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Lack of immune responses against multiple sclerosis–associated retrovirus/human endogenous retrovirus W in patients with 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 type W (HERV-W). Different lines of evidence appear compatible with a potential role of MSRV/HERV-W in the pathogenesis of MS. The authors therefore analyzed humoral and cellular immune responses against MSRV/HERV-W antigens in patients with MS, patients with other inflammatory and noninflammatory neurological diseases, and healthy controls, using indirect immunofluorescence and enzyme-linked immunospot assays. Antibodies against the HERV-W envelope (Env) protein, Syncytin-1, were found in one of 50 patients with MS and none of 59 controls, whereas antibodies against MSRV matrix and capsid (Gag) or Env proteins were not detectable in any of the patients or controls. Similarly, in a screening of human leukocyte antigen (HLA)-B7$^+$ patients with MS ($n = 23$) and controls ($n = 29$) for cytotoxic T-lymphocyte responses against 36 predicted HLA-B7–restricted MSRV/HERV-W Gag-, protease-, and reverse transcriptase–derived peptides, no such responses could be detected in any of the subjects studied. These data suggest that there are no appreciable humoral or cellular immune responses against MSRV/HERV-W in patients with MS. While this may be due to immunological tolerance of physiologically expressed HERV-W proteins, strategies other than measurement of immune responses will be required to further elucidate the relationship between MSRV/HERV-W and MS. *Journal of NeuroVirology* (2008) **14,** 143–151.

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Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system thought to result from an as yet incompletely understood complex interplay of genetic and environmental factors (Noseworthy, 1999). Particles with electron microscopical morphology of retroviruses containing reverse transcriptase activity (i.e., a characteristic property of retroviruses) were first discovered in supernatants of cell cultures from patients with MS more than 15 years ago (Perron *et al*,

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1989, 1991). After molecular characterization had revealed retroviral RNA sequences within such particles, they were named MS-associated retrovirus (MSRV) (Komurian-Pradel *et al*, 1999; Perron *et al*, 1997). Subsequently, MSRV was shown to be closely related to a human endogenous retrovirus (HERV) family, HERV-W (Blond *et al*, 1999). HERVs are sequences within the human genome that resemble present-day exogenous retroviruses and most probably represent surviving traces of ancestral germ line infections by active retroviruses, which have thereafter been transmitted in a Mendelian manner. In humans, approximately 8% of the genome consist of HERVs (de Parseval and Heidmann, 2005). Due to accumulation of numerous mutations and deletions most HERVs are nonfunctional; however, few HERVs have retained the capacity to code for viral proteins or even particles. For instance, a HERV-W provirus on chromosome 7q21 encodes a functional envelope (Env) protein (termed Syncytin-1) that likely plays a physiological role in human placenta formation (Blond *et al*, 2000). Whether MSRV represents an exogenous member of the HERV-W family or originates from endogenous HERV-W copies is currently unknown. For clarity, we refer in this article to nucleotide sequences, and the proteins they encode, originally obtained from extracellular particle–associated RNA as MSRV, whereas HERV-W designates a family of endogenous retroviral sequences present in the human genome.

Although definitive proof for specific pathogenic functions of HERVs in human diseases is still lacking, different lines of evidence suggest a possible involvement of MSRV/HERV-W in MS: Independent studies demonstrated a higher prevalence of virion-encapsidated MSRV RNA sequences in serum and cerebrospinal fluid (CSF) of patients with MS compared to patients with other neurological diseases and healthy controls (Dolei *et al*, 2002; Garson *et al*, 1998; Nowak *et al*, 2003; Perron *et al*, 1997). Also, the presence of MSRV RNA in CSF from patients with MS is associated with a more unfavorable disease course (Sotgiu *et al*, 2006). MSRV/HERV-W RNA levels were found to be higher in autoptic brain tissue from patients with MS than from controls, and neuropathological studies demonstrated expression of HERV-W Env antigens in astrocytes and microglia in actively demyelinating MS lesions (Antony *et al*, 2004, 2006, 2007; Mameli *et al*, 2007). A potential pathogenic relevance of these findings for MS is inferred from data showing that HERV-W Env (Syncytin-1) has indirect cytotoxic effects on oligodendrocytes in vitro and that expression of Syncytin-1 in mouse models results in demyelination *in vivo* (Antony *et al*, 2004, 2007). Likewise, intraperitoneal injection of purified MSRV particles in a hybrid animal model (severe combined immunodeficiency mice grafted with human lymphocytes) led to T cell–dependent acute neurological symptoms and death from brain hemorrhage, compatible with T cell–mediated immunopathogenic properties of such particles (Firouzi *et al*, 2003).

Infections with exogenous retroviruses (e.g., human immunodeficiency virus type 1), but also an aberrant expression of endogenous retroviral proteins (e.g., from the HERV-K family), can elicit the production of anti-retroviral antibodies as well as cellular immune responses (Rakoff-Nahoum *et al*, 2006; Sauter *et al*, 1995; Schiavetti *et al*, 2002). The latter are mainly mediated by $CD8^+$ cytotoxic T lymphocytes (CTLs), which recognize peptides (mostly of 9 to 10 amino acids length) produced by proteosomal cleavage of retroviral proteins and presented as a complex with human leukocyte antigen (HLA) class I molecules on virus-infected cells (Pamer and Cresswell, 1998). In view of the suspected role of MSRV/HERV-W in MS, an important and up to now unresolved question is whether MSRV/HERV-W antigens can induce immune responses that might be used as indirect markers for the expression of MSRV/HERV-W proteins or particles in patients with MS. We here report the results of a detailed study on antibody and CTL responses to MSRV/HERV-W antigens in patients with MS, patients with other inflammatory and non-inflammatory neurological diseases, and healthy controls.

Results

Humoral immune responses to MSRV/HERV-W

Immunoglobulin G (IgG) antibodies against MSRV/HERV-W were measured by indirect immunofluorescence assays using a recombinant baculovirus-based eukaryotic protein expression system in SF158-insect cells (see Materials and Methods for details). SF158 cells infected with recombinant baculoviruses carrying MSRV matrix and capsid (Gag), MSRV Env, or HERV-W Env (Syncytin-1) genetic sequences strongly expressed the respective proteins, as demonstrated by Western blot and indirect immunofluorescence using specific antibodies against these proteins (Figure 1A, B). Of note, the recombinant baculovirus expression system produces proteins with proper folding, disulfide bond formation, and oligomerization, which can also undergo post-translational modifications, e.g., cleavage or glycosylation (Kidd and Emery, 1993). Proteins expressed in this way are therefore structurally similar to their native counterparts and likely display antigenic epitopes similar to those presented *in vivo*.

A total of 50 patients with MS, 4 patients with a clinically isolated demyelinating syndrome (CIS), 20 patients with other inflammatory neurological diseases, 10 patients with other noninflammatory neurological diseases, and 29 healthy controls were screened for IgG antibodies to MSRV Gag, MSRV Env, and HERV-W Env (Syncytin-1). Except for one

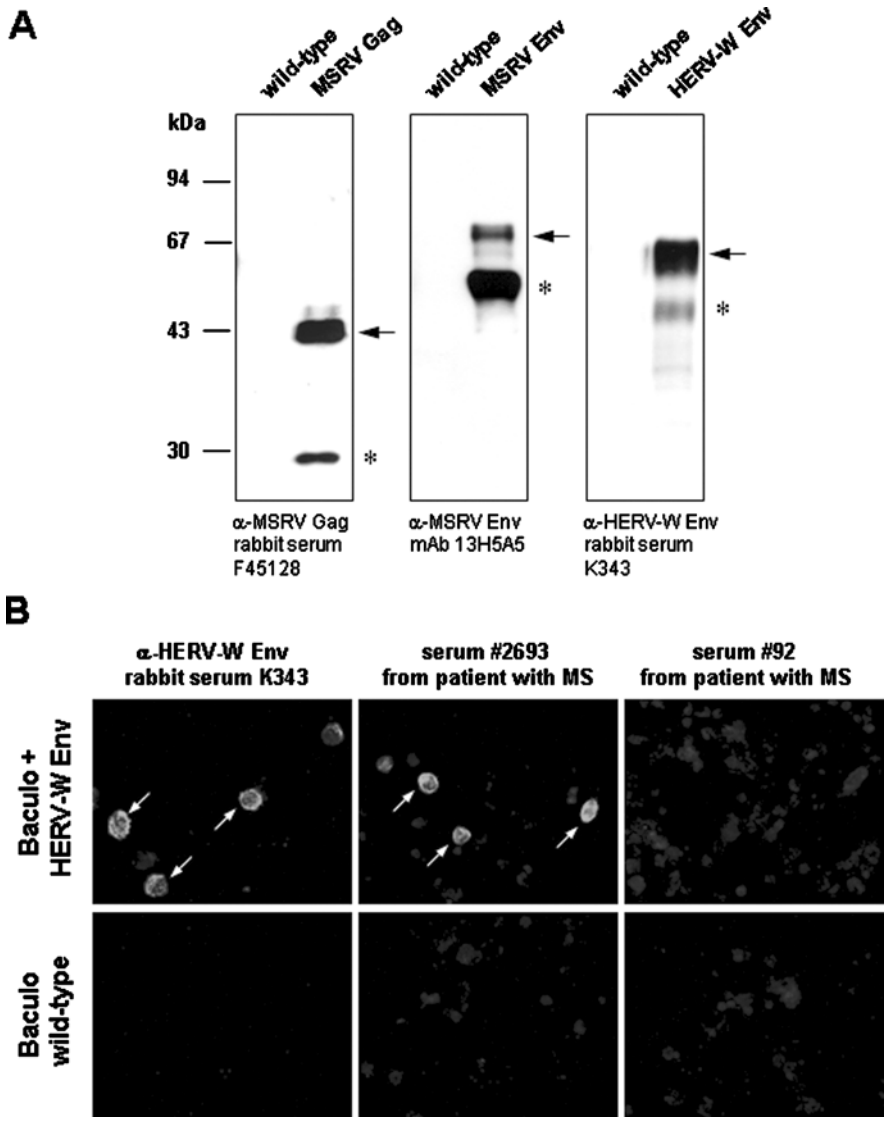

Figure 1 (**A**) Western blot analysis of MSRV/HERV-W proteins expressed in SF158 insect cells. Protein lysats from SF158 cells infected with wild-type baculoviruses or MSRV Gag, MSRV Env, or HERV-W Env recombinant baculoviruses were separated by SDS-PAGE, blotted onto PVDF membranes, and developed with specific antibodies against MSRV Gag, MSRV Env, or HERV-W Env. The upper band (*arrow*) visible in the MSRV Gag immunoblot (left panel) corresponds to a MSRV Gag precursor protein consisting of matrix and capsid domains, whereas the lower band (*asterisk*) appears compatible with a cleaved capsid protein. The upper bands (*arrows*) in the MSRV and HERV-W Env immunoblots (middle and right panels) represent the glycosylated MSRV Env or Syncytin-1 precursor proteins, respectively. The lower bands (asterisks) seem compatible with processed glycosylated surface (SU) subunits of MSRV Env and Syncytin-1 (see also Cheynet *et al*, 2005). The somewhat diffuse aspect of the MSRV and HERV-W Env bands likely results from heterogenous glycosylation. (**B**) Indirect immunofluorescence assay with SF158 insect cells expressing HERV-W Env (Syncytin-1). SF158 insect cells were infected with either recombinant baculoviruses carrying the HERV Env (Syncytin-1) gene (upper row) or wild-type baculoviruses as control (lower row). Cells were processed and stained as described in Materials and Methods with a polyclonal rabbit anti-HERV-W Env serum as positive control or human sera. Note the positive signals (*arrows*) in the HERV-W Env recombinant baculovirus-infected cells stained with the polyclonal rabbit anti-HERV-W Env serum and with serum #2693 from a patient with MS (i.e., the only serum in the entire investigation that contained antibodies to HERV-W Env), whereas no immunoreactivity above background is detectable with serum #92 from another patient with MS, and in the wild-type baculovirus infected control cells. Acquisition parameters (AxioVision 3.0) and digital processing (ImageJ) were identical for all images. Original magnification ×400.

serum from a patient with MS that contained antibodies against HERV-W Env (Figure 1B), no antibody reactivities could be observed. The HERV-W Env antibody positive MS serum had a titer of 1:320 and also reacted with HERV-W Env when tested in a confirmatory Western blot, in which 15 other MS sera analyzed in parallel revealed no reactivity to HERV-W Env (data not shown). Similarly, none of 20 CSF samples from patients with MS and CIS (including a CSF sample from the patient with MS who had antibodies to HERV-W Env in serum) and none of 30 CSF samples from patients with

other inflammatory and noninflammatory neurological diseases contained antibodies against MSRV Gag, MSRV Env, or HERV-W Env, as measured by indirect immunofluorescence assays. Because antibodies against Epstein-Barr virus (EBV) antigens were readily detectable in aliquots of the patient specimens used in this study (stored under the same conditions and tested by the time of [series A; see Materials and Methods] or up to 1.5 years after [series B] the MSRV/HERV-W antibody determinations [Gronen *et al*, 2006, and unpublished data]), poor sample quality or degradation of immunoglobulins can be ruled out as a possible cause for the nearly complete absence of antibodies against MSRV/HERV-W observed in this investigation.

The patient whose serum contained antibodies against HERV-W Env had highly active MS, as evidenced by worsening of clinical symptoms and gadolinium enhancing cerebral and cervical spinal cord lesions on magnetic resonance imaging at the time of blood/CSF collection. This study, however, was not specifically designed to detect differences in antibody levels between patients with active and stable MS, rendering the overall interpretation of these findings difficult. Although the precise cause of the presence of HERV-W Env antibodies in this patient's serum remains elusive, absence of such antibodies in a corresponding CSF sample argues against an intrathecal synthesis.

Cellular immune responses to MSRV/HERV-W

A cohort of 24 patients with MS and 29 healthy controls, all of them HLA-B7$^+$, were screened for CTLs specific to 36 predicted HLA-B7–restricted MSRV/HERV-W Gag-, protease (Pro)-, and reverse transcriptase (Pol)–derived peptides using interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assays (see Materials and Methods for details of the peptide prediction strategy and Table 1 for a list of the peptides used). Peptides from two proteins (U5/1 and U5/2) encoded by short open reading frames in the MSRV RU5 region (Komurian-Pradel *et al*, 1999) were included as well. One of the 24 patients with MS could not be evaluated for technical reasons. There were no CTL responses detectable to any of the peptides in any of the patients and controls investigated. As also already reported for a study that was performed in parallel to this investigation on the same peripheral blood mononuclear cell (PBMC) samples (Gronen *et al*, 2006), stimulation with phytohemagglutinin (PHA) resulted in strongly positive IFN-γ responses in all PBMC samples analyzed. Furthermore, 16 of 22 patients and 23 of 26 controls studied showed positive IFN-γ responses in ELISPOT assays when challenged with the common recall antigen tetanus toxoid. In addition, 16 of 23 patients and 21 of 26 controls investigated had positive IFN-γ responses to a HLA-B7–restricted peptide derived from the Epstein-Barr nuclear antigen (EBNA)-3A (Gronen *et al*, 2006). These different positive controls clearly indicate that the PBMC samples used were viable and functionally intact, and that the ELISPOT methodology employed in this investigation is sufficiently sensitive to detect HLA-B7–restricted peptide-specific CTL responses.

Discussion

We here describe a comprehensive search for humoral and cellular immune responses against MSRV/HERV-W antigens in patients with MS and controls. Converging results from antibody and CTL screenings suggest that overall no such immune responses are detectable in the population studied. There appear to be three possible explanations for these findings:

1. MSRV/HERV-W antigens were not present in the study population, precluding the generation of an immune response against these antigens.
2. MSRV/HERV-W antigens were present in the study population and caused an immune response, but this immune response escaped our attention because of insufficient sensitivity of the methods applied for its detection.
3. The detection methods used were sufficiently sensitive and MSRV/HERV-W antigens were present in the study population; however, expression of MSRV/HERV-W antigens may not be associated with an immune response against these antigens.

Several neuropathological studies have described a specific expression of MSRV/HERV-W Env (Antony *et al*, 2004, 2007; Mameli *et al*, 2007; Perron *et al*, 2005) and Gag (Perron *et al*, 2005) antigens in MS lesions from autoptic brain tissue of patients who had suffered from MS. Immunoreactivity was more prominent in acute lesions with active demyelination, but was detectable to a weaker extent in chronic lesions, too (Antony *et al*, 2004; Mameli *et al*, 2007). Despite of the fact that it is obviously impossible to state whether MSRV/HERV-W antigens have been present in MS lesions of the patients studied in this work, if one assumes that the findings of the mentioned postmortem analyses are to a certain degree representative of MS lesions *in vivo*, chances seem high that HERV-W antigens were expressed in some individuals of our patient population. Also, analyzing different cohorts of MS patients, independent groups have reported the detection of encapsidated MSRV RNA sequences in serum, plasma, or CSF in a high percentage (50% and above) of patients with MS (Dolei *et al*, 2002; Garson *et al*, 1998; Nowak *et al*, 2003; Perron *et al*, 1997). Under the premise that the detection of such encapsidated MSRV RNA sequences may be indicative of the presence of MSRV particles, it seems again likely, that some of our patients with MS harbored such particles. However,

Table 1 List of the 36 predicted HLA-B7–restricted peptides used in this study, the proteins they were derived from, and their SYFPEITHI binding scores

Peptide	Derived from protein	SYFPEITHI score for HLA-B*0702
NPDGYIDVL	MSRV gag*/HERV-W gag (AAF74213.1)**	24
KPPPCPCPL	MSRV gag	23
LPLGSEQNL	MSRV gag	22
VPPEAKMPL	MSRV gag	21
IPRLCPLQA	MSRV gag/HERV-W gag (AAF74213.1)	21
SPDSIEGQL	MSRV gag/HERV-W gag (AAF74213.1)	21
GPARVPVPF	MSRV gag/HERV-W gag (AAF74213.1)	20
TPNERSAAV	MSRV gag/HERV-W gag (AAF74213.1)	18
PPPYPSVPSPTPSST#	MSRV gag	19
IPPKAKTPL	HERV-W gag (AAF74213.1)	24
KEENPTAFLDRL	HERV-W gag (AAF74213.1)	21
MPSPSEPRVCL	MSRV pro (AAB66527)	28
MPESPTPLLGRDIL	MSRV pro (AAB66527)/HERV-W pro (AAF74214)	25
DPLNPTSQL	MSRV pol (AAB66528)	24
IPVRPDSQFLFA	MSRV pol (AAB66528)	22
SPHLFGQAL	MSRV pol (AAB66528)	22
LPTGQDFSL	MSRV pol (AAB66528)	22
FPYQRKYPL	MSRV pol (AAB66528)	21
KPNGQWRLV	MSRV pol (AAB66528)	19
TPIARPLYT	MSRV pol (AAB66528)	19
YPAVSSPYTLL	MSRV pol (AAB66528)	18
IPEEAEWFT	MSRV pol (AAB66528)	18
KVSKPKARL	MSRV pol (AAB66528)	18
WPHCLWVMA	MSRV pol (AAB66528)	18
ATLKPATFL	MSRV pol (AAB66528)	18
APAHAITLT	HERV-W pro (AF74214)	20
CPRQRSSRA	HERV-W pro (AF74214)	18
QPLSCDWGT	HERV-W pro (AF74214)	16
VPCLRKELI	HERV-W pro (AF74214)	16
NPHTPGTSTQLA	HERV-W pol (BAA78549)	21
ATLNPATFL	HERV-W pol (BAA78549)	17
GPCFLWLKL	MSRV U5/1+	22
RPLPLPIGLKA	MSRV U5/1	22
RPAADFHPF	MSRV U5/1	21
NPRSENTRL	MSRV U5/2+	23
IPFLGIRET	MSRV U5/2	17

*The MSRV Gag sequence used for the SYFPEITHI search corresponded to the sequence provided in Komurian-Pradel *et al* (1999) and was compiled from the deduced protein sequences of the 3 overlapping MSRV Gag clones, LB15 (AAD48374.1), CL2 (AAD48375.1), and CL17.
**Due to the high sequence homology between MSRV and HERV-W proteins, several of the identified HLA-B7-restricted peptides were identical in MSRV and HERV-W sequences.
#Because in some cases epitopes with high binding scores for HLA-B7 were located in overlapping nonamers, longer peptides (maximum 15-mers) that contained the overlapping HLA-B7 epitopes were chosen for synthesis. In these instances, the SYFPEITHI binding score of the nonamer with the highest avidity for HLA-B7 is indicated in the table.
+Sequences of the two short open reading frames from the RU5 region were from Komurian-Pradel *et al* (1999).

because no diagnostic method for the detection of MSRV antigenemia/viremia existed by the time this study was performed, we were unable to directly analyze and thus verify the presence of MSRV particles in our study population.

Does the observed absence of immune responses to MSRV/HERV-W result from poor sensitivity of the applied methodology? Indirect immunfluorescence assays with insect cells expressing endogenous retroviral proteins delivered by recombinant baculoviruses have previously been used extensively in our laboratory to study antibodies to HERV-K Gag and Env antigens. Detection of such antibodies in a high percentage of patients with germ cell tumors clearly demonstrates that this method is capable of measuring antibodies against HERV encoded antigens (Sauter *et al*, 1995). Although the lower limit of sensitivity of the immunfluorescence assay is unknown, the finding of anti HERV-W Env (Syncytin-1) antibodies in one patient with MS (Figure 1B) indicates that the assay methodology employed, in principle, can detect antibodies against HERV-W in biological samples.

The ELISPOT technology is widely used for measurement of antiviral CTLs and has also been applied to demonstrate the presence of HERV-K-specific CTLs in seminoma patients (Rakoff-Nahoum *et al*, 2006). The detection of positive IFN-γ responses to a well-characterized immunogenic HLA-B7–restricted EBNA-3A peptide (Hill *et al*, 1995), which served as a positive control, unambiguously shows that the ELISPOT methodology used in this work is able

to detect HLA-B7–restricted peptide–specific CTL responses. However, a limitation of our ELISPOT screen is that it does not exclude the presence of CTL responses against MSRV/HERV-W peptides presented by HLA alleles other than HLA-B7. Also, MSRV/HERV-W Env proteins were not included in the ELISPOT analysis. Therefore, although the lack of antibodies to MSRV/HERV-W Env and the complete absence of CTL responses against MSRV/HERV-W Gag-, Pro-, and Pol-derived peptides make it appear rather unlikely that a CTL screen with MSRV/HERV-W Env-derived peptides would have revealed any MSRV/HERV-W Env-specific CTL responses, we cannot rule out the possibility that such CTLs might exist. Nevertheless, for those proteins analyzed, the screen was rather comprehensive, and the number of predicted peptides used and their binding scores are comparable to previous successful applications of the SYFPEITHI epitope prediction algorithm. For instance, in a SYFPEITHI search for HLA-B*0702–restricted peptides within the EBNA-3A protein, the known immunogenic RPPIFIRRL peptide was found among the 2% of highest scoring peptides (binding score: 21); i.e., had we applied the search strategy and experimental protocol used in the present study to EBNA-3A, we would have identified this HLA-B7–restricted epitope. Overall, insufficient sensitivity of the detection methods does thus not seem to account for our data.

Antibody and CTL responses specifically targeted against proteins encoded by the HERV-K family have repeatedly been found in patients with certain tumors (e.g., germ cell tumors and melanomas), in which an increased expression of HERV-K antigens has also been described, suggesting that HERV-K proteins have the potential to elicit immune responses when expressed in a pathologic context (Rakoff-Nahoum *et al*, 2006; Sauter *et al*, 1995; Schiavetti *et al*, 2002). Others and ourselves have accordingly speculated that similar phenomena may also apply to MSRV/HERV-W in MS (Mattson and Taub, 2004; Ruprecht *et al*, 2006). Still, it needs to be emphasized that HERV-W Env (Syncytin-1) is primarily known for its expression in normal human placenta, where a physiological function of this protein in the formation of the syncytiotrophoblast layer seems plausible (Blond *et al*, 2000; de Parseval and Heidmann, 2005). Physiological expression of a HERV-W Gag protein has similarly been described in normal human placenta (Voisset *et al*, 2000) and brain (Perron *et al*, 2005). HERV-W Env (Syncytin-1) and HERV-W Gag are both highly similar to MSRV Env and Gag sequences at the protein level. Along this line, the absence of immune responses against MSRV/HERV-W may be due to immunological tolerance of such physiologically expressed HERV-W proteins, which would prohibit the generation of an immune response against MSRV/HERV-W antigens in the case of an altered or ectopic expression during disease. Such a scenario is indeed reminiscent of Jaagsiekte sheep retrovirus (JSRV), an exogenous retrovirus that causes pulmonary adenocarcinoma in sheep. There are approximately 20 endogenous copies of JSRV stably present in the genome of sheep, all of them being highly related to exogenous JSRV. Sheep infected with exogenous JSRV show a striking absence of antibody responses against this retrovirus (Ortin *et al*, 1998), most probably because endogenous JSRVs are immunologically tolerated and this extends to exogenous JSRV (Spencer *et al*, 2003).

While no difference in the overall low frequency (~4%) of antibodies against HERV-K could be observed between patients with MS and healthy controls (Boller *et al*, 1997), levels of antibodies against HERV-H–derived peptides were found to be significantly elevated in serum and CSF of patients with MS as compared to patients with autoimmune diseases and healthy controls, in which such antibodies were albeit also detectable (Christensen, 2005). This may be of interest as also HERV-H–related RNA sequences have been described in retrovirus-like particles from patients with MS (Christensen, 2005). However, oppositely to HERV-W, there are currently no data on the expression of HERV-H antigens in human tissues, neither under physiological conditions nor in association with MS, available, which makes it difficult to estimate the significance of the finding of elevated HERV-H antibodies in MS.

In conclusion, our results suggest that there are no appreciable humoral or cellular immune responses against MSRV/HERV-W in patients with MS. While this may be due to immunological tolerance of physiologically expressed HERV-W antigens, determination of immune responses as a surrogate marker for the presence of MSRV or an altered expression of HERV-W proteins in patients with MS appears not feasible. Strategies other than measuring immune responses will therefore be required to further elucidate the relationship between MSRV/HERV-W and MS.

Materials and methods

Patients and controls

The study was approved by the ethics committee of the faculty of medicine, Julius-Maximilians University Würzburg, and participants provided written informed consent. Two different series of patients and controls were included. Series A corresponded exactly to a previously reported cohort of 24 patients with MS (14 relapsing-remitting MS [RRMS], 5 secondary progressive MS [SPMS], 5 primary progressive MS [PPMS]) and 29 healthy controls (Gronen *et al*, 2006). In series A, the median (range) age of patients with MS was 39 (25–54) and of controls 35 (19–61) years. Further detailed demographic and clinical characteristics of this cohort have been described before (Gronen *et al*, 2006). Patients and controls of series A were HLA-B7$^+$ as determined by genotyping. PBMC and plasma samples were available

for analyses of CTL and antibody responses against MSRV/HERV-W from all individuals in series A. Plasma samples from series A had been stored at −20°C for a maximum of 8 months and had not been thawed prior to the present analysis.

Series B consisted of 26 patients with MS (16 RRMS, 8 SPMS, 2 PPMS), 4 patients with CIS, and 30 control patients with other inflammatory (3 Guillain-Barré syndrome, 2 chronic inflammatory demyelinating polyneuropathy, 4 bacterial meningitis, 7 viral encephalitis, 4 chronic meningoencephalitis) and noninflammatory (5 brain tumor, 4 meningeosis, 1 epilepsy) neurological diseases. From these patients, aliquoted samples of serum (complete series) and CSF (available for 17 patients with MS, 3 patients with CIS, and all 30 controls), previously stored at −20°C, were obtained for MSRV/HERV-W antibody testing from a local CSF/serum bank at the Department of Neurology, Julius-Maximilians University Würzburg. Serum and CSF samples from series B had been stored for a median of 3 (range 0.5–4) years and had not been thawed before the present investigation. The median (range) age of MS and CIS patients from series B was 36 (18–65) and of controls 42.5 (17–74) years. The female/male ratio of MS and CIS patients was 3.3 and of controls 1.1. Clinical data of all participants from series A and B were obtained by review of the medical records. All patients with MS had a diagnosis of definite MS according to Poser's criteria (Poser *et al*, 1983).

Detection of MSRV/HERV-W antibodies

For detection of IgG antibodies against MSRV/HERV-W antigens a recombinant baculovirus-based eukaryotic expression system using SF158 insect cells was employed. HERV-W Env clone 12 was constructed by cloning the full length HERV-W Env copy from chromsome 7q21 (ERVWE1, NM_014590, gene product: Syncytin-1), amplified from human PBMC genomic DNA, into the pGEM-T vector (Promega). Sequencing of HERV-W Env clone 12 showed an uninterrupted open reading frame for the entire 538–amino acid Syncytin-1 protein with one amino acid exchange (E402G) compared to the Syncytin-1 reference sequence (NP_055405.3). HERV-W Env clone 12 (nucleotides corresponding to nucleotides 1044 to 2671 in NM_014590) and the MSRV clones env pV14 (AF331500, nucleotides 1 to 1629) and Gag CL2 (AF123881, nucleotides 1 to 1060) were subcloned in the pAC360 baculovirus transfer vector. Cotransfection of SF158 insect cells with baculovirus DNA (baculoGOLD; BD Biosciences) and either of the different baculovirus transfer vectors resulted in the release of recombinant baculoviruses carrying MSRV Gag, MSRV Env, and HERV-W Env genetic sequences in the cell culture supernatants, which were harvested, filtered through 0.45 μm, and stored at 4°C. For indirect immunofluorescence, SF158 cells in logarithmic growth phase were separately infected with either MSRV Gag, MSRV Env, or HERV-W Env recombinant baculoviruses, or wild-type baculoviruses as negative control. Forty-two hours post infection, protein expressing insect cells were harvested, mixed with uninfected SF158 cells at 1:40, plated on microscope slides, and fixed in acetone. Slides were then incubated for 45 minutes with sera (1:40) or CSF (1:20) diluted in phosphate buffered saline and subsequently developed with fluoresceine isothiocyanate–conjugated goat anti-human IgG antibodies (1:50, Sigma). As positive controls, polyclonal rabbit antisera against MSRV Gag (F45128) and HERV-W Env (K343), and a mouse monoclonal antibody against MSRV Env (13H5A5) were used.

Western blot

Protein lysats from SF158 insect cells infected with recombinant baculovirus were separated by gel electrophoresis and blotted onto polyvinylidene difluoride (PVDF) membranes (millipore) as described (Boese *et al*, 2000). Membranes were probed with the MSRV Gag–, MSRV Env–, and HERV-W Env–specific antibodies, or patient sera (diluted 1:10), and developed with appropriate secondary peroxidase labeled IgG antibodies (1:2000, Sigma) and an enhanced chemoluminescence kit (Amersham) or 3,3′-diaminobenzidine.

Measurement of MSRV/HERV-W–specific T cells

The strategy for analysis of MSRV/HERV-W–specific CTLs consisted of screening a cohort of HLA-B7$^+$patients with MS and controls (series A) for CTLs specific to MSRV/HERV-W peptides, predicted to be presented in association with HLA-B7. To this end, amino acid sequences of MSRV Gag , HERV-W Gag, MSRV Pro, HERV-W Pro, MSRV Pol, HERV-W Pol, and two short proteins (termed U5/1 and U5/2) encoded by open reading frames in the RU5 region of MSRV were identified in the protein database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/sites/entrez?db=protein, February 2004) as well as in Komurian-Pradel *et al*, 1999. These sequences were analyzed with the SYFPEITHI peptide epitope prediction algorithm (Rammensee *et al*, 1999) to identify nonamer peptides likely to be presented in association with HLA-B*0702. Based on empirically determined known HLA binding motifs, SYFPEITHI assigns a binding score to each possible nonamer peptide in an amino acid sequence which is an indication of the peptide's binding strength to a given HLA class I molecule. Out of all peptides identified for each protein, the upper 2% of peptides with the highest binding score for HLA-B*0702 were chosen for synthesis (Rammensee *et al*, 1997). A list of the 36 MSRV/HERV-W peptides used in this study is provided in Table 1. High-performance liquid chromatography (HPLC)-purified peptides checked by mass spectometry analysis were purchased from NMI-Peptides (Reutlingen, Germany), dissolved in DMSO (final concentration per well 0.5%), and

assembled in 12 overlapping pools, each containing 5 to 7 peptides. Isolation of PBMCs and measurement of CTL responses against the predicted HLA-B7–restricted MSRV/HERV-W peptides by IFN-γ ELISPOT assays were performed exactly as described in detail in a previous study (Gronen *et al*, 2006). Positive controls included stimulation with PHA, a common recall antigen (tetanus toxoid), and a HLA-B7–restricted Epstein-Barr virus peptide (RPPIFIRRL) derived from the EBNA-3A protein (Hill *et al*, 1995), whereas medium containing 0.5% DMSO served as negative control (Gronen *et al*, 2006). In all ELISPOT assays, positive and negative controls were run together with MSRV/HERV-W peptides on the same ELISPOT plate. CTL responses to a given peptide were considered positive when after subtraction of the spot number from unstimulated control wells the spot number in peptide-stimulated wells was ≥ 20 per 10^6PBMCs. Except for 7 healthy controls from whom only previously frozen PBMCs (stored at -70°C) were available, ELISPOT assays for the remaining 22 healthy controls and all patients with MS were performed using fresh PBMCs immediately after isolation.

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