Vaccine 26 (2008) 5627–5632

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

# DNA sequence analysis of varicella-zoster virus gene 62 from subclinical infections in healthy children immunized with the Oka varicella vaccine

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ARTICLE INFO

*Article history:*
Received 12 February 2008
Received in revised form 4 July 2008
Accepted 28 July 2008
Available online 14 August 2008

*Keywords:*
Varicella vaccine
Gene 62
PCR

ABSTRACT

A live attenuated varicella vaccine, the Oka vaccine strain (vOka), is routinely administered to children in Japan and other countries, including the United States. vOka consists of a mixture of genotypically distinct variants, but little is known about the growth potential of each variants *in vivo*. We isolated varicella-zoster virus (VZV) DNA sequences from the peripheral blood mononuclear cells (PBMCs) of asymptomatic healthy children immunized with the Oka varicella vaccine. VZV gene 62 DNA fragments were detected in 5 of 166 (3.0%) PBMC samples by nested PCR within 5 weeks of the vaccination. Sequence analysis of VZV DNA from these five PBMC samples indicated that multiple viral clones in the vaccine could infect vaccinees and replicate *in vivo*. We also provide evidence that a nonsynonymous substitution at position 105356 may affect viral replication *in vivo*.



## 1. Introduction

Varicella-zoster virus (VZV) is a human herpesvirus that is the etiologic agent of chickenpox (varicella) and shingles (zoster). Varicella is highly infectious and associated with fever and generalized pruritic vesicular rash among children. The virus establishes latency in cells of the dorsal root ganglia during the primary infection. Herpes zoster is caused by the reactivation of VZV and is usually observed later in life.

A live attenuated varicella vaccine, the Oka vaccine strain (vOka), was originally developed by Takahashi et al. [1] in Japan and is routinely used to vaccinate children in Japan and other countries, including the United States. Among the elderly, the live vaccine, with a high viral titre, can enhance both humoral and cell-mediated immunity to VZV [2] and is useful for preventing herpes zoster [3]. In Japan, clinical reactions caused by this vaccine are very rare among healthy children, but some cases of chickenpox or shingles are found in immunocompromised individuals after vaccination.

vOka was derived from the Oka parental virus (pOka), isolated from a patient with typical varicella. vOka was developed by passaging pOka in guinea pig cells and human fibroblasts at a low temperature, to yield a live strain with extremely reduced virulence. We previously determined the complete DNA sequences of vOka and pOka, approximately 125,000 base pairs. We found 42 base substitutions, representing 20 amino acid differences, between them [4]. Thirty-one base substitutions of these in vOka were mixtures of two kinds of base at each of these positions in the sequencing analysis. These results showed that vOka is composed of a mixture of genotypically distinct viral strains. Interestingly, 15 of the 42 base substitutions and 8 of the 20 amino acid substitutions accumulated in a single region, that of gene 62, and were specific for vOka; that is, they were not present in wild-type strains derived from patients with varicella or zoster.

The gene 62 product, IE62, is a strong transactivator of VZV [5–8]. The production of infectious VZV, generated in cells transfected with purified viral genomic DNA, is robustly increased by the addition of an IE62-expressing plasmid [9]. The amino acid mutations in IE62 of vOka have been proposed to be responsible for its characteristic slower growth and less-efficient cell-to-cell spreading *in vitro* [4]. However, it is not clear that these mutations play an important

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doi:10.1016/j.vaccine.2008.07.069

role in the attenuation of vOka *in vivo*. In support of this idea, however, some mutations in gene 62 are associated with the occurrence of vaccine-associated rash, as recently reported [10].

In this study, we performed nested PCR to detect VZV gene 62 DNA sequences in the peripheral blood mononuclear cells (PBMCs) of asymptomatic healthy children after varicella vaccination. We then analyzed the obtained VZV DNA sequences to investigate whether the vOka that infected five children (of 166) who had detectable levels of VZV DNA was a clone that grew better *in vivo* than the original vaccine.

## 2. Materials and methods

### 2.1. Vaccine and vaccination

The subjects of this study were 166 healthy children who were vaccinated by subcutaneous injection of 0.5 ml vOka at Showa Hospital (Showa Hospital changed its name to Konan Kisei Hospital in 2008). The vaccine was manufactured by the Research Foundation for Microbial Diseases of Osaka University (Biken) and contained approximately 30,000 plaque-forming units per dose. Before the vaccination, EDTA-treated and -untreated blood samples were collected. Between 2 and 8 weeks after the vaccination, a single post-vaccination collection of EDTA-treated and -untreated blood was carried out.

This study was approved by the Institutional Review Board of Showa Hospital. Informed consent was obtained from the parents of the participants before this study's initiation.

### 2.2. DNA extraction and PCR

PBMCs were immediately fractionated from fresh EDTA-treated blood (1–2 ml) using Ficoll-Paque (GE Healthcare). The PBMCs were used directly for DNA extraction without cultivation. A 50-μl sample containing total DNA was extracted from the PBMCs by the QIAamp DNA Blood Mini Kit (Qiagen), and a portion of it was used as a template for the nested PCR assay. Gene 62 was amplified as three overlapping pieces by nested PCR, using three sets of outer primer pairs and three sets of inner primer pairs, shown in Table 1. The reaction mixtures for the first PCR contained 1.25 U of Ex Taq (Takara Bio), 200 μM (each) deoxynucleoside triphosphate, 0.3 μM each outer primer, and 15 μl of the template DNA in 50 μl of Ex Taq buffer, but only reaction for the region 1 contained with 6% dimethyl sulfoxide. The PCR reaction conditions were 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1.5 min, in an oil-free thermal cycler (Bio-Rad). Nested PCR was then performed using the same conditions, but with an inner primer pair and 0.5 μl of the product from the first PCR as the template.

Genomic DNA of vOka was extracted from the Biken vaccine by High Pure Viral Nucleic Acid Kit (Roche). Genomic DNA of pOka was also extracted from the infected cells as described previously [11]. The three overlapping pieces of vOka and pOka DNA were individually amplified using the outer primer pairs under the first PCR condition as described above.

### 2.3. Cloning of the PCR products

In PBMC samples, the nested-PCR products of the sizes predicted for the VZV gene 62 fragments were individually inserted into a pCR2.1 vector and then transformed into competent *E. coli* TOP10F′ cells by the TA cloning method (invitrogen). The first-round PCR products of vOka and pOka were also cloned by this method. More than 50 plasmids were extracted from respective transformants for DNA sequencing.

### 2.4. Estimation of allele frequencies

DNA sequencing of the plasmids was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the Genetic Analyzer 3100-Avant DNA sequencer (Applied Biosystems), and the sequences were compiled using the ATGC version 4 software (Genetyx). The allele frequencies at 15 base substitutions, which were specific for vOka [4-6], were estimated based on results of the sequencing.

### 2.5. Titration of gpELISA

Serum samples were obtained from the untreated blood. The assay to measure gpELISA antibody titre in each serum sample was performed as described previously [2,12].

## 3. Results

### 3.1. Detection of VZV gene 62 DNA in PBMC samples

Rashes following vaccination with the varicella Merck vaccine occur in about 5% of healthy children [13]. In contrast, vaccine-associated adverse events caused by the Biken vaccine are extremely rare in healthy individuals. In this study, we did not

**Table 1**
Primers used in the nested-PCR analysis

| Primer set | Name | Sequence | Position |
|---|---|---|---|
| Gene 62 (region 1) | | | |
| Outer primer pair 1 | 1-F1 | 5′-**TTTCCCAGTCACGACGTTG**TTCATAAAAACCGTTCCGC-3′ | 105121–105139 |
| | 1-R1 | 5′-**GGATAACAATTTCACACAGG**TTCTGATCATCTACGATCCG-3′ | 106600–106581 |
| Inner primer pair 1 | 1-F2 | 5′-TTTTATTAACAACAAACAGTCCGCG-3′ | 105140–105164 |
| | 1-R2 | 5′-CGTTGCCCGGAGAGGACATCAACGG-3′ | 106582–106558 |
| Gene 62 (region 2) | | | |
| Outer primer pair 2 | 2-F1 | 5′-**TTTCCCAGTCACGACGTTG**CAGGCACAACCGGTTACTCAG-3′ | 106455–106475 |
| | 2-R1 | 5′-**GGATAACAATTTCACACAGG**CAAATTCGGATGATTCGGAC-3′ | 107950–107931 |
| Inner primer pair 2 | 2-F2 | 5′-GGCTGCCAGGACCACAGACAGTCCC-3′ | 106476–106500 |
| | 2-R2 | 5′-TCAAATGATGGTGGGTCGACGAAAC-3′ | 107930–107906 |
| Gene 62 (region 3) | | | |
| Outer primer pair 3 | 3-F1 | 5′-**TTTCCCAGTCACGACGTTG**TTTGGTCTTACGAATCCTCGG-3′ | 107844–107864 |
| | 3-R1 | 5′-**GGATAACAATTTCACACAGG**AGCGGTCTCTCCTTAAACGC-3′ | 109381–109362 |
| Inner primer pair 3 | 3-F2 | 5′-GCTGATGGATCGATATCCCGGTTGG-3′ | 107874–107898 |
| | 3-R2 | 5′-CATGAACTTCCCGCCTCGAGTCTCG-3′ | 109350–109326 |

The design of these primers was based on the nucleotide sequence reported by Davison and Scott [26]. The nucleotide positions are as reported in ref. [26]. Bold letters indicate the linker sequences (M13 primers).