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Evidence of Presence of Poliovirus Genomic Sequences in Cerebrospinal Fluid from Patients with Postpolio Syndrome

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The postpolio syndrome (PPS) is characterized by new neuromuscular symptoms occurring 30 to 40 years after the acute episode of poliomyelitis paralysis. The presence of the poliovirus RNA genome in the cerebrospinal fluid from 10 patients with PPS and from 23 control patients was sought by using reverse transcription and a PCR specific for polioviruses and/or other enteroviruses. Poliovirus-specific genomic sequences in the 5′ untranslated region and in the capsid region (VP1) were detected by reverse transcription PCR in 5 of 10 patients with PPS but in none of the control patients. Sequencing confirmed the presence of mutated poliovirus sequences. This finding suggests persistent viral infection in the central nervous system related to the presence of poliovirus genomes.

The postpolio syndrome (PPS) is characterized by the delayed appearance of new neuromuscular symptoms in patients many years after their acute poliomyelitis paralysis (5, 6). The progressive aggravation of the motor deficiency is associated with myalgia, cramps, fasciculations, weakness, and amyotrophy in the affected and sometimes spared regions (23). Compression and entrapment neuropathies, plexopathies, or radicular syndromes, as well as the motor consequences of articular pathologies, are excluded (6). PPS occurs 30 to 40 years after the acute poliomyelitis attack and is observed in approximately 25 to 28% of patients (19); an increased prevalence of the syndrome in females is also noted (60% of cases). The etiology and mechanisms of PPS remain unclear; the current theory suggests the attrition and premature degeneration of surviving motor neurons with the loss of axonal terminals. Another hypothesis concerns the persistence of poliovirus in the central nervous system and is supported by data from Sharief et al. (24) demonstrating the presence of poliovirus immunoglobulin M (IgM) antibodies and poliovirus-sensitized cells in the cerebrospinal fluid (CSF) of PPS patients. However, conclusive demonstration of virus in the CSF from PPS patients has not yet been reported. The persistence of poliovirus has been shown in neuroblastoma cell culture (4) and in vivo in animals (16). Melchers et al. (15) examined CSF and muscular biopsy specimens from PPS patients for the presence of poliovirus RNA by PCR and probe detection and obtained negative results. In the present study, we tried to detect poliovirus RNA in CSF specimens from 10 PPS patients and 23 control patients using the highly sensitive reverse transcription PCR (RT-PCR) (11, 13, 28). Further characterization of the amplified products was obtained by sequencing.

Ten patients (patients 1 to 10) presenting with PPS were examined between 1991 and 1994. Strict criteria were adopted for the diagnosis of PPS, corresponding to those proposed by Dalakas and colleagues (5, 6): a clear history of acute paralytic poliomyelitis, functional stability or recovery for at least 15 years, residual muscle atrophy, weakness, and areflexia in at least one limb, with normal sensation and no sign of upper-motor-neuron weakness. None of the patients was vaccinated against poliomyelitis. For all patients, the following studies were performed: electrophysiologic studies including conventional electromyography and studies of nerve conduction velocities, immunologic tests with $CD4^+$ and $CD8^+$ T-cell counts, and immunoelectrophoresis of serum. Electrophoresis of CSF protein was done with freshly collected specimens in Bordeaux by using agarose gel electrophoresis and immunofixation. Ten patients (patients 14 to 23) with amyotrophic lateral sclerosis (ALS), 10 patients (patients 24 to 33) with other neurological diseases (cerebrovascular diseases, headache, or peripheral neuropathies), and 3 patients (patients 11 to 13) with stable poliomyelitis sequelae were included as controls in the study. For the three patients with stable poliomyelitis, the intervals between the acute paralytic poliomyelitis and analysis of CSF in the present study were 60, 62, and 72 years, respectively.

One CSF and one blood sample were taken from each patient except for patients 1, 2, 3, 4, and 14. For the latter group of patients, two CSF samples were taken at an interval of 1 year. For patients 3 and 4, two blood samples were taken at an interval of 2 years. The number of CSF samples repeatedly analyzed was limited because multiple lumbar punctures were not performed for each patient. Muscle biopsy specimens and other specimen types were not available for virological testing. All CSF samples (1.5 ml) were divided into three equal parts. For molecular biology purposes, CSF samples (500 μl per tube) were collected in two tubes containing proteinase K (100 μg/ml), and the tubes were sent to two laboratories, one in Lyon and one in Paris, and were analyzed separately. For viral isolation, one tube of CSF (500 μl) was sent to the National Reference Center for Enteroviruses in Lyon and was inoculated onto BGM K, Hep-2, and MRC 5 cells.

Both serum and CSF specimens from each PPS patient were examined for IgG and IgM antibodies to poliovirus by a neutralization assay. Antibodies were checked against types 1, 2, and 3 Sabin and wild prototype poliovirus strains provided by

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TABLE 1. Clinical characteristics of late-onset symptomatology in 10 patients with PPS[a]

Patient no.	Age (yr)	Sex	Age of patient at onset of APP attack (yr)	Aftermath	Interval between APP and PPS (yr)	PPS					CSF			
						Aggravation of the neuromuscular shortage		Myalgia	Weakness	Fasciculations	Protein concn (mg/dl)	Cell count	RT-PCR of 5′-NC	RT-PCR of VP1
						In the aftermath area	In other areas							
1	69	F	4	L Lb r P + D	37	+	L Lb r-L Lb l	+	+	0	33	1	+	+
2	67	F	10	Bilateral U Lb, P + D	57	+	0	+	0	+	28	2	+	+
3	55	F	20	L Lb P + D l > r	27	+	U Lb r > l	+	+	0	35	4	+	+
4	72	F	5	L Lb P + D 1 > 1	66	+	0	0	0	0	44	1	+	+
5	75	M	1	U Lb r-L Lb l	63	+	0	0	+	0	25	2	+	+
6	37	F	4	U Lb r	27	+	0	0	0	+	16	2	−	−
7	32	M	3	Bilateral L Lb U Lb l, scoliosis	28	+	0	+	+	0	28	1	−	−
8	44	F	7	Bilateral L Lb l > r Scoliosis	29	+	0	+	+	0	35	2	−	−
9	63	M	18	L Lb l > r	37	+	U Lb r D	0	+	+	40	1	−	−
10	59	M	9	L Lb r D, scoliosis	49	+	L Lb l P	0	+	+	20	2	−	−

[a] Abbreviations: Lb, limb; U, upper; L, lower; P, proximal; D, distal; l, left; r, right; F, female; M, male; 5′-NC, 5′ noncoding region.

the National Reference Center for Enteroviruses. Detection of antibodies to other organisms such as measles virus, mumps virus, cytomegalovirus, herpes simplex virus, or *Toxoplasma gondii* was not attempted. Separation of IgM and IgG in CSF and sera was performed on microcolumns by using an affinity chromatography gel (Seromed, Biochrom, Germany) according to the manufacturer's recommendations.

For molecular biology studies, CSF samples containing proteinase K were incubated for 1 h at 37°C and for 5 min at 95°C; RNA was extracted with RNAzol B from 100 μl of treated CSF samples (Bioprobe Systems, Montreuil, France) according to the manufacturer's recommendations, precipitated from the aqueous phase with isopropanol, washed with 70% ethanol, air dried, and dissolved in 20 μl of distilled diethylpyrocarbonate-treated water. The cDNA was synthesized with the universal oligo$(dT)_{15}$ primer. Two amplification runs were performed as described previously with negative (H_2O) and positive (coxsackievirus type B3) controls (12). PCR products were analyzed by electrophoresis in 1% agarose gels and were revealed by ethidium bromide staining. The sequences to be amplified were selected in the conserved part of the 5′ untranslated region (5′-UTR) and have been described elsewhere (11, 13, 28). Amplifications were also done in the poliovirus-specific VP1 region with primers D, E, and F in the Lyon laboratory (12) and with primers 7 (5′-GATAGTTTCACCGAAGGCG GA-3′; positions 2329 to 2349), 8 (5′-TCCAGGGGATTGTG GTGGC-3′; positions 3723 to 3741), and C1 (5′-CGCAGTG AACGTCATGTGG-3′; positions 3495 to 3513) in the Paris laboratory. Amplified products from the 5′-UTR were further analyzed by direct sequencing. PCR products were excised from the gel and were extracted by using the Magic PCR Prep DNA kit (Promega). The sequence was determined by cycle sequencing as described by Adams and Blaskeley (1), with slight modifications: 50 pmol of primer was end labelled with [γ-^{33}P] ATP by using polynucleotide kinase. Each of the four dideoxynucleotide sequencing reactions (10 μl) contained 40 to 80 fmol of template, 2.5 pmol of end-labelled primer, 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus), 30 mM Tris HCl (pH 9.0), 5 mM $MgCl_2$, 10 μM (each) deoxynucleoside triphosphate, 300 μM ddATP, 100 μM ddCTP, 50 μM ddGTP, and 400 μM ddTTP; the mixture was covered with one drop of paraffin oil. The reaction was done in a thermal cycler (PHC3; Techne) with 20 cycles of 95°C for 30 s, 55°C for 30 s, and 70°C for 60 s; this was followed by 10 cycles at 95°C for 30 s and 70°C for 30 s. The reaction was terminated by adding 5 μl of formamide dye solution. The samples were heated for 5 min at 95°C before being transferred to the 7% polyacrylamide–7 M urea gel. The migration was done at a constant power of 40 mA. The gels were dried in a vacuum dryer and were exposed to X-ray (Fuji) film for 24 to 48 h at room temperature.

All PPS patients (mean age, 57 years) had had a history suggestive of acute paralytic poliomyelitis in childhood or adolescence, i.e., a febrile illness associated with weakness and atrophy usually occurring during a poliomyelitis epidemic (Table 1). The acute attack had been followed by a partial recovery of motor deficit. A residual motor disability associated with amyotrophy was observed in at least one limb, with tendinous areflexia and normal sensitivity. The history of paralytic poliomyelitis was obtained from the patient and whenever possible was corroborated by medical records and information from relatives. The interval between acute poliomyelitis paralysis (APP) and the onset of PPS ranged from 27 to 66 years (mean time, 42 years). Neuromuscular symptoms were asymmetric motor deficit with amyotrophy in 8 of 10 patients, asymmetric motor deficit with amyotrophy restricted to the upper limbs in 2 of 10 patients, and trunk muscle deficiency with scoliosis in 3 of 10 patients. A progressive worsening of muscular weakness was observed in the muscles previously affected by APP in all patients. Four of the 10 patients exhibited weakness of the muscle previously spared by the APP attack. Muscular pains were mentioned by 5 of the 10 patients and muscle tiredness was mentioned by 6 of the 10 patients. None of these patients was affected with new bulbar, respiratory, or sleep difficulties. Electrophoresis of CSF protein was normal for all patients, and oligoclonal banding of immunoglobulins was not observed in any sample. Electromyographic studies showed abnormal motor units in the atrophic muscles, but also in many clinically unaffected muscles. Denervation potentials (fibrillations) were observed in two patients. Motor and sensory conduction velocities were normal. $CD4^+$ and $CD8^+$ T-cell counts were normal, as were the results of immunoelectrophoresis of serum, for all patients.

We did not find any specific neutralizing IgM antibodies to poliovirus in serum samples from the PPS patients. For all PPS

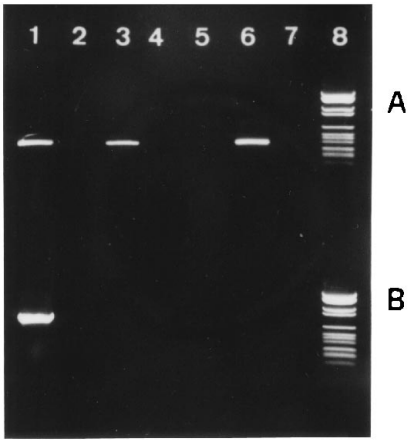

FIG. 1. RT-PCR of CSF samples from patients with PPS and control patients. Amplification of both the 5′-UTR region (A) and the VP1 region (B) was performed. Lanes 1 and 2, CSF from PPS patients; lanes 3 and 4, CSF from ALS patients; lane 5, CSF from a healthy subject; lane 6, nonpolio virus enterovirus-positive control; lane 7, negative control; lane 8, molecular weight marker VI (Boehringer Mannheim).

TABLE 2. Comparison of amplified sequences from CSF samples of PPS patients with sequences of polioviruses[a]

Virus source	% Sequence similarities		
	Poliovirus type 1 Sabin	Poliovirus type 2 Sabin	Poliovirus type 3 Sabin
Patient 1	90.7	**99.3**	82
Patient 2	**94**	90.7	82
Patient 3	74.7	75.3	**80**
Patient 4	**89.3**	87.3	86
Patient 5	**80**	76	73.3
Poliovirus type 1 Sabin		90.7	82.7
Poliovirus type 2 Sabin	90.7		81.3
Poliovirus type 3 Sabin	82.7	81.3	

[a] The poliovirus sequences from nucleotides 385 to 535 were compared (26). The sequence comparison was done by using the Clustal V program (9). Boldface indicates the highest percentage of similarities in each patient.

patients, IgG antibodies to the three poliovirus serotypes were detected. This result may indicate exposure to vaccine poliovirus strains by contact. We did not find specific neutralizing IgM and IgG antibodies to poliovirus in CSF samples from the PPS patients. All attempts to isolate in CSF samples a virus multiplying in cell culture remained unsuccessful.

Specific amplifications of sequences in the 5′-UTR and VP1 regions were repeatedly obtained for 5 of 10 CSF specimens from PPS patients (patients 1 through 5) in both laboratories, showing the presence of poliovirus genomes (Fig. 1). For patients 1 to 3, two CSF samples were taken at an interval of 1 year; positive PCR results correlated in patients 1 and 2, whereas for patient 3, the first CSF sample (1992) was positive by RT-PCR for amplification of the 5′-UTR and VP1 regions but the second (1993) was negative for amplification of both regions. Amplification of the 5′-UTR region was also obtained for 2 of 10 ALS specimens (from patients 14 and 15), but not for the VP1 region, indicating the presence of nonpoliovirus enterovirus sequences (Fig. 1). For patient 14, we obtained a positive PCR signal for two CSF samples collected at an interval of 1 year. CSF samples from patients with stable poliomyelitis and from control subjects were negative by RT-PCR for the 5′-UTR and VP1 regions. The sequences of the amplified products in the 5′-UTR region were obtained and compared with the nucleotide sequences of the prototype polioviruses (Table 2) and other common enteroviruses (data not shown) at positions 385 to 535. The results showed that the sequence obtained from the CSF of patient 1 was very close to the sequence of poliovirus type 2, and at position 481 the characteristic base of wild-type poliovirus type 2 was identified. In the CSF of patients 2, 4, and 5, sequences closer to that of poliovirus type 1 were found, and for patient 3, a sequence closer to that of poliovirus type 3 was found. However, all of these sequences differed from those of wild-type and vaccinal reference strains by 6 to 20%.

The finding that RNA viruses can persist in their host for life and can consequently induce a chronic disease is an important advance in virology (2, 25). The detection of enterovirus-specific RNA sequences in muscle biopsy specimens from patients with muscular diseases (myositis and heart diseases) has been reported by several groups of investigators (7, 12, 27). Chronic meningoencephalitis related to enterovirus infection has been observed (12, 20). Colbère-Garapin et al. (4) demonstrated that poliovirus can infect the neuroblastoma cell line and induce persistent infection in vitro.

A seminested amplification assay for the detection of enterovirus and poliovirus genomes, validated in different laboratories, allowed for the detection of 1 fg of viral RNA (11–13). Indeed, the use of another primer in the second amplification increases the sensitivity and the specificity of the test. All amplifications and sequencing assays were performed in two independent laboratories (in Lyon and Paris), and identical results were obtained. Molecular biology methods allowed for the detection of poliovirus sequences in 5 of 10 PPS patients without any correlation with the presence of IgM class antibodies and virus isolation in cell culture. The negative results with CSF samples obtained by Melchers et al. (15) could be explained by the use of a single amplification run, which is a less sensitive technique than our seminested RT-PCR. Like Salazar-Grueso et al. (22), but unlike Dalakas et al. (6), we did not find any oligoclonal patterns of proteins. These discrepancies could be related to the use of different techniques. Cell culture lacks the sensitivity required to detect enteroviruses in CSF during an acute enterovirus infection of the central nervous system (12, 20). Therefore, failure to isolate the virus from the CSF of PPS patients was an expected result. For patient 3, the first CSF sample was positive for the presence of viral RNA, but the second sample, obtained 1 year later, was negative. This could be explained either by infraliminar synthesis or the intermittent release of viral RNA from neural tissue in the CSF. Two results support this hypothesis. We previously described (12) the detection of the enterovirus genome only in cerebral biopsy specimens but not in the CSF from two agammaglobulinemic patients with meningoencephalitis. Henson et al. (8) described the detection of JC virus DNA by PCR in 17% of the CSF specimens that they studied and in 100% of the brain samples from the same patients with progressive multifocal leukoencephalopathy.

The 5′-UTR regions of all of the poliovirus genomes detected in our study were sequenced. For each poliovirus serotype, a major molecular marker, related to neurovirulence, is located in the 5′-UTR region, which makes it possible to distinguish between attenuated (Sabin) and wild-type poliovirus genomes (17). Because the sequencing results reveal a large number of point mutations all along the amplified fragment compared with the sequences of the reference strains, it was difficult to relate the sequence data to one type of poliovirus

except in one case. Indeed, for patient 1, the sequence proved to be closer to that of poliovirus type 2 and the base at position 481 corresponded to that in the wild-type strain. Taken together our findings favor the presence of the viral genome in the central nervous systems of patients with PPS. However, the link between the presence of poliovirus genomic sequences and the progressive course of the disease remains unclear. Two major hypotheses can be made. First, the persistence of poliovirus has been implicated in the development of an autoimmune disease, and three mechanisms are involved: molecular mimicry of cellular proteins by viral proteins, pathologically altered expression of cell membrane antigens by infected cells, and the alteration of immunosuppressive activity, leading to a loss of peripheral tolerance of self-reactive cytotoxic T cells (3). Anatomical studies of the spinal cord performed in deceased PPS patients have shown perivascular and/or parenchymal lymphocytic infiltrates, neuronal loss, and active gliosis suggesting an autoimmune reaction (18). Moreover, the presence of poliovirus-specific oligoclonal IgM in CSF and activated T cells in peripheral blood has been reported (24). However, these inflammatory lesions observed in the spinal cord were also observed in patients who had stable postpoliomyelitis deficits and could be the result of neuronal attrition. The test for poliovirus-specific IgM in CSF was negative both in our study and in the study of Jubelt et al. (10). Second, the persistence of poliovirus in the central nervous system could have altered the metabolism of motor neurons, particularly those which had been weakened in the acute attack. This action could be mediated either by excitotoxic injury or by a decrease in the level of expression of neurotrophic factors, mechanisms that are both involved in degenerative motor neuron diseases (14, 21).

The restricted number of patients (5 of 10) found to be positive for the presence of viral sequences suggests that the virus might persist in minute amounts in the central nervous system. Screening for the virus in patients with stable poliomyelitis was negative, but the number of patients in this group analyzed was too small to ascertain that the poliovirus genome could not be detected in some CSF specimens from patients with late stable sequelae or to draw conclusions regarding the link between PPS and the persistence of poliovirus.

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