Immune response to herpesvirus infections in immunocomprised children

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Chapter 7

Chronic varicella-zoster virus (VZV) detection in an immunocompetent child

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Submitted
Abstract

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 three weeks after the appearance of the exanthem. We here present a previously healthy, HIV-negative, 4-year-old boy admitted with severe varicella. High viral loads (>340,000 copies/ml) were found in his blood, which 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. By contrast, during the chronic 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.

Introduction

Children experiencing primary infection with varicella-zoster virus (VZV) develop chickenpox (varicella). Symptoms include fever and a generalized vesicular rash, which normally resolve within one to two 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 [1,2]. Both CD4+ and CD8+ T cells are believed to exhibit killing of infected target cells [3-8]. VZV DNA concentrations in peripheral blood are on average ~1600 copies/ml on day 2 (range 0-10) after onset of the rash [9], and can be detected until approximately week 3 after the onset of disease [10]. 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 [9,11]. 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 [12].

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 [13-16]. Unlike herpes-simplex virus (HSV)-1, viral protein expression has been demonstrated during latency, particular the ORF63 transcript [17-20]. The exact mechanism of establishing and maintaining latency are still largely unresolved [21-23]. 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 [13,24-26].

We here present a 4-year-old, HIV-negative, and 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, nor in the expression of MHC class I and II. During the chronic 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.
Patient, Materials, and Methods

Case report

A healthy, HIV-negative, Caucasian boy from non-consanguineous parents was admitted to the hospital at 4 years of age with a severe VZV infection since 2-3 days. He was known with a history of running ears (1-3 times yearly) and non-allergic bronchial hyper-reactivity for which he had used inhalation therapy in the past. He had not used any medication during the last 7-8 months prior to admission. The boy was not known with 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. Apart from the vesicles, the boy had approximately 20 large hemorrhagic blisters on his head and trunk. PCR analysis revealed a high VZV DNA load (>340,000 copies/ml). Liver enzymes ASAT and ALAT were maximal at admission at 564 U/ml and 324 U/ml respectively. The level of CRP was 33 mg/l. He was re-hydrated and treated with acyclovir intravenously for 10 days (30 mg/kg/day) in adjunct with flucloxacillin under suspicion of impetiginization and bacteremia. Oxygen support could be stopped after 48 hours of antiviral treatment. Blood cultures remained negative and after 5 days antibiotics were stopped. One day later he was discharged (CRP level < 3 mg/l). The levels of liver enzymes normalized within two weeks.

During follow-up the boy had various (sub-) febrile episodes lasting 1-2 weeks before full clinical recovery, which did not require hospitalization. During all these periods, bacterial pathogens or viral agents could not be detected by direct immunofluorescence tests, culture, serology or PCR, whereas VZV DNA was detectable in his blood for up to 1.5 years. At 6 weeks after discharge he developed an upper respiratory tract infection with suspected bronchitis and received broad-spectrum antibiotics for 10 days. A chest X-ray excluded pulmonary infiltrates. C-reactive protein (CRP) normalized from 100 mg/l to <3 mg/l within the following 2 weeks. Five months after discharge the boy showed a reactivation of CMV with positive cultures of saliva and urine, but negative CMV-PCR in whole blood. In the presence of a persistent VZV DNA load, he was treated for 3 weeks with valacyclovir. He still had large crustae on his head and trunk, some of which were surrounded by a vesicular rim. Bacterial culture of the vesicular fluid revealed Staphylococcus aureus. VZV culture and PCR on vesicular fluid were negative and yeast or fungi could not be detected. The vesicles did not respond to the re-instituted antiviral medication. Healing of the skin lesions only occurred after initiation of antibiotic medication, consisting of 7 days of oral flucloxacilnine, followed by intense and continuous local antibiotic treatment with mepiracine 0.1% cremor during three months.

Isolation of PBMC

Heparinized peripheral blood samples from the patient were collected longitudinally over a period of 2 years. Peripheral blood mononuclear cells (PBMCs) were isolated using standard density gradient centrifugation techniques by use of Lymphoprep (Nycomed, Pharma, Oslo, Norway). PBMCs were cryopreserved until use and thawed in accordance with standard procedures. All longitudinal samples were analyzed simultaneously. A control group consisting of 7 children experiencing a mild course of varicella was included.

Determination of VZV-specific CD4+ T cells by intracellular cytokine staining

VZV-specific CD4+ T-cell frequencies were determined as described elsewhere [27]. In brief, PBMCs were stimulated for 6 hours with VZV-antigen (20 µ/ml; Microbix Biosystems, Toronto, Canada), the final 5 hours in the presence of brefeldin-A (10 µg/ml). PBMCs were costimulated with CD28 (2 µg/ml; CLB 15E8) and CD49d (1 µg/ml; BD Biosciences, San Jose, CA). Cells were permeabilized using the BD-FACS intracellular cytokine staining kit (in accordance with the manufacturer's instructions) and stained for IFN-γ-FITC, CD4-PerCPCy5.5 (all BD Biosciences) and CD69-APC (Caltag Laboratories, Burlingame, CA). The CD4+CD69+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 of CD4+ T cells stimulated with Staphylococcus aureus enterotoxin B (SEB; Sigma, St
Chapter 7

Louis, MO). Analysis of cells was performed using a FACS Calibur flowcytometer and CellQuest software (BD Biosciences).

Figure 1 VZV virology. (A) VZV DNA loads (copies/ml) were determined in blood samples from the patient at various time points after onset of the rash (squares), as well as in blood from control patients experiencing acute varicella (circles) (n=7; mean load ± SD). Periods of anti-viral treatment are indicated (i.v. = intravenously; p.o. = per os). (B) VZV-specific IgG titers of the patient were determined and expressed in arbitrary units.

Immunofluorescent staining and flowcytometry
A total of 200,000 PBMCs were incubated with fluorescent label conjugated monoclonal antibodies (mAbs) (concentrations in accordance with the manufacturer’s instructions). Phenotyping of T cells was performed by staining with CD4-PerCPCy5.5 and CD8-APC in combination with FITC- or PE-labeled CD27, CD38, CD45RA, and HLA-DR (all BD Biosciences). B cells were detected by a combination of CD3-FITC and CD19-PE (BD Biosciences). Phenotyping of NK cells was performed by staining with CD3-PerCPCy5.5 and CD56-APC in combination with FITC- or PE-labeled CD16 (Sanquin, Amsterdam, The Netherlands), CD158a (KIR2DL1/KIR2DS1), CD158b (KIR2DL2/KIR2DL3/KIR2DS2), Nkp44, Nkp46 (all Beckman Coulter, Marseille, France), NKB1 (KIR3DL1), CD94 (BD Biosciences), or APC-labeled NKG2D (R&D Systems, Abingdon, UK).

Intracellular granzyme B and perforin staining
Intracellular granzyme B (GrB) and perforin stainings were performed by incubating 500,000 PBMCs with CD8-FITC (BD Biosciences), washing them once, fixing them with 50 µl of buffered formaldehyde acetone solution and subsequently permeabilising them by washing with 0.1% saponin 50 mM D-glucose. Cells were then incubated with anti-GrB (Sanquin, Amsterdam, The Netherlands) and anti-
perforin antibodies (Hölzel diagnostika, Köln, Germany) in accordance with the manufacturer's instructions.

**Cell fractions**

To determine in which cells VZV DNA was localized, PBMCs from 4 different time points during follow-up were fractionated by incubation with a combination of fluorescent labeled CD3, CD4, CD8, or CD3, CD19, CD56 mAbs (all BD Biosciences) for 30 minutes at 4°C. Cells were washed, and sorted by use of 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), CD3⁺CD56⁺ (i.e. NK cells) populations. VZV DNA loads were quantified in each cell fraction as described elsewhere [9]. Results are expressed as number of DNA copies per 10⁶ cells.

**VZV-specific virology**

VZV-IgM was determined by indirect immunofluorescence and VZV-IgG titers were determined in plasma as described by the use of miniVidas (Biomerieux, Marcy l'Etoile, France) [9]. Results are expressed as arbitrary units/ml serum. Quantitative PCR was performed in peripheral blood and cell fractions as described elsewhere [9]. The electrochemiluminescence (ECL) signal was measured by an M-8 analyzer (BIOVERS, Oxford, UK).

**Results**

![Graph A](attachment:image1.png)

**Figure 2 Lymphocyte subsets in peripheral blood.** (A) Absolute numbers of NK cells (circles), CD8⁺ T cells (triangles up), CD4⁺ T cells (triangles down), and B cells (diamonds) were determined during follow-up. Reference values (x10⁶ cells/ml blood) for children aged 2-10 years are 0.09-1.0 (NK cells), 0.3-1.8 (CD8⁺ T cells), 0.3-2.4 (CD4⁺ T cells), and 0.2-2.1 (B cells). (B) Activation status of CD4⁺ T cells (squares) and CD8⁺ T cells (triangles), determined by the co-expression of HLA-DR and CD38. (C) Intracellular detection of perforin (squares) and granzyme B (triangles) in CD8⁺ T cells.
At the time of admission, >340,000 VZV DNA copies/ml were detected in the blood from the patient, whereas on average only 6,000 VZV DNA copies/ml were detected at day 1-2 after onset of the rash in children experiencing mild varicella (n=7) (Fig. 1A). This in accordance with previously reported data using the same quantitative PCR for VZV DNA measurement [15]. Upon intravenous anti-viral 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, who cleared the virus within 3 weeks. Remarkably, VZV loads in blood from the patient remained high for over 1.5 years.

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 (CH50/AP50), serum opsonizing activity against Staphylococcus aureus, as well as specific antibodies of the IgG isotype to prior vaccinations (measles, mumps, rubella, tetanus toxoid) were all within the normal ranges (data not shown). After acute infection, VZV-specific IgM 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 otherwise healthy, immunocompetent children who controlled the virus within two to three weeks after appearance of the 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 age-matched controls, 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) [28]. 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 co-expression 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 of acute disease the proliferative capacity of lymphocytes against PHA, CD2/28 and CD3/28 was decreased to 7,000, 8,530, and 12,200 cpm respectively. Four months later these values were normal (>17,000 cpm, for all stimuli) (data not shown). CTL toxicity in a redirected killing of anti-CD3-preincubated P815 targets showed normal cytotoxicity in routine test systems described before (data not shown) [29,30].

Immunophenotypic changes and cytotoxic capacity of NK cells
In the past, NK cells have been shown to play a pivotal role in the early host defense against VZV [31]. 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 (NCR)
NKp44 could be detected directly ex vivo on a small fraction of circulating NK cells from our patient, in particular on the CD56\textsuperscript{bright} NK cell subset (Fig. 3A). The expression of the receptor declined during the chronic phase, but could be detected again after 25 weeks of infection, concomitant with the CMV reactivation.

Virtually all NK cells expressed CD16 (Fc\textgamma RIII\textalpha ) (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 [32], 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 chronic phase (Fig. 3B). Functional cytotoxicity of the patient's NK cells against the NK cell sensitive cell line K562 was normal compared with age-matched controls (data not shown).

![Figure 3 Phenotype of NK cells. (A) Expression of NKp44 on NK cells (CD3\textsuperscript{−}CD56\textsuperscript{−}) at 2, 13, 25, and 28 weeks of follow-up. All dot-plots are gated on CD3\textsuperscript{−}CD56\textsuperscript{−} 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)

**VZV-specific T cell response**

Normal T cell helper activity was already indicated by the normal IgM to IgG switch in VZV-specific antibodies, and the early presence and subsequent disappearance of anti-VZV antibody-mediated complement-binding reaction (CBR) in the presence of the usual rise in total anti-VZV antibody titers over time (data not shown). In support of an intact CD4\textsuperscript{+} T cell response, VZV-specific CD4\textsuperscript{+} T cells (i.e. CD69\textsuperscript{+}IFN-\gamma\textsuperscript{+}) were detected at high frequencies (up to 1.55% of total CD4\textsuperscript{+} T cells) during the acute phase of infection, which returned to background levels during the chronic phase of VZV (Fig. 4). Because class I-restricted VZV epitopes have yet to be determined, we were unable to detect circulating VZV-specific CD8\textsuperscript{+} T cells.
Localization of VZV in different immune cells

To localize VZV DNA in the different types of peripheral blood mononuclear cells over time, we performed cell fractionation and subsequent VZV DNA detection. Lymphocytes were sorted into B cell (CD3^-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 two weeks, showed that VZV DNA could be detected in limited amounts in CD4^+ T cells and CD8^+ T cells (Fig. 5).

**Figure 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 ag), medium (negative control), or SEB (positive control), at 2, 5, 13, 25, 28, and 40 weeks of follow-up. Dot-plots are gated on CD4^+ T cells. Numbers indicate the percentage of CD69^-IFN-γ^+ cells within the CD4^+ T cell pool.

**Discussion**

We here describe, for the first time, persistent VZV DNA detection following a severe primary VZV infection in an immunocompetent child. In normal courses of varicella, VZV DNA is detectable in the first two weeks after the onset of varicella, after which the virus is cleared from blood, and develops latency in neurons of the dorsal root ganglia [10,15,33]. VZV reactivation from latency is known as herpes zoster (shingles), which is limited to one or more dermatomes as mostly observed in elderly or in patients taking immunosuppressive medication, or may even generalize in the severely immunocompromised patients. In addition, recurrence of VZV infection of chronic nature with verrucous manifestations have been reported in patients with documented immunosuppression, most commonly HIV/AIDS [34,35]. Often the course of the infection, either due to 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 [36,37]. Cultures may remain negative in such patients whereas VZV DNA can be detected in blood.
On admission, high viral loads were detected in the patient's blood (>340,000 copies/ml). After start of intravenous acyclovir for 14 days, cultures from throat swabs became negative and remained so. In clinical terms, our patient showed episodic fever and non-healing ulcers but no verrucous lesions, neurologic or mental changes, or postherpetic pain syndrome. No abnormalities in expression of MHC class I and II, nor 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 were detectable. 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 [38]. 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 up to 1.5 years is extraordinary. We investigated in which cells VZV resided during acute and chronic infection. A study by Ito and co-workers showed that VZV DNA can be equally detected in B cells, and CD4⁺ and CD8⁺ T cells in otherwise healthy children experiencing varicella [12]. Also in our patient, during acute infection, VZV could be detected in the patient's B cells, CD4⁺ and CD8⁺ T cells, and in NK cells, with highest viral load in B cells. By contrast, the virus was predominantly localized within CD8⁺ T cells during chronic infection, in agreement with data obtained after infection of monkeys with simian varicella virus (SVV) [39].

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. The patient did show several (sub-) febrile episodes during follow-up, but typical clinical symptoms of varicella (or herpes zoster) were not detected during this period. VZV
infection without skin manifestations has been described as a clinical entity [40,41]. Nevertheless, 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 chronic 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. 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 chronic 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 switch to a latent infection is not known. Infectious virus can be recovered from ganglia during acute infection [42-44] 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, initiated by a percentage of the cells infected. 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, respectively. 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 from 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 [45]. Furthermore, memory CD8+ T cells survive much longer than memory CD4+ T cells [46,47]. The expression of particular genes only during latency and not during acute infection would enable us to determine whether the virus present during long-term follow-up is in a latent state. Unfortunately, these genes have not been described yet.

The use of 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.

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