, deriving from the activation of a pre-existing latent virus in the brain. From the bulk of published reports, it appears that HHV-6, as with other herpesviruses, has the potential to transactivate MSRV/HERV-W (Brudek et al., 2004; Lafon et al., 2002) and to exert pathogenic phenomena on brain tissues (Christensen, 2005; Fotheringham & Jacobson, 2005). Nonetheless, in the MS lesions studied, we found MSRV/HERV-W activation in the absence of latent or replicating HHV-6. Our data from blood cells from a wider cohort reinforce the data from brains. In fact, even though the presence of HHV-6 DNA occurs in a minority of individuals, with a slight, but not significant, increase in MS PBMCs, we detected HHV-6 RNA expression only in one MS sample and in no controls (2.8 and 0%, respectively; \( P > 0.05 \), not significant). It must be pointed out that the increase of HHV-6 DNA in the healthy has been reported to range from 0 to 60% in different populations (Fotheringham & Jacobson, 2005), therefore explaining discordant reports of HHV-6 DNA in the healthy has been reported to range from significant). It must be pointed out that the presence of HHV-6 DNA occurs in a minority of individuals, its reportedly increased presence is an epiphenomenon, derived from the activation of a pre-existing latent virus in the brain. From the bulk of published reports, it appears that HHV-6, as with other herpesviruses, has the potential to transactivate MSRV/HERV-W (Brudek et al., 2004) and to exert pathogenic phenomena on brain tissues (Christensen, 2005; Fotheringham & Jacobson, 2005). Nonetheless, in the MS lesions studied, we found MSRV/HERV-W activation in the absence of latent or replicating HHV-6.

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REFERENCES


