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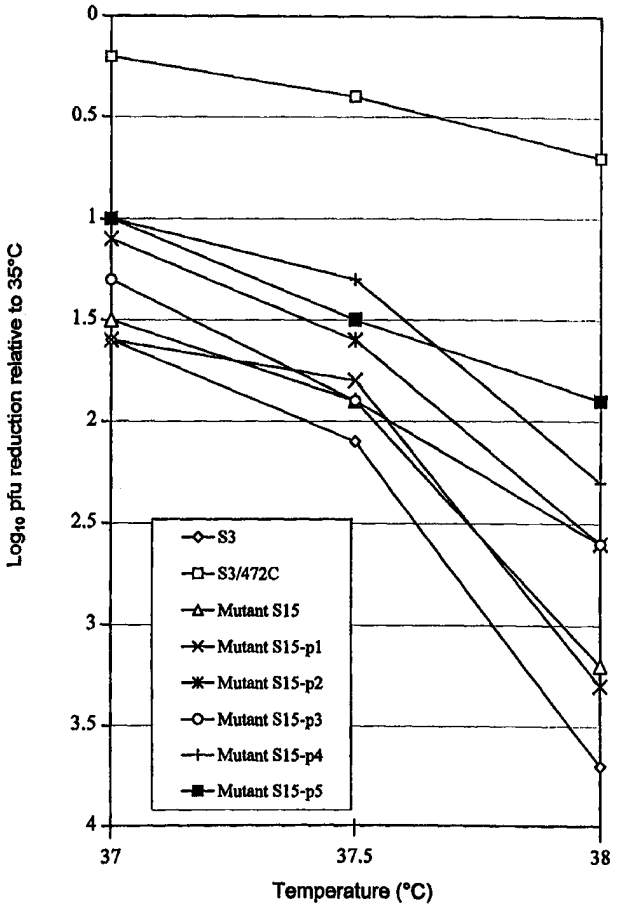
(57) Abstract

An attenuated poliovirus, suitable for vaccination against poliomyelitis, does not have a U–G base pair or other base pair mismatch in stem (a) or (b) of domain V of the 5' non–coding region of the poliovirus genome. The attenuated poliovirus may be a type 3 poliovirus whose 5' non–coding region contains the following U–A base pairs: (a) U–A at positions 472–537, U–A at positions 478–533 and U–A at positions 480–531; (b) U–A at positions 472–537, U–A at positions 480–531 and U–A at positions 481–530; or (c) U–A at positions 472–537, U–A at positions 480–531 and A–U at positions 482–529.



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ATTENUATED POLIOVIRUSES

Background of the invention

1. Field of the invention

This invention relates to attenuated polioviruses, to their preparation and to vaccines containing them. More specifically, the invention relates to polioviruses which are attenuated by the introduction of defined mutations into their genomes. These mutations attenuate the virulence of wild-type viruses and genetically stabilise existing live attenuated vaccine virus strains, thereby making them less likely to revert to virulence.

2. Description of the related art

The live attenuated poliovirus vaccines developed by Sabin in the 1950's have found great use throughout the world. Vaccine strains derived from each of the three poliovirus serotypes, known as Sabin types 1, 2 and 3, were prepared by passage of wild-type viruses in cell cultures and whole animals until attenuated strains were obtained. These attenuated viruses are substantially less able to cause poliomyelitis in humans than the original wild-type strains. They are administered orally and replicate in the gut to induce a protective immune response.

Although these vaccines are generally regarded as safe, their use is associated with a small incidence of paralysis in vaccinees. This is most often associated with type 2 and type 3 serotypes and rarely, if ever, with type 1. There is therefore a requirement for improved type 2 and type 3 vaccines which would be comparable in safety to the excellent type 1 strain.

The Sabin vaccine strains were developed by essentially empirical procedures. The genetic basis of their attenuation is not completely understood. Over the past several years, however, scientists have employed a number of molecular biological techniques in an attempt to elucidate the mechanism by which the neurovirulence of these vaccine strains is reduced. Most of the work has concentrated on serotypes 1 and 3. For both of these the complete nucleotide sequences of the vaccine strains have been compared with those of their neurovirulent progenitors.

In the case of poliovirus type 1, the vaccine strain differs from its progenitor at 47 positions in the 7441 base genome (Nomoto *et al.*, Proc. Natl. Acad. Sci. USA *79*, 5793-5797, 1982). All of these are simple point mutations and 21 of them give rise to

amino acid changes in virus- coded proteins. Although several mutations are thought to contribute to the attenuation phenotype of the vaccine strain, direct evidence has been presented that the mutation of A to G at position 480 in the 5' non-coding region of the genome has a marked attenuating effect on the virus (Nomoto *et al.*, UCLA Symp. Mol. Cell. Biol., New Series, *54* (Eds M.A. Brinton and R.R. Rueckert), 437-452, New York: Alan R. Liss Inc., 1987).

Analogous studies on poliovirus type 3 reveal just 10 nucleotide sequence differences in the 7432 base genome between the vaccine and its progenitor strain (Stanway *et al.*, Proc. Natl. Acad. Sci. USA *81*, 1539-1543, 1984). Just three of these give rise to amino acid substitutions in virus-encoded proteins. The positions of bases in the 5' non-coding region of the genome of type 3 poliovirus are numbered herein according to the numbering system of Stanway *et al.*, 1984.

The construction of defined recombinants between the type 3 Sabin vaccine strain and its progenitor strain has allowed the identification of the mutations which contribute to the attenuation phenotype. One of these is at position 2034 and causes a serine to phenylalanine change in virus protein VP3.

The other mutation of interest is C (progenitor) to U (vaccine strain) at position 472 in the 5' non-coding region of the genome. This 472U mutation has been observed to revert to the progenitor (wild type) 472 C rapidly upon replication of the virus in the human gut (Evans *et al.*, Nature *314*, 548-550, 1985). This reversion is associated with an increase in neurovirulence. C at position 472 has also been shown to be essential for growth of a mouse/human polio recombinant virus in the mouse brain (La Monica *et al.*, J. Virol. *57*, 515-525, 1986). More recently it has been observed that A changes to G at position 481 in poliovirus type 2, again upon replication of the virus in the gut of vaccinees (Macadam *et al.*, Virology *181*, 451-458, 1991).

A model for the secondary structure of the 5' non-coding region of the genome of poliovirus type 3 Leon strain has previously been proposed (Skinner *et al.*, J. Mol. Biol. *207*, 379-392, 1989). As concerns domain V (nucleotides 471-538), bases at positions 471 to 473 and 477 to 483 are paired with bases at positions 538 to 536 and 534 to 528 respectively as follows:

```
      471          477        483
...   UCC   ...    CCAUGGA    ...
...   AGG   ...    GGUGCCU    ...
      538          534        528
```

For convenience, the paired regions are termed stem (a) (471-473/538-536) and stem (b) (477-483/534-528). Previously, we found that a type 3 poliovirus with the base pair 472-537 reversed, i.e. 472 G and 537 C, is attenuated. Further, this attenuated virus had a slightly lower LD_{50} value than the corresponding poliovirus which only had the mutation C to G at position 472 but which retained the wild-type G at position 537. Attenuated polioviruses in which a base pair of stem (a) or stem (b) of domain V is reversed are disclosed in EP-A-0383433. However, subsequent experiments showed that the type 3 poliovirus in which the 472-537 base pair is reversed is not as attenuated as the type 3 Sabin vaccine strain.

It has remained a problem in this field to produce attenuated polioviruses which can have substantially the same attenuation as, or greater attenuation than, the Sabin vaccine strains (so that they are safe to use) but which are much more stable genetically. That is, these attenuated polioviruses can be approximately as attenuated as the Sabin vaccine strains but there is less risk that they may revert to the neurovirulence of the wild-type.

Summary of the invention

It has now been found that this combination of properties can be achieved by further alteration of domain V of the 5' non-coding region of the poliovirus genome. The solution of the present invention comprises providing an attenuated poliovirus which does not have a U-G base pair or other base pair mismatch in stem (a) or (b) of domain V of the 5' non-coding region of the poliovirus genome. (A departure from Watson-Crick base pairing is considered to be a mismatch.) The poliovirus may be a type 1, type 2 or type 3 poliovirus. A type 2 or type 3 poliovirus is preferred. Particularly preferred is an attenuated type 3 poliovirus in which the 5' non-coding region of the genome of the poliovirus contains a U-A base pair at position 472-537 or 480-531, or both. "Attenuated" means attenuated with respect to the wild type poliovirus which is the progenitor of the relevant Sabin vaccine strains (each strain has its own progenitor). Overall, the virus must be sufficiently attenuated to be safe

in use. One convenient definition of such safety is the "WHO monkey neurovirulence test", World Health Organisation (WHO): Requirements for poliomyelitis vaccine (oral), WHO Tech. Rep. Ser. *687*, 107-175, 1983.

Brief description of the drawings

FIGURE 1 shows the predicted RNA secondary structure of domain V (nucleotides 471-538) of the type 3 Sabin strain (adapted from Skinner et al., 1989). Arrows show attenuating nucleotide changes found in the three Sabin vaccine strains (attenuating bases are in bold, followed by the relevant serotype in parentheses). Due to slight differences in lengths of the 5' non-coding regions, nucleotide 480 in Sabin 1 is at position 483 in this Figure and nucleotide 481 in Sabin 2 is at position 484. The base-paired stem regions are designated (a), (b), (c) and (d).

FIGURE 2 shows the sequence of stems (a) and (b) of domain V of the Sabin vaccine strains of each type of poliovirus. Domain V of a type 3 poliovirus extends from positions 471 to 538. Domain V of a type 2 or a type 1 poliovirus extends from positions 468 to 535.

FIGURE 3 shows the sequence of stems (c) and (d) of domain V of the Sabin vaccine strains of each type of poliovirus.

FIGURE 4 to 6 show the results of genetic stability testing of the Sabin 3 strain with a point mutation to C at 472 (S3/472C), the Sabin vaccine strain (S3) and mutants S15 and S16 of the present invention, the three last-mentioned before passage and after passages 1-5 (p1-p5) in cells. The passaged virus was assayed and a parameter based on plaque-forming units (y axis) plotted against assay temperature (x axis). The graphs show loss of temperature sensitivity, used as a temperature attenuation phenotype, with increasing passage.

Description of the preferred embodiments

Attenuated polioviruses of the invention have been modified so that stems (a) and (b) of domain V do not contain a U-G base pair or other base pair mismatch such as the U-U mismatch in the type 1 Sabin vaccine strain. Preferably, stems (c) and (d) also do not contain a U-G base pair or other base pair mismatch. An alternative base pair is provided in place of the pair mismatch, chosen to ensure sufficient attenuation of the resulting poliovirus. This is as described above or, more preferably,

substantially the same level of attenuation as the Sabin vaccine strain of the same poliovirus type.

Preferably, a U-A base pair is present instead of a U-G base pair or a base pair mismatch. Thus, a U-A base pair is preferably present at positions 472-537 and/or 480-531 of domain V of a type 3 poliovirus, and at position 527-478 of a type 2 poliovirus, replacing U-G base pairs (refer to Figure 2).

Other mutations are present as necessary to modify the thermodynamic stability of domain V so that the above-mentioned desired level of attenuation is achieved. Preferably, but not necessarily, these mutations are in any one or more of stems (a), (b), (c) and (d) of domain V. They may include, for example, a base pair reversal and/or the replacement of a C-G base pair which is present in a neurovirulent wild-type poliovirus by a U-A or A-U base pair. Thus, a U-A or A-U base pair may also be present in domain V of poliovirus type 3 at positions 478-533 and/or positions 481-530 and/or positions 482-529. Preferred type 3 polioviruses of the invention contain the following U-A base pairs:

(a) U-A or A-U at 472-537, U-A at 478-533 and U-A at 480-531;

(b) U-A or A-U at 472-537, U-A at 480-531 and U-A at 481-530; or

(c) U-A or A-U at 472-537, U-A at 480-531 and A-U at 482-529.

It is possible to have the wild-type pairing C-G at 472-537 provided that other changes are made to increase attenuation and make these pairings more dissimilar from wild type. Examples of such viruses have the following changes from the type 3 positions:

(d) C-G at 472-537, U-A at 478-533, U-A at 480-531 and U-A at 481-532.

(e) C-G at 472-537, U-A at 478-533, C-G at 480-531 and U-A at 481-532.

Preferred type 1 and 2 polioviruses can be correspondingly derived from the sequence of stems (a) and (b) of the wild-type neurovirulent type 1 and 2 polioviruses. All strains are preferably Sabin.

The present invention provides a process for the preparation of an attenuated poliovirus of the invention, which process comprises:

(i) introducing the or each desired mutation by site-directed mutagenesis into a sub-cloned region, which includes the or each position it is wished to mutate, of a DNA copy of a poliovirus genome;

(ii) reintroducing the thus modified region into a complete copy DNA from which the region was derived; and

(iii) obtaining live virus from the copy DNA thus obtained.

A mutation can thus be introduced into a strain of a poliovirus, normally a Sabin strain, by site-directed mutagenesis of a copy DNA corresponding to the genomic RNA of a poliovirus. This may be achieved by sub-cloning an appropriate region from an infectious DNA copy of a poliovirus genome into the single strand DNA of a bacteriophage such as M13.

After the introduction of the or each mutation, the modified sub-cloned copy DNAs are reintroduced into the complete copy DNA from which they were derived. Live virus is recovered from the mutated full length copy DNA by production of a positive sense RNA typically using a T7 promoter to direct transcription *in vitro* (Van der Werf *et al.*, Proc. Natl. Acad. Sci. USA *83*, 2330-2334, 1986).

The recovered RNA may be applied to tissue cultures using standard techniques (Koch, Curr. Top. Microbiol. Immunol. *61*: 89-138, 1973). After 2-3 days of incubation, virus can be recovered from the supernatant of the tissue culture. The level of neurovirulence and thus of attenuation of the modified virus may then be compared with that of the unmodified virus using a standard LD50 test in mice or the above-mentioned WHO-approved vaccine safety test in monkeys.

Attenuation of domain V has also been shown to correlate approximately with temperature sensitivity in BGM cells (Macadam *et al.*, Virology *181*, 451-458, 1991) or in L20B cells (as described for CM-1 cells in Macadam *et al.*, Virology *189*, 415-422, 1992). The temperature sensitivity of modified virus can thus be determined as a preliminary screen to determine the level of attenuation expected. This can be expressed as the temperature (T) at which the number of plaque forming units (pfu) is reduced by a power of 10 (1.0 $\log_{10}$) from the number obtained at 35°C in the same cells. Preferably this temperature is the same as or within 0.2°C of that for the corresponding Sabin strain (when assayed concurrently). The lower the value of T, the greater is the degree of attenuation.

The attenuated polioviruses can be used as live vaccines. They may therefore be formulated as pharmaceutical compositions further comprising a pharmaceutically acceptable carrier or diluent. Any carrier or diluent conventionally used in live vaccine preparations may be employed. For example, the attenuated polioviruses can

be stabilised in 1M aqueous $MgCl_2$ and administered as a mixture of the three serotypes.

The attenuated polioviruses can therefore be used to prevent poliomyelitis in a human patient. For this purpose, they may be administered orally, as a nasal spray, or parenterally, for example by subcutaneous or intramuscular injection. A dose corresponding to the amount administered for a conventional Sabin vaccine strain, such as from 10^4 to 10^6 $TCID_{50}$, may be administered.

The following Examples illustrate the invention.

EXAMPLES

Material and Methods

Cells

HEp2C, BGM and Vero cells were grown in monolayers as described in Macadam *et al.*, Virology *181*, 451-458, 1991. L20B cells (mouse L-cells expressing the human poliovirus receptor) were grown as described in Pipkin *et al.*, J. Virol. Methods *41*, 333-340, 1993. MRC-5 cells were grown in monolayers in the same growth medium as BGM cells.

Virus passage

MRC-5, Vero or L20B cell monolayers in $25cm^2$ flasks were inoculated with viruses at multiplicities of infection (moi) of 0.1 or 10. Infected cell sheets were incubated in Minimum Essential Medium (MEM) containing antibiotics but no serum at 33°C or 37°C until complete cytopathic effect (cpe) was apparent. Flasks were frozen at –70°C and thawed, three times, and cell debris removed by centrifugation. Supernatants were used for further passage, as above, or for biological and molecular analysis.

Temperature sensitivity

BGM cells were used for temperature-sensitivity (ts) assays as described in Macadam *et al.*, 1991. L20B cells were used for ts assays as described for CM-1 cells in Macadam *et al.*, Virology *189*, 415-422, 1992. Briefly, viruses were assayed by plaque-formation at different temperatures. These were controlled by incubation of inoculated plates in sealed plastic boxes submerged in water baths whose

temperatures fluctuated by <0.01°C. All viruses were assayed at least twice and control viruses with known phenotypes were always included for validation.

Results were plotted on graphs of $\log_{10}$ (pfu reduction relative to 35°C) against temperature. The most accurate part of the curve is the initial reduction by 1.0 $\log_{10}$ and the figure obtained is unaffected by potential variants unless they are present at levels of more than 1 in 10.

Viruses with a Leon/Lansing genomic background were assayed in BGM cells; Sabin 3, Leon and mutants of these viruses were assayed in L20B cells. The range of temperatures over which variation in domain V stability influences viral growth depends on the cell substrate used (Macadam *et al.*, 1991 and 1992 cited above) and is lower in L20B cells than BGM cells. For Sabin 3, the major ts determinant is at VP3 91 (Minor *et al.*, J. Gen. Virol. *70*, 1117-1123, 1989). This influences ts at temperatures higher than domain V in L20B cells, but approximately the same temperatures as domain V in BGM cells. Consequently, ts assays in L20B cells can be used to distinguish viruses with different mutations in domain V irrespective of the residue at VP3 91.

Construction and recovery of site-directed mutants

Derivation of Leon/Lansing and construction of L3L2 472-537 UG and L2L2 492-537 UA were described in the above-cited paper Macadam *et al.*, 1992. All mutants listed in Table 2 were constructed in a similar way. Briefly, sub-clones of the Leon 5'-NCR in M13 were substrates for mutagenesis using the "Mutagene" kit (Bio-Rad). Correct sequences were reintegrated into full length clones as *Mlu*I-*Sst*I (279-751) fragments.

Derivation of Leon and Sabin type 3 cDNA clones was described in Westrop *et al.*, J. Virol. *63*, 1338-1344, 1989. Mutants Leon U-A and Leon U-G were constructed by partial *Sst*I/*Sal*I fragment exchanges between the relevant Leon/Lansing mutant and T7/Leon full-length clones. Mutants in Table 3 were constructed in two steps. *Mlu*I-*Sst*I fragments from relevant Leon/Lansing mutants were ligated into Sabin 3 clones digested to completion with *Mlu*I (279) and *Sst*I (751 and 1900). Since there are two *Sst*I sites in the Sabin 3 genome, resultant clones lacked the *Sst*I-*Sst*I (751-1900) region. Full-length infectious clones were generated

by digestion with SstI/*SmaI* (2768) and insertion of a partial *SstI/SmaI* (751-2768) fragment of the Sabin 3 clone.

Viruses were recovered by transfection of HEp2C monolayers with >2μg T7 transcripts (Van der Werf *et al.*, cited above), followed by incubation at 34°C for 24 to 48h, by which time complete cpe was apparent. Sequences of nucleotides 450-550 of all mutants were confirmed following RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR).

Neurovirulence

Viruses were assayed by the above-cited standard WHO approved test for vaccine safety (WHO, 1983) except that fewer animals were used per virus. It is expected that the results obtained using the required number of animals would not be materially different.

Results

Initial Studies

Neurovirulent revertants of Sabin type 3 isolated from vaccine-associated cases of poliomyelitis all have a C at nucleotide 472. Substitutions of G to A at 537 have never been observed in *in vivo* isolates, suggesting that a U-A base-pair at 472-537 confers little selective advantage relative to a C-G base-pair. A strain with a U-A base-pair at 472-537 would require two mutations to form a C-G base-pair; both mutations, individually, would weaken or disrupt the base-pair and would be selected against. Thus Sabin 3 with an A at nucleotide 537 might be more genetically stable in this region of the genome than the vaccine strain. However, the results of the present research, shown in Table 1 below (Leon-UA, Leon-UG), suggested that such a strain would be less attenuated than Sabin 3.

Table 1

Virus	472-537	T(L20B)[a]	Clinical Score[b]	MLS[c] (range)
T7/Leon	C-G	39.6	4/4[d]	2.71[d] (2.5-3.0)
Leon-UA	U-A	39.0	6/8	2.0 (1.07-2.48)
Leon-UG	U-G	38.4	4/8	1.78 (1.56-2.40)

a Temperature (T) in °C at which pfu were reduced by 1.0 $\log_{10}$ relative to pfu at 35°C in L20B cells.

b Number of paralysed animals/total number.

c Mean histological lesion score

d T7/Leon results from Westrop *et al.*, 1989, cited above.

Attenuation phenotypes in Leon/Lansing strains

Consequently, viruses were constructed with further mutations. These were first introduced into a Leon/Lansing (L3L2) genomic background so that temperature sensitivity data could be compared with those from previous studies (Macadam *et al.*, 1992). The mutants constructed and the ts phenotypes are shown in Table 2.

Mutant L1, with 472-537 U-A, confers a ts phenotype intermediate between those of Leon-Lansing, with C-G at 472-537, and Mutant L2 with U-G at 472-537, all being in a Leon-Lansing genomic background. A further mutation in the L3L2 472-537 U-A background of C-G to U-A or A-U at 478-533, 481-530 or 482-529 resulted in a large reduction of the T value, i.e. increased temperature-sensitivity, (compare mutant L1 with mutants L5, L8 and L10). This was considered likely to result in over-attenuation.

Introduction of one further mutation into strains L5, L8 and L10, of a G to A at nucleotide 531, converted a U-G base-pair at 480-531 to a U-A base-pair. Resultant viruses (mutants L14, L15 and L16) had ts phenotypes very similar to that of L3L2 472-537 UG. On the basis of the correlation of ts and attenuation, it was hoped that these combinations of mutations would not alter the attenuation phenotype of Sabin 3 significantly.

Table 2

Virus	Sequence of domain V										T(BGM)*
		stem a				stem b					
	471		473	477						483	
Leon/Lansing	U A	C G	C .... C G .... G		C G	A U	U G	G C	G C	A U	39.4
	538		536	534						528	
L2L2 472/537 UA (Mutant L1)		U A									39.1
L3L2 472/537 UG (Mutant L2)		U G									38.7
Mutant L5		U A			U A						38.0
Mutant L8		U A						U A			38.0
Mutant L10		U A							A U		38.0
Mutant L14		U A			U A		U A				38.7
Mutant L15		U A					U A	U A			38.7
Mutant L16		U A					U A		A U		38.7

* Temperature (T) in °C at which pfu were reduced by 1.0 log10 relative to pfu at 35° in BGM cells.

Attenuation phenotypes in Sabin strains

The mutations in domain V of mutants L14, L15 and L16 were introduced into a Sabin 3 genomic background and viruses were assayed for temperature-sensitivity in L20B cells. Ts phenotypes of these strains (mutants S14, S15 and S16) were similar to that of Sabin 3, although strain S14 was slightly more ts than Sabin 3 (Table 3). Mutants S15 and S16 were also tested for neurovirulence in monkeys and results are shown in Table 3 along with two sets of results for the cloned Sabin 3 strain ("T7/Sabin 3"), one set from a concurrent test and another derived from all

neurovirulence tests of this virus carried out to date. The attenuation phenotype of strain S15 hardly differed from that of T7/Sabin 3. No animals were paralysed by any of the viruses. The mean lesion score of strain S15 was almost identical to the score for T7/Sabin 3 in the concurrent test, although higher than the overall score. Strain S16 appeared slightly more attenuated than T7/Sabin 3. On the preliminary basis of these results both strains S15 and strain S16 would have passed the above-cited WHO monkey neurovirulence test. Strain S14, being more ts than Sabin 3, would also have done so.

Repeated passage of S15 and S16 in L20B cells has demonstrated a lack of reversion to virulence.

Genetic Stability

The current type 3 vaccine strain of poliovirus reverts rapidly in the human gut and is associated with a low level of paralytic poliomyelitis in vaccine recipients or their contacts. It has not yet been possible to compare the stabilities of these strains with that of Sabin 3 during growth in the human alimentary tract, so a cell culture model was sought. Conditions which favoured rapid reversion at nucleotide 472 were presumed to mimic selection pressures in the human gut. Reversion of nucleotide 472 from U to C on passage of the Sabin 3 strain in cell culture is temperature- and cell-dependent and it was found that growth at 37°C in L20B cells strongly selects for this reversion.

T7/Sabin 3 and strains S15 and S16 were passaged five times in L20B cells at 37°C using a multiplicity of infection (moi) of 10. All passage stocks were assayed for temperature sensitivity in L20B cells and fifth passage stocks were tested for neurovirulence. In addition, viral RNA was extracted and sequenced through domain V after RT-PCR. Sequencing and MAPREC (Mutant Analysis by Polymerase Chain Reaction and Restriction Enzyme Cleavage) assays (Lu *et al.*, PCR Methods Appl. *3*, 176-180, (1993) showed that by passage 3 over 50% of the Sabin 3 virus population had reverted to the wild type genotype of C at nucleotide 472, and by fifth passage almost 100% had a C at nucleotide 472. No other nucleotide changes were observed in domain V of these viruses. No nucleotide changes were observed in domain V of the fifth passage stocks of strains S15 or S16. In a temperature sensitivity assay, based on plaque formation and capable of distinguishing viruses with C at 472 from

those with U at 472, it was found that the ts phenotype of Sabin 3 was lost rapidly on passage (Fig 4) in parallel with the U to C mutation. In particular, passage 3 virus was only slightly more temperature-sensitive than the passage 5 or the fully reverted virus (S3/472C). In contrast, the ts phenotypes of strains S15 and S16 were much more stable (Figs 5 and 6). A gradual loss of temperature sensitivity did occur on passage of both these strains, which must have been due to mutation outside domain V. In a neurovirulence test, the fifth passage stock of Sabin 3 was significantly more virulent than the Sabin 3 vaccine strain, as shown by numbers of clinically affected animals and histological lesion scores, whereas the fifth passage stocks of strains S15 and S16 were as attenuated as the original stocks and the Sabin 3 vaccine strain (Table 3). Any mutations that did occur in strains S15 and S16 on passage therefore did not result in an increase in virulence. Thus, under conditions which rapidly selected neurovirulent variants of Sabin 3, the attenuation phenotypes of strains S15 and S16 were stable. These results indicate that S15 and S16 have highly suitable phenotypes to be regarded as new candidate vaccine strains.

Table 3

Virus	Domain V sequence	T(L20B)[a]	Clinical Score[b]	MLS (range)[c]
T7/Sabin 3 (all test results)	Vaccine-like (As mutant L2: see Table 2)	37.2	0/15	0.49 (0.07-2.04)
T7/Sabin 3 (concurrent test results)	Vaccine-like (As mutant L2: see Table 2)	37.2	0/4	0.63 (0.09-2.04)
T7/Sabin 3, 5th passage	Leon-like (472C)	Not done	1/4	1.92 (1.77-2.30)
Mutant S14	As mutant L14: see Table 2	37.0	Not done	Not done
Mutant S15	As mutant L15: see Table 2	37.3	0/4	0.61 (0.14-1.53)
Mutant S15, 5th passage	As mutant L15	Not done	0/4	0.53 (0.23-1.05)
Mutant S16	As mutant L16: see Table 2	37.1	0/4	0.42 (0.21-0.74)
Mutant S16, 5th passage	As mutant L16	Not done	0/4	0.68 (0.51-1.18)

a Temperature (T) in °C at which pfu were reduced by 1.0 log10 relative to pfu at 35°C, in L20B cells.

b Number of paralysed animals/total number

c Mean histological lesion score

CLAIMS

1. An attenuated poliovirus which does not have a base pair mismatch in stem (a) or (b) of domain V of the 5' non-coding region of its genome, which is sufficiently attenuated to pass the WHO monkey neurovirulence test (1983) and which is more stable than the corresponding Sabin strain against reversion to neurovirulence.

2. A poliovirus according to Claim 1, which has substantially the same degree of attenuation as the corresponding Sabin strain.

3. An attenuated poliovirus which does not have a base pair mismatch in stem (a) or (b) of domain V of the 5' non-coding region, in which a U-A base pair is present in domain V of the 5' non-coding region at positions 472-537 in type 3 or 469-534 in type 1 or 2, and which is sufficiently attenuated to pass the WHO monkey neurovirulence test (1983).

4. A poliovirus according to Claim 1, 2 or 3, which is of type 3 and in which a U-A base pair is present in domain V of the 5' non-coding region at positions 480-531.

5. A poliovirus according to Claim 4, in which a U-A base pair is present at positions 472-537 and 480-531.

6. A poliovirus according to claim 1 which is a type 3 poliovirus in which U-A base pairs are present in domain V of the 5' non-coding region as follows:

(a) U-A at positions 472-537, U-A at positions 478-533 and U-A at positions 480-531;

(b) U-A at positions 472-537, U-A at positions 480-531 and U-A at positions 481-530; or

(c) U-A at positions 472-537, U-A at positions 480-531 and A-U at positions 482-529.

7. A poliovirus according to any preceding Claim, for use in a vaccine.

8. A vaccine comprising an attenuated poliovirus as defined in any one of Claims 1 to 6 and a pharmaceutically acceptable carrier or diluent.

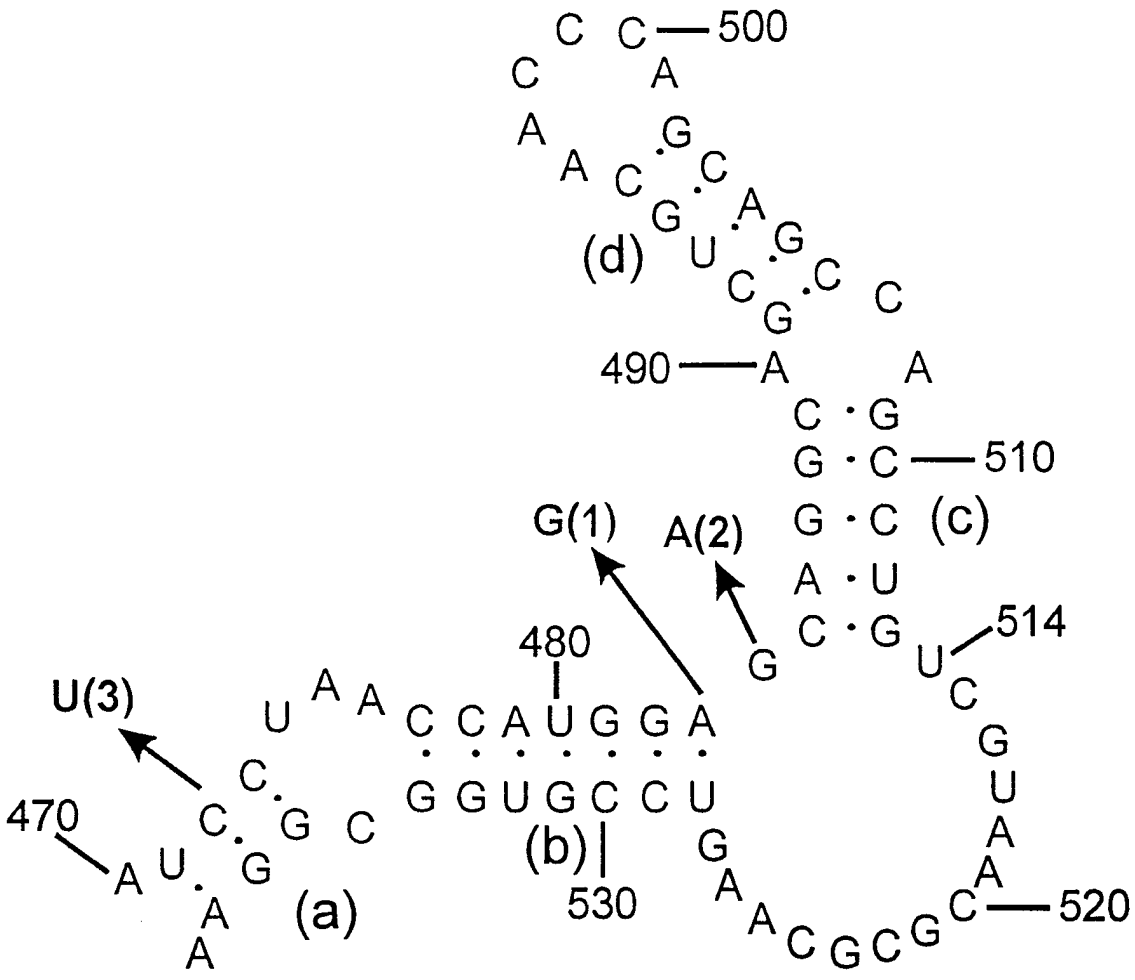

Fig. 1

Virus	Sequence of domain V									
			Stem a					Stem b		
	471		473	477						483
Sabin 3	**U**	*U*	**C** ... **C**		**C**	A	*U*	G	**G**	A
	A	G	**G** ... **G**		**G**	U	G	C	**C**	U
	538		536	534						528
			Stem a					Stem b		
	468		470	474						480
Sabin 2	**U**	C	**C** ... **C**		**C**	A	C	G	**G**	A
	A	G	**G** ... **G**		**G**	U	G	*U*	**C**	U
	535		533	531						525
			Stem a					Stem b		
	468		470	474						480
	U	C	**C** ... **C**		**C**	*U*	C	G	**G**	*G*
Sabin 1	**A**	G	**G** ... **G**		**G**	U	G	C	**C**	U
			533	531						
			Stem a					Stem b		
	U	C	**C** ... **C**		C	*U*	C	G	G	G
Consensus	**A**	G	**G** ... **G**		G	U	G	C	C	U

Bold Conserved base-pair

Italic Type-specific nucleotide

Fig. 2

Stem c
Stem d
Sabin 3
485
C A G G C C A A C C
G U C C G C G C A C
Sabin 2
482
C A G G C C G A A C
G U U C G C A G C
510 G
Sabin 1
482
C A G G U C A A C C
G U C C G C U G C
510 G
Bold UG (GU) base-pairs

Fig. 3

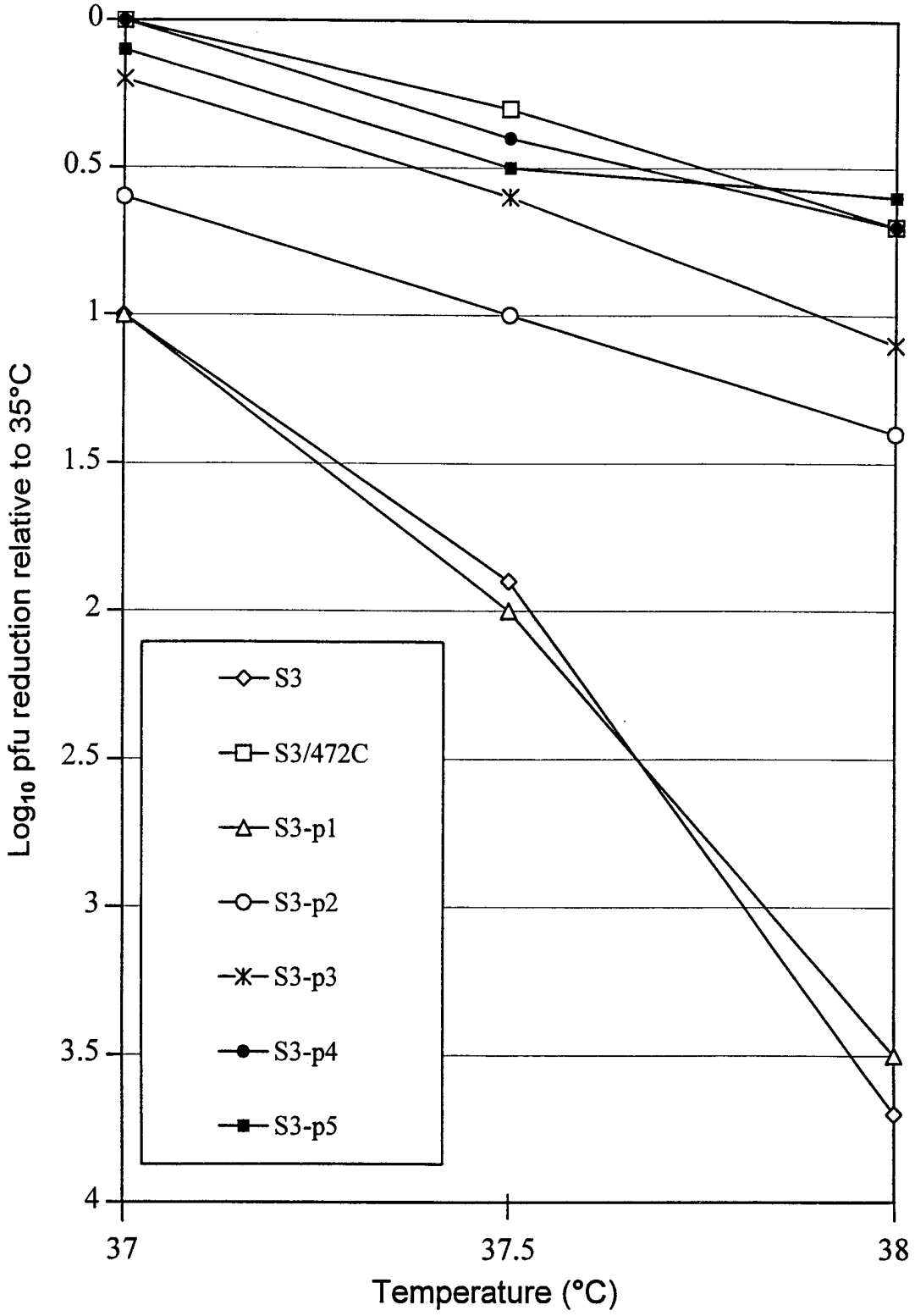
0
0.5
1
1.5
2
2.5
3
3.5
4
Log10 pfu reduction relative to 35°C
37
37.5
38
Temperature (°C)
S3
S3/472C
S3-p1
S3-p2
S3-p3
S3-p4
S3-p5

Fig. 4

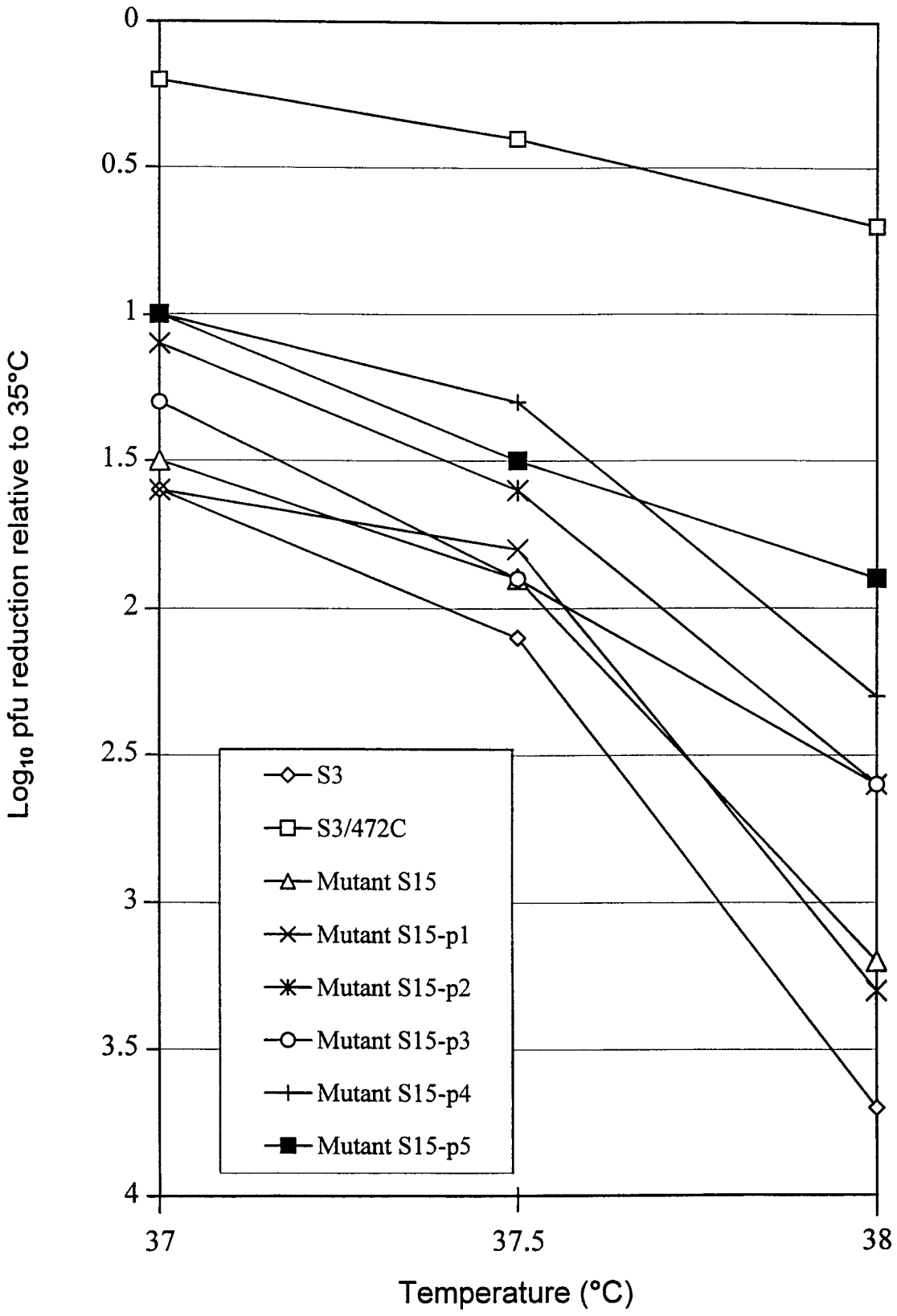
0
0.5
1
1.5
2
2.5
3
3.5
4
37
37.5
38
Log10 pfu reduction relative to 35°C
Temperature (°C)
S3
S3/472C
Mutant S15
Mutant S15-p1
Mutant S15-p2
Mutant S15-p3
Mutant S15-p4
Mutant S15-p5

Fig. 5

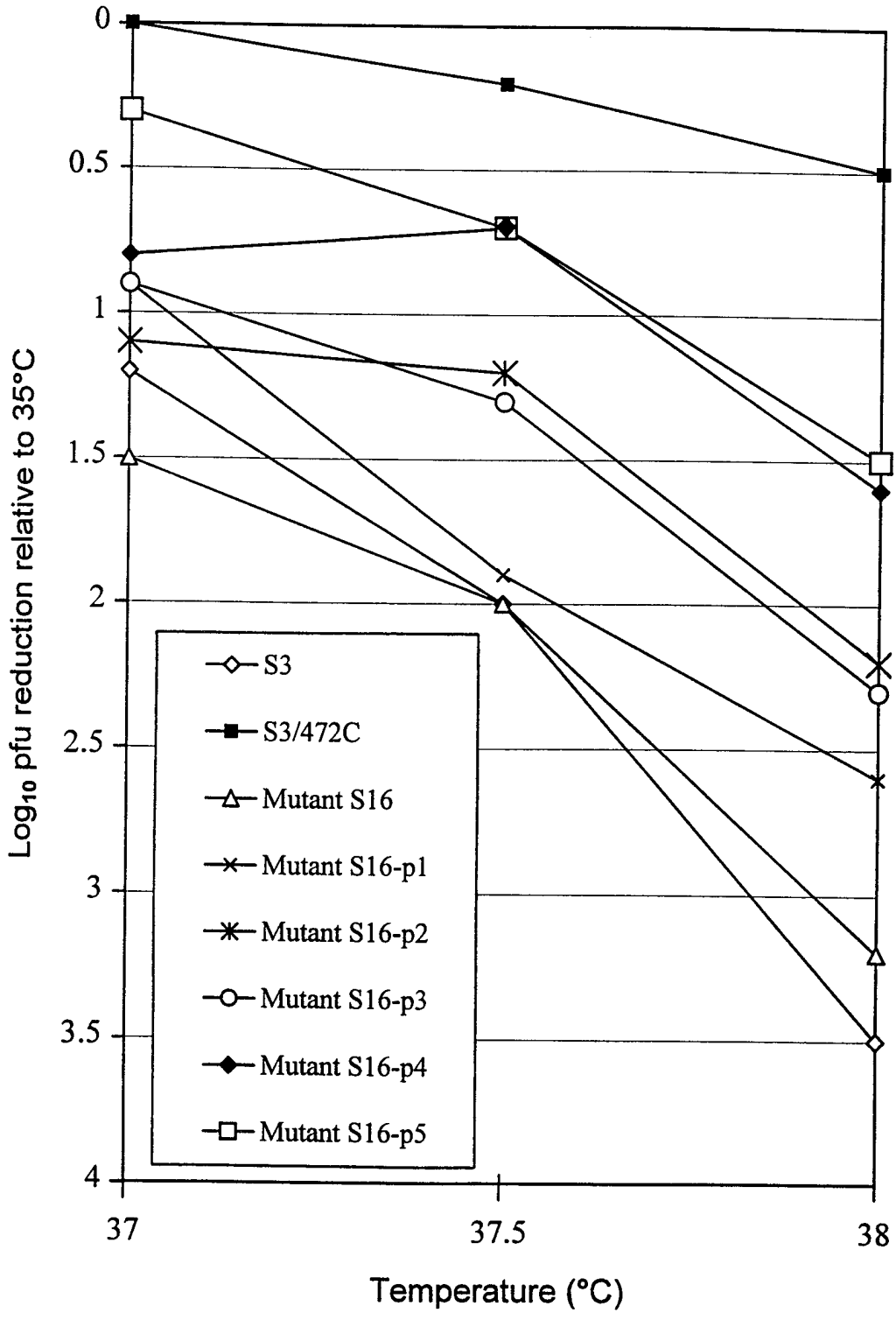
0
0.5
1
1.5
2
2.5
3
3.5
4
Log10 pfu reduction relative to 35°C
37
37.5
38
Temperature (°C)
S3
S3/472C
Mutant S16
Mutant S16-p1
Mutant S16-p2
Mutant S16-p3
Mutant S16-p4
Mutant S16-p5

Fig. 6

INTERNATIONAL SEARCH REPORT

Int .tional Application No
PCT/GB 98/00798

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N7/04 A61K39/13

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 508 783 A (BRITISH TECH GROUP) 14 October 1992 see example 1 ---	1-5,7,8
X	EP 0 325 768 A (AMERICAN CYANAMID CO) 2 August 1989 see page 5, left-hand column, paragraph 3; figure 1 ---	1,2,7,8
X	MACADAM A J ET AL: "CORRELATION OF RNA SECONDARY STRUCTURE AND ATTENUATION OF SABIN VACCINE STRAINS OF POLIOVIRUS IN TISSUE CULTURE" VIROLOGY, vol. 189, no. 2, August 1992, pages 415-422, XP002037412 cited in the application see Table 3 mutant LL480/531 480UËA --- -/--	1,7,8

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
13 July 1998	20/07/1998

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Int. tional Application No
PCT/GB 98/00798

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	HALLER A A ET AL: "ATTENUATION STEM-LOOP LESIONS IN THE 5' NONCODING REGION OF POLIOVIRUS RNA: NEURONAL CELL-SPECIFIC TRANSLATION DEFECTS" JOURNAL OF VIROLOGY, vol. 70, no. 3, March 1996, pages 1467-1474, XP002037413 see figure 1 ---	1-8
A	EP 0 383 434 A (BRITISH TECH GROUP) 22 August 1990 see the whole document -----	

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