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A Recombinant Virus between the Sabin 1 and Sabin 3 Vaccine Strains of Poliovirus as a Possible Candidate for a New Type 3 Poliovirus Live Vaccine Strain

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Biological tests including the monkey neurovirulence test performed on recombinants between the virulent Mahoney and attenuated Sabin 1 strains of type 1 poliovirus indicated that the genome region encoding mainly the viral capsid proteins had little correlation with the neurovirulence or attenuation phenotype of the virus. The results suggested that new vaccine strains of type 2 and type 3 polioviruses may be constructed in vitro by replacing the sequence encoding the antigenic determinants in viral capsid proteins of the Sabin 1 genome by the corresponding sequences of the type 2 and type 3 genome, respectively. Accordingly, we constructed recombinants between the Sabin 1 and Sabin 3 strains of poliovirus in which genome sequences of the Sabin 1 strain encoding most or all capsid proteins were replaced by the corresponding genome sequences of the Sabin 3 strain. One of the recombinant viruses thus constructed was fully viable and showed antigenicity and immunogenicity identical to those of type 3 poliovirus. The monkey neurovirulence tests and in vitro phenotypic marker tests (temperature sensitivity of growth, sodium bicarbonate concentration dependency of growth under agar overlay, and size of plaque) were performed on the recombinant virus. The stability of the virus in regard to the temperature sensitivity phenotype was also tested. The results suggested that the recombinant virus is a possible candidate for a new type 3 poliovirus vaccine strain.

Poliovirus, the causative agent of poliomyelitis (17), is a human enterovirus that belongs to the family *Picornaviridae*. The virus, a nonenveloped particle, consists of a single-stranded RNA of plus-strand polarity and 60 copies each of four capsid proteins, VP1, VP2, VP3, and VP4, and occurs in three serologically distinct types, type 1, type 2, and type 3. The severe paralytic disease caused by poliovirus has been effectively controlled in developed countries through the use of attenuated live (10, 17, 21) or killed (22) vaccines. Paralytic poliomyelitis, however, remains a serious threat in many countries of the world, especially in developing countries where effective vaccination against poliomyelitis is not sufficiently inclusive.

Although the virtue of the oral live poliovirus vaccines (Sabin vaccines; Sabin 1, Sabin 2, and Sabin 3 vaccine strains) has been widely recognized, the Sabin vaccine strains have the inherent problem of the risk, albeit low, of reversion from the attenuated to the neurovirulent phenotype upon repeated passages. Indeed, a very small number of cases of paralytic poliomyelitis continue to occur in countries with extensive oral poliovirus vaccine programs (10). Experimental evidence strongly suggests that most of these cases are caused by the vaccines themselves, especially type 2 (Sabin 2) and type 3 (Sabin 3) vaccines (4, 11).

To date, cDNA copies of the approximately 7,500-nucleotide 3′ poly(A)$^+$ RNA of both the virulent and attenuated strains of all three poliovirus serotypes have been sequenced and cloned in *Escherichia coli* (5, 9, 14, 18, 24, 25, 27). Racaniello and Baltimore (19) assembled a full-length clone of the genome of the virulent Mahoney strain of type 1 poliovirus from subgenomic clones and showed that transfection of plasmid DNA from this clone would produce infectious poliovirus in cultured primate cells. An infectious cDNA clone of the genome of the Sabin 1 vaccine strain of type 1 poliovirus, which is the safest of the Sabin vaccines, was isolated by Omata et al. (16). Virus recovered from HeLa S3 cells or African green monkey kidney (AGMK) cells transfected with the infectious Sabin 1 clone was shown to be indistinguishable from the Sabin 1 reference virus in regard to biochemical and biological properties including monkey neurovirulence (6, 16). Thus, the genetic information of the Sabin 1 vaccine strain appears to be stably stored in the infectious cDNA clone.

The attenuated Sabin 1 strain was derived from the virulent Mahoney strain of type 1 poliovirus by multiple passages through host cells of nonhuman origin (20, 21). In addition to their different potentials for causing disease, these two strains of virus differ in a number of biological characteristics that include the temperature sensitivity of growth (*rct* marker), bicarbonate concentration dependency of growth (*d* marker), and size of plaques.

The availability of the total nucleotide sequences and infectious cDNA clones of the genomes of the Mahoney and Sabin 1 strains made it possible to identify genome loci influencing the different biological characteristics by constructing and performing biological tests on recombinant viruses of the two strains. We recently constructed infectious recombinant plasmid clones from cDNA segments of the Mahoney and Sabin 1 RNAs and used them to analyze the influence of the genome regions on the biological properties of type 1 poliovirus (7, 13, 15). As a result of this

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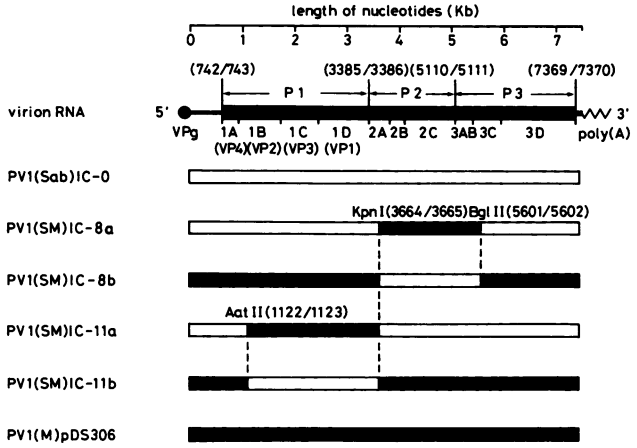

FIG. 1. Genome structure of recombinant type 1 poliovirus produced from the Mahoney and Sabin 1 strains. The expected genome structures of the recombinants between the Sabin 1 (▭) and Mahoney (▬) strains are shown. Numbers in parentheses indicate nucleotide positions from the 5′ end of the genome. The length (in kilobases) of the entire genome and the gene organization of poliovirus type 1 are indicated at the top of the figure. VPg is a small protein covalently attached to the 5′ end of the genome, and poly(A) is the 3′ terminal.

study, it was suggested that the genome loci including mainly the 5′ noncoding region harbor a relatively strong determinant(s) influencing the neurovirulence or attenuation phenotype, although the determinants influencing attenuation are spread over several areas of the entire viral genome, and that virion surface characteristics such as antigenicity and immunogenicity have little correlation with neurovirulence (7, 13, 15). These results suggest that it is possible to construct more stable (and hence safer) vaccine strains of type 2 and type 3 polioviruses in vitro by replacing only the sequence encoding the antigenic determinants of the Sabin 1 genome by the corresponding sequences of the type 2 and type 3 genomes, respectively.

In this report we prove by use of recombinant viruses of the Mahoney and Sabin 1 strains that the genome loci encoding mainly the viral capsid proteins only weakly influence the neurovirulence or attenuation phenotype. Furthermore, in vitro construction of intertypic recombinant viruses carrying the antigenicity of type 3 poliovirus is performed by using cDNA clones of the Sabin 1 and Sabin 3 strains. We also present data from monkey neurovirulence tests and in vitro phenotypic marker tests on a viable recombinant virus. The results suggest that the antigenically changed recombinant virus may be used as a possible candidate for a new type 3 poliovirus vaccine strain.

(Preliminary results have been presented at the meetings of the UCLA symposia on positive-strand RNA viruses [13] and the V International Conference on Comparative Virology [12].)

MATERIALS AND METHODS

DNA procedure. Both full-length cDNAs of the genomes of the virulent Mahoney and attenuated Sabin 1 strains have been inserted into the *Eco*RI site of bacterial plasmid pBR325 by using *Eco*RI linkers (15, 16, 23). To enhance the specific infectivity of these infectious cDNA clones, the full-length cDNAs of the Mahoney and Sabin 1 genomes were recloned into plasmid pSVA13, which carried the replication and transcription signals of simian virus 40 as well as the sequences encoding large T antigen (6), and were designated as pVM(1)pDS306(T) and pVS(1)IC-0(T), respectively. The Mahoney and Sabin 1 viruses [PV1(M)pDS306 and PV1(Sab)IC-0, respectively] (Fig. 1) produced in AGMK cells transfected with these two infectious cDNA clones showed the biological characteristics of their corresponding authentic parental virus strains (6, 7, 15, 16).

Allele replacement (7, 15) experiments were carried out on these two cDNA clones by using restriction cleavage sites of *Aat*II (nucleotide position 1122), *Kpn*I (position 3664), and *Bgl*II (position 5601). As a result, four different infectious cDNA clones from segments of the Mahoney and Sabin 1 genomes were constructed and designated pVSM(1)IC-8a(T), pVSM(1)IC-8b(T), pVSM(1)IC-11a(T), and pVSM(1) IC-11b(T). AGMK cells were transfected with 10 μg of closed-circular forms of these recombinant cDNA clones per plastic dish (diameter, 6 cm), and the corresponding viruses, designated PV1(SM)IC-8a, PV1(SM)IC-8b, PV1(SM)IC-11a, and PV1(SM)IC-11b, respectively (Fig. 1), were recovered from the cells by the procedure previously reported (6, 7, 15, 16). All recombinant viruses were grown at 33.5°C.

To construct intertypic recombinants between the Sabin 1 and Sabin 3 strains, we used plasmids pVS(1)IC-0(T), pVS(1)BB2 (14), and pVS(3)2603 (27) to assemble recombinant clones of the genomes of both the strains by the strategy shown in Fig. 2. As a result, two kinds of intertypic recombinant cDNA clones, pVSS(1/3)IC-AN and pVSS(1/3)IC-BN, were constructed. AGMK cells were transfected with 10 μg of these cDNA clones per plastic dish (diameter,

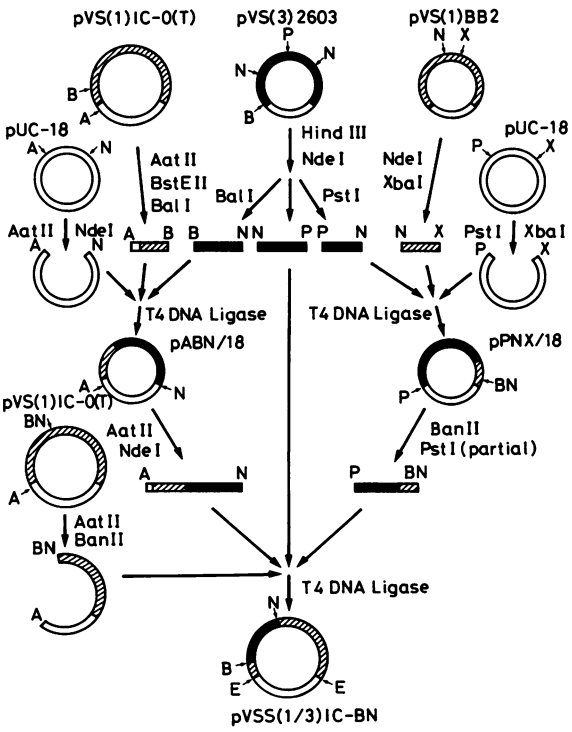

FIG. 2. Strategy for constructing a recombinant cDNA clone between the Sabin 1 and Sabin 3 strains. A recombinant cDNA clone, pVSS(1/3)IC-BN, was constructed by assembling segments of cDNAs from the Sabin 1 (▨) and Sabin 3 (▬) strains. ▭, Sequences derived from bacterial cloning vectors. B, A, N, P, X, BN, and E represent cleavage sites of the restriction enzymes *Bal*I, *Aat*II, *Nde*I, *Pst*I, *Xba*I, *Ban*II, and *Eco*RI, respectively.

6 cm) as previously described (15). The transfected cells were incubated at 33.5°C.

Restriction fragmentation of the recombinant cDNA clones with a number of restriction endonucleases (Takara Shuzo Co., Kyoto, Japan; Toyobo Co., Osaka, Japan) were carried out to confirm the nucleotide sequences of the recombinant cDNA clones expected from the correct allele replacement. T4 DNA ligase was purchased from Takara Shuzo Co., Kyoto, Japan, and calf intestine alkaline phosphatase was from Boehringer GmbH, Mannheim, Federal Republic of Germany. Enzymes were used as specified by the manufacturers.

Cells and viruses. AGMK cells were maintained in Dulbecco modified Eagle medium supplemented with 5% newborn calf serum. The cells were infected with the viruses recovered from the transfected cells at a multiplicity of infection of approximately 10^{-3} to make virus stocks. After an incubation at 33.5°C for 3 days, the virus was obtained by freeze-thawing three times followed by centrifugation to remove cell debris. Viruses thus obtained were used for monkey neurovirulence tests and in vitro marker tests (see below).

Viruses obtained as described above were passaged in AGMK cells with Hanks balanced medium 199 containing 0.1% bovine serum albumin and 0.225% sodium bicarbonate up to 10 more times at 37.5°C by using the procedure repeatedly used in the Japan Poliomyelitis Research Institute (6). Passaged virus was obtained by quick freeze-thawing twice followed by centrifugation to remove cell debris and was stored at −20°C. The passaged isolates were tested for their in vitro phenotypic markers (see below) at the passage number indicated in Fig. 5.

The reference virus of the Sabin 3 strain used in this study was F-310. This virus had been obtained by two passages of Leon $12a_1b$(SO; Sabin original strain) in primary cell cultures prepared from African green monkey kidney and is now being used as a type 3 oral poliovirus vaccine in Japan. Similarly, Leon is the reference for the virulent Leon strain of type 3 poliovirus.

Monkey neurovirulence tests. The titers of the viruses were determined by using primary-cultured cynomolgus monkey kidney cells (6, 15). For the test to estimate neurovirulence of recombinant viruses between the virulent Mahoney and attenuated Sabin 1 strains, 0.5 ml of the virus suspension (10^7 50% tissue culture infective doses/ml) was inoculated into both right and left thalamuses of each of the seronegative cynomolgus monkeys (15). For the test of an intertypic recombinant virus, PV1/3(SS)BN (6), 0.1 ml of the virus suspension (10^7 50% tissue culture infective doses/ml) was inoculated intraspinally into each of the seronegative cynomolgus monkeys. Monkeys were sacrificed 17 days after the inoculation. Monkeys showing severe clinical poliomyelitis were sacrificed at the peak of the disease. A total of 38 sections of the central nervous system were prepared to score the intensity of histological lesions as previously described (6, 15). Lesion scores were estimated by established procedures (2).

Antigenicity. Antigenicities of viruses were tested by a modified enzyme-linked immunosorbent assay with neutralizing monoclonal or polyclonal antibodies specific to the poliovirus serotype as described previously (7). Serological identification tests were performed as follows. A 0.7-ml portion of the virus solution (1×10^8 to 3×10^8 50% tissue culture infective doses/ml) was mixed with the same volume of hyperimmune serum specific to each poliovirus serotype. After incubation at 37°C for 2 h, 0.2 ml each of the virus-antibody mixture was added to AGMK cell cultures growing at the bottom of glass tubes. After incubation, the cell cultures were covered with 1 ml of Hanks balanced medium 199 containing 0.1% bovine serum albumin and 0.225% sodium bicarbonate and kept at 36°C for 7 days. The cytopathic effect displayed on AGMK cells was then estimated to identify the serotype of the virus.

In vitro phenotype marker tests. All tests were performed with primary-cultured cynomolgus monkey kidney cells. The reproductive capacity of viruses at different temperatures (*rct* marker) was investigated by measuring the virus titer on the cells at 36, 39, 39.5, and 40°C and at a sodium bicarbonate concentration of 0.225% after an incubation of 7 days as previously described (6, 7, 15, 16).

Delayed growth (*d* marker) of viruses in media containing a low concentration of bicarbonate was investigated by measuring virus titers on the cells at 36°C and at sodium bicarbonate concentrations of 0.225, 0.08, and 0.03% after an incubation of 4 days as previously described (6, 7, 15, 16).

For determination of plaque size produced by the different viruses, cells were infected and cultured under agar overlays at sodium bicarbonate concentrations of 0.225, 0.08, and 0.03% at 36°C. The diameters of approximately 100 plaques, observed on the days 3, 4, and 7 after infection, were measured as previously described (6, 7, 15).

RESULTS

Genome loci influencing attenuated phenotype of type 1 poliovirus. Our previous results (13, 15) have strongly suggested that a strong determinant(s) influencing the neurovirulent or attenuated phenotype of type 1 poliovirus resides in the 5′ noncoding sequence of the genome, and only a weak determinant(s) is present in the 1,840-nucleotide 3′-proximal sequence of the genome. However, the extent to which the genome region between positions 1123 and 5601 affects the

TABLE 1. Monkey neurovirulence tests and in vitro markers of recombinant viruses and their parental viruses

Virus	Lesion score (mean ± SE)	No. paralyzed/ no. injected	*rct*[a]			*d*[b]	Plaque size (mm) (mean ± SD)[c]
			36/39°C	36/39.5°C	36/40°C	0.225/0.03	
PV1(Sab)IC-0	0.07 ± 0.01	0/4	5.29	>8.58	ND[d]	5.40	7.5 ± 3.0
PV1(SM)IC-8a	0.12 ± 0.02	0/4	4.67	>8.07	>8.07	5.76	6.8 ± 2.9
PV1(SM)IC-8b	1.56 ± 0.42	2/4	0.70	1.33	1.35	0.37	10.8 ± 2.1
PV1(SM)IC-11a	0.08 ± 0.01	0/4	1.70	4.26	>8.65	0.57	14.8 ± 1.5
PV1(SM)IC-11b	1.73 ± 0.10	0/4	−0.13	0.18	3.83	4.44	4.9 ± 2.9
PV1(M)pDS306	2.48 ± 0.34	3/4	ND	0.03	0.12	0.60	15.0 ± 1.8

[a] *rct* marker values are the logarithmic differences of virus titers obtained at 36 and 39°C, 36 and 39.5°C, or 36 and 40°C.
[b] *d* marker values are the logarithmic differences of virus titers obtained at two different sodium bicarbonate concentrations of 0.225 and 0.03%.
[c] Diameter of plaques displayed on day 5 of growth.
[d] ND, Not done.

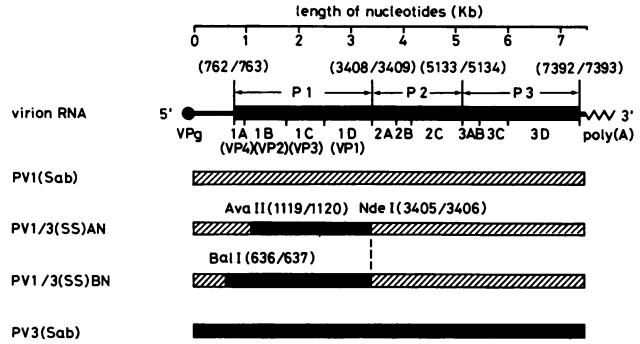

FIG. 3. Genome structure of recombinants between the Sabin 1 and Sabin 3 strains. The expected genome structures of the recombinants between the Sabin 1 (▨) and Sabin 3 (■) strains are shown. Virus PV1/3(SS)AN, however, was not recovered from the cells transfected with the corresponding cDNA clone, as described in the text. Nucleotide numbers shown in parentheses here follow those reported by Toyoda et al. (27). The length (in kilobases) of the entire genome and the gene organization of poliovirus are indicated at the top of the figure.

phenotype is not clear. This region of the genome includes sequences encoding most viral capsid proteins and whole P2 polyproteins.

To find the effect of the genome region on the attenuation phenotype, we constructed four recombinant viruses by using infectious cDNA clones as described previously (15). The genome structures of these recombinant viruses are shown in Fig. 1. These viruses were tested for their biological properties including monkey neurovirulence (Table 1). In vitro phenotypic marker tests were also carried out on these viruses to confirm our previous assumption about the relationship between the monkey neurovirulence and in vitro markers (15).

Viruses PV1(SM)IC-8b and PV1(SM)IC-11b showed fairly high lesion scores among the recombinants, although the lesion scores were clearly lower than that of the parental neurovirulent strain PV1(M)pDS306 (Table 1). In contrast, viruses PV1(SM)IC-8a and PV1(SM)IC-11a showed low lesion scores, similar to that of the attenuated PV1(Sab)IC-0 parental virus. These results suggest that a strong determinant influencing neurovirulence does not reside in either of the Mahoney genome regions represented by the *Aat*II-*Kpn*I fragment at nucleotide positions 1123 to 3664 and by the *Kpn*I-*Bgl*II fragment at positions 3665 to 5601. Thus, these regions of the genome were found to have only a weak influence on the neurovirulent or attenuated phenotype of type 1 poliovirus.

Virus PV1(SM)IC-8a showed an *rct* phenotype similar to that of the attenuated PV1(Sab)IC-0, although multiplication of PV1(SM)IC-8b was slightly more sensitive to the elevated temperatures than that of the virulent PV1(M)pDS306. These results suggest that mutations observed in the genome region represented by the *Kpn*I-*Bgl*II fragment at positions 3665 to 5601 have only a small influence on the *rct* phenotype of the Sabin 1 strain. Virus PV1(SM)IC-11a showed less sensitivity to elevated temperatures than the parental virus PV1(Sab)IC-0 did, and virus PV1(SM)IC-11b was more sensitive to high temperatures than the virulent PV1(M) pDS306 was. These results suggest that mutations in the genome region represented by the *Aat*II-*Kpn*I fragment at positions 1123 to 3664 contribute to some extent to the *rct* phenotype of the Sabin 1 strain. These data are compatible with the observation that the assembly of Sabin viral capsid proteins into capsomeres includes a temperature-sensitive step (3).

Our previous results (15) have suggested that determination of *d* and plaque size phenotypes map to the genome region encoding the viral capsid proteins. Viruses PV1(SM)IC-8a and PV1(SM)IC-11b showed the *d* phenotype and small-plaque phenotype like the attenuated virus PV1 (Sab)IC-0 (Table 1). In contrast, viruses PV1(SM)IC-8b and PV1(SM)IC-11a showed similar biological properties to that of the virulent virus PV1(M)pDS306 in regard to these phenotypes (Table 1). Since the first two and the last two viruses have Sabin- and Mahoney-derived capsid proteins, respectively (Fig. 1), the results presented here confirm our previous assumption described above.

Construction of intertypic recombinant viruses. Since the genome region encoding capsid proteins appears to have only a weak influence on the neurovirulent or attenuated phenotype of type 1 poliovirus, it is possible to construct safer vaccine strains of type 2 and type 3 polioviruses in vitro by replacing only the sequence encoding the antigenic determinants of the Sabin 1 genomes by the corresponding sequences of the type 2 and type 3 genomes, respectively. However, fully viable recombinants with junction points within the capsid sequences have not been isolated. Indeed, a cDNA construct in which the sequence encoding most of the VP1 region of the Sabin 1 strain was replaced by the corresponding sequence of the Sabin 3 strain did not yield virus on transfection of HeLa cells (26). We therefore constructed recombinant cDNAs in which much larger regions of capsid proteins of the Sabin 3 strain were engineered into the Sabin 1 strain. The recombinant cDNA clones thus obtained were designated pVSS(1/3)IC-AN and pVSS(1/3)IC-BN. The strategy for the construction of pVSS(1/3)IC-BN is shown in Fig. 2. A similar strategy was used to construct pVSS(1/3)IC-AN.

AGMK cells were transfected with these recombinant cDNA clones as described in Materials and Methods. The cells transfected with plasmid pVSS(1/3)IC-BN produced infectious virus particle, but those transfected with plasmid pVSS(1/3)IC-AN did not. The recovered virus was designated PV1/3(SS)BN. The genome structures of strain PV1/3(SS)BN and a putative strain, PV1/3(SS)AN, which was not produced in the cells transfected with pVSS(1/3)IC-AN are shown in Fig. 3. Thus, viable virus was obtained only by replacement of the entire capsid region of the Sabin 1 strain with that of the Sabin 3 strain. The generation time and final yield of PV1/3(SS)BN investigated in an in vitro culture system were very similar to those of the parent viruses (data not shown). Thus, the viability of the recombinant virus PV1/3(SS)BN appeared to be essentially the same as that of the Sabin 1 and Sabin 3 strains.

Antigenicity of PV1/3(SS)BN. Virus PV1/3(SS)BN should have antigenicities specific to the Sabin 3 strain, since the genome of this virus codes for P1 polyprotein identical to that of the Sabin 3. Accordingly, we investigated the antigenicity of the recombinant virus by enzyme-linked immunosorbent assay (7) with strain-specific neutralizing monoclonal and polyclonal antibodies (Fig. 4). PV1/3(SS)BN was recognized only by neutralizing monoclonal and polyclonal antibodies specific to type 3 poliovirus (Fig. 4). The antigenic properties of the recombinant virus were further analyzed by serological identification tests as described in Materials and Methods (Table 2). As expected, the virus PV1/3(SS)BN was effectively and specifically neutralized by hyperimmune se-

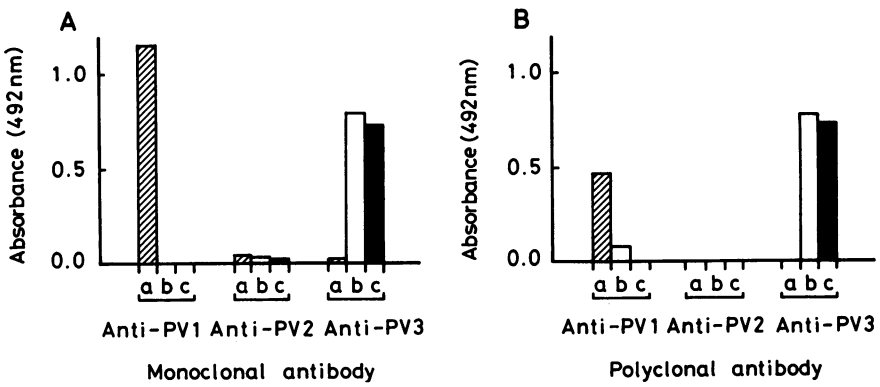

FIG. 4. Recognition of a recombinant poliovirus by strain-specific neutralizing monoclonal antibodies (A) or polyclonal antibodies (B). This was used to find the antigenicity of a recombinant poliovirus, PV1/3(SS)BN. a, b, and c represent Sabin 1, PV1/3(SS)BN, and Sabin 3 viruses, respectively.

rum specific to type 3 poliovirus and not by sera specific to type 1 and type 2 polioviruses.

Monkey neurovirulence test on PV1/3(SS)BN. Neurovirulence tests of the recombinant virus PV1/3(SS)BN were carried out by infecting cynomolgus monkeys intraspinally as described in Materials and Methods. The results are shown in Table 3. Eleven monkeys were used for the test of the reference virus, F-310, and seven monkeys were used for the recombinant virus, PV1/3(SS)BN.

The average lesion scores of PV1/3(SS)BN were lower than those of the reference virus. Furthermore, no paralytic syndrome was observed in the test animals injected with the recombinant virus or the reference virus (Table 3). These results suggest that the recombinant virus is attenuated enough to be considered a candidate virus for the oral live poliovirus vaccine.

In vitro phenotypic markers of PV1/3(SS)BN. Many differences in biological characteristics between the virulent and attenuated poliovirus strains have been recognized in addition to their different potentials for causing disease. Of these, *rct*, *d*, and plaque size markers are frequently used as in vitro phenotypic markers to analyze properties of poliovirus strains. Indeed, all three Sabin vaccine strains share these in vitro characteristics. It is therefore possible that these in vitro phenotypes of the attenuated Sabin vaccine strains have some correlation with the viral pathogenesis, which includes numerous biological processes. Thus, it may be important to know the in vitro properties of the recombinant virus PV1/3(SS)BN if the virus is considered to be a possible candidate vaccine for type 3 poliovirus.

The results of *rct* marker, *d* marker, and plaque size marker tests on PV1/3(SS)BN and reference viruses are shown in Tables 4, 5, and 6, respectively. The virus PV1/3(SS)BN showed temperature sensitivity similar to that of PV1(Sab)IC-0 and even greater than that of F-310 (Table 4). This result suggests that the segment exchanged in the recombinant virus genome (nucleotides 637 to 3405) of both the Sabin 1 and Sabin 3 strains harbors a determinant(s) influencing the temperature-sensitive growth of the virus.

The recombinant virus PV1/3(SS)BN showed a strong *d* phenotype (Table 5). The degrees of *d* phenotype among PV1(Sab)IC-0, PV1/3(SS)BN, and F-310 were indistinguishable from one another when compared with the logarithmic differences of virus titers obtained at two different sodium bicarbonate concentrations, 0.225% and 0.03%. However, the logarithmic difference of PV1/3(SS)BN virus titers at 0.225% and 0.08% sodium bicarbonate was the greatest among these three virus strains (Table 5). Thus, PV1/3(SS)BN appeared to have the strongest *d* phenotype of the

TABLE 2. Serological identification test

Virus	No. of samples showing CPE[a] with following serum/total no.			
	Anti-PV1 serum	Anti-PV2 serum	Anti-PV3 serum	Without antiserum
PV1(Sab)IC-0	0/6	6/6	6/6	6/6
PV1/3(SS)BN	6/6	6/6	0/6	6/6
F-310	6/6	6/6	0/6	6/6

[a] CPE, Cytopathic effect.

TABLE 3. Monkey neurovirulence test of recombinant virus and reference virus[a]

Virus and monkey no.	Lesion score[b]
F-310	
1	0.98
2	1.45
3	2.37
4	0.06
5	0.00
6	0.19
7	0.02
8	0.26
9	0.49
10	1.19
11	0.31
PV1/3(SS)BN	
1	0.09
2	0.22
3	0.14
4	0.46
5	0.30
6	0.48
7	0.13

[a] No paralysis was found for recombinant or reference virus.
[b] Average lesion scores were 0.67 for F-310 and 0.26 for PV1/3(SS)BN.

TABLE 4. Reproductive capacity of recovered viruses and reference viruses at different temperatures

Virus	Log_{10} PFU/ml at indicated temp (°C)				Difference in log_{10} PFU/ml between temps (°C)		
	36	39	39.5	40	36 and 39	36 and 39.5	36 and 40
PV1(Sab)IC-0	8.85	3.47	<0.39		5.38	>8.46	
PV1/3(SS)BN	8.31	2.74	<0.39		5.57	>7.92	
F-310	7.18	2.77	1.54		4.41	5.64	
PV1(M)pDS306	7.65		7.61	7.48		0.04	0.17
Leon	8.15		8.10	8.00		0.05	0.15

viruses tested. The reason is presently unknown. It is possible, however, that the *d* determinant(s) involved in capsid proteins of the Sabin 3 strain is stronger than that of the Sabin 1 strain and that the Sabin 1 virus carries a *d* determinant(s) in another region(s) than the capsid protein region in addition to the strong *d* determinant(s) residing in the capsid protein region (Fig. 1; Table 1).

Plaque sizes of the virus PV1/3(SS)BN and reference viruses measured on days 3, 4, and 7 after inoculation are shown in Table 6. PV1/3(SS)BN showed a small-plaque phenotype. The plaque size of this recombinant virus was smaller than those of both parent viruses, PV1(Sab)IC-0 and F-310. This observation is very similar to the results of *d* marker tests. The determinant(s) influencing plaque size, therefore, may reside in the same areas as those influencing *d* phenotype.

Stability in *rct* phenotype of PV1/3(SS)BN. It has been established that high-quality poliovirus vaccines have more stable biological characteristics of the *rct* marker upon serial passage at elevated temperature in tissue culture cells (1, 6). Accordingly, the recombinant virus PV1/3(SS)BN was passaged in AGMK cells up to 10 times at a temperature of 37.5°C, and the passaged isolates were then tested for their *rct* phenotype (Fig. 5).

Logarithmic differences of virus titers obtained at two different temperatures (36 and 39°C) at the indicated passage number are shown in Fig. 5A. The results show that PV1/3(SS)BN appears to be more stable than F-310 with respect to the *rct* marker during passaging. Similar results were obtained from the experiments done at 36 and 39.5°C (Fig. 5B). These data suggest that PV1/3(SS)BN might be a higher-quality poliovirus vaccine than F-310.

DISCUSSION

Allele replacement experiments were performed with the virulent Mahoney and attenuated Sabin 1 strains. The results confirmed our previous observation (15) that surface parameters such as antigenicity and immunogenicity were not the main determinants of the attenuation phenotype of the Sabin 1 strain. On the basis of this conclusion, we constructed two recombinant cDNA clones of the Sabin 1 and Sabin 3 strains in which the sequences encoding antigenicity were derived from the Sabin 3 strains. The cells transfected with the cDNA clone pVSS(1/3)IC-AN did not produce viable infectious virus. The reason for this is not known. It is possible, however, that the putative recombinant virus PV1/3(SS)AN has reduced efficiencies in certain viral replication steps, resulting in the production of no viable virus in the cells transfected with pVSS(1/3)IC-AN. Replacement of one part of a viral coat protein by elements of the coat protein region of other strains might cause alterations in the efficiency of capsid assembly or the correct folding required for the faithful processing of the precursor proteins. Indeed, Stanway et al. (26) have also been unsuccessful in constructing an antigenic recombinant virus of type 1 and type 3 polioviruses.

In contrast, the recombinant virus PV1/3(SS)BN had a viability indistinguishable from those of the parent viruses in vitro, although the genomes of the Sabin 1 and Sabin 3 strains differ in 51 nucleotides in the 5′ noncoding region downstream of the *BalI* cleavage site. These nucleotide differences, therefore, may not affect the functional structures probably formed in the 5′ noncoding sequence (8). This result is compatible with our previous observations that mutants lacking this genome region of highly variable sequence (27) were fully viable (8). It is also suggested that the capsid precursor protein P1 of the Sabin 3 virus is correctly processed by the virus-specific proteinases of the Sabin 1 virus, although a number of amino acid differences are observed around the cleavage sites of polyprotein P1 between both the strains.

The recombinant virus PV1/3(SS)BN carries capsid proteins identical to those of the Sabin 3 virus and therefore should have every neutralizing antigenic site of type 3 poliovirus. This characteristic may be very important for vaccine viruses, since antibodies elicited by the vaccine virus must be able to neutralize a wide range of antigenic variants of type 3 polioviruses. Monkey neurovirulence tests (Table 3) and in vitro phenotypic marker tests (Tables 4 to 6; Fig. 5) performed on the virus PV1/3(SS)BN revealed that

TABLE 5. Delayed marker test of recovered viruses and reference viruses

Virus	Log_{10} PFU/ml at indicated sodium bicarbonate concn (%)			Difference in log_{10} PFU/ml between concns (%)	
	0.225	0.08	0.03	0.225 and 0.08	0.225 and 0.03
PV1(Sab)IC-0	8.64	6.03	<1.39	2.61	>7.25
PV1/3(SS)BN	8.37	4.95	<1.39	3.42	>6.98
F-310	7.53	5.57	<1.39	1.96	>6.14
PV1(M)pDS306	7.83	7.86	6.86	−0.03	0.97
Leon	8.41	8.74	7.88	−0.33	0.57

TABLE 6. Plaque size of recovered viruses and reference viruses

Virus	Plaque size (mm) (mean ± SD) on following day after inoculation:		
	3	4	7
PV1(Sab)IC-0	1.5 ± 0.7	3.3 ± 2.3	11.3 ± 2.6
PV1/3(SS)BN	1.3 ± 0.4	2.6 ± 0.6	8.8 ± 1.5
F-310	2.3 ± 0.7	4.4 ± 1.7	13.3 ± 1.2
PV1(M)pDS306	5.0 ± 1.8	8.5 ± 2.4	17.5 ± 2.9
Leon	6.6 ± 1.8	11.0 ± 1.2	25.5 ± 1.9

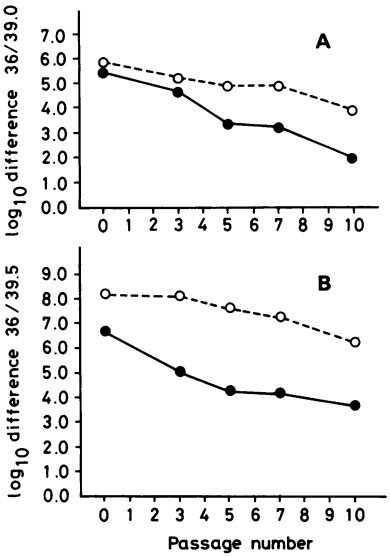

FIG. 5. Change of *rct* phenotype upon serial passage at an elevated temperature. Viruses PV1/3(SS)BN (○) and F-310 (●) were passaged up to 10 times at 37.5°C as described in Materials and Methods. At the indicated passage numbers, titers of passaged viruses were measured at 36, 39, and 39.5°C. Logarithmic differences of virus titers obtained at 36 and 39°C (A) or 36 and 39.5°C (B) are shown.

the recombinant virus might be a possible candidate for a new type 3 poliovirus vaccine strain. However, in addition to spinal neurovirulence characteristics and in vitro phenotypes, there are two important properties which should be present in the oral live vaccines. One is extensive multiplication in the human intestinal tract, and the other is limited change in neurovirulence of excreted virus at different times after ingestion of the vaccine virus. We have yet to test these properties of the recombinant virus PV1/3(SS)BN, and extensive clinical trials would be required before practical use even after these tests are carried out.

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