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NOTES

Persistent Poliovirus Infection in Mouse Motoneurons

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Poliovirus (PV) is the causal agent of paralytic poliomyelitis. Many survivors of the acute disease, after decades of clinical stability, develop new muscular symptoms called postpolio syndrome. It has been hypothesized that the persistence of PV in the spinal cord is involved in the etiology of this syndrome. To investigate the ability of PV to persist in the spinal cord after the onset of paralysis, we exploited a mouse model in which most animals inoculated with a mouse-adapted mutant survived after the onset of paralysis. Light microscopy and ultrastructural immunohistochemical studies and reverse transcription followed by nested PCR performed on spinal cord from paralyzed mice demonstrated that PV persisted in the mouse spinal cord for at least 12 months after the onset of paralysis. This mouse model provides a new tool for studying poliomyelitis evolution after the onset of paralysis.

Poliovirus (PV) is the causal agent of paralytic poliomyelitis, an acute human disease of the central nervous system. It is a member of the *Picornaviridae* family and it is classified into three serotypes (PV-1, PV-2, and PV-3). The viral genome is a single-stranded RNA of positive polarity enclosed in an icosahedral capsid composed of 60 copies of each of the four structural proteins, VP1, VP2, VP3, and VP4.

Humans are the only natural hosts of PV. The virus first infects the gut and then reaches the central nervous system (4). Paralysis results from PV replication and lysis of motoneurons in the spinal cord (4, 10). A postpolio syndrome which affects many poliomyelitic survivors after decades of clinical stability has been described (6, 13, 14). This syndrome involves a variable set of symptoms ranging from muscle and joint pain to slowly progressive muscle weakness.

One hypothesis proposed to explain postpolio syndrome is persistent PV infection in the spinal cord, as suggested by the presence of anti-PV immunoglobulin M (IgM) antibodies (32) and fragmentary genetic sequences resembling PV RNA (21, 22, 25) in spinal fluid from postpolio syndrome patients. In vitro, PV can persistently infect human neuroblastoma cells (9) and human fetal brain cells (27). However, an adequate animal model has yet to be developed to test whether PV can persist in the nerve cells of the spinal cord after the onset of paralysis.

Poliomyelitis can be induced experimentally in monkeys and sometimes in mice by inoculation of PV directly into the central nervous system. Normal mice are susceptible to infection with certain mouse-adapted PV strains, including PV-2/Lansing, but are resistant to infection with most of the other PV strains, including the wild-type PV-1/Mahoney strain. The human cellular receptor of PV (hPVR) has been identified as a member of the immunoglobulin superfamily (18, 23), and transgenic mice expressing hPVR are susceptible to infection with all wild-type PV strains. In normal mice that remain asymptomatic after PV inoculation, prolonged infections of the brain, lasting up to 4 months, have been described (15, 24) but PV was not found in the spinal cords of these mice. Most normal mice and transgenic mice expressing hPVR which develop paralysis following PV inoculation die (16, 19, 24), and it is thus difficult to investigate PV persistence in the mouse spinal cord after the acute phase of the disease.

We previously isolated mouse-adapted PV-1 mutants from the mouse central nervous system after a single passage of the PV-1/Mahoney strain inoculated by the intracerebral route (11). A single amino acid substitution in the capsid protein VP1 (Thr to Ile at position 22) confers a mouse-adapted phenotype to PV-1/Mahoney. Most animals inoculated with the mutant Mah-T1022I (previously named Mah-KK/NK-VP1 [11]) derived from PV-1/Mahoney and carrying this mutation survived after the onset of paralysis. We exploited this mutant to explore whether PV persists in the mouse spinal cord. Here, we show that PV persists for at least 12 months in the spinal cords of symptomatic mice. This animal model is the first exhibiting a persistent infection of PV in the spinal cord for several months after the onset of paralysis.

Most paralyzed mice infected with Mah-T1022I survived. To assess whether mice could survive after the onset of paralysis, we inoculated Swiss Webster mice (OF1; Iffa-Credo) with the mouse-adapted mutant Mah-T1022I and with the mouse-adapted reference strain, PV-2/Lansing (kindly supplied by V. Racaniello, Columbia University, New York, N.Y.). We determined the 50% lethal dose (LD_{50}) and the 50% paralytic dose (PD_{50}) as described previously (11). Both viruses caused flaccid paralysis (data not shown). The LD_{50} of PV-2/Lansing was close to the PD_{50} (Table 1). In contrast, the LD_{50} of Mah-T1022I was higher than the PD_{50} (Table 1). To evaluate more precisely the differences in mortality and morbidity between Mah-T1022I and PV-2/Lansing infection, we determined the percentage of paralyzed mice surviving 3 weeks after inoculation. Forty mice were inoculated with 10 times the PD_{50} of each virus. After inoculation with PV-2/Lansing, only 8% of the paralyzed mice survived (Table 1). In contrast, following

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TABLE 1. Mortality and morbidity in mice following infection with mouse-adapted strains of PV[a]

PV strain	PD_{50} (PFU)	LD_{50} (PFU)	% Paralyzed survivors[b]
PV-2/Lansing	$10^{4.5}$	$10^{4.7}$	8 (3/37)
Mah-T1022I	$10^{6.4}$	$10^{7.5}$	72 (29/40)

[a] Virus titers were determined with HEp-2c cells by plaque assay.
[b] The value in parentheses is the number of surviving paralyzed mice divided by the sum of the number of surviving paralyzed mice and the number of dead mice.

inoculation with Mah-T1022I, 72% of the paralyzed mice survived (Table 1).

Thus, the mice which survived after the onset of paralysis following Mah-T1022I infection provide a convenient animal model to investigate PV persistence in the mouse spinal cord after the acute phase of the disease. Mice were inoculated with 3×10^7 PFU of Mah-T1022I, and the surviving paralyzed mice were studied at 10 days and at 3, 6, 9, and 12 months after the onset of symptoms. Clinical analysis of surviving paralyzed mice did not reveal new apparent paralysis.

Histopathological findings in the spinal cords of surviving mice paralyzed following PV infection. Longitudinal sections from mice exhibiting paralysis in hind limbs were stained for Nissl bodies with cresyl violet to visualize degenerative changes in the lumbar spinal cord. At least two mice were examined at each time point after the onset of paralysis (10 days, and 3, 6, 9, and 12 months). Mice were killed under deep anaesthesia with Sagatal (60 mg/kg of body weight) and perfused with Bodian fixative (72% ethanol, 5% acetic acid, and 5% formaldehyde). The spinal cord was removed and the lumbar spinal cord was dehydrated and embedded in paraffin. Longitudinal sections (10 μm thick) of the spinal cord were cut and deparaffinized, and one section was stained with cresyl violet. Histopathological examination of the spinal cord revealed neuronal damage in a limited area of the ventral horn, which corresponds to the distribution of clinical paralysis. In this area, chromatolyzed motoneurons, as revealed by dark-staining of dispersed Nissl bodies, were detected in all mice analyzed (Fig. 1). Different cytopathological stages could be observed at each time point, ranging from mild to severe chromatolyzed motoneurons with the typical appearance of neuronophagia. In the same area, inflammatory cell infiltration was pronounced at 10 days and at 3 months after the onset of symptoms, and then decreased, but did not disappear entirely, at later time points, including the 12-month point (Fig. 1).

Viral antigens were detected in the spinal cords of surviving paralyzed mice for at least 9 months after the onset of symptoms. To investigate whether PV antigens could be detected in the affected area of the ventral horn of the spinal cord, immunofluorescence and immunoperoxidase stainings with monoclonal antibody C3, directed against the structural polypeptide VP1 of PV-1 (3), were performed on the two sections adjacent to those used for cresyl violet staining at 10 days and at 3 and 9 months after paralysis. Endogenous peroxidase was first blocked with 1% H_2O_2 in phosphate-buffered saline (PBS) for 30 min at room temperature, and then the sections were preincubated in PBS containing 3% normal goat serum with 0.1% Triton X-100 for 30 min, transferred to buffer containing monoclonal antibody C3 (1/200 of mouse ascites fluid), and incubated for 18 h at room temperature. The sections were rinsed and placed in goat anti-mouse IgG conjugated to biotin (1/100) and incubated for 2 h at room temperature. The first section was treated for indirect immunofluorescence. Primary monoclonal antibody C3 was visualized by incubation with fluorescein isothiocyanate-conjugated egg white avidin (Dakopatts) (1/25) for 60 min, then washed in PBS and distilled water, then mounted in mowiol. For the second section, antibody C3 was visualized with the avidin-biotin-peroxidase complex (Vectastain Elite ABC kit; Vector Labs) conjugated to horseradish peroxidase. The peroxidase activity was developed with 0.025% 3,3-diaminobenzidine and 0.01% H_2O_2 in 0.05 M Tris-HCl buffer, pH 7.6. The section was dehydrated and mounted with a neutral solution of polystyrene and plasticizers in xylene (DPX mountant; Fluka). To confirm the specificities of the immunofluorescence and immunoperoxidase stainings, a negative control was performed under the same conditions with monoclonal antibody C3 on sections from the unaltered contralateral ventral horn of the spinal cord from a mouse at 3 months postparalysis. An additional negative control was performed in the area of the lesion, the primary antibody being a monoclonal antibody (25E6) of the same Ig subclass (IgG2a) as C3 directed against rabies virus P protein (30).

Viral antigens were detected by immunofluorescence in the affected area at each time studied (Fig. 2). Some labeled cells appeared to be motoneurons. Staining of cellular debris and of other cells, probably inflammatory cells, was also observed. Immunoperoxidase labeling revealed the presence of viral antigens in motoneurons at each time point studied, as illustrated in Fig. 3 for the time point 3 months postparalysis. Small cells strongly immunoreactive for PV antigens were also observed within the affected area at all times studied. However, these cells remain to be identified.

PV RNA was detected in the spinal cords of surviving paralyzed mice for at least 12 months after the onset of symptoms. Replication of the PV genome requires transcription by the virus-encoded RNA polymerase of a minus-strand RNA intermediate from the positive viral genome template. To determine whether the PV genome persisted and replicated in the mouse spinal cord at times long after acute infection, positive and negative strands of viral RNA were detected independently by reverse transcription (RT) followed by nested PCR in spinal cord from paralyzed mice. Mice were anaesthetized with ether and exsanguinated by PBS perfusion before spinal cord dissection. Total RNA was extracted from the spinal cord with the RNA Plus reagent (Bioprobe Systems). RT-PCR was performed with the GeneAmp RNA PCR kit (Perkin-Elmer Cetus) according to the manufacturer's instructions in a DNA Thermal Cycler 9600 (Perkin-Elmer Cetus). PV plus- and minus-strand RNA molecules were detected specifically with primers corresponding to the antisense sequence of nucleotides 6494 to 6516 and to the sense sequence of nucleotides 5921 to 5940, respectively, of PV-1/Mahoney RNA. Total RNA (1 μg) in a total volume of 20 μl was reverse transcribed as follows: 30 min at 42°C, 5 min at 99°C, and 5 min at 4°C. The first PCR was performed with 20 μl of cDNA in a total reaction volume of 100 μl with both primers as follows: initial denaturation for 1 min at 94°C, 40 cycles consisting of denaturation for 15 s at 94°C and annealing and extension for 1 min at 60°C, and final extension for 7 min at 60°C. Nested PCR was then performed on 2 μl of the first PCR mixture in a total reaction volume of 50 μl with internal primers corresponding to nucleotides 6355 to 6376 and to nucleotides 6086 to 6108 of PV-1/Mahoney RNA. The limit of detection of the RT-nested PCR was 10^{-1} PFU. Purified infectious PV particles devoid of minus-strand RNA and RNA from a mock-inoculated mouse were used as negative controls.

RNA obtained from the spinal cords of two mice at 10 days and at 3, 6, 9, and 12 months postparalysis was analyzed. Results of the RT-nested PCR for one of the two animals for each time point are presented (Fig. 4). The specific 291-bp

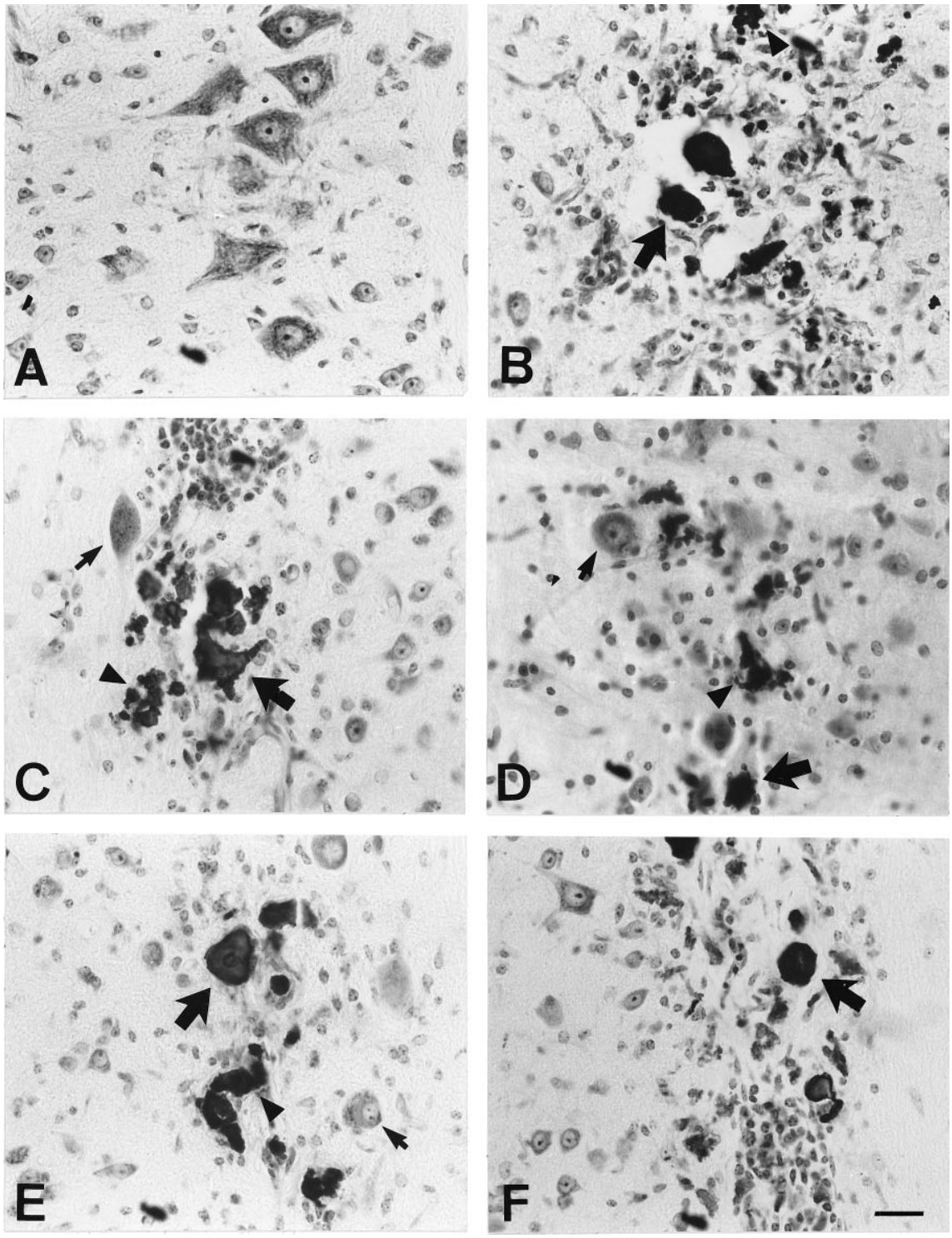

FIG. 1. Cresyl violet staining of longitudinal sections through the ventral horn of spinal cord taken from mice at different times postparalysis. (A) Control consisting of unaltered tissue contralateral to the lesion of the spinal cord from a mouse at 3 months postparalysis. (B to F) Sections through the lesions of spinal cord from mice at 10 days and at 3, 6, 9, and 12 months postparalysis, respectively. For each time point, note the inflammatory infiltrate and chromatolysis of motoneurons, ranging from mild (small arrows) to severe (large arrows), as well as neuronophagia (arrowheads). Bar, 10 μm.

fragment corresponding to the positive and negative RNA strands was detected at every time point examined in both mice. The identity of the 291-bp band was confirmed by sequencing with Sequenase (U.S. Biochemical) the nested PCR product from mice at 9 months postparalysis (data not shown). We also analyzed cytoplasmic RNA from the spinal cords of asymptomatic mice at 3 and 6 months after inoculation with mouse avirulent PV-1/Mahoney. Samples from these mice gave no detectable PCR product (data not shown).

Although we cannot rule out the possibility that viral RNA

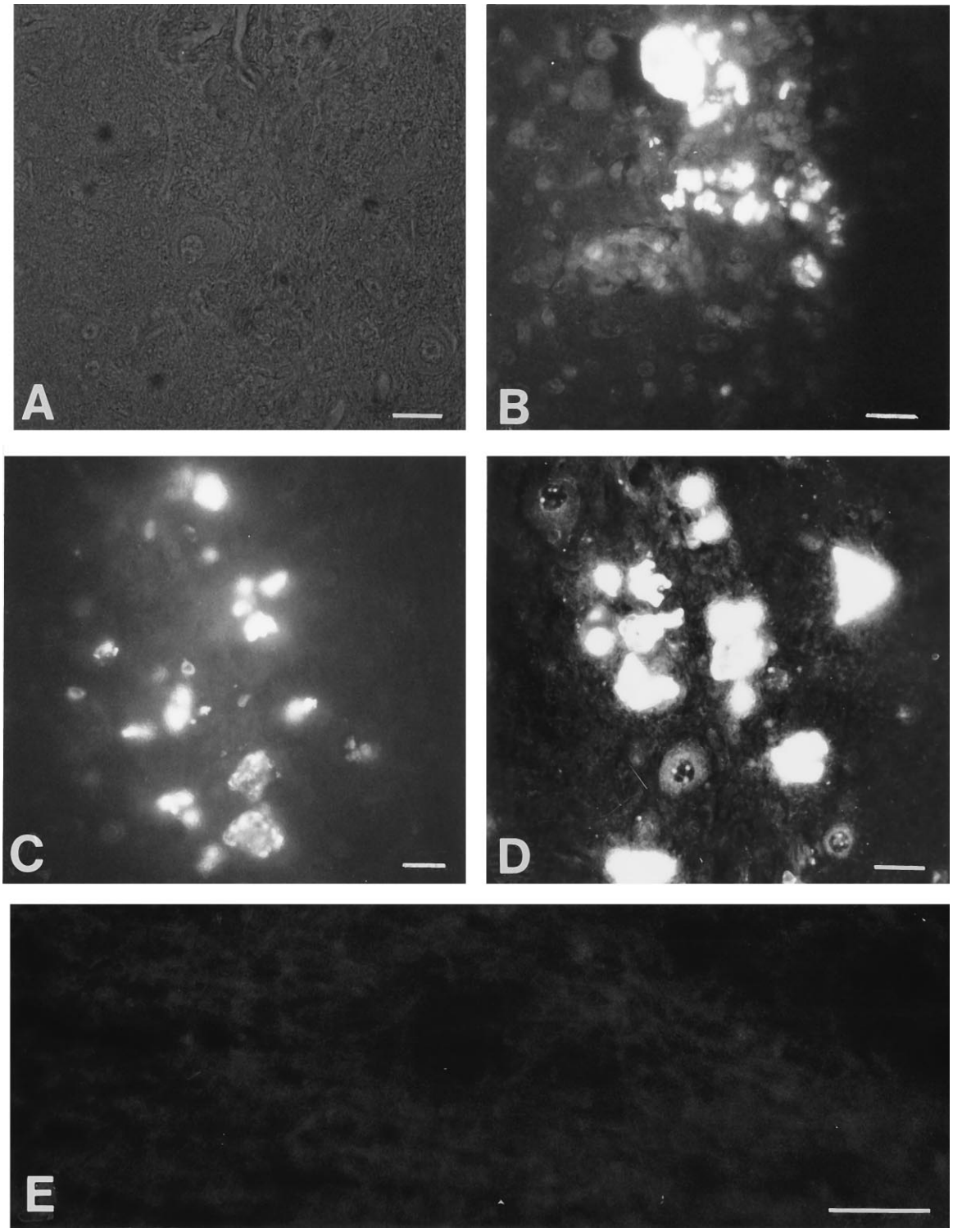

FIG. 2. Detection of PV capsid antigen VP1 by immunofluorescence labeling in the ventral horn of spinal cord sections adjacent to those shown in Fig. 1. (A) Negative control consisting of unaltered tissue contralateral to the lesion of the spinal cord from a mouse at 3 months postparalysis. (B to D) Sections through the lesions of spinal cord from mice at 10 days, and at 3 and 9 months postparalysis, respectively. (E) Additional negative control performed on a section through the lesion of the spinal cord from a mouse at 3 months postparalysis for which the primary antibody was a monoclonal antibody directed against rabies virus P protein. Bar, 10 μm.

detected in spinal cord from paralyzed mice might be deleted, these results suggest that the Mah-T1022I genome persists and that PV RNA replication continues in the spinal cords of paralyzed mice for at least 12 months after the acute disease. This would be consistent with the fact that continuous RNA replication is required for persistence of nonretroviral RNA viruses (2).

Infectious PV could not be recovered from homogenates of spinal cord from paralyzed mice beyond 10 days postparalysis.

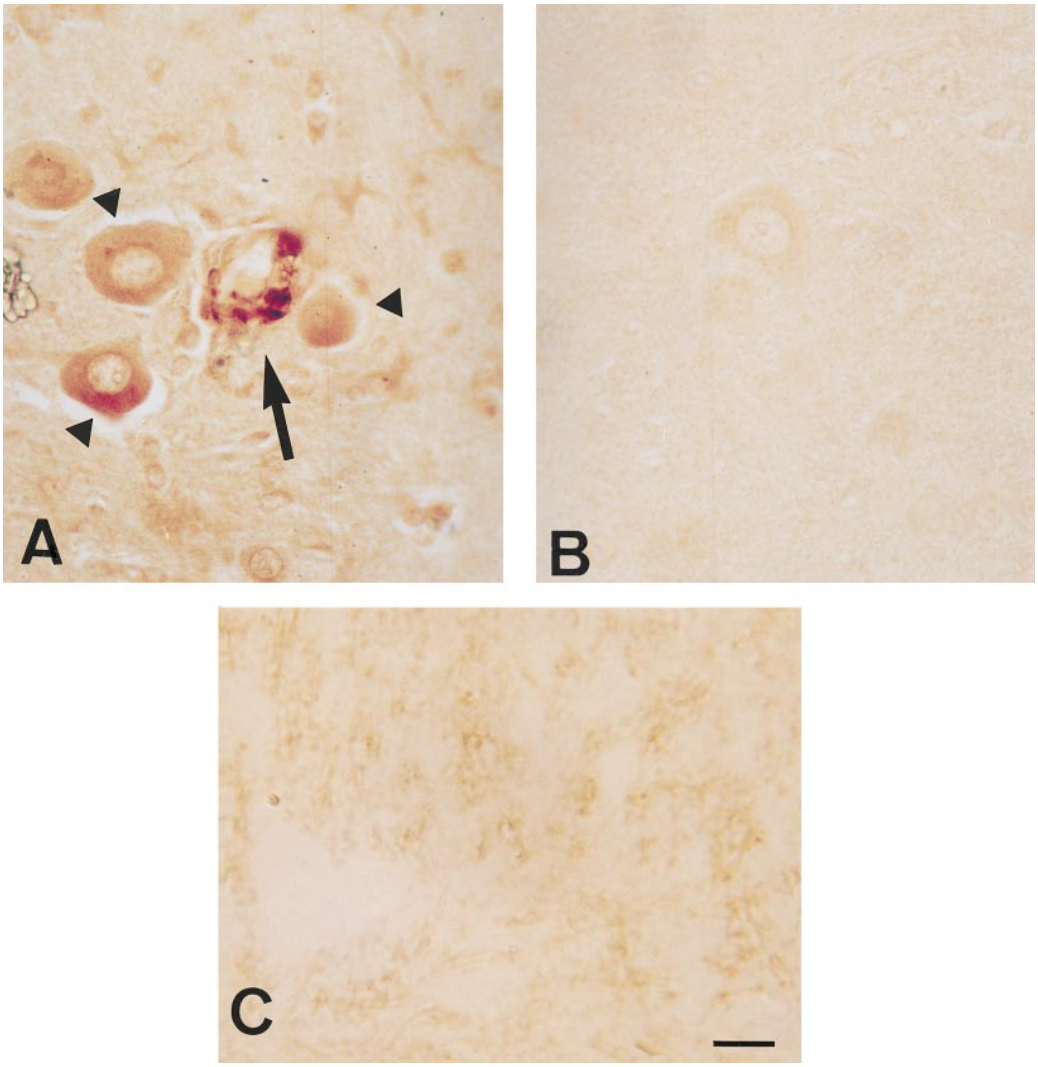

FIG. 3. Detection of PV capsid antigen VP1 by immunoperoxidase labeling in the ventral horn of spinal cord sections adjacent to those shown in Fig. 1 from a mouse at 3 months postparalysis. (A) Section through the lesion. Note the presence of viral antigen in motoneurons (arrowheads) and in small cells (small arrow). (B) Negative control performed on the unaltered tissue contralateral to the lesion. (C) Additional negative control performed on a section through the lesion for which the primary antibody was a monoclonal antibody directed against rabies virus P protein. Bar, 10 μm.

We investigated whether infectious virus could be isolated from paralyzed mice. Homogenates of spinal cord from mice at 10 and 20 days and at 3, 6, and 12 months postparalysis were prepared as described previously (11) and were used to inoculate cultures of human HEp-2c cells (commonly used to amplify PV) and cultures of mouse L-M(TK$^-$) cells. Two blind passages were performed after the initial infection. The only cytopathic effect was observed in HEp-2c cell cultures inoculated with homogenates of spinal cord from mice at 10 days postparalysis (data not shown). No cytopathic effect was observed either with spinal cord homogenates from mice beyond 10 days postparalysis or with supernatants from blind passages in HEp-2c or L-M(TK$^-$) cell cultures. To assess whether any PV particles from homogenates replicated without producing a cytopathic effect, cell cultures incubated for 5 h and for 1 and 5 days with spinal cord homogenates or with supernatants from blind passages were tested for the presence of viral antigen by immunofluorescence with monoclonal antibody C3. No viral antigens were detected in cells incubated with spinal cord homogenates removed from mice beyond 10 days postparalysis. We also inoculated intracerebrally both Swiss Webster mice and transgenic mice expressing hPVR (kindly provided by A. Nomoto, Tokyo University, Tokyo, Japan) with 0.03 ml of spinal cord homogenates or with 0.03 ml of supernatants from blind passages. Only mice inoculated with supernatants from passages of spinal cord from mice at 10 days postparalysis developed paralysis. Thus, infectious virus could not be recovered from homogenates of mouse spinal cord isolated beyond 10 days after the onset of symptoms of the disease.

Possibly, the PV genome acquired mutations during persistent infection, thus rendering the virus unable to grow in human HEp-2c cells. It has been shown that PV mutants recovered from persistent infection of human neuroblastoma cells exhibited modified cell specificity (5, 28). Alternatively and more likely, the inability to recover infectious virus from homogenates could be due either to the absence of infectious particles or to the fact that the number of infectious particles was too low to initiate an efficient infection of HEp-2c cell cultures. The absence or scarcity of infectious particles could be due to restricted PV replication in the central nervous

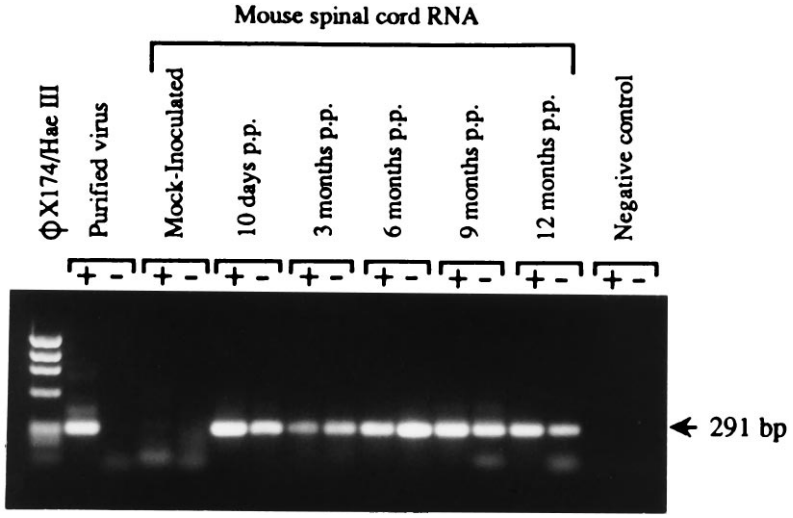

FIG. 4. Nested PCR products following RT of RNA extracted from mouse spinal cord at the indicated time points postparalysis (p.p.). Positive (+) and negative (−) RNA strands were specifically amplified with appropriate primers. One-third of each reaction mixture was electrophoresed on a 1.2% agarose gel, and the PCR products (596 bp for the first PCR and 291 bp for the nested PCR) were visualized by ethidium bromide staining. Purified infectious PV particles devoid of minus-strand RNA were used as a negative control for minus-strand RNA. A mock-inoculated mouse was used as a negative control for the mouse spinal cord. A negative control for RT-PCR, which contained water instead of RNA, was also included. An *Hae*III digest of ϕX174 (ϕX174/HaeIII; Pharmacia) was used as the source of DNA molecular weight markers.

system, which would allow the virus to persist beyond the acute phase of the disease. In fact, a prerequisite for the establishment and maintenance of persistent infection by a lytic virus is the limited expression of the viral genome, which can also allow the virus to avoid the immune system (26). Limited replication has been shown with other neurotropic viruses such as the measles virus (31) and mouse hepatitis virus (20), for which viral antigens and genome were detected although infectious virus could rarely, if ever, be isolated during persistent infection of the central nervous system.

PV particles were detected in motoneurons of the spinal cords of surviving paralyzed mice for at least 12 months after the onset of symptoms. To investigate whether PV particles were present in motoneurons of paralyzed animals, we performed ultrastructural studies with spinal cords from mice at 3, 6, and 12 months postparalysis. Mice were killed under deep anaesthesia with Sagatal (60 mg/kg) and perfused with a fixative solution of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The lumbar segment of the spinal cord was postfixed and incubated overnight in a 10% sucrose solution in PB. Longitudinal sections (50 μm thick) were cut with a vibratome (Lancer). Immunoperoxidase staining was performed on free-floating sections with monoclonal antibody C3 as described above to label PV particles. Sections were then washed in 0.1 M PB, pH 7.4, and postfixed in 1% osmium tetroxide for 45 min, stained with uranyl acetate, dehydrated, and flat-embedded in Epon between plastic coverslips. Blocks were trimmed from the lesioned areas of the ventral horn and were remounted on cylindrical support blocks of Epon. Ultrathin sections were collected on collodion-coated copper slot grids and were observed with an electron microscope (Jeol CX 100). PV-infected HEp-2c cell cultures were used as an ultrastructural control for PV particles.

PV particles were observed in the cytoplasm of motoneurons (Fig. 5A to C) which appeared mildly chromatolyzed, as assessed by light microscopy (Fig. 1), in the area of the lesion at each time point studied. The ultrastructure of severely chromatolyzed motoneurons did not allow particle detection. No PV particles were observed outside the area of the lesion. The sizes of the viral particles detected (between 90 and 120 nm) appeared to be larger than that expected for PV particles (30 nm). However these particles had the same size and the same appearance as those detected by the same procedure in the PV-infected HEp-2c cell cultures (Fig. 5D). No particles were detected in uninfected HEp-2c cell cultures (data not shown). The apparent sizes of the PV particles were probably due to the labeling procedure.

Although infectious PV could not be recovered from homogenates of spinal cord from paralyzed mice beyond 10 days postparalysis, PV persists for at least 12 months after the onset of paralysis in murine motoneurons, as shown by the presence of PV antigens, RNA, and particles. To our knowledge, this is the first description of viral persistent infection in motoneurons of immunocompetent mice.

Our in vivo study shows that Mah-T1022I persistently infects the mouse spinal cord. This could be a common characteristic of mouse-adapted strains of PV, since PV-2/Lansing RNA was also detected in the rare surviving mice (data not shown). PV is normally highly cytolytic, and thus how PV persistence is established is an interesting issue. This model now makes it possible to study molecular mechanisms of PV persistence in the central nervous system and, in particular, in motoneurons. The mechanisms by which nonretroviral RNA viruses persist in vivo with restricted replication include abnormal regulation of minus-strand production for positive-strand RNA viruses (7, 12, 17), generation of defective interfering particles (1, 33), and biased somatic hypermutations (8, 34). We are currently investigating whether one or more of these mechanisms are involved in PV persistence in the mouse model.

It has been suggested that late-onset neurological deterioration after poliomyelitis, leading to postpolio syndrome, may involve persistent PV infection of the spinal cord. Inflammation, neuronal atrophy, and chromatolysis were observed in spinal cord sections of patients 9 months to 44 years after acute poliomyelitis even in the absence of new muscular symptoms

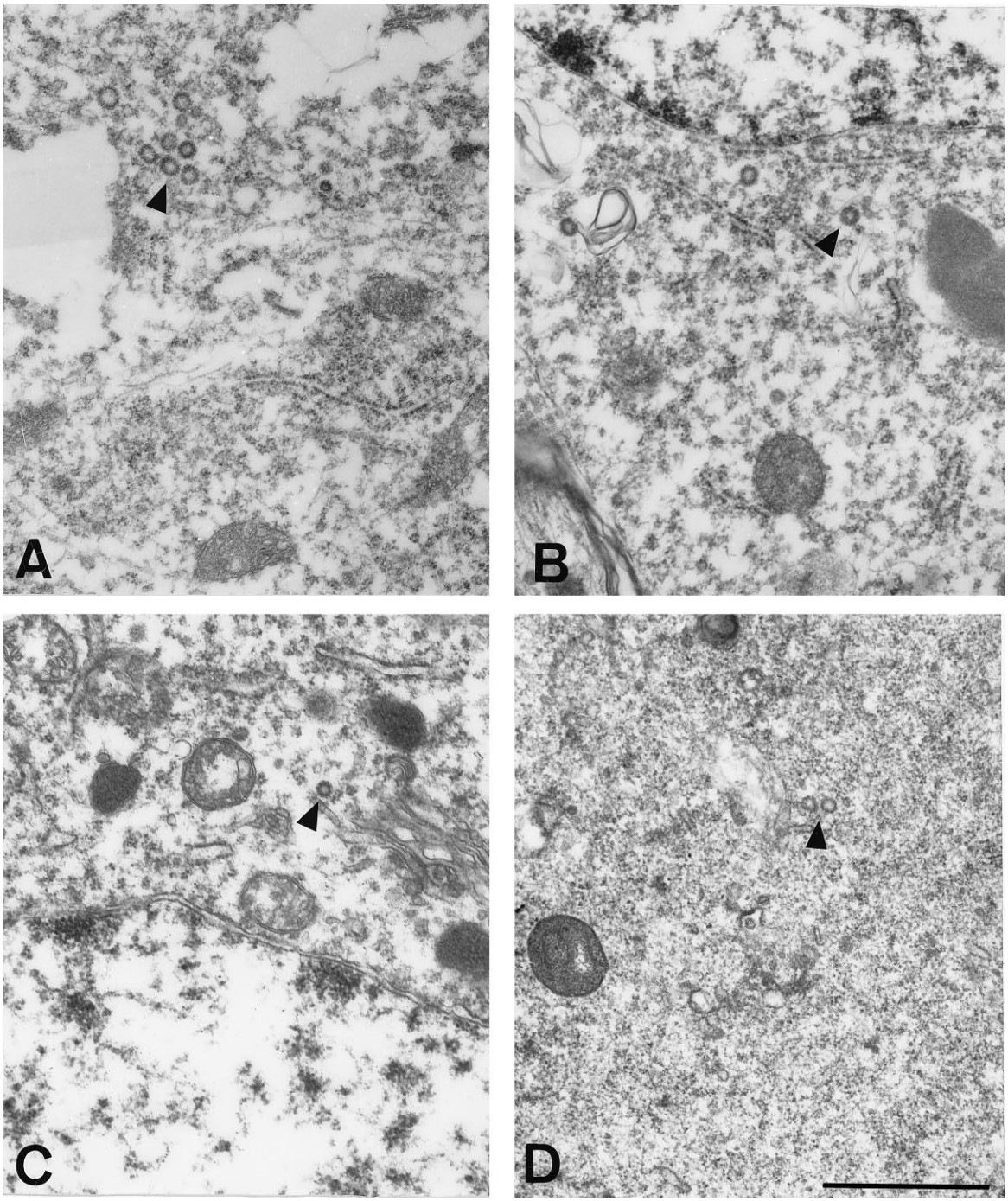

FIG. 5. Ultrastructural immunodetection of PV particles (arrowheads) in the cytoplasm of motoneurons of the spinal cord at 3 (A), 6 (B), and 12 (C) months after the onset of paralysis and in HEp-2c cell cultures infected with Mah-T1022I for 18 h at a multiplicity of infection of 0.01 (D). Bar, 1 μm.

(29). Our results showing continuing motoneuron damage in the spinal cords of paralyzed mice could be analogous to the histopathological observations in humans. These observations suggest some degree of ongoing neuronal necrosis in the spinal cord after the onset of paralysis in mice as well as in humans. Despite the relatively short life expectancy of mice, this model of PV persistence in the spinal cords of paralyzed animals provides a new tool to study the evolution of poliomyelitis after the onset of paralysis and could shed light on the etiology of postpolio syndrome.

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