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Human endogenous retrovirus (HERV)-W ENV and GAG proteins: Physiological expression in human brain and pathophysiological modulation in multiple sclerosis lesions

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Antigen expression of a human endogenous retrovirus family, HERV-W, in normal human brain and multiple sclerosis lesions was studied by immunohistochemistry by three independent groups. The HERV-W multicopy family was identified in human DNA from the previously characterized multiple sclerosis–associated retroviral element (MSRV). A panel of antibodies against envelope (ENV) and capsid (GAG) antigens was tested. A physiological expression of GAG proteins in neuronal cells was observed in normal brain, whereas there was a striking accumulation of GAG antigen in axonal structures in demyelinated white matter from patients with MS. Prominent HERV-W GAG expression was also detected in endothelial cells of MS lesions from acute or actively demyelinating cases, a pattern not found in any control. A physiological expression of ENV proteins was detected in microglia in normal brain; however, a specific expression in macrophages was apparently restricted to early MS lesions. Thus, converging results from three groups confirm that GAG and ENV proteins encoded by the HERV-W multicopy gene family are expressed in cells of the central nervous system under normal conditions. Similar to HERV-W7q ENV (Syncitin), which is expressed in placenta and has been shown to have a physiological function in syncytio-trophoblast fusion, HERV-W GAG may thus also have a physiological function in human brain. This expression differs in MS lesions, which may either reflect differential regulation of inherited HERV-W copies, or expression of "infectious" MSRV copies. This is compatible with a pathophysiological role in MS, but also illustrates the ambivalence of such HERV antigens, which can be expressed in cell-specific patterns, under physiological or pathological conditions. *Journal of NeuroVirology* (2005) **11,** 23–33.

Keywords: brain; endogenous retrovirus; HERV-W; immunohistology; multiple sclerosis; neuron; Syncitin

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Introduction

Previous studies on RNA associated with viral particles produced in choroid plexus or B-lymphocyte cultures from patients with multiple sclerosis (MS) had evidenced sequences corresponding to overlapping regions of a retroviral genome (Komurian-Pradel *et al*, 1999; Perron *et al*, 1997), which was provisionally named MSRV (multiple sclerosis–associated retrovirus element). MSRV was revealed to have genetically homologous elements in human DNA defining a novel family of human endogenous retroviruses HERV-W (Blond *et al*, 1999; Perron *et al*, 1997). HERVs are considered footprints of ancient retroviral germ-cell infections and comprise several percent of the human genome (Lower *et al*, 1996).

In normal human DNA, the HERV-W family is a multicopy gene family, most copies of which are truncated or lack open reading frames (orfs), with few ones retaining potential orfs for retroviral proteins. Thus, HERV-W proteins may be produced as complete or truncated proteins from various chromosomal copies in a tissue- or temporally restricted manner, as it occurs in other endogenous retrovirus families (Lower *et al*, 1993, 1996).

A complete HERV-W provirus is present on chromosome 7, HERV-W7q (Perron *et al*, 2000), in a region associated with genetic susceptibility to MS (Charmley *et al*, 1991; Wei *et al*, 1995). HERV-W7q encodes an envelope (ENV) protein that is strongly expressed in placenta (Blond *et al*, 1999), is involved in the physiological process of syncytiotrophoblast fusion (Blond *et al*, 2000), and was named "syncytin" (Mi *et al*, 2000). This HERV-W7q provirus is, nonetheless, not fully functional.

MSRV particles cannot be encoded by the defective endogenous HERV-W copies identified today in normal human DNA, unless under transcomplementation conditions (Girod *et al*, 1996). Thus, as in similar retroviral families such as mouse mammary tumour virus (MMTV) or murine leukemia virus (MLV) (Berlioz and Darlix, 1995; Xu *et al*, 1996), they may as well originate from a non ubiquitous endogenous copy—*de novo* retrotransposed or recombined—or from a transmissible member of the same family (Berlioz and Darlix, 1995; Christensen *et al*, 2002; Komurian-Pradel *et al*, 1999; Perron *et al*, 1992, 2000; Xu *et al*, 1996).

Independent studies have confirmed an association of MSRV virion RNA with both the occurence and the prognosis of MS (Dolei *et al*, 2002; Sotgiu *et al*, 2002). Differential MSRV/HERV-W RNA levels between MS and controls were also reported in lymphoid cells (Nowak *et al*, 2003).

Interestingly, the HERV-W pol copy number is not constant in DNA of human populations (Mirsattari *et al*, 2001), and active HERV-W proviruses can disseminate within human tumour cells (Yi *et al*, 2001, 2002). These data indeed indicate that HERV-W elements could retrotranpose in human cells and thus may generate novel and possibly recombined HERV-W copies in DNA of certain individuals or in certain cells of an individual, e.g., when triggered by cofactors. This assumption is also supported by a recent study showing significant differences in HERV-W pol copy number by a fluorescent *in situ* hybridization (FISH) technique, between MS patients and control individuals (Zawada *et al*, 2003). Moreover, HERV-W ENV retains properties of an infectious retrovirus envelope protein (An *et al*, 2001), which is consistent with our previous observation that MSRV virions could be transferred from "MSRV-positive" cultures to "MSRV-negative" cells *in vitro*, and further replicate in newly infected cells (Perron *et al*, 1992).

More recently, a differential RNA expression of various gag, pol, and env HERV-W copies from different chromosomes has been evidenced in normal and tumour human tissues (Yi *et al*, 2004), which suggests that the HERV-W family may express various antigens in human cells, other than the already characterized "syncytin" placental expression.

Therefore, we have designed the present study in order to characterize the expression of MSRV/HERV-W proteins in normal human brain and MS lesions. For this purpose, we have used a panel of antibodies specific for proteins encoded by the MSRV/HERV-W retroviral family (Komurian-Pradel *et al*, 1999) and several controls, including antibodies against rabies, human immunodeficiency virus (HIV)-1, and another family of human endogenous retroviruses (HERV-K), that can express proteins in human cells (Lower *et al*, 1993).

We here provide evidence that MSRV/HERV-W proteins are physiologically expressed in human brain and that this expression is modulated in MS lesions.

Results

A selection of antibodies that revealed appropriate for brain immunohistology, with clear labeling and reproducible patterns according to all results detailed below, is presented with labeling patterns in Table 1.

Austrian cohort

In our series we tested immunoreactivity of 10 different antibodies on brain autopsy material from acute MS, chronic active MS, chronic inactive MS, and controls. From these 12 antibodies, 6 did not show a specific staining in our technical conditions (3C1D5, 2A12A5, 6A2B2, 5B6F8, 4E4A11, and 4A9E3), they were consequently not used for immunohistological analysis. The other antibodies revealed three different, in part overlapping, staining patterns.

Reactivity for leucocytes, microglia, and endothelial cells: The paradigmatic antibody showing this staining reaction was 13H5A5, a monoclonal

Table 1 Selection of anti-HERV-W antibodies appropriate for brain immunohistology with consensus results obtained from all cases

		Antibodies				
	Brain regions	*13H5A5 monoclonal*	*5E9H9 monoclonal*	*2G5E12 monoclonal*	*F45128 polyclonal*	*Control*
Specificity		ENV protein	ENV protein	GAG protein	GAG protein	HIV-1, HHV6-A, rabies virus
Normal brain						*C. Pneumoniae*
Microglia	All	+++	++			
Endothelial cells	All	+	+		Negative	Negative
Neurons	All	Negative	Negative	Negative	++	Negative
Neurons	White matter axons	Negative	Negative	Negative	++	Negative
MS Brain						
Lymphocytes	All lesions	+++ *	++*			
Monocytes	All lesions	+++ *	++ *			
Activated macrophages	Recent demyelination	+++	++	Negative	Negative	
Foamy macrophages	Inactive lesions	Negative	Negative			Negative
Endothelial cells	All lesions	+	+			Negative
Endothelial cells	Acute/active lesions	(+)	(+)	+++**	+***	Negative
Neurons	Cortex	Negative	Negative	+	++	Negative
Neurons	White matter axons/lesions	Negative	Negative	+	++	Negative
Neurons	Dystrophic axons	Negative	Negative	Negative	++++	Negative

*Endogenous HERV-W ENV and GAG antigens are detected in normal lymphoid cells, but such cells are not detected in normal brains.
**In a subset of actively/acutely demyelinating progressive MS cases.
***In 8/12 of MS cases with actively demyelinating lesions, 0/4 chronic incative MS cases and 0/4 other CNS diseases controls.

antibody raised against the envelope protein (ENV) and targeting an epitope located in the N-terminus surface protein fragment (SU). In normal control brain, microglia was consistently stained both in the gray and white matter (Figure 1a, b). A similar "normal ENV" reactivity was also found in the normal appearing cortex and white matter of MS brains, irrespective the stage and type of disease. Additionally, in controls and normal brain tissue of MS patients, some blood vessel endothelial cells were immunoreactive. Within the MS plaques (Figure 1c to e), irrespective of demyelinating activity, an intense "ENV" labeling of lymphocytes was present.

Nonetheless and quite surprisingly, macrophages expressed the ENV antigen in areas of recent demyelinating activity only, whereas the classical foamy macrophages in inactive lesions were negative. Endothelial cells within the plaques expressed the ENV antigen in variable extent, both in active as well as in inactive lesions. Similar reactions pattern with weaker staining were found with the anti-ENV monoclonal antibody 5E9H9 (see Table 1).

Prominent endothelial reactivity in active MS cases: This pattern was found with the anti-GAG monoclonal antibody 2G5E12. The most characteristic and intense staining was a prominent GAG immunoreactivity of cerebral endothelial cells within demyelinated plaques in a subset of patients with acute or actively demyelinating chronic MS (Figure 2a to c). In these cases, it was present on most vessels, irrespective of their type (capillaries, arteries and veins). This prominent endothelial "GAG" staining was associated with weak immunoreactivity in the cytoplasm of cortical neurons and in some white matter axons. In contrast to the profound "anti-ENV" microglia staining described above, immunoreactivity for microglia with this anti-GAG antibody was absent in cases with actively demyelinating MS lesions. No accumulation of the HERV-W GAG epitope detected by 2G5E12 monoclonal antibody was found in dystrophic axons.

Immunoreactivity in neurons and axons with profound accumulation of reactivity in dystrophic axons of demyelinated plaques: This pattern was found exclusively with the polyclonal (Rabbit) anti-GAG antibody F45128. "Normal" axonal and neuronal staining was constantly present in control as well as MS cases (Figure 3 a to c). The staining was independent from lesional activity in MS patients. In the cortex, large neurons were stained with increased GAG detection in the dendrites. Similarly, axons in the white matter showed consistent GAG immunoreactivity, large axons being more intensely labeled than small caliber fibers.

The most prominent reactivity, however, was found in dystrophic axons within active or inactive MS plaques (Figure 3b). The staining pattern was similar to that of β-amyloid precursor protein, a protein that is produced within neurons and moved along the axon by fast axonal transport. In addition to axonal GAG immunoreactivity detected with F45128 antibody, we found weak GAG endothelial staining in 8 out of 12 MS cases with actively demyelinating lesions, but in none of the cases with inactive disease or in controls.

No immunoreactivity was found in brain sections when the primary antibody was omitted

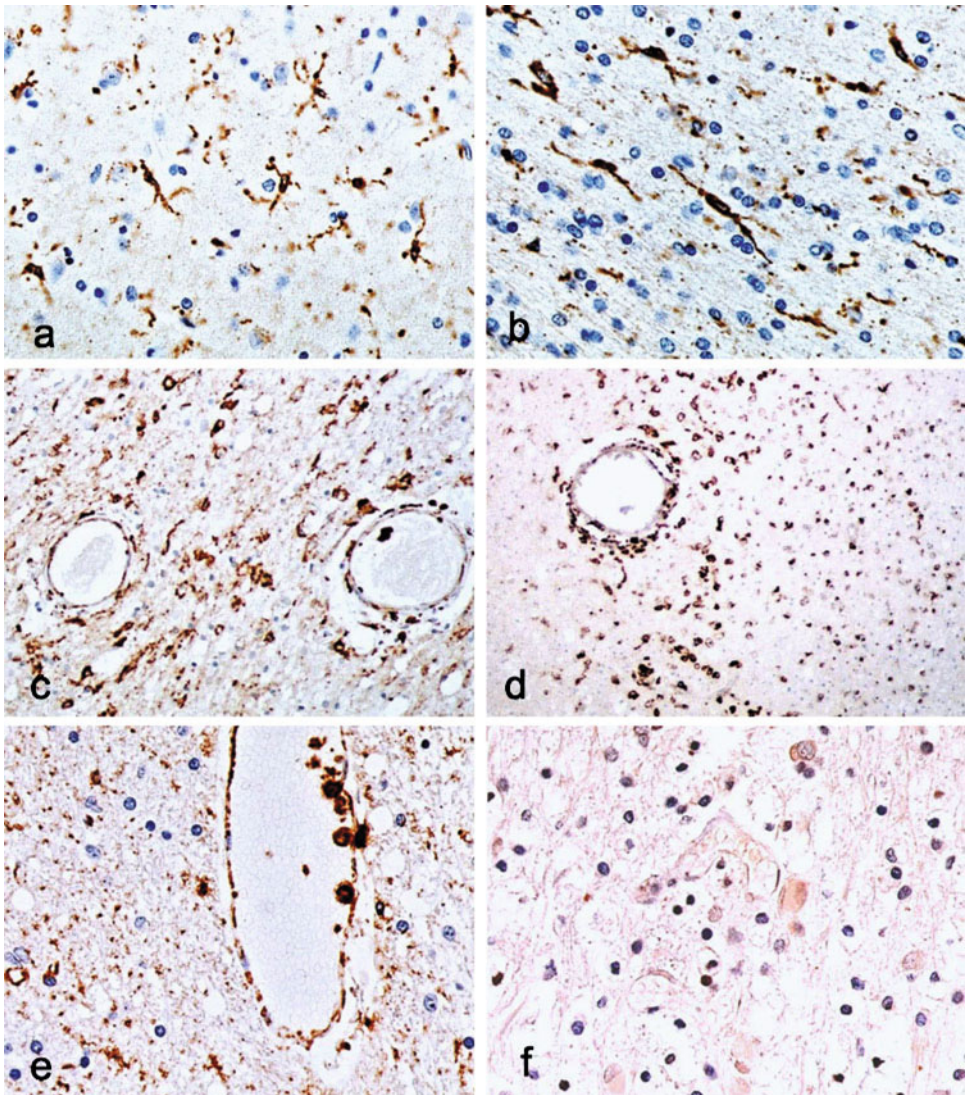

Figure 1 Staining pattern of anti-ENV monoclonal antibody 13H5A5. (**a**) Immunostaining of microglia in normal cortex of a control case; magnification ×100. (**b**) Immunostaining for microglia in normal white matter of a control case; magnification ×100. (**c**) Immunoreactivity of lymphocytes macrophages and microglia together with endothelial staining in a lesion of acute MS; magnification ×50. (**d**) Similar staining pattern in a lesion of chronic active MS; magnification ×50. (**e**) Similar staining pattern in an inactive MS plaque; magnification ×50. (**f**) Immunocytochemistry in the absence of primary antibody; no immunoreactivity; magnification ×100.

in the immunocytochemical procedure. In addition, absorption of the antibodies with the respective recombinant proteins completely abolished immunoreactivity, thus providing evidence of specific immunodetection.

French cohort

The origin of brain samples studied is detailed in Table 2. Because all monoclonals tested in the Austrian cohort were not available when the "French" study was performed and could not be further tested on the same samples, results presented below used a more limited panel of antibodies.

Non-MS control brains: A first screening by light microscopy of immunostained autopsy sections from normal brain hardly revealed any HERV-W GAG or ENV-"positive" cells—under sensitivity limits of the technique used for the French cohort—in gray or white matter. Nonetheless, GAG labeling of neurons was noticed with F45128 antibody, although intensity was weak and sometimes difficult to visualize on material with long post-mortem delay (cf. Table 2). No particular other HERV-W–positive cells were detected in cases with acquired immunodeficiency syndrome (AIDS) (HIV-1 encephalitis), Alzheimer's disease, amyotrophic lateral sclerosis, as well as in glial tumors.

HERV-W GAG in demyelinated axons of MS and PML lesions: Similar to the observations in the

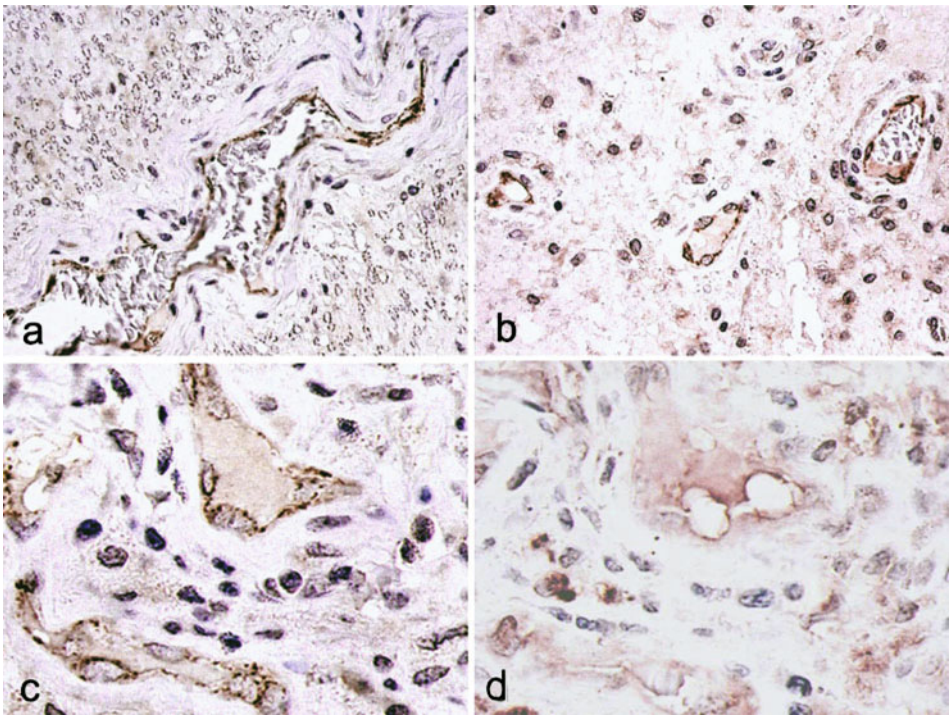

Figure 2 Endothelial staining of anti-GAG monoclonal antibody 2G5E12. (**a** to **c**) Immunoreactivity of endothelial cells in vessels of different caliber present in a case with very aggressive chronic active multiple sclerosis Magnification: **a**, ×50; **b**, ×100; **c**, ×400. (**d**) Absorption of antibody with specific GAG protein completely abolishes endothelial immunoreactivity; magnification: ×400.

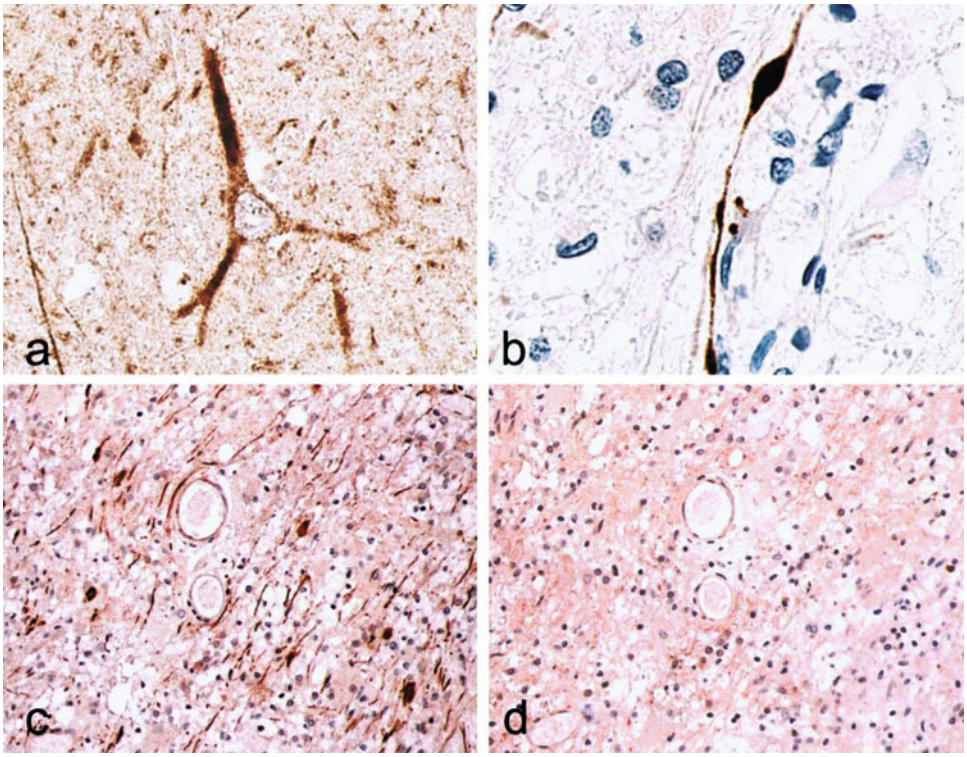

Figure 3 Neuronal and axonal staining obtained with anti-GAG polyclonal antibody F45128. (**a**) Cortical neuron in an MS patient, far distant from demyelinating lesion; strong immunoreactivity within dendrites, less pronounced in the perinuclear cytoplasm; magnification ×400. (**b**) Dystrophic axon in the center of an actively demyelinating MS lesion. Intense immunoreactivity in focal axonal swellings; magnification ×400. (**c**) Center of an actively demyelinating MS lesion with numerous dystrophic axons, showing very high immunoreactivity. In addition, there is weak endothelial immunoreactivity within the plaque vessels; magnification ×50. (**d**) Adjacent section to **c**, stained after absorption of the antibody with recombinant GAG protein. Immunoreactivity is completely abolished; magnification ×50.

Table 2 Clinical and pathological data (French series)

Case	*Sex*	*Age*	*Type of sampling*	*Diagnosis*	*Post-mortem delay*
1	M	37	Postmortem	Multiple sclerosis	51 h
2	F	39	Postmortem	Multiple sclerosis	60 h
3	M	66	Postmortem	Multiple sclerosis	50 h
4	F	74	Postmortem	Progressive multifocal leukoencephalopathy	17 h
5	M	73	Postmortem	Alzheimer disease	NR
6	M	33	Postmortem	AIDS encephalitis	NR
7	F	69	Postmortem	Amyotrophic lateral sclerosis	68 h
8	M	63	Postmortem	Normal control	29 h
9	M	55	Biopsy	Oligodendroglioma	0
10	M	62	Biopsy	Anaplastic oligoastrocytoma	0
11	F	52	Biopsy	Glioblastoma	0
12	F	45	Biopsy	Multiple sclerosis	0
13	F	38	Biopsy	Multiple sclerosis	0
14	F	69	Postmortem	Progressive multifocal leukoencephalopathy	27 h
15	M	53	Postmortem	Multiple sclerosis	7 h
16	M	NR	Postmortem	Multiple sclerosis	NR
17	F	NR	Postmortem	Multiple sclerosis	NR
18	M	NR	Postmortem	Amyotrophic lateral sclerosis	24 h
19	M	68	Postmortem	Control (oropharynx carcinoma, neuropathologically some hypertensive changes, argyrophilic grains)	48 h
20	M	38	Postmortem	Control (cardiocirculatory failure, no neuropathological alterations)	24 h

Note. M = male; F = Female. Age: in years. NR: not recorded.
Microscopic examination of brain tissue of the multiple sclerosis cases showed many plaques of demyelination with presence of inflammatory cells around blood vessels.

Austrian cohort, a striking HERV-W GAG antigen accumulation was observed with polyclonal F45128 antibody in axons within demyelinated white matter (Figure 4a to c). This pattern was not seen in white matter sections from unaffected regions of MS and PML brains, or from the other cases. In postmortem material from this series, positive axons were readily detected in PML lesions (Figure 4a). HERV-W GAG accumulation in axons was clearly detectable on slides obtained from MS lesions biopsies (Figure 4b, c). Double-labeling studies performed on this biopsy material revealed an isolated GAG detection with constant absence of ENV detection in the same demyelinated axons (Figure 4c).

GAG expression in microglial cells of active MS plaques: A strong labeling with anti-GAG antibodies was observed in biopsies obtained from acute lesions of severe MS cases. HLA-DR (class II) expression in GAG-positive microgliocyte-like cells was also seen (Figure 4b).

Control antibodies: Rabbit polyclonal antibodies and mouse monoclonal antibodies of the same isotype as anti-MSRV/HERV-W antibodies (IgG 1K), raised against various pathogens (rabies, HIV-1, HHV6 type A, *Chlamydia pneumoniae*), were tested in parallel under the same technical conditions. No immunoreactivity was detected neither in MS lesions, nor in any control sample with these antibodies.

Interestingly also, no significant detection of HERV-K proteins was observed in samples from pathological as well as normal brain white and gray matter from any source (not shown).

German cohort

Normal human brains: In this series we studied the expression pattern of HERV-W GAG proteins in normal human brain only, throughout different brain regions. Similar to the findings of the Austrian and French series, when analyzing HERV-W GAG expression with the F45128 antibody, a clear immunoreactivity was observed in neuronal cells and axons (Figure 5). In both cases investigated, this positive neuronal and axonal staining was consistently seen in all different brain areas studied, without obvious variations in the expression levels between different brain regions. Specifically, HERV-W GAG immunoreactivity was detected in cortical neurons of the supramarginal gyrus (neocortex); in pyramidal and dentate gyrus neurons in the hippocampus as well as in neurons of the cingulate gyrus (limbic cortex); in neurons and, prominently, axons of the globus pallidus, putamen, thalamus, and amygdala (subcortical nuclei); in the different neuronal populations and axons of the midbrain and pons, and in neurons of cranial nerve nuclei and olivary neurons in the medulla (brainstem); and finally in association with Purkinje cells in the cerebellum (see Figure 5).

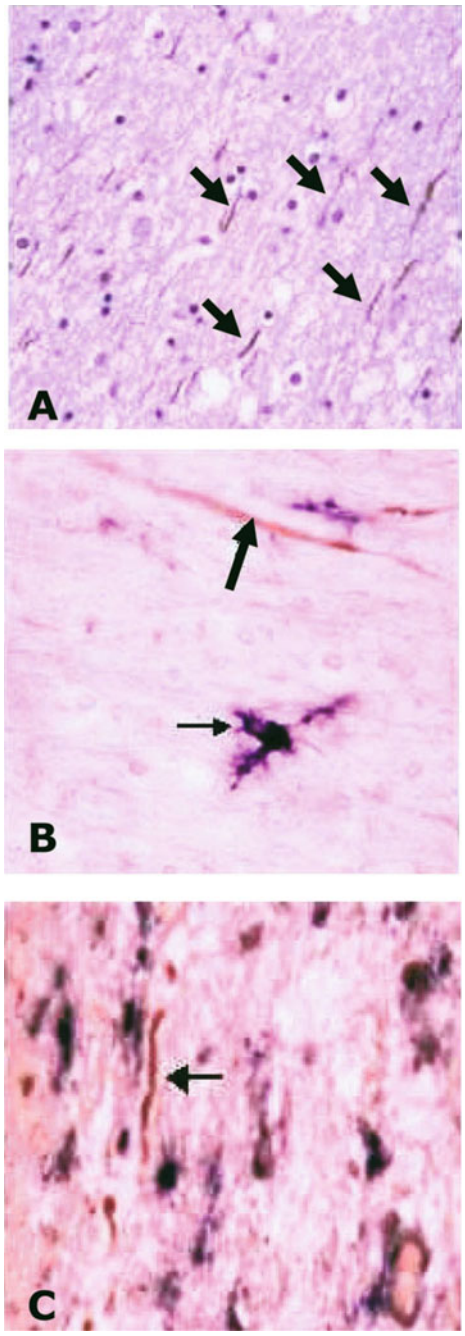

Figure 4 Demyelinated white matter lesions from MS and PML, necropsy and biopsy brain samples. (**a**) Progressive multifocal leukoencephalopathy brain white matter necropsy. HERV-W GAG protein is detected in axons with F4128 polyclonal immunostaining, case no. 14 (magnification ×250) (examples of positive axons are shown by *large arrows*). (**b**) MS plaque biopsy: Double labeling with antibodies against HERV-W GAG and HLA class II (DR; anti-CR3/43). HERV-W GAG is detected alone in axons from multiple sclerosis demyelinated white matter (*large arrow/brown staining*), whereas an activated microgliocyte/macrophage (*thin arrow/dark violet staining*) coexpresses HLA class II antigen in the vicinity of this positive axon. (Case no. 12, magnification ×50). (**c**) MS plaque biopsy: Double labeling with antibodies against ENV (*dark violet*) and GAG (*brown*). HERV-W GAG alone is detected in axons from multiple sclerosis biopsied demyelinated white matter (*large arrow*). (2A12A5mAb and F45128 polyclonal immunostaining, case no. 12, biopsy, magnification ×250).

Discussion

MSRV/HERV-W context

The pathogenic potential of retroviral elements belonging to genetic families with endogenous copies usually results from interaction between "pathogenic" elements and the other endogenous copies associated with the "physiological background" (Contag *et al*, 1989; Gardner, 1990; Kubo *et al*, 1996; Xu *et al*, 1996). Consequently, the contribution of other members of the same multicopy HERV family may be a key for genetic resistance or susceptibility to a disease induced by a pathogenic member. MSRV retroviral particles characterized in MS and genetically linked to the HERV-W endogenous family, thus raise questions on their respective pathophysiological roles in a similar context. But, before further studies can address the questions of MSRV particle formation, of their association with reverse-trancriptase activity, and of their circulation in body fluids in MS patients, an overview of HERV-W antigen expression in human tissues was necessary to learn about a possible physiological expression of the endogenous HERV-W family. This study became all the more pertinent after the recent evidence of HERV-W RNA expression from various chromosomal copies, in different healthy or cancerous human tissues, showing quite original HERV-W gag RNA expression in human brain (Yi *et al*, 2004).

Today, no antibody specific for an epitope unique to a virion-producing/pathogenic MSRV strain, not labeling any protein encoded by any of the normally expressed endogenous HERV-W DNA copies, has been identified. Therefore, only differences in the patterns of expression, if detectable within present technical limits, were a priori expected from this study.

The results of the present multicenter study thus provide evidence of general pathophysiological features. Rare and peculiar findings at the limits of interpretation (e.g., staining of one or two cells in brains from old individuals in a single series, without comparative material) were thus not taken into account for the present study, focused on common findings with a general meaning in physiology or disease.

Physiological expression of HERV-W ENV in human brain was detected in microglia of both gray and white matter and in certain blood vessel endothelial cells, whereas expression of HERV-W GAG antigens was observed in neurons (cell body, axons, dendrites).

In demyelinated brain lesions, a striking GAG antigen accumulation in dystrophic axons from MS and PML cases was observed. This antigen obviously corresponds to the GAG specificity detected in normal brain neuronal structures, which here favors a dysregulated expression/routing in demyelinated lesions, rather than an original expression.

De facto, a physiological role for this HERV-W GAG antigen in human neurons, as previously evoked,

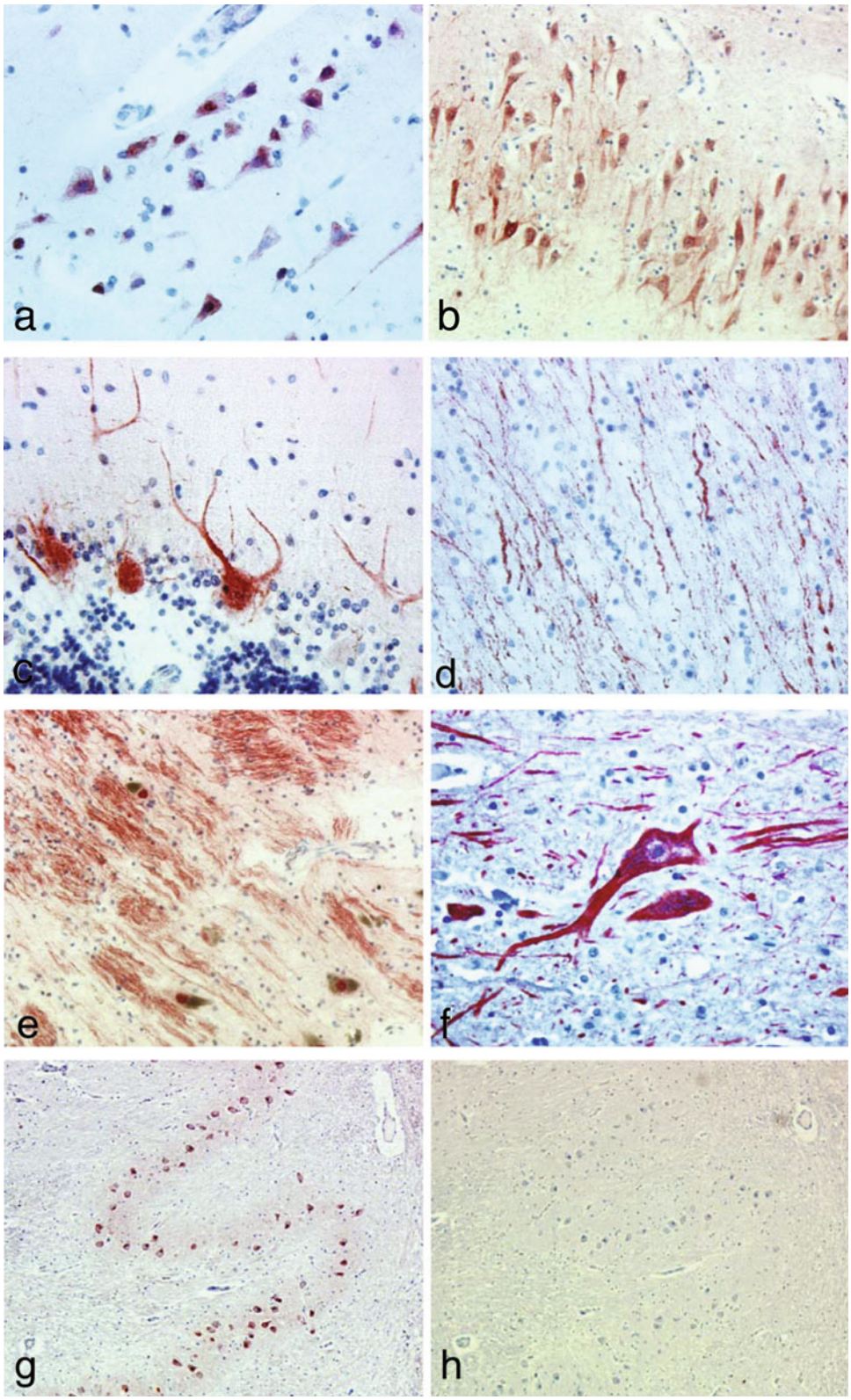

Figure 5 HERV-W GAG expression in normal human brain (F45128 antibody). (**a**) Immunostaining of cortical neurons in the supramarginal gyrus (case 19). (**b**) Immunostaining of pyramidal cells in the hippocampus (case 19). (**c**) Immunoreactivity in association with Purkinje cells in the cerebellum (case 19). (**d**) Axonal staining in the pallidum (case 19). (**e**) Immunostaining of axons and melanin containing neurons in the substantia nigra (case 19). (**f**) Immunoreactivity in neurons and axonal structures in the lower medulla/upper cervical cord (case 20). (**g**) Immunostaining of olivary neurons (case 20). (**h**) Absence of immunreactivity in a serial section stained with an isotype-matched control antibody (case 20).

becomes highly probable and comparable to that of HERV-W ENV7q *in placenta* (Mi *et al*, 2000). The most likely explanation for such an expression pattern is that HERV-W GAG is synthesized in low to moderate amounts within normal nerve cells and is axonally transported to the terminals, as described for amyloid precursor protein. Such neuronal proteins accumulate at sites of impaired axonal transport in demyelinated lesions (Ferguson *et al*, 1997; Kornek *et al*, 2000).

Elsewhere, no significant difference was noticed with age, sex, and years of MS disease, but with disease activity: GAG antigen expression on endothelial cells within active lesions of acutely/actively demyelinating progressive MS cases versus stable cases, as revealed by the Austrian series (Table 1). As it was not seen in non-MS controls as well, this GAG expression in brain endothelial cells evidenced by 2G5E12 antibody, may also be "MS-specific." An HERV-W copy origin different from that encoding the previous GAG antigen detected by F45128 polyclonal antibody in neurons would be compatible with differences in immunoreactivity and known amino acid sequence variants encoded by several HERV-W gag copies in human DNA (Voisset *et al*, 2000).

Interestingly also, a specific ENV pattern in activated microglial/macrophage cells was noticed in active MS plaques from the same series.

In conclusion, results from the present study have provided evidence that:

(i) A physiological expression of an HERV-W GAG antigen exists in certain human brain cells, but most prominently in neurons.
(ii) A physiological expression of HERV-W ENV antigens is also detected in human brain, but mainly associated with infiltrating lymphoid cells or brain macrophages.
(iii) "MS-specific" GAG and ENV patterns were detected in MS lesions, essentially at the level of endothelial and microglial cells.
(iv) In demyelinating diseases, an HERV-W GAG antigen accumulates in dystrophic axons within lesions.

These results illustrate the multifaceted aspects of human endogenous retroviruses in between physiology and pathology, genetics, and infection.

Moreover, links with demyelinating inflammatory diseases that have been evidenced here may point out connections with major immuno- and/or neuropathogenic mechanisms already evoked in progressive multifocal leukoencephalopathy (PML) or MS. Other links with brain diseases may thus reveal associated with HERV-W dysregulation in, e.g., neuronal cells, as suggested by recent studies on schizophrenia (Karlsson *et al*, 2001; Yolken *et al*, 2000). In particular, a physiological role for HERV-W GAG in neurons and its eventual dysregulation in shizophrenia frontal cortex, might be meaningful.

Further studies in these opening domains are now made possible and are required for tentatively elucidate the many questions remained unanswered after the present "first" study.

Methods

Subjects and tissue preparation

French cohort: A cohort of 18 patients, 16 from whom brain tissues were banked in the R. Escourolle Neuropathology Department of the Hôpital de La Salpêtrière (Paris, France) and 2 from whom a biopsy was made for particular diagnostic purpose in the Neuropathology Department of the Hôpital H. Mondor (Créteil, France), was selected for this study. Their list with corresponding neuropathological diagnoses and sampling conditions (biopsy/necropsy), is presented in Table 2. Samples from necropsic or biopsic samples had been fixed in formaldehyde, included in paraffin, cut at 7 μm thickness before staining. Serial sections were collected on SuperFrost Plus slides (Menzel-Glaser, Germany).

Austrian cohort: The cohort studied in Vienna consited of 20 patients. They included 7 patients with Marburg's type of acute MS (4 females and 3 males; mean age 45.8 years; disease duration 0.25 to 6 months); 5 patients with chronic active MS (3 females, 2 males; mean age: 37.8 years, disease duration 2.5 to 8 years), 4 patients with chronic inactive MS (3 females, 1 male; mean age: 71.5 years, disease duration >33 years), and 4 control patients without central nervous system (CNS) disease (2 females and 2 males; mean age 76.8 years). Brains were fixed in 4% buffered formaldehyde and routinely processed for paraffin embedding. Immunocytochemistry was performed on 5 μm thick paraffin sections, collected on SuperFrost Plus slides.

German cohort: Formalin-fixed paraffin-embedded 8-μm tissue sections from systematically sampled different brain regions (neocortex, limbic cortex, subcortical nuclei, brainstem, cerebellum) of autopsied brains from two patients were obtained from the Würzburg brain bank, Department of Neuropathology, University of Würzburg, Germany. Both patients had no evidence of neurological disease during live. Detailed neuropathological evaluation revealed some hypertensive changes and argyrophilic grains in one case; whereas the other showed no neuropathological alterations (see Table 1).

Generation of anti-MSRV antibodies

Anti-MSRV antibodies were produced by inoculating mice and rabbits with recombinant MSRV proteins. The following antibodies were selected for immunohistological applications. Monoclonals: 13H5A5, 3C1D5, 6A2B2, 5E9H9, and 2A12A5, (IgG1K) were selected after mice immunization with the SU and

partial TM portion encoded by MSRV ENV and produced in *Escherichia coli*; 2G5E12, 5B6F8, 4E4A11, and 4A9E3 (IgG1K) were selected after mice immunization with the capsid protein encoded by MSRV GAG and produced in *E. coli* by clone CL2 (AF123881). Polyclonals: F45128 was selected after rabbit immunization with the capsid recombinant protein encoded by CL2. Preimmune sera were kept as controls for each rabbit polyclonal. All antibodies were purified on protein-A sepharose columns. Recognition of MSRV recombinant proteins by polyclonal and monoclonal antibodies was investigated by enzyme-linked immunosorbent assay (ELISA) and Western blot experiments. The specificity of anti-MSRV ENV monoclonal antibodies was also evaluated in eukaryotic expression systems cells with a semliki virus expression system (not shown). Vectors without insert were used as controls.

Recombinant proteins for immunodepletion
Recombinant MSRV/HERV-W GAG and ENV proteins were produced and purified by Amplicon Express (Washington DC, USA). They were produced by transforming *E. coli* strains with plasmid constructions containing inserts with complete or partial GAG or ENV open orfs as already described (Blond *et al*, 1999; Komurian-Pradel *et al*, 1999) and permitting their procaryotic expression in fusion with a polyhistidine tail (HisTag). The proteins were further purified on nickel columns for His-tagged protein purification and their purification was controlled by WB analyses with anti-HisTag and antigen-specific monoclonal antibodies.

Because prokaryotic ENV and GAG recombinant proteins revealed difficult to solubilize, they were eluted in a buffer containing sodium dodecyl sulfate (SDS) (20 mM Tris, pH 8, 150 mM NaCl, 10 mM β mercaptoethanol, 1.5% SDS, 1 mM phenyl methylsulfonyl fluoride [PMSF], 50 mM EDTA).

Clones used for the production of recombinant proteins presented on Western blots have been deposited in Genbank database with following accession numbers: ENV pV14 clone (AF331500) and GAG CL2 (AF123881).

Immunohistology of brain sections
Austrian cohort: Immunocytochemistry was performed with a biotin/avidin/peroxidase technique as described in detail earlier (Bien *et al*, 2002). To increase sensitivity of the immune reaction, antigen retrieval was performed in a steamer in citrate buffer (pH 6.0) and the final avidine reaction was amplified by biotinylated tyramine enhancement (Bien *et al*, 2002). For control, immunocytochemistry was performed in the absence of the primary antibody. In addition, the respective antisera/antibodies were preabsorbed by adding 60 μg of the respective recombinant proteins (see above) to the final dilution of the antibodies and incubating the mixture for 60 min at 37°C. Immunocytochemistry was then performed as described above.

French cohort: MSRV immunoreactivity was examined in paraffin sections, using the avidin-biotin-peroxidase complex method and the diaminobenzidine (DAB) chromogen kit from Santa Cruz Biotechnologies (Santa Cruz, USA). Slides were microwaved (2×5 min in 10 mM sodium citrate buffer, pH 6) prior to immunostaining. Endogenous peroxidase was quenched in 2% H_2O_2 in methanol for 30 min. Antibodies were used at 5 μg/ml: anti-MSRV ENV 3C1D5 monoclonal, anti MSRV GAG 2G5E12 monoclonal, and anti-MSRV GAG F45128 rabbit polyclonal. The sections were incubated overnight at 4°C with the first antibody. The specificity of immunoreactivity was confirmed in all experiments by absence of peroxidase staining on identical tissue sections using normal serum instead of primary antibodies. Slides were counterstained with Gill's hematoxylin. For double labeling, we used combinations of F45128 (visualized with DAB) and CR3/43 (1/200; Dako, visualized by the avidin-biotin complex-alkaline phosphatase method and BCIP/NBT) for activated macrophages and microglial and astroglial cells. The BCIP/NBT and streptavidin-alkaline phosphatase were from Vector Lab, Burlingame, CA, USA.

Irrelevant control antibodies were tested in parallel on all MS samples: anti–rabies virus N-protein antibody (mouse IgG1K) (Montano-Hirose *et al*, 1995); anti-HERV-K ENV and c-orf rabbit polyclonals, provided by R. Löwer, Germany; anti-HIV-1 P24 (mouse IgG1K; Dako, clone Kal-1) and anti-Chlamydia pneumoniae–specific monoclonal (mouse monoclonal; Argene biosoft, Varilles, France, catalog no. 11-215).

German cohort: Slides were microwaved in 10 mM citrate buffer, pH 6, before immunostaining. Immunohistochemistry was performed with a commercial kit (Super Sensitive Detection Kit; BioGenex, San Ramon, CA) according to the manufacturer's instructions using AEC as chromogen. The primary antibody was the anti-MSRV GAG F45128 rabbit polyclonal at 5 μg/ml. Sections were counterstained with hematoxylin. In controls, the primary antibody was omitted or isotype-matched antibodies (IgG; Sigma, Deisenhofen, Germany) were used.

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