Characterization of Human Cytomegalovirus (HCMV) UL97 Mutations Found in a Valganciclovir/Oral Ganciclovir Prophylactic Trial by Use of a Bacterial Artificial Chromosome Containing the HCMV Genome

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A method based on manipulation of the human cytomegalovirus genome in a bacterial artificial chromosome was developed to determine the role played by 6 UL97 mutations of unknown significance. These mutations were found in blood samples from solid-organ transplant recipients in a trial comparing valganciclovir and oral ganciclovir prophylaxis. Recombinant viruses containing UL97 mutations P405L, A427V, M550I, A582V, Y617H, and A674T were generated in a bacterial system. Viral stocks were subsequently reconstituted in human fibroblasts, and ganciclovir susceptibilities were tested using a plaque-reduction assay. All recombinant viruses containing these unknown mutations were found to be susceptible to ganciclovir.

Human cytomegalovirus (HCMV) infections can cause serious morbidity and mortality among immunocompromised subjects, especially in patients with AIDS who have low CD4+ cell counts and in high-risk transplant recipients (i.e., those undergoing transplantation involving a CMV-seropositive donor and a CMV-seronegative recipient) [1]. The high incidence of HCMV infections in immunocompromised patients has resulted in the widespread use of anti-HCMV agents for either prevention or treatment of established HCMV diseases. Indeed, the prophylactic use of ganciclovir (either intravenously or orally) in solid-organ transplant (SOT) recipients has reduced the incidence of HCMV diseases [2]. Furthermore, a daily dose of ganciclovir, the valyl ester prodrug of ganciclovir, was found to be as clinically effective and well tolerated as a thrice-daily dose of oral ganciclovir for HCMV prevention in high-risk SOT recipients [3]. However, the long-term and widespread use of ganciclovir in immunocompromised patients has the potential to select for drug-resistant HCMV strains [4, 5]. Mutations in the UL97 (protein kinase) and, less frequently, UL54 (DNA polymerase) genes of HCMV have been shown to confer ganciclovir resistance, and several reports indicate that most drug-resistant viruses can retain their virulence [1].

In the absence of a viral isolate, it is difficult to distinguish between HCMV UL97 mutations associated with natural polymorphism and those conferring resistance to ganciclovir. The aims of this study were (1) to develop a rapid method based on initial manipulation of the HCMV genome in a bacterial artificial chromosome (HCMV/BAC)—to generate recombinant viruses containing UL97 mutations in a homogeneous viral background—and (2) to evaluate the drug phenotypes of those recombinant viral mutants in eukaryotic cells. Six UL97 mutations of unknown significance were found by genotypic analyses of blood samples during a prospective study comparing the efficacy of oral prophylactic antiviral agents in 301 high-risk SOT recipients. The prophylactic regimens consisted of either ganciclovir (1000 mg orally 3 times per day) or valganciclovir (900 mg orally per day), administered for 100 days after transplantation [6]. The specific HCMV mutants analyzed were identified either at the end of antiviral prophylaxis or subsequently at the time of suspected HCMV disease.

Materials and methods. Human foreskin fibroblasts (HFFs) were grown and maintained in Eagle’s minimal essential medium supplemented with 10% fetal bovine serum. The cells were used for transfection experiments and propagation of reconstituted viruses. The original HCMV/BAC plasmid (pBHS), derived from the AD169 strain, was provided by the laboratory of M. Messerle (Max von Pettenkofer-Institut, Munich, Germany). In this plasmid, the BAC cassette replaces the HCMV genes US2–US6, which are not essential for viral replication in cell culture [7]. Plasmids
Figure 1. Homologous recombination in the plasmid pH5, a bacterial artificial chromosome (BAC) containing the human cytomegalovirus (HCMV) genome. A, Inactivation of the UL97 gene in pH5. Most of the UL97 gene (codons -1 to 681, relative to the start codon of UL97) was replaced by a streptomycin resistance gene (Strep') flanked by HCMV regions, by use of homologous recombination (dashed lines) in Escherichia coli, which generated pH5UL97del. B, Reintroduction of the mutated UL97 gene (UL97*) in pH5UL97del. A linear DNA fragment containing the mutated HCMV UL97 gene preceded by a kanamycin selection marker (Kan') flanked by FLP recognition target (FRT) sites was introduced in pH5UL97del after a second homologous recombination, to generate pH5*FRT. C, Excision of the selection marker. Kan' was excised by site-specific homologous recombination between the FRT sites by use of the plasmid pCp20Ap, which codes for an FLP recombinase, leaving a short scar sequence of 36 bp preceding the start codon of UL97 by 4 bp. lr, internal repeat; Tr, terminal repeat; Ul, unique long; Us, unique short.

pKD13, pKD46, and pCp20, used in the construction of recombinant mutant viruses, were received from B. L. Wanner (Purdue University, West Lafayette, Indiana) [8].

Mutant HCMV/BAC plasmids were generated in the Escherichia coli BW25141 strain [8] by use of the HCMV/BAC plasmid pH5. First, a plasmid containing the entire UL97 sequence (707 codons) was constructed by cloning the UL95, UL96, and UL97 genes as well as part of the UL98 gene into the EcoRI and XhoI restriction sites of pSP72 (Promega), which generated pUL97. To create the inactivation cassette, a streptomycin-resistance gene (aadA1 taken from pLQ443, a gift from P. H. Roy; Laval University, Quebec City, Canada) was cloned into the BbsI (codon -1, relative to the start codon of UL97) and MscI (codon 681) restriction sites of pUL97, to obtain pUL97del, thus leaving 987 and 210 nt of homology with the HCMV sequences on the 2 sides of the selectable marker. The EcoRI/XhoI fragment digested from pUL97del was then used to delete the UL97 gene in pH5 by homologous recombination in E. coli by use of the Red recombinase expression plasmid pKD46 [8], which generated pH5UL97del (figure 1A). Therefore, only codons 681–707 of the UL97 gene remained in pH5UL97del after its inactivation. This first recombination event was required to ensure that subsequent homologous recombination steps would result in the integration of the desired mutation into the viral genome. Second, to generate wild-type and UL97-mutant viruses, an excisable, selectable marker (a kanamycin-resistance gene flanked by FLP recognition target [FRT] sites taken from pKD13 [8]) was cloned into the BbsI restriction site (4 bp before the start codon of UL97) in pUL97, to obtain pUL97FRT. The UL97 mutations (in comparison with AD169) of unknown significance—P405L, A427V, M550I, A582V, Y617H, and A674T, found in blood samples from patients who received prophylaxis with oral ganciclovir or valganciclovir [6]—and the well-described M460I UL97 mutation were introduced by site-directed mu-
togenesis in pUL97FRT. The EcoRI/XhoI fragments, containing the UL97 gene preceded by the excisable selectable marker, that were digested from wild-type or mutated pUL97FRT were introduced into pHBSUL97del by a second homologous recombination event, to generate pHBS*FRT (figure 1B). The excisable resistance marker was used to select for recombinant mutant HCMV/BAC plasmids, since preliminary experiments demonstrated that homologous recombination was ineffective at this step (data not shown). The resistance marker was then excised by FLP-mediated site-specific homologous recombination between FRT sites, by use of the plasmid pCp20Ap (modified from pCp20 by deletion of the chloramphenicol-resistance gene) [8], to introduce into pHB5 after migration on an agarose gel (data not shown). To demonstrate that homologous recombination was ineffective at this step (data not shown). The resistance marker was then excised by FLP-mediated site-specific homologous recombination between FRT sites, by use of the plasmid pCp20Ap (modified from pCp20 by deletion of the chloramphenicol-resistance gene) [8], which left a short scar sequence of 36 bp that preceded the start codon of the UL97 gene by 4 bp (figure 1C). Two independent recombinant HCMV/BAC plasmids were generated for each mutation analyzed.

Transfection of HFFs with recombinant HCMV/BAC plasmids was performed by electroporation, as previously described, by use of a pp71 expression plasmid to enhance infectivity of viral DNA [9]. Replication kinetics of reconstituted viruses RVHB5 and RVHB5s (which has the 36-bp scar sequence) were analyzed and compared in yield assays. In brief, HFFs were inoculated in duplicate, with 10^3 plaque-forming cells (pfc) of recombinant viruses per well in 12-well plates. Cell culture supernatants were collected at different time points after infection (days 0, 5, and 8), and viral titers were determined by a plaque-reduction assay. Susceptibility testing of recombinant viruses was performed in 24-well plates containing HFF cells. Wells were inoculated with 40 pfc. Final concentrations of ganciclovir (0–48 μmol/L, in triplicate) were used, and a standardized plaque-reduction assay was performed [10]. IC_{50} values were determined for each reconstituted viral mutant and for the wild-type virus (RVHB5). Recombinant viruses were considered to be resistant to ganciclovir if their IC_{50} value was ≥6 μmol/L or at least 3 times greater than that of RVHB5.

For all generated viruses, regions of the UL97 gene (codons 1–300 and 363–708) and of the UL54 gene (codons 170–1243) were amplified and sequenced, to confirm the appropriate genotype. The sequencing approach used in this study covered all reported ganciclovir-resistance mutations in the UL97 and UL54 genes [1]. In addition, homologous regions where recombination events occurred (nucleotide positions 139493–140480 and 142503–142713 of AD169) were sequenced in each recombinant virus.

Results. Recombinant HCMV/BAC plasmids containing UL97 mutations P405L, A427V, M550I, A582V, Y617H, A674T, and M460I (a resistant control) were generated by a 2-step homologous recombination method in E. coli BW25141, as described in Materials and methods. The integrity of the recombinant HCMV/BAC plasmids was first verified by enzymatic digestion with SspI. Digestion patterns of all recombinant viruses were considered to be resistant to ganciclovir if their IC_{50} value was ≥6 μmol/L or at least 3 times greater than that of RVHB5.

Table 1. Ganciclovir (GCV) susceptibilities of recombinant human cytomegalovirus UL97 mutants, as determined by plaque-reduction assay.

<table>
<thead>
<tr>
<th>Recombinant viruses</th>
<th>Day of transplantation after prophylaxis antiviral</th>
<th>GCV IC_{50} from 3 experiments, μmol/L</th>
<th>GCV IC_{50} mean ± SD, μmol/L</th>
<th>Fraction of WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>RVHB5 (WT)</td>
<td>Day 0 (VGCV)</td>
<td>0.20 ± 0.02</td>
<td>0.20 ± 0.02</td>
<td>1.00</td>
</tr>
<tr>
<td>RVHB5s</td>
<td>Day 0 (VGCV)</td>
<td>0.19 ± 0.03</td>
<td>0.19 ± 0.03</td>
<td>0.99</td>
</tr>
<tr>
<td>RVHB5M460I-1</td>
<td>Day 129 (GCV)</td>
<td>1.78 ± 0.04</td>
<td>1.78 ± 0.04</td>
<td>1.00</td>
</tr>
<tr>
<td>RVHB5P405s-1</td>
<td>Day 129 (GCV)</td>
<td>1.65 ± 0.03</td>
<td>1.65 ± 0.03</td>
<td>0.84</td>
</tr>
<tr>
<td>RVHB5A427V-1</td>
<td>Day 100 (GCV)</td>
<td>1.81 ± 0.04</td>
<td>1.81 ± 0.04</td>
<td>0.85</td>
</tr>
<tr>
<td>RVHB5A427V-2</td>
<td>Day 100 (GCV)</td>
<td>1.72 ± 0.04</td>
<td>1.72 ± 0.04</td>
<td>0.84</td>
</tr>
<tr>
<td>RVHB5M550I-1</td>
<td>Day 100 (GCV)</td>
<td>1.41 ± 0.03</td>
<td>1.41 ± 0.03</td>
<td>0.85</td>
</tr>
<tr>
<td>RVHB5M550I-2</td>
<td>Day 100 (GCV)</td>
<td>1.62 ± 0.03</td>
<td>1.62 ± 0.03</td>
<td>0.85</td>
</tr>
<tr>
<td>RVHB5A582V-1</td>
<td>Day 170 (VGCV)</td>
<td>1.69 ± 0.04</td>
<td>1.69 ± 0.04</td>
<td>1.00</td>
</tr>
<tr>
<td>RVHB5A582V-2</td>
<td>Day 170 (VGCV)</td>
<td>1.67 ± 0.04</td>
<td>1.67 ± 0.04</td>
<td>1.00</td>
</tr>
<tr>
<td>RVHB5Y617H-1</td>
<td>Day 100 (GCV)</td>
<td>1.58 ± 0.04</td>
<td>1.58 ± 0.04</td>
<td>0.85</td>
</tr>
<tr>
<td>RVHB5Y617H-2</td>
<td>Day 100 (GCV)</td>
<td>1.46 ± 0.03</td>
<td>1.46 ± 0.03</td>
<td>0.85</td>
</tr>
<tr>
<td>RVHB5A674T-1</td>
<td>Day 178 (VGCV)</td>
<td>1.83 ± 0.04</td>
<td>1.83 ± 0.04</td>
<td>0.85</td>
</tr>
<tr>
<td>RVHB5A674T-2</td>
<td>Day 178 (VGCV)</td>
<td>2.30 ± 0.04</td>
<td>2.30 ± 0.04</td>
<td>0.92</td>
</tr>
</tbody>
</table>

a “s” indicates that the virus has the 36-bp scar sequence; boldface type denotes UL97 mutations.

b NA, not applicable; VGCV, valganciclovir; WT, wild type.
reconstitute infectious viruses, HFFs were transfected with the recombinant HCMV/BAC plasmids. Viral plaques were generally observed 7–10 days after transfection. DNA sequencing confirmed the presence of the scar sequence (4 bp before the start codon of the UL97 gene) and each specific UL97 mutation. Furthermore, the absence of unintended mutations in the UL97 and UL54 genes and in the regions where recombination events occurred was confirmed for each recombinant virus.

Since the UL97 gene plays a role in HCMV replication [1], the impact of the 36-bp scar sequence preceding the UL97 gene was investigated. First, microscopical inspection of infected HFFs revealed plaques of morphological structure similar to that of the original virus (RVHB5) and the recombinant virus with the scar sequence (RVHB5s). Replication kinetics of RVHB5 and RVHB5s in HFFs were also similar, with viral titers of 10.4 × 10^6 and 9.4 × 10^5 pfu on day 0, 13.4 × 10^4 and 16.5 × 10^4 pfu on day 5, and 18.4 × 10^4 and 16.5 × 10^4 pfu on day 8, respectively. In addition, ganciclovir IC_{50} values of RVHB5 (1.95 ± 0.19 μmol/L) and RVHB5s (2.05 ± 0.28 μmol/L) were almost identical (table 1). These data suggest that the 36-bp scar sequence preceding the UL97 gene in RVHB5s does not alter the known functions of this gene.

The 2 recombinant mutant viruses containing the ganciclovir-resistance UL97 mutation M460I exhibited IC_{50} values of 7.90 and 7.46 μmol/L, which are ∼4 times the IC_{50} of the susceptible RVHB5 virus (table 1). All recombinant viruses containing mutations of unknown significance in the UL97 gene had ganciclovir IC_{50} values equal to or less than that of the RVHB5 control (i.e., 78%–100% of the control value) (table 1). Thus, these UL97 polymorphic variants of HCMV were considered to be susceptible to ganciclovir.

Discussion. In the present study, a new system was designed to rapidly generate recombinant viruses with mutations in the HCMV UL97 gene. The role played by several UL97 mutations of unknown significance, which were found during a prospective study comparing the efficacy of valganciclovir with that of oral ganciclovir in preventing HCMV disease in SOT recipients [6], was then evaluated with this approach. Recombinant viruses containing UL97 mutations P405L, A427V, M550I, A582V, Y617H, and A674T were first generated in a bacterial system and were then reconstituted in fibroblasts, at which time their susceptibility to ganciclovir could be determined. In contrast to the M460I ganciclovir-resistant control, all mutants generated in this study were found to be susceptible to ganciclovir.

Marker-transfer studies within the HCMV genome have been performed for many UL97 mutations associated with resistance to ganciclovir [1]. The major advantage of our newly designed system, compared with earlier systems, is the easy manipulation of the large HCMV genome in bacteria, followed by rapid and efficient reconstitution of viral stocks in eukaryotic cells for further phenotypic testing. Recombinant viruses with >1 UL97 mutation can be generated in a homogeneous viral background, and their genotypes can be easily verified in bacteria before reconstitution of infectious viruses. Furthermore, the BAC technology eliminates the possibility of contamination with wild-type viruses when infectious mutant viruses are reconstituted in eukaryotic cells, thereby avoiding drug selection [11], plaque-purification steps [11, 12], and generation of additional unintended mutations. Improvement of our method can be made by incorporating a reporter gene [12] in the HCMV/BAC, to facilitate phenotypic evaluation. Although unlikely, the possibility of generating unintended mutations was minimized in our study by sequencing the mutated UL97 and UL54 genes as well as the regions where recombination events occurred and by testing the phenotypes of 2 independent recombinant viruses for each mutation.

The ganciclovir IC_{50} values of the known drug-resistant M460I mutants generated in our study were comparable to those previously reported by other groups, with an ∼4-fold increase in resistance levels compared with that of the wild-type virus [10, 13, 14]. In contrast, all other mutants tested in this study were found to be susceptible to ganciclovir. The mutations were located in regions of the UL97 gene not previously implicated in ganciclovir resistance [1], and they were not associated with an adverse clinical outcome [6]. Thus, these results confirm our previous hypothesis regarding their role as viral polymorphisms or natural variations, as reported for other UL97 mutations [15].

There are other potential applications associated with this newly designed system aimed at evaluating the role played by HCMV mutations. For instance, the role played by UL54 (DNA polymerase) mutations of unknown significance and by double UL97 plus UL54 mutations could be evaluated. In this case, some modifications of our present system would be required, including the deletion of the UL54 conserved regions and the use of different excisable selection markers for both UL97 and UL54. With such a system, a map of all mutations conferring resistance to ganciclovir could be elaborated, with the aim of developing virtual drug phenotypes similar to those described for HIV.

References
5. Limaye AP, Raghu G, Koelle DM, Ferrenberg J, Huang ML, Boechk


