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Engineering a poliovirus type 2 antigenic site on a type 1 capsid results in a chimaeric virus which is neurovirulent for mice

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Poliovirus type 2 (PV-2) Lansing strain produces a fatal paralytic disease in mice after intracerebral injection, whereas poliovirus type 1 (PV-1) Mahoney strain causes disease only in primates. Atomic models derived from the three-dimensional crystal structure of the PV-1 Mahoney strain have been used to locate three antigenic sites on the surface of the virion. We report here the construction of type 1 - type 2 chimaeric polioviruses in which antigenic site 1 from the PV-1 Mahoney strain was substituted by that of the PV-2 Lansing strain by nucleotide cassette exchange in a cloned PV-1 cDNA molecule. These chimaeras proved to have mosaic capsids with composite type 1 and type 2 antigenicity, and induced a neutralizing response against both PV-1 and PV-2 when injected into rabbits. Moreover, a six-amino-acid change in PV-1 antigenic site 1 was shown to be responsible for a remarkable host-range mutation in so far as one of the two type 1 - type 2 chimaera was highly neurovirulent for mice.

Key words: neurovirulence/hybrid virus/capsid/nucleotide cassette

Introduction

Poliovirus, the causative agent of poliomyelitis, is a member of the family Picornaviridae. Three poliovirus serotypes can be distinguished on the basis of seroneutralization assays. Poliovirions are composed of a single-stranded RNA molecule of plus-strand polarity enclosed in an icosahedral capsid made of 60 copies each of the four structural polypeptides VP1, VP2, VP3 and VP4 (for a review, see Rueckert, 1985). The viral RNA of the attenuated (Sabin) strain and of at least one virulent wild-type strain of each of the three poliovirus serotypes have been cloned and sequenced (Racaniello and Baltimore, 1981a; van der Werf *et al.*, 1981; Nomoto *et al.*, 1982; Stanway *et al.*,1984; Toyoda *et al.*, 1984). As demonstrated by Racaniello and Baltimore (1981b), plasmids carrying genomic length poliovirus cDNA inserts are able to generate infectious poliovirus RNA and to initiate a complete virus cycle once transfected onto susceptible primate cells. Infectivity of such plasmids was greatly increased by using vectors carrying the SV40 origin of replication and enhancer sequence and transfecting COS-1 cells (Semler *et al.*, 1984), or vectors carrying the SV40 origin of replication and enhancer sequence with the SV40 T antigen gene (Kean *et al.*, 1986; Kuhn *et al.*, 1987). In the latter case, the plasmids generate high yields of poliovirus in cells of primate origin such as VERO or CV1 cells, due to their ability to replicate in these cells (Kean *et al.*, 1986).

The three-dimensional structures of the Mahoney strain of type 1 (PV-1; Hogle *et al.*, 1985) and the Sabin strain of type 3 (PV-3; Filman *et al.*, in preparation) poliovirus, of human rhinovirus 14 (Rossmann *et al.*, 1985), and of mengovirus (Luo *et al.*, 1987) have recently been elucidated. These studies, along with studies characterizing mutants which are resistant to neutralization by monoclonal antibodies, have made it possible to locate three major neutralization sites on the picornavirion. Site 1 is located in a highly exposed loop at the surface of the capsid near the 5-fold axes of the icosahedral virus particle (Hogle *et al.*, 1985). In PV-1, it forms a sequential epitope which is recognized specifically by a neutralizing monoclonal antibody (mAb), C3 (Crainic *et al.*, 1983; Blondel *et al.*, 1986). The C3 epitope was determined to lie within VP1 amino acids 93 - 103 (van der Werf *et al.*, 1983; Wychowski *et al.*, 1983). Variants that resist neutralization by C3 were isolated. These variants bear a mutation at VP1 amino acid 100 (Blondel *et al.*, 1986; Horaud *et al.*, 1987). This site has been shown to be an immunodominant site in type 2 and 3 polioviruses (Minor *et al.*, 1986).

The C3 epitope of poliovirus is located in a highly exposed, relatively flexible loop. This raises the possibility that poliovirus could be used as a vector for the expression of heterologous antigenic determinants by modifying or substituting the sequence of its C3 epitope, thus generating chimaeric virions expressing both PV-1 and a second, heterologous, antigenic specificity. As a first attempt, we chose to replace the sequence of the C3 epitope from the Mahoney strain of PV-1 with the equivalent sequence from the Lansing strain of PV-2. The Lansing strain has been adapted to mice (Armstrong, 1939) and induces a fatal paralytic disease in the animal upon intracerebral injection, whereas the Mahoney strain is unable to do so. In this manuscript, we show that a chimaeric virus in which the sequence of the C3 epitope from PV-1 Mahoney was replaced with the corresponding sequence from PV-2 Lansing expressed both type-1- and type-2-specific antigenicities and showed full neurovirulence for mice. A preliminary description of these results has already been published (Martin *et al.*, 1988).

Results

Construction of chimaeric type 1/type 2 polioviruses

In order to facilitate the replacement of the C3 epitope from PV-1, artificial restriction sites were created on either side of the C3 sequence by oligonucleotide-directed mutagenesis

Table I. Nucleotide sequence of the cDNA of the PV-1 Mahoney strain and mutations introduced by site-directed mutagenesis

	90										100					
PV-1	ATG	ACC	GTG	GAT	AAC	CCA	GCT	TCC	ACC	ACG	AAT	AAG	GAT	AAG	CTA	TTT
vKK17	MET	THR	VAL	ASP	ASN	PRO	ALA	SER	THR	THR	ASN	LYS	ASP	LYS	LEU	PHE
				HpaI										HindIII		
	90			93							100					
pAM4	ATG	ACC	GTG	GTT	AAC	CCA	GCT	TCC	ACC	ACG	AAT	AAG	GAT	AAG	CTT	TTT
pAM400	MET	THR	VAL	**Val**	ASN	PRO	ALA	SER	THR	THR	ASN	LYS	ASP	LYS	LEU	PHE
vAM4								PV-1								
	90			93	94						100		102			
pAM41	ATG	ACC	GTG	GTT	AAT	**GAT**	GCT	**CCA**	**ACA**	**AAG**	**CGT**	**GCC**	**AGT**	AAG	CTT	TTT
pAM410	MET	THR	VAL	**Val**	ASN	**Asp**	ALA	**Pro**	THR	**Lys**	**Arg**	**Ala**	**Ser**	LYS	LEU	PHE
v410								PV-2								
				EcoRV										HindIII		
	90				94						100					
pAM5	ATG	ACC	GTG	GAT	ATC	CCA	GCT	TCC	ACC	ACG	AAT	AAG	GAT	AAG	CTT	TTT
pAM500	MET	THR	VAL	ASP	**Ile**	PRO	ALA	SER	THR	THR	ASN	LYS	ASP	LYS	LEU	PHE
vAM5								PV-1								
	90				94						100		102			
pAM51	ATG	ACC	GTG	GAT	AAT	**GAT**	GCT	**CCA**	**ACA**	**AAG**	**CGT**	**GCC**	**AGT**	AAG	CTT	TTT
pAM510	MET	THR	VAL	ASP	ASN	**Asp**	ALA	**Pro**	THR	**Lys**	**Arg**	**Ala**	**Ser**	LYS	LEU	PHE
v510								PV-2								

The PV-1 cDNA sequence in the region of VP1 was modified by oligonucleotide-directed mutagenesis to create either a *Hpa*I and a *Hin*dIII sites (pAM4) or an *Eco*RV and a *Hin*dIII sites (pAM5) on either side of the sequence encoding capsid antigenic site 1. The sequence of the PV-2 Lansing strain (box) was then substituted for that of the PV-1 strain by oligonucleotide cassette exchange (see Materials and methods). Bold-type letters refer to nucleotide or amino acid changes. Numbering refers to amino acid position in the VP1 polypeptide of PV-1.

(Morinaga *et al.*, 1984) of a poliovirus cDNA fragment (nt 1–5601; see Figure 1 and Materials and methods). In one construction, a *Hpa*I site was created at nt 2756 (VP1 amino acid 93) and a *Hin*dIII site at nt 2786 (VP1 amino acid 103), generating plasmid pAM4. In another construction, an *Eco*RV site was created at nt 2756 and a *Hin*dIII site at nt 2786, generating plasmid pAM5 (Table I). The creation of the *Hpa*I site introduced an Asp 93 → Val mutation in VP1; similarly, the creation of the *Eco*RV site an Asn 94 → Ile mutation. In contrast, creation of the *Hin*dIII site did not lead to any amino acid change.

Plasmids pAM4 and pAM5 were treated with *Hpa*I and *Hin*dIII or *Eco*RV and *Hin*dIII respectively, after which the cleaved DNAs were mixed with two complementary, custom-made oligonucleotides that had been devised to form an artificial cDNA fragment coding for VP1 amino acids 94–102 from the Lansing strain of poliovirus, blunt-ended on the 5′ end and terminated by a *Hin*dIII acceptor site on the 3′ end. This allowed the precise in-frame docking of the substitute sequence into the sequence of the Mahoney genome (Table I), leading to plasmids pAM41 and pAM51.

The mutagenized cDNA fragments were recovered from plasmids pAM4, pAM5, pAM41 or pAM51 and reinserted in place of their wild-type counterparts into plasmid pKK17 (Kean *et al.*, 1986). In this plasmid, the genomic length Mahoney cDNA molecule is placed downstream from the SV40 late promoter (Figure 1). The plasmid also carries the SV40 origin of replication, the SV40 enhancer sequence and the whole SV40 early region, thus ensuring a high level of infectivity for monkey cells. The nucleotide sequences of the resulting plasmids were checked in the mutagenized region according to the method of Zagursky *et al.* (1985).

Monkey cells were transfected with these plasmids (Table I) and resulting viruses vAM4 (PV-1 with an Asp 93 → Val mutation in VP1), v410 (the same with VP1 amino acids 94–102 from PV-2), vAM5 (PV-1 with an Asn 94 → Ile mutation in VP1) and v510 (PV-1 with VP1 amino acids

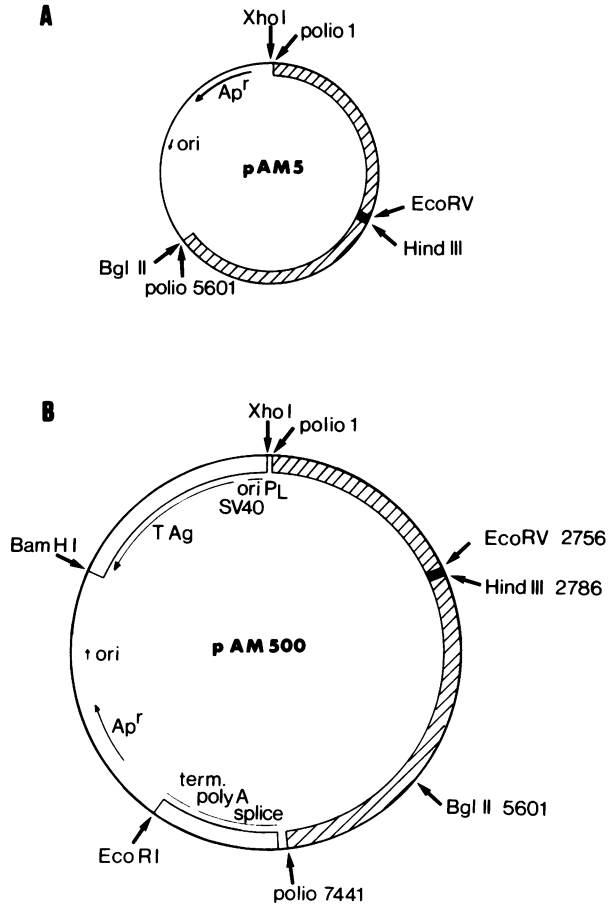

Fig. 1. Construction of type 1/type 2 poliovirus cDNAs. The *Xho*I–*Bgl*II fragment containing PV-1 nucleotides 1–5601 (hatched box) isolated from plasmid pKK17 (Kean *et al.*, 1986) was inserted into pBR327 derivative pAM2 (**A**), and unique *Hin*dIII and *Eco*RV (or *Hpa*I) sites were created by site-directed mutagenesis on either side of the sequence encoding VP1 antigenic site (black box). The sequence framed by these sites was then substituted by that of the PV-2 Lansing strain and the resulting hybrid cDNA fragment was reinserted into pKK17 (**B**). Sequences of the regions substituted are shown in Table I.

94–102 from PV-2) were grown into individual stocks. In each case, the nucleotide sequence of the RNA in the region encoding the new antigenic site (approximately from nt 2730 to nt 2810) was confirmed by dideoxynucleotide sequencing according to Geliebter *et al.* (1986). The Asp 93 → Val mutation generated by the creation of restriction site *Hpa*I was maintained in the genome of v410 (Table I), whereas the Asn 94 → Ile mutation introduced by the creation of restriction site *Eco*RV was eliminated from the genome of v510.

Antigenic properties of the chimaeric viruses

PV-1 mutants, vAM4 and vAM5, and chimaeric PV-1/PV-2 viruses, v410 and v510, grew on CV1, VERO, HeLa or HEp-2 cells. Virus yields were not appreciably affected by temperature over the range 33–39°C. However, whereas vAM4, vAM5 and v510 produced normal, large plaques (on CV1 cells), v410 showed a small plaque phenotype. The time course of v410 RNA synthesis, as measured from tritiated uridine incorporation in the presence of actinomycin D, was not significantly different from that of v510 or of vKK17 (the reference Mahoney strain). However, the v410 virus

Table II. Neutralization pattern of type 1/type 2 chimaeras

Virus	Neutralizing antibodies					
	Anti-type 1			Anti-type 2		
	Ic	C3	α-PV-1	IIo	HO2	α-PV-2
vKK17	1905	83	21 379	9	10	19
PV-2 Sabin	4	4	42	16	12 407	10 852
PV-2 Lansing	6	9	36	524	77 642	14 454
vAM4	1778	724	12 302	21	10	18
vAM5	1318	467	12 589	21	10	16
v410	407	4	29 512	4	15 488	23
v510	1318	5	8709	3162	9549	54

The Mahoney virus strain vKK17 isolated upon transfection of CV1 cells with pKK17, the PV-2 Sabin strain and a standard PV-2 Lansing strain were neutralized by the indicated mAbs or rabbit immune sera in parallel with PV-1 Mahoney mutant viruses vAM4 and vAM5 and chimaeric viruses v410 and v510 (see text and Table I). The figures are the highest dilution of serum or ascites fluid giving a 50% reduction in the number of plaques. mAbs Ic and C3 recognize specifically site 3b and site 1 of PV-1 Mahoney respectively. mAb IIo was generated against PV-2 MEF1 strain and specifically neutralizes Lansing but not PV-2 Sabin. mAb HO2 was described as mAbI-10C9E6 (Uytdehaag and Osterhaus, 1985) and is a broadly PV-2 neutralizing mAb.

Table III. Immunogenicity of v410 and v510

Rabbit	Virus injected	Serum titre			
		α-PV-1	α-PV-2	α-v410	α-v510
1	v410	5120	16	5120	ND
2	v410	640	1280	5120	ND
3	v410	10 240	64	20 480	ND
4	v410	5120	64	20 480	ND
5	v510	1280	32	ND	5120
6	v510	1500	640	ND	2560
7	v510	1280	160	ND	1280
8	v510	320	640	ND	1280
9	vKK17	> 10 240	32	ND	ND
10	vKK17	> 10 240	32	ND	ND

Neutralizing antibody titres in rabbits immunized with the indicated virus were determined as described in Materials and methods. Titres of pre-immune sera varied from < 8 to 16.
ND: not determined.

particles were found to be thermolabile. A loss of 2.7 $\log_{10}$ p.f.u. after 5 min incubation at 45°C was observed for v410, whereas neither the Mahoney strain nor v510 lost more than 1.6 $\log_{10}$ p.f.u. under the same conditions (results not shown). This suggests that the small plaque phenotype of v410 may be the result of an alteration in capsid stability.

All of the mutant and chimaeric viruses could be neutralized by an anti-PV-1 hyperimmune rabbit serum. In addition, both v410 and v510 could be neutralized, albeit to a very limited extent only, by an anti-PV-2 hyperimmune rabbit serum (Table II). Further neutralization studies were caried out using a panel of type-1- and type-2-specific mAbs. Whereas both mutants vAM4 and vAM5 were neutralized by mAb C3, which is directed against the antigenic site 1 of PV-1 (VP1 amino acids 93–103), neither of the two chimaeric viruses were neutralized by C3, confirming that both had been altered in this site. In contrast, the two chimaeric viruses were neutralized by mAb Ic, which is directed against PV-1 antigenic site 3b (VP3 amino acids 58–79) (Minor *et al.*, 1986). Two type-2-specific mAbs, IIo and HO2, were able to neutralize v510, but only HO2 was able to neutralize v410. These results demonstrate that v410 and v510 have acquired a type-2-specific neutralization epitope in a type 1 antigenic background, and confirm the mosaic nature of their capsid. The fact that mAb IIo was not able to neutralize v410 suggests that there may be two different epitopes within that region of VP1, only one of which is influenced by the nature of amino acid 93. HO2 is known to recognize PV-2 site 1. The neutralization epitope recognized by this mAb was indeed present on the capsid of both the PV-2 Sabin and Lansing strains, but was not found on the capsid of PV-2 Sabin escape mutants (Minor *et al.*, 1986) which map in antigenic site 1 (results not shown). Note that whereas both mAbs neutralized the PV-2 Lansing strain, only mAb HO2 was able to neutralize the PV-2 Sabin strain (Table II). The weak neutralization of chimaeric viruses by an anti-PV-2 MEF1 strain hyperimmune rabbit serum was rather surprising, since they were well neutralized by at least one type-2-specific mAb. It might happen that PV-2 site 1 is not or only slightly recognized by the rabbit immune system, whereas it is immunodominant for mice. Whether an anti-PV-2 mouse serum could better neutralize the chimaeric viruses has to be determined. An alternative hypothesis might be that antigenic site 1 of MEF1 strain (which was used to raise the anti-PV-2 rabbit serum) shows a conformation significantly different from that of site 1 on chimaeric viruses.

The immunogenicities of v410 and v510 were assessed by immunizing rabbits with clarified virus preparations and monitoring the neutralizing antibody response against PV-1 and PV-2 and against the chimaeric type 1/type 2 viruses. As shown in Table III, both v410 and v510 were able to elicit a neutralizing antibody response against PV-2 and against PV-1. Thus, not only was the Lansing antigenic determinant on the surface of the chimaeric virus capsid a target for existing type-2-specific neutralizing antibodies, but it was also recognized by the immune system of the rabbits as an appropriate immunogen for a type 2 neutralizing response. This property is of crucial importance when considering the possible use of chimaeric viruses for the development of multivalent vaccines.

Mouse neurovirulence

Groups of four 30-day-old male Swiss Webster mice were inoculated intracerebrally (i.c.) with serial dilutions of various virus stocks and the animals observed daily for 21 days for paralysis or death. A survival score was determined for each dilution by multiplying the number of mice surviving on a given day by the number of days the animals had survived. Thus, the longer the animal's survival, the higher the score. This representation was chosen to take into account not only the number of deaths observed in each of the individual lots of animals but also the rapidity of evolution of the disease in each lot.

As shown in Figure 2, v410 was as devoid of neurovirulence for mice as the control Mahoney type 1 virus. In contrast, v510 showed definite mouse neurovirulence, albeit to a somewhat lesser extent than the prototype Lansing virus strain. A more quantitative estimate of the degree of neurovirulence of v410 and v510 was obtained by determining the lethal dose 50 (LD_{50}) of each virus by the method of

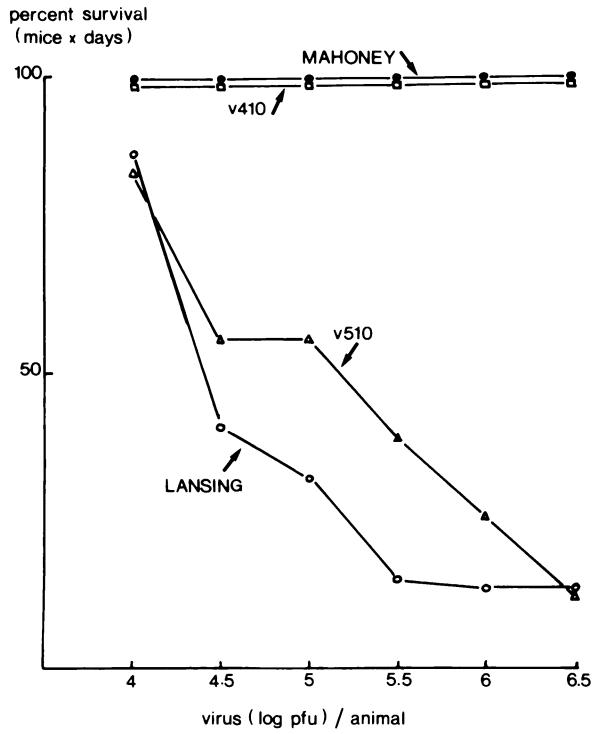

Fig. 2. Mouse neurovirulence of v410, v510 and parent viruses. Groups of four mice were injected by the i.c. route with 0.03 ml of PBS containing the indicated amount of virus and the animals were followed up for 21 days. Survival score was plotted as percentage mice × days of survival (see text).

Table IV. Antigenic characterization of poliovirus recovered from the spinal cord of paralysed mice

Monoclonal antibody	Virus neutralization index			
	v510		Lansing	
	Inoculum virus	Mouse spinal cord	Inoculum virus	Mouse spinal cord
Ic	2.00	3.00	1.50	1.00
C3	0.00	0.00	0.50	0.25
IIo	3.00	>3.50	6.50	>3.50
HO2	3.50	>3.50	7.00	>3.50

Virus recovered from paralysed mice that had been injected with either v510 or the PV-2 Lansing strain was analysed by neutralization with the indicated mAbs. Neutralization indices were calculated as described (Crainic *et al.*, 1983).

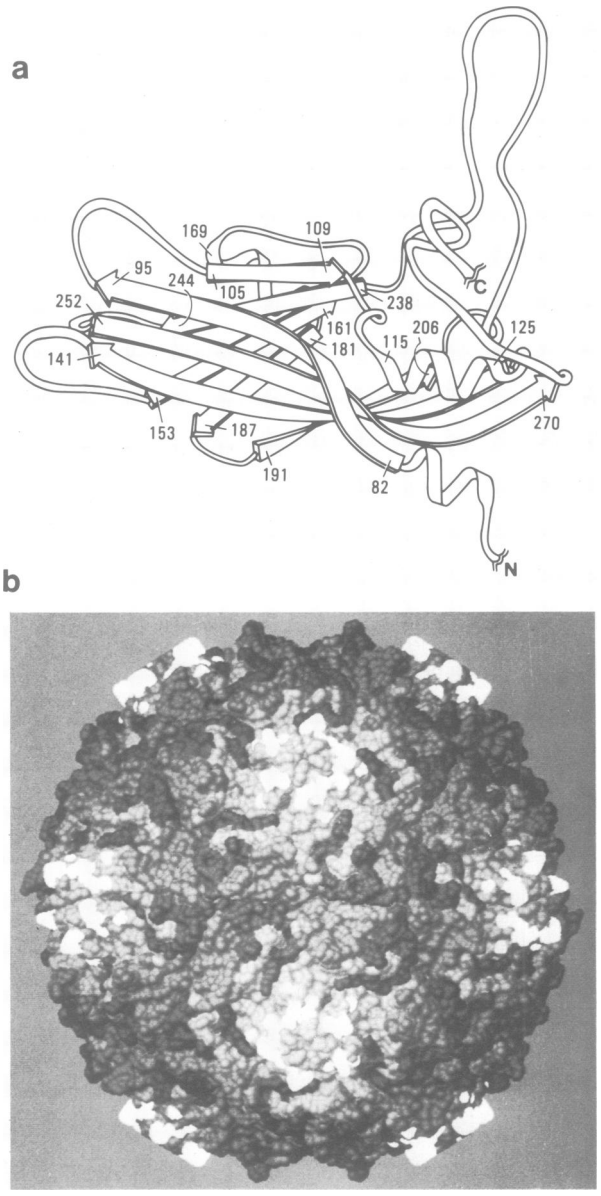

Fig. 3. Location of the residues which have been replaced in the type 1/type 2 chimaeras. (**a**) Schematic representation of VP1. The individual strands of the β-barrel are shown as ribbons. Residue numbers are indicated for reference with text. (**b**) Space-filling representation of the virus particle. The residues which have been replaced in the chimaeras are highlighted in white. The capsid proteins are distinguished by grey scale with VP1 light grey, VP2 grey and VP3 dark grey.

Kärber (1931). The LD_{50} of the Lansing strain and of v510 was found to be 4.0 $\log_{10}$ p.f.u. and 4.4 $\log_{10}$ p.f.u. respectively. In contrast, v410 and PV-1 Mahoney did not kill mice even at a dose of >7 $\log_{10}$ p.f.u.

Mice were injected i.c. with 6.7 $\log_{10}$ $TCID_{50}$ (tissue culture infectious doses 50) of either Lansing virus or v510. Once paralysed, the mice were killed and their brains and spinal cords were isolated and analysed for the presence of virus. The amount of virus recovered in spinal cords was 5.5 $\log_{10}$ $TCID_{50}$ and 5.0 $\log_{10}$ $TCID_{50}$ per mouse (0.08 g of spinal cord) for the Lansing virus and v510 respectively. Virus extracted from the spinal cords of the animals was serially diluted, mixed with a fixed amount of the mAbs indicated (Table IV) and neutralization indices were determined. As shown in the table, the virus recovered from the paralysed animals was identical in mAb neutralization pattern to the virus that had been used for inoculation.

Altogether, these results showed that v510 was able to grow in the central nervous system of mice and to cause fatal paralysis. In contrast, v410, which differs from v510 only by an Asp → Val mutation at VP1 position 93, was totally devoid of neurovirulence for mice.

Atomic structure of the chimaeric virus capsids

In an effort to understand better the significance of the Asp → Val substitution in v410, we have examined the environment of VP1 Asp 93 in the atomic models of the type 1 Mahoney (Hogle *et al.*, 1985) and the type 3 Sabin (Filman

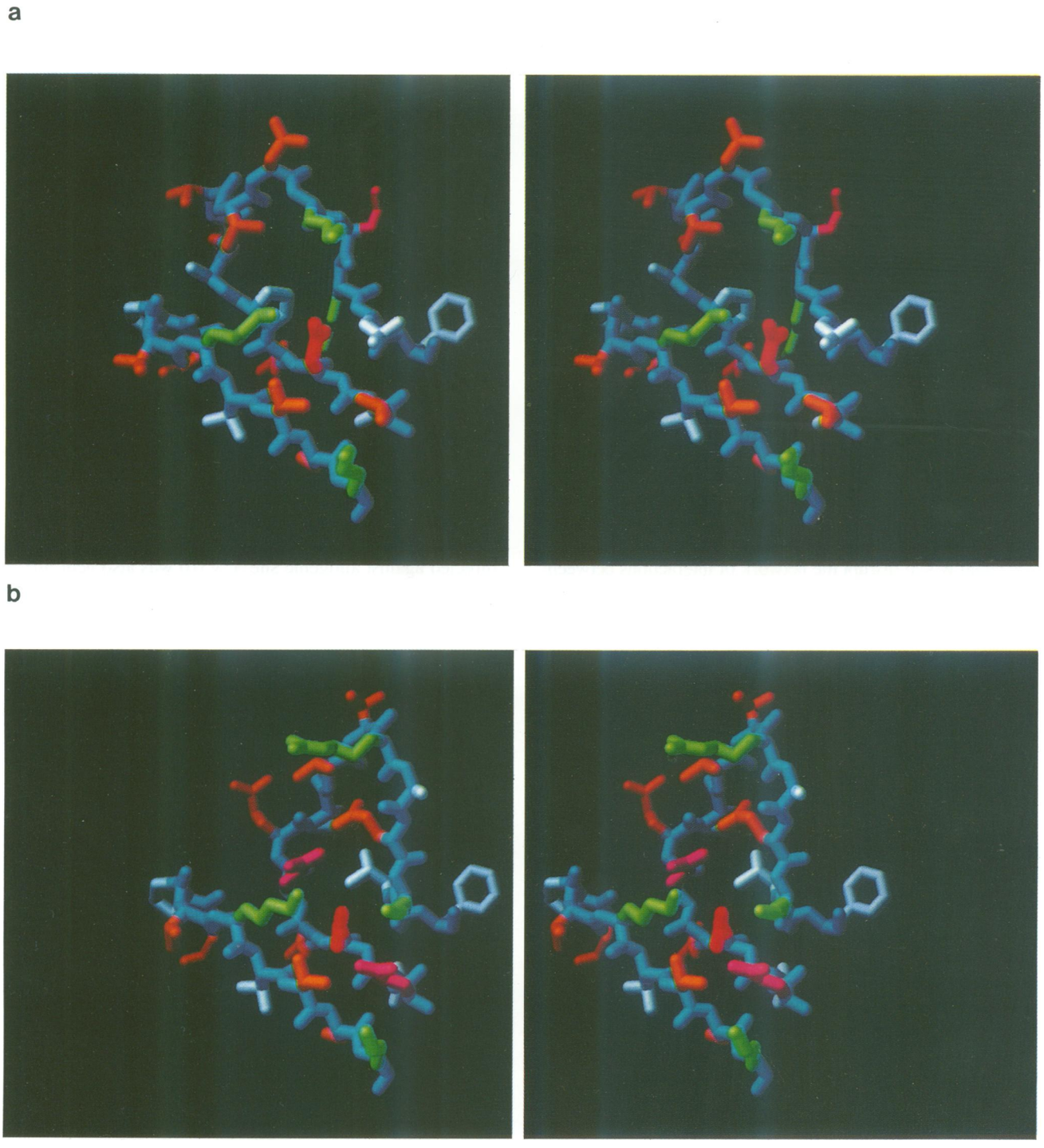

Fig. 4. Stereo view of the atomic models in the vicinity of Asp 93. The main chain is blue, the side chain of Asp 93 is red, other acidic side chains are magenta, polar side chains are orange, basic side chains are green and non-polar side chains are white. (**a**) The Mahoney model. The hydrogen bond between Asp 93 (centre) and the amide nitrogen of Leu 104 (immediately above) is not explicitly shown. The side chain of Asp 93 is bordered by the side chain of Lys 101 (top) and Lys 252 (bottom left) and by Pro 95 (immediately adjacent to the left of Asp 93) and Leu 104 (immediately adjacent to the right of Asp 93). (**b**) The Sabin 3 model. The Asp 93 side chain is part of a network of charged and polar side chains including Glu 91 and Lys 257 (lower right), Thr 255 (below Asp 93), Lys 253 and Glu 95 (to the left of Asp 93), Arg 100 (upper left) and Lys 103 (immediately above and to the right of Asp 93). Figure by David J.Filman, Research Institute of Scripps Clinic.

et al., in preparation) strains of poliovirus. Residue 93 is located near the carboxyl-terminal end of the first strand of the conserved β-barrel of VP1 (residues 82–95, Figure 3a). This β-strand immediately precedes a loop (residues 96–104) which contains the C3 epitope and which is highly exposed on the surface of the virion near the particle 5-fold axes (Figure 3b). This loop, together with several residues from the carboxyl-terminal end of the first strand, constitutes the major portion of antigenic site 1. The loop itself is the site of the largest structural differences between the Mahoney

1 and Sabin 3 structures (Filman *et al.*, in preparation).

As a result of the large structural differences in the loop, the aspartyl side chain of Asp 93 is involved in significantly different interactions in the two poliovirus structures. In the Mahoney structure one of the carboxylate oxygens of Asp 93 makes a hydrogen bond with the main chain nitrogen of Leu 104 which is located on the opposite side of the 96–104 loop. This hydrogen bond would be expected to contribute to the stability of the C3 loop. The negative charge of Asp 93 is further neutralized by the positively charged side chains of Lys 101 and Lys 252 (Figure 4a). In the Sabin 3 structure the side chain of Asp 93 has rotated such that it occupies a more exposed position on the surface of the virion. While the aspartyl side chain no longer participates in a hydrogen bond with the amide nitrogen of Leu 104, it participates instead in an even more extensive network of interactions between charged and polar side chains on the exposed surface of the loop, that includes Glu 91, Glu 95, Lys 101, Thr 254 and Lys 257 (Figure 4b).

In either structure the substitution of a valine for aspartate at residue 93 would be unfavourable. In the Mahoney structure a valine at position 93 would preclude the formation of the hydrogen bond with the amide nitrogen of Leu 104, and in both structures the presence of the hydrophobic valyl side chain would disrupt the network of interactions between polar and charged side chains on the exposed surface of the loop. The disruptive effect of the Asp → Val substitution at residue 93 would be expected to be more severe in the Sabin 3 structure. In the Mahoney structure favourable interactions with the hydrophobic side chains of Pro 95 and Leu 104 (Figure 4a) could partially compensate for the loss of the hydrogen bonds and disruption of the polar network. In the Sabin 3 structure, however, residue 95 is a glutamic acid, and as a result of the structural change in the loop, Lys 103 rather than Leu 104 makes a close approach to the side chain of Asp 93 (Figure 4b). The loss of hydrophobic contacts and the presence of additional charged residues on the exposed surface of the loop would make the presence of a valyl side chain at residue 93 particularly unfavourable. While it is not yet possible to predict accurately the structure of this loop in either the type 2 Lansing strain or in the type 1/type 2 chimaeras, certain similarities in the sequence of the Lansing 2 and Sabin 3 sequences in this loop (specifically the presence of an acidic side chain at residue 95, a Pro at residue 97, and an Arg at residue 100) suggest that the conformation of this loop in Lansing 2 and in the chimaeras is more likely to resemble the conformation of the loop in the Sabin 3 strain. Destabilization of the structure due to the disruption of the charge network by the Asp → Val substitution in v410 might be responsible for the heat lability and small plaque phenotype of this chimaera.

Crystallization of the v510 hybrid

A detailed knowledge of the structure of intertypic chimaeras would provide invaluable insight into the role of amino acid sequence in determining local loop structures in the capsid proteins of polio and related viruses. In addition, the apparent critical role of the C3 loop in determining host range makes this particular chimaera an excellent model for investigating structural factors controlling host range and possibly neurovirulence in poliovirus. We have succeeded in propagating several milligrams of the v510 chimaera. Immediately prior to crystallization the genomic RNA of the propagated virus was sequenced to confirm its identity as a *bona fide* chimaera. The virus was crystallized by dialysis versus low ionic strength buffer at neutral pH. The resulting crystals are apparently isomorphous with the crystals of the type 1 Mahoney strain (space group $P2_12_12$ with a = 323 Å, b = 358 Å and c = 380 Å), and they diffract to at least 2.5 Å resolution. Collection of three-dimensional diffraction data from these crystals is in progress.

Discussion

These results show that it is possible to use poliovirus as a vector for the expression of heterologous antigenic determinants on the surface of the capsid. Similar results have been independently obtained by Murray *et al.* (1988) and Burke *et al.* (1988), who have constructed viable type 1/type 3 intertypic hybrids. The type 1/type 2 chimaeric viruses described here were engineered by replacing the sequence of poliovirus antigenic site 1 (VP1 amino acids 94–102) in the cDNA of the PV-1 Mahoney strain with the corresponding sequence from the PV-2 Lansing strain. The resulting mosaic viruses v410 and v510 were neutralized by a type-2-specific mAb, HO_2, which is known to be directed against antigenic site 1, as well as by a type-1-specific mAb directed against antigenic site 3. v510 was also neutralized by a second type-2-specific mAb, IIo, presumably directed against antigenic site 1, and both chimaeras elicited an anti-type 2 and an anti-type 1 neutralizing antibody response when injected into rabbits. Moreover, in the v510 virus the substitution of the antigenic site 1 of the primate-specific PV-1 Mahoney strain with the corresponding sequence from the mouse-adapted PV-2 Lansing strain results in the ability to cause fatal paralysis in mice.

These results cannot be explained by accidental contamination of a PV-1 stock by PV-2, because both chimaeras show the unique loss of the type-1-specific antigenic site 1 (C3 epitope) and the compensatory acquisition of only a limited number of type-2-specific neutralization epitopes. Moreover, nucleotide sequencing of the appropriate regions of the RNA of the two chimaeric viruses showed the sequences expected for a PV-1 genome with a 27 PV-2 nucleotide substitution at position 2759–2785 (results not shown).

Although we cannot rule out possible effects of unknown mutations elsewhere in the v410 genome, the two type 1/type 2 hybrid viruses are thought to differ by a single amino acid substitution at amino acid 93 of VP1. In the v510 strain and the parental PV-1 Mahoney and PV-2 Lansing strains, this residue is an Asp. In the v410 strain, this residue is a Val. This substitution appeared to be associated with marked phenotypic differences between the two hybrids. v510 produced large plaques and was virulent in mice, whereas v410 produced small plaques, had heat-sensitive virions and was devoid of virulence in mice. Analysis of the atomic structures of polioviruses suggests that the substitution of a Val for an Asp at position 93 of VP1 would prevent the formation of a network of charged and polar side chains on the exposed surface of the C3 loop in the virion. The disruption of this network would be expected to destabilize significantly virions in the Mahoney 1 strain and especially in the Sabin 3 strain of poliovirus where the charge network is more extensive. Based on sequence similarities between the Lansing 2 and Sabin 3 strains we would predict that the

Asp → Val substitution in v410 would also result in significant destabilization of this chimaera. At the present time it is unclear whether the loss of mouse neurovirulence is a result of reduction of yield of the virus due to thermal instability, or whether the disruption of the extensive network of charged residues itself directly interferes with mouse virulence (for example, by interfering with the binding of the virus to its receptor in the mouse central nervous system).

Neurovirulence of poliovirus in mice is restricted to some poliovirus strains, notably the PV-2 Lansing and MEF1 strains. The Lansing virus was adapted to grow in mice by repeated passages (Armstrong, 1939). The study of hybrid viruses constructed by *in vitro* DNA recombination between cloned viral cDNAs from the PV-2 Lansing strain and the PV-1 Mahoney strain showed that the mouse-adapted phenotype mapped to the Lansing viral capsid proteins (La Monica *et al.*, 1986). These investigators (La Monica *et al.*, 1987b) also showed that neutralization escape mutants with an amino acid substitution at either VP1 positions 100 or 101, and to a lesser extent at position 99, showed considerably decreased neurovirulence. These amino acids appear therefore to be critical for mouse neurovirulence, although they do not seem to affect replication of the virus in cultured animal cells. A change of Asp 93 to Gly was observed by La Monica *et al.* (1987b) in two mAb-resistant variants of the PV-2 Lansing strain which exhibited reduced mouse neurovirulence. However, both of these variants also showed a mutation at position 100 and it was concluded that the mutation at position 93 was most probably irrelevant to the loss of neurovirulence.

The capsid of v510, the mouse neurovirulent type 1/type 2 chimaera that was constructed here, differed by six amino acids from that of the PV-1 Mahoney strain: the residues changed were those at VP1 positions 95, 97 and 99–102 (see Table I). It is remarkable that the mere substitution of these six amino acid residues conferred upon the virus a totally new host range phenotype. It has not yet been established whether the Lansing sequence is required in all six amino acid positions or whether a more limited number of substitutions (e.g. residues 99–101) would be sufficient to induce the host range modification.

In any case, these results confirm the earlier observation of La Monica *et al.* (1986, 1987b), and demonstrate for the first time that the Lansing sequence coding for residues 95–104 of VP1 is sufficient to confer mouse adaptation. This part of the capsid could, for example, be directly involved in the interaction with a receptor on the mouse neuron. It is also known that, in addition, mutations which lie outside the antigenic site 1 can affect mouse neurovirulence (La Monica *et al.*, 1986, 1987a,b). In that respect, v510 could provide a new and unique material to study and identify the molecular bases of attenuation of poliovirus neurovirulence without having to resort to costly experiments with monkeys.

The advantage of the cassette strategy adopted here is that it offers the theoretical possibility of substituting the sequence of the poliovirus antigenic site 1 by that of any other foreign epitope, using appropriate synthetic oligonucleotides. It will be of interest to determine the minimum number of amino acids that are required between VP1 residues 93 and 103 to maintain the structural organization of the capsid, and the largest number of amino acids that can be accommodated within that region without interfering with the viability of the virus. The expression of foreign antigenic determinants on the surface of a poliovirion is an attractive novel mechanism for the eventual development of new, multivalent recombinant vaccines, whether live oral vaccines, using a Sabin strain vector, or inactivated vaccines, using the Mahoney strain vector. Several experiments along these lines are now in progress.

Materials and methods

Bacterial strains and plasmids

Plasmids were propagated in *Escherichia coli* strains 1106 (803 r_k-m_k-) and HB101. Plasmids were amplified and their DNA was purified according to Maniatis *et al.* (1982). Restriction enzyme cleavage was performed according to the manufacturer's instructions.

Construction of plasmid pAM2

pBR327 plasmid DNA was linearized with *Hin*dIII, made blunt ended by treatment with Klenow enzyme (100 U/ml) in the presence of the four dNTPs (200 μM each), dephosphorylated with calf intestinal alkaline phosphatase (twice 0.02 U/μg of DNA for 30 min at 37°C in 50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM$ZnCl_2$) and ligated with a 100-fold molar excess of an *Xho*I synthetic linker (New England Biolabs) for 48 h at 4°C (or for 16 h at 15°C) in the presence of 1 U of T4 DNA ligase/μg of DNA. The resulting plasmid was linearized with *Eco*RV and ligated with a *Bgl*II synthetic linker.

Construction of plasmid pAM3

Plasmid pAM2 was treated with *Xho*I and *Bgl*II and the DNA fragment carrying the origin of replication and the ampicillin resistance gene was purified by electrophoresis on a 0.7% low gelling agarose gel (Sigma type VII; Wieslander, 1979). The *Xho*I–*Bgl*II fragment containing the first 5601 bp of the Mahoney poliovirus cDNA was similarly purified from pKK17 (Kean *et al.*, 1986). Eluted DNA fragments were purified by two phenol extractions, precipitated with 95% ethanol, resuspended into 10 mM Tris–HCl, pH 7.5, 1 mM EDTA and ligated together with T4 DNA ligase.

Site-directed mutagenesis of poliovirus cDNA

Plasmid pAM3 was cleaved with *Xba*I and the four resulting fragments were purified on a low gelling agarose gel. The smallest fragment (poliovirus nt 2546–2861) was eliminated, and the three larger fragments were mixed with a pAM3 DNA preparation which had been linearized with *Sal*I. To this mix (0.03 pmol of each DNA) were added two phosphorylated synthetic oligonucleotides (20 pmol each) each with a sequence complementary to that of poliovirus cDNA except for one mismatch necessary to create the desired *Eco*RV, *Hpa*I or *Hin*dIII restriction site. Oligonucleotides used were:

5′-GGAAGCTGGGATATCCACGG-3′ (*Eco*RV) or

5′-GCTGGGTTAACCACGGTC-3′ (*Hpa*I) and

5′-CCACACTGCAAAAAGCTTATCC-3′ (*Hin*dIII).

The mixtures were denatured by incubation for 3 min in a boiling water bath, cooled slowly, then treated overnight at 12.5°C with Klenow DNA polymerase (3 U) and T4 DNA ligase (3 U). HB101 cells were transformed with the ligated mixtures and resulting clones were screened by colony hybridization (Grunstein and Hogness, 1975) using the two ^{32}P-labelled oligonucleotides involved in the mutagenesis experiment as probes. Hybridization was at 42°C and washing at 53°C. Double mutant plasmids were checked for the presence of the two new restriction sites.

Construction of plasmids pAM41 and pAM51

Plasmids pAM4 or pAM5 were digested with endonucleases *Hpa*I and *Hin*dIII (or *Eco*RV and *Hin*dIII), dephosphorylated and mixed with a 20-fold molar excess of two complementary oligonucleotides, one with the sequence coding for VP1 amino acids 94–102 from the PV-2 Lansing strain, prolongated with an extra adenine residue at the 3′ end

(5′-AATGATGCTCCAACAAAGCGTGCCAGTA-3′)

and the other with the complementary sequence preceded by a *Hin*dIII adaptor sequence at the 5′ end

(5′-AGCTTACTGGCACGCTTTGTTGGAGCATCATT-3′).

Recombinant plasmids were screened by colony hybridization using the same oligonucleotides as labelled probes.

Construction of plasmids pAM400, 410, 500 and 510

Plasmids pAM4, pAM5, pAM41 and pAM51 were digested with *Xho*I and

*Bgl*II and the *Xho*I–*Bgl*II fragments containing the first 5601 bp of the poliovirus cDNA were purified on low gelling agarose gels. The *Xho*I–*Bgl*II fragment containing the last 1840 bp from the PV-1 Mahoney cDNA was similarly prepared from pKK17, and ligated to each of the above fragments. Resulting plasmids pAM400, pAM500, pAM410 and pAM510 were selected on the basis of their restriction maps.

DNA sequencing

Sequencing was performed directly on mini-preparations of plasmid DNA according to the method of Zagursky *et al.* (1985). The DNA (1 μg) was alkali denatured (0.4 N NaOH) and hybridized to an oligonucleotide primer (60 ng), the sequence of which is complementary to that of PV-1 nucleotides 2825–2839 (5′-CCTCCGTAACTGGAC-3). Sequencing reactions were carried out at 42°C in the presence of 15 μCi of [^{35}S]dATP and 14 U of reverse transcriptase using the dideoxynucleotide termination method (Sanger *et al.*, 1977).

Preparation of virus stocks

CV1 cells were transfected directly with mini-preparations of plasmid DNA in the presence of DEAE dextran according to Sompayrac and Danna (1981). When CPE was complete, the cells were scraped and lysed by five successive freeze–thawings. Virus stocks were first amplified on CV1 cells, then later on either HEp-2 cells in monolayer cultures or HeLa cells in suspension cultures. Chimaeric viruses proved to give higher titres on HEp-2 or HeLa cells than on monkey cells. Virus titration was using either HEp-2 or CV1 cells seeded in 6-well plates under 3 ml of 0.95% agarose in DMEM medium containing 2% fetal calf serum (FCS) and 10 mM $MgCl_2$. After 48 h incubation (or 96 h for v410) cell monolayers were stained with crystal violet and plaques were counted.

Viral RNA preparation and RNA sequencing

HEp-2 cells were infected with the appropriate virus stock at a m.o.i. of 10–20 p.f.u./cell. After 12 h of incubation at 37°C, the cells were scraped, pelleted at 2000 r.p.m., resuspended into PBS and mixed with 7 vol of 50 mM Tris–HCl, pH 7.5, 5 M guanidine monothiocyanate, 10 mM EDTA, 10% β-mercaptoethanol (Cathala *et al.*, 1983). The mixture was left at room temperature until complete dissolution, then centrifuged for 10 min at 8000 r.p.m. The supernatants were made 3.3–3.5 M with respect to LiCl, incubated for 24 h at 4°C, then centrifuged for 90 min at 12 000 r.p.m. The RNA pellets obtained were washed several times with 3 M LiCl, 2 M urea, then resuspended into 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.1% SDS. RNA sequencing was according to Geliebter *et al.* (1986). RNA (10 μg) in 10 mM Tris–HCl, pH 8.3, 250 mM KCl, was hybridized to the ^{32}P-labelled oligonucleotide primer (50 ng) for 45 min at 43°C. Sequencing was at 50°C in the presence of 12 U of reverse transcriptase and 0.6 μg of actinomycin D.

Virus neutralization

About 50 p.f.u. of virus in 0.2 ml of Leibowitz L-15 medium were incubated for 2 h at 37°C with equal volumes of 4-fold serial dilutions of mAb containing ascites fluids or rabbit anti-poliovirus immune sera in duplicate wells of a 24-well plate, after which 3×10^5 VERO cells were added in each well and the plates were further incubated for 3 h at 37°C. The cells were covered with 0.6 ml of a 1.6% carboxymethyl cellulose solution in L-15 culture medium with 2% FCS and further incubated for 3 days at 37°C. The cells were stained with crystal violet and plaques were counted. End points corresponding to 50% reduction in the number of plaques were calculated by least-squares linear regression from the slope of the best fitting straight.

The antigenicity of poliovirus extracted from spinal cords of paralysed mice was established by neutralization of serial virus dilutions with appropriate mAbs at a constant concentration. Virus–mAb mixtures were incubated for 2 h at 37°C, then overnight at 4°C. The mixtures were titrated by standard end-point assay on HEp-2 cells. Neutralization index was calculated as the difference between log virus titre in the absence and in the presence of the indicated mAb (Crainic *et al.*, 1983).

Immunization of rabbits

Approximately 10^8 p.f.u. of the appropriate virus stock were mixed with 1 vol complete Freund's adjuvant and injected intradermally on the back of each of four New Zealand white female rabbits. The animals were boosted 21 days later under the same conditions. Neutralizing antibody titres were determined 15 days after booster by a standard plaque reduction assay. 10^2 p.f.u. of appropriate virus were mixed with equal volumes of 2-fold serial dilutions of the rabbit sera, incubated for 2 h at 37°C and plated onto HeLa cell monolayers seeded in 6-well plates, using 2 wells/serum dilution. Titrations were performed as described above (see 'Preparation of viral stocks') and 50% plaque reduction end points were determined.

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Note added in proof

Since the submission of this manuscript, the structure of the chimaera has been elucidated. Detailed findings will be reported elsewhere.