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Differences in Replication of Attenuated and Neurovirulent Polioviruses in Human Neuroblastoma Cell Line SH-SY5Y

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A base change from C to U at position 472 of the 5′ noncoding region of the poliovirus genome is known to be a major determinant of attenuation in the P3/Sabin vaccine strain. To determine the biochemical basis for the attenuated phenotype imparted by this mutation, a cell line in which replication of neurovirulent and attenuated viruses could be distinguished was identified. A pair of P3/Sabin-P2/Lansing viral recombinants that differ only at position 472 was used; the viruses replicated equally well in HeLa cells, but the virus with a U at base 472 was attenuated in mice. In the human neuroblastoma cell line SH-SY5Y, recombinants with a U at base 472 replicated to approximately 10-fold-lower titers than did neurovirulent viruses with a C at this position. Analysis of viral RNA and protein synthesis indicated that translation of the attenuated viral RNA was specifically reduced in SH-SY5Y cells.

Poliovirus is well known as the causative agent of paralytic poliomyelitis. This virus is a member of the picornavirus family, whose members have a small nonenveloped virion that encloses a single-stranded positive-sense RNA genome of approximately 7 to 8 kilobases. Poliomyelitis has been successfully controlled by the use of live attenuated vaccine strains developed by A. B. Sabin. Recent studies have shown that mutations in the 5′ nontranslated region of the poliovirus genome play a particularly important role in the attenuation phenotype (for a review, see reference 8). For example, a single base change at position 472 from C in the neurovirulent P3/Leon/37 to U in the type 3 vaccine is a strong determinant of attenuation (4, 13). The same base change attenuates poliovirus neurovirulence in mice when incorporated into a mouse-adapted poliovirus–human poliovirus recombinant (6). Determinants of attenuation have also been identified in the 5′ noncoding region of the type 1 (7) and type 2 (6a; J. Almond, personal communication) vaccine strains. However, the mechanism by which these mutations attenuate poliovirus neurovirulence is not understood.

To further define the biochemical lesion imparted by the attenuating mutation at nucleotide 472, we sought a cell line in which replication of attenuated poliovirus would be impaired. For these studies, two previously isolated viral recombinants were used, PRV7.3 and PRV8.4, which differ only by the base at position 472: PRV7.3 contains a U, and PRV8.4 contains a C (5). We found that viruses containing a U at base 472 replicate to lower titers in human neuroblastoma cell line SH-SY5Y than do viruses with a C at this position. In infected SH-SY5Y cells, both viruses produce similar amounts of RNA, but translation of viral RNA containing a U at base 472 is reduced, suggesting that poliovirus attenuation may involve regulation of viral RNA translation.

PRV7.3 and PRV8.4 are viral recombinants in which the 5′ nontranslated region (742 nucleotides), together with 44 nucleotides encoding VP4 of P3/Leon $12a_1b$ and P3/119/70, respectively, is joined at nucleotide 786 to the rest of the genome of mouse-adapted P2/Lansing/37. Virus PRV7BE1 is a neural isolate recovered from a paralyzed mouse which had been inoculated intracerebrally with PRV7.3. PRV7BE1 differs from the parental virus PRV7.3 at position 472, where PRV7BE1 contains a cytosine (5). All three recombinants replicate with similar kinetics and to identical titers in HeLa cells; however, the virus with a U at base 472, PRV7.3, fails to replicate in the mouse central nervous system and does not cause paralytic disease (5). The change from C to U at base 472 is therefore a host range mutation that restricts replication in neural cells.

To identify a cell line capable of reproducing the host range phenotype of the recombinant viruses PRV8.4 and PRV7.3, the replication of these two viruses in several cultured cell lines was examined. The human neuroblastoma cell line SH-SY5Y was obtained from F. Alt, Columbia University, and grown in monolayers in RPMI 1640 medium containing 10% fetal bovine serum. The SH-SY5Y cell line was subcloned from the neuroblastoma line SKNSH, which was established from a bone marrow aspirate from a 4-year-old patient with metastatic neuroblastoma (10). SKNSH cells are considered to be of neuronal origin, since they contain high levels of dopamine-β-hydroxylase activity (2). One-step growth curves were plotted to compare replication of the viral recombinants. SH-SY5Y monolayers were dispersed by pipetting, centrifuged, and infected at a multiplicity of infection of 10 PFU per cell. After adsorption, cells were diluted to 2×10^6 cells per ml (in RPMI 1640–10% fetal bovine serum–50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], pH 7.0, for SH-SY5Y cells and in Joklik minimal essential medium–10% fetal bovine serum–50 mM HEPES, pH 7.0, for HeLa cells) and incubated in Spinner bottles at 37°C for 7 h. The cell suspension was sampled at hourly intervals, and total virus was determined by freezing and thawing samples three times, clarifying by centrifugation, and then using plaque assay on HeLa cell monolayers (6).

Although PRV8.4 and PRV7.3 have different neurovirulence levels in mice, they have identical growth kinetics and yields in cultured epithelioid (HeLa) cells (Fig. 1) (5). However, in SH-SY5Y cells, the final yield of PRV7.3 was consistently 10-fold lower than that of PRV8.4 (Fig. 1). The reduced replication capacity of PRV7.3 was specific for cells of neuronal origin, since the kinetics of virus production and

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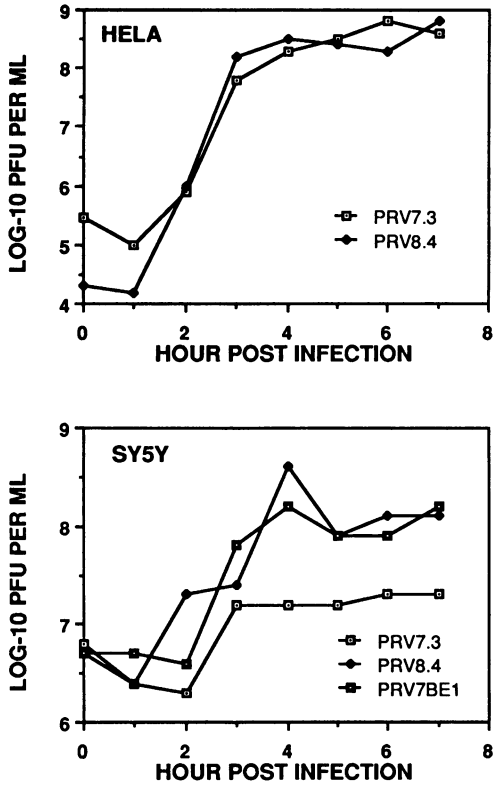

FIG. 1. Replication of recombinant polioviruses in HeLa and SH-SY5Y cells. Cells were infected at a multiplicity of infection of 10 and incubated at 37°C. Total virus titer was determined at each time point.

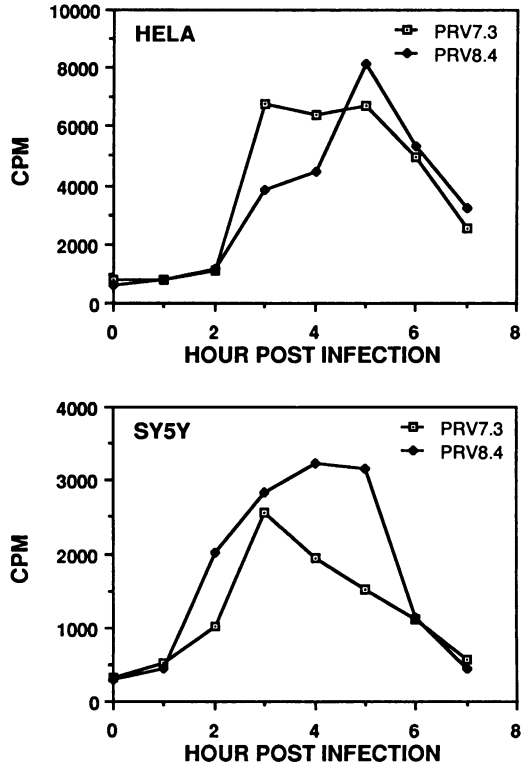

FIG. 2. Positive-strand viral RNA synthesis in HeLa and SH-SY5Y cells infected with poliovirus recombinants PRV7.3 and PRV8.4 at a multiplicity of infection of 10. RNA levels were determined by slot-blot analysis of total infected cell RNA by using a ^{32}P-labeled, negative-strand RNA probe.

the final yield of virus of these two recombinants were identical in a glial cell line (A172), in a lung carcinoma cell line (CALU-1), and in a neuroepithelioma cell line (CHP100L) (data not shown).

To further demonstrate that reduced replication of PRV7.3 in SH-SY5Y cells is linked to the nucleotide at position 472, replication of PRV7BE1 was studied. This virus is a neurovirulent revertant isolated from a paralyzed mouse that had been injected with PRV7.3 and contains a C at position 472 in the 5′ nontranslated region (5). Replication of PRV7BE1 and PRV8.4 in SH-SY5Y cells was identical, confirming that the base change at 472 is responsible for the difference in replication of the recombinants.

To elucidate the biochemical basis for the altered replication of PRV7.3 in SH-SY5Y cells, viral protein and RNA synthesis were examined. Cells were infected as described above and incubated in Spinner cultures, and samples of the cell suspension were taken at hourly intervals after infection. Virus-specific RNA synthesis was determined by extracting total cell RNA and subjecting it to slot-blot hybridization with ^{32}P-labeled RNA probes generated by in vitro transcription with SP6 RNA polymerase (9). Radioactivity on the nitrocellulose filters was determined by scintillation counting. To monitor protein synthesis, infected cells were collected by centrifugation, suspended in methionine-free Dulbecco minimal essential medium, and pulse-labeled with [^{35}S]methionine, and cytoplasmic extracts were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (9).

In HeLa cells, PRV7.3 and PRV8.4 positive-strand RNAs were synthesized with similar kinetics and to nearly the same levels (Fig. 2). PRV7.3 RNA levels were slightly higher than PRV8.4 RNA levels at 3 and 4 h postinfection, but apparently this difference does not affect protein production (see below) or viral yields, since both viruses replicated to similar titers in HeLa cells (Fig. 1). Patterns of viral RNA synthesis in SH-SY5Y cells were similar, except that levels of PRV8.4 RNA were slightly higher late in infection. Analysis of total viral RNA with a double-stranded DNA probe provided similar results in both cell types (data not shown). Furthermore, the relative amounts of positive- and negative-strand RNAs in the two recombinants did not differ in SH-SY5Y cells (data not shown).

The two recombinants showed different kinetics of viral protein synthesis in SH-SY5Y cells (Fig. 3). For example, at 1 h postinfection, many PRV8.4 viral proteins could be identified, yet only traces of PRV7.3 precursor polypeptides P1, P3, and 3CD were observed. At this time, levels of RNA of both viruses were similar (Fig. 2). At 2 h postinfection, all the PRV7.3 polypeptides could be observed, although densitometer tracings indicated that PRV7.3 proteins reached only 50% of the level of PRV8.4 proteins (data not shown). At later times postinfection, polypeptide production of both viruses decreased, although the synthesis of PRV7.3 polypeptides never equaled that of PRV8.4.

In contrast, no differences were observed in the patterns of PRV7.3 and PRV8.4 viral protein synthesis in HeLa cells (Fig. 4). Thus reduced protein synthesis of PRV7.3 was specific for SH-SY5Y cells.

These studies show that in the human neuroblastoma cell line SH-SY5Y, PRV7.3 replicates to lower titers than does

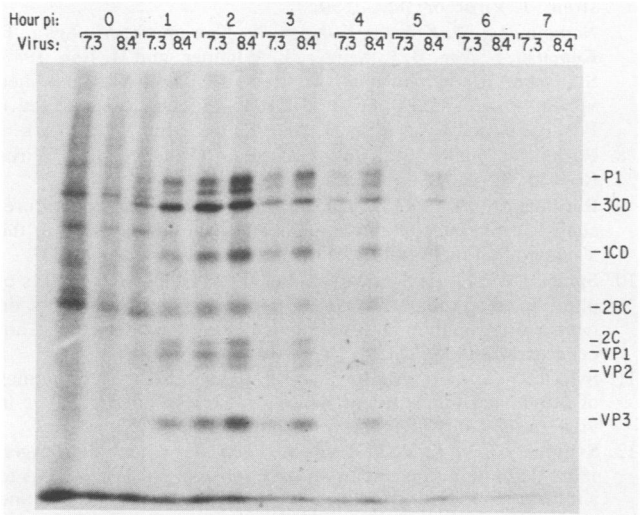

FIG. 3. Polypeptide synthesis in SH-SY5Y cells infected with PRV7.3 or PRV8.4. Cells infected at a multiplicity of infection of 10 were pulsed at hourly intervals with [^{35}S]methionine, and cytoplasmic extracts were fractionated on a 12.5% polyacrylamide gel which was then autoradiographed. Numbers at top refer to hours postinfection (pi). Some of the poliovirus-specific polypeptides are identified at right.

PRV8.4. In mice, PRV7.3 fails to replicate, while PRV8.4 replicates well and causes poliomyelitis (5). In contrast, both viruses replicate equally well in HeLa cells. The replication defect of virus PRV7.3 is linked to the presence of uracil at nucleotide position 472 both in mice and in SH-SY5Y cells. The correlation between the base at 472, the attenuation phenotype in mice, and the limited replication in SH-SY5Y cells suggest that this cell line may mimic the host restriction encountered by attenuated poliovirus in the central nervous system of the infected animal. Clearly these cells do not completely reproduce the conditions encountered by poliovirus during infection of the central nervous system. This difference is probably reflected in the observation that PRV7.3 replicates poorly, if at all, in the mouse brain (5), while in SH-SY5Y cells, final virus yield is just 10 times lower than that of PRV8.4. However, SH-SY5Y cells should be a suitable system for biochemical analysis of the effect of the base change at 472.

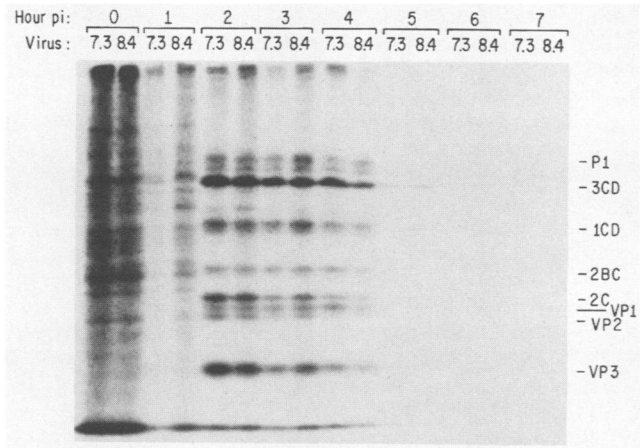

FIG. 4. Polypeptide synthesis in PRV7.3- and PRV8.4-infected HeLa cells. See legend to Fig. 3 for details.

The cytosine-to-uracil base substitution at nucleotide position 472 does not have a dramatic effect on PRV7.3 viral RNA synthesis in SH-SY5Y cells. On the basis of other studies of poliovirus mutants, it is not likely that the difference in RNA levels between PRV7.3 and PRV8.4 can account for the growth difference in SH-SY5Y cells. For example, a poliovirus mutant containing a lesion in 3C^{pro} has been shown to have a 50% reduction in the rate of RNA synthesis compared with that of wild-type virus, without showing any difference in the amount of virus produced during infection (3). In contrast, protein synthesis of PRV7.3 in SH-SY5Y cells is markedly delayed compared with that in PRV8.4 and subsequently reaches 50% of the level observed in PRV8.4-infected cells. Can this reduction of protein synthesis account for the 10-fold difference in titer observed between PRV7.3 and PRV8.4? The levels of viral capsid proteins likely have an important stoichiometric impact on the quantity of virus produced during infection. Each virion is formed by 60 copies of each of the four capsid proteins, but by only 1 copy of viral RNA. Furthermore, it is possible that proper assembly of virions in the infected cell takes place only in the presence of a certain concentration of structural proteins which can ensure the correct interaction between structural subunits. Viral capsid protein precursors appear to be synthesized in excess in infected cells, perhaps to maintain just such a critical concentration for assembly into virions (1). A decrease in the translation efficiency of viral RNA may have a magnified negative effect on the quantity of virus produced by limiting the amount of structural proteins available and altering the conditions necessary for assembly. However, we cannot rule out that other aspects of virus replication, such as genome packaging or RNA stability, are also affected by the base change at nucleotide 472.

The mechanism by which the mutation at position 472 might interfere with translation of viral RNA in neural cells is not known. Since the 5′ noncoding region directs internal binding of ribosomes to poliovirus RNA, it might be that the base change in some way alters interaction of this region with a translation factor. In support of this idea, it has been demonstrated that in vitro translation of viral RNA from attenuated vaccine strains is less efficient than translation of RNA from the neurovirulent viruses (11). Recently, a preparation from HeLa cells that restores the otherwise aberrant translation initiation of poliovirus RNA in reticulocyte lysates to the correct AUG was identified (12). This preparation, known as initiation-correcting factor, is less active in restoring correct initiation of attenuated viral RNAs than of neurovirulent RNAs, and this difference appears to be related, at least in the type 3 strains, to the base at 472. The initiation-correcting factor appears to be a complex of initiation factors eIF-2 and eIF-2B. We suggest that attenuating mutations in the 5′ noncoding region result in a poor interaction of the RNA with these initiation factors, leading to diminished translation. If such factors, for example, are reduced or are slightly different in neural cells, their interaction with the 5′ noncoding region of attenuated viruses might be altered, resulting in reduced translation and diminished multiplication of attenuated viruses in the central nervous system. Perhaps a similar defect is responsible for the reduced multiplication of PRV7.3 in SH-SY5Y cells. If the block to translation of PRV7.3 can be duplicated in in vitro extracts prepared from SH-SY5Y cells, it might be possible to restore translation with an extract from HeLa cells. If this is the case, it will be of interest to identify the

restoring factor and determining whether it is identical to initiation-correcting factor.

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LITERATURE CITED

1. **Baltimore, D., M. Girard, and J. E. Darnell.** 1966. Aspects of the synthesis of poliovirus RNA and the formation of virus particles. Virology **29**:179–189.
2. **Bielder, J. L., L. Helson, and B. A. Spengler.** 1973. Morphology, growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. Cancer Res. **33**:2643–2652.
3. **Dewalt, P. G., and B. L. Semler.** 1987. Site-directed mutagenesis of proteinase 3C results in a poliovirus deficient in synthesis of viral RNA polymerase. J. Virol. **61**:2162–2170.
4. **Evans, D. M. A., G. Dunn, P. D. Minor, G. C. Schild, A. J. Cann, Stanway, J. W. Almond, K. Currey, and J. V. Maizel.** 1985. Increased neurovirulence associated with a single nucleotide change in a noncoding region of the Sabin type 3 poliovaccine genome. Nature (London) **314**:548–550.
5. **La Monica, N., J. W. Almond, and V. R. Racaniello.** 1987. A mouse model for poliovirus neurovirulence identifies mutations that attenuate the virus for humans. J. Virol. **61**:2917–2920.
6. **La Monica, N., C. Meriam, and V. R. Racaniello.** 1986. Mapping of sequences required for mouse neurovirulence of poliovirus type 2 Lansing. J. Virol. **57**:515–525.

6a. **Moss, E. G., R. E. O'Neill, and V. R. Racaniello.** 1989. Mapping of attenuating sequences of an avirulent poliovirus Type 2 strain. J. Virol. **63**:1884–1890.

7. **Nomoto, A., M. Kohara, S. Kuge, N. Kawamura, M. Arita, T. Komatsu, S. Abe, B. L. Semler, E. Wimmer, and H. Itoh.** 1987. Study on virulence of poliovirus type 1 using in vitro modified viruses, p. 437–452. *In* M. A. Brinton and R. R. Rueckert (ed.), Positive strand RNA viruses. Alan R. Liss, Inc., New York.
8. **Racaniello, V. R.** 1988. Poliovirus neurovirulence. Adv. Virus Res. **34**:217–246.
9. **Racaniello, V. R., and C. Meriam.** 1986. Poliovirus temperature-sensitive mutant containing a single nucleotide deletion in the 5′-noncoding region of the viral RNA. Virology **15**:498–507.
10. **Spinelli, W., K. H. Sonnenfeld, and D. N. Ishii.** 1982. Effects of phorbol ester tumor promoters and nerve growth factors on neurite outgrowth in cultured human neuroblastoma cells. Cancer Res. **42**:5067–5073.
11. **Svitkin, Y. V., S. V. Maslova, and V. I. Agol.** 1985. The genomes of attenuated and virulent poliovirus strains differ in their in vitro translation efficiencies. Virology **147**:243–252.
12. **Svitkin, Y. V., T. V. Pestova, S. V. Maslova, and V. I. Agol.** 1988. Point mutations modify the response of poliovirus RNA to a translation initiation factor: a comparison of neurovirulent and attenuated strains. Virology **166**:394–404.
13. **Westrop, G. D., D. M. A. Evans, P. D. Minor, D. Magrath, G. C. Schild, and J. W. Almond.** 1987. Investigation of the molecular basis of attenuation in the Sabin type 3 vaccine using novel recombinant polioviruses constructed from infectious cDNA, p. 53–60. *In* D. J. Rowlands, M. A. Mayo, and B. W. J. Mahy (ed.), The molecular biology of the positive strand RNA viruses. Alan R. Liss, Inc., New York.