CONCISE COMMUNICATIONS

Early Detection of Ganciclovir-Resistant Human Cytomegalovirus in an Immunocompromised Child

Lothar Prix,† Klaus Hamprecht,† Birgit Holzhüter,† Rupert Handgretinger,‡ Thomas Klingebiel,‡ and Gerhard Jahn†

Children with innate immunodeficiencies may be at high risk for early development of ganciclovir-resistant human cytomegalovirus (HCMV) infection after bone marrow transplantation (BMT). For early and frequent monitoring of the occurrence of ganciclovir resistance-associated mutations in codons of the UL97 gene, a panel of previously described restriction assays was expanded for use on codons 591, 592, and 603. This technique enabled detection of suddenly emerging ganciclovir-resistant HCMV after BMT in a 7-year-old child with a T cell defect. Resistance emerged among the isolation of a ganciclovir-sensitive HCMV strain 32 days after transplantation, the first detection of genotypical resistance at day 44, and the isolation of resistant HCMV (ID₅₀ > 12 μM) at day 54. Simple and yet comprehensive methods for therapy surveillance may be important in this patient group, in which the restriction assays proved useful.

The use of ganciclovir to treat human cytomegalovirus (HCMV) retinitis in adults with AIDS frequently leads to the emergence of resistant virus after therapy for ≥3 months [1]. Recently, indications were found that immunodeficient children undergoing bone marrow transplantation (BMT) are at high risk for early development of ganciclovir resistance, even after short-term antiviral treatment [2]. This demands close and frequent monitoring of antiviral therapy in such patients, for rapid initiation of alternative treatment when resistance emerges.

Since phenotypical resistance determination using culture-based assays is time consuming, it can be done only retrospectively. In contrast, prospective detection of mutations in the HCMV UL97 gene associated with ganciclovir resistance is rapid with use of the polymerase chain reaction (PCR)–based restriction analysis in the relevant codons. This methodology has been described for screening of mutations in UL97 codons 520 [3], 460, 594, and 595 [4]. A mutation in codon 603, resulting in an amino acid substitution from cysteine to tryptophane, confers ganciclovir resistance [5]. Hence, we developed a restriction assay for specific identification of this mutation. The technique provided further mutation analysis in UL97 codons 591 and 592, which are also suspected to confer ganciclovir resistance [6]. Here we report the use of the comprehensive restriction analysis of sequential specimens, to detect the sudden emergence of a mutation in UL97 codon 603 (C603W) in an HCMV DNA specimen from an immunodeficient boy treated for 24 days with ganciclovir after BMT (cumulative 93 days of ganciclovir).

Material and Methods

Clinical summary: The subject was a 7-year old boy with an innate T cell defect, with agammaglobulinemia of unknown cause and growth inhibition since 1995. HCMV infection was first diagnosed in 1992 by virus isolation from a throat washing. Since the beginning of 1997, he was treated several times with intravenous (iv) ganciclovir (5 mg/kg 2/day) for pneumonitis and fever in the context of a positive HCMV diagnosis. With each round of ganciclovir, his condition improved (figure 1). However, because of serious recurrent infections, he underwent allogenic stem cell transplantation, despite HCMV DNAemia, in August 1997. Initial immunosuppression consisted of antilymphocyte globulin (20 mg/kg/day), administered from days −3 to −1. Haploidentical, positive selected CD34⁺ peripheral stem cells were transplanted. Because of the inherent T cell depletion, no further therapeutic immunosuppression was initiated for prophylaxis against graft-versus-host disease. HCMV infection was treated with foscarnet (3 × 40 mg/kg/day) for 30 days. After transplantation, treatment was switched to iv ganciclovir (5 mg/kg 2/day) for 22 days, followed by 5 mg/kg/day for 12 days. When ganciclovir resistance was detected, foscarnet induction therapy was initiated (3 × 40 mg/kg/day), followed by foscarnet maintenance therapy (1 × 40 mg/kg/day). The reduced dosage regimen was selected because of the child’s debilitated con-
Figure 1. Human cytomegalovirus (HCMV) screening and clinical follow-up of 7-year-old bone marrow–transplant (BMT) recipient. Arrows indicate first emergence of genotypical and phenotypical ganciclovir (GCV) resistance. C603W and A594V, point mutations detected by restriction fragment length polymorphism (RFLP) analysis in codons 603 and 594 of UL97 gene. RFLP analysis in codon 603 showed variant mixture of wild type and mutant sequences (see figure 2). BAL, bronchoalveolar lavage; iv, intravenous; neg, negative; PCR, polymerase chain reaction; PFA, foscarnet; +, positive; −, negative.

Cells and virus. Virus isolates were held on human foreskin fibroblasts, maintained in Eagle’s MEM, 10% fetal bovine serum, 0.05% L-glutamine, and 0.01% gentamicin.

Qualitative and semiquantitative PCR. HCMV DNA from serum, plasma, and leukocytes was determined by nested PCR, as described elsewhere [7]. The two nested primer pairs were derived from the fourth exon of the major immediate early gene of HCMV. For semiquantitative analysis, native plasma or serum was log diluted and used for nested PCR without DNA extraction [7]. The endpoint was read as the highest dilution, giving a visible band in 2% agarose gels.

Restriction analysis in the UL97 gene. Presence of mutations in UL97 was screened in DNA specimens and virus isolates by restriction analysis in codons 520 [3], 460, 594, and 595 [4]. In addition, for examination of codons 591, 592, and 603, a 189-bp fragment comprising the UL97 codons 564–625 was amplified by PCR with primers 595F (5′-CCTCATGGCGGCTGTTGGACC) and 595R (5′-CCATGGCTCCGCAGAGACA). Mutations in codon 591 result in the loss of a HaeIII site, whereas, because of mutations C592G and C603W, new FseI or AvaII sites are generated (figure 2A). The 189-bp amplification product was also digested with Hin6I for screening of the mutation A594V, based on a method of Chou et al. [4]. The restriction fragments were separated in 3% low-melting-point agarose gels and stained with ethidium bromide.

Drug sensitivity testing. Cell-associated virus isolates were tested for phenotypical drug resistance with a simplified plaque reduction assay [8], and the dose of antiviral required for 50% inhibition of plaque formation (ID50) was determined.

Ganciclovir and foscarnet sensitivity was defined as ID50 of <6 and <400 μM, respectively.

Sequence analysis. UL97 gene sequences were determined from PCR fragments comprising codons 439–696. The primers 460F (5′-GTTGGCCGAGCCTATCAAT) and 650R (5′-CGGTCCTCCTGGAGAAT) were used for PCR and sequencing. Sequencing reactions were done with a terminator cycle sequencing kit (BigDye; Perkin-Elmer Cetus, Norwalk, CT) and a genetic analyzer (ABI310; Perkin-Elmer).

Heterogeneity of virus isolates. PCR and restriction fragment length polymorphism (RFLP) analysis from the polymorphic regions UL10–UL13 and α-sequence were used to distinguish clinical strains of HCMV. A 2-kb amplification product of UL10–UL13 was digested with Rsul and Hin6I [9], and PCR products from the α-sequence [10] were digested with MnlI, BssHII, and AvaI. Restriction patterns were resolved in 2% agarose gels.
Figure 2.  

A. Schematic course of restriction fragment length polymorphism (RFLP) analysis of mutations in codons 591, 592, and 603 of UL97 gene. Mutations in codon 591 result in loss of HaeIII site, but, with mutations C592G and C603W, new FseI and AvaII sites are generated. Restriction sites are shaded; mutations and resulting amino acid substitutions are in bold.  

B. RFLP screening of sequential virus isolates and DNA extracts for C603W mutation. Virus isolates obtained at different times varied substantially in their signal for mutant sequence. Ganciclovir-sensitive isolate from leukocytes obtained at day +32 showed no mutation. In sequential DNA extracts obtained before and after bone marrow transplantation, mutation was first noticed at day +44 after transplantation. PCR, polymerase chain reaction.
Results

HCMV monitoring of a 7-year-old boy with an innate T cell defect was conducted for 7 months before and 5 months after BMT. For frequent and rapid screening of mutations in the UL97 gene associated with ganciclovir resistance, we used a comprehensive restriction assay to detect mutations in codons 591, 592, and 603, plus previously described principles for analysis of codons 460, 520, 594, and 595 [3, 4]. Eight sequential DNA specimens positive for HCMV were screened for resistance mutations in UL97 by RFLP analysis, beginning at day −184 before BMT. Before day +44 after BMT, in 4 sequential extracts, mutations were detected neither in codon 603 (figure 2B) nor in any other of the investigated codons (data not shown). However, at day +44, the C603W mutation emerged for the first time, obviously in the background of the wild type sequence. A 10-fold increase of the DNA titer in serum during ganciclovir therapy coincided with emergence of genotypical ganciclovir resistance (figure 1). Further sequential DNA specimens showed a strong mutant signal in two DNA extracts (days +80 and +122). However, at day +128, a shift from mutant to wild type was observed, resulting in a more prominent wild type signal shown by restriction analysis (figure 2B). This event coincided with a change in the fosmidnatre from induction therapy to maintenance therapy. Mutually, this switch augmented virus replication, since the DNA titer in plasma increased (figure 1). Nevertheless, the mutation in codon 603 disappeared and was not in the last leukocyte specimen.

Three virus isolates could be tested retrospectively for phenotypical drug resistance (figure 1). Two isolates were obtained at the time when genotypical resistance was noticed. The isolate obtained from leukocytes at day +32 was ganciclovir-sensitive (ID_{so}, 1.4 μM), whereas the urine isolate at day +54 was ganciclovir-resistant (ID_{so}, 13 μM) but susceptible to foscarnet (ID_{so}, 100 μM). The laboratory strain Towne, tested simultaneously as a control, had ID_{so} of 2.0 μM and 66 μM for ganciclovir and foscarnet, respectively. The mutation in codon 603 was not detected in the sensitive isolate from day +32 (figure 2B). A third virus isolate, obtained at day +149 from urine, was intermediately resistant to ganciclovir (ID_{so}, 9.2 μM); however, it remained sensitive to foscarnet (ID_{so}, 77 μM). Restriction analysis of the isolate showed only a weak signal for mutation C603W (figure 2B), but revealed an additional mutation in codon 594 (data not shown). It is noteworthy that this A594V mutation was seen in this isolate only—not in the other 2 virus isolates or in any of the leukocyte DNA extracts. All mutations observed in UL97 by restriction analysis were subsequently confirmed by sequence analysis, revealing no additional mutations in the UL97 region (data not shown).

Clinically, the ganciclovir-resistant HCMV infection was not noticed because of the child’s debilitated general condition and perhaps because of rapid initiation of the alternative foscarnet therapy after ganciclovir resistance was detected. The foscarnet therapy led to a sharp drop of plasma DNAemia as shown by semiquantitative PCR (figure 1).

To determine whether the patient was infected with hetero- geneic strains with possibly differing susceptibilities, molecular epidemiologic studies were performed on polymorphic genome regions of sequential virus isolates. The restriction patterns of the three sequential virus isolates showed no differences in the regions of UL10–UL13 and α-sequence (data not shown).

Discussion

Of interest was the short period (12 and 22 days) between the isolation of a clearly ganciclovir-sensitive HCMV strain at day +32, the first detection of genotypical resistance (day +44), and the isolation of fully resistant virus (ID_{so}, >12 μM) at day +54. This indicates that fully ganciclovir-resistant strains may emerge suddenly after a resistance mutation occurs. The patient was treated with ganciclovir for 24 days after BMT, when genotypical resistance was first detected. Because he received several courses of ganciclovir earlier, the cumulative time of treatment was 93 days, and the mutation event may have occurred before BMT but suddenly emerged after BMT, when strong viral replication under ganciclovir treatment may have selected the mutant. The reduced dosage of foscarnet treatment during BMT may have contributed to ongoing viral replication (figure 1) and selection of a resistant strain during subsequent ganciclovir therapy. Wolf et al. [2] recently demonstrated the emergence of UL97 mutations in immunodeficient children early after BMT. Thus, rapid diagnostic methods are necessary for mutation screening. Culture-based assays for determination of phenotypical drug resistance require at least 4–6 weeks for completion and are therefore inadequate. Most ganciclovir-resistant clinical isolates have mutations in one or more codons of the UL97 gene [11–13]. Restriction analysis testing for the most frequent of these mutations [6] has been described [3, 4].

In this report, a more stringent restriction analysis was designed and was useful for the rapid detection of the C603W mutation. The described assay is also useful for screening of mutations in codons 591 and 592, although the high GC content around this loci gives rise to some difficulties. The C592G mutation results in a new HaeIII site; however, loss of the adjacent HaeIII site also indicates a mutation in codon 591. Therefore, double mutants affected in both codons could not be identified with the assay, and the wild type would be assumed by mistake. Although such double mutants have not been described, this limitation should be kept in mind.

In the last virus isolate obtained at day +149 from urine, the signal for the mutation in codon 603 was very weak. Surprisingly, a second mutation conferring ganciclovir resistance (A594V) was detected in this isolate in codon 594 of the UL97 gene, although the patient had not been treated with ganciclovir for 3 months. It is not clear when and where this mutation first
occurred, since no urine samples could be examined between days +54 and +149. Different UL97 mutations in specimens from different body sites [14] or in sequential isolates [12] have been described elsewhere. Molecular epidemiologic studies on the sequential isolates from our patient indicated that sequential virus isolates that differ in their UL97 mutations may originate from the same parental strain, since RFLP analysis of highly polymorphic regions revealed no differences.

Currently, there is limited information about the persistence of resistant viruses in vivo. Rapid disappearance of ganciclovir-resistant strains after changes in therapy and long persistence have been reported [12, 15]. In our case, the C603W mutation was detectable during more than 3 months of foscarnet therapy. Further studies are needed to identify the reasons for variable persistence of certain UL97 mutants.

Acknowledgment

We thank James H. Cox, Federal Research Institute for Virus Diseases of Animals, Tübingen, Germany, for a critical reading of the manuscript.

References