VIROLOGY 210, 160–166 (1995)

Studies on Neurovirulence in Poliovirus-Sensitive Transgenic Mice and Cynomolgus Monkeys for the Different Temperature-Sensitive Viruses Derived from the Sabin Type 3 Virus

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Received January 3, 1995; accepted April 13, 1995

We have studied methods for testing the neurovirulence of live poliovaccine viruses by intraspinal inoculation into mice carrying the human poliovirus receptor gene (Tg mice). A comparison of the neurovirulence of Sabin type 3 vaccine virus and related viruses using the 50% paralysis dose determined after intraspinal inoculation into the Tg mice as an index revealed a close correlation between the results of the paralysis dose in Tg mice, the neurovirulence expressed by the histopathological lesion score in monkeys, and the temperature sensitivity of the viruses. The results of experiments in the Tg mice also showed a good correlation with the number of mutations at position 472 from U to C in the 5′ noncoding region in the genomes of the viruses tested. These results strongly suggest that the neurovirulence test for oral poliomyelitis vaccine using the Tg mice is an excellent method and may be used in place of the test using monkeys.

INTRODUCTION

Oral poliomyelitis vaccine (OPV) is one of the safest and most effective vaccines used in humans, as shown by the successful eradication of paralytic cases caused by wild virulent viruses in many developed countries (WHO Weekly Epidemiol. Rec., 1994). However, it is ironic that about one vaccine-associated case appears in about every 2,500,000 doses of OPV used in countries where the wild viruses have been eradicated (Prevots *et al.*, 1994). It is not clear why such cases occur. One possibility is that revertants derived from vaccine viruses during proliferation in the human gut may be responsible, the thought of which naturally has caused concern for some time regarding the use of OPV (Melnick, 1978). Recent progress in genetic technology has shown that viruses undergo changes at the gene level during proliferation in the human gut and also show increased neurovirulence. This is particularly so with the Sabin type 3 virus. There are 10- or 12-base point mutations in the virus genome between the Sabin type 3 strain and the parent virulent strain, Leon (Stanway *et al.*, 1984; Toyoda *et al.*, 1984; Tatem *et al.*, 1991). Evans *et al.* (1984) and Chumakov *et al.* (1992) reported that position 472 in the 5′ noncoding region in these point mutations closely correlates to neurovirulence in monkeys.

The neurovirulence test (NVT) in monkeys (WHO, 1990) requires special techniques and experience, and the monkeys used are expensive and a limited resource. Unlike small laboratory animals, monkeys show individual differences which sometimes may cause discrepancies in experimental results. Monkeys are also a reservoir of hazardous microorganisms, which presents a danger to those handling these animals. There are thus many problems involved in the use of monkeys.

For these reasons, if a simple, highly reliable NVT can be established in place of the current NVT using monkeys, such a development would have distinct advantages, namely, in the safety test performed on OPV and also with respect to the surveillance of polioviruses, which is indispensable in the task of eradicating poliomyelitis throughout the world. We feel the use of the NVT in mice instead of monkeys would assist in achieving this objective.

The poliovirus-sensitive transgenic mouse carrying the human poliovirus receptor (PVR) gene (Tg mouse), which was developed by Koike *et al.* (1991, 1994), has been studied with the aim of establishing such a new NVT. There are four lines of the Tg mouse, ICR-PVRTg1, ICR-PVRTg5, B10-PVRTg8, and ICR-PVRTg21. Varying susceptibility of the Tg mouse lines to poliovirus are dependent on the copy number of the PVR transgene and the level of PVR mRNA and cell-membrane-associated PVR protein in the central nervous system of the mouse lines. In preliminary tests the descending order of sensitivity of these mouse lines to type 1 virulent virus, Mahoney strain, was shown to be ICR-PVRTg1, B10-PVRTg8, ICR-

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0042-6822/95 $6.00

PVRTg21, and ICR-PVRTg5. ICR-PVRTg1 mice were inoculated with attenuated virus, Sabin type 1, by various routes: orally, intraperitoneally, intravenously, subcutaneously, intramuscularly, and intracerebrally. The results indicated that these routes of inoculation were unsatisfactory for testing vaccine viruses because they all showed an inadequate level of sensitivity (Horie *et al.*, 1994). As a result, the intraspinal inoculation method used in the NVT in monkeys was modified and tried in Tg mice. The sensitivity of ICR-PVRTg1 mice to the Sabin type 1 virus was so high that paralysis occurred following inoculation with very small amounts of virus. Thus, it was concluded that the ICR-PVRTg1 mice were unsuitable for evaluating the neurovirulence levels of type 1 vaccine viruses. Therefore, we chose to use ICR-PVRTg21 mice for the test. The results of the experiments by intraspinal inoculation of Sabin type 1 viruses using these mice showed good correlation with the histopathological lesion scores of the NVT in monkeys (Abe *et al.*, 1995). Furthermore, histopathological lesions in the central nervous system could be observed in all of the mice with paralysis. Accordingly, it was decided to test the neurovirulence of polioviruses in ICR-PVRTg21 mice.

In the present study, the NVT was performed with various type 3 viruses derived from the Sabin type 3 virus in IQI-PVRTg21 mice by intraspinal inoculation. These results were compared with the results of the NVT performed using monkeys, the temperature sensitivity test, reproductive capacity at different temperature tests (*rct* marker tests), and mutation analysis using the polymerase chain reaction and restriction enzyme cleavage method.

MATERIALS AND METHODS

Viruses

The viruses used in the present study were derived from the Sabin type 3 virus strain (Sabin *et al.*, 1973) except for a virulent strain. As reference strains having the standard biological properties of the vaccine virus, we used the WHO reference virus (Sabin type 3 Behringwerke AG, SO + 2) and F313, a Sabin type 3 reference virus prepared in Japan (SO + 8, including two plaque clonings). To obtain a virus with a higher attenuated phenotype, a clone was selected by plaque cloning twice from the Sabin type 3 virus (SO + 2) as an index of the temperature sensitivity. PV3-WV (SO + 10, including three plaque clonings and two limiting dilution cultures with monkey kidney cells), showing a lower virulence than the reference viruses, was also used. Mixed virus suspensions of F313 and PV3-WV at ratios of 1:99 and 10:90 were prepared and were studied to determine whether the theoretical properties at the ratios prepared were accurately reflected in the results obtained from determining temperature sensitivity and from the NVT using animals.

Serial passage of F313 in primary African green monkey kidney cells at 38° was performed to obtain a virus with higher neurovirulence and lower temperature sensitivity than F313. The second (F313-38/2) and fourth (F313-38/4) passages were discovered to show marked differences in temperature sensitivity from F313 and were used in the experiments presented here.

The Suwa strain was isolated from a paralytic polio patient in Japan. The isolate showed evidence in *in vitro* marker tests of being a more virulent phenotype than the Saukett strain (data not shown).

Tg mice

To achieve a steady and reliable supply of mice with uniform genetic backgrounds for use in poliovirus testing, the PVR gene of ICR-PVRTg21 (homozygote) was introduced into the inbred IQI mouse-line-derived ICR line. IQI-PVRTg21 (heterozygote) (Tg21) mice which were obtained after successive back-crosses to IQI line (Hioki *et al.*, 1993) were used in this study. The Tg21 line has been maintained under specific-pathogen-free conditions at the Central Institute for Experimental Animals. These mice showed no significant differences in sensitivity to the polioviruses by intraspinal inoculation compared to the results obtained using the ICR-PVRTg21 (heterozygote) mice (data not shown).

The mice were supplied at 4 weeks of age and were used for experiments at 5–8 weeks of age. Each group in the experiments contained about the same number of males and females.

NVT in mice by intraspinal inoculation

Intraspinal inoculation of Tg mice was performed by the method of Abe *et al.* (1995) as described below. The intraspinal inoculation was conducted using a 25-μl microsyringe and a needle specially made with a 33-gauge piercing tip (7 mm in length) and a 25-gauge part near the part connecting to the syringe (8 mm in length) (Exmire, Ito Corp., Shizuoka, Japan).

The cell culture infective dose ($CCID_{50}$) of the virus suspensions was determined using GMK2 cells (an African green monkey kidney cell line established in NIH Japan). Tenfold serial dilutions of virus suspension were prepared using medium 199 containing 0.225% $NaHCO_3$. A total of 5–10 mice were each inoculated with 5 μl of 3–5 dilutions. The inoculation sequence was as follows.

1. Preinoculation anesthesia was achieved by injecting about 0.4 ml of 5 mg/ml pentobarbital intraperitoneally into a mouse weighing about 20 g. When anesthesia was incomplete, inhalation anesthesia using ether was used.

2. After the hair and skin on the back of the mouse were disinfected with ethanol, scissors were used to cut along the midline for about 2 cm and the spine was exposed. The mouse was placed on a glass tube about 14 mm in diameter to expand the intervertebral space.

3. The needle was inserted into the enlargement of

the lumbar cord from the intervertebral space near the apex of the spinal curvature. Following insertion of the needle into the lumbar cord, correct insertion was indicated by the twitching of one or both hind limbs. A slight reaction was observed during the injection of 5 μl of virus suspension.

4. After the inoculation, the incised skin on the back was closed using a rapid-drying adhesive.

Mice that showed clinical injury within 24 hr after inoculation were excluded from the study as animals with mechanical injury due to the inoculation.

The clinical observations were performed daily for a period of 14 days. The mice were individually placed on an observation stand (about 60 × 60 cm), and their general condition, movements of the four limbs, and movements of the digits were observed in detail. Estimation of paralysis was carried out as follows:

0 No abnormality.
1 Weakness: The mice showed unnatural gait.
2 Paresis: The mice showed a gait with the heels of one or both hind limbs touching.
3,4 Paralysis: The mice crawled, dragging one or both hind limbs.

Paresis and paralysis were evaluated as positive paralysis.

The viral dose that causes paralysis in 50% of Tg mice (50% paralysis dose; PD_{50} in $CCID_{50}$) and serves as an index of neurovirulence was calculated by the method of Reed and Muench (1938).

NVT in monkeys

The test was performed in monkeys in accordance with the WHO method (WHO, 1990). Neurovirulence of the virus was expressed as the mean lesion score (MLS) calculated from histopathological observation of the central nervous system of monkeys inoculated intraspinally.

Temperature sensitivity test

The temperature sensitivity test used was the *rct* marker test (Omata *et al.*, 1984), conducted in primary cynomolgus monkey kidney cell cultures at temperatures of 36°, 39°, and 39.5°. Seven days after inoculation, plaques were counted. The titer in plaque forming units (PFU) was calculated for each titration at three temperatures. The temperature sensitivity of each virus was expressed as the difference of PFU ratios at 36° and at high temperatures (39 and 39.5°).

Analysis of mutation in the viral RNA genome

The abundance of virus particles showing mutation from uracil (U) to cytosine (C) at position 472 of the 5′ noncoding region of the genome RNA was determined by mutant analysis using the polymerase chain reaction and restriction enzyme cleavage (MAPREC) method (Chumakov *et al.*, 1992). In brief, viral RNA was isolated by phenol/SDS extraction from the virus sample. This was reverse transcribed by MoMuLV reverse transcriptase with random hexanucleotide primer, and polymerase chain reaction amplification of a segment of this cDNA was performed using sense primer (431) TGAGCTACAT GAGAGTGCTC CGGCCCCTGA ATGCGGCTGA (470) and antisense primer (513) CAGGCTGGCT GCTGGGTTGC AGCTGCCTGC (484) taken at a 10:1 ratio to ensure asymmetric amplification and generation of excess single-stranded DNA of sense polarity. The dsDNA was synthesized by DNA polymerase reaction using ^{32}P-labeled antisense primer, and the number of revertants was determined as the fraction of DNA that was digested with *Mbo*I restriction enzyme. After separation of the restriction fragments on polyacrylamide gel, the radioactivity of the DNA bands was quantitated by a beta imaging system.

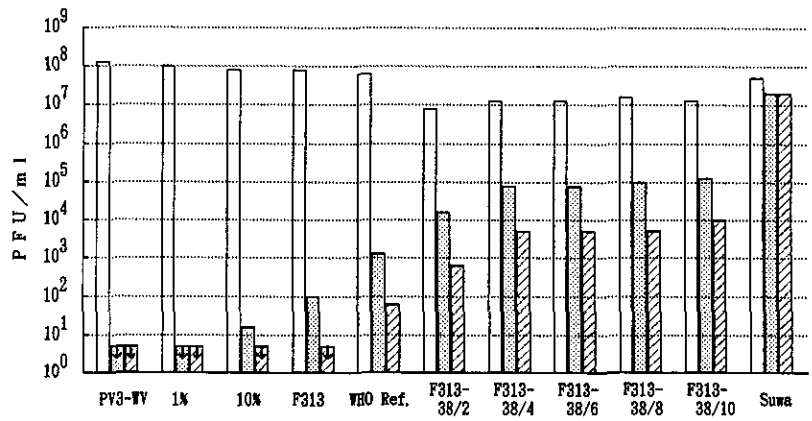

FIG. 1. Temperature sensitivity of Sabin type 3 viruses used in this study (plaque formation at 36°, 39°, and 39.5° — *rct* marker test). The infectious titer (PFU) of each virus at (□) 36°, (▩) 39°, and (▨) 39.5° is shown for type 3 viruses used in the study. The virulent Suwa strain showed low temperature sensitivity with high PFU at high temperatures (39 and 39.5°), almost the same as the PFU at 36°. In contrast, PV3-WV showed high temperature sensitivity with a PFU of about 10^8 at 36°, but a PFU of less than $10^{0.7}$ at temperatures of 39 and 39.5°. The 1% and 10% refer to the F313 virus content in PV3-WV. The sign (↓) in the columns indicates that no plaque formation by undiluted viral suspension (less than $10^{0.7}$ PFU) was observed at that temperature.

RESULTS

Temperature sensitivity (*rct* marker) of viruses

Figure 1 shows the results of the *rct* marker test of type 3 polioviruses used in this study. The WHO reference virus produced clear plaque formation ($10^{1.8}$ PFU) at 39.5 and at 39° more than 10^3 PFU were produced. F313 showed little plaque formation at 39.5° and 10^2 PFU at 39°. In comparison, PV3-WV showed no plaque formation at 39.5 and 39°. Mixed virus suspensions containing both F313 and PV3-WV did not show plaque formation at 39.5°, but the mixtures showed plaque formation corresponding to those of the individual ratios in the mixed suspension at 39°.

However, viruses derived from F313 passaged at 38° showed increased plaque formation capacity at high temperatures with increased passages. A dramatic change in the *rct* marker occurred at the 2nd passage but, thereafter, the degree of this change was markedly reduced until, by the 10th passage, its plaque forming capacity approached that of the *rct* marker of the virulent Suwa strain virus.

NVT in Tg21 and monkeys

As recently reported by Abe *et al.* (1995), degeneration of the nerve cells was very evident in histological specimens of the central nervous system of paralyzed Tg mice, but almost no lesions appeared in mice showing no paralysis. The lesions in Tg mice inoculated intraspinally with attenuated poliovirus showed weaker inflammatory reactions than those in monkeys, but there was obvious degeneration of the nerve cells. The lesions also spread from the lumbar cord to the cervical cord and the brain in almost all paralytic mice. Therefore, in NVT for poliovirus using Tg21, histopathological examinations would appear not to be required and an evaluation of the neurovirulence based solely on the incidence of paralysis seems feasible.

Following intraspinal inoculation, paresis of the digits on the hind limbs was seen as a mild symptom, and such mice were unable to grasp objects. As paresis progressed, dragging of the hind limbs occurred. Paralysis was bilateral in some cases, but in studies with low virulent virus, unilateral paralysis was common; in such cases reduced tonus also appeared on the opposite side in many cases. Mice with bilateral paralysis of the hind limbs were still able to use their fore limbs to move. They continued to take food and water and there was no evidence of the occurrence of sudden death. When subsequent paralysis of the fore limbs took place, the animals were unable to eat or drink and soon expired. The time interval from the onset of paralysis to death was reduced when virus with higher infectivity or of a more virulent nature was injected.

Figure 2 shows the clinical observations of Tg21 mice inoculated intraspinally with F313 and F313-38/2. As can be seen, the number of mice paralyzed or dying was wholly dependent on the quantity of virus inoculated.

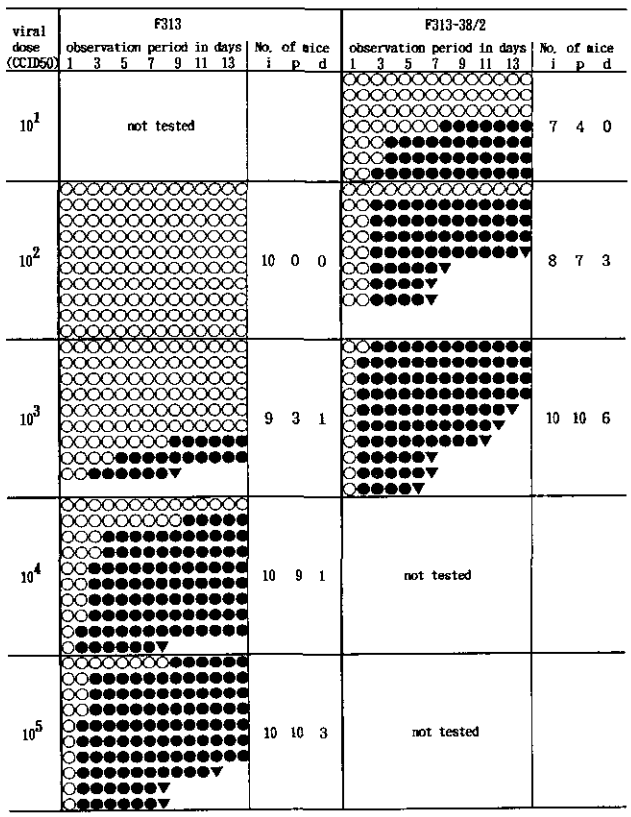

FIG. 2. Clinical observations of Tg21 mice inoculated intraspinally with F313 and F313-38/2. Each circle indicates a clinical sign of an individual mouse during the observation period (○, no abnormalities; ●, paralysis positive; ▼, death or sacrificed because moribund. Under No. of mice, (i) indicates number of inoculated mice, (p) indicates the number of paralysis-positive mice, and (d) indicates the number of dead mice or mice sacrificed because they were moribund. PD_{50} values were calculated from these numbers. The PD_{50} of the F313, for example, was $10^{3.3}$ $CCID_{50}$.

The *rct* marker, the PD_{50}, the MLS of NVT in monkeys, and the number of the mutations from U to C at position 472 of the tested viruses are all summarized in Table 1. The WHO reference virus showed a slightly more virulent phenotype than did F313 based on the MLS in monkeys and *rct* marker. PD_{50} values of F313 and PV3-WV were $10^{3.2}$ and $10^{4.8}$ $CCID_{50}$, respectively, and the PD_{50} values for the two mixtures of F313 and PV3-WV were intermediate between the PD_{50} values of the two original viruses. The PD_{50} values of the passaged viruses of F313, F313-38/2 and F313-38/4, were $10^{1.0}$ and less than $10^{0.6}$ $CCID_{50}$, respectively. The neurovirulence tended to increase markedly when the viruses were subcultured at 38°. F313-38/4 caused paralysis at very small doses in Tg21, and it was difficult to estimate the PD_{50} in Tg21. It may be that Tg21 is equally or slightly more sensitive to the revertant F313-38/4 than is the test based on CPE formation on GMK2 cells, which was used to determine $CCID_{50}$. The PD_{50} of these viruses showed a close correlation with the log difference of PFU observed at temperatures between 36 and 39° (temperature sensitivity) (Fig. 3).

The NVT results for F313 and PV3-WV and mixtures

TABLE 1

Comparison of Neurovirulence-Related Characteristics among Sabin Type 3-Derived Viruses

Sample	*rct*[a] 39.5°	*rct*[a] 39°	Tg21[b] PD_{50} in $CCID_{50}$	Monkey[c] MLS	472[d] (U → C)
(F313 + PV3 − WV)					
0 + 100	>7.5	>7.5	$10^{4.8}$ (n = 18/dil)	0.34 (n = 22)	0 (%)
1 + 99	>7.5	7.0	$10^{4.3}$ (n = 10/dil)	0.47 (n = 4)	0.003
10 + 90	>7.5	6.8	$10^{3.8}$ (n = 10/dil)	0.70 (n = 4)	0.03
100 + 0	7.4	5.8	$10^{3.2}$ (n = 30/dil)	1.03 (n = 22)	0.3
WHO reference	6.0	4.7	$10^{3.2}$ (n = 20/dil)	1.13 (n = 22)	n.t.
F313-38/2[e]	4.1	2.7	$10^{1.0}$ (n = 9/dil)	n.t.	77.8
F313-38/4[f]	3.5	2.2	$<10^{0.6}$ (n = 9/dil)	n.t.	94.1
F313-38/10[g]	2.7	2.0	n.t.	n.t.	99.1
SUWA[h]	0.4	0.3	n.t.	n.t.	n.t.

[a] Results of the temperature sensitivity test showed differences in reproductive capacity (titer in PFU) at 36 and 39 or 39.5°.

[b] NVT results in Tg mice are shown. The number of mice tested per dilution in a 10-fold serial dilution is shown in parentheses.

[c] NVT results in monkeys are shown. Monkeys were inoculated intraspinally with 10^6 $CCID_{50}$ of each virus. MLS, mean lesion score (WHO, 1990). The number of monkeys tested is shown in parentheses.

[d] The results of the MAPREC tests (Chumakov *et al.*, 1992) are shown as percentage of viral particles with C at position 472 (472-C) from 5′ terminus of the viral genome. The quantity of 472-C in viral suspensions of F313 and PV3-WV mixtures is calculated from the test data of 472-C content for F313 (0.3%) and PV3-WV (0%), respectively, by the MAPREC test.

[e,f,g] Virus obtained by passaging F313 2, 4, or 10 times at 38°, respectively.

[h] A virulent strain of type 3 poliovirus.

of the two in monkeys (Table 1) also showed a close correlation with the PD_{50} in Tg21 (Fig. 4). Since F313-38/2 appeared to have neurovirulence much higher than that of F313 from the results of NVT in Tg21, the NVT in monkeys was not performed.

Relationship between the abundance of 472-C and neurovirulence shown in Tg21 and monkeys

In the genomic RNA of the Sabin type 3 strain, U at position 472 in the 5′ noncoding region is considered to revert frequently to C. The mutation of U to C at position 472 has been reported to be closely related to the neurovirulence of the viruses in monkeys (Chumakov *et al.*, 1992).

Table 1 shows the results of a study of the level of viral particles with point mutation at position 472 to C by the MAPREC method for the viruses tested by the NVT in Tg21. The percentage of mutants of PV3-WV is shown as 0% because the results were less than those detectable by the MAPREC technique. The C mutation was

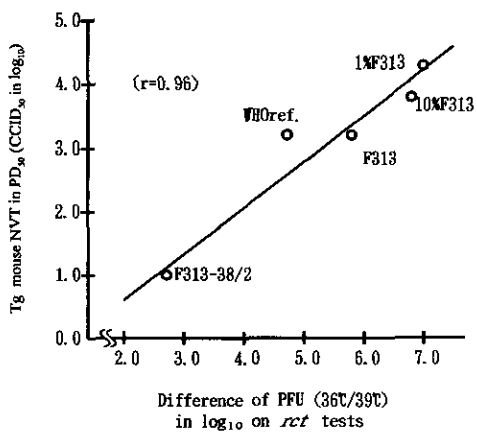

FIG. 3. Correlation between NVT in Tg21 mice and temperature sensitivity (*rct* marker). The ordinate shows the PD_{50} in Tg21 and the abscissa shows the log differences of the reproductive capacity of viruses PFU at 36 and 39°. The PD_{50} of the viruses in Tg21 showed a close correlation with the temperature sensitivity at a correlation coefficient of greater than 0.9.

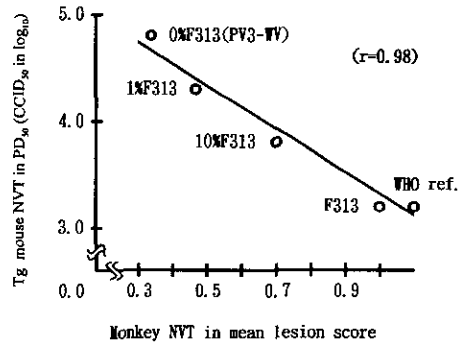

FIG. 4. Correlation between NVT in Tg21 and NVT in monkeys. NVT in monkeys was performed by inoculating cynomolgus monkeys intraspinally with 0.1 ml of 10^6 $CCID_{50}$ of a virus. Lesion scores for each monkey were determined from observations of histological specimens of the central nervous system 19 days after the inoculation, and the mean lesion scores were calculated for each group of monkeys in accordance with WHO requirements. F313, WHO reference, PV3-WV, and two mixtures of F313 and PV3-WV were plotted. The PD_{50} of the viruses in Tg21 mice and the monkey NVT showed a close correlation within this range.

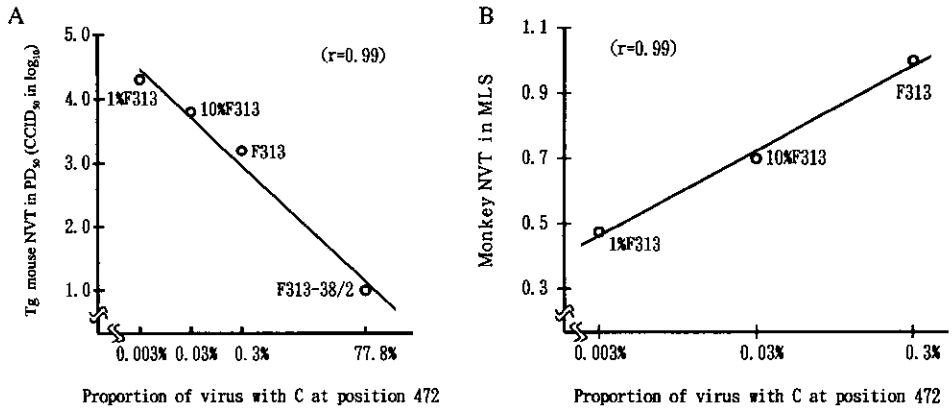

FIG. 5. Correlation between the percentage of viral particles with C at position 472 of the genome RNA (by the MAPREC method) and the NVT in Tg21 (A) or NVT in monkeys (B). In A, F313-38/2, F313, and two mixtures of F313 and PV3-WV were plotted. The 472-C proportions of 0.003 and 0.03% for the two mixtures are theoretical content by calculation. As shown, the percentage of viral particles with C at position 472 showed a good correlation with NVT results for both Tg21 mice and monkeys.

seen in 0.3% of F313 viral particles, and therefore, the mixed suspensions of these two viruses (mixtures containing 1 or 10% of F313) were calculated to contain 0.003 and 0.03% mutants, respectively. The viruses obtained by passaging F313 2, 4, and 10 times at 38° showed 77.8, 94.1, and 99.1% mutants, respectively, which indicated that a high rate of mutation occurs even when the virus has been passaged only a few times.

The percentage of C mutants ranging from 0.003 to 77.8% and the PD_{50} in Tg21 mice NVT showed a close correlation, as indicated in Fig. 5A. Although the C mutant percentages of 0.003 and 0.03% are merely calculated amounts, the MLSs of NVT in monkeys and the C percentages were also closely correlated, as shown in Fig. 5B.

DISCUSSION

The techniques employed for the NVT in monkeys, as a safety test for OPV virus, have already been established. However, it is becoming difficult to obtain large numbers of monkeys of uniform quality. To avoid the occurrence of differences in results due to this factor, WHO (1990) recommends that the neurovirulence of vaccine viruses be compared with that of the reference virus in repeated testing by each manufacturer and that the results be confirmed by statistical analysis based on lesion scores.

According to our experience, when more than 10 monkeys are inoculated intraspinally with 10^6, 10^5, or 10^4 $CCID_{50}$ of each concentration of virus, there are cases in which two groups of monkeys inoculated with 10^6 or 10^5 $CCID_{50}$ show a similar degree of neurovirulence clinically and histopathologically. It is difficult to estimate whether this is the result of individual differences among the monkeys or the properties of the virus. Differences also occur in the grade assigned to the lesions even in the same histological specimen, owing to the subjective nature of the observations (Wood *et al.*, 1994). Such problems arise with the NVT using monkeys. The NVT method for poliovirus using the viral dose showing 50% paralysis by intraspinal inoculation of Tg mice makes it possible to achieve a much more objective evaluation. Histopathological studies of Tg mice showing polio paralysis have confirmed that they are infected by the poliovirus and develop lesions in the central nervous system similar to those seen in humans and monkeys (Abe *et al.*, 1995). We have already confirmed that the NVT based on the intraspinal inoculation of Tg mice can be applied to Sabin type 1 virus (Abe *et al.*, 1995), and the results of the present study have shown that it is also applicable to Sabin type 3 virus. Research on Sabin type 2 virus is in progress and the results obtained so far are encouraging.

When plaque-cloning was performed for the Sabin type 3 virus (SO + 2) as an index of temperature sensitivity, the original virus fluids were found to consist of various populations with different properties such as plaque-forming ability at high temperature. Our studies have produced data that show that higher virus concentrations having plaque-forming efficiency at high temperature are more highly neurovirulent. Therefore, a NVT method that can reflect neurovirulence very precisely should be adopted to detect low levels of virus showing high virulence in a vaccine.

In the present study, it was found that a NVT based on the use of Tg21 mice can clearly differentiate Sabin type 3 viruses with slightly different properties such as artificial mixtures of PV3-WV (high temperature sensitivity and low neurovirulence) and F313 (reference virus for OPV in Japan) or viruses of F313 passaged at 38° (Fig. 1). Our results indicate a very close correlation between temperature sensitivity and results obtained using either the MLS value in monkeys or the PD_{50} value in Tg mice to show neurovirulence.

Intraspinal inoculation of Tg mice and evaluation of the neurovirulence by the 50% paralysis dose in inoculated mice provide a new experimental system for testing the neurovirulence of poliovirus. This method also has

the advantage of avoiding a number of problems (referred to above) that are associated with the use of monkeys. Since the Tg mice are an inbred mouse line (i.e., they have a homogeneous genetic background), individual differences among Tg mice are fewer than those among monkeys and the results of tests are therefore more reproducible. Furthermore, the test has the added advantages of being comparatively simple to perform and does not require the additional histological examination used in the monkey NVT.

We also studied the sequence heterogeneity at position 472 in Sabin type 3 poliovirus by the MAPREC method and examined the correlation between the number of mutants and the NVT results using Tg mice and monkeys. The minimum concentration of 472-C detectable by the MAPREC test is approximately 0.1%. But in these experiments exact 472-C content more than 0.3% and calculated contents lower than 0.3% in artificial mixtures showed a very close correlation with the NVT results in monkeys and Tg mice. The MAPREC method proved to be an important *in vitro* test for establishing the neurovirulence of Sabin type 3 poliovirus in the future.

The use of Tg mice should make it possible to use the NVT for the quality control of OPV and as a new tool for poliovirus surveillance, thereby conserving monkey resources. Moreover, this new technique could act as a forerunner in the development of a molecular NVT and, possibly, lead to the discontinued use of monkeys.

ACKNOWLEDGMENTS

We thank Dr. I. S. Levenbook (Center for Biologics Evaluation and Research, FDA) for recommending the manuscript, K. Hioki (Central Institute for Experimental Animals) for his cooperation in the constant supply of Tg mice, and K. Irisawa (Japan Poliomyelitis Research Institute) for his assistance in the intraspinal inoculation of Tg mice.

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