Review article

The molecular biology of poliovaccines

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Introduction

The earliest record of a viral disease is believed by some to be a funerary stele from Middle Kingdom Egypt, from the Ramesside period about 1300 BC, currently in the Ny Carlsberg Glyptothek, Copenhagen. It shows the priest Rom with his family, and the withered limb and dropped foot typical of paralytic poliomyelitis are clearly visible (Ghalioungui, 1973). During the 20th century poliomyelitis has occurred in epidemics in developed countries, which led to intensive and successful efforts to develop vaccines, firstly the formaldehyde-inactivated virus preparations of Salk (1960), then the live attenuated vaccine strains of Sabin & Boulger (1973). The effects of the vaccines, where they have been used appropriately, has been dramatic, and poliomyelitis is now extremely rare in countries such as the U.S.A. (Fig. 1) and U.K. In the U.K. the average number of cases was about 4000 per year during the 1950s, whereas now it is of the order of two or three. As a comparison, Creutzfeld-Jakob disease, which is often cited as a very rare condition, is responsible for about 25 deaths per annum. The continued occurrence of poliomyelitis in other parts of the world makes vaccination still necessary; the WHO has declared its goal of eliminating the disease from the world by the year 2000.

The efficacy of the vaccines has a number of implications. The first is that it demonstrates that paralytic poliomyelitis is indeed caused by poliovirus, with the corollary that the virus must be antigenically stable and not drift over time to any significant degree. Thus the vaccines, where they have been used appropriately, have been dramatic, and poliomyelitis is now extremely rare in countries such as the U.S.A. (Fig. 1) and U.K. In the U.K. the average number of cases was about 4000 per year during the 1950s, whereas now it is of the order of two or three. As a comparison, Creutzfeld-Jakob disease, which is often cited as a very rare condition, is responsible for about 25 deaths per annum. The continued occurrence of poliomyelitis in other parts of the world makes vaccination still necessary; the WHO has declared its goal of eliminating the disease from the world by the year 2000.

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Properties of poliovirus

Polioviruses are members of the Picornaviridae and are the archetypal members of the enterovirus genus. They occur in three distinct serotypes, designated type 1, type 2 and type 3, and replicate chiefly in the gut. The virus particle is about 27 nm in diameter as measured by negative staining electron microscopy, and consists of 60 copies each of the virion proteins VP1, VP2, VP3 and VP4 arranged with icosahedral symmetry and containing a single strand of infectious positive-sense RNA. The virion has no lipid layer, although a myristate group is covalently linked to each VP4 molecule, and a molecule of a 16 carbon lipid, possibly sphingosine, is inserted into each molecule of VP1. The atomic structures of several polioviruses have been elucidated (Hogle et al., 1985; Filman et al., 1989; J. Hogle, personal communication).

The genomic RNA is approximately 7500 nucleotides in length terminating at the 3' end in a polyadenylate tail of 70 to 100 residues, and at the 5' end in a small protein, VPG, which is covalently bound to the RNA. The functional layout of the genome is shown in Fig. 2. A 5' non-coding region of about 740 residues precedes a single large open reading frame; the 5' portion encodes the structural proteins. The remainder of the proteins are non-structural and include proteases such as 2A, which cleaves the structural from the non-structural proteins, is believed to be involved in the shut-off of host protein synthesis and can also cleave within the polymerase protein 3D. Protein 3C (whose active form may be the precursor 3CD), also has proteolytic activity, making most of the other cleavages including those which give rise to the individual structural proteins VP1, VP3 and VP0 (uncleaved VP2 and VP4). Other proteins are involved in RNA replication. The genome proper
concludes with a short 3' non-coding region of 70 bases, after which there is a poly(A) tract of 70 to 100 residues. A number of poliovirus genomes have been sequenced completely (Nomoto et al., 1982; Toyoda et al., 1984; Stanway et al., 1984; Cann et al., 1984; Hughes et al., 1986; Stanway, 1990) and the wealth of knowledge concerning the molecular biology of the virus suggests that it may be possible to understand virulence and attenuation in molecular terms.

**Molecular epidemiology of polioviruses**

A feature of the use of live attenuated poliovaccines, as shown in Fig. 1, is that although the incidence of disease can be reduced to very low levels, there remains a low number of cases that does not appear to fall further. It was suspected that at least some of the residual cases were due to the live vaccine itself (Assad & Cockburn, 1982) but this was technically difficult to prove because of the phenotypic and antigenic stability of the virus which make strain differentiation difficult. It was not until the use of molecular approaches such as T1 oligonucleotide mapping (Minor & Schild, 1981) and nowadays limited genomic sequencing (Rico-Hesse et al., 1987) that it was conclusively shown that the isolates were derived from the vaccine strains. Three doses of vaccine are given in a course of immunization, and the incidence of vaccine-associated cases of poliomyelitis is about one per 530000 for first-time vaccinees and about one per $2 \times 10^6$ in vaccinees overall (Nkowane et al., 1987). Vaccinees are almost always given trivalent vaccine containing one strain of each serotype, and 90% of vaccine-associated cases are attributable to either type 2 or type 3; type 1 accounts for the remaining 10%. In non-immunized populations in contrast, wild type 1...
poliovirus is responsible for up to 90% of cases of poliomyelitis (Assad & Ljungars Esteves, 1984). Wild-type viruses are readily distinguished from each other by molecular biological means, and isolates from cases drift generally during epidemics at a rate of about 10 mutations per month (Nottay et al., 1981).

The occurrence of vaccine-associated cases of poliomyelitis and the observation of genetic change in wild-type viruses reinforces the need for adequate safety testing of all poliovaccines before use. The current safety test involves primates and is cumbersome and expensive so that a viable alternative based on a real understanding of attenuation would be extremely attractive. Moreover it could be possible to design non-revertable vaccines based on the understanding of existing vaccines and the ecology of the viruses when they replicate in the host.

The molecular basis of the attenuation and reversion of the Sabin vaccine strains of poliovirus

The Sabin vaccine strains of poliovirus were obtained by passage of wild-type isolates under a variety of conditions, which differed for each of the serotypes. The resulting viruses were tested for virulence and stability in animals (Sabin & Boulger, 1973). For both the type 1 and type 3 strains the initial virus was highly virulent so that the attenuation process clearly involved the introduction of mutations.

It has been shown that full-length cDNA copies of the poliovirus genome were infectious (Racaniello & Baltimore, 1981) and that RNA produced from such cloned copies under the control of a suitable promoter was of similar infectivity to the genomic RNA itself (Van der Werf et al., 1986). Therefore it is possible, in principle, to define differences affecting virulence by generating recombinant viruses. This is done by exchanging parts of the genomes between virulent and vaccine strains, using recombinant DNA methods. The strategy has been to identify regions and mutations in the vaccine strain that will attenuate the virulent virus, and then to remove these mutations from the vaccine strain by site-directed mutagenesis, to generate a virus of comparable virulence to the parental or revertant strain. This strategy was first taken to its conclusion for the type 3 Sabin strain (Sabin) which is designated P3/Leon 12a,b and its virulent precursor, originally isolated from a fatal case of poliomyelitis in 1937 and designated P3/Leon/USA/1937 (Leon) (Westrop et al., 1989).

The Sabin strain and Leon differ by 10 base differences, summarized in Fig. 3 (Stanway et al., 1984). By use of convenient restriction sites a number of recombinant viruses were produced and tested in accordance with the neurovirulence test procedures used for poliovaccines (WHO, 1983) except that small numbers of animals were used. Virulence is assessed by the number of animals showing clinical signs in the course of the 22 days following inoculation of a single fixed dose of virus, and by the histological effects of the virus, including signs of damage to neurons as the virus replicates and spreads from the inoculation site. A summary of a typical series of experiments is given in Table 1 and the conclusions drawn were that there were two differences between Leon and Sabin contributing most to differences in neurovirulence. They were at base 2034, which produced a change at residue 91 of the capsid protein VP3, from a serine in the virulent virus to a phenylalanine in the vaccine strain, and in the 5' non-coding region either at base 220 or 472. The sequence of a virulent revertant strain isolated from a fatal case of vaccine-associated poliomyelitis showed only six differences from that of the Sabin strain, of which only the base at 472 was a straightforward back mutation to the...
Table 1. Neurovirulence of type 3 polioviruses derived by recombination between Sabin 3 and P3 Leon/USA/37

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Clinical signs</th>
<th>Mean histological lesion score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus</td>
<td>220 472 871 2034* 3333† 3464‡ 4064 6127 7165 7432</td>
<td></td>
</tr>
<tr>
<td>P3/Sabin</td>
<td>S§ S S S S S S S S</td>
<td>0/8</td>
</tr>
<tr>
<td>P3/Leon</td>
<td>S S S S S S S S S S</td>
<td>4/4</td>
</tr>
<tr>
<td>SV3/L</td>
<td>S S S S S S S S S</td>
<td>1/8</td>
</tr>
<tr>
<td>SV1/L</td>
<td>S S S S S S S S S</td>
<td>3/4</td>
</tr>
<tr>
<td>SP2/L</td>
<td>S S S S S S S S S</td>
<td>4/4</td>
</tr>
<tr>
<td>SCC/L</td>
<td>S S S S S S S S S</td>
<td>8/20</td>
</tr>
<tr>
<td>ST/L</td>
<td>S S S S S S S S S</td>
<td>1/3</td>
</tr>
<tr>
<td>ST'/L</td>
<td>S S S S S S S S S</td>
<td>3/4</td>
</tr>
<tr>
<td>SV3'/L</td>
<td>S S S S S S S S S</td>
<td>0/8</td>
</tr>
<tr>
<td>SLR1</td>
<td>S S S S S S S S S</td>
<td>0/3</td>
</tr>
<tr>
<td>SLR2</td>
<td>S S S S S S S S S</td>
<td>3/4</td>
</tr>
<tr>
<td>L472/S</td>
<td>S S S S S S S S S</td>
<td>0/4</td>
</tr>
<tr>
<td>LV3/S</td>
<td>S S S S S S S S S</td>
<td>1/4</td>
</tr>
<tr>
<td>L472V3/S</td>
<td>S S S S S S S S S</td>
<td>3/4</td>
</tr>
</tbody>
</table>

* Coding change in VP3 residue 91 from serine (Leon) to phenylalanine.
† Coding change in VP1 residue 286 from lysine (Leon) to arginine (Sabin).
‡ Coding change in P2A residue 30 from threonine (Leon) to alanine (Sabin).
§ S, Sabin type 3 nucleotide at the position indicated.

residue found in the virulent precursor strain Leon (Cann et al., 1984). As other isolates from vaccine-associated cases had inevitably reverted to the Leon nucleotide at base 472 but not at 220 (Evans et al., 1985), it was concluded that the residue at 472 was significant. Consequently a mutant was prepared from the Sabin clone, in which the bases at positions 472 and 2034 were mutated to those found in Leon. This construct was nearly as virulent as Leon, as shown in Table 1. Thus the largest part of the attenuated phenotype of the type 3 Sabin vaccine strain of poliovirus was attributable to only two mutations. Subsequently a third mutation at nucleotide 2493, which is believed to have an attenuating effect, was identified by other workers, leading to an amino acid change from isoleucine to threonine at residue 6 of capsid protein VP1 (Weeks-Levy et al., 1991; Tatem et al., 1992). This difference was not observed in our original clones probably because the virus from which they were derived has been plaque-purified and passaged twice in HEp2C cells, and it has been shown that the mutation is rapidly lost on passage. However the mutation is clearly present in vaccine batches which we have examined (A. J. Macadam, personal communication).

The type 2 vaccine strain was derived from an isolate from a healthy child. The virus was passaged in a chimpanzee and then found suitable for use with respect to stability and attenuation. Although the progenitor strain from the child is not generally available, and in any case is probably attenuated, the type 2 vaccine strain is capable of reversion and causing vaccine-associated poliomyelitis, especially in contacts of vaccinees. Virus isolated from one such case is designated P2/117 and differs from the type 2 vaccine strain at 23 bases (Pollard et al., 1989) as shown in Fig. 4. Recombinants between the Sabin vaccine strain of type 2 poliovirus and P2/117 have indicated that a major attenuating mutation lies in the 5' non-coding region of the genome at either base 437 or 481 or possibly both (Macadam et al., 1991 b). Other workers have used the mouse-adapted strain of type 2 poliovirus, Lansing, or transgenic mice carrying the human cellular receptor for poliovirus to define attenuating and reverting mutations between P2/117 and the Sabin type 2 vaccine strain. The transgenic mice, unlike normal mice, appear to be fully susceptible to poliovirus infection except possibly by the oral route and the clinical pathology closely resembles that found in primates. Using these models Ren et al. (1991) identified mutations at bases 481 and 2903 as significant. The difference at 2903 results in an amino acid change from isoleucine to valine at residue 143 of capsid protein VP1. A possible third mutation in or just before the sequence encoding VP4 might also have a slight effect. We have subsequently confirmed these findings with independent constructs tested in primates (A. J. Macadam et al., unpublished results). In addition, in isolates from vaccine-associated cases both residues 481 and 2903 have mutated, consistent with their importance for virulence and attenuation.

Whereas both the type 2 and type 3 Sabin vaccine strains of poliovirus appear to be attenuated or reverted by means of only two or possibly three mutations, work
by Nomoto and co-workers suggested that the attenu-ation of the type 1 strain compared to the precursor strain Mahoney was more complex. A major attenuating mutation in the 5' non-coding region at residue 480 was identified, but many mutations with an attenuating effect were scattered throughout the rest of the genome (Omata et al., 1986). This has been proposed as an explanation for the higher degree of safety of the type 1 Sabin vaccine strain of poliovirus compared to the type 2 and type 3 strains. However Christodoulou and co-workers reported that the Sabin type 1 strain could apparently revert to virulence by the introduction of only a few mutations. Vaccine virus was passaged at successively higher temperatures and became more virulent. On sequencing the isolates it was found that almost total reversion to the virulence of Mahoney was accomplished by two or three mutations: one at residue 525 in the 5' non-coding region which for reasons to be given later is believed to be equivalent to 480; one at base 6203 which introduces a change from histidine to tyrosine in residue 73 of the polymerase protein 3D; and one at the extreme 3' end of the genome (Table 2; Christodoulou et al., 1990). Variation in the 3' end of the genome is documented in type 3 (Cammack et al., 1989) where it has little effect on virulence. It is therefore possible that the attenuated phenotype of the type 1 Sabin vaccine strain of poliovirus is attributable to as few mutations as that of type 2 and type 3, although this awaits confirmation by the construction of a Sabin type 1 vaccine strain in which bases 480 and 6203 are reverted. It is striking that all three live attenuated poliovirus vaccine strains prepared by Sabin by different passage routes have attenuating mutations in the 5' non-coding region within the same short sequence; this presumably reflects the consistency of the criteria by which suitable vaccine strains were chosen.

### In vitro effects of attenuating mutations in the 5' non-coding region

The 5' non-coding region of poliovirus is about 740 nucleotides in length. Within the first 620 bases there are regions in which the sequence is totally conserved

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**Table 2. De-attenuation of the Sabin type 1 vaccine strain by passage at elevated temperatures**

<table>
<thead>
<tr>
<th>Virus*</th>
<th>Nucleotide/amino acid</th>
<th>Mean clinical signs</th>
<th>Mean histological lesion score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>525</td>
<td>2438</td>
<td>2741</td>
</tr>
<tr>
<td>Sabin 1</td>
<td>U</td>
<td>A</td>
<td>G</td>
</tr>
<tr>
<td>S137C1</td>
<td>C</td>
<td>A</td>
<td>G</td>
</tr>
<tr>
<td>S138C1</td>
<td>C</td>
<td>A</td>
<td>G</td>
</tr>
<tr>
<td>S139C8</td>
<td>C</td>
<td>G</td>
<td>U</td>
</tr>
<tr>
<td>Mahoney</td>
<td>U</td>
<td>U</td>
<td>A</td>
</tr>
</tbody>
</table>

* Viruses were isolated from parental type 1 virus by passage at elevated temperature followed by plaque purification. S137C1 was obtained from the Sabin 1 strain at 37 °C, S138C1 from S137C1 at 38 °C and S139C8 by passage of S138C1 at 39 °C. Data from Christodoulou et al. (1990). Variation in the 3' end of the genome is documented in type 3 (Cammack et al., 1989) where it has little effect on virulence. It is therefore possible that the attenuated phenotype of the type 1 Sabin vaccine strain of poliovirus is attributable to as few mutations as that of type 2 and type 3, although this awaits confirmation by the construction of a Sabin type 1 vaccine strain in which bases 480 and 6203 are reverted. It is striking that all three live attenuated poliovirus vaccine strains prepared by Sabin by different passage routes have attenuating mutations in the 5' non-coding region within the same short sequence; this presumably reflects the consistency of the criteria by which suitable vaccine strains were chosen.

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**Fig. 4. Comparison of the sequences of cDNA clones of the type 2 vaccine strain P2 Sabin and the virulent revertant strain P2117. The location of restriction sites used in generating recombinant viruses is indicated below the genome. Data from Pollard et al. (1989), Macadam et al. (1991b) and A. J. Macadam et al. (unpublished results).**
between all polioviruses and enteroviruses, whereas the 100 bases immediately preceding the start codon are the most divergent. The size of the region, its high degree of conservation in certain areas and the occurrence of a cluster of attenuating mutations within a small highly conserved sequence suggest that the 5' non-coding region has an important function in the viral life cycle.

The usual mechanism of initiation of translation in eukaryotic cells involves binding of the ribosome to a 5'-terminal cap structure, followed by scanning a brief sequence before translation begins at an initiation codon in the appropriate context. However, Pelletier & Sonnenberg (1988) demonstrated that the 5' non-coding region of poliovirus could act as an internal ribosome entry site if placed in the middle of a single piece of RNA between two reporter genes. Thus the initiation of protein synthesis for poliovirus includes binding of the ribosome in the absence of a free capped 5' end, probably to a large tertiary structure in the messenger RNA the nature and precise function of which are currently not clear.

A number of workers published secondary structure predictions for the first 620 bases at approximately the same time (Rivera et al., 1988; Skinner et al., 1989; Pilipenko et al., 1989; Lee & Zuker, 1990). An outline of the consensus structure is shown in Fig. 5. An enlarged diagram of domain V, in which the mutations involved in attenuation lie, is shown in Fig. 6. The structure was based on computer folding, comparisons of published sequences of polioviruses, other enteroviruses and rhinoviruses over the 5' non-coding region, which showed that a change in a base in a paired structure was accompanied by a compensating change in its proposed partner to conserve the pair, and chemical and biochemical analysis using single- and double-strand specific nicking conditions to confirm that particular bases were paired or not.

In the predicted structure shown in Fig. 6 the base attenuating the Sabin type 3 vaccine strain at position 472 resulted in the alteration of the C-G base pair found in the wild-type to a U-G base pair in the vaccine, and the base attenuating the Sabin type 1 vaccine strain at position 480 (483 in Fig. 6) resulted in the alteration of an A-U base pair to a G-U base pair. The attenuated strain base pair has not been found in any other polio- or enterovirus at either of these positions (Minor & Dunn, 1988). Moreover base 480 is predicted to pair with base 525 (528 in Fig. 6). In the revertant of the type 1 Sabin strain identified by Christodoulou et al. (1990) described
above, the G-U base pair is altered to a G-C base pair by a change at base 525. It therefore appears that, at least for type 1 and type 3, the attenuated phenotype is associated with a weakening of base pairing in a highly conserved structure.

Svitkin and co-workers demonstrated that in an in vitro translation system prepared from Krebs ascites cells the efficiency of translation of RNA from the type 1 Sabin vaccine strain of poliovirus was significantly less than that of Mahoney, the wild type 1 strain from which it was derived (Svitkin et al., 1985). The effect, which involves the measurement of protein synthesized when using equal, sub-saturating amounts of RNA, was even more striking with the type 2 and type 3 serotypes when RNA from the Sabin vaccine strain was compared to RNA from precursor and revertant strains (Svitkin et al., 1988, 1990). The subtle change of a single base pair from the strong G-C to the allowed but weaker G-U therefore appears to have a major effect on protein translation, at least in vitro.

La Monica & Racaniello (1989) showed that a neuroblastoma cell line could differentiate between the Sabin and Mahoney strains of type 1 poliovirus, producing 10-fold less Sabin virus than wild-type. It is possible that this is attributable to differences in the 5' non-coding region although this was not established. Macadam et al. (1991b) demonstrated that in certain cell lines the 5' non-coding region of the Sabin type 2 strain conferred a temperature-sensitive phenotype on virus growth. Subsequent studies (Macadam et al., 1992), involving site-directed mutants in which base pairing in the region 470 to 480 was disrupted to a greater or lesser extent, showed that the temperature-sensitive phenotype was most marked when the predicted disruption of the base-paired structure was most severe. This strongly supports a functional role for the structure shown in Fig. 6, at least in the region containing the attenuating mutations. The temperature-sensitive phenotype attributable to the 5' non-coding region is expressed more strongly in some cells in culture than others. The phenomenon does not appear to be affected by the species of origin, and may be due to the presence in different cells of different levels of factors involved in cap-independent initiation of translation on poliovirus RNA. Thus the reduced affinity between factor and RNA caused by reducing the stability of the RNA secondary structure could be compensated by a simple mass action effect of an excess of factor to produce the same amount of complex. A cell that did not allow the expression of the temperature-sensitive phenotype would presumably have an excess of such factors and therefore exert minimal selection against growth of the attenuated virus. This might be significant in the choice of cell substrate for the production of live poliovaccines.

**In vitro effects of the attenuating mutation in capsid protein VP3 of the Sabin type 3 strain**

Growth of the Sabin type 3 vaccine strain of poliovirus in HEp2C cells is sensitive to elevated temperatures. This is not attributable to the mutation in the 5' non-coding region, as these cells are highly permissive for known attenuating 5' non-coding mutations. Analysis of recombinant strains of the kind shown in Table 1 showed that the temperature-sensitive phenotype was attributable to the phenylalanine present at residue 91 of capsid protein VP3 of the attenuated virus; this was the second attenuating mutation identified in the type 3 vaccine strain. The location of this mutation in the structure (Filman et al., 1989; Minor et al., 1989) suggested that it might act by destabilizing interactions between adjacent protomers in the virion, and Macadam et al. (1991a) presented evidence that its effect was to prevent the assembly of the protomers, composed of one molecule each of VP0, VP1 and VP3, into pentamers.

Revertants of the temperature-sensitive recombinant strains were readily isolated in vitro. Moreover, isolates of type 3 poliovirus from vaccine-associated cases of poliomyelitis were found to have lost the temperature-sensitive growth phenotype. However, on examining the sequence of the capsid proteins it was found that most isolates retained the phenylalanine at residue 91 of VP3 while possessing mutations at other loci, some of which occurred in several independent isolates. A number of the mutations were located at the interface between protomers although some were not. In particular, of the in vitro selected mutants three out of four involved a single amino acid at residue 132 of VP1 which lines a pocket in the protein. This pocket is normally occupied by a lipid molecule believed to be sphingosine, and is the site in which drugs of the arildone family are inserted. The drugs inhibit picornavirus uncoating and the sites of insertion lie away from the interface. A third kind of mutation appears to occur in a region of the structure involved in stabilizing the interactions between pentamers in the intact virion (Filman et al., 1989), and a fourth category is internal. Site-directed mutants have been constructed and confirm that the four classes of second site mutations found suppress the temperature-sensitive phenotype associated with the phenylalanine in VP3. Some of the mutations found are given in Table 3 with their approximate location in the structure.

It is at first sight unexpected that the virus should revert the phenotype preferentially by second-site suppressor mutations. However, on closer analysis it became clear (Macadam et al., 1989) that the different virus strains had slightly different temperatures for optimum growth, and that on its own the back mutation to the Leon sequence at residue 91 of VP3 produced a
A final point arising from the location of the mutations and the mode of action deduced for them is that although the effect of the VP3 mutation is to inhibit the assembly of the protomers to pentamers, not all suppressor mutations act at this stage. One in particular in VP2 at residue 18 appears to affect the assembly of pentamers into capsids, which is presumably a late stage in the assembly process. The amino acid substitution involved has been confirmed by site-directed mutagenesis and is a change from leucine to isoleucine. The observation suggests that a mutation impairing the efficiency of an early step in the assembly process can be compensated by a second mutation which increases the efficiency of a separate, later step. In so far as the mutant phenotype involves failure to assemble capsids at high temperature, it could, in principle, be suppressed by mutations altering the equilibria or kinetics of transition between different assembly intermediates. Suppressor mutations need not therefore involve dramatic amino acid changes, nor directly illuminate the process affected by a particular mutation.

**In vivo growth of the Sabin vaccine strains of poliovirus**

Mutations known to attenuate the vaccine strains for primates can be shown to be reverted or suppressed in isolates made from vaccine-associated cases of poliomyelitis, consistent with their having an attenuating effect in humans. However, early work also indicated some degree of change in isolates obtained from healthy vaccinees. In particular isolates of type 3 tended to lose their temperature-sensitive growth phenotype and increase somewhat in neurovirulence, and isolates of type 1 changed slightly in their antigenic properties (WHO, 1969). It was therefore of interest to study the virus excreted by healthy vaccinees following routine immunization, using molecular biological methods. One child was studied in particular detail (Minor et al., 1986) although the results obtained have now been confirmed in at least 40.

Immunization was with trivalent vaccine containing each of the three Sabin vaccine strains given orally, and the first dose was given at 4 months of age. (The course of immunization in the U.K. now commences at 2 months of age.) Stool samples were collected and attempts to isolate virus made; type 1 virus was isolated for only a few days, whereas type 2 and type 3 virus could be isolated for 73 days. Virus excretion appeared to have finally ceased when there was evidence of infection with an adenovirus. The duration of the period of excretion of virus was rather longer than expected; while 1% of vaccinees are reported to excrete virus for 10 weeks as in this case, 50% no longer excrete virus after 5 or 6 weeks (Minor et al., 1986).

The nature of the type 3 virus excreted was surprising, however, as virus from the fourth specimen, collected 47 h post-immunization, had lost the attenuating mutation at base 472, and could be shown to be of increased although not wild-type neurovirulence (Evans et al., 1985). Later it was shown that 11 days after immunization the virus excreted was no longer temperature-sensitive in its growth properties. This was effected by a second site mutation in capsid protein VP2 at residue 18, a position involved in the interactions between pentamers in the virus structure as described above (Macadam et al., 1989; Filman et al., 1989). However, more dramatic genomic rearrangements also occurred; oligonucleotide mapping and sequence analysis showed that the isolate made at day 11 was a recombinant in

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**Table 3. Mutations in capsid proteins known to suppress the temperature-sensitive phenotype of the Sabin type 3 vaccine strain**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP3 91 Phe-Ser*</td>
<td>Protomer interface</td>
</tr>
<tr>
<td>VP2 215 Leu-Met*</td>
<td>Protomer interface</td>
</tr>
<tr>
<td>VP3 108 Thr-Ala</td>
<td>Protomer interface</td>
</tr>
<tr>
<td>VP3 175 Thr-Ala</td>
<td>Protomer interface</td>
</tr>
<tr>
<td>VP3 178 Gin-Leu</td>
<td>Protomer interface</td>
</tr>
<tr>
<td>VP2 18 Leu-Ile</td>
<td>Pentamer interface</td>
</tr>
<tr>
<td>VP1 132 Phe-Leu</td>
<td>Lipid/drug binding pocket</td>
</tr>
<tr>
<td>VP1 54 Ala-Val*</td>
<td>Internal</td>
</tr>
<tr>
<td>VP1 54 Ala-Thr*</td>
<td>Internal</td>
</tr>
</tbody>
</table>

* Found together in isolates from vaccinees.
which the structural proteins and some of the non-structural proteins were derived from type 3, whereas the non-structural proteins from the middle of P2C to the extreme 3' end were derived from the type 2 vaccine strain as shown in Fig. 7(a). Virus with this genomic structure was excreted until day 42, when a second recombination event occurred, resulting in the regaining of a portion of the 3' end of the type 3 genome (Fig. 7b), resulting in a virus in which the central portion of the region of the genome coding for the non-structural proteins derived from type 2, and the remainder was derived from type 3. The 5' recombination site in this second recombinant was nearer the 5' end of the genome than the first, suggesting that it may have arisen by recombination between the first type 3-type 2 recombinant and a type 2-type 3 recombinant, as illustrated diagrammatically in Fig. 7(c). No evidence for a suitable partner type 2-type 3 recombinant could be found in the type 2 isolates made from the child or as yet from other healthy vaccinees (Cammack et al., 1989; Dunn et al., 1990) but such recombinant type 2 strains have been identified in isolates from vaccine-associated cases (Lipskaya et al., 1991; A. J. Macadam, personal communication). The second recombination event was also associated with the loss of the 3'-terminal guanosine residue preceding the polyadenylate tract (Cammack et al., 1989). In addition to these changes, point mutations in antigenic sites defined by monoclonal antibodies and sequencing also occurred in the recombinant strains.

The extensive rearrangement and mutation of the genome is surprising in view of the proven high degree of safety of the vaccines, but studies in other vaccinees have shown that a very similar course of events is followed. In particular with one exception the 5' non-coding mutation at 472 is lost in all isolates studied from babies in the U.K. by 5 days post-immunization (Dunn et al., 1990; Minor & Dunn, 1988). The temperature-sensitive growth phenotype which as described above is suppressed by the same mutations as the attenuated phenotype, is lost by 11 days post-immunization (Macadam et al., 1989). Moreover all isolates of type 3 poliovirus made from vaccinees later than 11 days after vaccination have proved to be recombinant strains, either between type 3 and type 1 or type 3 and type 2, and where a type 3-type 2 recombinant is isolated a complex recombinant is generated at a later stage, when the 3'-terminal portion of the type 2 genome is replaced by the corresponding region of type 3 or type 1.

The same type of mutations that suppress the temperature-sensitive growth phenotype of type 3 have been found in isolates from healthy vaccinees and vaccine-associated cases of poliomyelitis, and the factors
determining whether an individual will develop disease following vaccination are not clear. However it has been shown (Macadam et al., 1989) that although the temperature-sensitive growth phenotype and the attenuated phenotype are suppressed by the same mutations, recombinant strains of virus from either healthy vaccinees or vaccine-associated cases are of slightly lower virulence than equivalent non-recombinant strains. The basis of this is not obvious in that no mutation attenuating the type 2 strain has been identified in the region of the type 2 genome incorporated into the recombinants. However, the revertant type 2 strains studied tend to be less virulent in animals than the type 3 strains, and this may be reflected in the attenuating effects of the exchanged segment. It is likely that the generation of the recombinants (which on the face of it is undesirable) may contribute to the safety of the vaccine. It would be predicted that vaccination with monovalent rather than trivalent vaccine should be associated with a higher incidence of vaccine-associated poliomyelitis.

The selective pressures leading to the emergence of the recombinant strains are not known. It would seem that some protein or combination of proteins encoded by the type 3 genome to the 3' side of protein P2C is selected against in the gut, and that the 3'-terminal portion of the type 2 genome is also selected against in the gut. One as yet untested hypothesis concerns an alternative proteolytic cleavage of the protease-polymerase protein 3CD. This cleavage, effected by the protease 2A, occurs in all type 1, type 2 and type 3 viruses examined other than those derived directly from the Sabin type 3 strain or its precursor strain (Minor, 1980). Recombinant type 3 viruses invariably perform this cleavage and the second recombinant site in the type 3-type 2 recombinants studied so far is such as to retain the type 2 cleavage site in 3CD. It is possible that the conservation of this site is of advantage to the virus in the host, by evading an immune response for example. Lee & Wimmer (1988) demonstrated that the site can be eliminated from type 1 virus without compromising its in vitro growth properties. They also presented evidence showing that a single amino acid substitution was sufficient to abolish the site in type 1. The type 3 strain is in theory able to change the amino acid to the cleavable form by a single mutation although it is not known whether this is in fact sufficient to render the type 3 protein cleavable or whether other mutations are also required. However if a single mutation is sufficient, and recombinants are selected on the basis of the ability of 3CD to undergo the cleavage, it implies that recombination occurs at a higher rate than mutation in the gut of vaccinees.

Finally, the mutation identified by Weeks-Levy et al. (1991) in VP1 apparently is also lost rapidly, being undetectable in four vaccinees after 4 days (A. J. Macadam, personal communication). The duration of attenuating mutations in excreted type 3 virus is summarized in Table 4 for four vaccinees.

<table>
<thead>
<tr>
<th>Vaccinee</th>
<th>Mutation at 472 (days)</th>
<th>Mutation at 2493 (days)</th>
<th>Temperature-sensitive phenotype (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM</td>
<td>2</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>EM</td>
<td>3</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>AM</td>
<td>5</td>
<td>&lt;2</td>
<td>12</td>
</tr>
<tr>
<td>CM</td>
<td>6</td>
<td>&lt;2</td>
<td>12</td>
</tr>
</tbody>
</table>

The type 1 and type 2 strains are also subject to selection and rapid modification in vaccinees. Whereas no recombinant type 1 or type 2 strains have yet been identified as the principal strains in healthy vaccinees, they have been found in isolates of type 2 virus from vaccine-associated cases (Lipskaya et al., 1991; A. J. Macadam, personal communication). The attenuating mutation in the type 2 strain at residue 143 of VP1 is lost in a significant proportion of isolates from healthy vaccinees, and in all isolates from vaccine-associated cases. The 5' non-coding region mutation in type 2 is lost slightly later than that in type 3, at about 7 days post-immunization, but in all vaccinees, whereas that in type 1 is lost at about 7 days post-immunization in only half of the vaccinees (Dunn et al., 1990).

**Practical implications and future developments**

The live attenuated vaccine strains of poliovirus developed by Sabin have proved to be extremely safe and effective in general use, but a number of issues remain. Firstly the occurrence of vaccine-associated cases of poliomyelitis makes it necessary to test each batch of vaccines for neurovirulence before use and currently this involves the use of primates which is undesirable. Secondly the infectivity of the three strains for vaccinees is significantly different, and the type 3 strain in particular tends to be outgrown by the other two serotypes. Thirdly the high degree of variability in the virus particularly with regard to mutations associated with attenuation and reversion to virulence is a matter for concern, although in practice it does not appear to pose a significant risk.

The understanding of the molecular basis of the attenuated phenotype of poliovirus outlined here suggests ways in which in vitro tests might be developed. Chumakov and co-workers have described a polymerase
chain reaction-based assay for the type 3 vaccine to measure the proportion of genomes which have reverted at base 472 (Chumakov et al., 1991, 1992). The assay involves amplification with a mismatched primer introducing a MboI restriction site into the revertant sequence and a HinfI site into the vaccine sequence. Fragments are resolved after digestion, and the ratio of cut to uncut fragments gives the proportion of reverted genomes present. Fig. 8 shows the relationship between the proportion of genomes reverted at 472 and the histological mean lesion score in the neurovirulence test of 50 commercial vaccines submitted over a number of years to NIBSC, and including several which failed the neurovirulence test assays variation in the proportion of genomes reverted at 472 and the histological lesion score are shown as open circles, those which failed as closed circles. Data from Konstantin Chumakov, CBER, U.S.A. and NIBSC, U.K.

Fig. 8. Correlation between the mean histological lesion score and percentage C at base 472 for commercial type 3 vaccines. Batches that passed the WHO neurovirulence test are shown as open circles, those which failed as closed circles. Data from Konstantin Chumakov, CBER, U.S.A. and NIBSC, U.K.

The immunogenicity and stability of the vaccine strains may be linked phenomena. It is conceivable that the poor growth of the Sabin type 3 strain is due to a degree of over-attenuation, such that the virus must either revert at base 472 or die out. This raises the possibility that a slightly less attenuated virus, produced by manipulation of base pairing in this region by site-directed mutagenesis (Macadam et al., 1992), might have a higher take rate and persist in the non-reverted form in the gut for longer, so paradoxically being more attenuated for the child than the existing over-attenuated strain. This remains speculative but is the subject of active investigation.

Conclusion

The molecular biology of the live attenuated Sabin vaccine strains of poliovirus has been studied extensively, and surprisingly few mutations are required to account for the greater part of the attenuated phenotype. The viruses are clearly capable of extremely rapid, extensive and precise variation in the vaccinee to adapt from the attenuated form to a form able to grow successfully in the host, yet despite this they cause almost no disease. The high degree of genetic variation in the face of general phenotypic stability in the wild-type suggests that polioviruses are extremely well adapted to their hosts and that vaccines exploit some aspects of the virus host ecology to be safe and effective. The precise mechanisms by which they do so raise possibilities of improving vaccine production and testing methods, and designing better vaccine strains.

The work described here was performed over several years as part of a collaboration between the group at NIBSC and the group of Professor J. W. Almond at the University of Leicester and the University of Reading. It involved a cast of thousands.

References


