Persistent Detection of Varicella-Zoster Virus DNA in a Previously Healthy Child after Severe Chickenpox

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In immunocompetent children with primary varicella-zoster virus (VZV) infection, peak viral loads are detected in peripheral blood near the onset of the vesicular rash. VZV DNA concentrations normally diminish and become undetectable within 3 weeks after the appearance of the exanthem. Here, we present a previously healthy, human immunodeficiency virus-negative, 4-year-old boy admitted with severe varicella. High viral loads (>340,000 copies/ml) were found in his blood, and the viral loads remained high for at least 1.5 years. Clinical recovery preceded complete clearance of the virus. General and VZV-specific immune reactivity were intact. NK cells and CD8+ T cells were activated during acute infection, and VZV-specific CD4+ T cells were detected at high frequencies. VZV DNA was initially detected in B cells, NK cells, and both CD4+ and CD8+ T cells. In contrast, during the persistent phase of VZV DNA detection, the viral DNA was primarily located in CD8+ T cells. For the first time, we describe the persistent detection of VZV DNA in a previously healthy child.

Children experiencing primary infection with varicella-zoster virus (VZV) develop chickenpox (varicella). Symptoms include fever and a generalized vesicular rash, which normally resolve within 1 to 2 weeks. Complications such as pneumonia, secondary bacterial infection of the lesions, and encephalitis are rare in otherwise healthy children, whereas the incidence of complications and mortality is increased in immunocompromised children.

Resolution of acute varicella is critically dependent on the coordinated action of natural killer (NK) cells and VZV-specific T cells (1–3). Both CD4+ and CD8+ T cells are believed to exhibit killing of infected target cells (11, 16, 21, 22, 26, 44). VZV DNA concentrations in peripheral blood are on average ~1,600 copies/ml on day 2 (range, 0 to 10) after the onset of rash (15) and can be detected until approximately week 3 after the onset of disease (30). It is generally assumed that viremia during VZV infections is cell associated, although viral DNA can also be detected in plasma and serum from a large proportion of patients with acute varicella (1, 15). Viral DNA has been previously described to be equally detected in B cells, CD4+ T cells, CD8+ T cells, and monocytes/macrophages during acute infection (27).

After acute infection, the virus develops latency in neuronal cells within trigeminal or dorsal root ganglia as shown by detection of VZV DNA at these sites (18, 31, 35, 36). Unlike herpes simplex virus type 1 (HSV-1), viral protein expression has been demonstrated during latency, particularly the ORF63 transcript (8–10, 28, 29). The exact mechanism of establishing and maintaining latency are still largely unresolved (32, 38, 39, 43). Upon waning cellular immunity, the virus may reactivate and cause herpes zoster (shingles), characterized by a painful vesicular rash usually confined to one or more sensory dermatomes (5, 18, 24, 45).

Here, we present a 4-year-old, human immunodeficiency virus (HIV)-negative, previously healthy boy with persistent VZV DNA concentrations in peripheral blood samples for up to 1.5 years after admittance because of severe varicella. Extensive immunologic evaluation did not reveal abnormalities in humoral immunity, in the numbers and activation of NK cells, CD4+, and CD8+ T cells, or in the expression of major histocompatibility complex (MHC) classes I and II. During the persistent phase of DNA detection, VZV DNA was predominantly detected in the CD8+ T cells, which may have resulted from the development of latency in immune cells.

CASE REPORT

A previously healthy, HIV-negative Caucasian boy from nonconsanguineous parents was admitted to the hospital at 4 years of age with a severe VZV infection at day 3 after the appearance of the exanthem. He was known to have a history of ear infections (one to three times yearly) and nonallergic bronchial hyperreactivity for which he had used inhalation therapy in the past. He had not used any medication during the last 7 to 8 months prior to admission. The boy was not known to have a bleeding tendency or prior signs of imperfect wound healing, as indicated by an uneventful adenoidectomy at the age of 2 years. The family history was uneventful. A younger sibling experienced a mild course of VZV shortly afterwards.

At presentation, he had a skin rash of more than 200 vesicles, high fever, signs of dehydration due to vomiting and diarrhea, and symptoms of coughing and dyspnea compatible with pneumonitis. According to the clinical scoring system of...
PBMCs were cryopreserved until use and thawed according to standard procedures from the patient longitudinally over a period of 2 years. Peripheral blood mononuclear cells (PBMCs) were subjected to PCR optimized by the lower limit of detection was 80 VZV DNA copies/ml of whole blood in the diagnostic procedure. Results are expressed as the number of DNA copies per 10^6 cells.

**Materials and Methods**

**Isolation of PBMCs.** Heparinized peripheral blood samples were collected from the patient longitudinally over a period of 2 years. Peripheral blood mononuclear cells (PBMCs) were isolated using standard density gradient centrifugation techniques by use of Lymphoprep (Nycoderm; Pharma, Oslo, Norway). PBMCs were cryopreserved until use and thawed according to standard procedures. All longitudinal samples were analyzed simultaneously. A control group consisting of seven children, diagnosed and monitored at the outpatient care unit for experiencing a mild to moderate course of varicella (Vazquez severity score: 5.9; n = 7; mean age, 3.6 years; three girls, four boys), was included. A limited number of blood samples from these control subjects was collected and stored when permitted by parental consent.

**Determination of VZV-specific CD4^+^ T cells by intracellular cytokine staining.** VZV-specific CD4^+^ T-cell frequencies were determined as described previously (50). In short, PBMCs were stimulated for 6 h with VZV antigen (20 µg/ml; Microbiex Biosystems, Toronto, Canada), the final 5 h in the presence of brefeldin-A (10 µg/ml). PBMCs were costimulated with CD28 (2 µg/ml; CLB 15EB) and CD49d (1 µg/ml; BD Biosciences, San Jose, CA). Cells were permeabilized using the BD-FACS intracellular cytokine staining kit (according to the manufacturer’s instructions) and stained for gamma interferon (IFN-γ)-fluorescein isothiocyanate (FITC), CD4-PerCP-Cy5.5 (all BD Biosciences), and CD69-allophycocyanin (APC) (Caltag Laboratories, Burlingame, CA). The CD4^+^ CD69^hi^ IFN-γ^- T cells (background levels subtracted) were designated VZV-specific CD4^+^ T cells. Negative controls consisted of CD4^+^ T cells stimulated with medium, and positive controls consisted of CD4^+^ T cells stimulated with Staphylococcus aureus enterotoxin B (Sigma, St. Louis, MO). Analysis of cells was performed using a FACS C flow cytometer and CellQuest software (BD Biosciences).

**Intracellular GrB and perforin staining.** Intracellular granzyme B (GrB) and perforin stainings were performed by incubating 500,000 PBMCs with CD8-FITC (BD Biosciences), washing cells once, and then fixing the cells with 50 µl of buffered formaldehyde acetone solution, and cells were subsequently permeabilized by washing with 0.1% saponin-50 mM n-gucose. Cells were then incubated with anti-GrB (Sanquin, Amsterdam, The Netherlands) and anti-perforin antibodies (Hölzel Diagnostika, Köln, Germany) according to the manufacturer’s instructions.

**Cell fractions.** To determine in which cells VZV DNA was localized, PBMCs from four different time points during follow-up were fractionated by incubation with a combination of fluorescence-labeled CD3, CD4, and CD8, CD3, CD19, and CD56 monoclonal antibodies (all BD Biosciences) for 30 min at 4°C. Cells were washed and sorted by a FACS Aria (BD Biosciences) into CD3^+^ CD4^+^ (i.e., CD4^+^ T cells), CD3^+^ CD8^+^ (i.e., CD8^+^ T cells), CD3^+^ CD19^+^ (i.e., B cells), and CD3^-^ CD56^-^ (i.e., NK cells) cells. VZV DNA loads were quantified in each cell fraction as described below. Results are expressed as the number of DNA copies per 10^6 cells.

**VZV-specific serology.** VZV immunoglobulin M (IgM) was determined by indirect immunofluorescence, and VZV IgG titers were determined in plasma as described previously using monoclonal antibodies (BioMérieux, Marcy l’Etoile, France) (15). Results are expressed as arbitrary units per milliliter of serum (a doubling in arbitrary units reflects a doubling in the amount of antibodies present in serum).

**Quantitative VZV PCR.** Quantitative PCR was performed on peripheral EDTA-anticoagulated whole-blood samples and the aforementioned cell fractions, according to a validated method previously described in great detail by de Jong et al. (15). DNA was purified from 50 µl peripheral EDTA-anticoagulated whole blood or from highly purified lymphocyte fractions according to the method described previously by Boom et al. (4), with the following modifications: 20 µl of size-fractionated silica particles were used in combination with 900 µl of lysis buffer (L6), and DNA was eluted in 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Five microliters (20 molecules) of internal control (IC) DNA mimicking the VZV target was included in the DNA extraction. The generation of IC DNA has been extensively described previously (4). Results are expressed as the number of DNA copies per 10^6 cells.

**Isolation of PBMCs.** PBMCs were cryopreserved until use and thawed according to standard procedures. All longitudinal samples were analyzed simultaneously. A control group consisting of seven children, diagnosed and monitored at the outpatient care unit for experiencing a mild to moderate course of varicella (Vazquez severity score: 5.9; n = 7; mean age, 3.6 years; three girls, four boys), was included. A limited number of blood samples from these control subjects was collected and stored when permitted by parental consent.
reactions due to carryover of amplimers. The probes used for subsequent hybridization (Perkin-Elmer B.V.) were the VZV-specific probe TBR-VZV-1 (5′-AAC GGT TTG GGT TTT CAC GCT GCC-3′) [the 5′ end was labeled with Tris-(2,2′-bipyridine)-ruthenium(II) chelate (TBR)] and the IC DNA-specific probe TBR-VZV-2 (5′-ACC TGT CGG ATC GTG ATG TGC GTT-3′) [the 3′ end was labeled with TBR]. The method used for hybridization was previously described in detail elsewhere (15). The electrochemiluminescence signal was measured by an M-8 analyzer (BIOVERIS, Oxfordshire, United Kingdom). The number of VZV DNA copies per milliliter of whole blood was calculated by use of an algorithm as described elsewhere previously (15).

The method used in the present paper has been shown previously to be highly sensitive for the quantification of VZV DNA (15). Extraction and amplification efficiencies were monitored by the inclusion of internal control DNA, mimicking the VZV target, in the DNA extraction. This PCR method used for quantification of VZV DNA has been extensively validated in the past and does not cross-react with a variety of herpesviruses, including HSV-1, HSV-2, Epstein-Barr virus, human herpesvirus 6, and CMV (15).

**Thymidine kinase resistance analysis.** DNA was isolated from the patient 5 months after discharge. The VZV gene encoding thymidine kinase and the adjacent sequence were amplified by a PCR assay described previously (34, 42). Data were compared with the data obtained using standard acyclovir-sensitive thymidine kinase as described previously (13).

**RESULTS**

**Virology.** At the time of admission, >340,000 VZV DNA copies/ml could be detected in the blood of the patient, whereas on average, only 6,000 VZV DNA copies/ml could be detected at day 1 to 2 after onset of the rash in children experiencing mild varicella (n = 7) (Fig. 1A). This agrees with previously reported data using the same quantitative PCR for VZV DNA measurement (36, 49). Upon intravenous antiviral therapy, a steady but slow decline in the VZV DNA levels was observed early during follow-up, in contrast to the rapid clearance of the virus in the controls, which cleared the virus within 3 weeks. Since the child had improved considerably due to the antiviral therapy, no other actions with regard to eradication of the virus were taken.

Remarkably, VZV loads in blood of the patient remained high for over 1.5 years. Also, oral valacyclovir, which was given 5 months after discharge at a therapeutic dosage to control for the antiviral effects on VZV and to improve the viral clearance whenever possible, did not result in an accelerated decline in VZV DNA levels. A PCR was performed to compare the thymidine kinase gene of the strain isolated from the patient with that of an acyclovir-sensitive VZV strain. This assay showed that the patient was not infected with an acyclovir-resistant strain.

**Humoral immunity and specific anti-VZV response.** In order to identify or exclude an immune defect responsible for the clinical presentation and high viral load on admission, various immunological tests were performed. Immunoglobulins, IgG subclasses, complement activity (50% hemolytic complement/ AP50, where AP50 is serum required for 50% lysis by alternative complement pathway activation), serum opsonizing activity against *Staphylococcus aureus*, and specific antibodies of the IgG isotype to prior vaccinations (measles, mumps, rubella, and tetanus toxoid) were all within the normal ranges (data not shown). After acute infection, VZV-specific IgM, which was already detectable on the day of admission, disappeared within 3 weeks, followed by a rise in VZV-specific IgG antibodies that remained positive during follow-up (Fig. 1B). Titers of VZV-specific IgG were within the range found in healthy immunocompetent children who controlled the virus within 2 to 3 weeks after the appearance of exanthem. VZV-specific IgM could not be detected in the patient’s blood samples during follow-up (data not shown).

**T-cell immunity and immunophenotypic changes.** Absolute numbers of lymphocyte subsets were determined. The numbers of NK cells and CD4+ T cells in peripheral blood were high early after infection in comparison with background data from age-matched controls who did not experience a course of varicella and normalized during follow-up (Fig. 2A). Characterization of circulating CD4+ and CD8+ T cells using CD45RA and CD27 as markers revealed progressive CD8+-T-cell differentiation over time (data not shown) (46). MHC class I and class II were expressed at normal levels, and the HLA type was A1/A1, B57/B62, Cw7/Cw7, DR17/DR13, DQ2/DQ6.

The CD8+-T-cell pool and, to a lesser extent, the CD4+-T-cell pool of the patient contained a high proportion of activated T cells during the early phase of infection, as shown by the coexpression of HLA-DR and CD38 (Fig. 2B). The levels of activated cells normalized during follow-up. During acute infection, the majority of the patient’s CD8+ T cells contained
the cytolytic mediators perforin and granzyme B, which progressively diminished during follow-up (Fig. 2C). In the first week during acute disease, the proliferative capacity of lymphocytes against phytohemagglutinin, CD2/28, and CD3/28 was decreased to 7,000, 8,530, and 12,200 cpm, respectively. Four months later, these values were normalized (7,000 cpm for all stimuli) (data not shown). Cytotoxic-T-lymphocyte toxicity in a redirected killing of anti-CD3-preincubated P815 targets showed normal cytotoxicity in routine test systems described previously (data not shown) (20, 33).

NK cells: immunophenotypic changes and cytotoxic capacity. In the past, NK cells have been shown to play a pivotal role in the early host defense against VZV (3, 49). We investigated the expression of activating and inhibiting receptors on the patient’s NK cells. The lymphocyte pool was highly enriched for CD3− CD56+ NK cells (up to 35% of total lymphocytes). During acute infection, the natural cytotoxicity receptor Nkp44 could be detected directly ex vivo on a small fraction of circulating NK cells from our patient, particularly on the CD56bright NK cell subset (Fig. 3A). The expression of the receptor declined during the persistent phase but could be detected again after 25 weeks of infection, concomitant with the CMV reactivation.

Virtually all NK cells expressed CD16 (FcγRIIIA) (Fig. 3B). The majority of NK cells expressed CD158b, whereas CD158a and NKB1 were expressed on a minority of cells (Fig. 3B). CD94, a member of the C-type lectin family associated with inhibitory NKG2A or activating NKG2C (25), was expressed on nearly all NK cells; the activating receptor NKG2D was detected on a substantial proportion of NK cells (Fig. 3B). The fraction of NK cells expressing Nkp46 was high during acute infection but diminished during the persistent phase (Fig. 3B). Functional cytotoxicity of the patient’s NK cells against the NK cell-sensitive cell line K562 was normal compared to that of age-matched controls (data not shown).

VZV-specific T-cell response. Normal T-helper-cell activity was already indicated by the normal IgM-to-IgG switch in VZV-specific antibodies and the early presence and subsequent disappearance of the anti-VZV antibody-mediated complement-binding reaction in the presence of the usual rise in total anti-VZV antibody titers over time (data not shown). In support of an intact CD4+ T-cell response, VZV-specific CD4+ T cells (i.e., CD69+ IFN-γ+) were detected at high frequencies (1.55% of total CD4+ T cells, after correction for background staining) during the acute phase of infection, which returned to background levels during the persistent phase of VZV (Fig. 4). Frequencies of VZV-specific CD4+ T cells in the control patients that experienced a mild to moderate course of varicella were <0.25% during the acute phase of infection (49, 50). Because class I-restricted VZV epitopes have yet to be determined, we were technically unable to detect circulating VZV-specific CD8+ T cells.

Localization of VZV in different immune cells. To localize VZV DNA in the different peripheral blood mononuclear cells over time, we performed cell fractionation and subsequent VZV DNA detection. Lymphocytes were sorted into B-cell (CD19−), NK cell (CD3+ CD56+), CD4+ T-cell (CD3+ CD4+), and CD8+ T-cell (CD3+ CD8+) fractions with >95% purity at various time points after the acute infection. During the early phase of the infection, VZV DNA (week 5, 15,000 copies/ml in whole blood) was present in all lymphocyte populations, with the highest viral DNA load per cell in B cells (Fig. 5). At later time points, viral DNA was predominantly present in CD8+ T cells (Fig. 5). A control sample derived from a pediatric individual with a mild course of varicella and normal clearance of VZV from the blood within 2 weeks showed that VZV DNA could be detected in limited amounts in CD4+ T cells and CD8+ T cells (Fig. 5).
DISCUSSION

We describe here, for the first time, persistent VZV DNA detection following a severe primary VZV infection in a seemingly immunocompetent child. During normal courses of varicella, VZV DNA is detectable in the first 2 weeks after the onset of varicella, after which the virus is cleared from blood, and develops latency in neurons of the dorsal root ganglia (19, 28, 30, 36). VZV reactivation from latency is known as herpes zoster (shingles), which is limited to one or more dermatomes, as mostly observed in elderly patients or in patients taking

![FIG. 3. Phenotype of NK cells. (A) Expression of NKp44 on NK cells (CD3⁻ CD56⁺) at 2, 13, 25, and 28 weeks (w.) of follow-up. All dot plots are gated on CD3⁻ CD56⁺ NK cells. (B) Expression of NK cell receptors at 2, 13, 25, and 28 weeks of follow-up, expressed as the percentage of positive cells within the NK cell pool.](image)

![FIG. 4. VZV-specific CD4⁺ T cells during follow-up. Detection of VZV-specific CD4⁺ T cells (i.e., CD69⁺ IFN-γ⁺) by intracellular cytokine staining after stimulation with VZV lysate (VZV antigen), medium (negative control), or Staphylococcus aureus enterotoxin B (SEB) (positive control) at 2, 5, 13, 25, 28, and 40 weeks (w.) of follow-up. Dot plots are gated on CD4⁺ T cells. Numbers indicate the percentages of CD69⁺ IFN-γ⁺ cells within the CD4⁺ T-cell pool.](image)
immunosuppressive medication or may even generalize in severely immunocompromised patients. In addition, recurrence of VZV infection of a chronic nature with verrucaous manifestations has been reported in patients with documented immunosuppression, most commonly HIV/AIDS (48, 53). Often, the course of the infection due to either wild-type VZV or the vaccine-derived Oka strain is indolent due to the underlying disease or development of thymidine kinase mutations causing acyclovir resistance (37, 40). Cultures may remain negative in such patients, whereas VZV DNA can be detected in blood.

Upon admission, high viral loads were detected in the patient’s blood (>340,000 copies/ml), and cultures from throat swabs and vesicles were positive for VZV. After the start of intravenous acyclovir for 14 days, vesicles disappeared and cultures from throat swabs became negative and remained so. In clinical terms, our patient showed episodic fever and non-healing ulcers but no verrucaous lesions, neurologic or mental changes, or postherpetic pain syndrome. No abnormalities in the expression of MHC classes I and II or in lymphocyte functions and phenotypes could be detected. The patient’s CD4+ T cells were activated, and high frequencies of VZV-specific CD4+ T cells could be detectable, in comparison to control patients, in which frequencies of VZV-specific CD4+ T cells remained below 0.25% (49, 50). CD8+ T cells were also activated in the acute phase of infection and expressed high levels of the cytolytic mediators granzyme B and perforin. Serology data showed a normal VZV-specific IgM-to-IgG switch and titers that were within the normal range. Nkp44 was expressed on a proportion of NK cells, indicating that these cells were activated (6). The patient’s NK cells were capable of lysing the NK cell-sensitive cell line K562.

The persistence of detectable VZV DNA in peripheral blood for at least 1.5 years is extraordinary. We investigated in which cells VZV resided during acute and persistent infection. A study by Ito and coworkers showed that VZV DNA can be equally detected in B cells and CD4+ and CD8+ T cells in otherwise healthy children experiencing varicella (27). Also, in our patient, during acute infection, VZV could be detected in the patient’s B cells, CD4+ and CD8+ T cells, and NK cells, with the highest viral load in B cells. In contrast, the virus was predominantly localized within CD8+ T cells during persistent infection, in agreement with data obtained after infection of monkeys with simian varicella virus (52).

Several explanations can account for the observations in our patient. First, the virus might continuously infect new cells, providing a dynamic reservoir for which infectious virus is needed. Although the patient did show several (sub)febrile episodes during follow-up, typical clinical symptoms of varicella (or herpes zoster) were not detected during this period. We believe that the persistent virus was not replicating. Cultures from throat swabs remained negative for VZV. Moreover, (virus-specific) CD4+ T cells, CD8+ T cells, and NK cells were activated during the early phase of infection, whereas signs of activation were no longer present during the persistent phase of virus detection. Finally, the patient did not respond to treatment with valacyclovir, with respect to the levels of VZV DNA in his blood, which was administered for 3 weeks at 6 months of follow-up. Since valacyclovir interferes with proliferation of the virus, and not clearance of the virus, this therapy is presumed not to have an effect on nonreplicating virus. Together, these observations make it unlikely that the persistence of VZV DNA in blood represents productive replication of an infectious virus.

Alternatively, the detection of VZV DNA during the persistent phase may be the result of the development of latency in the different immune cells. The precise mechanism that determines whether the virus will undergo productive replication or will switch to a latent infection is not known. Infectious virus can be recovered from ganglia during acute infection (7, 12, 51), indicating that infection of neural cells does not necessarily result in latency, and the development of latency may not be specific for the cell type infected. The development of the latent state may be a stochastic process, in which the virus develops latency only in a small percentage of infected cells. The higher the viral load, the higher the number of cells infected and the higher the chance that the virus develops latency in an unusual cell type. It is therefore not unlikely that in case of a severe infection in which many viral particles are involved, a proportion of infected cells will support the development of latency, even so in immune cells. As long as the cells survive, the virus remains detectable. The observation that VZV DNA could be detected primarily in CD8+ T cells during long-term follow-up may be the result of different decay rates of CD8+ T cells, CD4+ T cells, B cells, and NK cells. We propose that infected CD8+ T cells survive for a longer period than CD4+ T cells, B cells, and NK cells. It has previously been calculated using mouse models that naïve CD8+ T cells have a half-life of approximately 162 days, whereas CD4+ T cells have a half-life of 78 days (41). Furthermore, memory CD8+ T cells survive much longer than memory CD4+ T cells (14, 23).

The use of a validated quantitative PCR allowed us to describe for the first time a novel entity of persistent VZV DNA in immune cells. Although the persistent VZV DNA detection in this patient is abnormal, the relation to the clinical course is unclear, and an immune dysregulation cannot be fully excluded.

![Fig. 5. VZV DNA loads in PBMC fractions. PBMCs were fractionated into highly enriched populations of B cells (i.e., CD3−CD19+), NK cells (i.e., CD3−CD56+), CD4+ T cells (i.e., CD3+CD4+), and CD8+ T cells (i.e., CD3+CD8+). VZV DNA loads were quantified in each cell fraction at various time points after the onset of rash and are expressed as copies/10^6 cells. VZV DNA loads were also quantified in cell fractions of a control patient at 2 weeks after the onset of exanthem (control).](image-url)
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