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Short Communication

Regulation of human endogenous retrovirus W protein expression by herpes simplex virus type 1: Implications for multiple sclerosis

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The multiple sclerosis–associated retrovirus (MSRV), originally identified in cell cultures from patients with multiple sclerosis (MS), is closely related to the human endogenous retrovirus family W (HERV-W). Recently, HERV-W gag and env protein expression was demonstrated in MS lesions *in situ*. Here, the authors show that HERV-W gag and env proteins are induced by herpes simplex virus type 1 (HSV-1) in neuronal and brain endothelial cells *in vitro*. The transactivation of HERV-W proteins by HSV-1 could enhance their potential oligodendrotoxic and immunopathogenic effects, representing a mechanism by which HSV-1, and possibly also other herpesviruses associated with MS, may be linked to the pathogenesis of this disease. *Journal of NeuroVirology* (2006) **12,** 65–71.

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Although the etiology of multiple sclerosis (MS), an inflammatory demyelinating disease of the central nervous system (CNS), is as yet unknown, it is generally accepted that MS results from a complex interplay of environmental and genetic factors (Noseworthy, 1999). Based on various lines of evidence, viruses have thus repeatedly been implicated in the pathogenesis of MS (Cook *et al*, 1995; Kurtzke, 2000). Among these, herpesviruses such as the Epstein-Barr virus (EBV), human herpesvirus 6, and herpes simplex virus type 1 (HSV-1) have gained particular attention, although no *single* virus has so far been proven to be uniquely provocative (Dalgleish, 1997; Simmons, 2001).

The multiple sclerosis–associated retrovirus (MSRV) was originally identified in cell culture supernatants from patients with MS (Perron *et al*, 1989, 1997). Subsequent independent studies have confirmed an increased incidence (Dolei *et al*, 2002; Garson *et al*, 1998; Nowak *et al*, 2003) and a prognostic value (Sotgiu *et al*, 2002) of this retrovirus in MS. Interestingly, MSRV revealed to have genetically highly homologous counterparts in normal human DNA, the human endogenous retrovirus family W (HERV-W) (Blond *et al*, 1999). HERV-W most likely represents a proviral remnant of germline infection(s) by an ancestor active element 25 to 40 million years ago (Voisset *et al*, 1999) and is a multicopy gene family with at least 70 gag, 100 pro, and 30 env regions widely dispersed among human chromosomes (Voisset *et al*, 2000). To date, the precise origin of extracellular MSRV particles and their exact relation to HERV-W is not entirely clear. Available evidence, however, suggests that MSRV particles are not encoded by the regular HERV-W

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copies present in normal human DNA (Blond *et al*, 1999). Rather, they may originate from irregular (e.g., retrotransposed or recombined) endogenous copies or represent an exogenous member of the HERV-W family (Komurian-Pradel *et al*, 1999).

In contrast to the apparent inability of the HERV-W family for virion production, HERV-W–encoded proteins can be actively expressed in certain tissues or cells; the most prominent example for this being the HERV-W env protein (termed syncytin), which has been coopted during primate evolution to serve a physiological role in placentation (Mallet *et al*, 2004). Nevertheless, as HERVs may still possess some of the functions of infectious retroviruses, potentially pathogenic effects of HERVs have also been suggested, especially in presumed immune-mediated diseases such as MS (Christensen, 2005). Two recent studies have therefore analyzed immunohistochemically the expression of HERV-W proteins in normal human brain and MS lesions (Antony *et al*, 2004; Perron *et al*, 2005). The paper of Antony and coworkers describes HERV-W env expression in astrocytes and microglia of acute MS lesions, but not in control brains. Moreover, it additionally shows that conditioned medium of human fetal astrocytes in which a HERV-W env construct was expressed is cytotoxic to human and rat oligodendrocytes. Among the findings of the study of Perron and collegues was an apparently physiological expression of the HERV-W capsid (gag) protein in neuronal cells throughout different brain regions. Furthermore, HERV-W gag immunoreactivity was detected in brain endothelial cells in acute or actively demyelinating MS lesions, but not in endothelial cells of inactive MS lesions and normal control brains. The results of both studies seem compatible with a possible pathogenic role of HERV-W in MS and suggest that HERV-W–encoded proteins are regulated or inducible. As transactivation by herpesviruses is a known regulatory mechanism for exogenous and endogenous retroviruses (Kwun *et al*, 2002; Palu *et al*, 2001), we were interested to know whether HERV-W protein expression may be induced by HSV-1 *in vitro*.

Based on the detection of HERV-W gag in neuronal cells *in situ* (Perron *et al*, 2005), and pilot experiments that had suggested inducibility of HERV-W env by HSV-1 in IMR-32 cells (Lafon *et al*, 2002), we used this cell line to establish a cell culture model to analyze the regulation of HERV-W proteins by HSV-1 *in vitro*. IMR-32 neuroblastoma cells (ATCC CCL 127) were grown in RPMI1640 (Gibco), supplemented with 20% heat-inactivated fetal calf serum (FCS; Biochrom), 1× nonessential amino acids, as well as 100 U/ml penicillin/streptomycin, and infected by HSV-1 (strain Kupka, kindly provided by Prof. Peter Wutzler and PD Dr. Andreas Sauerbrei, University of Jena, Germany) at the indicated multiplicity of infection (moi) in serum-free medium. After a 1-h adsorption period, cells were washed and subsequently cultured in serum-containing medium. Cell lysates were prepared at different time points after infection using a buffer containing 1% NP-40, 125 mM NaCl, 50 mM HEPES, 1 mM EDTA (pH 7.4), and protease inhibitor cocktail (Roche). Lysates were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (protein concentration 50 μg per lane) and transferred to nitrocellulose membranes (Schleicher and Schuell). Membranes were then sequentially probed up to four times with different primary antibodies and developed according to standard protocols using appropriate secondary antibodies (Jackson Immunoresearch Laboratories) and enhanced chemoluminescence (Amersham Biosciences).

IMR-32 cells were highly susceptible towards infection with HSV-1, as demonstrated by the detection of the HSV-1 infected cell protein 0 (ICP0) as soon as 4 h post infection (p.i.) using a monoclonal ICP0 antibody (1:1200; Virusys Corporation) (Figure 1A). ICP0 is a nonstructural HSV-1 immediate-early protein synthesized in the infected cell soon after HSV-1 entry and functions as a pleiotropic transactivator of genes (Roizman and Sears, 1996). Coincident with this early sign of HSV-1 infection, a pronounced up-regulation of HERV-W env (~60 kDa) above weak baseline levels was observed using an anti-HERV-W env monoclonal antibody (3B2H4, 1 μg/ml). This antibody also reacted with the bacterially expressed recombinant MSRV env pV14 protein against which it was raised, showing an upper band of ~57 kDa, which corresponds to the expected length of the recombinant protein plus an additional His-tag, and a lower band of ~27 kDa, most likely representing an env fragment (data not shown). Of note, all antibodies directed against MSRV proteins generated so far crossreact with HERV-W epitopes, due to the high homology of MSRV and HERV-W at the protein level (Perron *et al*, 2005).

Next, expression and regulation of HERV-W gag was studied with the polyclonal anti-HERV-W gag F45128 antibody (2.5 μg/ml) (Perron *et al*, 2005). As seen in the first lane of Figure 1A, this antibody recognized the bacterially expressed recombinant MSRV capsid (gag clone 2) protein, which was used for rabbit immunization. Again due to a His-tag, the molecular weight of the recombinant gag protein is a bit above the calculated weight (42 kDa) of the MSRV gag clone 2 open reading frame (ORF) (Komurian-Pradel *et al*, 1999). In uninfected IMR-32 cells, the F45128 antibody reacted with a protein of ~55 kDa, whose expression levels, however, were not modified by infection with HSV-1. In contrast, parallel to the up-regulation of HERV-W env, a newly appearing ~70-kDa protein was detected by F45128 together with a fainter inconstant band slightly below this protein. A corresponding F45128 preimmune serum neither reacted with the recombinant gag protein nor with the ~55- and ~70-kDa proteins detected by the F45128 immune serum (data not shown). In preadsorption experiments, 3 μg of F45128 antibody

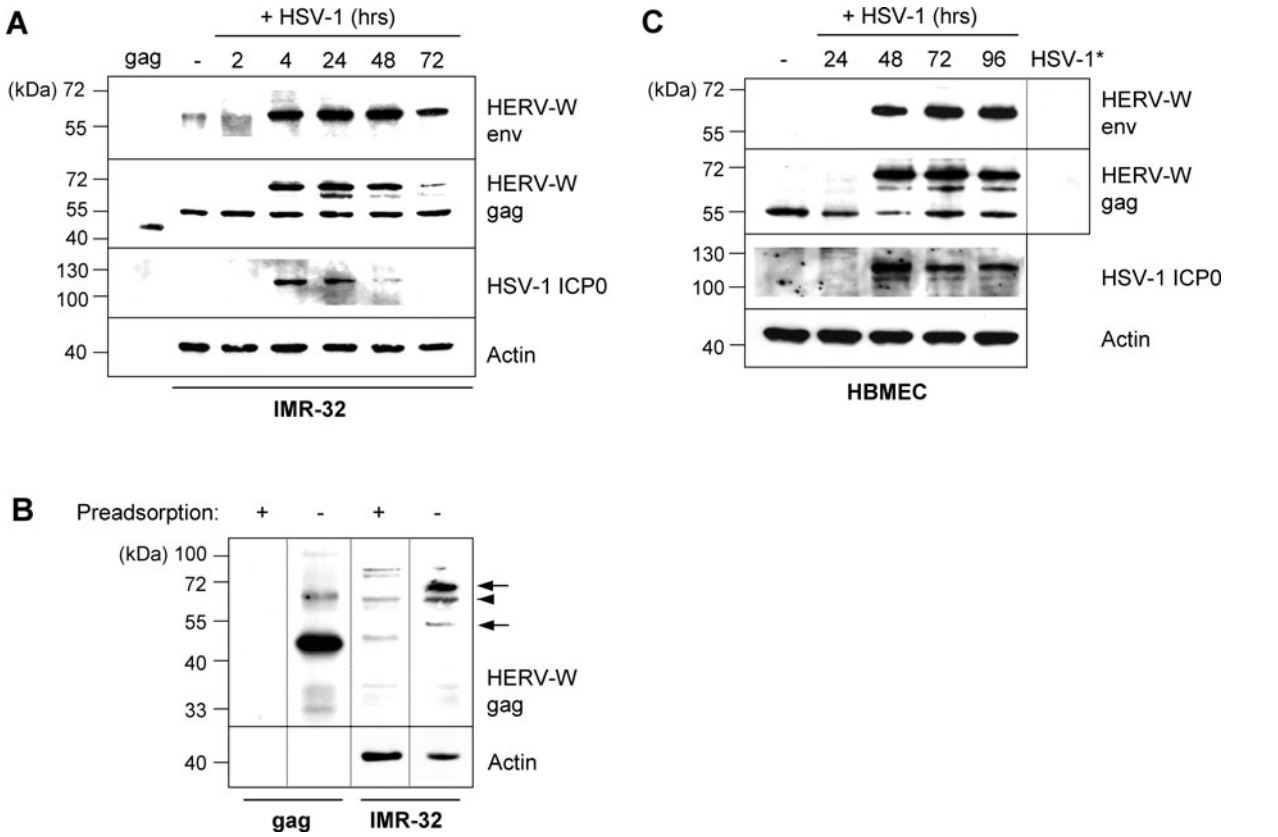

Figure 1 Regulation of HERV-W env and gag protein expression in IMR-32 cells and HBMECs by HSV-1. (**A**) IMR-32 cells were infected with HSV-1 (moi = 1) for 2 to 72 hours. Immunoblots were sequentially developed with the anti-HERV-W env mouse monoclonal antibody 3B2H4, the anti-HERV-W gag polyclonal rabbit antibody F45128, and a monoclonal antibody against HSV-1 ICP0 (124 kDa). Equal loading of lanes was proven with a polyclonal rabbit anti-actin antibody (42 kDa, Sigma). The protein weight marker (kDa) lanes are indicated on the left, gag = recombinant MSRV gag (clone 2) protein (25 ng). The experiment shown is representative of $n = 4$ independent experiments. (**B**) The anti-HERV-W gag F45128 antibody was preadsorbed (+) with the recombinant HERV-W gag protein or mock-preadsorbed (−) with PBS. Note that in IMR-32 cells (infected with HSV-1 for 24 hours), the ∼55 and ∼70 kDa HERV-W gag bands (*arrows*) are entirely abolished by the preadsorption procedure, as is the recombinant gag protein (= gag, 75 ng). A fainter band below the ∼ 70 kDa HERV-W gag protein (*arrowhead*) is also seen in the IMR-32 sample developed with the preadsorbed antibody, suggesting unspecific binding. (**C**) HBMECs were infected with HSV-1 (moi = 1) for 24 to 96 hours. HSV-1* = HSV-1 (2.5×10^4 PFU) was probed with anti-HERV-W gag and env antibodies in order to prove absence of crossreactive epitopes in HSV-1 preparations. The experiment shown is representative of $n = 3$ independent experiments.

were incubated with 15 μg recombinant gag protein and phosphate-buffered saline (PBS) in a volume of 100 μl or mock-preadsorbed with PBS only for 2 h at room temperature. This procedure completely abolished the recombinant gag protein band as well as the ∼55- and ∼70-kDa bands in HSV-1-infected IMR-32 cells, confirming the identity of the ∼55- and ∼70-kDa signals (Figure 1B). The weaker band observed below the ∼ 70-kDa band was unspecific, as it was also recognized by the preadsorbed antibody. As pilot experiments revealed a strong background in membranes developed with the preadsorbed antibody, probably due to low molecular substances present in the buffer of the recombinant gag protein interfering with enhanced chemiluminescence detection, both the preadsorbed and the mock-preadsorbed antibodies were microdialyzed (14- to 20-kDa pore size) against PBS before usage.

We further studied HERV-W protein expression in the SV40 large T antigen-transformed human brain microvascular endothelial cell line (HBMEC), in view of the disease-associated up-regulation of HERV-W gag in cerebral endothelial cells in acute MS lesions *in situ* (Perron *et al*, 2005). These cells were grown in RPMI 1640 containing 25 mM HEPES, GlutaMAX I (Gibco), 10% FCS, 10% NuSerum IV (Becton Dickinson), 1× nonessential amino acids and vitamins, 1 mM sodium pyruvate, 5 U/ml heparin, 30 μg/ml endothelial cell growth supplement (ECGS) (Sigma), and 100 U/ml penicillin/streptomycin on 0.5% gelatine (Sigma) coated flasks. Compared to IMR-32 cells, infection kinetics of HSV-1 in HBMECs were more prolonged with ICP0 becoming first detectable 48 h p.i. (Figure 1C). Otherwise, HSV-1–infected HBMECs behaved similar to IMR-32 cells, i.e., up-regulation of HERV-W env and a ∼70-kDa HERV-W gag protein coincided with the appearance of ICP0, whereas a ∼55-kDa gag protein expressed constitutively showed no consistent modulation. A fainter band below the ∼70-kDa HERV-W gag protein,

most likely corresponding to the unspecific band also observed in IMR-32 cells, was also visible in HBMECs. Although in some experiments very weak baseline levels of HERV-W env were observed (data not shown), this protein was not consistently detectable in resting HBMECs.

To exclude the possibility that HERV-W env and the ~70-kDa inducible gag protein may represent crossreactive epitopes from HSV-1 virions present in cell lysates of infected cells, seed virus stocks of HSV-1 were separated by electrophoresis, blotted, and developed with the F45128 and 3B2H4 antibodies. In these experiments, no bands of the appropriate size were detectable in HSV-1 stocks (Figure 1C), indicating that HSV-1 does not contain crossreactive HERV-W epitopes.

We could also investigate the expression of HERV-W in two different preparations (termed NH2904 and NH0205) of about 80% pure enriched primary human cerebral endothelial cells (HCECs). Following approval by the local ethics committee and informed consent, these cells were prepared from neurosurgical operation specimens, cultured, and characterized immuncytochemically as previously described (Ruprecht *et al*, 2001). In opposite to IMR-32 cells and HBMECs, no baseline expression of HERV-W gag was detectable in HCEC (Figure 2A). This discrepancy may be related to the SV40 transformation of HBMEC or a cell-type specific promotor activity of HERV-W long terminal repeats (LTRs), as reported in other human cell lines (Schon *et al*, 2001). HERV-W env was absent in one (Figure 2B), and showed low baseline levels in the other preparation (data not shown). Similar to HBMECs, ICP0 became visible in HCECs 48 h p.i. and coincided with the detection of a ~70-kDa HERV-W gag protein and a fainter, again probably nonspecific, band slightly below this protein (Figure 2A). Regardless of the somewhat unequal loading of lanes in the experiment shown, HSV-1 also up-regulated HERV-W env in HCECs (Figure 2B).

We here show that HERV-W env and gag proteins can be expressed in neuronal and brain endothelial cell cultures, providing a model to study their regulation *in vitro*. This is consistent with the previously described detection of HERV-W mRNA transcripts in several human tissues (including brain) and cell lines (de Parseval *et al*, 2003; Yi *et al*, 2004) and confirms that these transcripts may eventually be translated into proteins in some of those tissues or cell types. As only one of the 13 full-length HERV-W env elements identified in the human genome has a coding capacity, namely, the HERV-W copy on chromosome 7q (termed ERVWE1, OMIM 604659) (de Parseval *et al*, 2003), the HERV-W env protein recognized by the 3B2H4 antibody is likely to be encoded by ERVWE1. Accordingly, the molecular weight of the protein detected by 3B2H4 (~60 kDa) is compatible with the calculated molecular mass (59,565 Da, based on amino acid composition) of a nonglycosylated env precursor from the ERVWE1 ORF (Blond *et al*, 1999).

Although the putative complete gag gene from MSRV has an ORF for a gag protein with a calculated molecular weight of 54.9 kDa (Komurian-Pradel *et al*, 1999), a corresponding HERV-W ORF coding for a protein of such length has not been identified in normal human DNA (Voisset *et al*, 2000). Unexpectedly, the ~55- and ~70-kDa bands detected by F45128 are thus above the known coding capacities of HERV-W, the largest of which being a putative 45-kDa protein encoded on chromosome 3 (Voisset *et al*, 2000). The gag proteins detected by F45128 may therefore be produced by yet uncharacterized HERV-W gag genes in the human genome. Alternatively, mechanisms such as ribosomal frameshifting allowing expression of genes with in-frame stop codons or frameshifts in the DNA sequence should be considered. Finally, post-translational modifications such as protein glycosylation could also account for the elevated molecular weight observed on western blots.

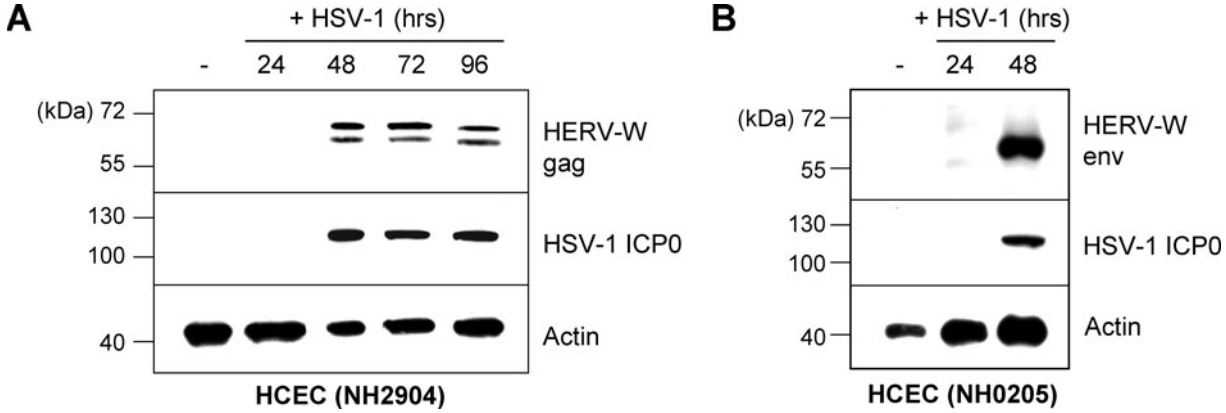

Figure 2 Regulation of HERV-W env and gag protein expression in primary HCECs by HSV-1. HCECs were infected with HSV-1 (moi = 2) for the indicated time periods. (**A**) HERV-W gag and (**B**) HERV-W env expression was analyzed by immunoblot as described for IMR32 cells and HBMEC. In (**B**) only a 48-hour kinetic analysis could be performed, as the amounts of primary cells were limited. Data shown are from separate experiments using two different HCEC preparations (NH2904 and NH0205) and are representative of $n = 2$ independent experiments.

The early timepoint of HERV-W protein upregulation upon HSV-1 infection, which coincided with the appearance of the HSV-1 immediate-early protein ICP0 in all cell types studied, is suggestive of a direct effect of HSV-1 on HERV-W expression. Indeed, using HERV-W LTR luciferase assays, it has been shown that HSV-1 can directly transactivate the HERV-W LTR and that this effect is partly mediated by ICP0 (Lee *et al*, 2003). The latter findings are also consistent with the previously reported enhanced MSRV particle production following HSV-1 infection or transfection with ICP0 plasmids in a cell line harboring MSRV (Perron *et al*, 1993). Increased transcription from retroviral elements may therefore be a general effect of ICP0 (Kwun *et al*, 2002; Lee *et al*, 2003). Although our data do not formally prove that up-regulation of HERV-W protein expression by HSV-1 results from an ICP0-mediated transactivation of the HERV-W LTR, they seem compatible with this assumption. Still, the inducibility of HERV-W proteins appeared to be variable, as only HERV-W env and the ~70-kDa HERV-W gag protein, but not the ~55-kDa gag protein, were up-regulated by HSV-1. An explanation for this could be that the ~55- and ~70-kDa gag proteins are coded for by different chromosomal HERV-W gag copies that are under the control of differentially activatable LTR promotors. HERV-W env and the ~70-kDa gag protein may thus underlie the same mechanisms of regulation, whereas the ~55-kDa gag protein does not. Future studies at the RNA level are warranted and should allow to address this question.

Because HERV-W env (syncytin) has been reported to have indirect oligodendrotoxic effects (Antony *et al*, 2004), toxic oligodendrocyte damage may be an important pathogenic consequence of HERV-W env up-regulation in the CNS. Although oligodendrocyte loss and demyelination have for a long time been recognized as neuropathological hallmarks of MS, recent data suggest that oligodendrocyte damage, i.e., extensive oligodendrocyte apoptosis, may in fact represent the earliest neuropathological feature of MS lesions, well before the appearance of infiltrating inflammatory cells (Barnett and Prineas, 2004). It is therefore tempting to speculate that transactivation of (endogenous) retroviral proteins with oligodendrotoxic properties could be related to, if not be the cause of, this oligodendrocyte damage. HERV-W env, the gene of which (ERVWE1), intriguingly, maps to a genetic susceptibility region for MS (Villoslada and Oksenberg, 2004), is an interesting candidate in this respect. The issue of HERV-W env–expressing cells in MS lesions is, however, not definitely resolved, because the pattern of HERV-W env immunoreactivity, possibly due to the different epitope specificities of the HERV-W env antibodies used, differed in the two neuropathological studies performed on this subject (Antony *et al*, 2004; Perron *et al*, 2005). As the HERV-W env–induced oligodendrotoxic activity is mediated by (a) soluble factor(s) (Antony *et al*, 2004), it seems nevertheless conceivable that activation of HERV-W env in neuronal and brain endothelial cells, but possibly also in other CNS cell types, e.g., astrocytes and (perivascular) microglia, or even outside the CNS, e.g., in immune cells bound to patrol the brain under normal conditions, may lead to oligodendrocyte damage, due to diffusion of the oligodendrotoxic factor(s) into the brain parenchyma.

Some retroviral env proteins are known to have superantigenic properties, i.e., they are capable of antigen-independent polyclonal activation of any T cells with a given T-cell receptor β-chain (Lafon *et al*, 2002). The transactivation of such a superantigen by common viruses, as exemplified by the EBV-induced up-regulation of a superantigen encoded by HERV-K18 env, which has been implicated in the pathogenesis of type I diabetes (Sutkowski *et al*, 2001), may thus be another mechanism linking exogenous viral triggers to presumed autoimmune diseases. Along this line, MSRV virions and a recombinant MSRV env protein have been shown to possess superantigenic functions (Perron *et al*, 2001). Whether HERV-W env has comparable superantigenic features is the subject of ongoing investigations.

Little is known about the potential physiologic or pathogenic functions of HERV-W gag. From an immunologic point of view, an important general consequence of an aberrant expression of endogenous retroviral proteins (e.g., HERV-W gag in brain endothelial cells) could be that these proteins may function as antigens. Indeed, processing of endogenous retroviral proteins in the HLA class I pathway may lead to their recognition by cytotoxic T lymphocytes (CTLs), resulting in an (auto-)immune attack. Such a phenomenon has been described in a patient with a melanoma who had CTLs targeting an endogenous retroviral antigen expressed in the melanoma (Schiavetti *et al*, 2002). Conversely, the presence of endogenous retroviruses may also lead to immune tolerance towards homologous exogenous retroviruses that may thus escape from immunosurveillance (Palmarini *et al*, 2004).

It has previously been suggested that the transactivation of an (endogenous) retrovirus could be a common pathogenic mechanism for several of the viruses that have been involved in MS (Lafon *et al*, 2002; Perron *et al*, 2001). The data provided here lend support to this concept. Although in the present report only HSV-1 was investigated, we consider it probable that transactivation of HERV-W may also be a feature of other (herpes)viruses. Indeed, the frequently evoked association of herpesviruses with MS may also reflect the genuine ability of *herpesviridae* to transactivate (endogenous) retroviruses (Palu *et al*, 2001; Sutkowski *et al*, 2001). Given the strong linkage between EBV and MS, we have recently reemphasized that EBV may play a particular prominent role in this context (Ruprecht and Perron, 2005).

The larger picture emerging is, however, complex and may involve the interaction of an endogenous

retrovirus family (HERV-W) with closely related exogenous retroviral elements (MSRV), which in turn may both be transactivated by common viruses, such as HSV-1. Along this line, several examples from animal retroviruses indicate that the pathogenic potential of retroviral families with endogenous and exogenous members results from a subtle interplay between the two variants (Fan, 1997; Xu *et al*, 1996).

Finally, beyond MS, HERV-W has been implicated in schizophrenia too (Karlsson *et al*, 2001). In view of intrauterine herpesviral infections as a risk factor for schizophrenia, and an association of HSV-1 seropositivity with cognitive deficits in schizophrenia (Yolken, 2004), expression of HERV-W gag and regulation by HSV-1 in neuronal cells may be of interest for further research into this disease as well.

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