Journal of NeuroVirology, 11: 237–246, 2005

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280590952745

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Poliovirus type 1 infection of murine *PRNP*-knockout neuronal cells

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Transfection of the prion protein gene (*Prnp*) into prion-deficient mouse cells was shown to reduce the replication of coxsackievirus B3, an enterovirus. Because mice can be susceptible to poliovirus infection by parenteral routes, the authors tested the susceptibility to poliovirus-1 (PV-1) of a panel of murine neuronal cell lines differing in their ability to express *Prnp*. The investigated cell lines (prionless HpL3.4 cells, HpL3.4 cells transfected with a *Prnp* vector, HpL3.4 cells transfected with a void vector, wild-type Hw3.5 $Prnp^{+/+}$ cells) expressed the murine homologue (Tage4) of human poliovirus receptor (CD155/hPVR). PV-1 infection of $Prnp^{-/-}$ HpL3.4 cells resulted in the production of high viral titers, though viral antigens could be detected in only 0.5% to 2% of cells. Wild-type $Prnp^{+/+}$ cells and prionless cells transfected with the *Prnp* gene were not permissive to PV-1. Results of viral titration and immunofluorescence were confirmed by conventional polymerase chain reaction (PCR) and quantitative real-time PCR. Exposure to PV-1 had no influence on the gene expression profile of $Prnp^{+/+}$ cells. In contrast, PV-1 infection was associated with upregulation of several genes in permissive $Prnp^{-/-}$ cell cultures: type I interferon (IFN) genes, IFN-related developmental regulator 1 (IFNRD1), tumor necrosis factor superfamily member 13b (TNFSF13b), interleukin (IL) — 7, granulocyte/macrophage colony-stimulating factors (CSFs), hepatocyte growth factor (HGF), vascular endothelial growth factor-A, transforming growth factors beta1 and beta3 (TGFb1, TGFb3), as well as a variety of bone morphogenetic proteins endowed with neuroprotective activity. Distinction of permissive from nonpermissive neuronal cells on the basis of *Prnp* expression suggests that prion-deficient mice could represent an extraordinarily sensitive animal model for poliovirus infection. *Journal of NeuroVirology* (2005) **11,** 237–246.

Keywords: BMP; CSF; HGF; hippocampal cells; prion gene; TGF; VEGF

Introduction

Prion diseases are neurodegenerative disorders caused by the conformational conversion of a normal host glycoprotein (PrP^{C}) into an infectious isoform (PrP^{Sc}) devoid of nucleic acid (Aguzzi and Polymenidou, 2004). PrP^{C} is encoded by the highly conserved *Prnp* gene; a *Prnp* homologue is denoted *Prnd* or *Dpl* (doppel) (DeArmond and Prusiner, 2003). PrP^{C} is expressed in neurons and glia of the brain and spinal cord, as well as in lymphoid cells and in a variety of peripheral tissues. *Prnp* transcripts can first be detected in the brains of mice and chickens in early embryogenesis and their level increases with development (Harris *et al*, 1993; Manson *et al*, 1992). In the adult central nervous system (CNS), PrP^{C} and its mRNA are widely distributed, with high concentrations in neocortical and hippocampal neurons, cerebellar Purkinje cells, and spinal motor neurons (DeArmond *et al*, 1987; Kretzschmar *et al*, 1986).

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This work was supported by Banca del Monte di Lombardia (BML, Milan, Italy); University of Tokyo (Tokyo, Japan); Neuroscience Center, University of Insubria. A. Baj is a PhD student of the University of Pavia, Italy; A. Bettaccini is supported by a fellowship from the Medical School of the University of Insubria, Varese, Italy.

Received 23 July 2004; revised 2 December 2004; accepted 13 January 2005.

Though PrP^C localization on the cell surface is consistent with roles in cell adhesion, ligand uptake, or transmembrane signaling, its function is unknown. PrP^C can interact with sulfated glycans (Caughey *et al*, 1994) and nucleic acids (Nandi and Leclerc, 1999), showing nucleic acid chaperoning properties previously described for retroviral nucleocapsid proteins (Derrington *et al*, 2002) and relevant in virus replication and assembly (Moscardini *et al*, 2001). PrP^C seems to be involved in copper metabolism, possibly as a copper-dependent superoxide dismutase that plays a role in cell survival (Brown *et al*, 2000; Lasmezas, 2003; Sakudo *et al*, 2004).

A few reports have analyzed the interaction of *Prnp* expression and viral infection: (a) infection of mice with adenovirus (Ehresmann and Hogan, 1986) or murine leukemia virus (Carp *et al*, 1999) was shown to accelerate the progression of scrapie; (b) herpes simplex virus (HSV) replication was retarded in $Prp^{-/-}$ mice, but these animals contained levels of latent virus higher than their wild-type counterpart. The data suggest that PrP^C may be involved in a metabolic pathway that favors apoptosis of virus-infected neurons (Thackray and Bujdoso, 2002); (c) transfection of the *Prnp* gene into prionless cells appeared to reduce the replication of coxsackievirus B3 in vitro (Nakamura *et al*, 2003). These findings prompted us to analyze the susceptibility to a neurotropic virus of a panel of murine neuronal cell lines differing in their ability to express the *Prnp* gene (Kuwahara *et al*, 1999; Weissmann and Flechsig, 2003).

Polioviruses (PVs) are important human neurotropic agents belonging to the family *Picornaviridae*. PVs enter the body through the enteric system and in up to 5% of cases reach the CNS. Interaction of PVs with human cells is mediated by the expression of surface receptors belonging to the immunoglobulin superfamily (Zhang and Racaniello, 1997). The human poliovirus receptor (hPVR; CD155) serves as a nectin-binding protein and its cytoplasmic domain is associated with Tctex-1, a component of the dynein motor complex (Mueller *et al*, 2002). Association of CD155 with Tctex-1 may offer an explanation for how poliovirus takes control of the cellular transport machinery to retrogradely ascend along the axon to the neuronal cell body (Ohka *et al*, 2004). CD155 is also involved in monocyte extravasation and is expressed at endothelial cell junctions (Reymond *et al*, 2004). CD155 has a murine homolog (mPVR; Tage4) that binds nectin-3 and could also serve as poliovirus receptor (Mueller and Wimmer, 2003; He *et al*, 2003; Ravens *et al*, 2003).

Mice can be susceptible to PV infection by parenteral routes (Gromeier and Nomoto, 2002). Pathologic alterations of the CNS have been observed not only in experimentally infected primates, but also in wild-type mice (Jubelt *et al*, 1980). The observed neuropathology of the wild-type mouse model more closely reflects what has been reported in infected humans and monkeys, than does the hPVR transgenic mouse model (Ford *et al*, 2002). Expression of hPVR in intestinal epithelial cells is not sufficient to permit poliovirus replication in the gut (Zhang and Racaniello, 1997), and PV replication in mouse motor neurons during paralytic poliomyelitis is associated with an apoptotic response (Girard *et al*, 1999). In paralyzed mice, PV may persist in the CNS for over a year after the acute phase of infection (Destombes *et al*, 1997; Girard *et al*, 2002). Persistence of enteroviruses is a recognized phenomenon in cultured cells (Conaldi *et al*, 1997) and is suspected to have a role in the human postpolio syndrome (Blondel *et al*, 1999).

These observations prompted us to investigate the influence of *Prnp* expression on the ability of the neurotropic PV-1 to infect mouse neuronal cells. If *Prnp* somehow confers resistance to virus infection, then it may be possible that hosts with altered PrP^C functions have a reduced ability to deal with viral agents affecting the CNS. Common viral infections might thus accelerate the progression of prion diseases.

Here we report that an established prionless ($Prnp^{-/-}$) murine neuronal cell line (HpL3.4) is susceptible to PV-1, whereas neuronal cell lines derived from wild-type ($Prnp^{+/+}$) mice are resistant. In addition, resistance to PV-1 is reintroduced into the prionless HpL3.4 cell line following transfection with the murine *Prnp* gene.

Results

Characterization of mouse neuronal cell lines

As shown in Figure 1, no signal was obtained when DNA from mouse neuronal cell lines was amplified with primers specific for human repeated *Alu* sequences (Charlieu *et al*, 1992). DNA from control human HeLa cells produced the expected 116-bp amplicon. As shown in Figure 2,mRNA transcripts encoding the human PVR (CD155) were expressed only by human HeLa cells, not by mouse cells. Taken together, the above results excluded the possibility that the mouse hippocampal cell lines used in this study were contaminated by human cells. As expected, when DNA from neuronal cell lines and control HeLa cells was amplified with primers specific for the murine *Prnp* gene (Nakamura *et al*, 2003), positive signals were only obtained with the wild-type Hw3.5 cells and with the prionless HpL3.4 cell line that was stably transfected with a *Prnp* vector (HpL3.4 RPN3 cells). DNA from the four mouse cell lines used in this study gave positive results when amplified with primers specific for the *Prnd* gene (Figure 1), showing that the *Prnd* gene was present in all of them. As expected, control human HeLa cells gave no signal with primers specific for mouse prion genes.

By reverse transcriptase–polymerase chain reaction (RT-PCR), the expression of mRNA transcripts

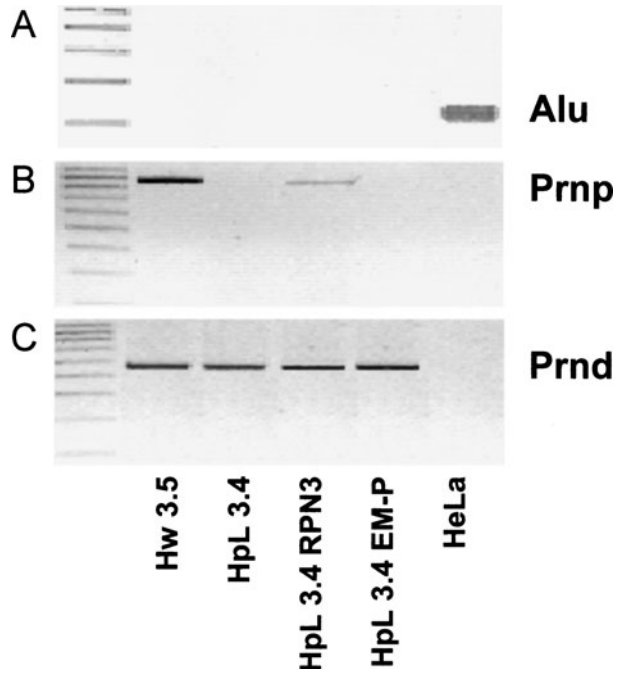

Figure 1 Amplification of genomic DNA extracted from the panel of 4 different murine cell lines investigated in this study and from human HeLa cells. PCR was carried out with primers specific for human repeated *Alu* sequences (**A**), as well as murine *Prnp* (**B**) and *Prnd* (**C**) genes. Left lane, DNA molecular weight ladder.

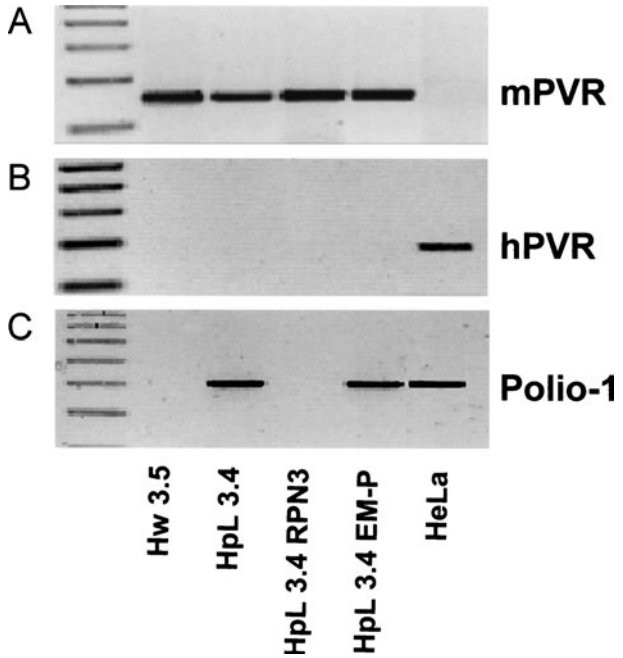

Figure 2 RT-PCR of total RNA extracted from the panel of four different murine cell lines investigated in this study and from human HeLa cells. Using primers specific for murine PVR, human PVR, and genomic poliovirus RNA, it is shown that (**A**) mPVR is expressed by the four murine cell lines, but not by HeLa cells; (**B**) hPVR is expressed only by HeLa cells; (**C**) 3 days post infection genomic poliovirus-1 RNA is produced in the supernatant of virus-infected HpL3.4, HpL3.4 EM-P, and HeLa cells, but not of virus-infected Hw3.5 and HpL3.4 RPN3 cells. Left lane, DNA molecular weight ladder.

for mPVR and hPVR was also evaluated (Figure 2). Whereas neuronal cell lines expressed mPVR at comparable levels, no signal was obtained with primers specific for hPVR. The reverse was true for the control human HeLa cells. RT-PCR also revealed that—upon infection with PV-1—viral genomic RNA was only detected in HeLa cells, prionless HpL3.4 cells, and prionless cells transfected with a vector devoid of the *Prnp* gene (HpL3.4EM-P). Notably, PV-1 genomic RNA was not detected in infected wild-type Hw3.5 cells and in HpL3.4 RPN3 cells. The latter cell line had been produced by stable transfection with a *Prnp* vector (Figure 1). By immunofluorescence, this cell line was shown to express the PrP^C protein (data not shown).

Poliovirus infection of neuronal cell lines

To investigate the susceptibility to PV-1 of the four neuronal cell lines, cell cultures were infected at an multiplicity of infection (m.o.i) of 3 with PV-1. Virus replication was followed over time by three methods: virus titration, detection of viral genomes by RT-PCR, and indirect immunofluorescence (IIF) for measuring the percentage of cells that were producing viral antigens. Elevated titers of PV-1 (i.e., over 10^5 tissue culture infectious $dose_{50}$ $(TCID)_{50}$/ml) were obtained in HpL3.4, but—under the same conditions—higher titers were obtained in human HeLa cells (approximately 10^7 $TCID_{50}$/ml). However, whereas HeLa cells underwent almost complete cytopathic effect (CPE) within 2 to 3 days of infection, HpL3.4 cells continued to produce PV-1 at levels of approximately 10^5 $TCID_{50}$/ml for at least 12 days post infection (p.i.) without overt CPE (data not shown).

As suggested by preliminary evidence, virus titration experiments (Figure 3A) showed that infectious virus failed to replicate in wild-type Hw3.5 cells and in prionless cells stably transfected with a *Prnp* vector (HpL3.4 RPN3). Virus production occurred in prionless HpL3.4 cells at titers over 10^5 $TCID_{50}$/ml for at least 6 days p.i. Transfection of the permissive HpL3.4 cell line with an empty vector (HpL3.4 EM-P) resulted in the production of infectious PV-1, but viral titers were significantly reduced (approximately 10^3 $TCID_{50}$/ml). CPE was not observed in virus-infected cultures.

The above results were confirmed by RT-PCR. As shown in Figure 3B, PV-1 RNA was detected in the supernatant of infected HpL3.4 cultures from day −2 onwards. Lower level amplification signals were obtained in PV-1–infected HpL3.4 EM-P cells. No amplification signal was obtained in either wild-type Hw3.5 cells or in HpL3.4 RPN3 cells that had been transfected with a *Prnp* vector. Quantitative results of real-time RT-PCR reinforced the data showing that (3 days p.i.) PV-1 genome equivalents were $10^{7.6}$/ml in supernatants of HeLa cells, $10^{6.1}$/ml in HpL3.4 cells, $10^{3.5}$/ml in HpL3.4 EM-P cells, and approximately 10^2/ml in both Hw3.5 and HpL3.4 RPN3 cells (data not shown).

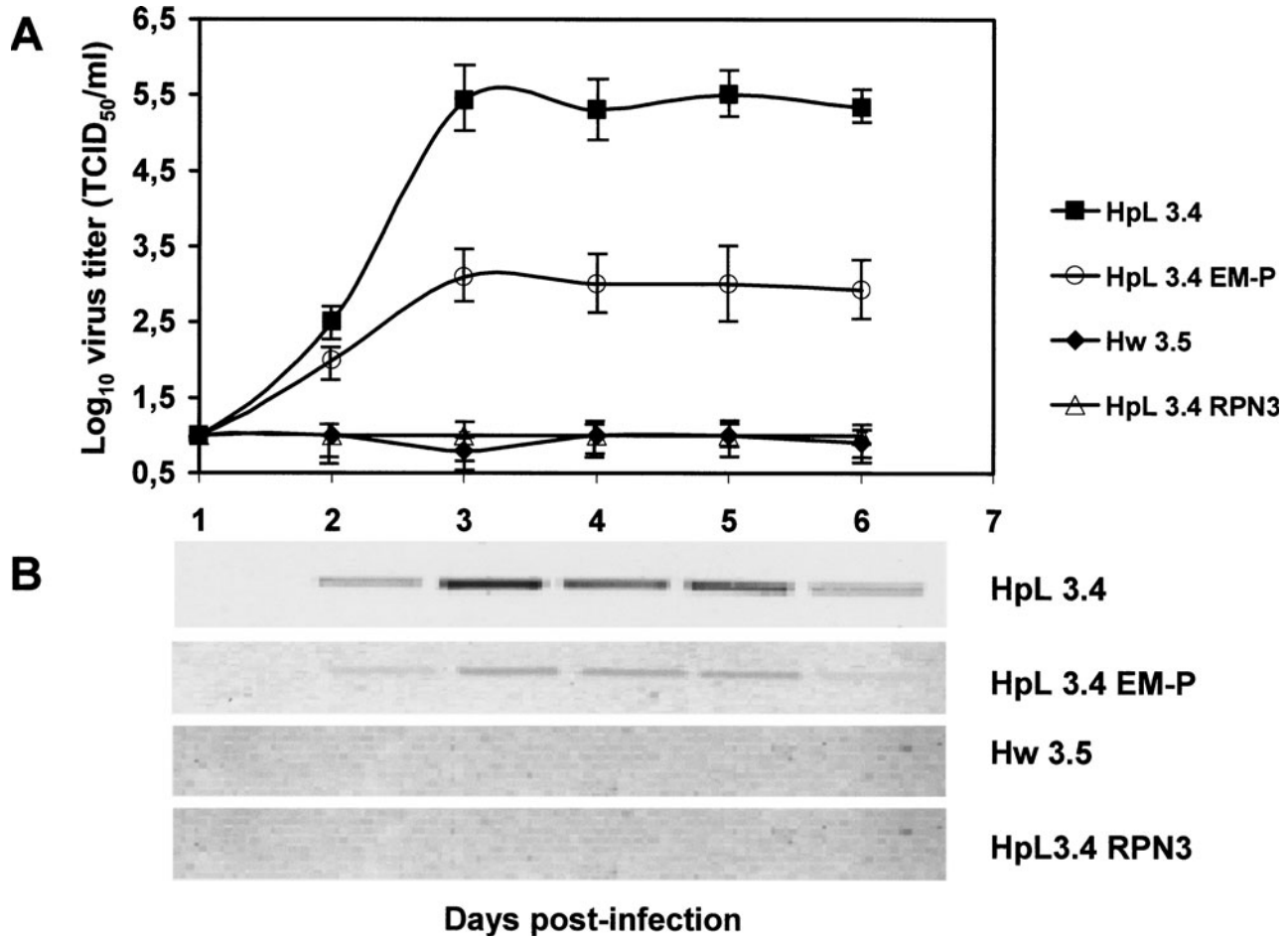

Figure 3 Time course of extracellular poliovirus-1 production in four different murine cell lines investigated in this study. Cell cultures were infected with PV-1 at time 0 (m.o.i. = 3). (**A**) Titer of infectious virus in the supernatant of infected cell cultures at different times post-infection ($TCDI_{50}$/ml). Each point represents the mean ± SD of four different experiments. (**B**) RT-PCR of total RNA extracted from the supernatant of poliovirus-infected cell lines. Genomic poliovirus-1 RNA was detected in the supernatant of HpL3.4 and HpL3.4 EM-P cells, but not in Hw3.5 and HpL3.4 RPN3 cells.

Staining infected cultures with a mix of pan-enterovirus antibodies at different times p.i. showed that in permissive cultures only a small percentage of cells was producing viral antigens. As shown in Figure 4, common enteroviral antigens were detected 6 days p.i. in 0.5% to 2% of HpL3.4 cells and in 0.1% to 0.5% of HpL3.4 EM-P cells. Enteroviral antigens could not be detected in infected Hw3.5 and HpL3.4 RPN3 cell lines.

Analysis of gene expression in PV-1–infected cell cultures

Gene-arrays were used to compare gene expression in PV-1–infected *Prnp*$^{+/+}$ Hw3.5 cells and in permissive prionless HpL3.4 cells. Table 1 lists genes whose expression was up-regulated at least two-fold as compared to uninfected cultures either at an early phase (6 h) or at a late phase (6 days) of infection. Up-regulation is expressed as change (n-fold) over uninfected control cultures. Upon infection of permissive prionless HpL3.4 cells WITH PV-1, 14 of 96 common cytokine genes (14.6%) were up-regulated, from 2.0- to 6.8-fold. Notably, no change in gene expression could be detected upon infection of the non-permissive Hw3.5 cell line.

In permissive HpL3.4 cells, the early phase of poliovirus infection was associated with the increased expression of (a) interferon alpha genes 11 and -B; (b) tumor necrosis factor superfamily member 13b (ligand); (c) interleukin-7. The late phase of infection was associated with the increased expression of (a) interferon-related developmental regulator 1, a factor associated with neuronal growth processes and also activated in response to ischemia (Alexander *et al*, 2003); (b) a variety of growth factors (colony-stimulating factor [CSF]-1, granulocyte-macrophage CSF, hepatocyte growth factor, vascular endothelial growth factor-A); (c) proteins belonging to the superfamily of transforming growth factor beta (TGFb1, TGFb3, bone morphogenetic protein-1, -5, and -7).

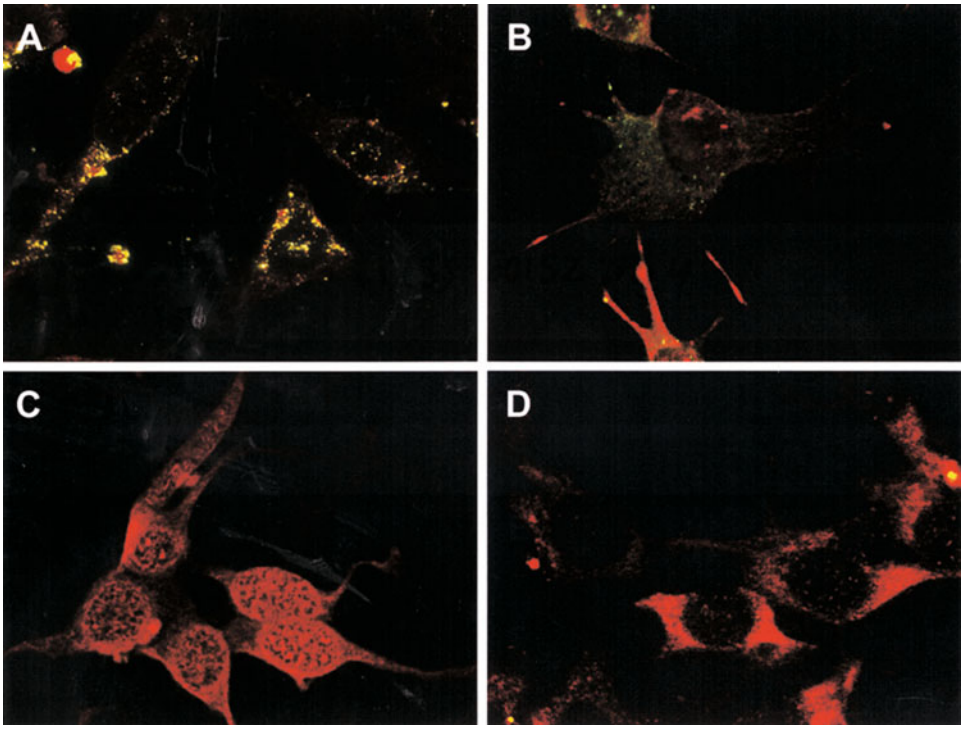

Figure 4 Monolayers of poliovirus-infected HpL3.4 (**A**), HpL3.4 EM-P (**B**), Hw3.5 (**C**), and HpL3.4 RPN3 (**D**) cells stained with antibodies to common enteroviral antigens. Indirect immunofluorescence 6 days post infection (100× objective). Virus antigens were detected in a small percentage of prion-deficient HpL3.4 and HpL3.4 EM-P cells. Viral antigens could not be detected in Hw3.5 and HpL3.4 RPN3 cells.

Discussion

PVs are important neurotropic pathogens that cause poliomyelitis. Though experimental infection of monkeys represents the most relevant animal model for demonstrating histopathologic lesions and the attenuation level of different viral strains, infection of mice with PV-2 is becoming an increasingly accepted animal model to investigate pathogenetic events (Jubelt *et al*, 1980; Gromeier and Nomoto, 2002). PV-2–infected Swiss mice show anterior motor neuron destruction and flaccid paralysis closely mimicking human disease (Ford *et al*, 2002), i.e., infection of motor neurons of anterior horns of the spinal cord and of motor nuclei of brain stem with minor lesions in the forebrain. An apoptotic response of neuronal cells follows PV infection (Girard *et al*, 1999) and is reminiscent of what observed in CNS infections produced by other RNA neurotropic viruses such as reovirus type 3 (Richardson-Burns and Tyler, 2004). In the reovirus model, treatment with caspase inhibitors reduced virus growth in cortical neuronal cells, suggesting that cells with a reduced sensitivity to programmed death may have reduced susceptibility to neurotropic viruses.

The four cell lines investigated in the present study are of the same genetic background (i.e., they have been derived from inbred C57BL/6 mice) and express markers of the neuronal lineage. The four cell lines expressed comparable levels of mPVR transcripts. mPVR is the murine homologous of hPVR/CD155 (Mueller and Wimmer, 2003) and—though it has not been demonstrated—could represent the murine receptor involved in the cell entry of PVs (He *et al*, 2003). The investigated cell lines expressed markedly different levels of *Prnp* transcripts. By testing the in vitro susceptibility to PV-1, it was concluded that neuronal cells expressing *Prnp* were not susceptible to PV-1.

This study suggests three possible roles for PrP^C in PV infection: (a) one possibility is that PrP^C increases endogenous antiviral activity in neuronal cells; (b) the second one is that lack of PrP^C expression somehow boosts up apoptotic pathways, thus favoring virus replication; (c) the third one is that PrP^C expression is associated with promotion of cell survival.

The first possibility may be ruled out, at least in part, by the results of gene array experiments showing that uninfected $Prnp^{+/+}$ and $Prnp^{-/-}$ cells expressed undetectable levels of mRNA transcripts encoding antiviral factors such as IFNs alpha/beta and their receptors as well as factors belonging to the TNF superfamily and their receptors. With regard to the

Table 1 Effect of poliovirus infection on gene expression of wild-type (Hw3.5) and prionless (HpL3.4) neuronal cell lines

			Poliovirus-infected cell lines: fold change in gene expression at different times post infection[a]			
			Hw3.5 + PV-1		*HpL3.4 + PV-1*	
Gene name	*GenBank accession no.*	*Description*	*6 hours*	*6 days*	*6 hours*	*6 days*
IFNa11	M68944	Interferon alpha family, gene 11	1.00	0.90	5.99	1.00
IFNaB	NM008336	Interferon alpha family, gene B	1.00	1.00	3.36	2.00
IFNRD1	V00756	Interferon-related developmental regulator 1	1.00	1.34	0.86	2.37
TNFSF13b	AF119383	Tumor necrosis factor (ligand) superfamily, member 13b	1.00	1.10	4.03	2.00
IL-7	NM 008371	Interleukin-7	1.00	1.14	4.66	2.99
M-CSF	X05010	Mouse mRNA for colony stimulating factor-1 (CSF-1)	1.00	1.00	0.53	4.82
GM-CSF	X03020	Colony-stimulating factor, granulocyte macrophage	1.00	1.21	1.00	2.22
HGF	X84046	Hepatocyte growth factor	1.00	0.90	1.00	4.98
VEGF-A	M95200	Vascular endothelial growth factor-A	1.00	1.20	1.35	2.50
TGFb1	M13177	Transforming growth factor, beta1	1.00	1.51	1.00	2.50
TGFb3	M32745	Transforming growth factor, beta3	1.00	1.22	1.00	6.84
BMP1	L24755	Bone morphogenetic protein-1	1.25	1.30	1.00	5.81
BMP5	NM 007555	Bone morphogenetic protein-5	1.00	1.00	1.00	2.25
BMP7	NM 007557	Bone morphogenetic protein-7	1.00	0.72	1.00	3.45

[a]Mean densitometric values are expressed for each gene as fold difference for cultures infected with poliovirus-1/uninfected control cultures. Negative (pUC18) and positive controls (GAPDH, cyclophillin A, RPL13A, beta-actin) were used to normalize the data. Data are reported only for genes whose expression was altered at least 2.0-fold in either cell line following exposure to poliovirus.

second possibility, we have previously shown that transfection of the *Prnp* gene into prionless cells suppressed the apoptotic response to coxsackievirus B3 infection and reduced viral replication (Nakamura *et al*, 2003). In apparent contrast with this view are the observations that expression of PrP^C favored the lytic infection of mice by HSV, whereas lack of PrP^C supported the establishment of latent HSV infection (Thackray and Bujdoso, 2002). It should be noted, however, that viral replication pathways and the cell compartments involved in the reproduction and assembly of large DNA viruses are completely different from those utilized by small RNA viruses. Because only approximately 1% of $Prnp^{-/-}$ susceptible cells allowed productive infection by PV-1, in our model it was impossible to analyze the apoptotic response of infected cell cultures. It has been recently proposed that endogenous PrP^C could regulate p53-dependent caspase-3–mediated neuronal cell death (Paitel *et al*, 2004), but the association of this pathway with RNA virus replication is unclear. That PrP^C may play a role in cell survival is indicated by several lines of evidence (Lasmezas, 2003): (a) PrP^C binds the anti-apoptotic factor Bcl-2; (b) PrP^C protects neurons against Bax-mediated cell death; (c) PrP^C has the activity of a superoxide dismutase that protects cells from oxidative stress; (d) PrP^C binds a poorly characterized neuroprotective factor. The gene array technology utilized for these experiments failed to demonstrate differences in the ability of uninfected $Prnp^{+/+}$ and $Prnp^{-/-}$ cell lines to express mRNA transcripts encoding growth factors of several different families (colony stimulating factor, fibroblast growth factor, insulin-like growth factor, transforming growth factor alpha, transforming growth factor beta, and bone morphogenetic proteins). In any case, it should be borne in mind that viral antigens and viral genomes could not be detected in infected $Prnp^{+/+}$ cells, indicating that virus replication was made impossible at an early step of virus-cell interaction.

Evaluation of gene expression upon infection with PV-1 revealed that exposure to the virus had no influence on the gene expression profile of $Prnp^{+/+}$ cells. This confirmed virus titration experiments showing that $Prnp^{+/+}$ cells (like prionless cells transfected with the *Prnp* gene) were not permissive to PV-1. In contrast, $Prnp^{-/-}$ cells allowed productive infection by PV-1 and produced virus at titers surprisingly high when considering that only 1% of the cell population expressed viral antigens. The reason for this behavior is unclear, but it appears that expression of PrP^C makes mouse neuronal cells not susceptible to PV-1.

The clear distinction of permissive from nonpermissive neuronal cells on the basis of *Prnp* expression makes the present model peculiar and suggests that infection of prionless mice could represent a potential animal model for investigating the pathogenetic events in poliovirus infection (Ford *et al*, 2002).

In our model, poliovirus infection profoundly altered the gene expression profile of permissive $Prnp^{-/-}$ cells. As expected, the early phase of infection was associated with the activation of type I interferon (IFN) genes endowed with antiviral activity (Biron *et al*, 2002), of a proinflammatory cytokine (TNFSF13b), and of interleukin (IL)-7, a cytokine that promotes B-cell proliferation and neuronal cell growth (Michaelson *et al*, 1996). Later stages of infection were associated with activation of the

IFN-related developmental regulator 1 (a nerve growth factor–inducible gene that has been associated with neuronal development) (Roth *et al*, 2003). In the later phase of infection, up-regulation of a variety of growth factor genes was observed: granulocyte/macrophage CSFs that act on the hematopoietic compartment, hepatocyte growth factor, vascular endothelial growth factor-A, TGFb1, and TGFb3. It is of interest that a variety of bone morphogenetic proteins (BMP1, BMP5, BMP7) were also activated. BMPs play a role in calcium regulation and are involved in cartilage and bone formation. More recently, BMPs have been also recognized as important neurotrophic and neuroprotective factors (Allan *et al*, 2003; Angley *et al*, 2003).

Materials and methods

Virus and cell culture

If not otherwise specified, tissue culture reagents and chemicals were from Sigma Chemical (St. Louis, MO); plasticware was obtained from Falcon (Oxnard, CA). Attenuated PV-1 (Chat strain) and reference horse serum against PV-1 were obtained from ATCC (Promochem, Teddington, UK). Virus stocks were prepared in HeLa cell cultures grown in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 2 mM L-glutamine and 2% heat-inactivated fetal calf serum (HI-FCS). Supernatants were collected 3 days p.i. and clarified by low-speed centrifugation. Virus titers were determined with a microtitration assay in 96-well plates using HeLa cells as targets. Triplicate wells were infected with 50 μl of serial 10-fold dilutions of virus. CPE was read microscopically on day 5 p.i. Virus titers were expressed as $TCDI_{50}$. PV-1 preparations were neutralized by the reference anti–PV-1 horse serum using 100 $TCID_{50}$/well in a microdilution assay at a titer $\geq$1:1280. Virus aliquots were stored at -70°C.

Prionless mice were generated by homologous recombination replacing the entire *Prnp* gene open reading frame (ORF) and the 3′-end of intron 2 of ES cells with a *pgk-neo* cassette (Yokoyama *et al*, 2001). Heterozygous ($Prnp^{+/-}$) mice were obtained by crossing chimeras with C57BL/6 mice. Homozygote ($Prnp^{-/-}$) mice were obtained by crossing heterozygous animals. The wild-type neuronal cell line Hw3.5 was established from cultures of hippocampal cells prepared from inbred $Prnp^{+/+}$ C57BL/6 mice on embryonic day 14. The neuronal prionless cell line HpL3.4 was established as above from $Prnp^{-/-}$ congenic C57BL/6 mice. HpL3.4 and Hw3.5 cell lines were grown in 5% CO_2 atmosphere using Neurobasal medium additioned of B-27 supplement (Gibco-Invitrogen, Carlsbad, CA), 2 mM L-glutamine, and 10% HI-FCS. As seen by IIF with the anti-PrP6H4 antibody (Prionics AG, Schlieren, Switzerland), HpL3.4 cells did not express immunoreactive PrP^{C}, whereas Hw3.5 cells expressed PrP^{C} both on the surface (Kuwahara *et al*, 1999) and in the cytoplasm.

Virus infection of neuronal cell lines

HpL3.4 and Hw3.5 cell lines were cultured in T-25 flasks and infected with PV-1 (m.o.i. = 3). After infection, cell monolayers were washed 3$\times$ with warm medium and incubated at 35°C. Cultures were observed by phase-contrast microscopy in order to evaluate the development of CPE. Cell cultures prepared in 2-well chamber slides (Nalgene-Nunc, International PBI, Milano, Italy) were used for detecting viral antigens in infected cells by IIF. At different times p.i., cell monolayers were fixed with cold acetone for a few seconds, then with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min. Monolayers were washed in PBS containing 1% FCS, permeabilized with Triton X100 (0.05% in PBS, 10 min), briefly immersed in distilled water, and dried out. A blend of two monoclonal antibodies directed to common enteroviral antigens (clones 2E11 [immunoglobulin M; IgM) and 9D5 (IgG3)] obtained from Chemicon (Temecula, CA) was used to detect immunoreactive PV-1 by indirect immunofluorescence. A rabbit fluorescein isothiocyanate (FITC)-labeled antibody to total mouse IgG (Sigma) was used as secondary reagent upon dilution with Evans Blue in PBS. Slides were examined with a fluorescence microscope (BX-60; Olympus, Tokyo, Japan) equipped with a digital camera (Nikon, Tokyo, Japan).

Transfection of HpL3.4 cells with a Prnp *gene construct and cell line characterization*

If not otherwise specified, molecular biology reagents were from Applied Biosystems (Monza, Italy). As reported (Nakamura *et al*, 2003), the coding region of *Prnp* cDNA was amplified by PCR, cloned into the pT7Blue vector, and subcloned into pIREShyg (Clontech, Palo Alto, CA). Two different constructs carrying *Prnp* (pIREShyg-PrP) or devoid of *Prnp* (pIREShyg) were transfected into HpL3.4 cells using Lipofectamine plus (Gibco-Invitrogen). Transfected cells were selected for 10 to 20 days in complete medium containing 400 μg/ml hygromycin B (Wako, Japan). The transfected HpL3.4-RP-3 cell line was selected for the stable expression of PrP^{C} by surface staining with the 6H4 antibody (Ab). The 6H4 antibody (Ab) was diluted 1:500 in PBS supplemented with 1% FCS and 0.05% sodium azide and revealed by IIF with a FITC-labeled antibody. The control cell line carrying the void plasmid vector (HpL3.4-EM-P) gave no signal upon staining with 6H4. By RT-PCR, it was shown that transcripts of the glial fibrillary acidic protein (a glial cell marker) were not expressed in the

four cell lines investigated (Kuwahara *et al*, 1999). Transcripts of the *NF68K* and *NF200K* genes (encoding either 68-kDa or 200-kDa neurofilament) were detected both in $Prnp^{+/+}$ and $Prnp^{-/-}$ cell lines. Thus, the investigated cell lines appeared to belong to the neuronal cell lineage.

Response of neuronal cell lines to infection by PV-1 and detection of viral sequences by RT-PCR

The sensitivity to PV-1 of four different cell lines (HpL3.4, Hw3.5, Hpl3.4-RP-3, and HpL3.4-EM-P) was evaluated by determining the virus CPE by phase-contrast microscopy and by measuring extracellular virus titers at different times p.-i. The number of cells expressing PV-1 antigens was evaluated by IIF.

PV-1 replication was also evaluated by detecting viral genomes using RT-PCR. Briefly, total RNA was extracted from tissue culture supernatants at different times p.i. using the QIAmp viral kit (Qiagen, Valencia, CA). cDNA was obtained from RNA with Moloney-murine leukemia virus (Mo-MLV) reverse transcriptase in conjunction with random hexamer primers (Clontech). A real-time PCR instrument (Smart Cycler; Cepheid, Sunnyvale, CA) was used. Table 2 lists the primers and probes used in this study. For real-time PCR, 25-μl reactions contained AmpliTaq Gold polymerase with 1 × reaction mix, 500 nM of each primer, 100 nM of 5′FAM- and 3′BHQ-1–labeled probe, 4 mM $MgCl_2$ in a final volume of 25 μl. Thermal cycler conditions consisted of an initial 10-min hold at 95°C followed by 40 cycles of 15 s at 95°C followed by 30 s at 60°C. Real-time data were collected during the extension step of each cycle. Amplification of mouse GAPDH was used as a control. RNA extracted from a PV-1 stock containing 6.8×10^6 $TCID_{50}$/ml was used as a quantitative reference for viral RNA amplification. The threshold cycle number (Ct value) represented the cycle number at which a fluorescence signal significantly different from the baseline was obtained.

Detection of DNA and mRNA sequences in uninfected cell lines

Primers listed in Table 2 have been used to amplify murine *Prnp*, murine *Prnd*, and human *Alu* sequences by conventional PCR. Expression of human PVR and mouse PVR mRNA transcripts was evaluated by RT-PCR. Applied Biosystems model 2400 thermal cyclers were used for PCR reactions using AmpliTaq Gold polymerase (2 units) in a final volume of 50 μl. Samples were denatured at 94°C for 10 min before cycling for 22 to 26 cycles. Amplicons were analyzed on 2% agarose gel using ethidium bromide staining and photographed on a ultraviolet (UV) transilluminator (Kodak Image Station 440). Amplicons were quantified using the Kodak 1D 3.5 software using beta-actin and glyceraldehyde phosphate dehydrogenase (GAPDH) transcripts to normalize the data.

Analysis of gene expression by gene arrays

GE Array Q series membranes (MM-003N common cytokines nonradioactive kit; Superarray, Bethesda, MD) were used to characterize gene expression profiles associated with PV-1 infection. Membranes contained 96 cDNA tetraspots of mouse genes; pUC18 DNA was used as a negative control, and housekeeping genes (GAPDH, cyclophillin A, RPL13A, beta-actin) as positive controls. To evaluate what genes were induced or suppressed in response to infection, side-by-side hybridizations with samples from uninfected and PV-1–infected cultures were performed. Total RNA was extracted with the Trizol method (Gibco-Invitrogen), from confluent cell monolayers grown in T-25 flasks and infected or not (6-h and 6-day exposures). Biotinylated probe synthesis was performed using mixtures of primers proper to each gene set. Before probe synthesis, RNA samples were

Table 2 Oligonucleotide primers used in this study

Primer designation	*Sequence*	*Reference*
CVA16	fwd: TCCTCCGGCCCCTGA rvs1: AATTGTCACCATAAGCAGCCA rvs2: GATTGTCACCATAAGCAGCCA probe1: [FAM]-CGGAACCGACTACTTTGGGTGTCCGT-[BHQ1] probe2: [FAM]-CGGAACCGACTACTTTGGGTGACCGT-[BHQ1]	Nijhuis *et al*, 2002
Human picornaviruses	fwd: AAGCACTTCTGTTTCC rvs: CATTCAGGGGCCGGAGGA	Hyypia *et al*, 1989
Prnp	fwd: CGAATTCCGCCACCATGGCGAACCTTGGCTACTGGCTG rvs: CACGAATTCCACCTCAATTGAAAGAGCTACAGGTGG	Nakamura *et al*, 2003
Prnd	fwd: GCGTCGACCGCCACCATGAAGAACCGGCTGGGTACATGG rvs: CTCGTCGACCTCTGTGGCTGCCAGCTTGATTGA	T Onodera, personal communication
Alu	fwd: GCCTCCCAAAGTGCTGGGATTA rvs: TTGCAGTGAGCCGAGATCGCGCC	Charlieu *et al*, 1992
mPVR AI325026	fwd: CTGTGTGGTTCCACTTGTCC rvs: GAAACACCAAGTTCCACCCT	UniSTS: 159606
hPVR	fwd: CGAGCCATGGCCGCCGCGTGGCCG rvs: GGTCTGAGTGCCAGGTGATTTGGGCT	Solecki *et al*, 2002

analyzed and quantified by agarose gel electrophoresis. RNA samples (1 to 5 μg) combined with the primer mix were added with a prewarmed labeling mix containing 50 units of Mo-MLV reverse transcriptase, 20 units of RNase inhibitor, and a dNTP mix containing 2 nmol of biotin-16-dUTP (Roche Applied Science, Monza, Italy) and incubated at 42°C for 90 min. GE Array membranes were prehybridized for 1 h with GEAhyb buffer containing 100 μg/ml heat-denatured salmon sperm DNA (Life Technologies) to prevent nonspecific hybridization. Membranes were hybridized overnight at 60°C with denatured cDNA probes using the kit hybridization buffer and hybridization bottles rotating at 10 rpm. After extensive washing (60°C) at low- and high-stringency conditions in rotating hybridization bottles (10 rpm), membranes were incubated for 10 min with alkaline phosphatase–conjugated streptavidin (AP-streptavidin 1:7500). Gene expression was detected by chemiluminescence using the AP substrate CDP-Star. Chemiluminescence signals were recorded on X-OMAT film (Kodak, Rochester, NY) using exposure times of 0.5 to 8 min. After development, X-OMAT films were scanned with a high-definition scanner (Coolscan 4000 ED; Nikon). Dedicated software (Gene-Analyzer, Superarray) was used for densitometric analysis. Experiments were repeated at least twice.

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