Poliovirus pathogenesis in a new poliovirus receptor transgenic mouse model: age-dependent paralysis and a mucosal route of infection

Shane Crotty,† Laura Hix, Luis J. Sigal and Raul Andino

1 Department of Microbiology and Immunology, University of California, Box 0414, San Francisco, CA 94143, USA
2 Basic Science Division, Fox Chase Cancer Center, Philadelphia, PA 19111, USA

We constructed a poliovirus receptor (PVR) transgenic mouse line carrying a PVRδ cDNA driven by a β-actin promoter. We refer to this model as the cPVR mouse. The cPVR mice express Pvr in a variety of tissues (including small intestines, brain, spinal cord, muscle, blood and liver) and are susceptible to infection after intraperitoneal, intracerebral or intramuscular inoculation of poliovirus. After intraperitoneal inoculation, poliovirus replication is observed in cPVR muscle, brain, spinal cord and, notably, small intestine. The cPVR mice exhibit a striking age-dependent paralysis after intramuscular infection, with 2-week-old mice being 10 000-fold more susceptible to paralytic disease than adult mice. The cPVR mice are also susceptible to paralysis following intranasal infection with poliovirus. After intranasal infection, virus replication is observed in the olfactory bulb, cerebrum, brain stem and spinal cord, suggesting that intranasal infection of cPVR mice is a model for bulbar paralysis. Intranasally infected mice frequently display unusual neurological behaviours. The PVR transgenic mouse reported here provides the first available model for a mucosal route of infection with poliovirus.

Introduction

A poliovirus receptor (PVR) transgenic mouse was the first transgenic mouse model of a human viral pathogen (Ren et al., 1990). Those PVR transgenic mice are susceptible to poliovirus infection, and succumb to lethal paralytic poliomyelitis after intracerebral, intraspinal, intramuscular, or intraperitoneal inoculation of wild-type poliovirus. Several additional PVR transgenic mouse lines have since been generated, all of which have similar phenotypes (Deatly et al., 1998; Koike et al., 1991; Zhang & Racaniello, 1997). Unfortunately, none of these transgenic mice are susceptible to any mucosal route of infection with poliovirus, including the oral route (Koike et al., 1991; Ren et al., 1990; Zhang & Racaniello, 1997), which is the natural route of poliovirus infection in humans. Though the PVR transgenic mice support poliovirus infection in most of the tissues that are thought to be susceptible in humans (namely, muscle, spinal cord and brain), they do not support poliovirus infection in gut tissues (Koike et al., 1993; Ren & Racaniello, 1992a; Ren et al., 1990; Zhang & Racaniello, 1997).

In normal human poliovirus infections, however, the gut appears to be the site of greatest virus replication, as large quantities of virus are shed in human faeces; and in experimentally inoculated chimpanzees the majority of infectious virus was isolated from the gastrointestinal tract (Bodian, 1955; Nathanson & Ahmed, 1997; Sabin, 1956; Sabin, 1986).

Currently (as of 1999) 20 000 cases of paralytic poliomyelitis occur per year in the world, corresponding to an estimated 2 million total poliovirus infections annually (WHO, 1998b). These numbers are rapidly decreasing due to extensive efforts by the World Health Organization to vaccinate the world population with the Sabin poliovirus vaccine (WHO, 1998b).

Studying transgenic mouse models of poliovirus infection and their reasons for inaccurately mimicking the disease in humans is important for several reasons. Poliovirus is a major virus model system, with excellent reverse genetics, biochemistry and structural systems available; an accurate small
animal model system would allow one to bring much more of this knowledge to bear on viral pathogenesis in vivo. Moreover, it has generally been difficult to generate accurate transgenic mouse models of human infectious diseases, as evidenced by the extensive efforts to develop mouse models of human immunodeficiency virus (HIV) infection and measles (Blixenkrone-Moller et al., 1998; Browning et al., 1997; Hanna et al., 1998; Horvat et al., 1996; Jamieson & Zack, 1999; McCune, 1997; Mrkic et al., 1998; Oldstone et al., 1999; Rall et al., 1997; Speck et al., 1998). Experiences with PVR transgenic mice may shed light on generalities about the complexities of developing such model systems.

We therefore generated a new PVR transgenic mouse line, which we refer to as cPVR mice, in which the PVRα cDNA is driven by a β-actin promoter. These animals are susceptible to paralysis following intranasal infection with wild-type poliovirus. Using this model we demonstrate that, in spite of the fact βPVR mRNA was present in many tissues, poliovirus only replicates in specific tissues which resemble the pattern observed in humans. In addition, we demonstrated that young cPVR mice are much more susceptible to paralytic disease than adult cPVR mice.

Methods

DNA procedures and recombinant poliovirus construction. To construct pPVR7-1, human RNA was isolated from HeLa cells and RT–PCR was performed to generate PVR cDNA. The primers specifically amplified the shorter β form membrane-bound PVR isoform with a predicted 8 amino acid cytoplasmic tail. The βPVR cDNA was cloned into the HindIII/Sall sites of a mammalian expression plasmid, pKS25, containing a 4.3 kb rat β-actin promoter, β-actin 5’ UTR and an 880 bp β-actin 3’ UTR (Fig. 1A) in a pBluescript background (Sturm et al., 1999; Trainor et al., 1999). This construct (pPVR7-1) was sequenced, and the PVRα sequence was identical to that previously published (Mendelson et al., 1989). The Xbal–AIII fragment of the plasmid was used for standard mouse embryonic stem cell microinjections (Brinster et al., 1985).

The plasmid pMov2.8-EGFP contains a full-length poliovirus cDNA (Mahoney strain) (originally derived from the plasmid pXPα) with a T7 promoter at the 5’ end. The poliovirus genome cDNA sequence has been modified to contain a hammerhead ribozyme at the 5′ end (Herold & Andino, 2000), a poly(A) tail 80 nucleotides in length at the 3′ end (Herold & Andino, 2000), and a cloning site identical to that in pMov2.11 (Tang et al., 1997) between the capsid protein VP1 and the protease 2A.

Poliovirus stocks. Stocks of Mahoney type 1 poliovirus, Sabin 1 poliovirus and Sabin 2 poliovirus were each generated from a molecular genomic cDNA clone in a plasmid. Mahoney type 1 poliovirus stocks were made from plasmid pXPα (Racaniello & Baltimore, 1981). Sabin 1 stocks were made from plasmid pS1, derived from Nomoto’s molecular clone of Sabin 1 (Nomoto et al., 1982b; Omata et al., 1984), which we resequenced and modified the plasmid backbone in minor ways (Crotty et al., 2001). Sabin 2 stocks were made from plasmid pS2F, which was constructed and sequenced in our laboratory (Crotty et al., 2001).

Poliovirus stocks were generated by linearization of the appropriate plasmid followed by T7 transcription and HeLa electroporation (Gohara et al., 2000). Electroporated cells (P8 viral stock) were grown on 6 cm plates in 3 ml of DMEM/F12 medium (Gibco) containing 10% FCS (Gibco), until lysis (approximately 12 h). Cells and supernatant were harvested, freeze–thawed three times with dry ice–ethanol and 37 °C baths, and supernatant was transferred to a fresh tube after the cellular debris was pelleted at 300 g for 5 min. P1 stocks were generated by infecting 50–80% confluent 10 cm dishes of HeLa cells with 1 × 107 p.f.u. (m.o.i. = 0.005). When 100% CPE was observed, cells and supernatant were harvested and freeze–thawed. Stocks were titred on HeLa cells as described (Crotty et al., 1999). All passaging and titrations of Sabin 1 were done at 32 °C; all passaging and titrations of Mahoney and Sabin 2 were done at 37 °C.

Mice. cPVR mice were initially made and bred at the Gladstone Institute Transgenic Core facility in 1994, using standard microinjection techniques (Brinster et al., 1985). The cPVR mice were later transferred to the UCSF specific pathogen-free Transgenic Mouse facility and have been continuously maintained there since. Currently the cPVR mice are at generation ~18. The cPVR mice are derived from an ICR outbred strain purchased from Simonsen (Gilroy, CA, USA). Due to supply problems with Simonsen, we maintain our own colony of nontransgenic ICR mice (originally purchased from Simonsen) in the UCSF clean mouse facility as our controls. The gPVR mice (TgPVR1-17) (Deatly et al., 1998; Ren & Racaniello, 1992a; Ren et al., 1990) were the gift of V. Racaniello (Columbia University, USA) and are maintained as a separate colony at UCSF. C57BL/6 mice were purchased from Charles River Laboratory, and CD1 mice were purchased from Jackson Laboratory. cPVR mice exhibited no unusual characteristics, physically or behaviourally, compared to nontransgenic ICR mice. cPVR mice are good breeders as both homozygotes (PVR+/PVR) and heterozygotes (PVR+/ PVR−), with normal litter sizes.

Genotyping and RT–PCR. Genomic DNA from tail bleeds (Ausubel et al., 1995) was used as template in PCR genotyping assays. PCR primers corresponding to positions 799–817 (GAGGCCACCTGT-ACCTGCG) and 983–1000 (GAGGTCCCTCTTTGACCT) (reverse complement) of the PVR open reading frame were used as forward and reverse primers respectively. PCR reactions used rTth polymerase (Perkin Elmer) with conditions as recommended by the manufacturer, with ~50 ng of template and 30 cycles of amplification.

For RNA analysis, tissues were removed from transgenic animals, snap-frozen in a dry ice–ethanol bath, and frozen at −80 °C until ready for use. Tissues were then weighed, quickly thawed, and homogenized with an Ultra-Turrax T8 (IKA Laboratory) motorized disruptor, in cell lysis buffer (Qiagen). Poly(A)+ RNA was isolated from homogenized tissue using a Qiagen poly(A)+ RNA isolation kit. Approximately 10 µg RNA was isolated per 200 mg tissue, which was resuspended in 100 µl. Samples were treated with DNase I (Roche) (10 U) at 37 °C for 1 h to remove any contaminating genomic DNA. RNA quality was confirmed by gel electrophoresis. cDNA was synthesized using Superscript II (Life Technologies) with random hexamer primers and 1 µg RNA as template. PVR PCR was done as described above, except for 40 cycles. PCR for 18S RNA expression was done from the cDNA as a positive control, and from the poly(A)+ RNA as a negative control, using mouse 18S primers obtained from Ambion.

Fluorescence activated cell sorting (FACS) analysis. Spleen cells from the indicated mice were depleted of red blood cells by hypotonic lysis. The remaining white cells were counted, and washed twice in FACS staining buffer (PBS containing 2% FCS and 0.04% sodium azide). For primary labelling, the cells were incubated in 100 µl of FACS staining buffer containing the anti-PVR monoclonal antibody D171 (a gift from Eckard Wimmer, Stony Brook, NY, USA) or, as controls, the
MHC 1 anti-H-2K\(^b\) mAb Y3 or an irrelevant mouse anti-chicken ovalbumin. MC57G-PVR was a mouse cell line stably transfected with the PVR\(^{-}7\)-1 expression construct, and this cell line was used as a positive control for Pvr expression on mouse cells. Following incubation with the primary antibody, the cells were washed twice and incubated in 100 \(\mu\)l of FACS staining buffer containing as secondary antibody FITC-labelled goat anti-mouse IgG, gamma chain-specific (KPL), at a final dilution of 1/300. Finally, the splenocytes were washed twice with PBS and resuspended in 200 \(\mu\)l of 0.5% paraformaldehyde in PBS. The stained cells were analysed for fluorescence using a FacsCalibur apparatus and the Cellquest program (both from Beckton Dickinson).

### Poliovirus infections.

All of the poliovirus infections reported in this study used 6- to 8-week-old adult mice, unless otherwise indicated.

Intranasal (i.n.) inoculations were done as 50 \(\mu\)l injections into the thigh of the right hind leg using a 26 gauge syringe. Intraperitoneal (i.p.) inoculations were done as 100–300 \(\mu\)l injections using a 26 gauge syringe. Intracerebral (i.c.) inoculations were done in anaesthetized animals (200 \(\mu\)l Avertin injected i.p. in adult animals) as 15–30 \(\mu\)l injections into the mid-brain (between the ears of the mouse, along the skull midline) using a 27 3\(\mu\)l gauge syringe. (In 10-week-old mice, i.c. inoculations were done into the same region from the left temple, on the side of the head, because the skull was too thick to penetrate consistently.) Intravenous (i.v.) inoculations were done as 200 \(\mu\)l tail vein injections using a 26 gauge syringe after warming the mice for 20 min under a heating lamp and topically treating the tail with 70% isopropanol. Intragastric inoculations of 100 \(\mu\)l were done using a steel 2 inch long round-balled gavage (a curved needle with a rounded end) attached to a syringe. The gavage tube was inserted down the throat of the mouse to the stomach, at which point the 100 \(\mu\)l volume was released and the gavage retracted from the mouse. Oral infections were done in a 50 \(\mu\)l volume by depositing the inoculum into the mouth using a standard 200 \(\mu\)l micropipetter. Intranasal (i.n.) inoculations were done in mice briefly sedated with Halothane (but not fully unconscious) (Halocarbon Laboratories, River Edge, NJ, USA). Virus inoculum (7.5 \(\mu\)l) was placed at the opening of each nostril sequentially using a micropipette. The pipette tip was not inserted into the nostril, and generally did not come in direct contact with the skin. Rapid breathing of the mouse led to the 15 \(\mu\)l inoculum being rapidly sucked up into the nose. Mice were active and alert again within 2 min. Rectal inoculations were done as 100 \(\mu\)l (in cPVR mice) or 50 \(\mu\)l (in C57BL/6 and derivatives) injections using a micropipetter with a fine plastic tip (‘yellow tip’) coated with a thin layer of vaseline. Mice were fasted for 3–4 days and then anaesthetized with Avertin (see above) and the inoculating pipette tip was slowly inserted ~0.5–1.5 cm into the rectum of a prone animal. The inoculum was then slowly released and the tip withdrawn. This technique was pilot tested using trypan blue as an indicator. Trypan blue was injected rectally, and mice were sacrificed 2 h later. Trypan blue was present throughout the colon and large intestine, though it did not appear to go into the small intestines of the mice. No bleeding or tearing of the colon wall was observed, and the presence or absence of faeces in the colon did not grossly affect the spread of the trypan blue.

A series of LD\(_{50}\) experiments (i.c., i.m. and i.p.) was also done using cPVR \(\times\) C57BL/6 F1 mice, with comparable LD\(_{50}\) results to that obtained when using homozygous cPVR mice (data not shown).

In pathogenesis experiments, all mice inoculated with poliovirus or PBS (control) were observed for at least 21 days for signs of disease: lethargy, ruffled fur, arched back, flaccid paralysis of a limb or back region, and death.

### Tissue isolations and virus quantification.

For tissue tropism experiments, mice were sacrificed every 24 h and tissues were removed surgically. Whole tissues were stored at \(-80\) °C until a series of samples was collected. Tissues were weighed and then homogenized in PBS containing penicillin–streptomycin using an Ultra-Turrax T8 motorized homogenizer. Samples were then frozen at \(-20\) °C for long-term storage. Poliovirus was quantified in these tissue samples by plaque assay on HeLa cells in six-well plates. Tissue from at least three cPVR mice and two ICR mice was used for each data-point, and the graphed data (see Fig. 3) represent the average titre from the multiple samples. At least six cPVR mice were used for most time-points for tissues that exhibited poliovirus replication (muscle, spinal cord, brain, small intestine).
Results
cPVR mouse construction and transgene expression

Prior to this study, there were three major PVR transgenic mouse lines available. The laboratories of V. R. Racaniello and A. Nomoto each developed several lines of transgenic mice containing multiple copies of the human PVR locus integrated into the mouse genome (Deatly et al., 1998; Koike et al., 1991; Ren et al., 1990). We currently refer to Racaniello’s TgPVR-17 mice as gPVR mice and have used them in several studies (Mandl et al., 1998, 2001; Sigal et al., 1999, Tang et al., 1997). The mouse lines based on the genomic PVR locus express all four isoforms of Pvr, two membrane bound and two soluble receptors, in varying amounts, driven by the natural human PVR promoter. In transgenic mice, the natural human PVR promoter drives substantial amounts of PVR RNA and Pvr protein expression in the brain, muscle, spinal cord, lung and genitalia. Tenfold lower levels are expressed in tissues such as small intestine, lung and kidney, with undetectable amounts of RNA in liver and spleen (Deatly et al., 1998; Koike et al., 1991). It was reasoned that these lower levels of expression might limit poliovirus infection in some mouse tissues or cell types and prevent the mice from being orally susceptible to poliovirus. Therefore, Zhang & Racaniello (1997) developed a PVR transgenic mouse line (TgFABP-PVR) expressing Pvr from an intestinal epithelium specific promoter; but in spite of the good expression, that mouse was also not susceptible to oral poliovirus infection. The authors did confirm that Pvr was expressed on the surface of M cells and gut epithelial cells in that mouse line, and that TgFABP-PVR mice were susceptible to i.c. infection with poliovirus (Zhang & Racaniello, 1997).

We reasoned that the lack of expression of membrane-bound Pvr on certain unidentified intestinal or lymphatic cell types (i.e. Peyer’s patch residents) may be the reason that none of the previous PVR transgenic mice lines were orally susceptible to poliovirus infection. Therefore, we set out to generate a transgenic mouse that expresses membrane-bound Pvr in a wide range of cell types. Human RNA was isolated and RT–PCR was performed to generate PVR cDNA. The primers specifically amplified the shorter δ form membrane-bound PVR isoform with a predicted 8 amino acid cytoplasmic tail. The δPVR cDNA was cloned into a mammalian expression plasmid containing a β-actin promoter, β-actin 5’ UTR and β-actin 3’ UTR (Fig. 1A). This construct was sequenced and then used to generate an ICR transgenic mouse line using standard embryonic stem cell technology (Brinster et al., 1985). Founder

Fig. 2. PVR expression in cPVR mice. (A) RT–PCR analysis of PVR RNA expression in tissues from cPVR mice. RT–PCR for 18S RNA expression was done from the same cDNA as a positive control (middle). As a negative control (bottom), a reaction was done containing purified cPVR poly(A)+ RNA, primer and nucleotides, but lacking reverse transcriptase (RT−), to control for potential DNA contamination. (B) Expression of Pvr on splenocytes. FACS analysis of whole spleen from cPVR × C57BL/6 F1 mice and nontransgenic C57BL/6 mice. MC57G-PVR cells were used as a positive control for Pvr expression. Binding of an irrelevant antibody (i), anti-Pvr antibody (ii) and anti-MHC I antibody (iii) are all shown.
mice were screened for the presence of transgene by PCR, and two positive mice were used to generate a homozygous PVR+/PVR+ mouse line called cPVR, referring to the fact that this mouse has the PVR gene as an intron-less cDNA. Mice are genotyped using PCR primers specific for PVRδ cDNA sequence (Fig. 1B).

The β-actin promoter was expected to drive near universal expression of the transgene. RT–PCR was done to determine the expression of PVR RNA in various tissues (Fig. 2A). PVR RNA was detected in brain, brain stem, spinal cord, blood, liver, small intestine and muscle. Very low levels were detected in kidney. This distribution of expression was different than that for gPVR and Nomoto et al., 1991). Pvr protein expression likely closely parallels the RNA expression profile in cPVR mice as the cDNA is flanked by β-actin 5’ and 3’ UTRs.

A series of FACS analyses was performed using splenocytes from F1 cPVR × C57BL/6 mice (Fig. 2B). Expression of Pvr was detected on the surface of the splenocytes, with a median fluorescent intensity 11-fold higher than background staining in C57BL/6. MC37G-PVR, a mouse cell line stably transfected with PVR, was used as a positive control.

### Poliomyelitis in cPVR mice

The classic routes of poliovirus inoculation were tested in the cPVR mice. The results are displayed in Table 1. The cPVR mice are susceptible to lethal poliomyelitis following i.c., i.m. i.p. and i.v. infection. Disappointingly, these mice are not susceptible to poliomyelitis following oral or intragastric inoculation of up to 1 × 10⁵ p.f.u. In adult mice (6- to 8-weeks-old), after i.m. inoculation, the cPVR mice develop a rapid paralysis (2–5 days) and generally die within 3–6 days at the LD₅₀ of 1–10⁶ p.f.u. Paralysis was consistently seen initially in the leg that was inoculated. I.c. poliovirus inoculation of cPVR mice led to a slower paralysis with a 20-fold higher LD₅₀ than i.m. infection. Paralysis after i.c. inoculation was generally observed in 5–13 days. I.p. infection had an LD₅₀ of 1 × 10⁸ in cPVR mice, with paralysis and death seen in 3–7 days post-infection (p.i.). Generally, this paralysis presented as a flaccid uni- or bilateral paralysis of the hind legs in conjunction with flaccid paralysis of the lower back. I.v. poliovirus infection had a similar LD₅₀ of 2 × 10⁸, presenting as a flaccid paralysis of the lower back at approximately day 5 (Table 1). It was uncommon for any animal not to die within a couple of days of the onset of paralysis, but approximately 5% of paralysed mice did survive the infection and could survive with permanent paralysis of a hind leg or the lower back for a minimum of 6 months with no additional complications. This is in contrast to the pathogenesis of the virus in humans, where only a minority of paralysis cases are lethal (Nathanson & Ahmed, 1997; Sabin, 1986).

We analysed the tissue tropism of wild-type poliovirus in cPVR mice after i.p. inoculation. Mice were inoculated with 5 × 10⁸ p.f.u. wild-type poliovirus (Mahoney strain) and tissue

### Table 1. Classic susceptible routes of wild-type poliovirus infection

<table>
<thead>
<tr>
<th>Route</th>
<th>LD₅₀ *</th>
<th>Day of paralysis†</th>
<th>Inoculum used (p.f.u.)</th>
<th>Paralysis summary‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intramuscular</td>
<td>2 × 10⁵</td>
<td>2–6</td>
<td>≥ 1 × 10⁷</td>
<td>18/18</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 × 10⁶</td>
<td>5/6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 × 10⁵</td>
<td>2/6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5 × 10⁴</td>
<td>4/6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5 × 10³</td>
<td>14/25</td>
</tr>
<tr>
<td>Intracerebral</td>
<td>4 × 10⁶</td>
<td>5–13</td>
<td>5 × 10⁴</td>
<td>2/16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5 × 10³</td>
<td>3/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5 × 10²</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 × 10¹</td>
<td>4/5</td>
</tr>
<tr>
<td>Intrapерitoneal</td>
<td>1 × 10⁸</td>
<td>3–6</td>
<td>2 × 10⁸</td>
<td>12/15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>≤ 5 × 10⁷</td>
<td>2/17</td>
</tr>
<tr>
<td>Intravenous</td>
<td>2 × 10⁸</td>
<td>5–7</td>
<td>2 × 10⁸</td>
<td>3/5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>≤ 1 × 10⁷</td>
<td>0/10</td>
</tr>
<tr>
<td>Oral</td>
<td>&gt; 1 × 10¹⁰</td>
<td>–</td>
<td>1 × 10⁸</td>
<td>0/5</td>
</tr>
<tr>
<td>Rectal</td>
<td>&gt; 1 × 10¹⁰</td>
<td>–</td>
<td>1 × 10⁸</td>
<td>0/5</td>
</tr>
<tr>
<td>Intragastric</td>
<td>&gt; 1 × 10¹⁰</td>
<td>–</td>
<td>1 × 10⁸</td>
<td>0/5</td>
</tr>
</tbody>
</table>

* LD₅₀ is in p.f.u. of wild-type poliovirus.
† No. of days p.i. at which paralysis was observed.
‡ Mice paralysed/total no. of mice inoculated.
samples were taken daily from a series of infected mice. The amount of infectious poliovirus present in each tissue was quantified by plaque assay. After i.p. infection with poliovirus, there is a rapid decline in circulating virus in nontransgenic ICR mice and the virus is completely cleared from most tissues by day 2 p.i. (Fig. 3). In the cPVR mice, poliovirus is readily detected above background in small intestine, spinal cord, muscle and brain tissue beginning on days 1, 2, 2 and 3 p.i., respectively, and the virus is present in each of those tissues on all days thereafter (Fig. 3A–D). We observed significant variability in muscle titres between individual mice (ranging from 3 p.f.u. to 45 000 p.f.u./mg on day 4; data not shown), resulting in large daily variations in the average muscle titre (Fig. 3B). Virus was not detected at significant levels in kidney or blood (Fig. 3E–F), or lung, heart, spleen or liver (data not shown). The maximum poliovirus titres (starting at day 2) detected in various cPVR and ICR mouse tissues after i.p. inoculation are shown in Fig. 4(A) for direct comparison. Spinal cord and muscle supported the highest levels of virus replication, with maximum titres reaching 38 500 p.f.u./mg in spinal cord and 12 500 p.f.u./mg in muscle. Brain reached a maximum titre approximately 10-fold lower (1 700 p.f.u./mg).
New poliovirus receptor transgenic mouse

Fig. 4. Poliovirus titres in specific tissues. (A) Highest average poliovirus titres (p.f.u./mg) in cPVR mouse tissues after i.p. inoculation, at day 2 p.i. or later. (B) Comparison of small intestine viral titres in two PVR transgenic mouse lines. cPVR and gPVR mice were infected i.p. with $2 \times 10^8$ poliovirus. Nontransgenic control mice (ICR and C57BL/6 mice, respectively) were given $2 \times 10^8$ poliovirus as well. Viral titres in small intestine were determined from daily tissue samples. As a control we determined viral titres in brain. Both cPVR and gPVR mice had substantial replication in brain tissue (data not shown), demonstrating that productive infections occurred in all transgenic animals used, consistent with our observation that $2 \times 10^8$ p.f.u. is approximately the LD$_{50}$ of poliovirus for both cPVR and gPVR adult mice.

and small intestine reached maximum titres 25% that of brain (501 p.f.u./mg).

We were intrigued by the presence of substantial poliovirus titres in cPVR small intestine, as this had not been reported with previous transgenic mice. Therefore, we compared the poliovirus titres found in small intestine after i.p. inoculation of cPVR, ICR, gPVR and C57BL/6 (a nontransgenic control for gPVR) mice. We found substantially higher levels of poliovirus in cPVR mice than ICR mice, most strikingly on day 2, where cPVR mice had 500 p.f.u./mg, compared with background levels of 3 p.f.u./mg in nontransgenic ICR mice (Fig. 4B). We were able to detect virus for at least 5 days, suggesting that the virus is replicating in this site. In contrast, there was no detectable virus in small intestine in gPVR and C57BL/6 on day 2 or any day thereafter.

Phenotype of Sabin poliovirus vaccine strains in cPVR mice

As anticipated, the attenuated Sabin poliovirus vaccine strains Sabin 1 and Sabin 2 are both highly attenuated in cPVR (Table 2). No adult cPVR mice were paralysed at the maximum dose of Sabin vaccine strain virus that we could inoculate by any route. The LD$_{50}$ after i.m. inoculation with Sabin 1 strain is at least 2000 $\times$ higher than that for wild-type poliovirus. The LD$_{50}$ after i.c. inoculation with Sabin 1 is at least 50 $\times$ higher than for wild-type (Table 2). Sabin 2 was also highly attenuated, at least 150 $\times$ less neurovirulent than wild-type after i.m. inoculation (Table 2).

Intranasal susceptibility

Interestingly, cPVR mice are susceptible to poliovirus infection via an i.n. route of inoculation (Fig. 5), whereas gPVR mice are not susceptible to this route of inoculation (Fig. 6A). Adult cPVR mice develop a lethal paralysis within 5–8 days following i.n. infection with $5 \times 10^8$ p.f.u. poliovirus. These mice frequently display an atypical poliomyelitis, showing a highly arched back and neurological disorders in 4–8 days. Some mice become antisocial, display a lack of coordination, walk in tight circles, or exhibit head or forelimb twitches. We determined that the i.n. infection LD$_{50}$ was $5 \times 10^7$ p.f.u. (Fig. 5). cPVR mice are therefore more sensitive to paralysis following i.n. infection than i.v. infection.

We then examined poliovirus tissue tropism following i.n. inoculation, in two experiments. The first experiment involved i.n. inoculation of cPVR mice with a sublethal dose of poliovirus. High levels of virus were observed strictly in the olfactory bulb of the mice (Fig. 6B). Replication was not observed in the rest of the brain (cerebrum, cerebellum, brainstem), spinal cord, lungs or intestines. Virus was observed for $\sim$ 7 days before clearance.

In the second experiment, mice were inoculated i.n. with a lethal dose of poliovirus. Virus was observed at very high titres in the olfactory bulb early in infection ($1 \times 10^6$ p.f.u./mg at day 3), with fairly constant levels of virus observed in the main body of the brain throughout the course of the experiment ($1 \times 10^6–1 \times 10^7$ p.f.u./mg) (Fig. 6C). Strikingly, virus was not observed at all in the spinal cord until day 5 p.i., after which very high titres were observed ($1 \times 10^7$ p.f.u./mg) that closely correlated with the onset of clinical disease and paralysis in the animals.

Tracking poliovirus spread after intracerebral inoculation

To be able to track poliovirus replication and spread through cells in the central nervous system, we constructed a recombinant poliovirus expressing green fluorescent protein.
Table 2. Attenuated phenotype of Sabin vaccine strain polioviruses in cPVR mice

<table>
<thead>
<tr>
<th>Virus/route</th>
<th>Inoculum used (p.f.u.)</th>
<th>Paralysis summary*</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt;†</th>
<th>Attenuation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sabin 1/i.m.</td>
<td>2 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>0/6</td>
<td>&gt; 2 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>&gt; 2000x</td>
</tr>
<tr>
<td>Sabin 1/i.c.</td>
<td>1 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>0/6</td>
<td>&gt; 1 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>&gt; 50x</td>
</tr>
<tr>
<td>Sabin 2/i.m.</td>
<td>8 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0/6</td>
<td>&gt; 8 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>&gt; 150x</td>
</tr>
<tr>
<td>Sabin 2/i.c.</td>
<td>2.5 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0/6</td>
<td>&gt; 2.5 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>&gt; 5x</td>
</tr>
</tbody>
</table>

* Mice paralysed/total no. of mice inoculated.
† LD<sub>50</sub> is in p.f.u. of the appropriate virus.

Fig. 5. Mucosal route of poliovirus infection. Survival curves of cPVR mice given intranasal inoculations of (A) 5 × 10<sup>8</sup> p.f.u. (B) 1 × 10<sup>8</sup> p.f.u., (C) 1 × 10<sup>7</sup> p.f.u. or (D) 1 × 10<sup>6</sup> p.f.u. poliovirus. Control, nontransgenic ICR mice exhibited no mortality or clinical symptoms at any i.n. dose of poliovirus (data not shown).

As polio–GFP spread in the brain it exhibited genetic instability, reverting to wild-type poliovirus that no longer carries and expresses GFP. Such genetic instability is a hallmark of replication-competent recombinant polioviruses (Crotty et al., 1999; Tang et al., 1997), and is particularly pronounced with GFP (S. Crotty, M. Neagu & R. Andino, unpublished results), possibly because GFP’s unusual protein fold interferes with poliovirus polyprotein folding and processing. However, we were able to track spread of polio–GFP for 3–4 days p.i. in the brain, after which time GFP<sup>+</sup> cells were no longer visible,
though viral plaques in the brain were apparent (data not shown), indicating that the poliovirus was still spreading, but was no longer expressing GFP. Infected cells were seen broadly distributed in various regions of the grey matter in different mice, indicating no strict restriction of poliovirus infection and replication to a few discreet areas of the brain in cPVR mice. The virus did not spread extensively in the brain during the first 3–4 days p.i. (we did not observe any focus of polio–GFP$^+$ infected cells more than twice the size of the one shown in Fig. 7C), corroborating our previous data that virus replication and/or spread was slow after i.c. inoculation (Table 1).

**Age dependence of poliovirus susceptibility**

All of the experiments described thus far were done using 6- to 8-week-old adult mice. We next used cPVR mice at other ages to determine whether there was age dependence to the susceptibility to poliovirus infection and paralysis in these transgenic animals. Ten- to 12-week-old cPVR mice were inoculated i.c. with wild-type poliovirus, and these mice exhibited comparable susceptibility to infection as 6-week-old mice (Table 3); 2-week-old mice were then inoculated i.c., and these younger mice exhibited greater susceptibility to paralysis, with an LD$_{50}$ 25-fold lower than that of adult mice.

We then explored the most susceptible route of infection, intramuscular. Young mice were remarkably more susceptible to infection and paralysis after i.m. inoculation than adult mice, with a surprising LD$_{50}$ of 50 p.f.u., which was 10000-fold lower than the LD$_{50}$ for 6-week-old adult cPVR mice (Table 3). With such a highly enhanced susceptibility, 2-week-old mice inoculated i.m. should be an excellent system for testing the attenuation of various poliovirus mutants in future in vivo studies using cPVR transgenic mice (S. Crotty & R. Andino, unpublished data).
Fig. 7. For legend see facing page.
Table 3. Age dependence of poliovirus susceptibility

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Intramuscular</th>
<th>Intracerebral</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inoculum (p.f.u.)</td>
<td>Frequency of paralysis*</td>
</tr>
<tr>
<td>2</td>
<td>1 x 10^4</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>1 x 10^3</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>1 x 10^2</td>
<td>11/11</td>
</tr>
<tr>
<td></td>
<td>1 x 10^1</td>
<td>1/5</td>
</tr>
<tr>
<td>≥ 1 x 10^7</td>
<td>18/18</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1 x 10^8</td>
<td>5/6</td>
</tr>
<tr>
<td></td>
<td>1 x 10^5</td>
<td>2/6</td>
</tr>
<tr>
<td>10</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Six 2-week-old C57BL/6 control mice inoculated with 5 x 10^7 p.f.u. of poliovirus intracerebrally had no paralysis or death.
† LD_{50} is in p.f.u. of wild-type poliovirus.
ND, Not determined.

Discussion

Pvr is expressed in a wide variety of tissues in cPVR mice, driven by a heterologous promoter, but poliovirus only replicates in select tissues in the cPVR mice. We found this somewhat surprising, and we were further surprised to see, after i.v. or i.p. inoculation, poliovirus replicated only in the tissues where it replicates in humans – muscle, spinal cord, brain and intestine. Importantly, we observed both PVR mRNA (RT–PCR, Fig. 2A) and expression of the receptor on the surface of the splenocytes (Fig. 2B) and yet these cells were not susceptible to infection, indicating that additional blocks to infection exist in this cell type (S. Crotty, L. J. Sigal & R. Andino; unpublished data). This shows that the poliovirus receptor is necessary but not sufficient to confer susceptibility to poliovirus infection in vivo, and that there are additional cell type- and tissue type-specific post-entry blocks to translation, replication and/or packaging.

The cell tropism of HIV has been studied more extensively than any other virus, and it has been determined that, in the mouse, HIV requires the expression of at least four human proteins to allow a full HIV replication cycle, and three of those factors have been identified. CD4 (Dalgleish et al., 1984; Klatzmann et al., 1984; Maddon et al., 1986) and the coreceptor CXCR4 or CCR5 are required for virus entry (Choe et al., 1996; Feng et al., 1996). HIV also requires an intracellular factor, the TAR binding protein cyclin T1 (hCycT1), that is crucial for mediating Tat’s activation of HIV transcription elongation (Bieniasz et al., 1998; Wei et al., 1998). As murine cells expressing CD4, CCR5 and hCycT1 do not support the full HIV life-cycle (Bieniasz & Cullen, 2000; Garber et al., 1998), there is at least one additional human factor missing in mice. Poliovirus appears to require the intracellular factors PCBP, La, Unr, PTB, Sam68 and/or PABP for translation and replication (Andino et al., 1999; Gamarnik & Andino, 1996; Herold & Andino, 2001; McBride et al., 1996), but their tissue expression and effects on tissue tropism are unknown. One or more of these factors may be important additional blocks to poliovirus replication in mice. Additionally, it has recently been reported that the innate immune system can determine tissue tropism, as interferon-α/β receptor knockout mice show a massive expansion in the tissue tropism of Sindbis virus (Ryman et al., 2000). Whether aspects of the innate immune system are important for poliovirus tissue tropism is unknown.

Oral resistance

The resistance of PVR transgenic mice to oral infection with poliovirus was of particular interest, as it is a constant theme in all PVR+ strains. We attempted to infect the mice intrarectally to circumvent the possibility that the gastric environment of mice was somehow inhospitable to poliovirus (be it pH, the presence of a certain digestive enzyme or the presence of an inactive small molecule). But the mice (both cPVR and gPVR) were resistant to poliovirus infection following intrarectal inoculation (Table 1). The fact that we...
observed poliovirus titres in cPVR small intestine after i.p. inoculation (Figs 3, 4B) indicated that the problem may be the apical surface of the intestine. We propose three hypotheses to explain the failure of small intestine infection from the luminal/apical side: (1) poliovirus may not infect human intestinal epithelium but simply be transported through human M cells, and mice lack factors required for efficient transcytosis of poliovirus particles through M cells; (2) Pvr does not receive a post-translational modification necessary for poliovirus entry in the target gastrointestinal cell type; or (3) there may be cellular factors in mouse intestinal cells different than in human cells that either block or fail to support poliovirus replication, as discussed in the previous paragraph. Data and theories of Zhang & Racaniello (1997) using TgFABP-PVR mice are consistent with these proposals.

It would be valuable to have a small animal model where the replication and tropism of the Sabin oral poliovirus vaccine (OPV) can be studied. Our initial results indicate that the mouse intestine can support poliovirus replication (Figs 3 and 4), and the cPVR mouse model should hopefully allow for a more detailed analysis of Sabin virus replication and spread in vivo.

Poliovirus spread

Data from all of our infection experiments were compiled and analysed to develop a working model of poliovirus replication and spread in cPVR mice. Virus is first seen in the small intestine after i.p. or i.v. inoculation. The virus then rapidly spreads to muscle, spinal cord and brain. Paralysis and death are closely correlated with high viral titres in spinal cord. It does not appear that replication in muscle is obligatory for spread to the CNS in cPVR mice, as replication was detected in the CNS in several cPVR mice without any detectable titres in muscle (data not shown). However, this could be due to ‘hidden’ replication in muscle tissue that we did not biopsy. Alternatively, the virus presumably may directly infect peripheral nerves accessible in the peritoneal cavity. Notably, cPVR mice are most susceptible to paralysis after i.m. inoculation of poliovirus (Table 1).

Retrograde axonal transport appears to be very rapidly utilized by poliovirus (Ren & Racaniello, 1992b), as evidenced in cPVR mice by the rapid paralysis and death after i.m. inoculation. The virus likely replicates in the muscle tissue, infects neurons at the neuromuscular junctions, and then utilizes the cellular active retrograde transport system to quickly reach motor neurons of the spinal cord. Forward axonal transport appears to be poorly used, if at all, by poliovirus, as evidenced by the long lag time to paralysis after i.c. or i.n. inoculation. The surprising difference in the time to paralysis between i.m. and i.c. infection suggests that virus in the cerebrospinal fluid in the brain (deposited in the ventricles during injection of the virus inoculum) is unable to infect accessible cells in the spinal cord during circulation. Instead the virus apparently uses cell-to-cell spread via neuronal dendrits and axons to move through the brain to the motor neurons of the spinal cord. But since the infection spreads so slowly in this direction it appears that the virus does not have the capacity to use active forward axonal transport and must instead rely on passive diffusion. This theory is supported by the observation that after i.n. inoculation the virus quickly spreads through the olfactory bulb to the cerebellum, but spreads slowly thereafter. Olfactory neurons are oriented such that transport from the nasal cavity to the cerebrum is retrograde axonal transport. Once the virus accesses the cerebrum after infection via the olfactory pathway, it is forced to use forward transport or diffusion to spread to the spinal cord. As mentioned above, given the long time lag to paralysis following i.c. inoculation, though the virus efficiently uses active retrograde transport it does not appear to be able to utilize forward axonal transport mechanisms.

It is also possible that the virus accesses the CNS by entering the bloodstream after i.p. inoculation and then trafficking across the blood–brain barrier, but that is unlikely for three reasons: (1) i.c. inoculation (which accesses the cerebrospinal fluid) has a long time lag before paralysis; (2) the LD50 after i.v. inoculation is similar to the LD50 after i.p. inoculation and therefore the quantity of virus in the blood does not appear to be an indicator for subsequent paralysis; and (3) we do not see a viraemic phase of infection after i.p. inoculation.

Susceptibility to infection in young mice

We were surprised that 2-week-old cPVR mice exhibited such a massive increase in susceptibility to infection and paralysis after i.m. but not i.c. inoculation (Table 3). This result indicates that there is a specific increase in susceptibility of muscle tissue and/or spread to the spinal cord, but not in brain, in young mice. We propose two possible explanations for this result: (1) higher expression of a cellular factor necessary for poliovirus replication (including Pvr), selectively expressed in young muscle tissue or in neurons that innereve the muscle but not in brain; (2) the absence in young mice of a factor necessary to limit replication in muscle and/or spread to the spinal cord from muscle. It is also possible that spinal cord neurons accessible to the virus after i.m. inoculation are particularly susceptible to poliovirus infection in young mice. With such a highly enhanced susceptibility, 2-week-old mice inoculated i.m. should be an excellent system for testing the attenuation of various poliovirus mutants in future in vivo studies using cPVR transgenic mice.

Mucosal route of infection

The cPVR mouse model reported here is the first description of a small animal model of poliovirus that is susceptible to a mucosal route of infection. This result is significant because poliovirus is transmitted mucosally in humans, and the very successful Sabin oral poliovirus vaccine (OPV) elicits a highly
protective mucosal immune response that is poorly understood. Possessing a mouse susceptible to a mucosal route of poliovirus infection should allow us to examine and test aspects of OPV-elicted protective mucosal immunity.

Interestingly, the i.n. infection of cPVR mice appears to be comparable to i.n. infection of cynomolgus macaque monkeys, which leads to ‘bulbar paralysis’ following rapid, extensive replication in the olfactory bulb. Whether this alternative route of infection exists in humans remains unclear. Though infection via the olfactory pathway has been predicted not to occur in humans (Sabin, 1956), too few poliomyelitis autopsies examining the location of viral plaques in the brain have been performed to clearly establish that olfactory pathway infection does not occur in a small number (< 5%) of cases (Faber, 1955).

The cPVR mice are useful for a variety of studies. We have used these mice to identify poliovirus-specific CTLs (Sigal et al., 1999), and we are pursuing additional immunological studies in these mice. These mice should also be a useful model system for studying poliovirus replication in the small intestines, and Sabin vaccine virus cell tropism and replication in vivo. Additionally, the World Health Organization has recently approved the use of transgenic mice to assess the safety of human CD4 and CCR5 are susceptible to HIV infection. Proceedings of the National Academy of Sciences, USA 94, 14637–14641.


Received 7 November 2001; Accepted 25 February 2002