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Mutations in the UL97 ORF of ganciclovir-resistant clinical cytomegalovirus isolates differentially affect GCV phosphorylation as determined in a recombinant vaccinia virus system

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Abstract

Mutations in the human cytomegalovirus (HCMV) UL97 phosphotransferase have been associated with ganciclovir (GCV) resistance due to an impairment of GCV monophosphorylation. Vaccinia virus recombinants (rVV) were generated that encoded different HCMV UL97 proteins (pUL97) with mutations previously detected in resistant HCMV clinical isolates at codons 460, 520, 592, 594, 595, 598 and 607. These rVVs allowed quantification of GCV phosphorylation catalyzed by the different mutated pUL97s. When compared to rVV-UL97 wild type, mean levels of residual intracellular GCV phosphorylation differed by a factor of 10 for the mutated UL97 proteins ranging from 5.2 to 51.8%. Mutations M460V (located in a UL97 region homologous to domain VIb of protein kinases) and H520Q (located in a cytomegalovirus-specific, functionally critical domain) were responsible for the lowest levels of residual GCV phosphorylation (9.3 and 5.2%). Mutations in a region homologous to the domain IX had a lower impact on GCV phosphorylation (15.8–51.8%). The relevance of pUL97 mutation G598S in inducing GCV resistance was demonstrated for the first time.

Keywords: Ganciclovir resistance; Cytomegalovirus; UL97; Recombinant vaccinia virus

1. Introduction

Ganciclovir (GCV) is a first-line drug for the therapy of human cytomegalovirus (HCMV) disease in immunocompromised patients. However, in the last decade the emergence of GCV-resistant

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HCMV strains during treatment has become a substantial problem in patients with AIDS (Drew et al., 1991; Jacobson et al., 1991; Stanat et al., 1991; Gerna et al., 1992; Lurain et al., 1994; Baldanti et al., 1995, 1996, 1998b; Boivin et al., 1996; Hanson et al., 1995; Chou et al., 1995a,b, 1997; Jabs et al., 2001; Wolf et al., 1995), in transplant recipients (Knox et al., 1991; Lurain et al., 1996; Rosen et al., 1997; Alain et al., 1997; Baldanti et al., 1998a; Limaye et al., 2000) and in other immunosuppressed patients (Erice et al., 1989; Wolf et al., 1998). At the moment, there is no consensus on the definition of GCV-resistance in clinical HCMV isolates. In fact, either GCV $IC_{50} > 6.0$ μM or an increase of > 3.0-fold in IC_{50} values with respect to HCMV strains from untreated immunocompromised patients have been associated with GCV resistance and lack of response to treatment (Erice, 1999). However, the different biological assays utilized for measurement of drug susceptibility still suffer from lack of standardization (Erice, 1999). In addition, determination of drug susceptibility is complicated by the potential presence of a mixed virus population in clinical HCMV isolates recovered from immunocompromised patients (Erice, 1999; Gerna et al., 1992; Baldanti et al., 1998a,b).

Mutations in the UL97-encoded phosphotransferase (pUL97) have been shown by marker transfer to play a key role in altering HCMV drug susceptibility via inhibition of intracellular GCV monophosphorylation (Sullivan et al., 1992; Lurain et al., 1994; Hanson et al., 1995; Chou et al., 1995a,b; Baldanti et al., 1995, 1998c). However, no systematic quantitative data are available on the influence of different mutations in UL97 proteins on GCV phosphorylation. Strikingly, all available data indicate that the pUL97 is not a nucleoside kinase (Michel et al., 1996, 1998; He et al., 1997) and sequence alignments as well as mutational analysis have shown that pUL97 contains functional regions homologous to conserved domains characteristic of protein kinases (Michel et al., 1998, 1999). To date, the biological function and the natural substrate of the UL97 phosphotransferase are unknown. It has been shown recently that the homolog of murine cytomegalovirus (MCMV) M97 differs significantly in several aspects from UL97 and is not responsible for the GCV-sensitive phenotype of MCMV (Wagner et al., 2000). However, the importance of both the UL97 and M97 function in virus replication is demonstrated by the dramatic growth impairment shown by recently obtained knock-out mutants (Prichard et al., 1999; Wagner et al., 2000) and also by the selective UL97 inhibition by the benzimidazole derivative 1263W94 (Davis et al., 1998). The importance of UL97 phosphotransferase for viral replication is further supported by the observation that drug-resistant clinical HCMV isolates either lacking the UL97 ORF or carrying extended deletions in the UL97 ORF or deletions interfering with the autophosphorylation of pUL97 have not been reported. In addition, a mathematical model has been developed showing a reduced in vivo fitness of GCV-resistant UL97 mutants in the absence of the drug (Emery and Griffiths, 2000). It is not clear so far if UL97 mutations conferring GCV resistance may interfere with an unknown important biological function of UL97.

The aim of this study was to quantify the level of GCV phosphorylation by UL97 proteins carrying mutations which have been detected in different putative UL97 domains of phenotypically resistant clinical isolates. This work was performed using recombinant vaccinia viruses (rVV) expressing the mutated pUL97 in order: (i) to better standardize the quantification of GCV phosphorylation, and (ii) to overcome the potential problem of mixed HCMV populations and of differential growth properties of different clinical HCMV isolates.

2. Materials and methods

2.1. UL97 mutations detected in GCV-resistant HCMV clinical isolates

A panel of ten mutations (M460V, M460I, H520Q, C592G, A594V, L595S, L595F, L595del, G598S, C607Y) was selected. The role of nine of them in determining GCV resistance has already been proven by marker transfer (Lurain, et al., 1994; Hanson et al., 1995; Chou et al., 1995a,b, 2000; Baldanti et al., 1995, 1998a). The remaining substitution G598S detected in a GCV-resistant

HCMV clinical isolate (Baldanti et al., 1998b) but not confirmed by marker transfer was also included in the study. In detail, UL97 mutations C592G, A594V, L595F, L595del, G598S and C607Y, were detected in VR4991, VR4955, VR5438, VR3480, VR5406, and VR4990 isolates recovered in Pavia, Italy. GCV susceptibility of Pavia isolates was determined by an immediate-early plaque reduction assay (Gerna et al., 1992) which documented GCV ID_{50} values in the range of 18–50 μM corresponding to an increase of 3–8-fold in IC_{50} values with respect to HCMV strains from treatment-naive immunocompromised patients (Gerna et al., 1992; Baldanti et al., 1995, 1996, 1998a,b,c). Similar GCV resistance levels were reported to be associated with three additional UL97 mutations (M460V, M460I and L595S) investigated in this study (Lurain et al., 1994; Chou et al., 1995a,b). The only exception was represented by the clinical isolate carrying the H520Q mutation with a GCV IC_{50} of >200 μM which was explained by the presence of an additional mutation in the DNA polymerase (Hanson et al., 1995).

2.2. HCMV UL97 cloning and expression in the vaccinia virus system

The primer set pri.5-*Kpn*I and pri.6-*Eco*RI (Metzger et al., 1994) was used to amplify UL97 gene fragments of 626 bp (nt 1498–nt 2124) from GCV-resistant clinical HCMV isolates VR4991, VR4955, VR5438, VR3480, VR5406, VR4990. The UL97 fragment carrying the H520Q substitution was amplified from the HCMV isolate CMV-6 (kindly provided by A. Erice), as previously described (Michel et al., 1996). Amplified UL97 fragments were inserted into the p7.5K-UL97 vector by substitution of the wild type UL97 *Kpn*I–*Eco*RI or SalI–*Kpn*I fragments, respectively, as previously reported (Metzger et al., 1994). In addition, M460V, M460I, and L595S substitutions were introduced in the p7.5K-UL97 vector by site-directed mutagenesis, as described previously (Landt et al., 1990; Metzger et al., 1994; Michel et al., 1998) using the mutagenized primers: 5′-ATTACACCCGTGAACGTGC-3′, 5′-ATTACACCCATCAACGTGCT-3′ and 5′-TTCTCCGACGCGCGGCA-3′. After cloning the UL97 gene fragments in p7.5K-UL97, the entire gene was sequenced to verify that no additional changes had been introduced during the amplification or cloning steps.

The expression of the different pUL97 in CV1 cells was verified by Western blot analysis using a polyclonal antiserum specific for the UL97 phosphotransferase (Michel et al., 1996) and the enhanced chemiluminescent technique (ECL, Amersham, Braunschweig, Germany). In addition, the correct nuclear localization of the pUL97 was verified by immunological staining of shell vial cultures of 143B (tk−) cells infected with the indicated vaccinia recombinants using the same antiserum and the immunofluorescence technique (Michel et al., 1996).

Some previously reported rVV were used as controls: (i) rVV-UL97 expressing the wild type HCMV UL97; (ii) rVV-AACR590–593del expressing the pUL97 carrying the AACR590–593 deletion; (iii) rVV-234, whose pUL97 lacking 66 amino acids (amino acids 305–365) is transported to the nucleus, but is neither phosphorylated itself nor gives rise to phosphorylated GCV (Metzger, et al., 1994; Michel et al., 1996, 1998).

2.3. GCV phosphorylation activity and phosphorylation of pUL97 in cells infected by wild type and mutated UL97 vaccinia recombinants

GCV phosphorylation activity was analyzed in infected 143B (tk−) cells by HPLC as reported, whereby fractions containing all GCV phosphates (mono-, di-, and triphosphates) and the unphosphorylated compound were collected according to their retention times as determined by external standard runs (Michel et al., 1996; Zimmermann et al., 1997). In order to standardize the quantitative determination of GCV phosphorylation capacity by wild type and mutated pUL97 in the heterologous vaccinia virus system, the correlation between different multiplicities of infection (m.o.i.), the amount of protein expressed and GCV phosphorylation was analyzed. In detail, 143B cells were infected with rVV-UL97 wild type, rVV-M460I, rVV-L595F or rVV-G598S, us-

ing m.o.i. between 0.5 and 40 in at least three separate experiments. Twenty-four hours post-infection (p.i.), Western blot analysis with crude cell extracts and quantification of GCV phosphorylation was performed from the same samples in parallel. The expressed UL97 proteins were visualized by autoradiography after immunostaining with the specific UL97 antiserum. Signal intensity of the 80 kDa bands representing the expressed UL97 protein were quantified by densitometry. GCV phosphorylation activity was analyzed by HPLC as reported earlier.

If not stated otherwise, all further experiments were performed after infecting cells with the indicated rVV at an m.o.i. of 5–10. In addition, for each rVV, GCV phosphorylation assay experiments were replicated 7–18 times and median values were calculated. In each experiment, GCV phosphorylation mediated by the rVV-UL97 wild type was set at 100%, while GCV phosphorylation mediated by rVVs carrying mutated HCMV UL97 was expressed as a fraction of UL97 wild type. Finally, the low background counts of reference VV Copenhagen, which was very similar to that in mock-infected cells, were subtracted in each experiment.

The pUL97 phosphorylation was determined in the nuclear matrix fraction of CV1 cells following a reported protein kinase assay (He et al., 1997).

2.4. Statistical analysis

GCV phosphorylation and pUL97 phosphorylation data by wild type and mutated UL97 phosphotransferase were evaluated by the Wilcoxon 2-sample test.

3. Results

3.1. Generation of rVV carrying the different mutated UL97 coding regions and expression of the UL97 proteins

As described above in Section 2, ten recombinant vaccinia viruses were generated which contained different HCMV UL97 ORFs carrying mutations originally detected in GCV-resistant clinical isolates. Two separate recombinant vaccinia virus clones were plaque-purified for each UL97 mutant after recombination. Sequence analysis confirmed the presence of the mutated UL97 sequences in the respective vaccinia virus vectors pK7.5-UL97 in the absence of additional mutations.

Using our polyclonal UL97 antiserum, a clear spotty nuclear signal was detected by indirect immunofluorescence in 143B cells at 17–24 h after infection with rVV-UL97 wild type and all rVVs expressing mutated pUL97 derived from GCV-resistant clinical isolates. No specific staining was found in cells which were mock-infected or infected with the VV Copenhagen strain (data not shown). Western blot analysis demonstrated comparable expression of UL97 proteins (pUL97) in 143B cells infected with all rVV expressing mutated UL97 ORFs (Fig. 1). Furthermore, nuclear matrix extracts from cells infected with the rVV carrying wild type or mutated UL97 ORFs were analyzed for phosphorylation of pUL97 itself, as published previously (Michel et al., 1998). Again, none of the mutations found in clinical HCMV isolates qualitatively inhibited the pUL97 phosphorylation in the vaccinia system (data not shown). The 143B cells infected with VV Copenhagen strain (lacking UL97) as well as with our

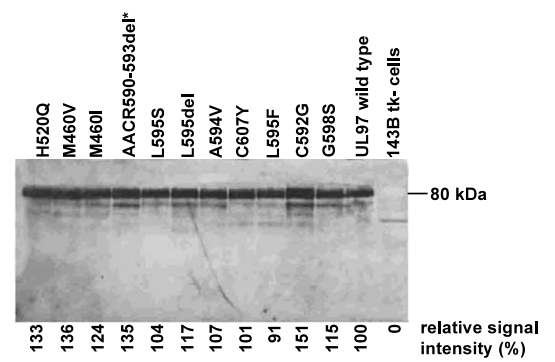

Fig. 1. Western blot analysis of pUL97 expressed in 143B cells after infection with the different rVVs. The figures on top of the lanes indicate the mutated amino acid position; letters indicate amino acids; del, deletion. The figures at the bottom of the lanes indicate relative signal intensity of the 80 kDa bands quantified by a phosphoimager (% of UL97 wild type). *, deletion AACR590–593 was first reported in an in vitro selected laboratory strain (Sullivan et al., 1992).

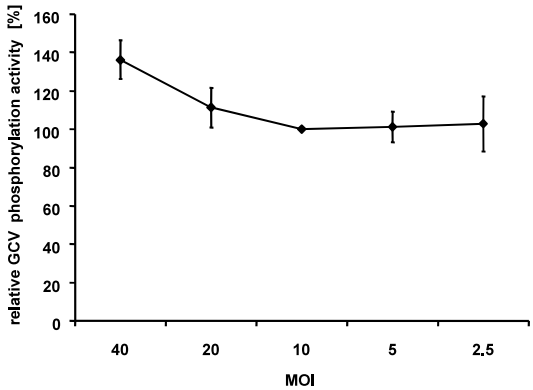

Fig. 2. GCV phosphorylation in 143B (tk −) cells after infection with recombinant vaccinia viruses at different m.o.i. Each point represents the mean value, while vertical bars indicate the standard error values from four independent experiments using four different rVVs (rVV-UL97 wild type, rVV-M460I, rVV-G598S, and rVV-L595F). Infections were performed with the indicated m.o.i. The GCV phosphorylation activity using an m.o.i. of 10 was set at 100%.

negative control for pUL97 phosphorylation (rVV-234) exhibited no phosphorylated pUL97 band (data not shown)

3.2. Standardization of the GCV phosphorylation assay using rVV expressing wild type and mutated HCMV UL97

Quantitative Western blot values given in Fig. 1 show that the amount of expressed protein was comparable for all rVVs. In fact, the mutated proteins were expressed between 91 and 151% as compared to pUL97 wild type (100%). To rule out an hypothetical relevant influence of inevitable minor inter test variations in the multiplicities of infection (m.o.i.) used, we analyzed in more detail the correlation between different m.o.i. and GCV phosphorylation using four of the rVV (rVV-UL97 wild type, rVV-M460I, rVV-L595F, rVV-G598S). In Fig. 2 the cumulative result of all experiments is presented. The GCV phosphorylation was not significantly affected when using an m.o.i. between 2.5 and 10. In contrast, a significant decrease in pUL97 expression and a higher variability of the GCV phosphorylation results were observed when using an m.o.i. of 0.5 and lower (data not shown). With further increase of the m.o.i. to 20 and 40 an increase in GCV phosphorylation could be observed. Thus, GCV phosphorylation by rVVs carrying mutated UL97 proteins was evaluated including experiments where the m.o.i. was between 5 and 10.

3.3. Phosphorylation of GCV in cells infected by rVVs expressing different pUL97

The rVV-A5 carrying the AACR deletion derived from an HCMV strain which had originally been selected in laboratory, and all the rVVs expressing UL97 genes with single mutations derived from clinical isolates consistently showed impaired, yet never completely abolished GCV phosphorylation (Fig. 3). The H520Q substitution resulted in the lowest mean value of residual GCV phosphorylation (5.2%), while the maximum residual mean phosphorylation value detected with the mutation G598S was ten times higher

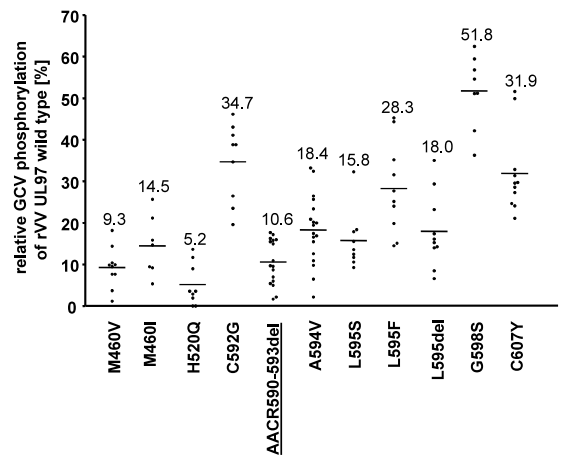

Fig. 3. Quantitative GCV phosphorylation in 143B tk − cells infected with different rVV expressing the mutated pUL97. Each dot represents a GCV phosphorylation (mono-, di-, and triphosphates) value obtained in a single experiment performed by infecting cells in duplicate with the indicated rVV (rVV-M460V to rVV-C607Y) at an m.o.i. of 5–10. The values are given as percent of UL97 wild type. The mean value for all experiments and each rVV is shown. The figures on the *x*-axis indicate the mutated amino acid position; letters indicate amino acids; del, deletion. The deletion AACR590–593 (underlined) was first reported in an in vitro selected laboratory strain (Sullivan et al., 1992).

(51.8%). Both of these pUL97 mutants showed mean residual GCV phosphorylation levels which were statistically different from all other values ($P < 0.05$). Arbitrarily, three groups of mutants could be defined: (i) pUL97 carrying the mutations M460V and H520Q exhibited a very low mean residual GCV phosphorylation (< 10%); (ii) pUL97 carrying M460I, A594V, L595S, L595del showed a mean residual GCV phosphorylation between 10 and 20%; and (iii) pUL97 with C592G, L595F, G598S, C607Y mutations exhibited a mean GCV phosphorylation in the range of 30–50%. Interestingly, in two cases, different mutations at the same codon (M460V vs. M460I, and L595del and L595S vs. L595F) induced significantly different mean values of inhibition of GCV phosphorylation. It has to be stressed that there was no correlation between the comparable amounts of expressed UL97 proteins (Fig. 1) and the respective mean values of GCV phosphorylation.

4. Discussion

The mechanism of GCV resistance induced by several reported mutations in the UL97 ORF of HCMV is the impairment of the UL97 protein in phosphorylating GCV to its monophosphate (Stanat et al., 1991; Littler et al., 1992; Sullivan et al., 1992; Lurain et al., 1994; Hanson et al., 1995; Chou et al., 1995a,b; Baldanti et al., 1995, 1996, 1998b,c). We used the rVV system to express pUL97 from HCMV wild type and from several GCV-resistant clinical isolates carrying mutations in different pUL97 regions. These regions of UL97 ORF are homologous to different domains of protein kinases (Michel et al., 1999; Chee et al., 1989). Our aim was to analyze the quantitative impact of each UL97 mutant on the phosphorylation of GCV.

The vaccinia virus system exhibits several advantages: (i) recombinant vaccinia virus strains can be generated easier than HCMV recombinants and UL97 proteins can be expressed to high amounts; (ii) after cloning single UL97 genes in the vaccinia virus vector, the biochemical activity of mutated pUL97 in infected 143B cells is not influenced by the presence of mixed HCMV populations; and (iii) standardization of a quantitative assay for testing GCV phosphorylation is independent of other biological properties (e.g. replication rate) of the original HCMV isolates, since rVVs can be used at high titers and quantification of infectious virus is easily achieved and highly reproducible.

In this study, rVVs carrying the different UL97 mutations consistently showed impaired GCV phosphorylation as compared to rVV expressing the wild type HCMV UL97 and significant differences between the GCV phosphorylation levels induced by individual UL97 mutants could be observed. In our system, the mutation H520Q was the most efficient in inhibiting GCV phosphorylation. Mutation M460V (homologous to domain VIb of protein kinases) similarly exhibited a very low mean residual GCV phosphorylation level (< 10%). All other mutations reported from GCV-resistant HCMV strains are clustered between codons 590 and 607, a region homologous to domains IX and X of protein kinases (Chee et al., 1989). Mutations in this part of the protein can be separated into two groups with respect to GCV phosphorylation: (i) mutations A594V, L595S, L595del showed a quite low residual GCV phosphorylation (10–20%) as compared to pUL97 wild type; (ii) substitutions C592G, L595F, G598S, C607Y showed much higher mean residual GCV phosphorylation levels (30–50%). In particular, mutation G598S was detected in a GCV-resistant clinical isolate from an AIDS patient after treatment failure (Baldanti et al., 1998b), which showed reduced levels of phosphorylated GCV in human fibroblast cultures (Baldanti et al., 1998b). However, so far, repeated attempts failed to rescue recombinant HCMV strains under GCV selection after homologous recombination of UL97 fragments carrying the G598S mutation into the AD169 genetic background. The relevance of this mutation in reducing intracellular GCV anabolism has now been verified in the heterologous vaccinia virus system, confirming its role in conferring a moderate degree of GCV resistance. In addition, the difficulty in selecting the recombinant HCMV by GCV selection may be explained by the relatively high

residual GCV phosphorylation by this mutant, which is about ten-fold higher than that of the most inhibitory mutation H520Q.

It has been shown that pUL97 is important for viral replication and that the still unknown crucial biological function of pUL97 is not strictly related to GCV phosphorylation (Michel et al., 1996; He et al., 1997). Our results confirm previous data that a complete loss in the phosphorylation of pUL97 itself was never observed in cell cultures infected with rVV expressing the wild type or the pUL97 carrying mutations associated with GCV resistance of clinical strains (Michel et al., 1999). This led to the conclusion that pUL97 phosphorylation might be associated with the biological function of UL97 (Wagner et al., 2000; Michel et al., 1999; Prichard et al., 1999). In addition, it has been shown that the phosphorylation of the UL97 protein itself is also a prerequisite for the GCV phosphorylation (Michel et al., 1999). However, at the moment we are neither able to quantify autophosphorylation of pUL97, nor can we exclude that the different mutations in pUL97 additionally lead to different degrees of impairment of the biological function of pUL97. Other investigators have shown, by applying a mathematical model, a reduced in vivo fitness of GCV-resistant UL97 mutants in the absence of the drug. They have also shown a different degree of fitness loss for different UL97 mutations (Emery and Griffiths, 2000). The mechanisms responsible for the reported reduction of the in vivo replication of GCV-resistant HCMV isolates as well as a possible correlation with different mutations again rely on the biological function of pUL97 and remain to be elucidated.

Although in view of the low standardization of the biological assays, it is not possible to strictly correlate the level of GCV resistance reported for the respective HCMV isolates with the reduction in GCV phosphorylation as measured in the rVV system, it is remarkable that mutations M460V, M460I, A594V and L595S, which highly impair GCV phosphorylation, are the most frequently detected in GCV-resistant clinical HCMV strains (Chou et al., 1995a,b; Jabs et al., 2001). The M460V mutation could be selected also in vitro quite easily by GCV and acyclovir resulting in highly resistant HCMV strains (Michel et al., 2001). On the other hand, the mutation H520Q which maximally reduces GCV phosphorylation occurs less frequently than mutations at positions 460 and 594 or 595. As we have reported earlier, H520 is part of a sequence (YHPAF 519–523) in a putative domain which is highly conserved exclusively among cytomegaloviruses and is located between domains VII and VIII of other protein kinases where no homologous region does exist (Michel et al., 1999). The critical relevance of this motif was proven by the fact that a F523C mutation generated in our laboratory resulted in a complete loss not only of GCV phosphorylation but also of pUL97 phosphorylation (Michel et al., 1999).

In conclusion, the reduction of GCV anabolism by mutations in different UL97 domains present in GCV-resistant HCMV clinical isolates was measured in a recombinant vaccinia virus system showing that the system is a valid technique for quantification of the GCV phosphorylating capacity of mutated UL97 ORF from phenotypically resistant HCMV strains. The residual capacity of GCV monophosphorylation by the different pUL97 mutants may vary even by a factor of 10. In addition, our data support the relevance of an additional UL97 mutation (G598S) for GCV resistance. The data show that mutations which are most frequently selected in treated patients reduce GCV phosphorylation to a greater extent.

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References

Alain, S., Honderlick, P., Grenet, D., Stern, M., Vadam, C., Sanson-Le Pors, M.J., Mazeron, M.C., 1997. Failure of ganciclovir treatment associated with selection of a ganciclovir-resistant cytomegalovirus strain in a lung transplant recipient. Transplantation 63, 1533–1536.

Baldanti, F., Silini, E., Sarasini, A., Talarico, C.L., Stanat, S.C., Biron, K.K., Furione, M., Bono, F., Palu, G., Gerna, G., 1995. A three-nucleotide deletion in the UL97 open reading frame is responsible for the ganciclovir resistance of a human cytomegalovirus clinical isolate. J. Virol. 69, 796–800.

Baldanti, F., Simoncini, L., Sarasini, A., Zavattoni, M., Grossi, P., Revello, M.G., Gerna, G., 1998a. Ganciclovir resistance as a result of oral ganciclovir in a heart transplant recipient with multiple human cytomegalovirus strains in blood. Transplantation 66, 324–329.

Baldanti, F., Simoncini, L., Talarico, C.L., Sarasini, A., Biron, K.K., Gerna, G., 1998b. Emergence of a ganciclovir-resistant human cytomegalovirus strain with a new UL97 mutation in an AIDS patient. AIDS 12, 815–818.

Baldanti, F., Underwood, M., Talarico, C.L., Simoncini, L., Sarasini, A., Biron, K.K., Gerna, G., 1998c. The Cys607 → Tyr change in the UL97 phosphotransferase confers ganciclovir resistance to two human cytomegalovirus strains recovered from two immunocompromised patients. Antimicrob. Agents Chemother. 42, 444–446.

Baldanti, F., Underwood, M.R., Stanat, S.C., Biron, K.K., Chou, S., Sarasini, A., Silini, E., Gerna, G., 1996. Single amino acid changes in the DNA polymerase confer foscarnet resistance and slow-growth phenotype, while mutations in the UL97-encoded phosphotransferase confer ganciclovir resistance in three double-resistant human cytomegalovirus strains recovered from patients with AIDS. J. Virol. 70, 1390–1395.

Boivin, G., Chou, S., Quirk, M.R., Erice, A., Jordan, M.C., 1996. Detection of ganciclovir resistance mutations and quantitation of cytomegalovirus (CMV) DNA in leukocytes of patients with fatal disseminated CMV disease. J. Infect. Dis. 173, 523–528.

Chee, M.S., Lawrence, G.L., Barrell, B.G., 1989. Alpha-, beta- and gammaherpesviruses encode a putative phosphotransferase. J. Gen. Virol. 70, 1151–1160.

Chou, S., Erice, A., Jordan, M.C., Vercellotti, G.M., Michels, K.R., Talarico, C.L., Stanat, S.C., Biron, K.K., 1995a. Analysis of the UL97 phosphotransferase coding sequence in clinical cytomegalovirus isolates and identification of mutations conferring ganciclovir resistance. J. Infect. Dis. 171, 576–583.

Chou, S., Guentzel, S., Michels, K.R., Miner, R.C., Drew, W.L., 1995b. Frequency of UL97 phosphotransferase mutations related to ganciclovir resistance in clinical cytomegalovirus isolates. J. Infect. Dis. 172, 239–242.

Chou, S., Marousek, G., Guentzel, S., Follansbee, S.E., Poscher, M.E., Lalezari, J.P., Miner, R.C., Drew, W.L., 1997. Evolution of mutations conferring multidrug resistance during prophylaxis and therapy for cytomegalovirus disease. J. Infect. Dis. 176, 786–789.

Chou, S., Meichsner, C., Sisson, B., Michels, K., 2000. Human cytomegalovirus (CMV) strains with unique restriction sites for transfer of drug resistance mutations. Twenty-fifth International Herpes Virus Workshop. Portland, Oregon July 29–August 4 2000. Abstract 8.17.

Davis, M.G., Talarico, C.L., Underwood, M.R., Baldanti, F., Biron, K.K., 1998. Mapping of a mutation in human cytomegalovirus resistant to 1263W94. Third International Herpesvirus Workshop. York, UK, 1–7 August 1998. Abstract no. 314.

Drew, W.L., Miner, R.C., Busch, D.F., Follansbee, S.E., Gullett, J., Mehalko, S.G., Gordon, S.M., Owen, W.F. Jr., Matthews, T.R., Buhles, W.C., DeArmond, B., 1991. Prevalence of resistance in patients receiving ganciclovir for serious cytomegalovirus infection. J. Infect. Dis. 163, 716–719.

Emery, V.C., Griffiths, P.D., 2000. Prediction of cytomegalovirus load and resistance patterns after antiviral chemotherapy. Proc. Natl. Acad. Sci. USA 97, 8039–8044.

Erice, A., 1999. Resistance of human cytomegalovirus to antiviral drugs. Clin. Microbiol. Rev. 12, 286–297.

Erice, A., Chou, S., Biron, K.K., Stanat, S.C., Balfour, H.H. Jr., Jordan, M.C., 1989. Progressive disease due to ganciclovir-resistant cytomegalovirus in immunocompromised patients. N. Engl. J. Med. 320, 289–293.

Gerna, G., Baldanti, F., Zavattoni, M., Sarasini, A., Percivalle, E., Revello, M.G., 1992. Monitoring of ganciclovir sensitivity of multiple cytomegalovirus strains coinfecting blood of an AIDS patient by an immediate-early antigen plaque assay. Antiviral Res. 19, 333–345.

Hanson, M., Preheim, L.C., Chou, S., Talarico, C.L., Biron, K.K., Erice, A., 1995. Novel mutation in the UL97 gene of a clinical cytomegalovirus strain conferring resistance to ganciclovir. Antimicrob. Agents Chemother. 39, 1204–1205.

He, Z., He, Y., Kim, Y., Chu, L., Ohmstede, C., Biron, K.K., Coen, D.M., 1997. The human cytomegalovirus UL97 protein is a protein kinase that autophosphorylates on serines and threonines. J. Virol. 71, 405–411.

Jabs, D.A., Martin, B.K., Forman, M.S., Dunn, J.P., Davis, J.L., Weinberg, D.W., Biron, K.K., Baldanti, F., 2001. Mutations conferring ganciclovir resistance in a cohort of patients with acquired immunodeficiency syndrome and cytomegalovirus retinitis. J. Infect. Dis. 183, 333–337.

Jacobson, M.A., Drew, W.L., Feinberg, J., O'Donnell, J.J., Whitmore, P.V., Miner, R.D., Parenti, D., 1991. Foscarnet therapy for ganciclovir-resistant cytomegalovirus retinitis in patients with AIDS. J. Infect. Dis. 163, 1348–1351.

Knox, K.K., Drobyski, W.R., Carrigan, D.R., 1991. Cytomegalovirus isolate resistant to ganciclovir and foscarnet from a marrow transplant patient. Lancet 337, 1292–1293.

Landt, O., Grunert, H.-P., Hahn, U., 1990. A general method for site-directed mutagenesis using the polymerase chain reaction. Gene 96, 125–128.

Limaye, A.P., Corey, L, Koelle, D.M., Davis, C.L., Boeckh, M., 2000. Emergence of ganciclovir-resistant cytomegalovirus disease among recipients of solid-organ transplants. Lancet 356, 645–649.

Littler, E., Stuart, A.D., Chee, M.S., 1992. Human cytomegalovirus UL97 open reading frame encodes a protein that phosphorylates the antiviral nucleoside analog ganciclovir. Nature 358, 160–162.

Lurain, N.S., Spafford, L.E., Thompson, K.D., 1994. Mutation in the UL97 open reading frame of human cytomegalovirus strains resistant to ganciclovir. J. Virol. 68, 4427–4431.

Lurain, N.S., Ammons, H.C., Kapell, K.S., Yeldandi, V.V., Garrity, E.R., O'Keefe, J.P., 1996. Molecular analysis of human cytomegalovirus strains from two lung transplant recipients with the same donor. Transplantation 62, 497–502.

Metzger, C., Michel, D., Schneider, K., Lüske, A., Schlicht, H.-J., Mertens, Th., 1994. Human cytomegalovirus UL97 kinase confers ganciclovir susceptibility to recombinant vaccinia virus. J. Virol. 68, 8423–8427.

Michel, D., Pavic, I., Zimmermann, A., Haupt, E., Wunderlich, K., Heuschmid, M., Mertens, Th., 1996. The UL97 gene product of the human cytomegalovirus is an early–late protein with a nuclear localization but is not a nucleoside kinase. J. Virol. 70, 6340–6347.

Michel, D., Schaarschmidt, P., Wunderlich, K., Heuschmid, M., Simoncini, L., Mühlberger, D., Zimmermann, A., Pavic, I., Mertens, Th., 1998. Functional regions of the human cytomegalovirus protein pUL97 involved in nuclear localization and phosphorylation of ganciclovir and pUL97 itself. J. Gen. Virol. 79, 2105–2112.

Michel, D., Kramer, S., Höhn, S., Schaarschmidt, P., Wunderlich, K., Mertens, Th., 1999. Amino acids of conserved kinase motifs of the cytomegalovirus protein UL97 are essential for autophosphorylation. J. Virol. 73, 8898–8901.

Michel, D., Höhn, S., Haller, T., Jun, D., Mertens, Th., 2001. Aciclovir selects for ganciclovir-cross-resistance of human cytomegalovirus in vitro which is only in part explained by known mutations in the UL97 protein. J. Med. Virol. 65, 70–76.

Prichard, M.N., Gao, N., Jairath, S., Mulambia, G., Krosky, P., Coen, D.M., Parker, B.O., Pari, G.S., 1999. A recombinant human cytomegalovirus with a large deletion in UL97 has a severe replication deficiency. J. Virol. 73, 5663–5670.

Rosen, H.R., Benner, K.G., Flora, K.D., Rabkin, J.M., Orloff, S.L., Olyaei, A., Chou, S., 1997. Development of ganciclovir resistance during treatment of primary cytomegalovirus infection after liver transplantation. Transplantation 63, 476–478.

Stanat, S.C., Reardon, J.E., Erice, A., Jordan, C., Drew, W.L., Biron, K.K., 1991. Ganciclovir-resistant cytomegalovirus clinical isolates: mode of resistance to ganciclovir. Antimicrob. Agents Chemother. 35, 2191–2197.

Sullivan, V., Talarico, C.L., Stanat, S.C., Davis, M., Coen, D.M., Biron, K.K., 1992. A protein kinase homologue controls phosphorylation of ganciclovir in human cytomegalovirus-infected cells. Nature 358, 162–164.

Wagner, M., Michel, D., Schaarschmidt, P., Vaida, B., Jonjic, S., Messerle, M., Mertens, T., Koszinowski, U., 2000. Comparison between human cytomegalovirus pUL97 and murine cytomegalovirus (MCMV) pM97 expressed by MCMV and vaccinia virus: pM97 does not confer ganciclovir sensitivity. J. Virol. 74, 10729–10736.

Wolf, D.G., Smith, I.L., Lee, D.J., Freeman, W.R., Flores-Aguilar, M., Spector, S.A., 1995. Mutations in human cytomegalovirus UL97 gene confer clinical resistance to ganciclovir and can be detected directly in patient plasma. J. Clin. Invest. 95, 257–263.

Wolf, D.G., Yaniv, I., Honigman, A., Kassis, I., Schonfeld, T., Ashkenazi, S., 1998. Early emergence of ganciclovir-resistant human cytomegalovirus strains in children with primary combined immunodeficiency. J. Infect. Dis. 178, 535–538.

Zimmermann, A., Michel, D., Pavic, I., Hampl, W., Lüske, A., Neyts, J., De Clercq, E., Mertens, T., 1997. Phosphorylation of acyclovir, ganciclovir, penciclovir and S2242 by the cytomegalovirus UL97 protein: a quantitative analysis using recombinant vaccinia viruses. Antiviral Res. 36, 35–42.