Molecular Analysis of the Oka Vaccine Strain of Varicella-Zoster Virus

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Live attenuated Oka vaccine was subjected to molecular analysis, with the long-term goal of identifying genes in the vaccine strain responsible for its attenuation. Complete genomic sequences for both the Oka parent virus and the Oka vaccine virus were determined and compared. There were differences in only 42 bases between the 2 viruses. More than one-third (15/42) of the nucleotide substitutions in V-Oka were found in open reading frame (ORF) 62. These differences were also observed when sequences of Japanese low-passage clinical isolates of varicella-zoster virus (VZV) were compared with the Oka vaccine strain. The ORF62 gene encodes an immediate early (IE) protein, IE62, that is the major transactivator of VZV and, as such, is critical to initiating the VZV gene expression cascade. Several insertions and deletions were also observed in comparing the 2 sequences, largely in the internal tandem repeat units. Functional differences between the 2 types of Oka virus were also examined. Oka vaccine was found to consist of a mixture of different subpopulations, and the parental Oka virus replicates more efficiently than does the vaccine strain. Development of a bacterial artificial chromosome for VZV expression should lead to the discovery of additional differences in wild and vaccine types and, thus, enhance our understanding of the genetic basis for attenuation of the Oka vaccine strain.

Live attenuated varicella vaccines derived from the parent Oka (P-Oka) strain of varicella-zoster virus (VZV) are used routinely to prevent or modify the severity of varicella in children in Japan and many other countries [1–4]. The vaccine Oka strain (V-Oka) was developed by Takahashi and colleagues, using the empirical approach of passing P-Oka, which is a clinical isolate recovered from skin lesions of a child with varicella, in cultured human and guinea pig cells [5]. Attenuation of V-Oka was established clinically by inoculating susceptible children. These children had no signs of varicella, developed VZV IgG antibodies, and were protected from illness when they were exposed to wild-type VZV. Attenuation was also demonstrated by the reduced virulence of V-Oka in immunocompromised children with leukemia or other immunosuppressive conditions [5–9]. Finally, V-Oka exhibits a low secondary transmission rate in comparison with wild-type VZV, with secondary transmission occurring only when the vaccine recipient had cutaneous lesions, and cases of varicella resulting from secondary V-Oka transmission are characteristically mild [10, 11]. The development of live varicella vaccine was accomplished before methods were available to investigate the genetic basis for V-Oka attenuation. New tools can now be used to clarify what molecular changes were introduced that distinguish P-Oka and V-Oka and which of those changes are likely to be associated with the reduced pathogenicity of V-Oka. Conceivably, the results from such studies could drive the future development of a molecularly engineered immunogenic vaccine that lacks any functional VZV virulence genes. Here, we summarize our work related to understanding the molecular mechanisms of V-Oka attenuation.

COMPARISON OF THE DNA SEQUENCES OF V-OKA AND P-OKA VIRUSES

The first approach that we used to investigate V-Oka attenuation was to sequence the complete genomes of
V-Oka and P-Oka, on the basis of the hypothesis that correlations between DNA sequence and virulence could be identified. The full-length DNA sequences of V-Oka and P-Oka were determined by amplifying regions across the whole genome by polymerase chain reaction (PCR), followed by cloning and direct sequencing of the PCR products [12]. When the complete DNA sequences of V-Oka and P-Oka viruses were compared, only 42 bases were found to be different (figure 1). These differences did not include the substitutions in the terminal repeat segment (TRS), because these changes are duplicated in the internal repeat segment (IRS) of the VZV genome.

The DNA sequence information was used to predict amino acid sequences of predicted VZV gene products [12]. This analysis revealed that base substitutions at 20 nucleotide positions in V-Oka would result in amino acid conversions, and base substitutions at 17 nucleotide positions were silent mutations. The remaining 5 base substitutions occurred in noncoding regions. At 30 positions, alternative base substitutions appeared in V-Oka, indicating that V-Oka contained a mixture of genomes that differed with respect to the particular nucleotide (figure 1). Fifteen of these substitutions resulted in differences between the amino acid residues in the respective V-Oka gene products, so that V-Oka was predicted to contain viruses with variations in the amino acid at each of these positions. Surprisingly, more than one-third (15/42) of the nucleotide substitutions in V-Oka were found in open reading frame (ORF) 62 and therefore in the duplicated gene, ORF71, and its flanking regions. Approximately half (8/20) of the predicted amino acid substitutions resulted from nucleotide substitutions within ORF62. This observation of enhanced variability in the ORF62 region was of particular interest because ORF62 and ORF71 encode the immediate early (IE) 62 protein, which is the major viral transactivator of VZV and directs the transcription of all VZV genes required for its replication and for the production of progeny virions. In addition to the changes in IE62, 12 other amino acid substitutions were identified on the basis of differences in the V-Oka and P-Oka DNA sequences. These mutations occurred in ORFs 6, 9A, 10, 21, 31, 39, 50, 52, 55, 59, and 64. Among these, the gene products for which the characteristics, functions, or predicted functions are known include ORF6 (helicase primase complex), ORF10 (tegument/regulatory protein), ORF21 (nucleocapsid), ORF31 (glycoprotein B), ORF39 (integral membrane protein), ORF50 (glycoprotein M), ORF55 (helicase primase complex), and ORF59 (uracil DNA glycosylase).

In addition to the nucleotide substitutions, the V-Oka sequence had several deletions and insertions, compared with P-Oka in certain regions, including the tandem repeat regions and the origin of DNA replication. The VZV genome has 5 repeat regions, designated R1, R2, R3, R4, and R5, that appear...
a single rV01 and a recombinant adenovirus, AxCANCre. The VZV rV02 contains the gpt vector, the unique long (UL), internal repeat (IR), and unique short (US) DNA domains.

Figure 2.  Construction of the varicella-zoster virus (VZV) genome bacterial artificial chromosome (BAC) in *Escherichia coli*. The VZV genome (A) is approximately 125 kbp long and consists of terminal repeat (TR), unique long (UL), internal repeat (IR), and unique short (US) DNA domains. The recombinant plasmid, PHA-2/VZV11-12 (C), contained 2.0 kbp each of 2 sequences from VZV (shaded boxes 11 and 12), as well as the BAC vector, the gpt gene, and the gfp gene, flanked by \( \text{lox}^P \) sites. The open reading frame (ORF) 11 and ORF12 fragments of the genome of the VZV wild-type strain, P-Oka (shaded boxes 11 and 12) were generated by polymerase chain reaction amplification using the appropriate primers. The recombinant VZV rV01 (D) was generated by transfection of the VZV BAC plasmid into HEL cells. The recombinant VZV rV02, which lacked the BAC vector sequences (E), was generated by superinfection of VZV rV01 and a recombinant adenovirus, AxCANCre. The VZV rV02 contains a single \( \text{lox}^P \) site. The circle enclosing an L represents the \( \text{lox}^P \) site. Adapted from [15].

in ORFs 11, 14, and 22 and in the intergenic regions between ORFs 62 and 63 (and the corresponding region between 70 and 71), and between ORFs 60 and 61, respectively. The length of the repeats in regions R1, R3, and R4 were variable in V-Oka. The VZV genome contains an origin of replication in the IRS, which is duplicated in the TRS; each origin region is ~270 bp in length. The P-Oka genome contained 269 bp in this region and was 8 bp longer than V-Oka. The average full-length genome of V-Oka was slightly longer than that of P-Oka because of these insertions and deletions.

**BIOLOGICAL COMPARISON OF V-OKA AND P-OKA VIRUSES**

When the sequencing of V-Oka revealed that it consisted of a mixture of subpopulations with some sequence differences, viruses were isolated from V-Oka by plaque purification [13, 14]. Thirteen V-Oka isolates were obtained. When ORF62 was amplified and sequenced from each isolate, one clone, designated S7-01, had all of the 8 predicted amino acid substitutions compared with P-Oka. In contrast, another clone, S7-13, had the minimum number of substitutions compared with P-Oka, with only 5 predicted changes in amino acid residues. Therefore, these 2 clones were chosen to determine whether the ORF62 mutations correlated with differences in the replication of S7-01 and S7-13. A comparison of the plaque sizes of S7-01, S7-13, V-Oka, and P-Oka showed that P-Oka produced the largest plaques. V-Oka and S7-13 showed relatively larger plaques than S7-01. In particular, S7-01 showed significantly smaller plaques compared with P-Oka (P < .01). When these V-Oka progeny viruses, V-Oka, and P-Oka were compared by infectious center assay using human embryonic lung fibroblasts, the number of cells infected by P-Oka was 2.1-fold, 4.2-fold, and 2.6-fold higher than the number infected by V-Oka, S7-01, and S7-13, respectively. These results suggested that the efficiency of viral replication differed slightly among the V-Oka clones and that P-Oka replication was relatively more efficient than V-Oka replication.

To further assess the biological significance of differences at ORF62 of the V-Oka clones and P-Oka, the capacity of each version of IE62 to transactivate the ORF28 (VZV polymerase) promoter was evaluated. IE62 has well-established transactivating effects on this promoter. A reporter plasmid, pLuc-Pol, that had the DNA polymerase promoter upstream of the luciferase ORF was constructed and used for transient luciferase assays. The mutant IE62s expressed by the V-Oka variants were predicted to have less transactivating activity than IE62 encoded by P-Oka. The results of the luciferase assays showed trends that were consistent with the differences observed between P-Oka and V-Oka variants in mean plaque size and in infectious center assays. These findings support the possibility that both viral replication and cell-cell spread may be diminished in V-Oka because of altered potency of IE62 for inducing VZV gene transcription.

ORF10 protein, which is a homologue of herpes simplex virus type 1 VP16, is a virion-associated transactivator that can stimulate transcription from the ORF62 promoter. The base substitution identified in ORF10 caused a predicted amino acid conversion. Given the importance of IE62 as the major VZV transactivating protein, this change had the potential to affect V-Oka replication. However, when the ORF10 proteins encoded by V-Oka and P-Oka were compared for their capacities to enhance transcription from the ORF62 promoter, the P-Oka ORF10 gene product was somewhat more potent, but this difference was not significant.

**GENETIC DIFFERENTIATION OF V-OKA VIRUS FROM OTHER CLINICAL ISOLATES OF VZV**

To demonstrate that the changes in ORF62 were associated with the process of P-Oka attenuation by passage in cultured cells to generate V-Oka, clinical isolates were evaluated to determine the nucleotide sequence of ORF62 [13, 14]. All of the low-passage clinical isolates of VZV differed from V-Oka at the same 15 bases that were found to be different between V-Oka and P-Oka. The differentiation of V-Oka from other clinical
isolates, including P-Oka, was also evident from changes that altered the cleavage sites for BssHII and NaeI to a unique pattern in V-Oka. V-Oka was successfully identified using 7 of 8 enzymes, with the exception of MaeII. On the basis of PCR and restriction enzyme digestion, all 54 VZV clinical isolates were shown to have sequence differences from V-Oka. Thus, the empirical attenuation of V-Oka introduced genetic changes in V-Oka that resulted in a mixture of genetic variants that appear to differ consistently from wild-type VZV.

DEVELOPMENT OF A BACTERIAL ARTIFICIAL CHROMOSOME (BAC)

A more complete analysis of the mutations in V-Oka that lead to its attenuation may be accomplished by introducing the changes observed by PCR and sequencing as single mutations or combinations of mutations into the P-Oka genome. Toward this end, we have developed a BAC that contains the P-Oka genome (figure 2) [15]. After electroporation into E. coli strain DH10B, the VZV BAC was stably propagated over multiple generations of its host. Human embryonic lung cells transfected with VZV BAC DNA that was recovered from DH10B showed cytopathic effects, and spread of the virus to neighboring cells was observed. The BAC vector sequences are flanked by loxP sites, and coinfection of the reconstituted virus with a recombinant adenovirus expressing Cre recombinase removed the residual bacterial sequences. The resulting recombinant, designated rV02, replicated as well as the parental virus in cultured cells. This P-Oka BAC should facilitate the next phase of investigations of the molecular mechanisms of V-Oka attenuation.

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References


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