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NOTES

A Mouse Model for Poliovirus Neurovirulence Identifies Mutations That Attenuate the Virus for Humans

NICOLA LA MONICA,[1] JEFFREY W. ALMOND,[2] AND VINCENT R. RACANIELLO[1]*

Department of Microbiology, Columbia University College of Physicians and Surgeons, New York, New York 10032,[1] and Department of Microbiology, University of Reading, Reading RG1 5AQ, England[2]

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A mutation in the genome of poliovirus type 3 that is known to reduce neurovirulence in humans similarly reduces neurovirulence in mice when incorporated into a mouse-adapted–human poliovirus recombinant. Viral recombinants with a uracil at nucleotide position 472 in the 5′-noncoding regions of their genomes are unable to replicate in the mouse brain. Viral recombinants with a cytosine at this position are neurovirulent in mice. Neurovirulence of poliovirus in mice may therefore prove to be a useful indicator of the genetic stability of new attenuating mutations created by site-directed mutagenesis.

Polioviruses exist as three distinct serotypes, each of which causes an identical disease in humans. These viruses have a single-stranded, positive-sense RNA genome of approximately 7,400 bases enclosed in a capsid composed of four viral proteins, VP1 through VP4 (for a review, see reference 15). The oral poliovirus vaccines in current use are derived from strains of the attenuated virus developed by Albert Sabin in the 1950s (16). Although the vaccines have been very successful, countries using them experience a low frequency of poliomyelitis caused partly by occasional reversion mainly of type 2 and type 3 vaccine viruses to a neurovirulent phenotype (2, 3, 7, 10). Knowledge of the molecular biology, structure, and genetics of polioviruses has increased in recent years (6, 8, 12, 13) to the extent that the construction of completely safe, alternative, vaccine strains is regarded as a realistic goal. However, a significant obstacle to the development of a new vaccine is the cost and availability of the large numbers of monkeys that would be required for neurovirulence testing of candidate strains. An important initial objective therefore is to identify a more convenient and less expensive laboratory animal that would allow preliminary identification of attenuated strains.

Recent studies on the poliovirus type 3 vaccine have identified a base change from cytosine to uracil at position 472 in the 5′-noncoding region of the genome as a strong determinant of attenuation. Neurovirulent revertants of the type 3 vaccine have reverted to a cytosine at this position (1, 3). Replication of the type 3 vaccine in the human gut after routine administration to healthy infants results in rapid selection for viruses of increased neurovirulence that contain a cytosine at position 472 (4). Furthermore, neurovirulence studies on a series of recombinants between the type 3 vaccine and its progenitor indicate that the presence of uracil at position 472 is a strong determinant of attenuation in monkeys (G. D. Westrop, D. M. A. Evans, M. A. Skinner, M. Ferguson, D. Magrath, G. C. Schild, P. D. Minor, and J. W. Almond, *in* M. A. Brinton and R. R. Rueckert, ed., *Positive Strand RNA Viruses*, in press). In an attempt to develop a mouse model for neurovirulence which would be valuable for the development of safer vaccines, we have investigated whether the mutation at position 472 would also reduce poliovirus neurovirulence in the mouse. Our approach was to construct chimeric viruses containing the protein-coding region, which confers the mouse-adapted phenotype (9) of a mouse neurovirulent strain (P2/Lansing/37), and the 5′-noncoding region of either the type 3 vaccine (P3/Leon12a_1b), its neurovirulent progenitor (P3/Leon/37) (16) or a neurovirulent revertant derived from the type 3 vaccine (P3/119/70) (10).

A common *Eco*RI restriction endonuclease site at position 786 in the infectious cDNA clones of P2/Lansing/37, P3/Leon 12a_1b, P3/Leon/37, and P3/119/70 was used to construct recombinant cDNA clones (Fig. 1). The recombinant plasmids were constructed as follows. Plasmid pVN-9 containing the 810-nucleotide *Pst*I-*Bam*HI fragment representing the 5′ end of the P2/Lansing/37 genome (9) was partially digested with *Eco*RI, and linear molecules were isolated and cleaved with *Pst*I. The large DNA fragment lacking type 2 sequences from nucleotides 1 to 786 was purified and ligated to type 3 cDNA fragments from pOLIOSabin3 (17), pOLIOLeon3 (18) and pOLIO119 (3) representing nucleotides 1 to 786. The resulting plasmids pVN-20, pVN-21, and pVN-24 containing P3/Leon 12a_1b, P3/Leon/37, and P3/119/70 5′-end sequences, respectively, were joined at the *Eco*RI site to P2/Lansing/37 cDNA from nucleotides 787 to 810. Plasmid pSV20(204), containing a full-length cDNA copy of the P2/Lansing/37 genome (9), was cleaved with *Bam*HI, and a 7.5-kilobase DNA fragment was purified. This DNA fragment extends from nucleotide 810 through the 3′ end of the P2/Lansing/37 genome up to the *Bam*HI site in the vector DNA (9). This DNA fragment was then ligated to plasmids pVN-20, pVN-21, and pVN-24, all of which had been partially digested with *Bam*HI. The resulting plasmids pVN-22, pVN-23, and pVN-25 consist of the 5′-nontranslated region (742 nucleotides) together with the first 44 nucleotides encoding VP4 of P3/Leon12a_1b, P3/Leon/37, or P3/119/70 joined at nucleotide 786 to the rest of the P2/Lansing/37 cDNA. Because of the high level of homology between the viruses, the exchange of the 44-nucleotide coding sequence did not result in amino acid

* Corresponding author.

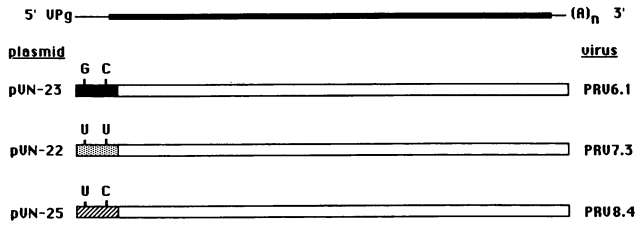

FIG. 1. Maps of chimeric poliovirus cDNAs used to isolate recombinant viruses. Viral RNA is shown at the top; (——) untranslated regions; (▬), open reading frame. The structures of DNA inserts from three chimeric plasmids are shown below; the name of each plasmid is shown at the left, and the name of the virus derived by transfection with that plasmid is shown at the right. The nucleotide differences between the vaccine virus (P3/Leon $12a_1b$), its progenitor (P3/Leon/37), and a virulent revertant (P3/119/70) at bases 220 and 472 are shown. ▭, P2/Lansing/37; ▬, P3/Leon/37; ▩, P3Leon/$12a_1b$; ▨, P3/119/70.

changes in VP4 of the recombinant viruses compared with that of P2/Lansing/37. The 5'-noncoding regions derived from P3/Leon $12a_1b$, P2/Leon/37, or P3/119/70 differ only in the bases at positions 220 and 472 (Fig. 1) (3, 18). The structures of all three recombinant plasmid DNAs were verified by cleavage with restriction endonucleases (data not shown).

Monolayers of HeLa S3 cells were transfected with closed circular forms of pVN-22, pVN-23, and pVN-25, and the corresponding viruses PRV7.3, PRV6.1, an PRV8.4 were recovered (9). The recombinant viruses were shown to be serotype 2 by neutralization assay with homotypic antisera (9). The nucleotide sequences at positions 220 and 472 in the 5'-noncoding region of the viral recombinants were then determined by the primer extension method (5) with purified viral RNA (manuscript in preparation) and with two synthetic oligonucleotide primers which annealed to the RNA from positions 255 to 273 and 509 to 527. As expected, the sequences of PRV6.1, PRV7.3, and PRV8.4 viral RNAs at these positions were identical to the sequences of P3/Leon/37, P3/Leon $12a_1b$, and P3/119/70, respectively (Table 1) (18).

The neurovirulence of the recombinant viruses was determined by intracerebral inoculation of 18- to 21-day-old Swiss Webster mice. Groups of 10 mice were inoculated in 10-fold increments with 0.05 ml of phosphate-buffered saline containing from 10^2 to 10^7 PFU of virus. The method of Reed and Muench (14) was used to calculate the 50% lethal dose (LD_{50}) for each virus, using paralysis or death as an endpoint. Mice were observed for 21 days. Seven plaque-purified laboratory stocks of P2/Lansing/37 virus, derived by transfection with cDNA (11), had LD_{50} values from 1.6×10^3 to 1×10^5 PFU (Table 1). Recombinant viruses PRV6.1 and PRV8.4 were both neurovirulent in mice ($LD_{50} < 6 \times 10^2$ and 9×10^3 PFU, respectively; Table 1). In contrast, recombinant virus PRV7.3 was markedly less neurovirulent, as was shown by its LD_{50} of $>2 \times 10^7$ PFU. These results showed that recombinant viruses that contain a cytosine at position 472 are neurovirulent, while those that contain a uracil at this position are attenuated. The 15-fold difference in the neurovirulence of recombinants PRV6.1 and PRV8.4 (which differ in the base at position 220) suggests that the nucleotide at position 220 may also play a role in determining the level of neurovirulence in mice. It is not clear whether this difference is significant, since the neurovirulence of P2/Lansing/37 plaque isolates varies over a wide range of values (Table 1). Furthermore, in no case has a change in the base at position 220 been associated with an increase in neurovirulence of poliovirus type 3 (2, 3). Determination of the neurovirulence of viruses with a guanine at position 220 and a uracil at position 472 might provide further information on the role of the base at position 220 in neurovirulence in mice.

When 2×10^7 PFU of PRV7.3 was inoculated into each of 10 mice, one animal was paralyzed and two were found dead; whereas another 10 mice were completely free of disease after inoculation with 1/10 of this amount (data not shown). To investigate whether the disease in mice inoculated with a high dose of the attenuated virus PRV7.3 was due to a low level of neurovirulence of this strain or due to the selection of a neurovirulent variant, virus was recovered from the central nervous system of a paralyzed mouse, as described below. The neurovirulence of virus isolated from either the brain (PRV7BE.1) or spinal cord (PRV7SC.1) was at least 1,000 times higher than that of the inoculum PRV7.3 (Table 1). The LD_{50} of these neural isolates was similar to that of P2/Lansing/37, PRV6.1, and PRV8.4 (Table 1). Nucleotide sequence analysis of viral RNAs revealed that the neural isolates contained a uracil at position 220, as in the parent PRV7.3, but in both cases a uracil to cytosine mutation had occurred at nucleotide 472 (Table 1). In contrast, no change at position 472 (or at 220) was observed in the RNA of virus recovered from paralyzed mice that had been inoculated with the virulent PRV6.1 (Table 1). These results provide further evidence that the base at position 472 plays a major role in the determination of neurovirulence. Although it is possible that the increased neurovirulence of the neural isolates is due to changes elsewhere in the viral RNA, the results obtained with recombinants PRV8.4 and PRV7.3 show that a change from uracil to cytosine at nucleotide 472 is sufficient to change an attenuated virus to a neurovirulent one. Thus, it appears that disease in mice induced by high doses of the attenuated PRV7.3 is caused by a highly neurovirulent revertant containing a cytosine at nucleotide 472, which probably constitutes a subpopulation of the virus inoculum.

To investigate the physiological basis for the attenuated phenotype of recombinant PRV7.3, its capacity to replicate in the central nervous system of mice was compared with that of three neurovirulent viruses, P2/Lansing/37 and recombinants PRV6.1 and PRV8.4 (Fig. 2). Mice were inoculated intracerebrally with 3×10^4 PFU, and at 24-hour intervals, three mice were sacrificed per virus and their

TABLE 1. Bases at positions 220 and 472 and neurovirulence of recombinant poliovirus and neural isolates in mice

Virus	Base at position:		LD_{50}[a]
	220	472	
P2/Lansing/37	NA[b]	NA	1.6×10^3–1×10^5
PRV6.1	G[c]	C	$<6 \times 10^2$
PRV7.3	U	U	$>2 \times 10^7$
PRV8.4	U	C	9×10^3
PRV7BE.1	U	C	3×10^4
PRV7SC.1	U	C	4×10^4
PRV6SC.1	G	C	ND[d]

[a] LD_{50} is the amount of virus required to cause paralysis or death in 50% of mice by day 21.
[b] NA, Not applicable; the sequence of the P2/Lansing/37 5'-noncoding region does not align perfectly with those of P3/Leon/37 and P3/Leon $12a_1b$.
[c] G, Guanine; and C, cytosine.
[d] ND, Not determined.

brains were removed and homogenized in phosphate-buffered saline. Viral titers in brain homogenates were determined by plaque assays on HeLa cell monolayers (9). The results show that both P2/Lansing/37 and the neurovirulent recombinants PRV6.1 and PRV8.4 replicate in the brains of infected mice to titers of between 10^6 and 10^7 PFU/g by day 2, and the titers then slowly decline to 10^5 to 10^6 PFU/g. In contrast, the titer of the attenuated recombinant PRV7.3 was approximately 1×10^3 to 6×10^3 PFU/g until day 3 and then rapidly declined to less than 100 PFU/g. These low levels of PRV7.3 most likely represent residual inoculum, which was expected to be approximately 7×10^4 PFU/g. Therefore, it appears that virus PRV7.3 replicates poorly, if at all, in the mouse brain.

Viral replication was examined in cultured cells to determine whether the failure of PRV7.3 to replicate in mice was caused by a general defect in viral growth. Monolayers of 4×10^6 HeLa cells were prepared as described previously (9), and infected at a multiplicity of infection of 0.01 PFU per cell. After adsorption, virus was removed by washing with phosphate-buffered saline and cell culture medium was added. At different times postinfection, both cells and medium were frozen and thawed three times and virus titer was determined by plaque assay (9). The results show that the growth of all three recombinants is indistinguishable from that of wild-type P2/Lansing/37 in HeLa cells (Fig. 2). The growth curves of all four viruses were also indistinguishable when higher multiplicities of infection were used, resulting in one-step multiplication cycles (data not shown). Furthermore, none of the recombinants were temperature sensitive (data not shown). Therefore, the inability of PRV7.3 to replicate in mice represents a host restriction, not a general defect in viral growth. Presumably a similar restriction limits the replication of poliovirus vaccine strains in the human gut (4).

These experiments show that a single-base change at position 472 in the 5'-noncoding region of the poliovirus genome which reduces neurovirulence in primates also drastically affects neurovirulence in mice. The results thus provide further evidence that the mutation of cytosine to uracil at nucleotide 472 in the type 3 vaccine is important for its attenuated phenotype. The role of this part of the 5'-noncoding region in influencing neurovirulence is further underscored by the identification of an attenuating mutation at nucleotide 480 in the poliovirus type 1 vaccine P1/LS-c, 2ab (A. Nomoto, M. Kohara, S. Kuge, N. Kawamura, M. Arita, T. Komatsu, S. Abe, B. L. Semler, E. Wimmer, and H. Itoh, *in* M. A. Brinton and R. R. Rueckert, ed., *Positive Strand RNA Viruses*, in press).

The mechanism by which a mutation in the 5'-noncoding region of poliovirus RNA results in a virus that cannot replicate in the central nervous system is unknown. Since the mutation of the base at position 472 affects replication only in neural tissues and not in cultured epithelioid (HeLa) cells, the altered viral function most likely involves a host cell component. Perhaps the secondary structure of the region, which appears to be altered by the mutation at position 472 (4), is important. Possible functions of the 5'-noncoding region include control of viral RNA synthesis, translation, packaging, or virion uncoating. There is some evidence to suggest that the efficiency of translation of viral RNA may be lower in attenuated strains (19). In any case, the ability of the mutation at position 472 to attenuate a mouse-adapted strain of poliovirus suggests that the viral function in question is common to neural cells of different animal species.

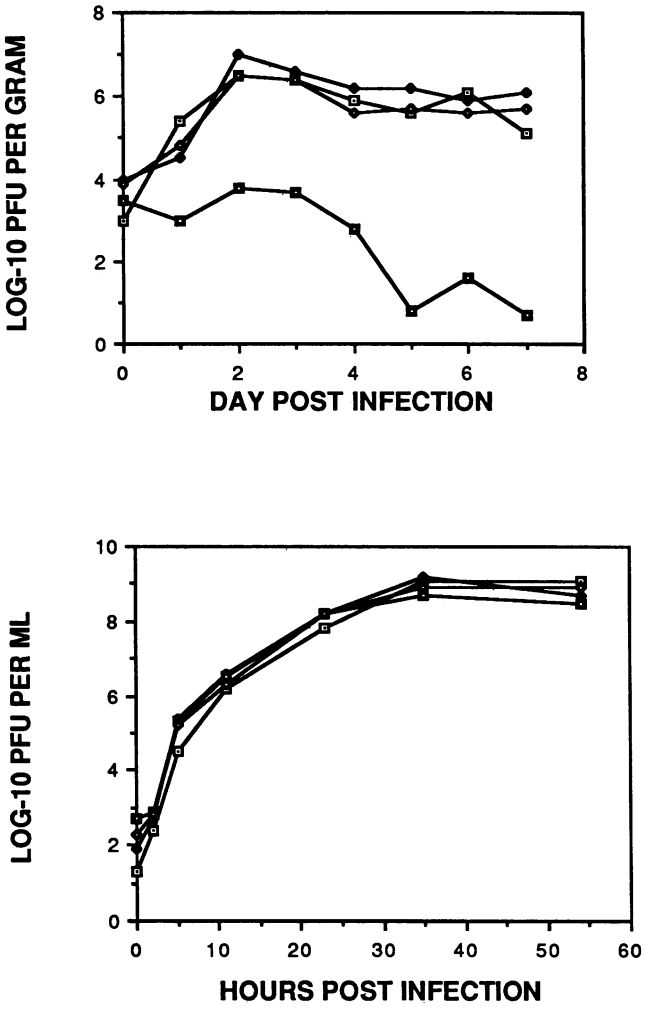

FIG. 2. Replication of P2/Lansing/37 virus (□) and viral recombinants PRV6.1 (◆), PRV7.3 (■), and PRV8.4 (◇) in mouse brain (top panel) and in cultured HeLa cells (bottom panel). In the top panel, each point represents the mean of virus titers in the three mouse brains (calculated by first converting viral titers to the $\log_{10}$ base and then determining the mean of the three titers).

The observation that a mutation known to attenuate poliovirus in primates also produces an attenuated phenotype in mice is extremely useful for future studies, which may lead to the development of new vaccines that cannot revert to neurovirulence. Development of such vaccines might involve construction of viruses, by manipulation of infectious cDNA, in which either mutations known to cause attenuation were modified so as to be less likely to revert, e.g., by deletion, or in which new attenuating mutations were introduced at other sites. The cost of testing such an array of vaccine candidates in monkeys at the experimental stage would be prohibitive. However, since our results suggest that a given mutation would produce a similar phenotype in monkeys and in mice, it should be feasible to build altered versions of polioviruses with capacities to replicate in mice (i.e., those containing sequences encoding the P2/Lansing/37 viral capsid proteins). The most stable mouse-adapted avirulent viruses could later be fully examined by neurovirulence testing in monkeys, after replace-

ment of the Lansing capsid with sequences from the appropriate vaccine strains.

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