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Random selection: a model for poliovirus infection of the central nervous system

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Mixed infections occur in the natural environment, and also result from the use of mixed live vaccines. Some recipients of the trivalent oral poliovirus vaccine develop vaccine-associated paralytic poliomyelitis (VAPP). Numerous serotypes and recombinant genotypes of vaccine-derived polioviruses may be found in stool samples from such cases. To investigate the relationship between the multiplication of various genotypes at the primary replication site in the gut and the infection outcome in the central nervous system (CNS), the viruses excreted on consecutive days by two patients with VAPP were compared with the viruses isolated from the CNS. The genotypes from stools were numerous and varied with time in both cases, suggesting a multiplication of the viruses in multiple foci in the gut. Where the CNS isolated virus clearly corresponded to one of the many viruses detected in stool, this virus was unexpectedly less neurovirulent than others isolated from stool. To assess the mechanism by which viruses with different degrees of neurovirulence are selected in the CNS, transgenic mice sensitive to poliovirus infection were inoculated extraneurally with mixtures of two phenotypically different viruses at different neuropathogenic doses. The virus(es) inducing neurological disease was then isolated from the CNS. At less than 100% input neuropathogenic dose of both inoculated viruses, individual mice were affected stochastically by the virus variants from the mixture. Extrapolated to humans, this selection pattern might explain the occurrence of CNS infections with less neurotropic viruses derived from an extraneural pool containing also highly neurotropic viruses.

Introduction

Poliovirus, the aetiological agent of poliomyelitis, is a nonenveloped, positive-stranded RNA virus belonging to the family *Picornaviridae*. The genomic RNA is approximately 7·5 kb in length and has a single open reading frame coding for the structural and nonstructural viral proteins flanked by two noncoding regions involved in the translation and the replication of the viral genome (Wimmer *et al.*, 1993).

The natural host of poliovirus is man. Poliovirus infects by the oral route and its primary multiplication site is the digestive tract. As a result, it can be isolated from nasopharyngeal swabs and routinely from stools. Gut infection is asymptomatic in most cases. In 1–2% of cases, paralytic poliomyelitis occurs due to the destruction of motor neurons in the spinal cord or brain stem (Bodian & Horstmann, 1965). The mechanism of virus spread to the central nervous system (CNS) from the digestive tract is still controversial. Two possibilities have been envisaged for poliovirus invasion of the CNS: by crossing the brain–blood barrier during viraemia (Bodian & Howe, 1955) or by migration along nerve fibres from putative secondary replication sites in muscle (Wyatt, 1990; Ren & Racaniello, 1992).

Two vaccines are used to prevent poliomyelitis: the inactivated poliovirus vaccine and the oral attenuated poliovirus vaccine (OPV). The OPV is widely used and is the preparation of choice for interrupting the circulation of wild poliovirus in human populations. It consists of three attenuated poliovirus strains (the Sabin strains), one of each serotype (Sabin & Boulger, 1973). Following the administration of the OPV, a small number of individuals develop vaccine-associated paralytic poliomyelitis (VAPP) (1 case/520000 first doses) (Nkowane *et al.*, 1987). VAPP is believed to be due to migration to the CNS of vaccine-derived strains which have lost the attenuated phenotype during multiplication in the

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digestive tract. In a recent study, half of the strains isolated from the CNS of serologically immunocompetent VAPP patients did not have a high neurovirulence, as tested in transgenic mice sensitive to poliovirus infection, suggesting that the increased neurovirulence may not be the only mechanism causing disease (Georgescu *et al.*, 1994).

In cases of classical epidemic poliomyelitis a single virus strain generally establishes the infection. Recipients of OPV receive simultaneously large doses of the three poliovirus serotypes. In addition, each vaccine serotype is a mixed population (Chumakov *et al.*, 1991) formed, as for all RNA virus populations, of related genomes differing by one or several mutations (quasispecies) (Holland *et al.*, 1982). During multiplication in the gut, the genomes of the attenuated vaccine strains both revert to the wild type at some genomic loci and also recombine (Minor, 1992). In recipient VAPP cases, both various different serotypes and also complex mixtures of genotypes (recombinant and nonrecombinant) of the same serotype can be isolated simultaneously from stool samples (Georgescu *et al.*, 1994). Thus, the administration of the trivalent OPV leads to *in vivo* mixed infection. It is, however, unclear which of the several genotypes detected in stools causes paralysis and by what mechanism.

In this study, we investigated the impact of a mixed infection at the primary replicative site for the development of the CNS infection. We thus analysed in detail two recipient VAPP cases of which both CNS samples and stool samples containing mixtures of viruses were available. To identify the relationship between neurovirulence and virus spread to the CNS from an extraneural location, we first analysed the neurovirulence of individual strains isolated from a stool mixture. This parameter did not appear to be sufficient for the selection of a variant in the CNS, as the strain detected in the CNS in one case did not correspond to the most neurovirulent variant from the stool mixture. The mechanism of selection in the CNS was further analysed by using transgenic mice as an *in vivo* model in which mixtures of viruses with known neurovirulence were intraperitoneally inoculated. Using this animal model, we also investigated whether the evolution of the CNS infection differs after inoculation of isolated strains or of mixtures of strains.

Methods

■ **Cells and viruses.** HEp-2c cells were grown in monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal calf serum.

Viruses from stool and CNS (cerebrospinal fluid) samples of VAPP cases were isolated on primary monkey kidney cells and viral stocks obtained after two passages on HEp-2c cells at 34 °C.

Viruses were recovered from the spinal cord of paralysed or dead infected mice. The spinal cord was homogenized in 0·6 ml DMEM, subjected to two cycles of freeze–thawing and clarified. HEp-2c cells seeded in 35 mm dishes were infected with 100 µl of spinal cord supernatant. The cells and supernatants were collected when complete cytopathogenic effect was observed and viral stocks were prepared.

To obtain high-titre viral stocks for the inoculation of mice, virus was concentrated by ultra-speed centrifugation (Georgescu *et al.*, 1994). Plaque titration was performed on HEp-2c cell monolayers, at 34 °C.

■ **Genomic analysis.** Reverse transcription of viral RNA and PCR were performed as previously described (Balanant *et al.*, 1991). RFLP of PCR-amplified fragments was analysed to detect recombinant genotypes (Furione *et al.*, 1993) or the recombination site (Georgescu *et al.*, 1994). Genomic RNA sequencing (Fichot & Girard, 1990) was used to map precisely the site of the recombination junction and to investigate genomic positions known to revert to wild genotype in vaccine strains. The nucleotide positions for the Sabin strains are indicated according to their genomic maps (Toyoda *et al.*, 1984).

■ **Analysis of mixtures of viruses.** After virus isolation from clinical specimens, mixtures of viruses of different serotypes were separated by a standard microplate assay in the presence of type-specific neutralizing sera (serotyping). For each serotype, viral suspensions were recovered independently from the last three tenfold dilution wells showing a cytopathogenic effect in the presence of heterotypic sera, passaged once and retested for the serotype.

The mixtures of homotypic strains with recombinant and non-recombinant genotypes were detected by an RFLP assay in the $3D^{pol}$ genomic region (Georgescu *et al.*, 1994). Ten to 20 individual viruses were isolated from the mixture by plaque purification followed by PCR–RFLP analysis. Viral stocks for the cloned genotypes were prepared after an additional plaque purification.

The relative amounts of different recombinant genotypes in mixtures were determined by adapting the mutant analysis by PCR and restriction enzyme cleavage method (MAPREC) (Chumakov *et al.*, 1991). A 290 bp fragment was amplified from the $3D^{pol}$ region using the universal primers UG7 and UG8 (Furione *et al.*, 1993). PCR and ^{32}P-labelling of the PCR product were performed as previously described (Chumakov *et al.*, 1991). The labelled PCR fragments were purified from excess labelled primers using the Wizard PCR Preps Purification System (Promega) and cut with *Hae*III restriction endonuclease. The components from mixtures obtained from clinical samples were quantified by this technique after virus isolation. Mixtures from mouse spinal cord were characterized both directly from the specimen and after virus isolation.

■ **Rct marker.** The reproductive capacity at high temperature (Rct marker) was evaluated using an end point titration micromethod (Georgescu *et al.*, 1994). The Rct value was calculated as the difference, after 6 days of incubation, between the $\log_{10}$ virus titre ($TCID_{50}$/ml) at 37 °C and that at 39·5 °C. Viruses were considered thermosensitive (ts) if the Rct value (between 37 °C and 39·5 °C) was $> 4{\cdot}00$ and thermoresistant (non ts) when the Rct value was $< 2{\cdot}00$.

■ **PVR-Tg mouse neurovirulence.** Viruses were tested for neurovirulence in homozygous transgenic mice (PVR-Tg21) susceptible to poliovirus (Koike *et al.*, 1991). Groups of six 5 to 6-week-old mice were inoculated intraperitoneally (IP) with a fixed dose of each virus: 10^8 p.f.u. per mouse in 0·5 ml (IP-MHT test) (Georgescu *et al.*, 1994). The mice were observed daily for 14 days for the onset of paralysis or death and, for each virus, the mean healthy time (MHT) was recorded. The MHT is the mean period of disease-free survival for all the mice inoculated with the same virus. Viruses were considered highly neurovirulent for MHT < 8 days, intermediary for MHT between 8 and 14 days, and attenuated for MHT $= 14$ days.

For two viruses (2-IVs′3 and 2-IVs″3), groups of six male mice were inoculated intraperitoneally with tenfold virus dilutions in PBS (0·5 ml per mouse) so as to cover the virus titre range causing disease in 100% to 0% of mice (IP-PD_{50} test). For these two viruses an intracerebral PD_{50} test was also performed by inoculating 30 µl of virus in groups of six 5-

week-old mice (three males and three females). When the 100% paralytogenic dose was not available, the highest obtainable titre was inoculated (10^9 p.f.u. per mouse). The virus dose inducing paralysis or death in 50% of mice (PD_{50}) after a 21 day follow-up was calculated by the method of Reed and Muench (Reed & Muench, 1938). For the paralysed mice, the disease outcome and duration were recorded.

Controlled mixtures of viruses were prepared by mixing in different proportions two individual virus stocks of known titre and were used to inoculate intraperitoneally groups of male mice. All the suspensions were titrated before and immediately after inoculation. Infected mice showing nonevolving paralysis for 3 days were sacrificed and dead mice collected. Their spinal cords were removed and virus was isolated.

Results

Analysis of the viruses from the human clinical specimens

Two VAPP cases (2-III and 2-IV) for which mixtures of poliovirus strains were present in the stool specimens were used to investigate the selection of poliovirus in the human CNS. The clinical and epidemiological features of these cases were previously described (Georgescu *et al.*, 1994). For each case, three stool specimens recovered on consecutive days and one CNS specimen (cerebrospinal fluid) were available. An isolate from nasopharyngeal swab was also available from case 2-IV. Various poliovirus serotypes in the samples were detected and separated by neutralization with polyclonal sera. Homotypic viruses of different genotypes, defined by their recombination patterns, were identified by PCR–RFLP analysis of the $3D^{pol}$ genomic region and separated by plaque purification. The genotype of each cloned virus was determined by PCR–RFLP analysis and partial genomic RNA sequencing (Fig. 1).

From the case 2-III, viruses of two serotypes, type 2 and type 3 were isolated from the stool and CNS samples (Fig. 1). Within each serotype, many genotypes were identified. Only one genotype, an S2 × S1 recombinant was detected in all stool specimens (s′2, s′3 and s′5). Other genotypes were detected in two of the three stool samples: the S2 × S3 × S1 (s″3 and s″5) and S2 × S3 × S2 × S3 (s‴3 and s‴5) genotypes from the day 3 and 5 post-paralysis samples, and the S2 × S3 (s°2 and s°5) and S3 × S1 × S3 (s⁗2 and s⁗5) genotypes from the day 2 and 5 post-paralysis samples. Some genotypes were detected only in one stool sample: s″2, s⁗2 and s⁗5. The genotypes isolated from the CNS were different from all the genotypes isolated from stools. In the S3 × S2 × S3 recombinant genotype isolated from the CNS (c′2), neither the S3 × S2 nor the S2 × S3 junction (within the fragment 6825–6865) corresponded to any of the cross-over sites identified in other genotypes (s⁗5 or s°2, s°5). In case 2-III, some genotypes were repeatedly identified in different samples whereas others appeared or were lost rapidly demonstrating a high variation of virus excretion. This might explain why the genotypes found in the CNS sample were not detected in the stool. However, the insufficient sensitivity of the methods for the detection of rare genotypes relative to much more abundant ones within a mixture could also account for this lack of identification.

In the case 2-IV, the pattern of virus excretion over three consecutive days (day 3, 4 and 5 post-paralysis) was investigated and the genotypes of viruses were precisely determined by viral RNA sequencing of the junction regions (Fig. 1). A type 3 recombinant virus was detected only in the first stool isolate. Among the three type 2 genotypes detected in the first stool isolate, only the nonrecombinant virus (s°3) was detected in the day 4 sample (s4). It was not found in the third stool isolate which contained two recombinant S2 × S1 genotypes also isolated from the first stool sample (s′3, s″3 and s′5, s″5). As for case 2-III, this showed that the genotypes excreted during poliovirus enteral infection may vary on consecutive days. The nasopharynx isolate contained a different recombinant S2 × S1 strain (n3), evidence that the different serotypes administered had multiplied in the upper digestive tract. The nasopharynx strain was different from the stool strains and from the strain isolated from the CNS. The CNS isolate (c3) was similar to one of the S2 × S1 recombinant viruses isolated from stool suggesting that it migrated to the CNS from the lower digestive tract.

Genomic sequencing was also used to investigate the positions involved in reversion to neurovirulence [481 and those of the codon 143 of VP1 for Sabin 2 (Ren *et al.*, 1991; Macadam *et al.*, 1993), and 6203 for Sabin 1 (Christodoulou *et al.*, 1990)]. All had reverted to the wild genotype in each virus tested. Other mutations were also detected. In one virus (s′5) there was a missense mutation in an antigenic site (position 2776 changing Lys-1099 → Gln). Mutations were used to ascertain the genotype identity (for the nonrecombinant viruses there were mutations in positions 398 and 6678 in both s°3 and s4) or to distinguish isolates [there was a mutation in position 5350 in the CNS isolate but not in two viral clones sequenced for each of the stool corresponding genotype (s″3 or s″5)]. In contrast, the reversion at position 7441 and the silent mutation in position 2865 were found in only one of the two clones of the same genotype from stool day 3 (s″3), demonstrating a 'quasispecies' population within the genotype.

The presence and relative amounts of the type 2 genotypes in samples from case 2-IV were evaluated by a quantitative RFLP assay: MAPREC (Fig. 2). The S2 × S1 recombinant genotype (s″3 and s″5) corresponding to that isolated in the CNS (c3) was the most abundant genotype in the stool samples recovered the third and the fifth day post-paralysis, accounting for 71% and 93% of all type 2 genotypes, respectively. Although absent from the fourth day sample, this finding suggested a possible dose-related passage to the CNS of this genotype.

The phenotypic characteristics of the two recombinant S2 × S1 viruses (s′3 and s″3) isolated from the day 3 stool sample of case 2-IV were compared to the CNS isolate (c3)

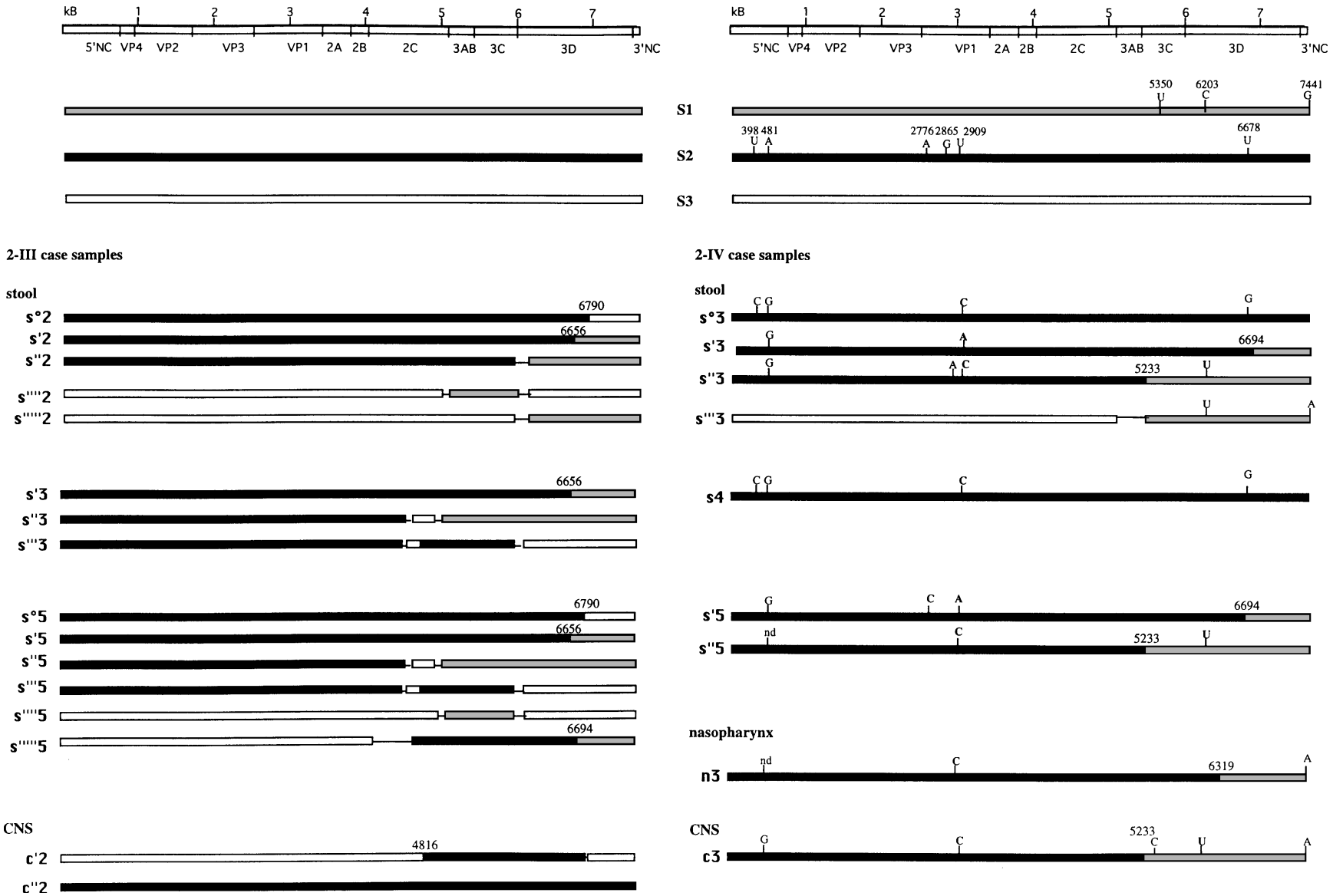

Fig. 1. Genetic organization of viruses isolated from two VAPP cases. The poliovirus genome is represented on the top line showing the 5′ noncoding (5′NC) and 3′ noncoding (3′NC) regions and the regions coding for structural capsid proteins (VP1 to VP4) and nonstructural proteins (2A to 3D). The genome length is indicated (kb). Schematic representations of the Sabin 1, 2 and 3 strains (S1, S2, S3) are shown (▨, ■, □, respectively). Some of the nucleotide positions known to revert to the wild genotype are indicated for S1 and S2 strains (for references, see text). Other positions corresponding to mutations in the VAPP isolates are also indicated. The genomes of poliovirus strains isolated from the two VAPP cases are represented. The viruses are identified by the initial of the respective sample followed by primes (′) when several viruses were isolated from the same sample and by a number indicating the day after paralysis when the sample was collected. The structure of the genomes was deduced from RFLP patterns and from partial genomic RNA sequencing. Lines indicate junction regions determined by RFLP and numbers specify the downstream nucleotide position of the recombination junction as determined by genomic sequencing. The nucleotide positions screened for reversion are indicated for the strains of case 2-IV only where different from those of the Sabin strains.

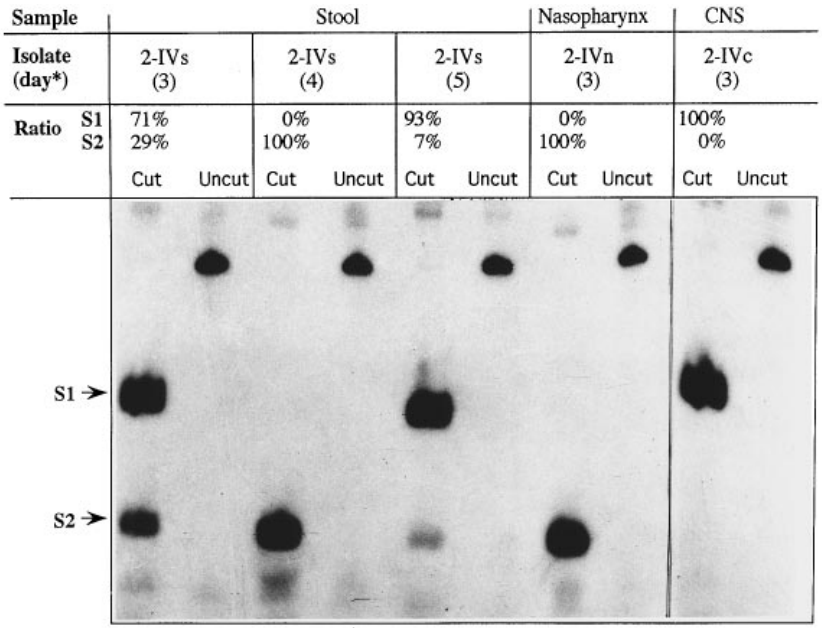

Fig. 2. Proportions of different type 2 genotypes in multiple clinical samples collected from the VAPP case 2-IV. Five type 2 poliovirus isolates recovered 3, 4 and 5 days after the onset of the paralysis (*) in this VAPP case were subjected to quantitative RFLP analysis (MAPREC). The amounts of nonrecombinant and S2 × S1 recombinant genotypes of the type 2 polioviruses present in mixtures were determined by an RFLP assay (*Hae*III endonuclease digestion) of the PCR fragment from between nucleotides 6086 and 6376 of the $3D^{pol}$ region.

Table 1. Phenotypic characteristics of the CNS and stool isolates from case 2-IV day 3 post-paralysis

Isolate	Viruses*	Rct at 39·5 °C (Δlog_{10} $TCID_{50}$/ml)	Neurovirulence		
			MHT (days)	IP-PD_{50}† (log_{10} p.f.u. per mouse)	Disease outcome in mice
2-IVs(3)	s′3 = H	0·50	4·17	6·82 ± 0·34	Immediate death
	s″3 = C	4·16	9·00	⩾ 8·82 ± 0·02	Paralysis
2-IVc(3)		2·75	8·67		Delayed death

* These viruses are named H (hot) and C (cold) based on their phenotypic properties.
† Values are means ± standard deviation from two tests on male mice.

(Table 1). As evaluated by the Rct test performed at 39·5 °C, these viruses had different thermosensitive phenotypes: s′3 was non ts whereas s″3 was ts. The neurovirulence evaluated by the IP-MHT test correlated with the ts phenotype: the neurovirulence of s′3 was high and that of s″3 low. Similarly, their PD_{50} values following intracerebral (data not shown) and intraperitoneal inoculation differed by at least 3 and 2 log_{10} p.f.u. per mouse, respectively. The clinical courses of the disease caused by these two viruses in mice differed regardless of the inoculated dose. All the mice paralysed after inoculation with s′3 died rapidly (mean survival time of 1·3 days) and all but one (9/10) inoculated with s″3 survived with paralysis until the end of the test. Therefore, considering their different phenotypes, these viruses are hereafter referred to as H (hot) for s′3 and C (cold) for s″3. It was intriguing that the genotype of the CNS strain (c3) corresponded to that of C. Phenotypically, c3 was also closer to C although less ts and more neurovirulent than the C virus (as evaluated by the MHT value and the disease outcome in paralysed mice where death occurred after a mean delay of 4·3 days) (Table 1). It was thus interesting to determine what chances of spread to the CNS had the C virus from a mixture with the H virus.

Analysis of the selection of viruses in the CNS of mice

To study the mechanism of selection in the CNS for viruses from an extraneural pool, various mixtures of viruses were inoculated intraperitoneally into homozygous PVR-Tg21 mice. To exclude a possible bias introduced by sex, for these experiments, only male PVR-Tg mice were used. The inoculated mixtures contained different ratios of the twice-plaque purified H and C S2 × S1 recombinant viruses. The H and C viruses were chosen for the following reasons: (i) they

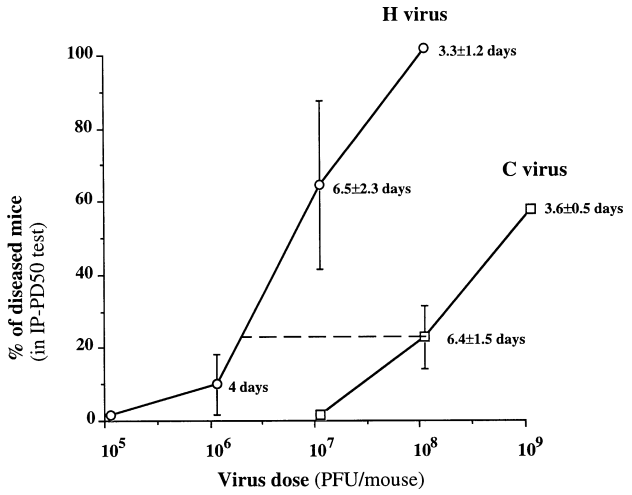

Fig. 3. Neurovirulence of the H and C viruses inoculated intraperitoneally in male PVR-Tg mice. The neurovirulence levels for the H and C viruses are represented as the mean percentage, shown with standard deviation, of diseased mice calculated by the Reed and Muench method as a function of the inoculated dose following two IP-PD_{50} tests. Mean values and standard deviations for the disease incubation time are indicated for each virus at the inoculated doses. The dotted line indicates the H virus neuropathogenic dose which is equivalent to the 10^8 p.f.u. per mouse dose of the C virus.

were simultaneously present in stools but only the one corresponding to the C genotype was isolated from the CNS of a patient with VAPP; (ii) they caused different dose-independent disease outcomes in mice, providing a clinical marker for their specific pathogenicity (Table 1); (iii) they were of the same serotype, such that different immune responses to different serotypes could not interfere with virus selection and multiplication in the CNS; (iv) they had different genotypes precluding a transition from one to the other by mutation within the CNS. Viruses multiplying in the CNS after inoculation were isolated from the spinal cord of the paralysed or dead mice and their genotype(s) were identified by PCR–RFLP analysis and quantitative MAPREC. In all these tests, to exclude recombination between the C and H viruses, the fragment containing the VP1–143 codon was sequenced for each individual genotype isolated from the spinal cord. This fragment has different nucleotide sequences for the two viruses (see Fig. 1) and is situated 5′ to the common recombination sites. The sequences of the spinal cord isolates matched the RFLP profile in the $3D^{pol}$ region as in input C and H viruses, attesting that there had been no detectable recombination between the two genotypes in the inoculated animals.

To compare the input neuropathogenic doses for the two viruses at different concentrations, the neurovirulence of the C and H viruses was assessed in two IP-PD_{50} tests and expressed as the mean percentage of diseased mice calculated by the Reed and Muench method at each inoculated dose (Fig. 3). An equivalent paralysis incidence was induced by the 10^8 p.f.u. per mouse dose of the C virus and a dose between 10^6 and 10^7 p.f.u. per mouse of the H virus as indicated by the intersection of the neurovirulence curves of the C and H viruses. Another parameter important in estimating the neurovirulence is the mean incubation time following inoculation. This was approximately equal, 6·4 days and 6·5 days at 10^8 p.f.u. per mouse for C virus and at 10^7 p.f.u. per mouse for H virus, respectively (Fig. 3). As the MHT value reflects both the incidence and the incubation time of the disease, it may be a better indicator than the percentage in the IP-PD_{50} test for evaluating competition between viruses. This indicator was further used.

Four mixtures were inoculated (M8/5, M8/6, M8/7, M8/8), each containing a fixed amount of C virus (10^8 p.f.u. per mouse) and one of a series of amounts of the H virus (from 10^5 to 10^8 p.f.u. per mouse) (Table 2). In parallel, the same doses of single viruses were inoculated as controls. After inoculation of mixtures, the neurovirulence was determined individually for C and H viruses considering only the mice having the disease outcome specific for C (paralytic residue) or H (death), respectively (individual neurovirulence) or globally for the whole mixture by considering all the diseased mice (overall neurovirulence). In all cases where a single genotype was detected by RFLP, the disease outcome corresponded to that genotype. When two genotypes were detected in equal amounts in the CNS, the mouse died, suggesting a dominance of the H virus phenotype.

The pathogenicity of the H virus present in mixtures was dose-dependent and paralleled that of the single H virus (Fig. 4). Its effects were thus independent of the presence of the C virus.

The effects of the C virus inoculated in a mixture with the H virus were more complex. Its phenotype expression was dominated by the H phenotype as seen for the M8/7 mixture. In M8/8 inoculated mice, only trace amounts of the C genotype (less than 5 %) were evidenced by MAPREC and in only one of the five dead mice. In a preliminary experiment (unpublished results), two of three dead mice inoculated with an 8/8 mixture and one dead mouse inoculated with an 8/6 mixture presented trace C genotype in addition to the major (> 95 %) H genotype. The two spinal cord isolates from the 8/8 mixture with trace amounts of the C genotype were plaque-purified and one C clone was identified in each. There was a reversion at nucleotide position 7441 in one of these C clones indicating that the C genotype also replicated in the mouse CNS. At 10^8 p.f.u. per mouse, the C virus had a mean incubation time of 6·4 days whereas that of the H virus was 3·3 days (Fig. 3). At this dose, the H virus killed quickly 100 % of mice which might explain why only small amounts of the C genotype, although replicating, were found in these animals.

The incidence of the C genotype in M8/8 and M8/6 mice was similar to that in the controls. In M8/7 and M8/5 mice, the C genotype-induced neurovirulence was only slightly higher than that in mice inoculated with C virus alone (Fig. 4). However, the incidence of C genotype was significantly higher (Table 2). This difference might be due to variations within the test (the titre of the M8/5 mixture was higher than that of the

Table 2. Selection of poliovirus genotypes in the CNS of male PVR-Tg mice inoculated intraperitoneally with various mixtures of two strains

Test	Inoculum		Dose p.f.u. per mouse C*	Dose p.f.u. per mouse H*	Diseased/ inoculated mice	Genotypes recovered from the CNS of diseased mice† C	Genotypes recovered from the CNS of diseased mice† H	Genotypes recovered from the CNS of diseased mice† Mixed	Neurovirulence (MHT, days)‖ Individual C	Neurovirulence (MHT, days)‖ Individual H	Neurovirulence (MHT, days)‖ Overall
1	Controls		10^8		3/6				10·5		
				10^5	0/7					14·0	
	Mixture	M8/5	10^8	10^5	7/10	6‡	0		8·4	14·0	8·4
2	Controls		10^8		1/5				12·4		
				10^6	0/6					14·0	
	Mixture	M8/6	10^8	10^6	3/10	2	1		12·6	13·0	11·6
3	Controls		10^8		1/5				12·2		
				10^7	2/5					10·8	
				10^8	5/5					2·8	
	Mixture	M8/7	10^8	10^7	5/6	2	2	1§	11·6	9·5	7·1
		M8/8	10^8	10^8	5/5	0	5		–¶	2·6	2·6

* C and H represent inoculated viruses (see Table 1 for details).
† Refers to the isolated genotype as determined by PCR–RFLP analysis.
‡ Only six of the seven paralysed mice were sacrificed. The remaining mouse survived with paralytic residue.
§ The proportions of the two recovered genotypes were roughly equal. As the mouse died, its incubation time was included only in the individual MHT value for the H virus.
‖ Individual MHT considers only mice having a specific disease outcome: paralytic residue for the C virus or immediate death for the H virus. Overall MHT considers all the diseased animals regardless of the disease outcome.
¶ The MHT value for the C virus could not be calculated since all animals died shortly after inoculation.

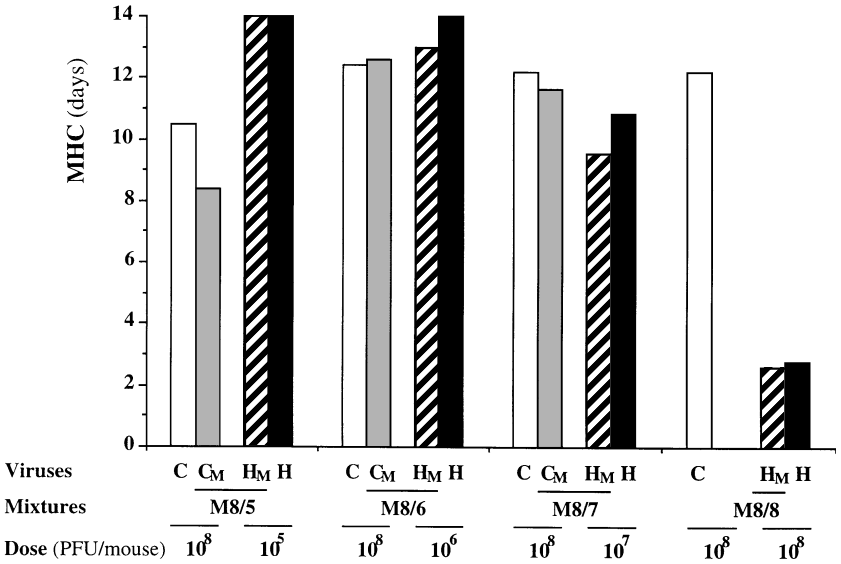

Fig. 4. Effects of C and H viruses inoculated in mixtures or independently. The neurovirulence is expressed as mean healthy time (MHT) calculated for each virus as the mean of life spans without clinical signs for all inoculated mice. The MHT values (see Table 2) for viruses after inoculation in mixtures (C_M and H_M) or for their corresponding single-inoculated controls (C and H, respectively) are represented for each mixture. In the four inoculated mixtures as well as in the controls, the dose of the H virus varied whereas that of the C virus was kept constant.

control) or to enhanced permissivity for the C virus in the presence of H virus.

For the mixtures where both clinical phenotypes were observed in diseased animals (M8/6 and M8/7), the overall neurovirulence of the mixture was higher than that of the individual viruses or of the controls (Table 2).

Discussion

In this study, we examined the evolution of mixed infections, with special emphasis on the mixed infection encountered in VAPP. Beside the mixed infection with vaccine strains developing in OPV recipients, mixed vaccine and wild-

type poliovirus infections can occur during the intensive oral vaccination programmes in epidemics, and wild-type mixed infections are not improbable in endemic areas. There are other natural infections, for example human immunodeficiency virus (HIV) and influenza virus infections, that may be induced simultaneously by many genotypes (Katz & Webster, 1988; Sala *et al*., 1994).

In VAPP, the stools from recipient VAPP patients frequently contain mixtures of homotypic vaccine-derived polioviruses (Georgescu *et al*., 1994). We report large variation in excreted genotypes in stool samples from consecutive days in two cases of VAPP where such mixtures were present. The pattern of genotype excretion analysed in the two VAPP cases varied within a short period of time (3 or 4 days). Several genotypes were replaced by others and some even reappeared. An excreted genotype can be replaced by a new genotype in OPV recipients after a longer period of time (Minor *et al*., 1986; Minor, 1992). This slow emergence of new variants could be explained by temporal evolution due to competitive selection although the selection pressures leading to these changes are unclear. However, this cannot account for the abrupt and complex changes we observed and which led us to imagine a spatial evolution of the infection. For case 2-IV, the isolation from the nasopharynx of a strain different from those excreted in stool suggested at least two digestive replicative foci. In HIV infection different genotypes evolve in adjacent splenic foci (Cheynier *et al*., 1994). By analogy, enteral poliovirus infection may consist of multiple replicative foci. In these foci, viruses of one or several viral populations may multiply competitively and intermittently release different variants which may successively seed other intestinal areas or be excreted in the faeces. The multitude and turnover of the foci may result in a mixed and fluctuating excretion of viruses with various phenotypic properties. This is consistent with the pattern observed in our two VAPP cases. Alternatively, the presence of a single strain in stools after vaccination may reflect a better fitness of the strain and consecutive invasion of the remaining intestinal areas or the shutoff of previous replicative foci. The individual host response may also affect the pattern and duration of virus excretion (Minor *et al*., 1986).

We investigated the neurovirulence of the viruses from the stool samples in an attempt to predict the strain establishing the CNS infection. We found that the CNS strain did not correspond to the most neurovirulent virus from the gut. Possibly, the more neurovirulent variants in the gut appeared after CNS invasion. The hypothesis of focal evolution of the intestinal infection could imply invasion of the CNS by whichever strain(s) escaped from a focus to the systemic circulation. Nevertheless, it is possible that several viruses compete to invade the CNS or that the neurotropic characteristics (neuroinvasiveness, neurovirulence) of the virus influence its selection in the CNS (Morrison & Fields, 1991). The mechanism of virus selection in the CNS was further analysed by extraneural inoculation of mixtures of viruses in PVR-Tg mice. The intraperitoneal inoculation of the viruses respected the CNS as a separate compartment and permitted assessment of neuroinvasiveness by both routes of entry (nervous and haematogenic), mimicking the secondary viraemia incriminated for poliovirus infection of the CNS (Sabin, 1956). Although we were interested in reproducing the spread of poliovirus from the digestive tract this could not be studied because PVR-Tg mice are not sensitive to poliovirus infection by the oral route (Koike *et al*., 1991). To minimize the effects of host variables, we inoculated homotypic strains and used intratest controls and hosts of the same sex and age. We used two viruses with different genotypes present within the viral population isolated from stool the same day post-paralysis as the CNS strain (VAPP case 2-IV). Although both viruses displayed the genomic mutations causing full reversion to the neurovirulence of the parental Sabin 2 virus, they expressed different degrees of neurovirulence by both intraperitoneal and intracerebral inoculation of PVR-Tg mice. This was probably due to the different recombination patterns or to the presence of unidentified suppressive mutations.

When the input neuropathogenic dose of one of the viruses was null, the other virus was responsible for 100% of the induced pathology (M8/5). When both viruses multiplied in the same host, the pathology corresponded to that caused by the most virulent (in our case the H virus) regardless of the inoculated dose. As expected, this was true for the cases where the H virus had a shorter incubation period (M8/8) but also for the case with equal incubation times for the two viruses (M8/7). In the cases where neither of the strains were 100% paralytogenic (M8/6 and M8/7), each of them could induce paralysis, depending on their input individual neuropathogenic dose. More important, even when one of the strains had an input neuropathogenic dose higher than the other but below 100% paralytogenic, the low neurovirulent strain was detected in some hosts (M8/7). Schematically, the selection of strains inoculated at less than 100% paralytogenic doses is depicted in Fig. 5. These findings suggest a random selection in the CNS of the virus variants from an extraneural pool. This selection was dose-dependent but mixture-independent: that is, mice reacted as if they were separately inoculated with each virus at a given dose. If these results can be extrapolated to humans, they may explain observations such as for case 2-IV, where the more abundant but not the most neurovirulent virus from the gut spread and multiplied in the CNS, or case 2-III, which presented a dual infection of the CNS.

In a study of the *in vivo* selection of virus from an intracranially inoculated mixture of two viruses with different adaptations for the mouse CNS, the most adapted outcompeted the less adapted virus (Holland *et al*., 1991). The input neuropathogenicity of these viruses was unknown but, as all the inoculated mice died with neurological disease, this may correspond to our M8/8 case, with the more adapted strain being 100% neuropathogenic. In the field, there are few if any situations in which an infection with a neuropathogenic virus

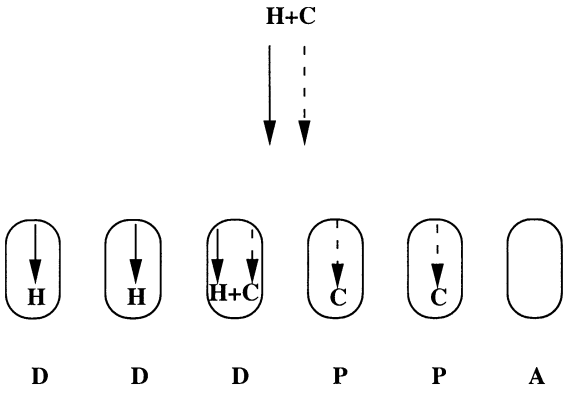

Fig. 5. Selection and multiplication in the CNS of viruses inoculated at below 100% neuropathogenic doses. Solid and dotted arrows follow the respective courses of each of two viruses (H and C, see text) used together to inoculate transgenic PVR-Tg mice. The CNS of each mouse is represented by an oval. Outcome of the disease: dead (D), paralysed (P) or asymptomatic (A). This figure depicts the results obtained by inoculation of the M8/7 mixture.

is induced by a 100% neuropathogenic dose. Even in epidemic poliomyelitis, the incidence of paralytic disease is 1–2% of poliovirus infections. Thus, the selection of neurotropic strains is more pertinently studied at less than 100% paralytogenic doses, as for example our 8/5, 8/6 and 8/7 genotype mixtures. To our knowledge, this is the first time that the selection in the CNS of viruses from an extraneural pool has been investigated as a function of input parameters.

Our unpublished experiments with mixtures of mutant viruses suggested that the multiplication in the CNS of mutant variants may also depend on random selection. For mutant viruses, selection should be distinguished from the accumulation of mutations during multiplication within the CNS. The mutation rate *in vivo* may be monitored following inoculation of infectious cDNA (Pelletier *et al.*, 1995). Random selection might thus explain the observation of CNS strains being less virulent than those in stools also for VAPP patients presenting a single pathogenic strain (Georgescu *et al.*, 1994). Therefore, considering RNA quasispecies infections as mixed infections, the random selection mechanism might lead to the selection of certain variants and account for differences of pathology within a host population that are generally supposed to be due to variability in individual reactivity.

The selection of the variants within a population was studied using *in vitro* models (de la Torre & Holland, 1990; Clarke *et al.*, 1994). The selection of variants by numerous passages in cell culture depends on the initial relative fitness and the population size of each of the virus populations in competition (de la Torre & Holland, 1990). These *in vitro* studies have contributed to our understanding of the basic laws governing the selection of strains but the *in vivo* mechanisms of selection could only be inferred from the *in vitro* data. Our study offers insights into the mechanisms of *in vivo* selection that can determine the disease outcome of infected individuals.

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