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A Mutation Present in the Amino Terminus of Sabin 3 Poliovirus VP1 Protein Is Attenuating

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The attenuated phenotype of Sabin 3 poliovirus compared with its neurovirulent progenitor strain has been largely accounted for by mutations in the genome at positions 472 and 2034 (G. D. Westrop, K. A. Wareham, D. M. A. Evans, G. Dunn, P. D. Minor, D. I. Magrath, F. Taffs, S. Marsden, M. A. Skinner, G. C. Schild, and J. W. Almond, J. Virol. 63:1338–1344, 1989). By sequencing vaccine virus RNA, we recently identified another Sabin 3-specific mutation at position 2493 (U → C), which predicts an Ile → Thr change at the sixth residue of VP1 (C. Weeks-Levy, J. M. Tatem, S. J. DiMichele, W. Waterfield, A. F. Georgiu, and S. J. Mento, Virology 185:934–937, 1991). Viruses generated by using cDNAs which represent the vaccine sequence (LED3) and a derivative (VR318) possessing a single base change to the wild-type nucleotide (U) at 2493 were used to determine the impact of the 2493 mutation on virus phenotype. The VP1 proteins of LED3 and VR318 viruses were distinguishable by denaturing electrophoretic analysis. LED3 produced smaller plaques in Vero cells than VR318 virus did. Neurovirulence testing of these cDNA-derived viruses in monkeys demonstrated that the 2493 mutation in LED3 virus is attenuating.

The live oral poliovirus vaccine currently administered to children is based on the attenuated strains of types 1, 2, and 3 poliomyelitis virus developed in the 1950s by A. Sabin. Despite widespread use of these strains for more than 40 years, the molecular basis of their attenuation is still largely unknown (15).

The genome of poliovirus is a single strand of RNA of positive polarity approximately 7,500 nucleotides long. Compared with double-stranded DNA genomes, the error frequency during replication of single-stranded RNA is high (3). Because of this inherent property, every preparation of poliovirus, including the Sabin Original (SO) strains, is genetically heterogeneous.

Several reports document that the attenuated phenotype of the Sabin 3 strain is less genetically stable than the type 1 and 2 vaccine strains (4, 7, 12). In 1964, an alternative type 3 manufacturing seed (RSO) was derived from the original Sabin 3 virus through plaque selection after primary African green monkey kidney cells were transfected with viral RNA (19). As suggested by Kohara and coworkers for the Sabin 1 seed (10), an infectious cDNA clone might be used to further benefit the constancy and quality of the type 3 seed.

Until recently, the literature contained two cDNA sequences for Sabin 3 which differed at 18 nucleotide positions (17, 22). To define the consensus Sabin 3 genome present in the oral poliovirus vaccine distributed in the United States (Lederle Laboratories), our laboratory directly analyzed the RNA of the vaccine virus (24). The RNA sequence (LED3) contained the same 10 mutations reported by Stanway and coworkers that distinguish the cDNA sequence of Sabin 3 from Leon, the neurovirulent progenitor strain (18), as well as mutations at positions 2493 and 6061. Although the silent genetic change at 6061 was identified previously (22), the T → C switch at 2493, which predicts a coding change within the amino terminus of VP1, is new.

Reverse transcriptase and standard techniques were used to construct a full-length cDNA clone of Sabin 3 vaccine virus RNA (Lederle Laboratories) in pBR322. The cDNA was positioned under the control of the bacteriophage T7 promoter so that positive-sense RNA transcripts were synthesized in vitro by T7 RNA polymerase. This initial cDNA construct was not infectious. When the complete sequence of the cDNA was determined by using Sequenase enzyme (U.S. Biochemical) and compared with the LED3 sequence, four nucleotide differences were identified. By using oligonucleotide-directed mutagenesis (16), positions 198 (G to A), 2493 (T to C), 4466 (T to C), and 6334 (T to C) were corrected to generate pLED3. The cDNA sequence of clone pVR318 was identical except for the Leon-like nucleotide (T) at position 2493.

Transcription reaction mixes were prepared as described elsewhere (13) and yielded 20 to 25 μg of RNA approximately 7.6 kb in length from 1 μg of the 8.1-kb *Pvu*I fragment of pLED3 or pVR318. DNA template and RNA product were quantified by agarose gel electrophoresis and densitometric scanning. The sequence of the transcripts was analyzed directly as described before (21) and demonstrated the presence of two extra guanines at the 5′ end immediately before the first poliovirus nucleotide and 133 extraneous bases at the 3′ end after the poly$(A)_{27}$ tail. When these RNA transcripts were introduced into Vero cells (20) by transfection with DEAE-dextran (23), they had a specific infectivity of 1×10^2 to 2×10^2 PFU/μg (about 3% of that of vaccine virus RNA). LED3+1 and VR318+1 viruses were harvested 48 to 72 h (+4 cytopathic effect) after Vero cell monolayers (10^7 cells) were transfected with aliquots of the transcription reaction mix containing 25 μg of full-length RNAs. The LED3 and VR318 viruses used in these studies were passaged again in Vero cells at 33.5°C at a multiplicity of infection of 0.1. Sequence analysis of viral RNA from these

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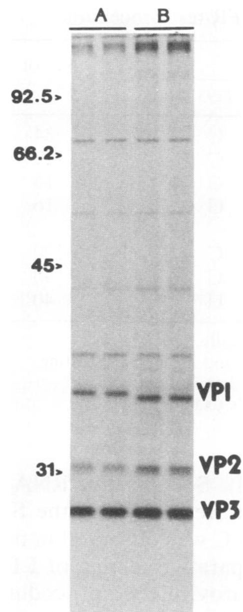

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of LED3 and VR318 cDNA-derived viruses. A [^{35}S]methionine-labeled sample was prepared as described in the text and resolved by electrophoresis in a 15% polyacrylamide gel. The gel was dried, and the protein bands were visualized by autoradiography. Lanes: A, LED3 virus; B, VR318 virus. The positions of prestained molecular mass markers (in kilodaltons) are indicated on the left. Positions of viral capsid proteins are identified on the right.

stocks confirmed them to be identical to the LED3 sequence except that VR318 had a U at position 2493.

The mutation identified at position 2493 in vaccine virus predicts that the sixth residue of VP1 is threonine (Thr-6 [24]) instead of isoleucine (Ile). To determine whether the biochemical properties of VP1 would be altered by this substitution, the VP1 proteins of LED3 and VR318 viruses were compared. To do this, Vero cells were infected at a high multiplicity of infection (>1) with LED3 or VR318 virus and then maintained at 33.5°C in methionine-free minimal essential medium (Select Amine Kit; GIBCO) supplemented with [^{35}S]methionine (60 μCi/ml, >1,000 Ci/mmol; Amersham). Radioactive virus was harvested at +4 cytopathic effect (within 24 h), pelleted from the fluid after clarification as described before (21), and then boiled in Laemmli sample buffer prior to analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (11). Even though the molecular weights are similar, the VP1 possessing Ile-6 migrated faster than that with the Thr-6 substitution (Fig. 1). The decrease in the mobility of VP1 correlating to the U → C change at 2493 is likely due to a concomitant decrease in the hydrophobicity of the protein (8).

According to the three-dimensional structure of native poliovirus, residue six within the N terminus of VP1 is buried inside the virion in close association with terminal regions of the other capsid proteins, forming a network which is likely to direct and stabilize virion assembly (6). To assess whether the Ile-6 to Thr substitution alters virion stability, LED3 and VR318 viruses were compared for susceptibility to thermal inactivation at several temperatures. Virus stocks (approx. $10^{7.3}$ PFU/ml) were incubated at room temperature (22°C), 37°C, or 42°C, and samples were taken at 24-h intervals for 5 days. The virus was incubated in

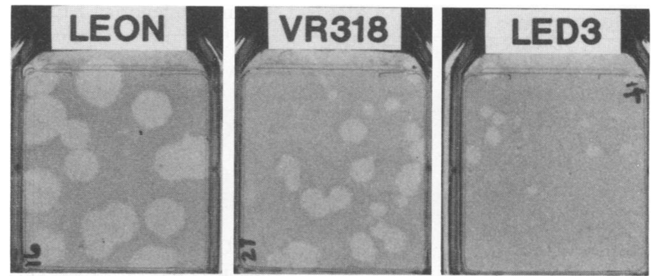

FIG. 2. Plaque phenotype of Leon (wild type), VR318, and LED3 viruses on Vero cells. After incubation at 33.5°C for 3 days under 1.0% nutrient agar, cells were stained with neutral red to visualize plaques.

modified Earle's lactalbumin hydrolysate maintenance medium (pH 7.3), which lacks stabilizers. As measured by plaque titration in Vero cell monolayers, the titer of neither virus stock was reduced after 5 days at room temperature (22°C). At 37 and 42°C, the thermal inactivation curves were identical for both viruses; a 2.3 and 5.8 log reduction in titer was measured after 5 days at each temperature, respectively (data not shown). The 2493 mutation had no apparent effect on the thermal stability of the virus.

Small plaque size is often used to differentiate attenuated vaccine strains from virulent strains (14). Plaque titration of the virus stocks on Vero cell monolayers demonstrated that VR318 produced plaques that were intermediate in size between the small plaques of LED3 and the large plaques produced by Leon (Fig. 2). The C to U switch at position 2493 correlated with partial loss of a phenotypic marker (small plaque size) linked with attenuation.

Restriction of virus growth at 40°C (T marker) and at acidic pH (d marker) are two other in vitro biological tests used to evaluate the quality of vaccine strains (14). By these assays, the LED3 and VR318 viruses were the same; compared with Leon, both were as attenuated as vaccine virus (data not shown).

LED3 and VR318 were compared with appropriate controls for neurovirulence as tested in *Macaca mulatta* monkeys by procedures defined in either the U.S. Code of Federal Regulations (CFR) (2a) or World Health Organization (WHO) requirements for the acceptance of vaccine lots (26). Differences between these procedures include the route of inoculation (intrathalamic and intraspinal versus only intraspinal) as well as amount (volume and titer) of the sample injected. Upon histological examination of brain and spinal cord tissue from each monkey, the severity of neuronal damage is scored from 0 (none) to 4 (high). The mean lesion score (MLS) is the average of scores recorded for all positive monkeys within a group.

Table 1 compiles the neurovirulence data from two intraspinal tests in which LED3 and VR318 were tested concurrently by a CFR method. Included for comparison are results from tests on actual vaccine produced by using rederived Sabin seed in primary monkey kidney cells [RSO+2 (PCMK)] as well as vaccine produced experimentally in Vero cells [RSO+2 (Vero)]. The MLSs produced by RSO+2 (PCMK), RSO+2 (Vero), and LED3+2 (Vero) were 0.52, 0.36, and 0.34, respectively. These data indicate that Vero cells can be used to produce vaccine virus while preserving the attenuated phenotype and that LED3 is at least as attenuated as the current vaccine strain. In contrast, the MLS of monkeys receiving VR318 was 1.31, which was significantly higher ($P < 0.01$) than the other scores.

TABLE 1. Neurovirulence of LED3 and VR318 strains with the CFR test procedure

Injection route	Group	Virus	Cell substrate	Nucleotide at:		No. of monkeys	MLS or % positive
				472	2493		
Intraspinal[a]	1	RSO+2	PCMK	U	C	24	0.52
	2	RSO+2	Vero	U	C	12	0.36
	3	LED3+2	Vero	U	C	16	0.34
	4	VR318+2	Vero	U	U	16	1.31[b]
Intrathalamic[c]	5	RSO+2	PCMK	U	C	120	4%
	6	LED3+2	Vero	U	C	40	0%
	7	VR318+2	Vero	U	U	40	13%[d]

[a] 0.2 ml of virus (titer, ≥7.6 log 50% tissue culture-infective doses [$TCID_{50}$]/ml [1]) administered intraspinally.
[b] Group 4 MLS significantly greater than MLSs for groups 1, 2, and 3 ($P < 0.01$) by analysis of variance and mean range testing.
[c] 0.5 ml of virus (titer, ≥7.6 log $TCID_{50}$/ml) administered intracerebrally into the thalamic region of each hemisphere.
[d] Percent positive for group 7 significantly greater than percents for groups 5 and 6 ($P < 0.05$) by Fisher's exact test.

The data in Table 1 also represent two tests that were carried out to compare the neurovirulence of LED3 and VR318 after intrathalamic injection into monkeys. The percentage of monkeys exhibiting polio lesions rather than an MLS was used to compare the neurovirulence of the viruses because so few animals exhibited lesions. The data compiled from four CFR release tests on the RSO+2 vaccine demonstrated that the percentage of reactive monkeys is typically low (4%). Of the 40 monkeys receiving LED3, none exhibited lesions. VR318, however, produced lesions in 13% (5 of 40) of the animals. Both methods used in the CFR test showed that the cDNA-derived LED3 virus is indistinguishable from the RSO+2 vaccine and that virus possessing a C at 2493 (LED3 and RSO+2) is more attenuated than virus with a U at 2493 (VR318).

For the WHO test procedure, LED3 and VR318 were evaluated concurrently with NC1, which is a vaccine strain (SO+2) produced from the SO seed and used as an attenuated reference strain in this test. Compared with the CFR intraspinal test discussed above, these monkeys received 10-fold less virus. With the WHO method, the MLSs for LED3 and VR318 were 0.21 and 1.51, respectively (Table 2). The reference virus, NC1, produced an MLS of 1.08. Although this is a high reference score, the comparison of reactivity between NC1, LED3, and VR318 is valid because they were tested concurrently. Although VR318 produced a greater MLS than NC1, the difference was not statistically significant. On the other hand, the MLS for LED3 was shown to be significantly lower than that for VR318 or the NC1 reference strain ($P < 0.05$).

We recently reported that the RSO seed is a purified derivative of the SO strain with respect to nucleotide composition at 2493 (24). Specifically, RNA sequence analysis detected a U/C mixture at 2493 in the SO+2 vaccine (i.e., NC1), whereas only C was observed in the RSO+2 vaccine. In this report, comparative testing of LED3 and VR318 by CFR and WHO neurovirulence procedures correlated C at 2493 with increased attenuation. The level of variants with U at 2493 in the SO+2 vaccine (approx. 50%) may explain why, in the WHO test, the neurovirulence of the reference strain was greater than that of LED3 but less than that of VR318. Although this report does not address how the proportion of the U-2493 variant affects the acceptability of vaccine lots, the neurovirulence test comparisons made between viruses LED3 (C-2493), VR318 (U-2493), RSO+2 (C-2493), and SO+2 (U/C-2493) suggest that the reference strain used to assess attenuation will influence the outcome.

TABLE 2. Neurovirulence of LED3 and VR318 strains with WHO test procedure[a]

Group	Virus	Cell substrate	Nucleotide at:		No. of monkeys	MLS
			472	2493		
1	LED3+2	Vero	U	C	6	0.21[b]
2	NC1[c]	PCMK	U	U/C	6	1.08
3	VR318+2	Vero	U	U	6	1.51

[a] 0.1 ml of virus (titer, 6.5 to 7.5 log $TCID_{50}$/ml) administered intraspinally.
[b] MLS for group 1 significantly lower than MLSs for groups 2 and 3 ($P < 0.05$) by analysis of variance and mean range testing.
[c] NC1 (SO+2) is an attenuated type 3 reference for the WHO neurovirulence test.

The presence of C-472 variants at levels exceeding 1.17% of total virus in vaccine lots was recently correlated with failure in the WHO neurovirulence test by a detection method incorporating the polymerase chain reaction (2). Determination of equivalence at position 472 for LED3 and VR318 was based on sequence analysis of the viral RNA. We have shown that a subpopulation of ≤10% is below the detectable limit by this method (21). Reevaluation of LED3 and VR318 by the polymerase chain reaction method cited above demonstrated that these samples were equal with respect to the nucleotide at 472 (data not shown).

The degree to which the C-2493 mutation attenuates Sabin 3 in comparison to the attenuating mutations at 472 and 2034 is difficult to assess because all the cDNA-derived Sabin 3 recombinants tested previously lack the attenuating C at 2493 (25). The additional difference in Sabin 3 sequences at position 6061 further complicates comparison. Derivatives of LED3 containing single mutations at positions 472 (C), 2034 (C), and 6061 (C) in the genome are being constructed to address this point.

The mechanism by which the change of Ile-6 to Thr in VP1 attenuates LED3 compared with VR318 is unclear. Recent reports suggest that the N terminus of VP1 plays a role in cell entry. One study showed that upon attachment of virus to susceptible cells, this region of VP1 is externalized and this exposure is required for attachment to liposomes (5). Others correlated deletion within the N terminus of VP1 to a defect in release of viral RNA from the capsid during infection (9). According to Fricks and Hogle, a periodicity of hydrophobic amino acids occurs in this region of VP1 that is capable of forming a helix with one distinctly hydrophobic side (5). If the function associated with exposure of the N terminus of

VP1 is mediated through a hydrophobic interaction, it may be weakened by the Ile-6 to Thr substitution.

This report demonstrates that the biological properties associated with attenuation of the Sabin 3 vaccine strain were preserved in LED3. Compared with seeds derived from viral plaques, a seed in the form of a cloned, genetically defined cDNA would be more homogeneous and not limited in supply. Passage studies comparing LED3 with other seeds used to manufacture Sabin 3 vaccine are in progress to assess whether LED3 constitutes a more genetically stable seed.

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