Isolation of escape mutants of a hybrid poliovirus with the aid of insert-specific polyclonal antibodies

Tapani Hovi,[1]* Anita Huovilainen,[1] Andrew Murdin[2]† and Eckard Wimmer[2]

[1] *Enterovirus Laboratory and Molecular Biology Programme, National Public Health Institute, Mannerheimintie 166, FIN-00300 Helsinki, Finland and* [2] *Department of Microbiology, State University of New York, Stony Brook, NY, USA*

We constructed a hybrid type 1/type 3 poliovirus comprising the BC-loop of capsid protein VP1 of PV3/Finland/60212/84 and the rest derived from PV1/Mahoney, and cultured the virus in the presence of diluted rabbit antiserum to PV3/Finland/60212/84. Several strains isolated under this selection showed point mutations in the inserted type 3 poliovirus sequence but only in one case in the flanking PV1/Mahoney-derived RNA. These results indicate that, with the use of recombinant cDNA technology, it may be possible to study molecular interactions of defined regions of virus capsid proteins with neutralizing polyclonal antibodies.

Several of the surface-exposed regions of poliovirus capsid proteins contribute to the designated neutralization antigenic sites (NagI–IV) defined on the basis of amino acid substitutions in mutant virus strains resistant to monoclonal antibodies (Diamond *et al.*, 1985; Minor *et al.*, 1986; Page *et al.*, 1988; Wiegers & Dernick, 1992). One of these regions is the loop between beta strands B and C of capsid protein VP1, the BC-loop of VP1. In the three-dimensional virion structure, the VP1 BC-loops form a prominent pentameric bulge around the 5-fold axis at each of the vertices of the icosahedral virion (Hagle *et al.*, 1985). The VP1 BC-loop of type 2 and 3 polioviruses is highly immunogenic in mice (Icenogle *et al.*, 1986; Minor *et al.*, 1986, 1987; Roivainen *et al.*, 1990*b*) and humans (Roivainen & Hovi, 1987, 1988) after parenteral immunization while other antigenic sites appear to be relatively more important in type 1 poliovirus (Diamond *et al.*, 1985; Minor *et al.*, 1986). Interestingly, several hybrid viruses based on type 1 poliovirus structure, and which express heterologous sequences at the VP1 BC-loop, readily induce antibodies to the insert sequence (Burke *et al.*, 1988; Martin *et al.*, 1988; Murray *et al.*, 1988*a*, *b*).

The VP1 BC-loop of type 3 poliovirus shows considerable sequence variation between natural independent isolates (Minor *et al.*, 1987; Kinnunen *et al.*, 1990), which can be taken as a sign of antigenicity of this region in natural infections in man. Most type 3 strains, however, have conserved a sensitivity to host serine proteases that cleave after arginine or lysine, such as trypsin and plasmin (Minor *et al.*, 1987; Roivainen *et al.*, 1990*a*). Cleavage at a single residue in the BC-loop results in significant antigenic changes in the virions (Fricks *et al.*, 1985; Icenogle *et al.*, 1986; Roivainen & Hovi, 1987; Roivainen *et al.*, 1990*a*). The cleaved virions remain infectious and, after binding to host cell receptors, show an enhanced rate of uncoating (L. Piirainen, A. Airaksinen, T. Hovi and M. Roivainen, unpublished results), perhaps an advantageous property in competition.

An outbreak of poliomyelitis occurred in Finland in 1984–85 (Hovi *et al.*, 1986) and was caused by a specific type 3 poliovirus strain (Magrath *et al.*, 1986; Hughes *et al.*, 1986) genetically related to isolates previously found in the Mediterranean region (Pöyry *et al.*, 1990). Most of the strains isolated during the outbreak were not sensitive to trypsin but, in the follow-up of individual excretors, the virus often evolved and strains isolated late in the infection had frequently, but not always, regained sensitivity to trypsin (Huovilainen *et al.*, 1987, 1988). We postulated several years ago that sensitivity to trypsin was beneficial to the virus in one way or another and, therefore, mutants with a substitution enabling cleavage by trypsin were enriched in the intestines of infected people (Minor *et al.*, 1987; Huovilainen *et al.*, 1987, 1988).

We wanted to reproduce the 'trypsin cleavage-driven selection procedure' in cell culture with target sequence-specific antibodies in the presence of trypsin. Since

* Author for correspondence. Fax +358-0-4744355. e-mail Tapani.Hovi@ktl.fi

† Present address: Connaught Laboratories Ltd, Willowdale, Ontario, Canada.

Table 1. *Neutralization of parental type 1 and type 3 polioviruses and type 1/type 3 hybrid viruses with selected rabbit antisera**

Virus	Antiserum...	Neutralization titre AP3F	AP3S	AP1	Amino acids 87–103 of VP1†
PV1/Mahoney		< 4	< 4	65000	V T I M T V D N P A S T T N K D K
PV3/FIN/60212/84		> 32000	> 32000	< 4	- A - I E - - - E Q P - - - V Q -
PV3/Sabin		8192	> 32000	< 4	- A - I E - - - E Q P - - R A Q -
PV1M/3FK		512	64	65000	- - - - E - - - E Q P - - - V Q -
PV1M/3L		8	> 32000	65000	- A - I E - - - E Q P - - R A Q -

* Reciprocals of highest dilutions showing complete neutralization on day 6 are shown. Antisera were raised in rabbits by immunizing with purified virions (Ukkonen *et al.*, 1986) as follows: AP3F, PV3/Finland/60212/84; AP3S, PV3/USA/Saukett G/50; PV1, PV1/USA/Brunhilde.

† Positions according to PV1/Mahoney; the underlined region represents the major part of the designated antigenic site 1 (Minor *et al.*, 1986).

monoclonal antibodies specific for the VP1 BC-loop of a trypsin-resistant epidemic strain were not available, we decided to exploit hybrid virus technology to obtain a targeted immunological pressure. Intertypic and heterologous swapping of antigenic regions into this location in both type 1 and type 3 poliovirus has frequently – but not always – been successful (Stirck & Thornton, 1994). We started our cDNA construction work with the pT7XLD transcription vector, derived from pT7PV1-5 described previously (van der Werf *et al.*, 1986) and containing the entire genomic sequence of poliovirus type 1 Mahoney under the T7 promoter. Using the cartridge mutagenesis principle basically as described before for the PV1/Mahoney–PV3/Leon hybrid (PV1M/3L; Murray *et al.*, 1988*a*) a 12 amino acid stretch of the BC loop of PV1/Mahoney was replaced by the corresponding sequence of PV3/Finland/60212/84 (Kinnunen *et al.*, 1990) (Table 1).

The hybrid cDNA was transcribed with T7 RNA polymerase and the transcripts were transfected into HeLa cells using the Lipofectin technique according to the manufacturer's (Gibco) recommendations. The hybrid virus produced by the cells (PV1M/3FK) was found to have the expected sequence at the VP1 BC-loop coding region of RNA. Like several other hybrid polioviruses (e.g. Murray *et al.*, 1988*a*), PV1M/3FK replicated somewhat less rapidly than PV1/Mahoney, but reached moderate to high titres in primate cells in the second passage (not shown). The virus was neutralized by both type 1-specific and type 3-specific poliovirus antisera (Table 1). However, it is remarkable that the titre of the antiserum raised against PV3/FIN/60212/84 (AP3F) was considerably lower to the PV1M/3FK hybrid virus than to the parental PV3/FIN/60212/84 virus. This suggests that either the VP1-BC loop of the PV3/FIN/60212/84 virus had not been highly immunogenic in this rabbit or that the antigenicity of the loop was not fully retained in the PV1M/PV3 hybrid. In contrast, the PV1M/3L hybrid was still neutralized at high dilutions of an antiserum raised against the PV3/Saukett virus (AP3S) (Table 1). Titres of antisera were determined using a standard microneutralization assay with 30–300 $TCID_{50}$ of the test virus and 15000 Vero cells per well as described in detail previously (Huovilainen *et al.*, 1987).

It is interesting that some cross-reactivity could be demonstrated between the two PV1/3 hybrids (PV1M/3L and PV1M/3FK) with the use of polyclonal antisera raised against type 3 virions with sequence differences in this region (Table 1). In contrast, both PV3/FIN/60212/84 (Huovilainen *et al.*, 1987) and PV1M/3FK (not shown) are resistant to five separate VP1 BC-loop-specific neutralizing monoclonal antibodies induced by PV3/Sabin or PV3/Leon (gifts from Dr Morag Ferguson, NIBSC, UK). The low degree of cross-reactivity between the polyclonal sera might be considered somewhat surprising, as 10 out of the 12 amino acids in the insert are identical. Note, however, that the type 3-specific insert in PV1M/3L is three amino acids longer than that in PV1M/3FK, and that there is one amino acid difference between the PV1M/3L insert sequence and the immunogen (PV3/Saukett G) used to generate the AP3S serum.

In pilot experiments propagation of the PV1M/3FK hybrid virus in the presence of the PV3/FIN/60212/84-specific polyclonal rabbit serum (AP3F, dilution 1:512; Ukkonen *et al.*, 1986) was found to result in selection of variants that were reproducibly poorly neutralized with the serum. Two different approaches were used in attempts to demonstrate the effect of trypsin on the pattern of mutants selected. In the first approach, we aimed at generating a growth advantage to trypsin-sensitive variants by inoculating multiple parallel cultures of HeLa-Ohio and Vero cells with a low multiplicity of PV1M/3FK and supplementing the otherwise serum-free culture medium with AP3F serum at a 1:512 dilution and trypsin at 5 μg/ml. Other infected cultures were incubated with trypsin alone, antiserum alone or

without additions. Cultures yielding replicating viruses were harvested at complete CPE and, after three cycles of freezing and thawing, subjected to 'plaque purification' as described (Huovilainen *et al.*, 1988). There was no difference in the frequency of resistant strains isolated from the trypsin- and antiserum-containing cultures (AST-set), as compared with that from cultures containing antiserum but without trypsin (AS-set). We cannot be sure, however, if the trypsin concentration used was sufficient to cleave, under the culture conditions, the putative trypsin-sensitive mutants generated during replication. Higher concentrations could not be used because they caused detachment of the cell monolayers.

In the second approach, we tried to protect the putative trypsin-sensitive variants presumably present in a virus stock from neutralization by pretreating the stock preparation with high concentrations of trypsin (80 μg/ml) (Roivainen & Hovi, 1987). It was assumed that a trypsin-cleaved PV1M/3FK hybrid virus would be resistant to the VP1 BC-loop-specific antibodies analogous to the situation with PV1M/3L (Roivainen *et al.*, 1990*b*). After trypsin treatment and subsequent incubation of the stock with undiluted AP3F serum, the preparation was inoculated into HeLa cells without further dilution. The culture was harvested after complete CPE and the progeny virus was subjected to the same two-phase treatment. This was repeated 30 times. Plaques were purified from progeny virus samples harvested after the first round (TS1-set) and the 30th round (TS30-set) and tested for sensitivity to AP3F.

Several AP3F-resistant strains from each of the four sets (see above) were characterized further with polyclonal and monoclonal antibodies and by partial sequencing after PCR amplification (Huovilainen *et al.*, 1994) and cloning. While none of the strains was completely resistant, in the following we refer to as resistant all the strains that reproducibly showed a reduction of at least 4-fold in the neutralization titre of the AP3F serum, as compared with that obtained with the original PV1M/3FK hybrid virus. Several of the strains selected for resistance to AP3F were also poorly neutralized with the AP3S serum raised against the PV3/Saukett strain (Table 2), an observation indicating that the mutations had altered binding sites for antibodies from both sera. As expected, none of the strains was neutralized with the five PV3-specific monoclonal antibodies targeted to the BC-loop of VP1 (not shown).

About 200 nucleotides were sequenced in one-third of strains retaining their AP3F resistance upon passaging. In order to do this, RNA was extracted from about 10^6 infected cells and the VP1 BC-loop-coding region was amplified by RT–PCR essentially as described by Huovilainen *et al.* (1994), except that PV1/Mahoney-specific primers targeted to nucleotide positions 2612–2640 and 2846–2862 were used. The PCR product was cloned into the TA Cloning kit (Invitrogen) and the Sequenase 2.0 kit (United States Biochemical) with T7 and SP6 promotor specific primers (Promega) was used for sequencing. Nine separate classes of mutants were identified with different amino acid substitutions in the inserted PV3 sequence (Table 2). A substitution in the flanking PV1/Mahoney sequence was seen in only one mutant strain (B1, with lysine at position 109 replaced by glutamic acid). This was also the only mutant with a theoretical sensitivity to trypsin, because of the substituted lysine in the insert, but it was isolated from a culture incubated without trypsin in the medium.

Our failure to demonstrate trypsin-driven selection of trypsin-sensitive variants in cell culture does not prove that this kind of selection could not take place *in vivo*. It is obvious that several parameters in the microenvironment of the replicating virus can be different in the intestines from those *in vitro*. Poliovirus type 1/Mahoney is not trypsin-sensitive under the conditions used (Fricks *et al.*, 1985), even though its VP1-BC loop contains a suitable target amino acid. The conformation of the PV3/FIN/60212/84 insert in the PV1M/3FK hybrid may also prevent the action of trypsin and, hence, might not facilitate enrichment of trypsin-sensitive mutants under our culture conditions. Furthermore, the low titre of the PV3F antiserum used might not provide a selecting pressure strong enough to favour the replication of the putative resistant mutants. Indeed, several of the originally 'resistant' mutants isolated turned out, in the next passage, to be neutralized by the selecting antiserum like the parental hybrid virus.

As for the mutants selected in the present study, some observations require a comment. Substitution of aspartic acid for the homologous glutamic acid at position 95 (E95; variant E1) brought about a 4-fold decrease in the titres of both antisera suggesting slight alteration of a common epitope. Substitution of a basic lysine residue at the same position (variant B1), together with a substitution of an acidic aspartic acid for an asparagine at position 100 (N100), resulted in a virus with a neutralization pattern similar to that caused by the N100 to D substitution alone (variant F1). This is interesting as the glutamic acid is very conserved among natural type 3 isolates (E93 in type 3 poliovirus; Minor *et al.*, 1987) and is considered to be important in stabilization of the BC-loop structure of the Sabin 3 virus (Filman *et al.*, 1989). Glutamic acid-95 is hydrogen bonded to glutamine-102, and the carboxylate ion is likely to contribute to neutralization of the positive charge of lysine-103 (Filman *et al.*, 1989). Of course, we cannot know if this kind of intra-loop stabilization exists in the PV1M/3FK hybrid. Substitution of serine for proline-97 (variant 5A)

Table 2. *Neutralization and VP1 BC-loop sequences of hybrid poliovirus PV1M/3FK mutants relatively resistant to a rabbit antiserum to the PV3 parental strain PV3/FIN/60212/84**

Virus	Selection set	Antiserum...	Neutralization titre AP3FK	AP3S	AP1	Amino acids 87–103 of VP1†
PV1M/3FK			512	64	65000	V T I M E V D N E Q P T T N V Q K
5A	AST		256	8	130000	- - - - - - - - - - S - - - - - -
8A	AST		16	8	65000	- - - - - - - - - - - A - - - - -
B1	AS		4	8	65000	- - - - - - - - K - - - - D - - -
DP11	AS		8	64	130000	- - - - - - - - - - - - - D - - -
E1	AS		128	16	65000	- - - - - - - - D - - - - - - - -
F1	AS		8	16	130000	- - - - - - - - - - - - - D - - -
TBu1	TS-1		8	8	65000	- - - - - - - - - - - I - - - - -
TBa	TS-30		4	8	130000	- - - - - - - - - - - - - D A - -
PV1M/3L			8	> 32000	65000	- A - I - - - - - - - - - R A - -

* For experimental details see Table 1. Thirteen strains sequenced fell in the indicated categories. Selection set refers to the conditions used for mutant isolation: AST and AS, low multiplicity of infection in the presence of the AP3F serum (AS) supplemented with 5 μg/ml trypsin (AST); TS, treatment of the virus with 80 μg/ml trypsin followed by neutralization with AP3F and inoculation into cell cultures; progeny virus analysed after one (TS-1) or 30 rounds (TS-30) of treatment and replication.

† Positions according to PV1/Mahoney; the underlined region represents the major part of the designated antigenic site 1 (Minor *et al.*, 1986).

had no effect on neutralization by the PV3F serum but affected that by the PV3S serum. On the other hand, replacement of the adjacent threonine-98 by neutral isoleucine (TBu1) or alanine (8A) strikingly reduced the titre of both antisera. The latter mutant had an insert sequence identical with the corresponding region of the reference strain of the Finnish outbreak (Hughes *et al.*, 1986). Positions 100–101 appeared important for the neutralizing activity of the AP3F serum as a neutral to acidic substitution in either of these positions almost abolished the activity. A substitution of aspartic acid for the neutral amino acid at position 100 (F1), but not that at 101 (DP11), also affected the AP3S activity. These two positions were found to be the most frequently mutated ones in the BC-loop of VP1 among virus strains isolated during the Finnish outbreak in 1984–85 (Huovilainen *et al.*, 1988; Kinnunen *et al.*, 1990). A neutral valine → alanine substitution at 101, which is known to affect the activity of some monoclonal antibodies on the PV3/Fin strains (Magrath *et al.*, 1986; Huovilainen *et al.*, 1987, 1988) had no effect when present in a double mutant containing an acidic substitution at position 100 (TBa vs F1). It is interesting to note that no substitutions were seen at positions 99 and 102, in contrast to mutants selected with monoclonal murine antibodies *in vitro* (Evans *et al.*, 1983). Likewise, natural type 3 poliovirus isolates rarely show variation at these locations (Magrath *et al.*, 1986; Kinnunen *et al.*, 1990).

In conclusion, in spite of failing in the principal goal of these studies, we demonstrated in this paper that by exploiting recombinant cDNA technology it may be possible to obtain information on molecular interactions of selected regions in a virus capsid protein when selective pressure is applied with specific neutralizing antibodies. Naturally, this approach has obvious limitations but may be useful in situations like ours where it is difficult to obtain a suitable set of monoclonal antibodies.

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