Development of Resistance to Acyclovir during Chronic Infection with the Oka Vaccine Strain of Varicella-Zoster Virus, in an Immunosuppressed Child

Myron J. Levin,1 Karen M. Dahl,1 Adriana Weinberg,1 Roger Giller,2 Amita Patel,3 and Philip R. Krause 3

1Sections of Pediatric Infectious Diseases and 2Pediatric Hematology-Oncology and Bone Marrow Transplantation, University of Colorado Health Sciences Center, and Children’s Hospital, Denver; 3Center for Biologics Evaluation and Research, Drug Administration, Bethesda, Maryland

(See the editorial by Gershon on pages 945–7.)

A 1-year-old boy was vaccinated with the Oka strain of varicella just prior to the discovery of a tumor that required intensive antitumor therapy. Three months later he developed herpes zoster, which developed into chronic verrucous lesions that were refractory to treatment with acyclovir and which subsequently disseminated. DNA from a biopsy specimen of a chronic herpes-zoster lesion indicated that the Oka vaccine strain of the virus caused this severe complication. Analysis of this viral DNA demonstrated a mutation in the viral thymidine kinase gene. Plasmids containing this altered gene were unable to produce functional thymidine kinase in an in vitro translation system. The presence of this mutation would explain the clinical resistance to acyclovir. This is the first report of Oka-strain varicella virus causing severe disease after reactivation and of resistance to acyclovir during an infection caused by this virus.

Varicella can cause severe and persistent infections in immunocompromised patients who have inadequate varicella-zoster virus (VZV)–specific T cell–mediated immune (CMI) responses [1–8]. In some of these patients, the VZV infection does not heal and persists in the form of chronic skin lesions, despite acyclovir (ACV) therapy [6, 7, 9–12]. In addition, VZV that has remained latent in sensory ganglia after primary infection can cause recurrent disseminated VZV infection or herpes zoster (HZ) in immunocompromised patients, and these reactivations also can lead to skin lesions that fail to regress after treatment with ACV [6, 7, 9–17]. These refractory skin lesions, which have a characteristic hyperkeratotic, verrucous appearance, indicate chronic VZV infection and serve as a strong indicator of the presence of ACV-resistant VZV [10–13, 15–18].

In severely immunocompromised patients, the Oka strain of VZV, although attenuated, can rarely cause significant disease, including pneumonitis, hepatitis, and encephalitis [19, 20]; however, the Oka vaccine strain of VZV, which is susceptible to ACV [15, 19–23], has not previously been implicated in chronic, nucleoside-refractory infection. The patient described below developed HZ due to the Oka vaccine strain of VZV, which subsequently resulted in chronic verrucous skin lesions and disseminated disease with an ACV-resistant mutant virus.

MATERIALS AND METHODS

Histology of biopsy specimen. The biopsy specimen was stained with standard hematoxylin-eosin and was fixed for electron microscopy.

Identification of VZV strain. Typing for VZV-strain identity was based on the polymorphism at nucleotide position 106262 of open reading frame 62 [24, 25].
Measurement of VZV antibody. VZV antibody was measured by a latex-agglutination assay (VZVScan; Becton-Dickinson).

Measurement of VZV-specific T cell–mediated immunity. VZV-specific T-cell–mediated immunity was measured by a lymphocyte-proliferation assay using peripheral-blood mononuclear cells from heparinized blood. The cells were collected on ficoll-hypaque gradients, washed, counted, and resuspended in RPMI 1640 with glutamine-containing 10% human AB+ serum and with 1% antibiotics. Mononuclear cells (10^5 cells/well) were added to triplicate wells containing either VZV antigen, mock-infected control antigen, pokeweed mitogen (PWM) (Sigma) (5 μg/mL), Candida antigen (Greer) (50 μg/mL), tetanus toxoid (Connaught) (2.5 μg/mL), or medium alone. After 6 days of incubation at 37°C in a CO2 incubator, wells were pulsed with [3H]-thymidine (50 μCi/well) for 6 h, and the contents were harvested and counted in a scintillation counter. The stimulation index (SI) was calculated by dividing the median counts per minute in the stimulated wells by the median counts per minute in the control wells. A positive assay result was defined as that having an SI ≥3.0.

Cloning and sequencing of the thymidine kinase (TK) gene from Oka vaccine and from a clinical isolate. The Oka vaccine strain of VZV was obtained from the manufacturer (Merck). DNA was extracted from MRC-5 cells infected with the vaccine strain and from serum and cerebrospinal fluid (CSF), by use of the QIAamp DNA Blood Midi Kit (Qiagen), and from skin-biopsy specimens, by mincing and treating them, with proteinase K (2.5 mg/mL), for 10 min at 65°C, 2 min at 25°C, and 2 min at 65°C, and 5 min at 97°C. Polymerase chain reaction (PCR) primers (forward primer, 5’-CCT AAG AC GGT TTG TCT ACA ATA AAC-3’; reverse primer, 5’-CGT ACA CGC GAG TAT GAC AAT GTG-3’) that encompass the entire coding sequence of the TK gene were used to amplify TK sequences from both strains [26]. PCR products were cloned into plasmid pCR4-TOPO by use of the TOPO TA Cloning Kit for Sequencing (Invitrogen). Sequences were determined on both strands by use of the forward primer, M13 reverse primer, and internal VZV TK primers (forward primer, 5’-GTA TTG GCG GTA TAA CCT TGC-3’; reverse primer, 5’-GAA TAA CGT GTC TTC AAT C-3’). Clones from 3 independent PCRs were used for each strain, to ensure that potential Taq polymerase errors would not lead to erroneous sequences.

In vitro assays of TK. Plasmids containing TK-gene sequences cloned in the orientation downstream from the T7 promoter were used as a substrate for in vitro translation, by use of Single Tube Protein System 3 (Novagen). A plasmid that expresses β-galactosidase under the control of the T7 promoter was used as a negative control. TK assays were performed on translation products from these 3 plasmids, in triplicate (in each case, with a product from a separate in vitro translation experiment), by use of either ATP or CTP as substrate [27]. A 20-μL portion of translation product was incubated with 20 μL of an assay mix consisting of Tris (0.02 mol/L (pH 7.8),
Figure 2. Chronic herpes-zoster infection with acyclovir-resistant Oka-strain varicella-zoster–virus vaccine

\[^{3}H\]-thymidine (2 × 10^{-6} mol/L [85 Ci/mmol]), MgCl\(_2\) (0.002 mol/L), KCl (0.2 mol/L), NH\(_4\)Cl (0.1 mol/L), mercaptoethanol (0.005 mol/L), and either ATP or CTP (0.002 mol/L), for 60 min at 37\(^\circ\)C; 25 \(\mu\)L of this material was spotted onto diethylaminoethyl membranes (Whatman) and washed by laying the filters in a vacuum filter flask unit (Nalgene) and washing them 3 times (5 min each) with 0.001 mol ammonium formate/L and then washing them with distilled water and fixing them with absolute ethanol. Filters were dried and were counted by use of 5 mL of Optiphase scintillation fluid (Perkin Elmer Life Sciences).

RESULTS

Case history. An apparently healthy boy was vaccinated, in his right thigh, with the Oka strain of varicella, just prior to his 1-year, routine, well-child examination. Subsequently at that visit, an abdominal mass was noted, and a computed-tomography scan revealed a large retroperitoneal mass obstructing the left renal collecting system. Biopsy revealed a neuroblastoma. Chemotherapy was begun 5 days later (5 days after vaccination). Antitumor therapy was complicated by a nonfunctioning left kidney, hypertension, and multiple episodes of fever and neutropenia. Three months later, the boy developed HZ lesions at the site of vaccination. Treatment with ACV while chemotherapy cycles continued produced improvement in the skin lesions, but these never resolved completely. ACV at several different dosages was continued as indicated in figure 1.

Four months after the onset of HZ, the boy’s lesions increased in both number and area of involvement; the lesions developed a verrucous appearance and spread over his right anterior and lateral thigh and buttock (figure 2). Continuing antitumor therapy included infusion of autologous stem cells collected >1 month after completion of the second and third cycles of induction chemotherapy. The boy continued to receive ACV for 1 month after stem-cell infusion, without any change in his rash. He then developed fever and multiple erythematous papules involving his scalp, face, and trunk. A lumbar puncture was performed because of irritability. Results of PCR performed on serum and CSF (protein, 102 mg/dL; 11 cells/mm\(^3\) [98% were lymphocytes]) were positive. Foscarnet was started (40 mg/kg every 12 h), and the lesions improved. ACV was discontinued.

Over the next 3 months, foscarnet therapy was frequently altered because of variation in creatinine clearance (figure 1). Worsening HZ lesions, disseminated lesions, fever, and irritability recurred when the foscarnet dose was decreased or when the dosing interval was increased. Four months after transplantation, additional therapy included another infusion of autologous frozen stem cells and VZV immune globulin. Within 10 days, the boy’s rash was improved, and he was dis-
Infection with the ACV-Resistant Oka Vaccine Strain of VZV

Figure 3. Identification of clinical isolate as being the Oka-strain varicella-zoster virus (VZV) vaccine. The Oka-strain VZV vaccine may be differentiated from wild-type strains by a polymorphism at nucleotide position 106262. This polymorphism enables restriction endonuclease Smal to cleave vaccine-strain DNA amplified by polymerase chain reaction using primers AGG TTG GCA AAC GCA GTC and ATT ACT GTC GAC CCG AGA CC to be cleaved at this site, causing a 230-bp fragment to be cut from the 302-bp PCR product, from both the vaccine strain and the wild-type strain. "118/112 bp" denotes a doublet.

charged, with foscarnet therapy (50 mg/kg/day) being continued. Gradual healing occurred with continuing intravenous foscarnet therapy at home, for 3 months. Weight gain and resumption of developmental milestones were observed and have continued during the 310 posttherapy days preceding submission of this report. During the healing phase, the lymphocyte responses to PWM and VZV antigen were present, but responses to Candida and tetanus antigens have remained negative.

Laboratory findings. The skin biopsy showed verrucous-appearing skin with intraepidermal vesicles and necrosis. Electron microscopy showed intranuclear particles consistent with herpesviruses. VZV was not isolated from either a swab of the chronic HZ lesions or from a subsequent skin-biopsy specimen, but the VZV polymerase gene was detected by PCR performed on DNA extracted from the biopsy specimen (figure 3). Further PCR analysis of this DNA indicated the presence of the Oka vaccine strain of VZV. The VZV DNA amplified from CSF was also identified as being that of the Oka vaccine strain. The VZV TK gene was amplified by PCR performed on DNA extracted from the skin, and its nucleotide sequence was compared with the TK-gene sequence amplified from cells infected with the Oka vaccine strain. The TK gene was studied because the inhibitory activity of ACV against VZV requires its initial phosphorylation by a VZV-specified TK. The TK genes in the specimen DNA and in the Oka vaccine strain were identical in sequence, except that, at VZV nucleotide position 65074 (on the basis of the Dumas-strain sequence), there was a T in the amplified specimen DNA whereas there was a C in the DNA of the Oka vaccine strain. This substitution introduces, in the TK-coding sequence, at amino acid position 90 in the 341-aa TK protein, a TAG stop codon in place of a CAG glutamine codon.

The effect of this mutation was determined in vitro by comparing the TK activity induced by sequences from the DNA of the Oka vaccine strain versus the TK activity induced by sequences from the amplified specimen DNA, by use of sequences encoding β-galactosidase as a negative control. This was accomplished by in vitro translation of plasmids containing these sequences. The presence of TK was detected by phosphorylation of radiolabeled thymidine (with either ATP or CTP used as a donor) and by binding the resulting thymidine monophosphate to a filter; nonphosphorylated thymidine substrate was washed through the filter. In this experiment, a small amount of filter-associated residual radioactivity was observed in the β-galactosidase negative control, whereas protein translated from the TK gene of the Oka vaccine strain was associated with robust TK activity (figure 4). Protein translated from the TK gene of the VZV DNA extracted from the skin-biopsy specimen showed very low TK activity indistinguishable from that of the negative control. Similar results were obtained when either ATP or CTP was used as the phosphate donor. Thus, the stop-codon mutation completely abrogated TK activity as determined by this assay.

DISCUSSION

Oka-strain varicella vaccine is an attenuated virus licensed for use in immunocompetent individuals; however, its pathogenic potential in immunocompromised patients had been demonstrated in the immediate postvaccine period, after inadvertent administration [19, 20]. In healthy vaccinees, the vaccine strain of VZV originally had been known to become latent and to reactivate as HZ, albeit less frequently than does wild-type VZV [28, 29]. The case in the present study demonstrates that the Oka vaccine strain of VZV is capable of causing severe HZ and systemic disease in immunocompromised patients after reactivation from latency. Chronic ACV therapy in the immunocompromised boy reported in the present study resulted in selection of an ACV-resistant mutant that caused the chronic hyperkeratotic, verrucous skin lesions and disseminated infec-
Figure 4. In vitro determination of activity (in disintegrations per minute [dpm]) of thymidine kinase encoded by sequences cloned from the Oka-strain varicella-zoster virus (VZV) vaccine, a VZV clinical isolate, and a negative control expressing β-galactosidase (Beta-gal). In vitro–translated sequences were incubated with [3H]-thymidine and a phosphate donor (either ATP or CTP). Thymidine kinase activity was detected by scintillation counting of [3H]-thymidine monophosphate bound to diethylaminoethyl filter paper.

Isolation of VZV from chronic skin lesions, even by tissue biopsy, has often been problematic [15]. However, DNA extracted from the biopsy specimen from the boy reported in the present study contained sequences characteristic of the Oka vaccine strain of VZV. This VZV DNA contained a TK-gene mutation that was predicted to code for a truncated TK enzyme that had limited activity and that would result in failure of ACV therapy. The absence of appreciable TK activity was confirmed by in vitro analysis of the protein translated from the mutant TK gene. More than 35 different mutations have been reported in ACV-resistant wild-type varicella in immunocompromised patients.

Isolation of VZV from chronic skin lesions, even by tissue biopsy, has often been problematic [15]. However, DNA extracted from the biopsy specimen from the boy reported in the present study contained sequences characteristic of the Oka vaccine strain of VZV. This VZV DNA contained a TK-gene mutation that was predicted to code for a truncated TK enzyme that had limited activity and that would result in failure of ACV therapy. The absence of appreciable TK activity was confirmed by in vitro analysis of the protein translated from the mutant TK gene. More than 35 different mutations have been reported in ACV-resistant wild-type varicella in immunocompromised patients [22, 23, 30–35], half of which produce a premature stop codon in the TK gene. Half of the mutations are the result of point mutations; 40% result from nucleotide deletions, and the remainder result from nucleotide additions. Mutations responsible for resistance to ACV have not been reported for the Oka strain of VZV.

The conditions conducive to selection of the ACV-resistant Oka strain of VZV were severe immunosuppression produced by chemotherapy and prolonged antiviral therapy, possibly with periods of suboptimal dosing (see figure 1). Successful resolution of both local and systemic VZV infection was probably facilitated by foscarnet therapy, since this antiviral drug works directly on the VZV DNA polymerase and does not require activation by VZV-specific TK. However, the boy reported in the present study did not recover completely until after 12 weeks of foscarnet therapy, consistent with the observation that cure of chronic VZV in severely immunocompromised patients requires and heralds the return of VZV-specific immune responses. Although these responses were not detected by our methods by the time that the boy’s lesions had completely healed, they were clearly present at the next testing episode (figure 1). VZV-specific CMI was reconstituted at the same time that response to mitogens was detected—and before responses to other antigens (Candida and tetanus)—suggesting that persistent exposure to VZV antigens accelerated reconstitution of immune response to this antigen. The importance of VZV-specific CMI is demonstrated both by the failure of some cases of ACV-resistant VZV infection to respond to foscarnet and by the frequent recrudescence of VZV in patients thought to have been treated successfully with ACV [6, 19]. The initial response to foscarnet therapy is also followed by recrudescence in one-half of successfully treated patients, with the mean time to recurrence being 110 days [18]. The boy reported in the present study has remained symptom free for 10 months, while maintaining a strong VZV-specific immunological response. This clinical correlation with recovery of immune response is consistent with the observation (1) that VZV reactivation can be prevented with an inactivated Oka vaccine strain of VZV after human stem-cell transplantation and (2) that the success of that vaccination correlates with the return of lymphocyte proliferation in response to VZV antigen [36]. The role that adjunctive autologous stem-cell immunotherapy plays in enhancing VZV-specific CMI in the boy reported in the present study is not clear, although the rationale for such a role is sound. The cells infused into this patient did not have detectable VZV-specific CMI (data not shown).

The prolonged absence of an adequate anti-VZV immune response in patients such as the boy reported in the present study suggests that high doses of ACV should be used for initial therapy, and the slow return of these responses argues for prolonged therapy in some patients [16]. The occurrence and persistence of a chronic rash after VZV infection in an immunocompromised patient who has received ACV therapy for 2–3 weeks suggests that the VZV is resistant to ACV. Thus, when a clear therapeutic response has not occurred after this interval, especially when hyperkeratotic, verrucous lesions are present, attempts at virus isolation and at susceptibility testing should be made, and a change in antiviral therapy should be considered [35]. This suggestion is applicable to disease caused by the Oka vaccine strain of VZV, as well as to that caused by wild-type VZV.
Acknowledgments

We gratefully acknowledge Sanjay Gummalla, for assisting with the molecular experiments and for proofreading the manuscript, and Julie Patterson, for assisting with the immunological assessments. Ralph Quinones and Brian Greffe, the clinical investigators who cared for this patient, assisted in the research evaluation.

References