

# Oncolytic Treatment and Cure of Neuroblastoma by a Novel Attenuated Poliovirus in a Novel Poliovirus-Susceptible Animal Model

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## Abstract

Neuroblastoma is one of the most common solid tumors in children. Treatment is of limited utility for high-risk neuroblastoma and prognosis is poor. Resistance of neuroblastoma to conventional therapies has prompted us to search for a novel therapeutic approach based on genetically modified polioviruses. Poliovirus targets motor neurons leading to irreversible paralysis. Neurovirulence can be attenuated by point mutations or by exchange of genetic elements between different picornaviruses. We have developed a novel and stable attenuated poliovirus, replicating in neuroblastoma cells, by engineering an indigenous replication element (*cre*), copied from a genome-internal site, into the 5'-nontranslated genomic region (mono-*crePV*). An additional host range mutation (A<sub>133</sub>G) conferred replication in mouse neuroblastoma cells (Neuro-2a<sup>CD155</sup>) expressing CD155, the poliovirus receptor. Crossing immunocompetent transgenic mice susceptible to poliovirus (CD155 tg mice) with A/J mice generated CD155 tgA/J mice, which we immunized against poliovirus. Neuro-2a<sup>CD155</sup> cells were then transplanted into these animals, leading to lethal tumors. Despite preexisting high titers of anti-poliovirus antibodies, established lethal s.c. Neuro-2a<sup>CD155</sup> tumors in CD155 tgA/J mice were eliminated by intratumoral administrations of A<sub>133</sub>Gmono-*crePV*. No signs of paralysis were observed. Interestingly, no tumor growth was observed in mice cured of neuroblastoma that were reinoculated s.c. with Neuro-2a<sup>CD155</sup>. This result indicates that the destruction of neuroblastoma cells by A<sub>133</sub>Gmono-*crePV* may lead to a robust antitumor immune response. We suggest that our novel attenuated oncolytic poliovirus is a promising candidate for effective oncolytic treatment of human neuroblastoma or other cancer even in the presence of present or induced antipolio immunity. [Cancer Res 2007;67(6):2857–64]

## Introduction

Neuroblastoma is one of the most common solid tumors in children (1). Available treatment is of limited utility for high-risk neuroblastoma and prognosis is therefore poor (2). The high incidence of resistance of advanced-stage neuroblastoma to conventional therapies has prompted investigators to search for novel therapeutic approaches. Replication-competent viruses that replicate in tumor cells and lytically kill them with limited side

effects have been reported to have great potential in antitumor therapy (3–6).

Poliovirus has recently been added to the list of viruses that hold promise as possible agents in tumor therapy (7, 8). A nonenveloped, plus-stranded enterovirus of the *Picornaviridae*, poliovirus replicates in the gastrointestinal tract causing little, if any, clinical symptoms. Rarely (at a rate of 10<sup>-2</sup> to 10<sup>-3</sup>), the virus invades the central nervous system (CNS) where it targets predominantly motor neurons, thereby causing paralysis and even death (poliomyelitis; ref. 9). Generally, poliovirus replicates efficiently in nearly all tumor cell lines tested, which has led to the suggestion that it may be suitable for the treatment of different cancers. However, the possibility that poliovirus can cause poliomyelitis calls for significant neuroattenuation to avoid collateral neurologic complications in cancer treatment. The aim of our study was to develop highly attenuated polioviruses that may be suitable for the treatment of neuroblastoma in children. It was of concern, however, that the high coverage of antipolio vaccination in early childhood in the United States and other countries may interfere with the application of poliovirus in tumor therapy. Therefore, we also aimed to develop an immunocompetent animal model that would allow us to investigate the oncolytic capacity of neuroattenuated polioviruses for the treatment of neuroblastoma in the presence of high titers of poliovirus neutralizing antibodies.

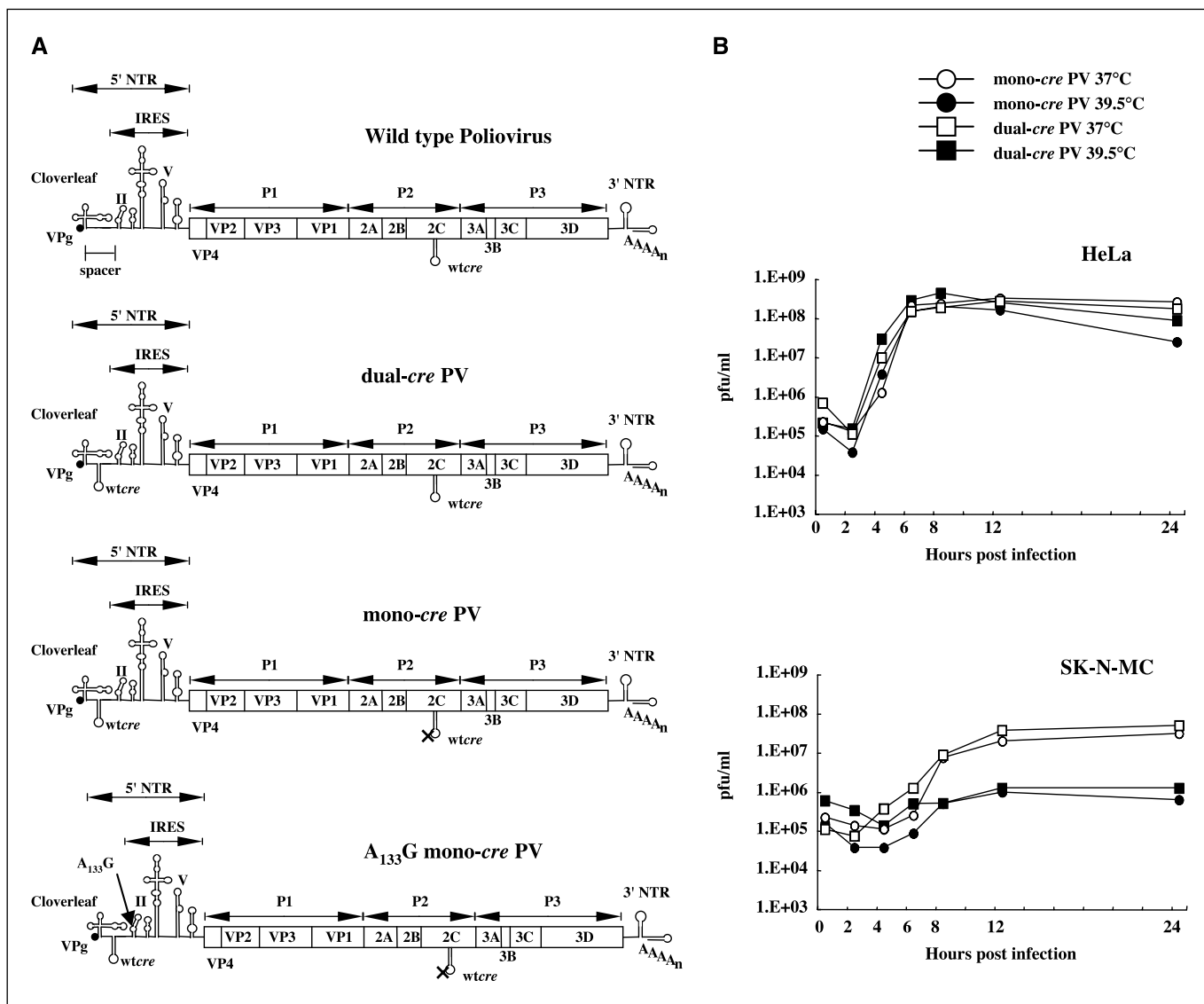
Pathogenesis of poliovirus and of other neurotropic viruses can be controlled by translation (7, 10, 11). In poliovirus, an exchange of the internal ribosomal entry site (IRES) within the 5'-nontranslated region (NTR) with its counterpart from human rhinovirus type 2 (HRV2), another picornavirus, yielded viruses [called PV1(RIPO)] that are highly attenuated in transgenic mice for the human poliovirus receptor (PVR) CD155 (CD155 tg mice; refs. 10, 12) yet replicate efficiently and lytically in cell lines derived from malignant glioma and breast cancer (7, 8, 10, 13). However, PV1(RIPO) and PVS(RIPO), a derivative of PV1(RIPO) that is currently under investigation for the treatment of glioma, grow poorly in neuroblastoma cells (7, 10).<sup>1</sup> This observation prompted us to search for other poliovirus derivatives with oncolytic properties against neuroblastoma. The whole genome synthesis of poliovirus (14) has produced the surprising observation that a point mutation (A<sub>103</sub>G) in a "spacer region" between the cloverleaf and IRES in the 5'-NTR that was introduced as genetic marker (Fig. 1A) attenuated poliovirus 10,000-fold (15). We found that the A<sub>103</sub>G variant of poliovirus replicates well in human neuroblastoma cell lines at 37°C (15). However, the attenuating mutation A<sub>103</sub>G in the spacer region was unstable on replication and direct revertant variants that had acquired the

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**Figure 1.** Genomic organization of poliovirus and one-step growth curves for mono-crePV and dual-crePV. **A**, structure of the PV1(M), dual-crePV, mono-crePV, and A<sub>133</sub>Gmono-crePV genome. The single-stranded RNA is covalently linked to the viral-encoded protein VPg at the 5'-NTR. The 5'-NTR consists of two *cis*-acting domains, the cloverleaf and the IRES, which are separated by a spacer region. The IRES controls translation of the polyprotein (*open box*), consisting of a structural region (*P1*) and nonstructural regions (*P2* and *P3*) specifying the replication proteins. Within the 2C<sup>ATPase</sup> coding region, the *cis* replication element (*cre*) is indicated. The 3'-NTR contains a heteropolymeric region and is polyadenylated. RNA replication requires all three structural elements: cloverleaf, *cre*, and the 3'-NTR. The duplicated *cre* was inserted into the spacer between cloverleaf and IRES (dual-crePV). The native *cre* in 2C<sup>ATPase</sup> was inactivated by mutation as indicated by an X (mono-crePV). A point mutation (A<sub>133</sub>G) was engineered into domain II of the 5'-NTR in mono-crePV (A<sub>133</sub>Gmono-crePV). **B**, one-step growth curves for mono-crePV and dual-crePV in HeLa cells (*top*) and SK-N-MC cells (*bottom*). Cells were infected at a MOI of 10 and incubated at 37°C or 39.5°C. The virus titer was determined by plaque assay on monolayers of HeLa cells as described in Materials and Methods.

neurovirulent phenotype of wild-type (wt) poliovirus type 1 (Mahoney) [PV1(M)] were readily scored (15). We reasoned that a stable attenuation phenotype could be generated if the spacer region would be interrupted by an essential RNA replication element that the virus cannot afford to delete. Such an element is the *cre*, a stem-loop structure mapping to the coding region of viral protein 2C<sup>ATPase</sup> (Fig. 1A; ref. 16). Based on this concept, we have developed a stable attenuated poliovirus, replicating in neuroblastoma cells, by introducing the *cre* element into the spacer region between the cloverleaf and IRES in the 5'-NTR (mono-crePV) at the 104-nucleotide locus.

Using the nude mice model, we have shown previously that tumors of human origin can be successfully treated with neuro-

attenuated poliovirus strains, that is with PV(RIPO) derivatives (7), or with the Sabin vaccine strains (17). However, the lack of a possible immune response to the oncolytic agents mitigates the importance of the results. In this study, we have therefore constructed fully immunocompetent mice (*CD155* tgA/J mice) that express CD155 and accept Neuro-2a<sup>CD155</sup> cells for the formation of lethal neuroblastoma. Neuroblastoma bearing *CD155* tgA/J mice that were fully protected by immunization against lethal doses of wt PV1(M) could be cured by intratumoral administration of a variant of mono-crePV (A<sub>133</sub>Gmono-crePV). Remarkably, the tumor-bearing mice, which were cured through treatment with A<sub>133</sub>Gmono-crePV, resisted attempts to reestablish neuroblastoma with Neuro-2a<sup>CD155</sup> cells. These data offer promise

to develop viral oncolytic therapy against high-risk neuroblastoma in the general pediatric population and perhaps against other forms of human cancer.

## Materials and Methods

**Cells and viruses.** The neurovirulent poliovirus type 1 [Mahoney; PV1(M)] is the strain being used routinely in the laboratory (14). The mouse neuroblastoma cell line stably expressing CD155 $\alpha$  (Neuro-2a<sup>CD155</sup>) has been described (18). Neuro-2a<sup>CD155</sup> cells, which are susceptible to poliovirus infection, were maintained in DMEM containing 1% penicillin/streptomycin and 10% fetal bovine serum. HeLa cells and human neuroblastoma cell lines SK-N-MC, SK-N-SH, and SH-SY5Y were obtained from the American Type Culture Collection (Manassas, VA) and maintained according to the manufacturer's specification.

**Construction of plasmids and DNA manipulation.** The poliovirus cDNA sequence was that used by Cello et al. (2002) for cDNA synthesis (plasmid pT7PVM; ref. 19). "pT7PVM *cre*(2C<sup>ATPase</sup>) mutant" is a full-length poliovirus cDNA clone in which the native *cre* element in the 2C<sup>ATPase</sup> coding region was inactivated by introducing three mutations at nucleotides 4,462 (G to A), 4,465 (C to U), and 4,472 (A to C; refs. 20–22). Dual-*cre*PV is a derivative of pT7PVM carrying two active *cre* elements: one at nucleotide 102/103 of the 5'-NTR in which a new *NheI* restriction site was created and the other in the 2C<sup>ATPase</sup> coding region (Fig. 1A; ref. 20). Mono-*cre*PV, the construction of which has also been described (20), has an active *cre* in the spacer region, whereas the native *cre* in the 2C<sup>ATPase</sup> coding region has been inactivated (Fig. 1A; ref. 20). To construct A<sub>133</sub>Gmono-*cre*PV, which has a single A<sub>133</sub>G mutation in the 5'-NTR, site-directed mutagenesis was done with the QuikChange Mutagenesis kit from Stratagene (La Jolla, CA) using primers 5'-CAAGTTCAATAGGAGGGGTA-CAAAC-3' and 5'-CTGGTTTGTACCCCTCCTATTGAAC-3'. Mutations and final constructs were verified through sequencing using the ABI Prism DNA Sequencing kit.

**In vitro transcription, transfection, and one-step growth curves.** All plasmids were linearized with *DraI*. RNAs were synthesized with phage T7 RNA polymerase, and the RNA transcripts were transfected into HeLa cell monolayers by the DEAE-dextran method as described previously (19). The incubation time was up to 2 days, and virus titers were determined by a plaque assay (23). One-step growth curves in HeLa, Neuro-2a<sup>CD155</sup>, SK-N-MC, SK-N-SH, and SH-SY5Y were carried out as follows. Cell monolayers (1 × 10<sup>6</sup> cells) were infected at a multiplicity of infection (MOI) of 10. The plates were incubated at 37°C or 39.5°C, as indicated, and the cells were harvested at 0, 2, 4, 6, 8, 12, and 24 h after infection. The plates were subjected to three consecutive freeze-thaw cycles, and the viral titers of the supernatants were determined by plaque assay on HeLa cell monolayers as described before (23).

**Serial passages of mono-*cre*PV in Neuro-2a<sup>CD155</sup> cells.** The selection of mono-*cre*PV variants capable of efficient replication in Neuro-2a<sup>CD155</sup> and SK-N-MC cells was carried out according to the following procedure: Neuro-2a<sup>CD155</sup> and SK-N-MC cells were infected with the mono-*cre*PV at a MOI of 10 and incubated at 39.5°C for 48 h. Infected cells were then lysed by three freeze-thaw cycles, and the supernatant fluid was harvested and clarified by low-speed centrifugation. Virus stock from each passage was obtained by growing the virus in HeLa at 37°C. After 15 passages, RNA extracted from the viral cell lysate served as template for reverse transcription-PCR (RT-PCR) and purified PCR amplicons were used for sequencing reactions. Isolation of viral RNA, RT-PCR, purification of PCR products, and sequencing were carried out as described previously (14).

**Animal studies.** The transgenic mice that express human CD155 under its original promoter (ICR-CD155/Tg21) were kindly provided by Dr. Akio Nomoto (University of Tokyo, Tokyo, Japan; ref. 24). The CD155 tg mice were kept in the homozygous state. A/J mice, which express the MHC haplotype H-2<sup>a</sup>, were purchased from The Jackson Laboratory (Bar Harbor, ME). A/J mice carrying CD155 gene were obtained by outcrossing A/J mice with CD155 tg mice and called CD155 tgA/J mice. The CD155 tgA/J mice are heterozygous for CD155 and H-2<sup>a</sup>. Mice were at least 6 weeks of age before use. All procedures involving experimental mice were

conducted according to protocols approved by the institutional committees on animal welfare.

**Neurovirulence assays.** Groups of four CD155 tg mice or CD155 tgA/J mice (equal number of male and females) were inoculated with any given amount of virus ranging from 10<sup>1</sup> to 10<sup>7</sup> plaque-forming units (pfu; 30  $\mu$ L/mouse) i.c. or i.m. with mono-*cre*PV, A<sub>133</sub>Gmono-*cre*PV, dual-*cre*PV, and wt PV1(M). Mice were examined daily for 21 days after inoculation for paralysis and/or death. The virus titer that induced paralysis or death in 50% of the mice (PLD<sub>50</sub>) was calculated by the method of Reed and Muench (25).

**Immunization and microneutralization assay.** CD155 tgA/J mice were immunized by i.p. injection of mono-*cre*PV (1 × 10<sup>8</sup> pfu) thrice with intervals of 1 week. For the neutralizing antibody assay, blood was collected from the tail vein before immunization and on day 21 after the last immunization. Titers of poliovirus neutralizing antibodies in mouse serum samples were determined by microneutralization assay with 100 pfu of challenge virus done according to the recommendations of WHO (26).

**Experimental tumor model.** Neuro-2a<sup>CD155</sup> cells (1 × 10<sup>7</sup>) were s.c. implanted in the right flank of each CD155 tgA/J immunized mouse (day 21 after the last immunization). When the s.c. tumor volumes were ~170 mm<sup>3</sup> (approximately 7–12 days after implantation), mice were inoculated intratumorally with A<sub>133</sub>Gmono-*cre*PV or PBS, respectively. Tumor growth was determined by measuring the tumor volume (length × width × height) every day. Mice were sacrificed when their tumors measured reached >17 mm in any diameter. Mice were followed for up to 6 months after treatment. For experiments of rechallenging with tumor cells those animals that survived without signs of cancer cells for 6 months, survivors were inoculated with 1 × 10<sup>7</sup> Neuro-2a<sup>CD155</sup> cells in the contralateral flank. For CD155 expression assays, tumor tissue was suspended in 2 volumes of PBS with 1% of Triton X-100 and a protease inhibitor cocktail (Roche, Indianapolis, IN). Tumor tissue was lysed with 8 to 12 strokes of a 15-mL Dounce homogenizer with a type B pestle (Bellco, Vineland, NJ) and incubated on ice for 30 min. Cell debris and nuclei were removed by centrifugation at 8,000 × *g* for 10 min at 4°C. Cell lysate (100  $\mu$ g) was separated on a 10% SDS-PAGE followed by Western blot analysis with CD155-specific antiserum NAEZ-8 (1:5,000)/anti-rabbit horseradish peroxidase (HRP; 1:10,000) or anti-actin mouse monoclonal antibody JLA20 (1:1,000)/anti-mouse HRP (1:10,000).

## Results

**Development and characterization of novel neuroattenuated poliovirus strains.** We have recently described that a single point mutation in the 5'-NTR of the genome neuroattenuates poliovirus 10,000-fold in CD155 tg mice (15). Importantly, this variant, named GG PV1(M), replicates in and kills human neuroblastoma cells (SK-N-MC) at 37°C. This growth property of GG PV1(M) is different from that of PV(RIPO), another attenuated poliovirus variant under investigation for the treatment of human glioma whose replication in SK-N-MC cells is greatly restricted (7, 10).<sup>1</sup> GG PV1(M), however, is not useful in tumor therapy because revertants rapidly emerge whose neurovirulence matches that of wt PV1(M) (15). The GG dinucleotide mutation maps to a region (nucleotide 102/103) in the poliovirus genome that previously had not been implicated in poliovirus pathogenesis. To genetically stabilize the attenuated phenotype of GG PV1(M), we made use of poliovirus constructs in which the *cre*, an essential *cis*-acting replication element mapping to the coding region of protein 2C<sup>ATPase</sup> (Fig. 1A), was placed into the nucleotide 102/103 locus. The insertion of the duplicated *cre* element into the 102/103 locus (dual-*cre*PV; Fig. 1A) does not interfere with virus replication in HeLa cells (Fig. 1B; ref. 20). Moreover, inactivation of the endogenous *cre* by three point mutations (mono-*cre*PV; Fig. 1A) yielded a variant replicating also with a wt phenotype in HeLa cells



(Fig. 1B; ref. 20). Although both mono-*cre*PV and dual-*cre*PV replicated in human neuroblastoma SK-N-MC cells at 37°C, they are strongly restricted at 39.5°C (Fig. 1B), a phenotype reminiscent of GG PV1(M) (15). We reasoned that loss of the *cre* element at 102/103 was less likely to occur with mono-*cre*PV than with dual-*cre*PV because elimination of the only functional *cre* in mono-*cre*PV would result in loss of viability. I.c. injection of mono-*cre*PV or dual-*cre*PV into *CD155* tg mice revealed a very strong attenuation phenotype (Table 1), and neurovirulent variants of mono-*cre*PV have never been isolated from infected animals (data not shown). Therefore, mono-*cre*PV was chosen for further studies.

**Generation of fully immunocompetent mice *CD155* tgA/J mice.** Testing mono-*cre*PV as candidate in antineuroblastoma therapy requires that neuroblastoma can be generated in a mouse model susceptible to poliovirus. *CD155* tg mice (strain ICR-*CD155*/Tg21; ref. 24) are suitable for our studies of poliovirus pathogenesis because they are susceptible to poliovirus infection via the i.c., i.p., i.m., s.c., and i.v. routes (24). Infected mice develop a paralytic disease resembling human poliomyelitis (24). We have recently developed a cell line (Neuro-2a<sup>CD155</sup>) that is susceptible to poliovirus infection (18). Neuro-2a<sup>CD155</sup> cells, however, cannot establish tumors in *CD155* tg mice because the original Neuro-2a cell line was developed from a spontaneous tumor in A/J mice. In contrast to *CD155* tg mice, A/J mice express the MHC H-2<sup>a</sup> (data not shown). Accordingly, Neuro-2a<sup>CD155</sup> cells were rejected from *CD155* tg mice (data not shown). We therefore introduced the *CD155* gene into A/J mice via outcrossing and obtained *CD155* tgA/J mice that responded to poliovirus infection indistinguishable from *CD155* tg mice. The PLD<sub>50</sub> value of *CD155* tgA/J mice inoculated i.c. with wt PV1(M) was nearly identical to that of *CD155* tg mice (Table 1), and both mono-*cre*PV and dual-*cre*PV expressed the same striking attenuated phenotype in these new transgenic animals (Table 1). Importantly, s.c. injection of  $1 \times 10^7$  Neuro-2a<sup>CD155</sup> cells into the hind flank of *CD155* tgA/J mice established tumors in 80% of the animals. The tumors progressed to a mean tumor volume of 570.6 mm<sup>3</sup> after 2 weeks, and all tumor-bearing mice were sacrificed when their tumors reached >17 mm in maximal diameter.

**Effect of mono-*cre*PV on tumor grafts *in vivo* and its adaptation through repeated passaging *in vitro*.** We originally assumed that the replication of mono-*cre*PV would be sufficient for oncolytic treatment of Neuro-2a<sup>CD155</sup> tumor-bearing mice. This was wrong. Treatment of four *CD155* transgenic A/J mice

bearing s.c. tumors with a dose of  $1 \times 10^8$  pfu of mono-*cre*PV did not lead to tumor regression (data not shown). Indeed, we subsequently observed that mono-*cre*PV does replicate poorly in mouse Neuro-2a<sup>CD155</sup> cells (Fig. 2D). However, although none of the treated mice developed paralysis, virus recovered from tumors of these mice revealed what seemed to be adaptive mutations scattered over a wide range of the genome: A<sub>133</sub>G, C<sub>2575</sub>A, A<sub>3719</sub>C, C<sub>5584</sub>G, A<sub>6427</sub>G, and U<sub>6607</sub>A. We speculated that one or several of the observed mutations may increase the replication efficiency in Neuro-2a<sup>CD155</sup> and perhaps even in SK-N-MC cells. Therefore, we passaged mono-*cre*PV 15 times on SK-N-MC or on Neuro-2a<sup>CD155</sup> cells and sequenced the total RNAs of putative variants after RT-PCR. The analyses showed that the *cre* element in the 5'-NTR was retained after passages in both cell lines. However, seven mutations accumulated in variants after serial passage in SK-MC (A<sub>133</sub>G, A<sub>807</sub>G, G<sub>1264</sub>A, A<sub>3787</sub>G, C<sub>5699</sub>U, A<sub>6260</sub>C, and U<sub>6261</sub>G) and five mutations (G<sub>101</sub>A, A<sub>133</sub>G, A<sub>145</sub>C, C<sub>2607</sub>U, and G<sub>3543</sub>C) after serial passage in Neuro-2a<sup>CD155</sup> cells. Only the A<sub>133</sub>G transversion was observed in both cell culture-adapted and tumor-adapted mono-*cre*PV, an observation suggesting that this mutation is responsible for the increased replication. Therefore, an A<sub>133</sub>G transition was engineered into mono-*cre*PV, yielding A<sub>133</sub>Gmono-*cre*PV. This variant replicated with significantly increased efficiency in mouse Neuro-2a<sup>CD155</sup> cells (Fig. 2D), whereas in SK-N-SY5Y and SK-N-MC cells the increase was less remarkable (Fig. 2B and C). The increased replication in Neuro-2a<sup>CD155</sup> cells of A<sub>133</sub>Gmono-*cre*PV covaried with an increase of neuropathogenicity in both *CD155* tg mice and *CD155* tgA/J mice, although the virus was still attenuated compared with wt poliovirus (Table 1). By comparing the two other human neuroblastoma cell lines with SK-N-MC cells, we observed that both mono-*cre*PV and A<sub>133</sub>Gmono-*cre*PV replicate more efficiently in SK-N-SH and SH-SY5Y cells (Fig. 2A-C). Moreover, the temperature-sensitive phenotype of mono-*cre*PV is absent or weak in SK-N-SH or SH-SY5Y cells, respectively (Fig. 2A-C). Interestingly, at 39.5°C, A<sub>133</sub>Gmono-*cre*PV replicated better than mono-*cre*PV in SH-SY5Y, SK-N-MC, and Neuro-2a<sup>CD155</sup> cells (Fig. 2A-C). These results suggest that the A<sub>133</sub>G mutation is responsible for an increased replication at 39.5°C not only in mouse neuroblastoma cells but also in human neuroblastoma cells.

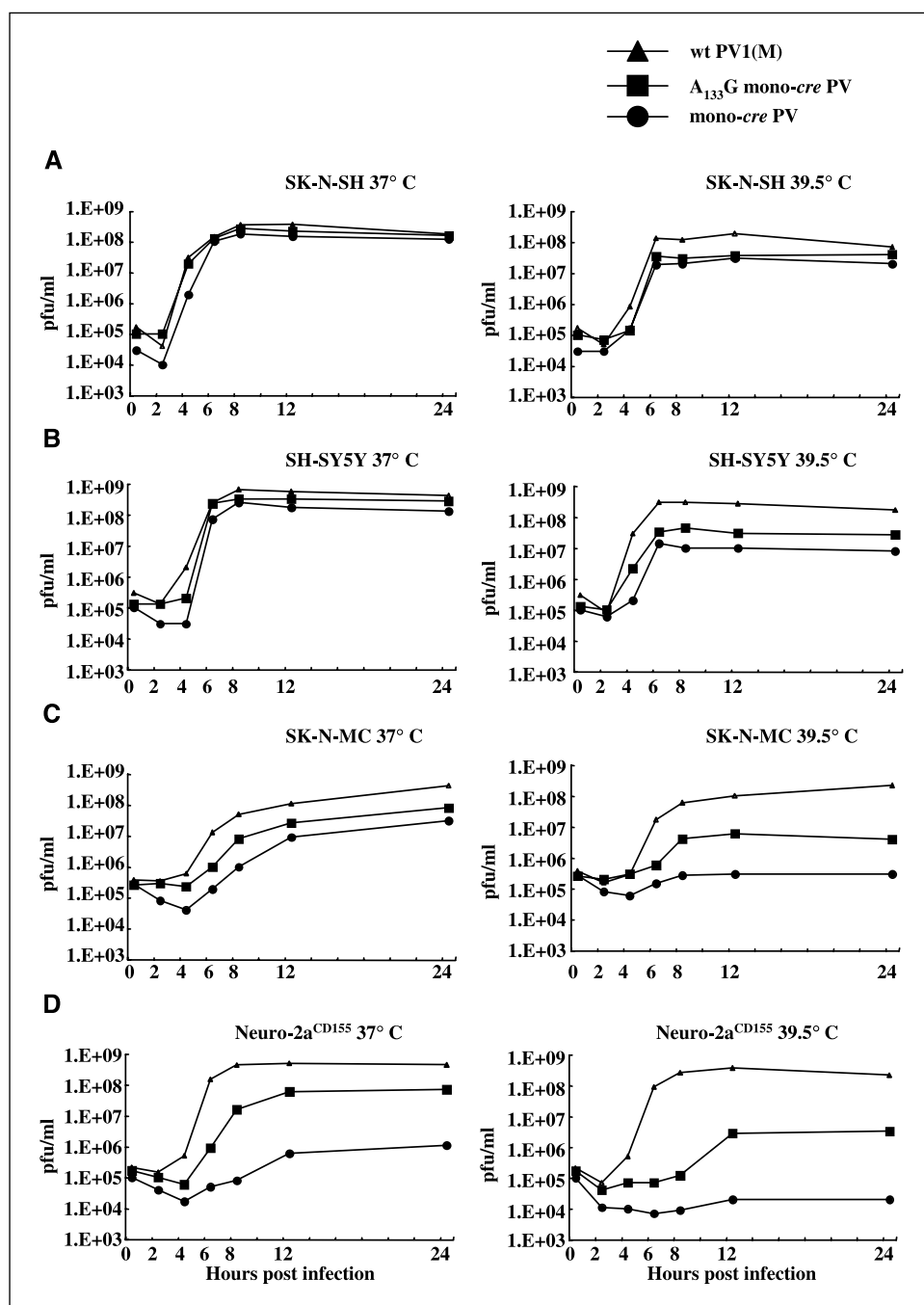
A single intratumoral injection of  $1 \times 10^6$  pfu of A<sub>133</sub>Gmono-*cre*PV into four *CD155* tgA/J mice, bearing a s.c. Neuro-2a<sup>CD155</sup> tumor, caused dramatic tumor regression within 5 days. However, two of four animals treated with A<sub>133</sub>Gmono-*cre*PV showed paralysis and died ~7 days after virus injection (data not shown). This result suggested that A<sub>133</sub>Gmono-*cre*PV can efficiently replicate in s.c. neuroblastoma but can also spread to the CNS causing paralysis.

**Treatment of s.c. Neuro-2a<sup>CD155</sup> tumors in poliovirus-immunized *CD155* tgA/J mice by A<sub>133</sub>Gmono-*cre*PV.** We hypothesized that the unacceptable side effect of A<sub>133</sub>Gmono-*cre*PV could be prevented by the presence of serum neutralizing antibodies. Therefore, *CD155* tgA/J mice were immunized with mono-*cre*PV ( $1 \times 10^8$  pfu) i.p. thrice at 1-week intervals (Fig. 3J). High titers of neutralizing antibodies against poliovirus (in the range of 256 to 2,048) were detectable in all mice at day 21 after immunization (data not shown). Immunized and control animals were challenged by the i.m. route with  $1 \times 10^6$  pfu of PV1(M) to examine whether the antipolio antibodies were protected from poliovirus CNS invasion. None of immunized *CD155* tgA/J mice showed signs of paresia and paralysis, whereas all of the control

**Table 1.** Neuropathogenicity of wt poliovirus PV(M), dual-*cre*PV, mono-*cre*PV, and A<sub>133</sub>Gmono-*cre*PV

Virus	PLD <sub>50</sub> (pfu)* in PVR transgenic mice	PLD <sub>50</sub> (pfu)* in PVR transgenic A/J mice
wt PV1(M)	10 <sup>1.8</sup>	10 <sup>2.0</sup>
Dual- <i>cre</i> PV	>10 <sup>7.0</sup>	>10 <sup>7.0</sup>
Mono- <i>cre</i> PV	>10 <sup>7.0</sup>	>10 <sup>7.0</sup>
A <sub>133</sub> Gmono- <i>cre</i> PV	10 <sup>4.5</sup>	10 <sup>4.8</sup>

\*Defined as the amount of virus that causes paralysis or death in 50% of PVR transgenic mice or PVR transgenic A/J mice after i.c. inoculation.

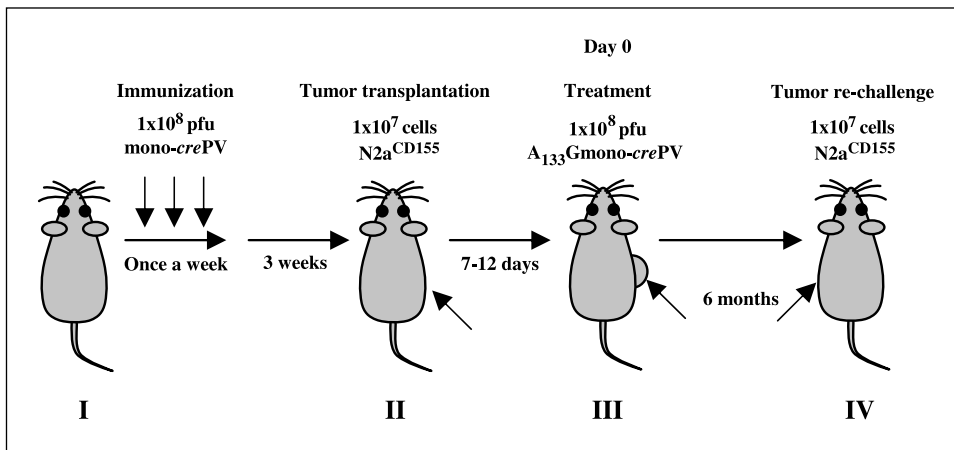


**Figure 2.** One-step growth curves of polioviruses in different human and mouse neuroblastoma cells. Cells were infected as described in Fig. 1B with PV1(M) ( $\blacktriangle$ ), mono-crePV ( $\bullet$ ), and  $A_{133}$ Gmono-crePV ( $\blacksquare$ ). A, human SK-N-SH at 37°C and 39.5°C. B, human SH-SY5Y at 37°C and 39.5°C. C, human SK-N-MC at 37°C and 39.5°C. D, mouse Neuro-2a<sup>CD155</sup> at 37°C and 39.5°C.

CD155 tgA/J mice died of flaccid paralysis within 5 days after injection of PV1(M) with lethal dose.

Following the schedule outlined in Fig. 3, we then established s.c. Neuro-2a<sup>CD155</sup> tumor in polio-immunized mice. After the mean tumor volume had reached a volume of  $\sim 170 \text{ mm}^3$  (day 0; Fig. 3, III), the mice were randomized and injected intratumorally with either PBS or  $A_{133}$ Gmono-crePV on 4 consecutive days (Figs. 3, III and 4). By day 8, the tumors had grown in all PBS-treated mice to a diameter of  $>17 \text{ mm}$  and the animals were euthanized (Fig. 4). In contrast, injection of  $A_{133}$ Gmono-crePV resulted in marked regression of tumors in all of the 12 treated mice, and the mean tumor volume for these virus-treated animals was  $128.8 \text{ mm}^3$  after 8 days. Moreover, none of  $A_{133}$ Gmono-crePV-treated mice showed

paralysis. Of the 11  $A_{133}$ Gmono-crePV-treated mice, 9 animals showed no evidence of recurrent tumors by day 180. One mouse showed a residual tumor mass, which started growing on day 20. Another mouse, although initially presenting complete regression, showed recurrence of the tumor on day 61. Although the two mice with the recurrent tumors were treated again with  $1 \times 10^8$  pfu of  $A_{133}$ Gmono-crePV, tumor regression was not observed (data not shown). The two animals were euthanized when the tumor reached a diameter of  $>17 \text{ mm}$ . Western blot analysis with anti-CD155 rabbit polyclonal antibody NAEZ-8 was done to examine CD155 expression in the recurrent tumor cells as well as in cells harvested from poliovirus-treated mice on day 8 (Fig. 4, dotted arrow). Proteins extracted from the tumors that had not been subjected to



**Figure 3.** Schematic presentation of  $A_{133}Gmono-crePV$  therapy on  $Neuro-2a^{CD155}$  tumors in  $CD155$  tgA/J mice with established immunity against poliovirus. Stage I,  $CD155$  tgA/J mice were immunized i.p. with live  $mono-crePV$  ( $1 \times 10^8$  pfu) thrice with an interval of 1 wk. Stage II, 21 d after the last immunization,  $1 \times 10^7$   $Neuro-2a^{CD155}$  cells were transplanted s.c. in the animals. Stage III, intratumoral treatment of the s.c. tumor with  $A_{133}Gmono-crePV$  ( $1 \times 10^8$  pfu) or PBS at days 0, 2, 4, and 6. Stage IV, mice that survived without signs of tumors for 6 mo were rechallenged with  $Neuro-2a^{CD155}$  cells ( $1 \times 10^7$  cells) in the contralateral flank.

$A_{133}Gmono-crePV$  treatment were used as positive controls. Our results indicated that  $CD155$  expression in the residual and recurrent tumor cells was very low compared with cells of nonrecurrent tumors (Fig. 5).

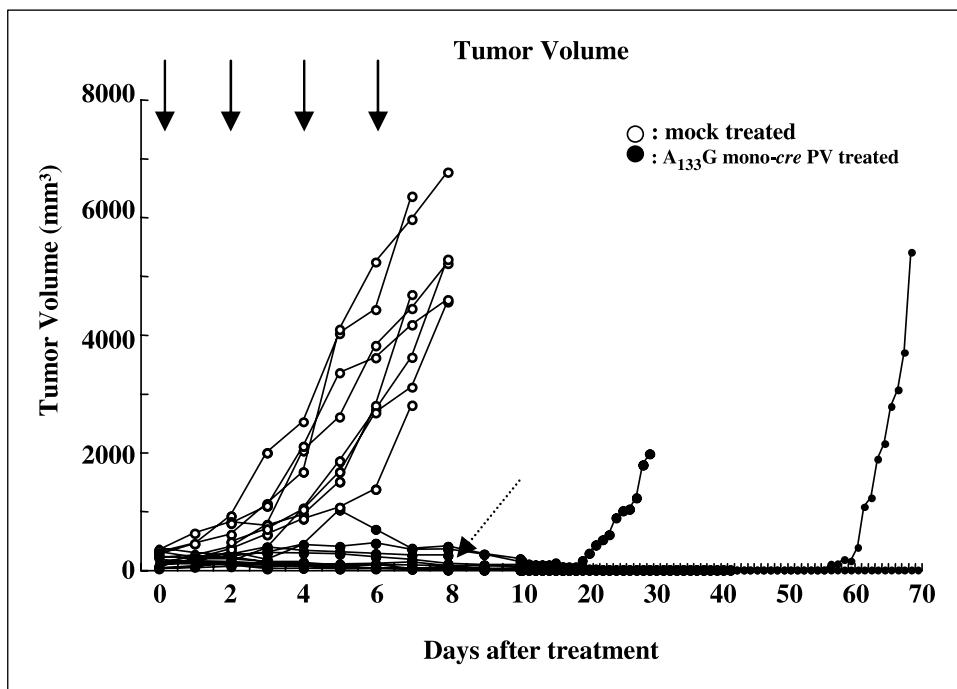
**Rechallenge of neuroblastoma-cured  $CD155$  tgA/J mice with  $Neuro-2a^{CD155}$ .** It has been shown that antigen-presenting cells might internalize antigen released from virus-infected tumor cells, leading to specific peptide presentation and generation of CTL, which in turn may facilitate tumor killing (27, 28). We hypothesized that the  $CD155$  tgA/J mice cured from neuroblastoma may have acquired antineuroblastoma immunity. To test this hypothesis,  $A_{133}Gmono-crePV$ -treated mice with no evidence of recurrent tumors 6 months after virus injection were rechallenged with  $Neuro-2a^{CD155}$  cells (Fig. 3, IV). Specifically,  $1 \times 10^7$   $Neuro-2a^{CD155}$  cells (the same number of cells as in the original challenge) were injected into the opposite flank of nine animals. Significantly, none of the rechallenged animals developed tumors at the site of  $Neuro-2a^{CD155}$  reinoculation or elsewhere.

These data suggest that the oncolytic therapy by  $A_{133}Gmono-crePV$  activated the immune system against  $Neuro-2a^{CD155}$  cells, leading to an antitumor activity that 6 months later is likely to be independent of  $A_{133}Gmono-crePV$ .

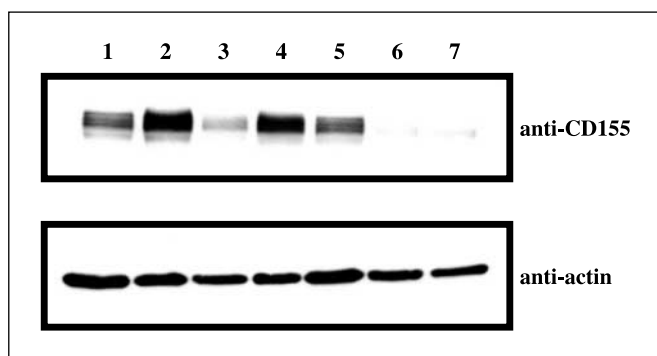
### Discussion

Currently, children with high-risk neuroblastoma are treated with radiotherapy, dose-intensive cycles of multidrug chemotherapy, or, if patients responded poorly, myeloablative dose of chemotherapy supported by stem cell rescue. Despite an aggressive treatment strategy, disease relapse occurs frequently and both short- and long-term toxicities, including treatment-related acute myelogenous leukemia, occur in a significant percentage of disease survivors (29, 30). Novel therapeutic strategies are therefore essential to improve the prognosis of patients with high-risk neuroblastoma.

Replication-competent poliovirus is an attractive candidate agent for neuroblastoma treatment because this enterovirus is



**Figure 4.** Abolition of established neuroblastoma implants in  $CD155$  tgA/J mice with  $A_{133}Gmono-crePV$ .  $Neuro-2a^{CD155}$  was introduced as a tumor implant s.c. in  $CD155$  tgA/J mice, and multiple intratumoral injections of  $1 \times 10^8$  pfu of  $A_{133}Gmono-crePV$  (solid arrows) were given when the tumor volume reached  $\sim 170$  mm<sup>3</sup> (day 0).  $\circ$ , control animals were given PBS;  $\bullet$ , virus-treated animals showed regression of the tumors. One of the 12 virus-treated animals was sacrificed at day 8 (dotted arrow) for tumor analysis. Two of the 11 mice observed long term developed tumors as indicated.



**Figure 5.** Expression of CD155 in tumor cells. Whole-cell lysates of tumors from mice untreated with  $A_{133}G$ mono-*cre*PV (lanes 1–4), and tumor from the mouse that was treated with  $A_{133}G$ mono-*cre*PV and sacrificed at day 8 (Fig. 4, dotted arrow; lane 5) and tumors from two mice with recurrent tumors (lanes 6 and 7) were resolved on a 10% SDS-PAGE gel followed by Western blotting with anti-CD155 antibody NAEZ-8 (top) or anti-actin antibody (bottom).

benign except for its rare and accidental invasion of the CNS. Certainly, the most important characteristic of any oncolytic virus is that, ideally, it must propagate efficiently in tumors but is highly restricted in those target tissues in which the destruction could be disastrous for the patient. Because paralysis caused by poliovirus is largely irreversible, the attenuation of the neurovirulence of this agent, if chosen for tumor therapy, is of utmost importance. We have in the past described a poliovirus/HRV2 chimera [PV1(RIPO) or PVS(RIPO)] that seems to meet this requirement. PVS(RIPO) is, in fact, under consideration for brain tumor therapy (7, 8).<sup>1</sup> With the exception of Raji cells, a Burkitt's lymphoma cell line harboring a transcriptionally inactive *CD155* gene (31), wt poliovirus kills all human tumor cells tested, including neuroblastoma cell lines established from patients (17). PVS(RIPO), however, replicates very poorly in human neuroblastoma cells, which disqualified it from consideration in neuroblastoma therapy. We describe here the properties of a novel, highly attenuated poliovirus (mono-*cre*PV) with excellent (SK-N-SH and SH-SY5Y) to modest (SK-N-MC) replication properties in different human neuroblastoma cell lines. Neuroblastoma therapy with a poliovirus derivative may produce less toxicity often associated with chemotherapy and radiotherapy, and complications, such as second malignant neoplasm, cardiopulmonary sequelae, renal dysfunction, and endocrine consequences, may not occur.

In constructing a suitable mouse model to study neuroblastoma therapy *in vivo* with mono-*cre*PV, we made the unexpected observation that replication of this poliovirus variant is restricted at 37°C in Neuro-2a<sup>CD155</sup>, a mouse neuroblastoma cell line that we developed to produce the tumors in *CD155* tgA/J mice. Thus, animals carrying lethal neuroblastoma could not be cured by intratumoral treatment with mono-*cre*PV. Virus recovered from tumors into which mono-*cre*PV was injected, however, revealed several different mutations scattered throughout the genome. We considered it likely that these are adaptive mutations allowing increased replication in mouse neuroblastoma cells. If correct, then serial passage of mono-*cre*PV in Neuro-2a<sup>CD155</sup> cells is also expected to lead to adaptive mutations. This was indeed the case. Of several nucleotide changes found, however, only the  $A_{133}G$  transition in domain II of the IRES (Fig. 1A) was common to those identified in tumor-adapted virus. Engineering just this  $A_{133}G$  transition into mono-*cre*PV yielded the variant  $A_{133}G$ mono-*cre*PV whose replication in Neuro-2a<sup>CD155</sup> cells increased by two logs compared with

mono-*cre*PV (Fig. 2D). Nomoto et al. (32) have described a related observation before. PV1(M), although replicating in mouse L<sup>CD155</sup> cells at 37°C with wt kinetics, is highly restricted in these cells at 40°C (32). The temperature-sensitive phenotype in mouse cells is ablated by the same  $A_{133}G$  transition described here (32). It is noteworthy, however, that the host cell restriction of PV1(M) in mouse L<sup>CD155</sup> cells is apparent only at 40°C, whereas mono-*cre*PV is restricted in Neuro-2a<sup>CD155</sup> cells already at 37°C. Because a stimulating effect conferred by the  $A_{133}G$  mutation is also observed in one of the human neuroblastoma cell line (SK-N-MC), we conclude that the  $A_{133}G$  transition is not strictly a host range mutation.

Potential problems about viral tumor therapy in immunocompetent hosts are (a) preexisting immunity, a result of natural infection or vaccination, or (b) a response of the immune system to the oncolytic agent. In either case, the required oncolytic effect may be suppressed because virus spread is impaired (33). To address the question whether established immunity against poliovirus will interfere with viral tumor therapy, we vaccinated *CD155* tgA/J mice with the highly attenuated mono-*cre*PV before transplantation of the tumor cells. The presence of high titers of anti-poliovirus antibodies was expected to eliminate the anticipated complications in therapy due to the neurovirulence of  $A_{133}G$ mono-*cre*PV. By intratumoral administrations of  $A_{133}G$ mono-*cre*PV, this sequence of steps (Fig. 3) indeed led to the complete regression of the established lethal s.c. Neuro-2a<sup>CD155</sup> tumors without neurologic side effects (Fig. 4). This result suggests that the large amount of oncolytic virus delivered locally into the tumor escaped the circulating anti-poliovirus antibodies until the substrate for viral proliferation (the tumor cells) was exhausted. Similar observations have been reported with other oncolytic viruses in mice and humans (34–36).

Two of 11 tumor-bearing animals treated with  $A_{133}G$ mono-*cre*PV had recurrent tumors. It is possible that this resulted from tumor-founding Neuro-2a<sup>CD155</sup> cells in which expression of *CD155* was disrupted, making the tumor cells resistant to  $A_{133}G$ mono-*cre*PV. This is highly likely because we have observed previously that cells transformed to express a foreign gene are likely to produce some rare variants lacking expression of this gene. The nearly undetectable levels of *CD155* expression in the two recurrent tumors strongly support this hypothesis (Fig. 5), as selection for the virus resistant cells forming the tumors would be favored. Other unknown mechanisms (e.g., intratumoral heterogeneity) may also have contributed to the observed tumor recurrence. Because most monotherapeutic approaches may not lead to complete tumor control, combination therapy may be needed in the oncolytic treatment of neuroblastoma.

A potentially important result from our studies is the apparent enhancement of an antitumor immune response that was evoked by the infection and lysis of neuroblastoma cells. The lack of tumor growth in those *CD155* tgA/J mice, which were cured from neuroblastoma, on rechallenging the animals with Neuro-2a<sup>CD155</sup> suggests that a long-term carcinostatic effect developed from antitumor immunity after  $A_{133}G$ mono-*cre*PV treatment. Previous investigations in mice have shown that treatment with oncolytic viruses can result in the enhancement of antitumor immune response (27, 37). We presume that destruction of tumor cells by  $A_{133}G$ mono-*cre*PV can increment the release of tumor antigens, which may induce a more efficient antigen presentation, and the development of a robust antitumor immunity. We are currently testing this hypothesis.



The animal model described here is likely to serve as a guide for an effective oncolytic treatment of human neuroblastoma. In our animal model, we used a single cell line to generate s.c. tumor. Therefore, future work on *in vivo* oncolytic capacity of neuroattenuated poliovirus using several neuroblastoma cell lines is needed. Moreover, an orthotopic neuroblastoma model with tumor development in the adrenal gland is necessary to evaluate the therapeutic efficacy of ultrasonography-guided poliovirus injection into orthotopic tumor. Although A<sub>133</sub>Gmono-*cre*PV may be the candidate of choice, its residual neurovirulence will require the presence of high anti-poliovirus titers to protect the patient from neurologic complications. It should be noted, however, that mono-*cre*PV, which is highly attenuated, replicates well in human neuroblastoma cell lines. In the

presence of anti-poliovirus immunity, mono-*cre*PV may be the preferred virus in possible human clinical trials for neuroblastoma. It may be effective also for the treatment of other human cancers.

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