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Reduced Mouse Neurovirulence of Poliovirus Type 2 Lansing Antigenic Variants Selected with Monoclonal Antibodies

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The Lansing strain of poliovirus type 2 is a mouse-adapted virus that induces a fatal paralytic disease in mice after intracerebral inoculation. Our previous results indicated that the mouse-adapted phenotype maps to the Lansing viral capsid. To further define regions of the capsid that are specifically involved in the infection of mice, antigenic variants resistant to neutralization with monoclonal antibodies were selected, and their mouse neurovirulence was studied. The monoclonal antibodies used were directed against antigenic site 1, an immunodominant loop of capsid polypeptide VP1 located on the virion surface. Ten of twenty-two variants selected had lower intracerebral neurovirulence in mice when compared to the parental virus. Four of the ten antigenic variants with reduced neurovirulence were temperature sensitive (ts) for replication in HeLa cells, while the remaining six variants replicated in HeLa cells as well as the parent virus. Two ts$^+$ variants that were studied had a reduced ability to replicate in the mouse brain. There was no difference in the histopathology and pattern of involvement in the central nervous system of one variant compared to the parent virus. In three variants, reduction of neurovirulence correlated with specific amino acid substitutions at positions 100 and 101 of VP1, located within antigenic site 1. The ts phenotype in three variants was associated with a single amino acid deletion at position 105. Virus recovered from the brain of paralyzed mice that had been inoculated with the antigenic variants was characterized to identify the virus causing disease. In most cases, brain isolates resembled the inoculated virus in neurovirulence and amino acid sequence at the antigenic site. Virus recovered from brains of paralyzed mice that had been inoculated with the ts variants was either ts$^+$ or cold sensitive, and had become more neurovirulent. These results suggest that specific amino acid changes within an antigenic site on the virion surface may result in reduction of mouse neurovirulence without affecting viral replication in cultured cells.

INTRODUCTION

The Lansing strain of poliovirus type 2, which was isolated from a fatal case of poliomyelitis, was adapted to mice after repeated passages (Armstrong, 1939). Mice inoculated intracerebrally with P2/Lansing develop a fatal paralytic disease which clinically and histologically resembles human poliomyelitis (Jubelt *et al.*, 1980). In contrast, mice inoculated with the P1/Mahoney strain of poliovirus do not develop disease (La Monica *et al.*, 1986). Viral recombinants between P2/Lansing and P1/Mahoney, constructed by manipulating cloned, infectious viral cDNAs, were used to show that the mouse-adapted phenotype of P2/Lansing maps to the viral capsid proteins (La Monica *et al.*, 1986). Therefore adaptation of P2/Lansing to mice most likely involved selection of viral variants containing changes in the capsid protein that enabled the virus to enter cells of the murine central nervous system.

One of our goals is to identify specific regions of the P2/Lansing capsid required for the mouse-adapted phenotype. An approach to this problem is to introduce mutations in the capsid proteins which do not affect viral replication in cultured cells but reduce or abolish mouse intracerebral neurovirulence. In other virus systems, it has been demonstrated that antigenic variants resistant to neutralization with monoclonal antibodies often contain single amino acid changes. Such variants have been used to show that areas of viral glycoproteins are determinants of animal pathogenicity (Dalziel *et al.*, 1986; Dietzschold *et al.*, 1983; Fleming *et al.*, 1986; Löve *et al.*, 1985; Spriggs and Fields, 1982). Here we show that P2/Lansing variants selected for resistance to neutralization with monoclonal antibodies possess different levels of mouse intracerebral neurovirulence. Some variants were as neurovirulent as the parent virus while others were markedly less neurovirulent. No difference in the histopathology or pattern of involvement of the central nervous system was observed between one attenuated variant and the parental virus. However, antigenic variants of low neurovirulence had a reduced ability to replicate in the mouse brain. In some cases, the attenuated phenotype of the variants correlated with specific amino acid substitutions within the antigenic

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site recognized by the selecting antibodies, suggesting that this region is a determinant of P2/Lansing neurovirulence in mice.

MATERIALS AND METHODS

Cells, virus, and virus assays

V676P3 is a plaque isolate derived by transfection of HeLa S3 cells with a P2/Lansing/37 infectious cDNA clone (Racaniello, 1984). V667 is a uncloned P2/Lansing/37 stock prepared by multiple passages in HeLa S3 cells of a virus obtained from the American Type Culture Collection (Racaniello, 1984). Preparation of viral stocks, plaque assays, and neutralization tests were performed using HeLa S3 cell monolayers as described (La Monica *et al.*, 1986).

Selection of mutants

Antigenic variants of P2/Lansing/37 virus resistant to neutralization were selected with a panel of anti-poliovirus type 2 monoclonal antibodies (mab; provided by M. Ferguson and P. Minor, National Institute for Biological Standards and Control, London, UK). These antibodies were raised against the P2/P712,Ch,2ab strain and are directed against antigenic site 1 of poliovirus (Minor *et al.*, 1986). Antigenic site 1 was originally defined in type 3 poliovirus as VP1 amino acids 89 to 100 (Evans *et al.*, 1983) and its equivalent was subsequently identified in type 2 poliovirus as amino acids 91–102 encoded by nucleotides 2689 to 2784 on the P2/Lansing viral genome (Minor *et al.*, 1986; the numbering of VP1 differs because the length of this protein is variable among the serotypes).

Ascitic fluids were used at a final dilution which was determined to reduce viral titer by approximately 3 $\log_{10}$ units. Tenfold dilutions of virus (V676P3) containing from 10^2 to 10^5 plaque-forming units (PFU) were prepared in 0.1 ml of phosphate-buffered saline (PBS) plus 0.2% horse serum (HS), and were incubated for 60 min at room temperature with an equal volume of the diluted monoclonal antibody. The virus–antibody mixture was titered on HeLa cell monolayers to isolate viruses that escaped neutralization. The infected cells were overlayed with 5 ml of 0.9% Bacto-Agar in Dulbecco's minimal essential medium (DME) supplemented with 5% HS and the selecting mab. Individual plaques were then picked and expanded into virus stocks. These stocks were then subjected to another round of incubation with mab and plaque purification. A plaque was picked and expanded into virus stock, and the resistance of the virus to neutralization with the selecting mab was assessed by incubating 10-fold dilutions of virus with the mab. If the mab reduced the titer of the virus by less than 1 $\log_{10}$ unit, the isolate was considered to be resistant to neutralization. The serotype of the selected mutants was confirmed as type 2 by neutralization assay with rabbit anti-P2/Lansing antiserum (provided by J. Miller, Department of Neurology, Columbia University).

Temperature sensitivity test

Tenfold dilutions of virus were used to infect HeLa cell monolayers in duplicate. After adsorption at room temperature for 45 min, the inoculum was removed, and the monolayers were overlayed with 5 ml of 0.9% Bacto-Agar in DME containing 5% HS (La Monica *et al.*, 1986). One set of plates was incubated at 33° for 3 days and the duplicates were incubated at 39.5° for 2 days. The efficiency of plating (e.o.p.) of each virus was calculated as the titer at 39.5° divided by the titer at 33°. Viruses with an e.o.p. of less than 0.1 were considered temperature-sensitive (ts) mutants while viruses with an e.o.p. greater than 10 were considered cold-sensitive (cs) mutants.

Neurovirulence assay

Groups of eight Swiss Webster mice 21 days old (four male and four female) were inoculated intracerebrally (ic) with 0.05 ml of virus diluted in PBS plus 0.2% HS. Tenfold increments of virus concentration were used so that each group of mice received from 10^3 to 10^7 PFU (La Monica *et al.*, 1986). Mice were observed daily for 21 days for paralysis or death. The amount of virus which caused paralysis or death in 50% of mice (LD_{50}) was calculated by the method of Reed and Muench (1938). At least two LD_{50} determinations were performed for each variant.

Virus replication in mouse brain and HeLa cells

To study replication of antigenic variants in the mouse brain, mice were inoculated ic with 3×10^4 PFU and at the indicated times three mice were sacrificed per virus, and the brains were removed and homogenized in 1 ml of PBS. Viral titers in brain homogenates were determined by plaque assay on HeLa cell monolayers.

To study the replication of the antigenic variants in HeLa cells, monolayers of 4×10^6 cells were infected at various multiplicity of infections, and after adsorption, virus was removed by washing with PBS and cell culture medium was added. At the indicated times, both cells and medium were frozen and thawed three times and the lysate was clarified by centrifugation. The virus titer was determined by plaque assay on HeLa cell monolayers.

Histology

Groups of six mice were inoculated with 10^6 PFU of 433R19.1, V676P3, or V667. Paralyzed animals were sacrificed and the brain and spinal cord were removed and fixed with 10% formalin prior to paraffin embedding. Brains were sectioned coronally at four to eight levels. Spinal cords were sectioned horizontally at four to eight levels. Tissues were stained with hematoxylin and eosin.

Isolation of virus from infected mouse brain

To recover virus from paralyzed mice that had been inoculated with antigenic variants, the brain was removed and homogenized in 1 ml of PBS. Viral stocks were prepared by infecting HeLa cell monolayers with the homogenate, and the serotype of the recovered virus was determined by neutralization assay with the selecting mab and with rabbit anti-P2/Lansing antiserum. Since the neural isolate from a paralyzed mouse inoculated with 433R27.1 was heterogeneous with respect to mab sensitivity, this isolate was plaque purified, and two plaque isolates (433R27BE1S and 433R27BE1L) were studied further.

RNA isolation and nucleotide sequence analysis

Viral RNA was prepared by infecting three 140-mm cell culture dishes, each containing 1×10^7 HeLa cells, with the appropriate virus. Cells were incubated at 37° until the monolayer was completely destroyed. The contents of the plates were frozen and thawed three times and clarified by low-speed centrifugation (1500 *g;* 10 min), followed by centrifugation at 40,000 rpm for 45 min at 4° in a Beckman 60Ti rotor. The pellet was resuspended in 3.0 ml of 6 *M* guanidine thiocyanate/0.1 *M* sodium acetate, pH 5.0/5 m*M* EDTA/71 m*M* β-mercaptoethanol/0.5% Sarkosyl. The resuspended material was layered on a 1.6-ml cushion of 5.7 *M* cesium chloride/0.1 *M* sodium acetate, pH 5.0, and centrifuged in a SW50.1 rotor at 33,000 rpm for 16 hr at 20°. The supernatant was discarded and the pelleted RNA was washed with ice-cold 70% ethanol, resuspended in 0.2 ml TES (10 m*M* Tris–HCl, pH 7.4, 5 m*M* EDTA, 1% SDS), transferred to a 1.5-ml microfuge tube, and incubated at 45° for 30 min. The RNA solution was then extracted with an equal volume of 4:1 chloroform:butanol. RNA was recovered from the aqueous phase by two ethanol precipitations and resuspended in H_2O.

Nucleotide sequence of viral RNA was determined by the chain termination method (Evans *et al.,* 1985), using reverse transcriptase and a synthetic oligonucleotide primer extending from nucleotide 2818 to nucleotide 2834 of the P2/Lansing genome.

RESULTS

Selection of monoclonal antibody-resistant variants

P2/Lansing antigenic variants resistant to neutralization were selected with mabs that had been obtained from mice inoculated with the P2/P712,Ch,2ab strain of poliovirus (Minor *et al.,* 1986). Based on sequence analysis of mab-resistant variants of P2/P712,Ch2ab, it was concluded that most of the mabs are directed against antigenic site 1 of poliovirus, which is composed of VP1 amino acids 91 to 102. This amino acid sequence is identical in P2/Lansing and P2/P712,Ch,2ab. Neutralization assays showed that six of the eight mabs tested neutralized P2/Lansing (Table 1). The epitope recognized by mab 437 has not been mapped, and therefore its failure to neutralize P2/Lansing could be explained if the amino acid sequence at the epitope differs in these two strains. mab 269 recognizes antigenic site 1, so its failure to neutralize P2/Lansing suggests that the presentation of the antigenic site differs in the two strains.

The six mabs that neutralized P2/Lansing were then used to screen for monoclonal antibody-resistant (*mar*) variants as described under Materials and Methods. Twenty-two neutralization-resistant variants were isolated at frequencies of 10^{-4} to 10^{-5} using mabs 433, 435, 436, and 467 (Table 1). No neutralization-resistant variants were obtained with mabs 267 and 268, as previously observed for P2/P712,Ch,2ab (Minor *et al.,* 1986). The *mar* variants were subjected to two rounds of plaque purification in the presence of mab to ensure that they were truly resistant. The nomenclature for the *mar* variants includes the number of the mab used for selection (first three digits) and an isolate number (last two digits). Four independent virus isolates (called V676P3.1a, V676P3.2a, V676P3.3a, and V676P3.4a), obtained by subjecting P2/Lansing to the

TABLE 1

SENSITIVITY OF P2/LANSING/37 VIRUS TO NEUTRALIZATION WITH MONOCLONAL ANTIBODIES AND NUMBER OF NEUTRALIZATION ESCAPE VARIANTS SELECTED WITH EACH ANTIBODY

Antibody	Neutralization of P2/Lansing	*mar* variants
267	+	0
268	+	0
269	–	0
433	+	15
435	+	3
436	+	3
437	–	0
467	+	1

Note. +, 90–100% reduction in titer. –, No reduction in titer.

isolation procedure in the absence of mab, were used as controls.

Neurovirulence of *mar* variants

As an initial screen for the neurovirulence of the antigenic variants, groups of four to six Swiss Webster mice were inoculated ic with 10^5 PFU of each virus. The variants which caused disease in the fewest animals, as well as two which appeared to be as lethal as the parent virus, were then studied further by determination of the LD_{50} (Table 2). For comparison, the LD_{50} of the parental P2/Lansing virus (V676P3) and four viruses that had been subjected to the variant isolation procedure in the absence of mab (V676P3.1a, V676P3.2a, V676P3.3a, and V676P3.4a) was also determined. The parent P2/Lansing virus had LD_{50} values of 3.7 and 4.1 $\log_{10}$ PFU, while the LD_{50} of the unselected isolates ranged between 4.1 and 5.0 $\log_{10}$ PFU. The LD_{50} of the *mar* variants ranged from 3.9 to over 7.1 $\log_{10}$ PFU. Variants with LD_{50} of approximately 6 $\log_{10}$ PFU or higher were considered to have reduced neurovirulence, since the highest LD_{50} observed for unselected isolates was 5.0 $\log_{10}$ PFU. By these criteria, at least 10 out of the 22 *mar* variants were less neurovirulent than the parent virus.

TABLE 2

NEUROVIRULENCE OF *mar* VARIANTS AND PARENT P2/LANSING/37

Virus	Survivors/inoculated mice[a]	LD_{50}[b]
433R1.1	3/8	3.9
433R2.1	1/4	ND
433R3.1	1/8	4.6
433R4.1	2/5	ND
433R5.1	1/5	ND
433R6.1	1/4	ND
433R7.1	4/5	>7.3, 6.9
433R8.1	5/5	>7.3, 6.8
433R12.1	5/6	>7.0, 6.8
433R16.1	5/5	6.7, >6.4
433R17.1	5/6	6.1, 6.0
433R19.1	5/5	6.5, 6.8, 6.8
433R22.1	0/6	ND
433R26.1	3/6	6.4, 6.2
433R27.1	6/6	>7.1, >7.1
435R1.1	4/5	>7.0, 7.0, >7.0
435R2.1	0/5	ND
435R5.1	5/5	5.8, 5.8
436R1.1	0/5	ND
436R2.1	0/5	ND
436R4.1	0/5	ND
467R1.1	3/5	ND
V676P3[c]	0/6	4.1, 3.7
V676P3.1a[d]	ND	4.2
V676P3.2a	ND	4.1
V676P3.3a	ND	4.5, 5.0
V676P3.4a	ND	4.7

Note. ND, not determined.

[a] Mice were injected with 10^5 PFU.

[b] Each value, shown as $\log_{10}$ PFU/LD_{50}, represents an independent measurement.

[c] cDNA derived P2/Lansing used to select *mar* variants.

[d] Unselected isolates.

Temperature sensitivity of the *mar* variants

To determine whether the reduced neurovirulence of the *mar* variants might be caused by a temperature-sensitive defect, the e.o.p. in HeLa cells at 33° and 39.5° was determined. All but four of the *mar* variants had e.o.p. values similar to those of the parent virus and the unselected isolates (Table 3). *mar* variants 433R12.1, 433R27.1, 435R1.1, and 435R5.1 were ts mutants since their e.o.p. values were 14- to 500-fold lower than those of the parental virus. Therefore the reduced neurovirulence of these *mar* variants is most likely due to their inability to replicate in mice, whose body temperature is 38° (Doll and Johnson, 1985).

Incubation of the ts *mar* variants 433R12.1, 433R27.1, and 435R1.1 for 45 min at 39.5° and subsequent plating at 33° lead to a 200-fold reduction in viral titer (data not shown). Therefore these three ts *mar* variants are thermolabile and probably contain a mutation in the viral capsid.

Histopathology

The reduced neurovirulence of non-ts *mar* variants might be related to a difference in the pattern of infection in the central nervous system. To address this possibility, the brain and spinal cord from paralyzed mice which had been inoculated with *mar* variant 433R19.1 or the parental P2/Lansing were examined. Since the parental P2/Lansing used for selection of *mar* variants was derived by cDNA transfection (Racaniello, 1984), the histopathology of this virus (V676P3) was also compared with that of the P2/Lansing isolate (V667; Racaniello, 1984) that had been used to construct the cDNA clone.

The inflammatory response in the brain and spinal cord consisted of cuffs of lymphocytes and monocytes clustered around blood vessels lined by hypertrophic endothelial cells. Reactive monocytes and microglial cells, many with prominent rod-shaped nuclei, infiltrated the parenchyma and could be seen surrounding neuronal perikarya or forming small nodules associated with fragments of cellular debris (neuronophagia; see Fig. 1A).

Leptomeningeal inflammation was sparse and usually present in association with a superficial lesion in the underlying parenchyma. Involvement of cerebral

TABLE 3

EFFICIENCY OF PLATING (EOP) OF P2/LANSING AND *mar* VARIANTS

Virus	Titer at 33°	Titer at 39.5°	e.o.p.[a]
433R1.1	2.3×10^8	4.3×10^8	1.9
433R3.1	5.1×10^8	5.1×10^8	1.0
433R7.1	1.4×10^8	1.4×10^8	1.0
433R8.1	2.8×10^8	1.3×10^8	0.5
433R12.1	1.3×10^8	1.3×10^6	0.01
433R16.1	6.9×10^8	1.2×10^9	1.7
433R17.1	6.7×10^7	7.2×10^7	1.1
433R19.1	5.0×10^7	6.6×10^7	1.3
433R26.1	1.7×10^8	1.5×10^8	0.9
433R27.1	1.7×10^8	4.2×10^5	0.002
435R1.1	1.0×10^8	2.2×10^6	0.02
435R5.1	3.2×10^8	2.2×10^7	0.07
V676P3	3.0×10^8	2.1×10^8	0.7
V676P3.1a	8.5×10^8	1.0×10^9	1.2
V676P3.2a	1.2×10^9	1.4×10^9	1.2
V676P3.3a	7.5×10^8	6.7×10^8	0.9
V676P3.4a	1.5×10^9	1.5×10^9	1.0

[a] e.o.p. values represent viral titer at 39.5°/33°.

plained by a change in disease pattern produced in the central nervous system.

Replication of *mar* variants in mouse brain and HeLa cells

To determine the basis for the reduced neurovirulence of the *mar* variants, the replication of 433R16.1 and 433R19.1 in the mouse brain was examined. These isolates were selected for study because they had significantly reduced neurovirulence (Table 2) and were not ts (Table 3).

Viral titers in the brains of mice inoculated with P2/Lansing virus rose to $10^{6.1}$ PFU g^{-1} of brain by 3 days postinfection (the residual inoculum at Day 0 is approximately 10^4 PFU g^{-1}), and subsequently fell to about 10^5 PFU g^{-1} (Fig. 2, top panel). In mice inoculated with *mar* variants 433R16.1 and 433R19.1 the viral titer hovered at about 10^4 PFU g^{-1} until Day 4, and thereafter rapidly declined to $10^{1.9}$ PFU g^{-1} of brain by Day 6.

cortex was patchy and most frequent in the frontal and parental regions. The hippocampus was moderately to severely involved in all animals examined. The occipital cortex, septum, hippocampus, and hypothalamus were severely involved in two animals infected with cDNA-derived P2/Lansing, but involvement of the septum, basal ganglia, thalamus, and hypothalamus was otherwise generally sparse or absent.

Involvement of the brainstem was patchy, the most severe changes occurring with severe cerebral involvement. The midbrain and pons were more frequently afflicted than the medulla. Cerebral involvement was absent in all cases.

The spinal cord was severely involved and lesions were localized to the ventral horns (Fig. 1B), though perivascular inflammatory cells and reactive microglial nuclei were often present in the intermediate and intermediolateral gray matter structures and in the lateral and ventral white matter bordering the ventral horns.

In summary, the histopathology and pattern of involvement of all three viruses were similar to previously reported patterns for P2/Lansing virus infection of mice (Jubelt *et al.*, 1980; Jubelt and Meagher, 1984), except that the thalamus and cerebellum were less involved and the occipital cortex was more involved than in previous studies. These differences were observed for all three viruses examined: *mar* variant 433R19.1, transfection-derived virus V676P3, and the original P2/Lansing stock. Thus it does not appear that the reduced neurovirulence of 433R19.1 can be ex-

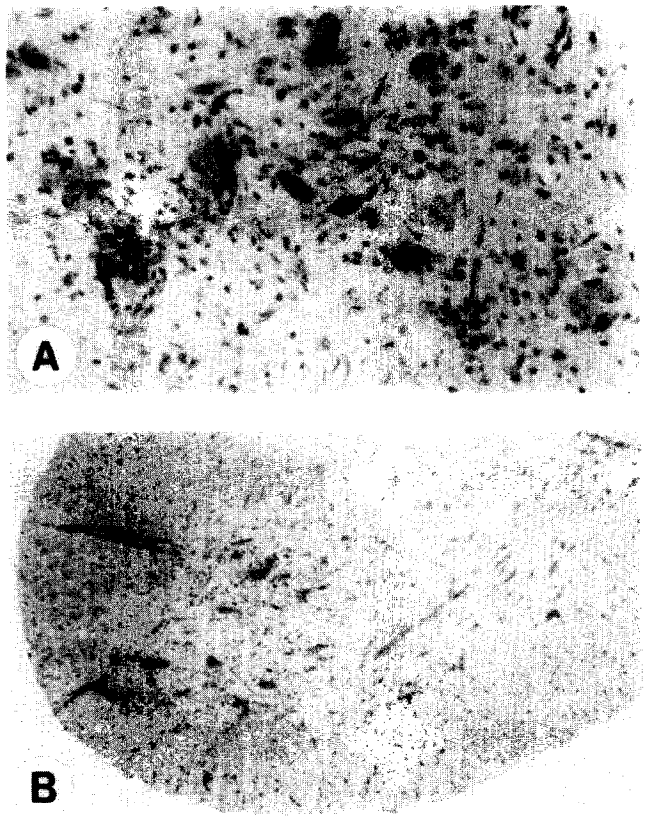

FIG. 1. Anterior horn of spinal cords from animal infected with *mar* variant 433R19.1. Horizontal section. Hematoxylin and eosin stain. (A) Microglial reaction, neuronophagia, and perivascular mononuclear cell infiltration in moderately severely involved area. Cervical region. ×180. (B) Localization of lesion to ventral horn with prominent perivascular mononuclear cell infiltrates. Lumbar region. ×70.

To determine whether the limited ability of these *mar* variants to multiply in the mouse brain was due to a general defect in the viral infectious cycle or to a host range mutation, the replication of variants 433R16.1 and 433R19.1 was examined in HeLa cells. At a m.o.i. of 0.04, the kinetics of viral production and the final yield of virus was identical both in *mar* variants and in P2/Lansing (Fig. 2, bottom panel). Similar results were also obtained when a higher m.o.i. was used (4.0; data not shown). Therefore *mar* variants 433R16.1 and 433R19.1 are not defective for replication in HeLa cells but are unable to reach wild-type levels in the mouse brain.

Nucleotide sequence of *mar* variants at antigenic site 1

To determine whether there was a correlation between reduction of mouse neurovirulence and mutations responsible for resistance to neutralization, the nucleotide sequence of *mar* viral RNA was determined in the region of capsid polypeptide VP1 known to comprise antigenic site 1. Previous studies established that resistance of poliovirus type 2 to neutralization with the mabs used here is due to mutations within antigenic site 1, composed of amino acids 91–102 (Minor *et al.*, 1986).

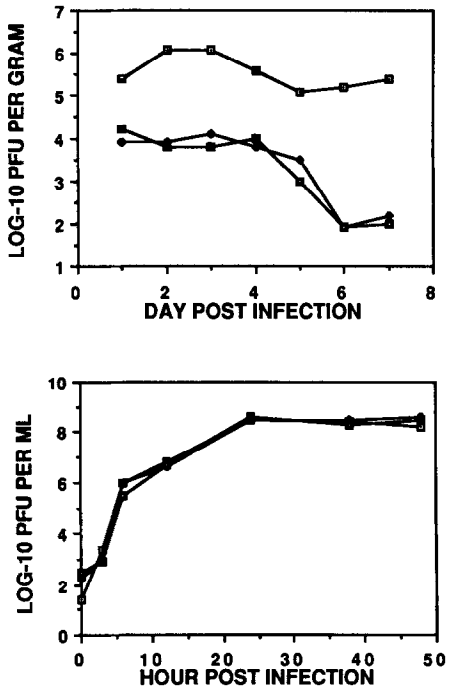

FIG. 2. Replication of P2/Lansing/37 virus (□) and *mar* variants 433R16.1 (■) and 433R19.1 (♦) in mouse brain (top panel) and in cultured HeLa cells (bottom panel). In the top panel, each point represents the geometric mean of the virus titers in three mouse brains.

Amino acid changes in or near the antigenic site 1 were found in all *mar* variants examined with one exception (Table 4). The ts *mar* variants 433R12.1, 433R27.1, and 435R1.1 contained a deletion of amino acid 105, and ts *mar* variant 435R5.1 had a Lys to Glu substitution at amino acid 99. The remaining *mar* variants contained one to three amino acid substitutions at positions 93, 95, 96, 99, 100, or 101.

Characterization of neural isolates

It was important to determine whether disease in mice inoculated with *mar* variants was caused by the inoculated virus or by the viral variants selected for during replication in the murine CNS. Therefore viruses were isolated from the brains of paralyzed mice that had been inoculated with some of the *mar* variants and characterized with respect to sensitivity to neutralization with the selecting mab, temperature sensitivity, mouse neurovirulence, and amino acid sequence in the region of antigenic site 1.

Viruses recovered from the brains of mice inoculated with the ts *mar* variants were markedly more neurovirulent than the inoculum (Table 5). These viruses were no longer ts; three were ts^+ and two were cold sensitive. In one of these ts^+ neural isolates, 433R27BE1L, the deleted Phe residue at position 105 had been restored; this was the only isolate of all those studied that had regained sensitivity to the selecting mab. The two other ts^+ and two cs isolates did not contain amino acid changes in the area of antigenic site 1. Therefore the loss of the ts phenotype in these isolates must be due to a second site mutation.

Neural isolates obtained from paralyzed mice which had been injected with the non-ts *mar* variants showed no changes in e.o.p. and sensitivity to the selecting mab. Furthermore, the neurovirulence of the neural isolates had not changed significantly (Table 5).

The brain isolate from a mouse inoculated with virus 433R16.1 differed from the inoculum in the amino acid sequence at antigenic site 1. This virus contains a change from Gly to Asp at position 93 which represents a reversion to the parental sequence. This change, however, was not accompanied by an increase in neurovirulence (Table 5). These results suggest that paralysis observed in mice after inoculation with the non-ts *mar* variants is probably caused by the inoculated viruses and not by revertants selected in the mouse CNS.

DISCUSSION

We recently reported that the mouse-adapted phenotype of P2/Lansing maps to the viral capsid proteins. This conclusion is based on the observation that

TABLE 4

AMINO ACID SEQUENCE AT ANTIGENIC SITE 1 OF *mar* VARIANTS

Amino acid no.	93	94	95	96	97	98	99	100	101	102	103	104	105
WT:	Asp	Asn	Asp	Ala	Pro	Thr	Lys	Arg	Ala	Ser	Lys	Leu	Phe
Variant													
433R1.1								Leu					
433R3.1							Asn						
433R7.1							Asn						
433R8.1	Gly							Pro					
433R12.1													Del
433R16.1	Gly							Pro					
433R17.1			Glu	Ser			Glu						
433R19.1									Asp				
433R22.1								Ser					
433R26.1							Glu						
433R27.1													Del
435R1.1													Del
435R2.1													
435R5.1							Glu						

Note. Antigenic site 1 is composed of amino acids 91–102 of VP1; amino acids 91–92 are not shown, since they were identical in all variants. Del, deleted.

a viral recombinant containing P2/Lansing capsid sequences on a P1/Mahoney background is infectious in mice (La Monica *et al.*, 1986). To identify specific regions of the P2/Lansing capsid sequences involved in infection of mice, the neurovirulence of antigenic variants resistant to neutralization with monoclonal antibodies was studied. The mabs used are directed at antigenic site 1, an immunodominant loop located on the surface of the virion and composed (in poliovirus type 2) of amino acids 91 to 102 of protein VP1 (Minor *et al.*, 1986).

Many of the *mar* variants were less neurovirulent than the parent P2/Lansing, as judged by their higher LD_{50} values (Table 2). Although the highest LD_{50} value observed with unselected P2/Lansing was 5.0 $\log_{10}$ PFU, many of the *mar* variants had an LD_{50} of 6.0 $\log_{10}$ and higher. We considered any virus with an LD_{50} of 6.0 $\log_{10}$ PFU or higher of significantly reduced neurovirulence. Not all of the *mar* variants had reduced neurovirulence; for example, variants 433R1.1 and 433R3.1 are clearly as neurovirulent as the parent virus. At least 10 other *mar* variants appeared to be quite lethal as judged by preliminary testing, and the LD_{50} of these isolates was not determined. However, the high frequency of isolation of *mar* variants with low neurovirulence (10 out of 22) suggests that reduction in neurovirulence is linked to mutations that cause resistance to neutralization.

TABLE 5

CHARACTERIZATION OF NEURAL ISOLATES FROM PARALYZED MICE INOCULATED WITH *mar* VARIANTS

			Brain isolate			
Brain isolate	LD_{50}	LD_{50} of inoculum	Titer at 33°	Titer at 39.5°	e.o.p.	Sequence change
433R27BE1L	4.1	>7.1 (ts)	1.0×10^8	5.4×10^8	5.7	Del 105 to Phe
433R27BE1S	5.4	>7.1 (ts)	1.1×10^7	1.5×10^8	13.0	Second site
435R1BE1	5.8	>7.0 (ts)	1.4×10^7	6.3×10^7	4.5	Second site
433R12BE1	5.1	6.8, >7.0 (ts)	2.0×10^6	7.3×10^7	36.5	Second site
433R8BE1	6.2	6.8, >7.3	6.9×10^8	4.3×10^8	0.6	No change
433R19BE1	6.5	6.5, 6.8	5.0×10^8	8.8×10^8	1.8	No change
433R16BE1	>6.6	6.4, 6.7	5.7×10^8	7.4×10^8	1.3	Gly 93 to Asp
433R7BE1	6.6	6.9, >7.3	6.3×10^8	6.3×10^8	1.0	No change
433R17BE1	5.8	6.0, 6.1	1.1×10^8	8.5×10^7	0.8	No change
435R5BE1	5.1	5.8 (ts)	7.0×10^8	5.2×10^8	0.8	No change

The reduced neurovirulence of four variants, 433R12.1, 433R27.1, 435R1.1, and 435R5.1, can be ascribed to their temperature-sensitive phenotype. Three ts *mar* variants contained a deletion of amino acid 105, while a fourth contained a substitution of Glu for Lys at amino acid 99. The replication of these viruses was reduced in cell culture at 39.5°, and the virions are also thermolabile at this temperature. These ts variants probably do not cause disease in mice because the mouse body temperature is 38° (Doll and Johnson, 1985). This conclusion is supported by the observation that the only viruses isolated from paralyzed mice inoculated with the ts variants had lost the ts phenotype and had become more neurovirulent.

The ts phenotypes of 433R12.1, 433R27.1, and 435R1.1 appear to be caused by the deletion of amino acid 105. This conclusion is based on the location of amino acid 105 in the atomic structure of poliovirus (see below) as well as our observation that in neural isolate 433R27BE1L, the deleted amino acid 105 is restored. This isolate is ts^+ and is neutralized by mab 433, indicating that the deletion of amino acid 105 in 433R27.1 is responsible for both the ts phenotype and resistance to neutralization. The ts^+ phenotype of another neural isolate, 435R1BE1, is due to a second site suppressor since the amino acid 105 deletion is still present. Two other neural isolates recovered from animals inoculated with ts mutants had become cold sensitive (433R27BE1S and 433R12BE1). These cs isolates still contained the 105 deletion, and therefore they must contain a second site suppressor mutation that renders them defective at low temperatures. It will be of interest to identify these second site suppressor mutations because they will provide information on how polypeptides interact in the poliovirus capsid. The ts phenotype of 435R5.1 is due to a mutation outside antigenic site 1, since two other variants with a substitution of Glu for Lys at amino acid 99 (433R17.1 and 433R26.1) were not ts.

Examination of the atomic structure of the P1/Mahoney capsid (Hogle *et al.,* 1985) indicates that amino acid 105 is not part of the exposed polypeptide loop that constitutes antigenic site 1, but rather is part of a β-sheet that anchors one end of the loop and forms the core of VP1. Therefore deletion of amino acid 105 must lead to an alteration of the structure of the antigenic loop and consequent resistance to neutralization, as well as alteration of the VP1 core leading to thermolability.

Variants 433R16.1 and 433R19.1, which were not ts, had significantly reduced neurovirulence and replicated to low levels in the mouse brain (Fig. 2). The histopathology and pattern of involvement in mice inoculated with *mar* variant 433R19.1 were similar to those previously reported for P2/Lansing (Jubelt *et al.,* 1980; Jubelt and Meagher, 1984). Neuropathological changes in infected mice included neuronal degeneration, inflammation with mononuclear and microglial rod-shaped cells, microglial nodules, and neuronophagia. The extent of involvement of several regions of the mouse brain differed from previous reports—the thalamus and the cerebellum showed decreased histopathological lesions while the occipital cortex was more affected. However, these differences cannot explain the reduced neurovirulence of 433R19.1, since the same patterns were observed with the cDNA-derived P2/Lansing as well as the uncloned P2/Lansing parent.

Amino acid substitutions at positions 100 and 101 within antigenic site 1 correlated with reduction of mouse neurovirulence (Table 4). In two different isolates, 433R8.1 and 433R16.1, substitution of Arg with Pro at amino acid 100 resulted in decreased neurovirulence. However, viruses in which Arg was replaced with Ser (433R22.1) or Leu (433R1.1) were fully neurovirulent. Variants 433R8.1 and 433R16.1, which contained the Pro mutation, also contained a change at amino acid 93 from Asp to Gly. The Gly substitution does not appear to play a role in the phenotype of these variants, since virus 433R16BE1, which had been isolated from a mouse inoculated with 433R16.1, has reverted to Asp at amino acid 93 while maintaining the Pro residue at amino acid 100 (Table 5). The neurovirulence of this isolate is similar to that of the inoculum. A change from Ala to Asp at amino acid 101 of 433R19.1 also resulted in reduced neurovirulence.

Three different changes were observed at amino acid 99. Substitution of Lys with Leu in variant 433R1.1 produced a fully neurovirulent virus. Variants 433R3.1 and 433R7.1 contained a change to Asn at this position; the neurovirulence of the first was very high and that of the latter was very low. Perhaps the attenuated phenotype of 433R7.1 is caused by a mutation outside of antigenic site 1. Three *mar* variants with reduced neurovirulence contained a change from Lys to Glu at amino acid 99: 433R17.1, 433R26.1, and 435R5.1. Variant 433R17.1, in addition, had amino acid changes at positions 95 and 96, but its neurovirulence was similar to that of variants 433R26.1 and 435R5.1 which lacked these changes. The ts phenotype of variant 435R5.1 is probably caused by a mutation outside of antigenic site 1, since the neurovirulent brain isolate 435R5BE1 is ts^+ but does not contain a change in this region. Curiously, the LD_{50} of these three variants was not as high as observed in variants

with other amino acid changes; perhaps changes at amino acid 99 are less disruptive than changes at amino acid 100.

Although in some cases it is possible to correlate specific amino acid substitutions in antigenic site 1 with reduction in neurovirulence, it is important to determine whether amino acid changes have occurred elsewhere in the capsid of the *mar* variants. To address this question, sequencing of viral RNA encoding the entire capsid of several attenuated *mar* variants is in progress. To determine whether specific amino acid substitutions result in reduction of neurovirulence, it will be necessary to introduce the mutation into P2/Lansing cDNA by site-directed mutagenesis, and study the neurovirulence of viruses that contain only the introduced mutation.

The amino acid sequence of antigenic site 1 of P2/Lansing and the mouse avirulent P2/P712,Ch,2ab are identical, and therefore this antigenic loop cannot be the sole determinant of mouse infectivity. Other determinants within the viral capsid proteins clearly must play a role in the mouse-adapted phenotype. However, since we did not observe LD_{50} values in the range of 6–7 $\log_{10}$ PFU for unselected P2/Lansing isolates, it is unlikely that the reduction of neurovirulence of the *mar* variants is due to mutations in noncapsid regions of the viral RNA.

Attenuated variants 433R16.1 and 433R19.1 showed growth rates in HeLa cells similar to those of the parent virus, but replicated to low levels in the mouse brain (Fig. 2). This observation indicates that reduction of neurovirulence of 433R16.1 and 433R19.1 is due to a host range mutation, and not to a general defect in viral growth. How might mutations associated with resistance to neutralization affect viral neurovirulence in the mouse? One possibility is that such changes alter the interaction of the virus with a mouse brain receptor and reduce the efficiency of infection. Indeed, any function mediated by the viral capsid, such as penetration, uncoating or RNA packaging, might be affected. Alternatively, the mutations might affect the stability of the virion capsid in the mouse brain, where the environment is quite different from that in a culture dish containing HeLa cells. A study of the functional block in the attenuated *mar* variants awaits identification of a cell culture model that mimics the host range phenotype of the variants.

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