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Multiple sclerosis–associated virus-related *pol* sequences found both in multiple sclerosis and healthy donors are more frequently expressed in multiple sclerosis patients

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In the etiopathogenesis of multiple sclerosis (MS), both genetic and environmental factors play an important role. Among environmental factors, viral infections are most likely connected with the etiology of MS. There are many evidence suggesting possible involvement of retroviruses in the development of autoimmune diseases including MS. Multiple sclerosis–associated retrovirus (MSRV) seems to be the important candidate for viral etiology of MS. The aim of the study was to analyze MSRV *pol* sequences in patients with MS. As control, groups of myasthenia gravis, Parkinson's disease, and migraine patients, and healthy individuals have been studied. The MSRV *pol* sequences have been analyzed in RNA isolated from the serum and in DNA and RNA of peripheral blood lymphocytes from untreated MS patients and control groups. The MSRV *pol* sequences have been detected by reverse transcriptase–polymerase chain reaction (RT-PCR) and PCR technique, using specific oligonucleotide primers. In the serum RNA (cDNA), MSRV *pol* sequences have been identified in 31/32 MS patients. MSRV *pol* sequences were detected in serum cDNA of 9/17 myasthenia gravis patients, 7/16 Parkinson's disease patients, 10/21 migraine patients, and 13/27 healthy individuals. MSRV *pol* sequences were observed also in RNA from lymphocytes of all MS patients, 12/17 myasthenia gravis patients, 9/16 Parkinson's disease patients, 14/21 migraine patients, and 18/27 healthy donors. In the DNA from peripheral blood lymphocytes of all studied patients and healthy individuals, MSRV *pol* sequences have been found. The observed pattern of fiber–fluorescence *in situ* hybridization (FISH) signals suggests the presence of multiple copies of MSRV *pol* sequences, most likely tandemly dispersed in the genome. It can be concluded that MSRV *pol* sequences are endogenous, widespread in lymphocytes DNA, and transcribed into RNA of MS patients as well as of other studied patients and healthy individuals. However, more frequent expression of MSRV sequences detected in lymphocytes RNA (cDNA), as well as their presence in higher frequency in the serum of MS patients, may suggest the involvement of MSRV in the etiopathogenesis on MS. *Journal of NeuroVirology* (2003) **9,** 112–117.

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Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease with multifocal destruction of central nervous system (Ffrench-Constant, 1994; Steiman, 1996). The pathology of MS is heterogeneous and therefore

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various factors, both genetic and environmental, may lead to development of MS (Sadovnik *et al*, 1996; Steiman *et al*, 1995). Among environmental factors, viruses may contribute to the etiology of MS (Azoulay Cayla, 2000; Dalgeisch, 2000; Perron, 1998). Many epidemiological studies indicate that the onset of development of MS is greatly affected by viral or bacterial infection (Rasmussen and Clausen, 1997; Tuke *et al*, 1997). Many viruses were postulated to cause MS, but no single virus has been shown as a causative agent of MS (Rasmussen and Clausen, 1997; Rieger *et al*, 2000; Tuke *et al*, 1997). It seems that a combination of viruses acting synergistically, along with some genetic factors, may be required to induce the development of MS (Aliel *et al*, 1998c; Tienari *et al*, 1992). There is much evidence that strongly indicates autoimmune response, in which viral infection plays a crucial role in the pathogenesis of MS (Perron *et al*, 2001; Rasmussen and Clausen, 1997). The concepts of autoimmune and viral etiology of MS are commonly recognized. The best hypothesis explaining etiology of MS seems to be viral infection along with some genetic factor leading to autoimmune response, which finally causes demyelination in central nervous system.

MSRV (multiple sclerosis–associated retrovirus), described by Perron *et al* (1997) seems to be a good candidate for a virus directly involved in the pathogenesis of MS. Recently a new family of human endogenous retroviruses, HERV-W, that is related to MSRV has been identified (Blond *et al*, 1999; Kim and Crow, 1999; Kim *et al*, 1999; Voisset *et al*, 1999). Endogenous retrovirus sequences are widespread in human genome (Rasmussen, 1997). Endogenous retroviruses may play important role in human diseases, especially those of autoimmune origin. There is increasing evidence to suggest that retroviruses affect immune function and very likely participate in the development of autoimmune diseases (Alliel *et al*, 1998b; Gaudin *et al*, 2000; Rasmussen and Clausen, 1997). The effect of endogenous retrovirus greatly depends on their site of localization (Rasmussen, 1997). The retroviruses integrated close to long terminal repeats (LTRs) may drive the expression of adjacent genes or modify other genes in their close vicinity. Retroviruses may also cause gene splicing. The best example is alternation of the splicing of *Fas* transcript, resulting in lymphocyte differentiation defect, leading to lupus-like autoimmune disease. It was also postulated that the products of endogenous retrovirus may play a role as a superantigen (Rasmussen, 1997). The expression of endogenous retrovirus sequences most likely reflexes the function or state of the cells. This is supported by the finding that the expression of some endogenous retrovirus increases with age. Various changes in the normal function of cells have also been associated with alternation in expression of endogenous retrovirus (Rasmussen, 1997). All these data strongly suggest certain role of endogenous retroviruses in the development of MS.

The aim of this paper was to study the presence of MSRV *pol* DNA and mRNA sequences in serum and peripheral blood lymphocytes of MS patients and of control groups of myasthenia gravis, Parkinson's disease, migraine patients, and healthy individuals. MSRV mRNA *pol* sequences were analyzed to detect either virus particle or expression of endogenous MSRV *pol* sequences.

Results

MSRV has been detected by reverse transcriptase–polymerase chain reaction (RT-PCR) technique according to the detailed procedure kindly provided by Perron. Primers used were specific for *pol* sequence of MSRV. MSRV *pol* sequences in the serum of MS patients have been found in 31 out of 32 cases (Table 1). Unexpectedly almost 50% (13/27) of healthy blood donors and 9/17 myasthenia gravis patients, 7/16 Parkinson's disease patients, and 10/21 migraine patients appeared to be positive for MSRV *pol* sequence in the serum. To exclude the possible contamination with DNA, RNA isolates without reverse transcription was subjected to PCR test. Five serum RNA isolates from MS patients and healthy donors were negative when tested in PCR with specific MSRV primers. Additionally, we have detected MSRV in cerebrospinal fluid of MS patients (only three samples were available). All three samples were positive in RT-PCR. The relative high numbers of MSRV-positive sera prompted us to analyzed the MSRV *pol* sequence expression in peripheral blood lymphocytes. RNA isolated from lymphocytes were

Table 1 Incidence of MSRV *pol* sequences in MS patients and healthy individuals

Material	*Multiple sclerosis*	*Myasthenia gravis*	*Parkinson's disease*	*Migraine*	*Healthy individuals*
Serum RNA (cDNA)	31/32*	9/17	7/16	10/21	13/27
	96.9%	52.9%	43.8%	47.6%	48.1%
Peripheral blood lymphocytes RNA (cDNA)	32/32*	12/17	9/16	14/21	18/27
	100%	70.6%	56.3%	66.7%	66.7%
Peripheral blood lymphocytes DNA	32/32	17/17	16/16	21/21	27/27
Cerebrospinal fluid RNA (cDNA)	3/3	*ND*	*ND*	*ND*	*ND*

*Statistically significant difference in comparison to all other groups ($P < .05$; Student's t test).

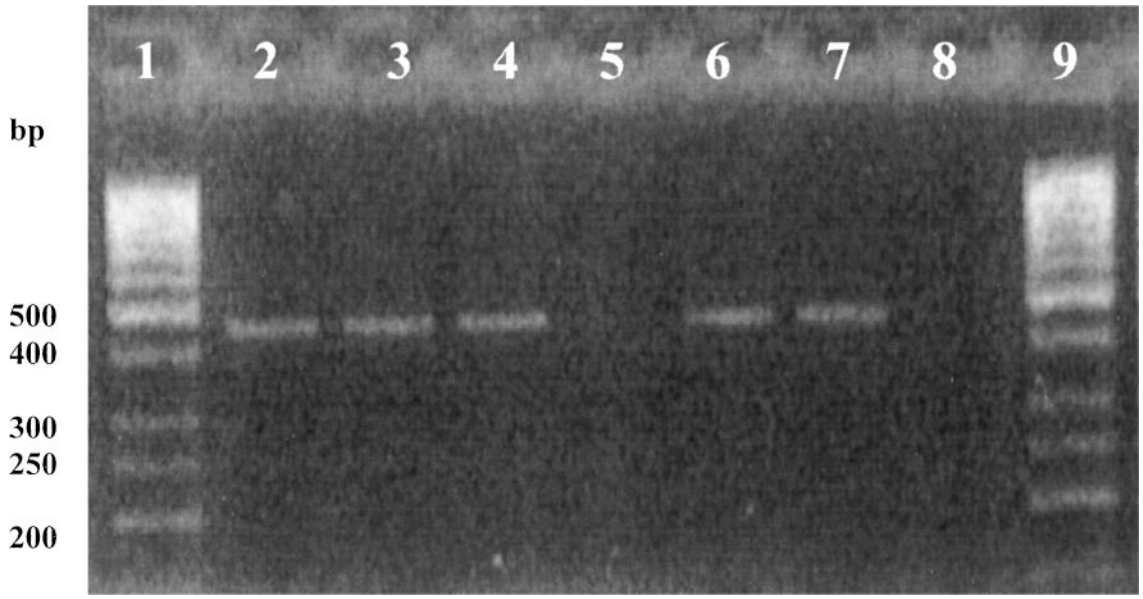

Figure 1 RT-PCR analysis of MSRV *pol* sequence expression in serum, lymphocytes, and cerebrospinal fluid of MS patients and healthy individuals. Lanes 1, 9: 50-bp DNA molecular marker (Fermentas); lane 2: MS serum; lane 3: MS lymphocytes; lane 4: MS cerebrospinal fluid; lane 5: PCR on RNA without reverse transcription; lane 6: healthy donor serum; lane 7: healthy donor lymphocytes; lane 8: negative control (PCR without cDNA). Size of the amplified products is 430 bp.

transcribed to cDNA and tested by RT-PCR under the same technical conditions as described by Perron *et al* (1997). MSRV *pol* sequence in peripheral blood lymphocytes was expressed in all MS patients, 12/17 myasthenia gravis patients, 9/16 Parkinson's disease patients, 14/21 migraine patients, and 18/27 healthy donors. The expression of MSRV in peripheral blood lymphocyte strongly argues that the MSRV *pol* sequences are present in human genome. To prove this, MSRV *pol* sequences have been identified in genomic DNA. PCR performed in MS and other patients as well as healthy donors gave positive results with all DNA samples tested. Representative results of MSRV detection with PCR with all negative controls are given in Figure 1.

To further investigate the presence of MSRV in human genome, high-resolution fluorescence *in situ* hybridization (FISH) to chromatin fibers (linearly extended chromatin fibers [ECFs]) has been applied. Biotinylated PCR product, obtained with specific primers for MSRV on serum cDNA, hybridizes to chromatin fibers in all samples tested. Representative results of fiber-FISH are presented in Figure 2. Under standardized condition of chromatin DNA stretching, the signal lengths were similar in all samples tested, including MS and healthy donors. The FISH results evidently indicate the presence of MSRV *pol* sequence in human genome.

Discussion

There is increasing evidence that retroviruses in human can affect immune response and may act as a genetic susceptibility factors in MS (Rasmussen *et al*, 1997; Rieger *et al*, 2000). MRSV, described by Perron *et al* (1997) seemed to be a favorite candidate in MS. Perron *et al* (1997) found MSRV *pol* sequence in the serum of 9 of 17 MS patients and 3 out of 44 healthy individuals. On the basis of obtained results, they postulated that MSRV is an exogenous retrovirus playing an important role in MS development. Following Perron's procedure, we have found the high frequency of MSRV presence in blood serum of MS patients (96.9%). In all other groups, the frequency

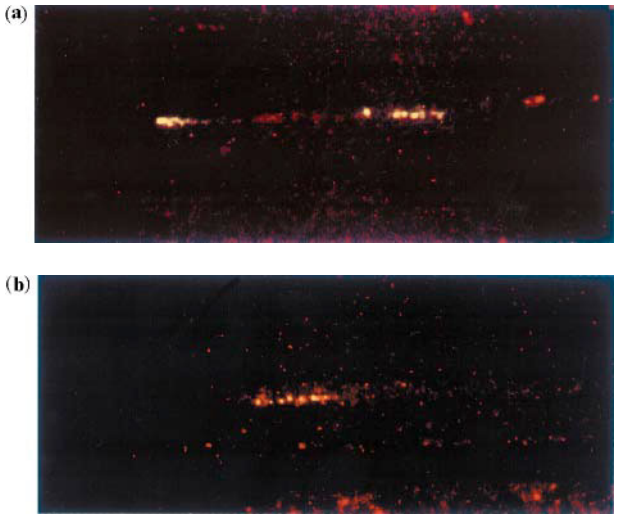

Figure 2 High-resolution fiber-FISH with MSRV *pol* sequence probe (green signals: FITC fluorescence) to chromatin fibers (red signals: proopidium iodide) of MS patient (**a**) and healthy individual (**b**).

of MSRV incidence in the serum does not exceed 53% (myasthenia gravis). In addition to the serum, MSRV *pol* sequences were detected in lymphocyte RNA of all MS patients and in also high frequency in other control groups. These findings are difficult to explain by retrovirus of exogenous origin. It is more likely that detected MSRV *pol* sequence is a product of the gene present in human genome. Finding the MSRV *pol* sequence in DNA from all patients and healthy individuals proved this assumption. The fiber-FISH results gave additional evidence of the existence of MSRV *pol* sequences integrated in the human genome. The observed pattern of fiber-FISH signals suggests the presence of multiple copies of MSRV *pol* sequences, most likely tandemly dispersed in the genome.

Human genome contains many endogenous viral sequences. Several human endogenous retroviruses have been sequenced and characterized. It is interesting that many of the human endogenous retroviruses are present in multiple copies (Perron *et al*, 2000; Rasmussen, 1997). Our fiber-FISH results may indicate on multiple copies of MSRV *pol* sequence in the genome.

MSRV *pol* sequence is related to ERV-9 HERV (Blond *et al*, 1999; Komurian-Pradel *et al*, 1999). ERV-9 sequences were detected with MSRV in plasma or cell culture supernatant of MS patients (Alliel *et al*, 1998a; Garson *et al*, 1998) . MSRV *pol* sequences were also found in the supernatant of synoviocyte culture of rheumatoid patients (Gaudin *et al*, 2000). All these results reported by others as well as our own data strongly indicate the presence of endogenous MSRV sequence. MSRV RNA sequences detected by us are most likely the products of the endogenous DNA sequences. Initially Perron *et al* (1997) suggest that the identified MSRV sequences may represent extracellular particles resulting from virus infection. In one of the latest papers, he claims that, "due to multiple endogenous HERV copies it is technically difficult to perform standardized test to detect MSRV" (Perron *et al*, 2000). The detected MSRV *pol* sequences are, therefore, most likely produced by genes of endogenous virus. Belonging to HERV family, MSRV may represent a virion producing exogenous member of an endogenous family. Alternatively, MSRV present in the serum or cerebrospinal fluid could be composed of defective retroviral elements cooperating via *trans*-complementation.

Many observations indicate that several endogenous viruses are normally transcribed in human peripheral blood leukocytes (Rasmussen, 1997). Previous studies using Northern blood analysis showed the expression of number of endogenous retroviruses in peripheral blood leukocytes from healthy donors and patients suffering from autoimmune muscle diseases, without evidence of significant differences between the two groups examined (Rasmussen, 1997). Transcription activity of endogenous retroviruses may influence the cell function and may also contribute to the development of the autoimmune diseases.

Rasmussen *et al* (1997) reported the expression of endogenous retroviruses in peripheral blood nuclear cells and brain tissue from MS patients. They found the expression of proviral sequences, including HERV-K, HRES-1, and ERV3. It is interesting to note that ERV3 was more frequently expressed in peripheral blood lymphocytes of healthy donors than in MS patients.

In our study, we have found the expression of MSRV *pol* sequence in all lymphocyte RNA and in all but one serum RNA of MS patients. Higher frequency of expression of MSRV sequences was detected in lymphocyte RNA as well as in the serum of MS patients in comparison to other groups. On the basis of obtained results, we can claimed that expression of MSRV *pol* sequence is specific for MS. It may be speculated that integration of MSRV to human genome like other HERV is not entirely accidental and may have effect on gene transcription. It is possible that MSRV sequences could have promoting effect in the pathogenesis of MS. This effect could be an indirect one, for example, influencing susceptibility to viral or bacterial infection. It can be imagined that the expression of MSRV may affect destruction of myelin by protease or tumor necrosis factor (TNF). Endogenous MSRV sequences could contribute also for the recognition of similar peptide epitopes shared with exogenous viruses.

Summarizing our results, it can be concluded that MSRV *pol* sequences are widespread in lymphocyte DNA and are transcribed into RNA of MS patients as well as of other studied groups and, therefore, MSRV *pol* sequences are of endogenous origin. Higher frequency of expression of MSRV *pol* sequences in MS patients than in all other groups studied suggests the involvement of MSRV in the etiopathogenesis on MS.

Material and methods

Peripheral blood was obtained from 32 patients with clinically definite, untreated MS, aged 19 to 57 years (median 37.7 years). To prove the diagnosis of MS, magnetic resonance imaging examinations have been performed in the majority of patients. The control group consisted of 17 myasthenia gravis patients, 16 Parkinson's disease patients, 21 migraine patients, and 27 healthy adults, aged 23 to 46 years. Additionally, cerebrospinal fluid has been obtained from three MS patients. The Ethics Committee of the Academy of Medical Sciences in Poznan approved the study.

DNA has been isolated from peripheral blood lymphocytes using QIAamp DNA Blood Mini Kit (Qiagen). RNA from lymphocytes was obtained with QIAamp Viral RNA Mini Kit (Qiagen). Isolation of RNA from the serum and supernatant of cerebrospinal fluid has been performed according

detailed procedure kindly provided by Perron. The sequence of primers, reverse transcription, and PCR conditions were the same as described by Perron *et al* (1997). To exclude the possible contamination of DNA in RNA isolates, PCR test has been performed without reverse-transcribed RNA.

To investigate the presence of MSRV integrated to human genome, high-resolution FISH analysis to DNA fibers (ECFs) has been applied. In our experiment, the protocols by Florijn *et al* (1995) and Lestou *et al* (1996), with some modifications, have been used. Briefly, peripheral blood lymphocytes were centrifuged onto a clean microscope glass slides at 700 r.p.m. for 3 min using a Cytospin. Immediately after centrifugation, the air-dried cells were lysed by incubating the slides with high-salt solution and Carnoy's fixative, methanol:acetic acid (3:1). The probe for FISH was biotinylated PCR product (430 bp), obtained with specific primers for MSRV on serum cDNA. Hybridization signals were amplified with rabbit antibiotin/fluorescein isothiocyanate (FITC) goat anti-rabbit immunoglobulin G (IgG) (Vector Lab). Hybridization signals were detected under a fluorescence microscope Axiophot (Zeiss) at a magnification of ≈1000. The obtained images have been processed by MetaSystems (GmbH) program. High-resolution DNA fiber-FISH of the entire MSRV genome has been carried out in four patients with MS and in two healthy individuals (all of them were women with normal karyotype).

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