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In addition to the genetic hypervariability of the HSV-I TK gene, ACV resistance is mostly associated with specific mutations in the drug-targeted viral TK protein [31]. The current study demonstrated that for each participant the sequential HSV-I strains shed were identical within and between oral HSV-I shedding episodes and expressed an overall individual-unique genotype. The data are in line with a recent study demonstrating that paired trigeminal ganglia are latently infected with a person-specific HSV-I strain [11]. Recurrent symptomatic ACV^R HSV-I infections have been described in ocular infections demonstrating that ACV^R HSV-I can reactivate from latency [6]. Four of 14 (28.6%) participants shed HSV-I strains with ACV^R-associated TK protein variants, including 2 patients on two different episodes, suggesting that the inferred ACV^R HSV-I strains have established latency and reactivated from latency [6, 11]. Recurrent therapy-induced ACV^R HSV-I have been described in immunocompromised individuals, illustrating the importance of local immunity in viral clearance from infected mucosal tissues [50-53]. Due to the low prevalence of ACV^R HSV-I in the population [54], the participants likely developed ACV^R HSV-I during the course of ACV treatment prior to study entry. Alternatively, ACV^R HSV-I could arise locally as the result of natural variation. HSV-I TK genotyping can be an additional diagnostic tool to determine the anti-viral sensitivity of clinical samples [6, 30]. Rationalized selection of the appropriate antiviral agents may prevent the development of severe herpetic disease in immunocompromised patients, including individuals infected with HIV.

The limitations of the current study are the relatively small sample size with male predominance and the relatively long swabbing interval. Assessment of herpesvirus shedding in the oral cavity in persons infected with HIV can be improved by a dense time interval of mucosal sampling in addition to a detailed description of clinical symptoms (e.g., cold or flu-like symptoms) and dental procedures that may be associated with herpesvirus reactivation. Moreover, a next generation sequencing approach would allow monitoring of drug-resistant minority virus variants present in the isolate.

In summary, the current study demonstrated that short episodes of oral HSV-I reactivations occur frequently in individuals infected with HIV. Within a daily swabbing interval, HSV-I shedding episodes were detected with a median of 2 days. HSV-I TK genotyping demonstrated that each individual sheds a unique HSV-I strain among episodes, which can express ACV^R-associated mutations. VZV shedding was detected in two of 22 participants at very low copy numbers, demonstrating the low incidence of VZV shedding even in immunocompromised individuals. Future studies should address the mechanism underlying the different shedding kinetics between these two closely related human alpha-herpesviruses.

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CHAPTER 5

Latent acyclovir-resistant herpes simplex virus type 1 in trigeminal ganglia of immunocompetent individuals

Monique van Velzen, Freek B. van Loenen, Roland J.W. Meesters,
Miranda de Graaf, Lies Remeijer, Theo M. Luider,
Albert D.M.E. Osterhaus, Georges M.G.M. Verjans

ABSTRACT

Specific mutations within the hypervariable herpes simplex virus (HSV) gene thymidine kinase (TK) gene lead to acyclovir (ACV) resistance. To uncover the existence of latent ACV-resistant (ACV^R) HSV-I, we determined the genetic and functional variability of the HSV-I TK gene pool in paired trigeminal ganglia (TG) of 5 immunocompetent individuals. The latent virus pool consisted of a donor-specific HSV-I quasispecies, including one major ACV-sensitive (ACV^S) and multiple phylogenetic-related minor ACV^S and ACV^R TK variants. Contrary to minor variants, major TK variants were shared between paired TG. The data demonstrate the coexistence of phylogenetic-related ACV^S and ACV^R latent HSV-I in human TG.

Introduction

Herpes simplex virus type 1 (HSV-1) is a highly prevalent human pathogen that causes a variety of diseases, including the potentially sight-threatening ocular disease herpetic keratitis [1]. HSV-1 establishes a lifelong latent infection in sensory neurons that innervate the anatomic site of primary infection and reactivates intermittently to cause recurrent lesions [1]. The trigeminal ganglion (TG) is a major site of HSV-1 latency. Recurrent HSV-1 infections are successfully treated with the nucleoside analogue acyclovir (ACV), in part due to the prerequisite conversion of the prodrug ACV to ACV monophosphate (ACVmp) by HSV-1 thymidine kinase (TK) [2].

Compared with other HSV-1 genes, the TK gene is highly polymorphic, and mutations within specific locations of the gene result in a functionally deficient TK protein or altered substrate specificity leading to ACV resistance [2–4]. Despite the extensive use of ACV for >3 decades, the incidence of ACV resistance is relatively low in the general population (0.1%–0.7%) but is more common in the immunocompromised (4%–14%) and in corneas of patients with herpetic keratitis (6.4%) [5]. Although TK is nonessential for viral replication *in vitro*, reactivation of latent TK-deficient HSV-1 is impaired in mice [6]. An analogous reactivation deficiency is assumed in humans. By contrasting, we have recently shown that genetically identical ACV^R HSV-1 strains can be reisolated years later from the same cornea of patients with recurrent herpetic keratitis, suggesting that ACV^R HSV-1 establishes latency and reactivates from the innervating TG [7]. The aim of the current study was to determine the prevalence of ACV^R HSV-1 in latently infected human TG. Therefore, we analyzed the genetic and functional variability of the latent HSV-1 TK gene pool in the left and right TG of 5 immunocompetent individuals.

Materials and Methods

Clinical specimens

The left and right TG from 5 immunocompetent individuals (median age, 82 years; range, 74–93 years), 2 female and 3 male, were obtained at autopsy with a median postmortem interval of 5:15 hours (range, 5:05 to 7:30 hours). All donors had a neurologic disease history affecting the central nervous system (mainly Alzheimer's and Parkinson's disease) and the cause of death was not related to herpesvirus infections. The TG specimens were collected by the Netherlands Brain Bank (Netherlands Institute for Neuroscience, Amsterdam) from donors from whom a written informed consent for brain autopsy and the use of the material and clinical information for research purposes had been obtained. All study procedures were performed in compliance with relevant laws and institutional guidelines and in accordance with the ethical standards of the Declaration of Helsinki.

HSV-1 TK sequence analyses

The amplification of the whole HSV-1 TK gene (1128 bp) from DNA extracted from fragmented TG was performed as described elsewhere [3, 7]. Agarose gel-purified TK amplicons were ligated into the TOPO-TA cloning vector (Invitrogen) and used to transform DH5 α bacteria (Invitrogen). Per transformation up to 48 recombinant bacteria co-

lonies were picked to sequence the TK insert using primers, located at the 5' and 3' end of the vector's multiple cloning site, provided by the TOPO-TA cloning kit (Invitrogen). The TK sequences were aligned to the consensus TK sequence of the reference HSV-I strain H129 (GenBank accession No. GU_734772). A maximum likelihood phylogenetic tree of TG-derived TK nucleotide sequences was estimated under the general time reversible model using PhyML 3.0 software. The HSV-I TK sequences obtained were deposited in the GenBank database under accession numbers HQ667768–HQ667782 and HQ685966–HQ686038.

HSV-I TK functional assay

Representative HSV-I TK variants were cloned into the pcDNA3 expression vector (Invitrogen) and transfected into Cos-7 cells (American Tissue Culture Collection; CRL-1651) using the FuGene-6 reagent according to the manufacturer's instruction (Roche). After 48 hours of incubation at 37°C, transfected cells were stained intracellularly with goat antiserum direct against HSV-I TK (sc-28037) and an isotype control (sc-2028), followed by allophycocyanin-conjugated donkey anti-goat serum (sc-3860) according to the manufacturer's instructions (all Santa Cruz Biotechnology). Fluorescence was detected on a FACS Canto II and analyzed using FACS Diva software (BD Biosciences).

The surplus transfected cells were lysed by freeze-thawing. Cell lysates were incubated with 1 volume of 200 $\mu\text{mol/L}$ ACV in phosphate-buffered saline (acycloguanosine; Sigma-Aldrich) at 37°C. After 0, 30, 60, and 120 minutes, proteins were precipitated by adding 4 volumes of methanol. ACV and ACVmp levels were measured in quintuple by applying matrix-assisted laser desorption/ionization tandem mass spectrometry (MALDI-QqQ-MS/MS; AB Sciex) [8]. Analyses were carried out by selected reaction monitoring. The monitored ion transitions were m/z 226 \rightarrow m/z 152 for ACV and m/z 306 \rightarrow m/z 152 for ACVmp. Ratios of ACVmp to ACV were calculated to determine the ACV-converting activity of the in vitro-produced TK proteins in time. The ACVmp/ACV ratios were corrected for the concentration of TK protein. The ACV-converting activity of the selected HSV-I TK minor variants was calculated by normalizing the corrected ratios to the activity of the ACV-sensitive (ACV^S) cognate major variant, which was set at 100% at 120 minutes incubation. The prevalence of latent ACV^R HSV-I was defined by determining the frequency of TK sequences that encode a TK protein unable to convert ACV to ACVmp.

Results

The aim of the study was to determine the prevalence of ACV^R HSV-I in human TG. Hereto, the genetic and functional variability of the latent HSV-I TK gene pool in the left and right TG of 5 individuals was analyzed. In total, 81 of 447 TK DNA sequences (18.1%) obtained were unique, with a mean of 8.6 distinct TK variants (range, 4–12 variants) per TG (data not shown). About three-fourths of the TK sequences contained nonsynonymous mutations resulting in amino acid mutations of the HSV-I TK protein. Each TG contained 1 major TK protein variant, on average 85% (range, 71%–96%) of all TK protein variants per TG, and a variable number of minor TK protein variants (mean, 5.7; range, 2–9 minor TK variants per TG) (Table 1). The major TK protein variant was

always genetically identical between paired left and right TG but distinguishable between the TG donors. The minor TK variants, however, were genetically related to the donor-associated major TK variant but were not shared with the contralateral TG of the same donor. Phylogenetic analyses demonstrated that both the major and minor TK protein variants, irrespective of left or right TG origin, clustered into distinct TG donor-specific clades (Figure 1A).

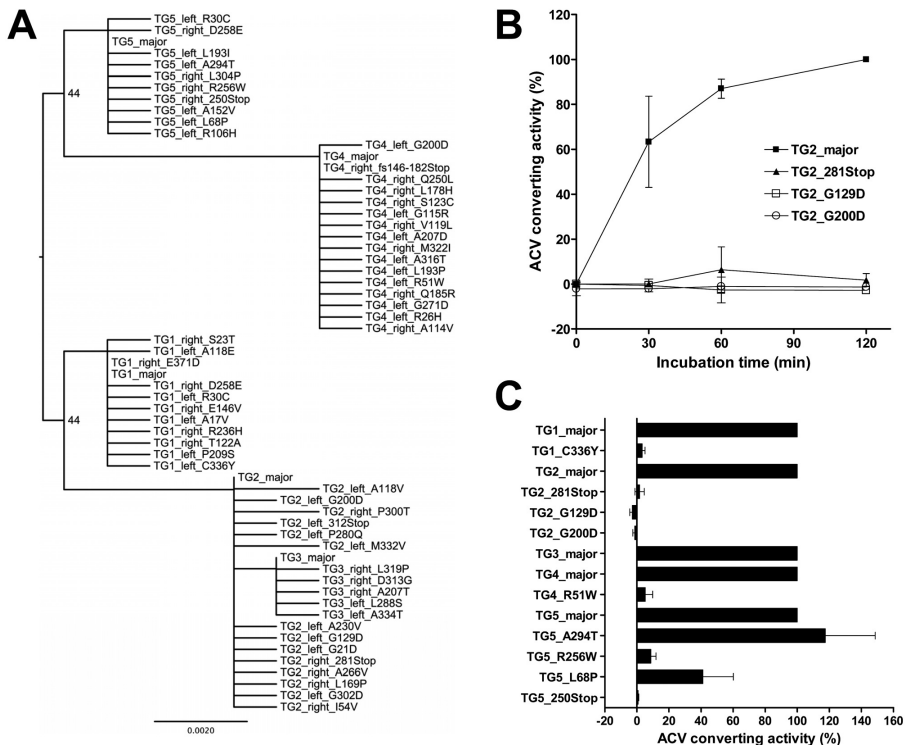


Figure 1. Phylogenetic tree and functional characteristics of human trigeminal ganglion (TG)-derived herpes simplex virus type 1 thymidine kinase (TK) variants. (A) Maximum likelihood unrooted phylogenetic tree was estimated under the general time-reversible model using PhyML software (version 3). The HSV-1 TK variants shown are coded by corresponding TG donor number (TG1-TG5), anatomical site of the originating TG (left or right), and the amino acid change compared to the cognate major HSV-1 TK variant (see Table 1; e.g. R30C, arginine at amino acid position 30 changed to cysteine). Selected bootstrap values are given. Scale bar represents number of nucleotide substitutions per site. (B) Results of a representative mass spectrometry experiment demonstrating the acyclovir (ACV)-converting activity of selected HSV-1 TK variants of donor TG2 in time. Dominant TK sequence variant is referred to as ‘major’ (Table 1). TG2_281Stop refers to a minor TK variant with a premature stop codon at amino acid position 281. The other minor TK variants represent amino acid substitutions at the indicated amino acid position. (C) ACV-converting activity of selected HSV-1 TK variants of 5 donors, measured by mass spectrometry (coded TG1-TG5). Values and standard deviations are calculated from ≥ 2 independent experiments and expressed as percentages measured after 120-minute incubation of the *in vitro*-produced TK proteins with ACV, corrected for the concentration of TK protein and normalized to the respective ACV-sensitive major TK variant of the same donor that was arbitrarily set to 100% (see Materials and Methods section for details). Amino acid changes are listed that differ from the major TK variant of the respective donor.

Table 1. Herpes simplex virus type 1 thymidine kinase (TK) variants detected in human trigeminal ganglia (TG).

TG	Amino acid changes in TK variant (% with variant in corresponding TG specimen)
TG1 left	Major variant: D14G, <u>1138Y</u> , <u>G240E</u> (85) Minor variants: <u>A17Y</u> (2.5); R30C (2.5); A118E (5); P209S (2.5); C336Y [4] (2.5)
TG1 right	Major variant: D14G, <u>1138Y</u> , <u>G240E</u> (85.4) Minor variants: S23T (2.1); T122A (2.1); E146V (2.1); R236H (2.1); D258E (2.1); E371D (4.2)
TG2 left	Major variant: <u>1138Y</u> (71.1) Minor variants: G21D (2.6); A118V (5.2); G129D [3] (5.2); G200D (2.6); A230V (2.6); P280Q (2.6); G302D (2.6); 3125stop (2.6); M322V (2.6)
TG2 right	Major variant: <u>1138Y</u> (85) Minor variants: <u>154V</u> (2.5); L169P (2.5); A266V (2.5); 281Stop [3] (2.5); P300T (2.5); R366H (2.5)
TG3 left	Major variant: R18C, <u>1138Y</u> (95.8) Minor variants: L288S (2.1); <u>A334T</u> (2.1)
TG3 right	Major variant: R18C, <u>1138Y</u> (93.5) Minor variants: A207T (2.2); D313G (2.2); L319P (2.2)
TG4 left	Major variant: S23N, E36K, Q89R, <u>1138Y</u> , <u>G240E</u> , R281Q (80.9) Minor variants: R26H (2.1); R511W [11] (2.1); G115R (4.2); L193P (2.1); G200D (2.1); A207D (2.1); G271D (2.1); A316T (2.1)
TG4 right	Major variant: S23N, E36K, Q89R, <u>1138Y</u> , <u>G240E</u> , R281Q (80.9) Minor variants: A114V (2.1); V119L (2.1); S123C (2.1); FS146-182Stop [3] (4.2); L178H (2.1); Q185R (2.1); Q250L (2.1); M322I (2.1)
TG5 left	Major variant: L42P, <u>1138Y</u> , <u>G240E</u> (87) Minor variants: R30C (2.2); L68P (2.2); R106H (2.2); A152V (2.2); L193I (2.2); A294T (2.2)
TG5 right	Major variant: <u>L42P</u> , <u>1138Y</u> , <u>G240E</u> (87.2) Minor variants: 2505top (2.1); R256W [10] (2.1); D258E (2.1); L304P (4.2)

For major variants, the amino acid changes listed are different from the HSV-1 TK reference sequence (Genbank accession No. GU_734772). Minor variants represent sequences that contain the amino acid changes of the major variant in addition to the listed changes. Parenthetical values represent percentage of colonies per sample with the respective amino acid change. Undefined amino acid changes are known not to affect acyclovir sensitivity of the respective HSV-1 strain [3, 4, 7, 9] (based on Figure 1C). Boldface changes are published acyclovir resistance-associated amino acid mutations [3, 4, 10, 11] (based on Figure 1C). (Reference citations are given in brackets). Abbreviations: FS, frameshift; Stop, premature stop codon at the indicated amino acid position.

TK mutations conferring ACV resistance are commonly localized to the adenosine triphosphate-binding (amino acids 51–63) and nucleoside-binding site (amino acids 168–176), 5 designated TK gene regions conserved among Herpesviridae (amino acids 55–66, 79–91, 162–178, 212–226, and 218–292), and a 7-Gs homopolymer repeat region located at nucleotides 430–436 [3, 4, 7]. The majority of the TG-derived HSV-I TK variants had mutations outside these TK function-related regions and have been documented to confer an ACV^S phenotype of the respective HSV-I strain, for example, S23N, L42P, I138V, and G281Q (Table 1) [3, 4, 7, 9]. Notably, several ACV resistance-associated TK variations described elsewhere were identified, consisting of mutations leading to premature stop codons (eg, amino acids 312stop and 250stop) or single amino acid substitutions, such as C336Y and R51W (Table 1) [3, 4, 10, 11].

To confirm the predicted ACV^S and ACV^R phenotype of the TG-derived HSV-I TK protein variants, we developed a novel TK functional assay. The assay involves longitudinal mass spectrometric measurements of ACV and ACVmp concentrations in reaction mixtures containing ACV and an in vitro-produced TK protein variant (Figure 1B). The ACV-converting activity of all 5 major TG-derived TK variants was comparable to that of TK derived from the ACV^S HSV-I reference strain KOS (data not shown). Among the minor TK variants, several variants had amino acid mutations suggesting an ACV^R phenotype of the respective latent HSV-I strain [3, 4, 10, 11]. Indeed, except for the A294T variant, all suspected TK amino acid mutations resulted in deficient or (in the case of L68P) impaired TK activity (Figure 1C).

Discussion

The sequence and functional variability of the HSV-I TK pool in latently infected TG specimens of 5 immunocompetent individuals was assayed to determine the prevalence of latent ACV^R HSV-I. Analogous to mucocutaneous HSV-I infections, the latent HSV-I pool is a mixed virus population. About 85% of the TK sequences in each TG represented one single major ACV^S TK variant, identical between paired TG but distinguishable between TG donors. In addition, all TG contained multiple minor TK variants, both ACV^S and ACV^R TK variants, which were phylogenetically related to the cognate major TK variant.

In addition to the causative role of TK mutations in ACV resistance, the hypervariability of the TK gene provides insight into the clonal composition of an HSV-I isolate [12]. In each TG donor, the latent virus pool consisted of a donor-specific HSV-I quasi-species, of which only the major TK variant was detected in the paired left and right TG. This, along with the position of the major TK variant at the trunk HSV-I quasi-species, of which only the major TK variant was detected in the paired left and right TG. This, along with the position of the major TK variant at the trunk of each donor-specific clade in the phylogenetic tree, suggests that the minor TK variants are derived from this major TK variant. The data strengthen the dogma that a single HSV-I strain colonizes the TG and persists as a perennial source of latent virus and do not support TG superinfection with another virus strain during life [13, 14]. Colonization of both TG with the major

variant may have occurred during primary infection or alternatively by autoinoculation of the contralateral anatomic site during recurrent infection. In contrast to the major TK variants, the phylogenetically related minor TK variants were not shared with the contralateral TG, suggesting that the minor variants are the result of intrahost virus evolution. Given their discordant presence between the paired TG, we postulate that the minor variants have evolved from the major variants within the respective TG, potentially during reactivation, and, less likely, within the TG-innervating mucosa during recurrent infection.

Although all major TK variants were ACV^S, several minor TK variants had amino acid mutations suspected of having an ACV^R phenotype of the cognate HSV-I strain. We developed a novel TK functional assay and demonstrated that these TK variants were unable to convert ACV to ACVmp, which implies that the respective latent HSV-I was ACV^R. The prevalence of latent ACV^R HSV-I was estimated at 2.8%. Because we do not have information on the donors' ACV treatment history, the ACV^R variants detected could be due to the selective pressure of ACV treatment. Alternatively, the host immune system or other currently unknown selective factors of host or virus origin may account for the uncommonly high polymorphism of the HSV-I UL23 TK gene resulting in both ACV resistance-associated and natural mutations of the progeny virus during the donor's life. The presence of ACV^R TK mutants in ganglia has been described elsewhere in a case report on an 18-year-old with a severe congenital immunodeficiency disorder [15]. The patient suffered from multiple episodes of severe HSV-I infection, starting at 17 months of age and was extensively treated with ACV. The frequency of TK mutants suspected to be ACV^R (25%) was much higher than that reported here and was most likely attributable to the patient's deprived immune status and the continuing ACV selection pressure resulting in the emergence and persistence of ACV^R viruses early in life [15]. In contrast, the TG donors analyzed here did not have a history of immunodeficiency or severe HSV-I infections, reflecting the presence of latent ACV^R HSV-I in the general population. Latent ACV^R virus may reactivate by itself from human TG, or functional TK from a co-reactivating latent ACV^S virus could complement the reactivation deficiency of the ACV^R virus in trans to spread to the innervating mucosa, leading to recurrent disease [16, 17]. The coexistence of both ACV^R and ACV^S phylogenetically related HSV-I in individual neurons within the same human ganglion supports the latter option [15].

In conclusion, we report on the presence of phylogenetically related ACV^S and ACV^R HSV-I in latently infected TG of immunocompetent individuals. Reactivation of latent ACV^R HSV-I from its ganglionic stronghold poses a risk of developing ACV refractory clinical disease in immunodeficient patients or at immunoprivileged anatomic sites, such as the cornea [7].

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CHAPTER 6

Acyclovir-resistant herpes simplex virus type 1 in intra-ocular fluid samples of herpetic uveitis patients

Monique van Velzen, Tom Missotten, Freek B. van Loenen,
Roland J.W. Meesters, Theo M. Luider, G. Seerp Baarsma,
Albert D.M.E. Osterhaus, Georges M.G.M. Verjans

ABSTRACT

Acyclovir (ACV) is the antiviral drug of choice to treat patients with herpes simplex virus type 1 (HSV-1) uveitis. The prevalence of intra-ocular ACV-resistant (ACVR) HSV-1 in herpetic uveitis is unknown and may have clinical consequences. In addition to its predictive value on ACV susceptibility, the polymorphic HSV-1 thymidine kinase (TK) gene facilitates differentiation between HSV-1 strains. The objective of this study was to determine the genetic composition and ACV susceptibility of the causative virus in intra-ocular fluid samples (IOF) of HSV-1 uveitis patients. The intra-ocular HSV-1 pool from 11 HSV-1 uveitis patients was determined by sequencing IOF-derived viral TK genes. The ACV susceptibility profile of the cloned intra-ocular TK variants was defined by mass spectrometry. In addition, the ganciclovir (GCV) susceptibility of the ACV^R HSV-1 TK variants was defined. Intra-ocular fluid samples of HSV-1 uveitis patients contain HSV-1 quasispecies, principally consisting of one major and multiple genetically related minor patient-specific TK variants. Four of 10 patients analyzed had an intra-ocular ACV^R HSV-1 of which 3 were cross-resistant to GCV. The ACV^R profile of intra-ocular HSV-1 did not correlate with symptomatic ACV treatment. Affected eyes of HSV-1 uveitis patients are commonly infected with a patient-specific HSV-1 quasispecies, including one major and multiple genetically related minor variants. A relatively high prevalence of intra-ocular ACV^R HSV-1, mainly ACV/GCV cross-resistant viruses, was detected in HSV-1 uveitis patients.

Introduction

Herpes simplex virus type 1 (HSV-1) is an endemic human alphaherpesvirus that causes a variety of diseases, including the sight-threatening ocular diseases keratitis and uveitis [1, 2]. HSV-1 establishes a lifelong latent infection in sensory neurons that innervate the site of primary infection and reactivates intermittently to cause recrudescence disease [1].

HSV-1 uveitis is due to an initial cytopathic effect of the virus on uveal-resident cells followed by a local inflammatory response to the inciting virus. Treatment is therefore aimed at inhibiting viral replication with antiviral drugs and dampening the intra-ocular immune response with anti-inflammatory agent [2]. The antiviral drug acyclovir (ACV), highly selective for human alphaherpesviruses and low in cytotoxicity, is the gold standard to treat HSV-1 uveitis. Acyclovir is converted to ACV-monophosphate (ACVmp) by virus-encoded thymidine kinase (TK) protein, which is subsequently converted by cellular enzymes to the active compound ACV-triphosphate that inhibits viral replication [3, 4]. ACV can be applied topically and systemically and is given as prophylaxis to prevent recurrent ocular HSV-1 diseases [5].

The prevalence of ACV resistant (ACV^R) HSV-1 is generally low for immunocompetent individuals (<1%), but higher in the immunocompromised (4-14%) [3, 6-8]. This difference illustrates the pivotal role of the host's immune system to control HSV-1 infections [4]. Recently, we reported on the relatively high incidence of ACV^R HSV-1 in otherwise healthy HSV-1 keratitis patients [7]. Notably, patients with corneal ACV^R HSV-1 were refractory to ACV therapy and had poor visual outcome [7, 9]. Resistance to ACV is commonly due to specific mutations in the viral TK and incidentally in the viral DNA polymerase gene. ACV^R HSV-1 viruses may have a deficient TK protein or altered substrate activity [3, 4, 10, 11]. Moreover, the natural hypervariability of the HSV-1 TK gene facilitates differentiation of HSV-1 strains [12, 13].

The prevalence of intra-ocular ACV^R viruses in HSV-1 uveitis is unknown and may have clinical consequences. The objective of this study was to determine the genetic composition and ACV susceptibility of intra-ocular HSV-1 in herpetic uveitis patients. Additionally, we determined the cross-resistance of intra-ocular ACV^R HSV-1 to ganciclovir (GCV).

Materials and Methods

Clinical specimens

The samples analyzed were surplus intra-ocular fluid (IOF) samples, either aqueous humor or vitreous fluid, obtained from 11 immunocompetent uveitis patients during diagnostic paracentesis or therapeutic vitrectomy (patients UV2, UV5 and UV7), respectively (Table 1). HSV-1 was identified as the causative virus by real-time PCR analysis on the respective IOF samples [14]. The clinical variables scored were age at sampling date, gender, ACV therapy regimen and clinical picture of ocular disease at presentation and in the preceding years. No detailed clinical information of patient UV3 was available. The

median patient age was 49 years (range 30-69 years) and 7 patients were female. Eight patients (73%) received systemic ACV (n=4) or valacyclovir (valACV; n=4) treatment, of variable duration (3-52 days, average 15 days), directly before IOF sample collection (Table 1). Two patients started valACV therapy after sample collection (UV3 and UV5). Two patients received complementary topical ACV ointment (UV4 and UV11). All patients received topical corticosteroids at time of sampling. After IOF sample collection, systemic (val)ACV treatment was started, continued and/or tapered until the clinical picture improved. None of the patients received topical or systemic ACV treatment in the 2 years preceding sampling and no patients were treated before or during sampling with (val)ACV analogues like ganciclovir (GCV). During follow-up all patients, except for patient UV7, restored visual acuity to baseline levels (data not shown). Patient UV7 lost vision due to ocular hypotonia after therapeutic vitrectomy (Table 1). Study procedures were performed in compliance with Dutch laws and institutional guidelines and in accordance with the ethical standards of the Declaration of Helsinki.

HSV-I thymidine kinase sequence analysis

The entire HSV-I TK gene was amplified by PCR using DNA isolated from IOF samples as described [7]. From 5 patients (UV6-UV10), the intra-ocular HSV-I TK amplicons were directly used for pool sequencing [7]. From the remaining 6 HSV-I uveitis patients, the intra-ocular TK gene pool was determined by colony sequencing as described [12, 13]. In short, TK amplicons were ligated into the TOPO-TA cloning vector (Invitrogen), transformed into bacteria and a variable number of colonies were sequenced. All sequences were aligned to a reference HSV-I TK sequence (strain HI29; Genbank GU734772). A maximum-likelihood phylogenetic tree of IOF-derived TK nucleotide sequences was estimated under the general time-reversible model using PhyML 3.0 software. The IOF-derived HSV-I TK sequences obtained were deposited in GenBank under the accession numbers HQ707581-HQ707642 and JX392955-JX392980 (Table 2 and Table 3).

HSV-I thymidine kinase functional assay

The functional effect of a selected set of HSV-I TK variants was assayed by a described mass spectrometry assay [13]. In brief, HSV-I TK sequences were cloned into the pcDNA3 expression vector (Invitrogen) and expressed as recombinant TK proteins in Cos-7 cells. After 48 hours of incubation at 37°C, TK protein expression was determined by flow cytometry using a HSV-I TK-specific antibody. Cell lysates were incubated with 100 µM ACV (Sigma-Aldrich) for 0, 30, 60, and 120 minutes at 37°C. Acyclovir and ACVmp levels were measured in quintuple by MALDI mass spectrometry [13, 15]. ACV/ACVmp-ratios were corrected for the amount of TK protein as measured by flow cytometry (data not shown) [13]. The ACV-converting activity was normalized to TK activity derived from the ACV sensitive (ACV^S) HSV-I reference strain KOS [13], which was set at 100% after 120 minutes of incubation. Cut-off value for ACV-resistant TK variants in the HSV-I mass spectrometry assay was >10% of the TK activity of the ACV-sensitive HSV-I strain KOS.

In vitro ganciclovir sensitivity testing

Ganciclovir susceptibility assay used was adapted from a recent study by Shiota et al. [16] In short, a human cornea-derived ACV/GCV cross-resistant HSV-I strain [7] (GenBank EU541365) was plaque-purified in vitro under 50 µM ACV and 50 µM GCV (Ro-

che) selection [17]. Cloned IOF-derived HSV-I TK variants were transfected into Cos-7 cells. After 24 hrs of incubation at 37°C, transfected cells were infected with the ACV/GCV cross-resistant HSV-I clone at a multiplicity of infection of 0.01. Cells were incubated with 0, 0.1, 0.5, 1, 5, 10, and 50 µM of GCV, or solely 50 µM ACV, for 18-24 hrs at 37°C. Cell lysate's HSV-I DNA load was determined by real-time PCR using primers and probes as described [7, 18]. An ACVS HSV-I (strain KOS) served as positive control for the efficacy of ACV and GCV to inhibit HSV-I replication. Untransfected Cos-7 cells infected with the ACV/GCV cross-resistant HSV-I strain served as negative control. All HSV-I TK variants were assayed in triplicate.

Median inhibitory concentration (IC50) was defined as the concentration of the antiviral drug that reduced viral DNA copies by 50%. HSV-I TK variant transfection efficiency was checked by TK protein staining and flow cytometry [13] and was always >75% (data not shown). Relative viral replication was normalized per TK variant to the viral replication in the absence of antiviral drugs, set at 100%. HSV-I TK variants were considered ACV^R or GCV^R if viral replication levels were >1% in the presence of 50 µM ACV and IC50 values ≥ 1 µM GCV [7, 18], respectively.

Results

Genetic characteristics of intra-ocular HSV-I in herpetic uveitis patients

To gain insight into the incidence and clinical significance of intra-ocular ACV^R HSV-I in HSV-I uveitis, we determined the genetic composition and ACV susceptibility of IOF-derived HSV-I. Intra-ocular HSV-I was genotyped by sequencing viral TK genes in IOF samples from 11 HSV-I uveitis patients. From 6 IOF samples (UV1-UV5 and UV11), the HSV-I TK gene was PCR amplified, cloned and on average 54 colonies per IOF sample were sequenced (range 45-74 colonies/sample). From the remaining 5 patients (UV6-UV10), the HSV-I TK amplicon itself was directly used for pool sequencing. In total, 88 of 326 (27%) TK nucleotide sequences obtained were unique. Eighty-two percent contained non-synonymous mutations resulting in 72 unique intra-ocular HSV-I TK protein variants.

Whereas the pool-sequenced IOF samples revealed one patient-specific TK protein variant, colony-sequencing identified 6 to 26 different HSV-I TK protein variants per IOF sample (Table 2). One major TK protein variant was identified in 4 of 5 colony-sequenced IOF samples, accounting for on average 72% (range 37-90.5%) of all TK protein variants/sample. Except for patients UV1 and UV4, all major HSV-I TK variants were patient-unique at the nucleotide level. No minor TK variants were shared between the IOF samples. Notably, IOF-derived HSV-I TK sequences obtained from patient UV11 consisted of one dominant and a subdominant TK protein variant (accounting for 37% and 15% TK nucleotide sequences, respectively), and 23 minor TK variants (Table 3). Pool sequencing of the IOF samples of patients UV1 and UV3 revealed a TK variant identical to the major TK variant identified by colony sequencing of the respective sample (data not shown), demonstrating the applicability of pool sequencing to identify the dominant TK variant in IOF samples. Phylogenetic analyses demonstrated that the major and mi-

Table 1. Characteristics of patients with herpes simplex virus type 1 (HSV-1) uveitis.

Information on preceding ocular disease history ^a				Information on uveitis episode studied ^b			
Donor	Age, sex	Clinical disease	Oral or topical ACV	Ocular disease	Duration disease before sampling	Time on ACV until sample collection	Total disease duration
UV1	68; F	-3 yrs:Ant. Uveitis	No ACV	Keratouveitis	57 days	3 days	87 days
UV2	30; M	No history ocular disease	No ACV	ARN	28 days	24 days	44 days
UV3	66; F	-30 yrs till sampling date: recurrent Ant Uveitis eci	No ACV	Ant. Uveitis	Unknown	0 days	Unknown
UV4	62; M	-12 yrs: Ant. Uveitis eci	No ACV	Ant. Uveitis	74 days	52 days	141 days
UV5	62; F	-14 yrs: Ant. Uveitis eci	No ACV	Panuveitis	1 day	0 days	40 days
UV6	39; F	-16 yrs: HSV-1 IEK -14 yrs: keratouveitis	No ACV	Keratouveitis	18 days	18 days	41 days
UV7	69; M	No history ocular disease	No ACV	ARN	4 days	4 days	vision loss after 2.5 mth
UV8	41; F	-8 yrs:Ant. Uveitis eci -3 yrs:Ant. Uveitis eci	No ACV 5x400 mg/day (1 mth)	Ant. Uveitis	3 days	3 days	35 days
UV9	42; F	No history ocular disease	No ACV	Ant. Uveitis	20 days	10 days	31 days
UV10	49; F	-3 yrs:Ant. Uveitis eci	No ACV	Ant. Uveitis	28 days	6 days	83 days
UV11	43; M	-5 days:stromal keratitis eci	No ACV	Keratouveitis	5 days	0 days	45 days

Abbreviations: UV, uveitis; F, female; M, male; Age, age at time of uveitis episode sampled (years); ACV, acyclovir; ARN, acute retinal necrosis; Ant. Uveitis, anterior uveitis; eci, disease of unknown cause; IEK, infectious epithelial keratitis; yrs, years; mth, month.

a. Information on history of ocular disease, which preceded the sampled uveitis episode, are indicated. The time point e.g. "-3yrs: Ant. Uveitis" refers to an episode of anterior uveitis 3 years before the sampled uveitis episode.

b. Underlined time intervals indicate patients who were on valacyclovir treatment. Duration of the sampled uveitis episode was calculated from the first clinical symptoms presented to the ophthalmologist until resolution of the clinical symptoms.

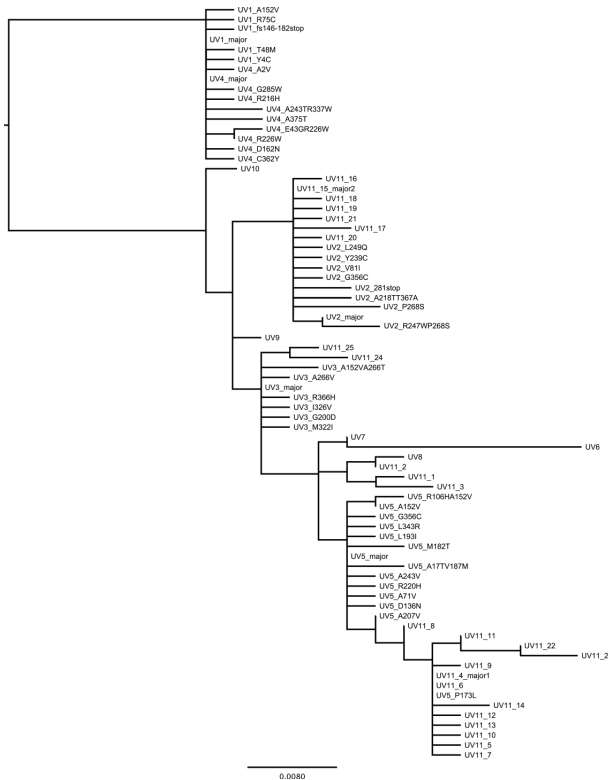


Figure 1. Phylogenetic tree of intra-ocular HSV-1 TK variants in HSV-1 uveitis patients. A maximum likelihood unrooted tree was estimated under the general time-reversible model using PhyML software 3.0. HSV-1 TK variants shown are coded by the corresponding HSV-1 uveitis patient (UV1–UV11) and the amino acid change compared with the cognate major HSV-1 TK variant (see Table 2; e.g., A152V, alanine at amino acid position 152 changed to valine). In case of patients UV6–UV10 only one HSV-1 TK sequence was obtained by pool sequencing of the intra-ocular fluid-derived DNA. The TK variants of patient UV11 are numbered according to Table 3. Scale bar represents number of nucleotide substitutions per site.

nor TK protein variants clustered into patient-specific HSV-1 clades indicating that the affected eyes contained a mixture of genetically related HSV-1 viruses (Figure 1). HSV-1 TK quasiespecies in patient UV11 contained a mixed TK variant spectrum genetically related to either major TK variant (Table 3 and Figure 1).

Locations of ACV^S- and ACV^R-associated polymorphisms within the HSV-1 TK protein have been described [3, 4, 9–11, 13, 19–26]. Among the 76 unique IOF-derived TK polymorphisms detected, 17 (22%) and 9 (12%) residue changes have been reported earlier to confer an ACV^S and ACV^R phenotype of the corresponding HSV-1 strain, respectively [3, 4, 9–11, 13, 19–26] and 48 new HSV-1 TK polymorphisms were identified (Table 2 and Table 3).

Functional characteristics of intra-ocular HSV-1 TK variants from herpetic uveitis patients

To validate the predicted ACV^S and ACV^R phenotype of IOF-derived HSV-1 TK variants, the ability of a selected set of TK variants to convert ACV to ACVmp was determined by mass spectrometry [13]. Four of 11 IOF-derived major TK variants, R41H (UV1 and UV4), C336Y (UV8) and P173L (UV11), were deficient to convert ACV into ACVmp, confirming the predictive ACV^R phenotype of the corresponding HSV-1 strain (Table 2 and Figure 2). Furthermore, several previously described ACV^S-associated (e.g., Q44H, Q89R, I138V, G240E, and S345P) and ACV^R-associated (I182Stop, P173L, R216H, R220H,

Table 2. Amino acid variation of the predicted herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) protein sequences obtained from intra-ocular fluid samples of HSV-1 uveitis (UV) patients.

Donor	Amino acid changes (percentage of sequenced colonies)
UV1	Major variant: C6G, R41H, Q89R, 1138V, A192V, G251C, V267L, P268T, D286E, N376H (90.5) Minor variants: Y4C (1.4); T48M (1.4); R75C (1.4); A152V (1.4); F5146-182Stop (4.1)
UV2	Major variant: Q44H, 1138V, S345P (83) Minor variants: V811 (2.1); A218T, T367A (2.1); Y239C (2.1); R247W, P268S (2.1); L249Q (2.1); P268S (2.1); 281Stop (2.1); G356C (2.1)
UV3	Major variant: 1138V (85.4) Minor variants: A152V, A266T (4.2); G200D (2.1); A266V (2.1); M322I (2.1); I326V (2.1); R366H (2.1)
UV4	Major variant: C6G, R41H, Q89R, 1138V, A192V, G251C, V267L, P268T, D286E, N376H (68.9) Minor variants: A2V (2.2); E43G, R226W (2.2); D162N (2.2); R216H (4.4); R226V (2.2); A243T, R337W (2.2); G285W (6.7); C362Y (2.2); A375T (6.7)
UV5	Major variant: 1138V, G240E (67.9) Minor variants: A71T, V187M (5.7); A71V (1.9); R106H, A152V (1.9); D136N (3.8); A152V (1.9); P173L (1.9); M182T (1.9); L193I (1.9); A207V (1.9); R220H (1.9); A243V (3.8); L343R (1.9); G356C (1.9)
UV6	S23N, E36K, Q89R, 1138V, G240E, R281Q
UV7	1138V, G240E
UV8	D14G, 1138V, G240E, C336Y
UV9	1138V, E210D
UV10	Q89R, 1138V, M322L
UV11	Mixed population Major 1: 1138V, P173L, G240E (37) Major 2: Q44H, 1138V, S345P (15) Minor variants: mixture of genetically divergent sequences (see Table 3)

For major variants, the amino acid changes listed are different from the HSV-1 TK reference sequence (GU_734772). Minor variants represent sequences that contain the amino acid changes of the donor's major variant in addition to the listed mutations. Parenthetical values represent percentage of colonies per sample with the respective amino acid changes. Fs, frameshift; and Stop, premature stop codon at the indicated amino acid position.

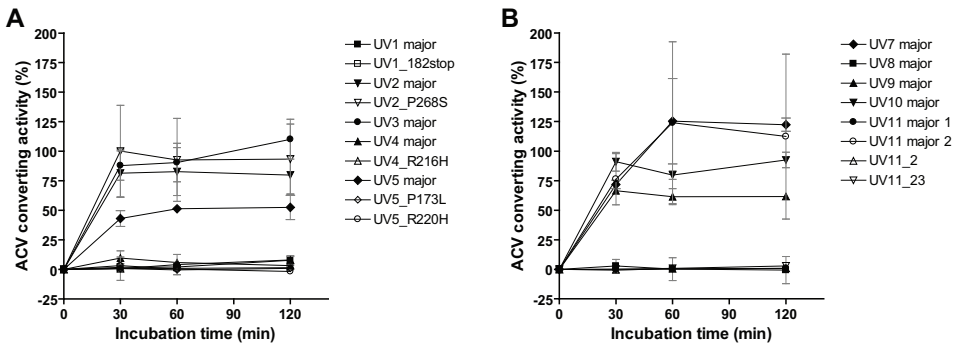


Figure 2. Functional characteristics of intra-ocular HSV-1 TK variants in HSV-1 uveitis patients. Kinetics of the (acyclovir) ACV-converting activity of a subset of HSV-1 TK variants from 10 HSV-1 uveitis patients (UV1-UV5 and UV7-11) was measured by mass spectrometry. Dominant TK variant is referred to as 'major' (Table 2). The minor TK variants represent amino acid substitutions at the indicated positions that differ from the major TK variants of the respective uveitis patient. UV11_major I and II represent the two dominant TK variants identified in the sample of uveitis patient 11. The minor TK variants of patient UV11 are numbered according to Table 3. Values are calculated from at least 2 independent experiments and are expressed as percentages normalized to data obtained using the TK variant from the ACV-sensitive HSV-1 reference strain KOS, which was arbitrarily set at 100% (see Materials and Methods for details). The cut-off value for ACV-resistant TK variants in the HSV-1 mass spectrometry assay was $>10\%$ of the TK activity of HSV-1 strain KOS. Values are expressed as means \pm standard error. 182Stop, premature stop codon at residue 182 of the TK protein.

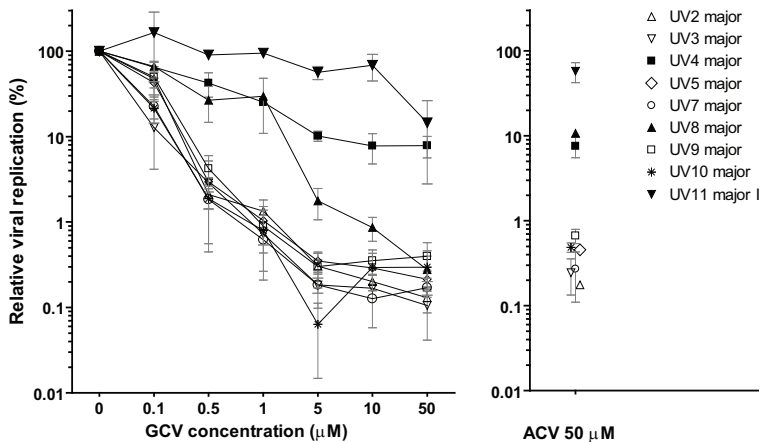


Figure 3. Ganciclovir sensitivity of intra-ocular HSV-1 TK variants in HSV-1 uveitis patients. The inhibitory effect of increasing concentrations of ganciclovir (GCV), and 50 μM acyclovir (ACV), on viral replication of a GCV/ACV cross-resistant HSV-1 strain in transfected Cos-7 cells expressing intra-ocular fluid-derived HSV-1 TK variants from 9 HSV-1 uveitis patients (UV2-UV5 and UV7-UV11) was measured by real-time PCR. Dominant TK variant is referred to as 'major' (Table 2). Relative viral replication was normalized per TK variant to the viral replication in the absence of antiviral drugs, set at 100%. Values are expressed as means \pm standard error. HSV-1 TK variants were considered ACVR or GCVR if viral replication levels were $>1\%$ in the presence of 50 μM ACV and if IC_{50} values $\geq 1 \mu\text{M}$ GCV, respectively. The GCV IC_{50} values for the tested TK variants were $<0.1 \mu\text{M}$ (i.e., UV2, UV3, UV5, UV7, UV9 and UV10), $1.2 \mu\text{M}$ (UV4), $0.5 \mu\text{M}$ (UV8) and $10.9 \mu\text{M}$ GCV (UV11).

Table 3. Herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) protein variants detected in the intra-ocular fluid (IOF) sample of patient UVI 1^a

TK variant in phylogenetic tree (see Figure 1)	Amino acid changes in TK protein variant (% with variant among colonies sequenced; n= 54 total)	Genbank accession no.
1	D14G, I138V, R216L, G240E, S345P (1.9)	JX392955
2	D14G, I138V, G240E, C336Y (7.4)	JX392956
3	D14G, I138V, R216L, G240E, <u>A365S</u> (1.9)	JX392958
4 (major variant 1)	I138V, P173L , G240E (37)	JX392959
5	<u>A133V</u> , I138V, P173L , G240E (1.9)	JX392960
6	I138V, Fs155-I82Stop , P173L , G240E (1.9)	JX392961
7	I138V, P173L , A192V, G240E (1.9)	JX392962
8	I138V, P173L , <u>A207V</u> , G240E (1.9)	JX392963
9	I138V, P173L , G240E, <u>A266V</u> (1.9)	JX392964
10	I138V, P173L , G240E, <u>P274S</u> (1.9)	JX392965
11	I138V, P173L , G240E, <u>A365S</u> (1.9)	JX392966
12	I138V, P173L , G240E, <u>A375D</u> (1.9)	JX392967
13	I138V, P173L , <u>A207T</u> , G240E (1.9)	JX392968
14	I138V, <u>E146G</u> , P173L , <u>L178P</u> (1.9)	JX392969
15 (major variant 2)	Q44H, I138V, S345P (14.8)	JX392970
16	Q44H, I138V, <u>L193I</u> , S345P (1.9)	JX392971
17	Q44H, I138V, Fs198-377Stop , S345P (1.9)	JX392972
18	Q44H, I138V, <u>P333T</u> , S345P (1.9)	JX392973
19	Q44H, I138V, Fs146-I82Stop , S345P (1.9)	JX392974
20	Q44H, I138V, <u>P199S</u> , S345P (1.9)	JX392975
21	Q44H, I138V, <u>R247W</u> , S345P (1.9)	JX392976
22	Q44H, I138V, P173L , G240E, <u>A365S</u> (1.9)	JX392977
23	Q44H, I138V, P173L , G240E (1.9)	JX392978
24	R51W , I138V, <u>A168V</u> , S345P (1.9)	JX392979
25	<u>A117T</u> , I138V, S345P (1.9)	JX392980

^aThe left column indicates the TK variant ID presented in Figure 3. Amino acid (aa) changes are listed that are different from the HSV-1 TK reference sequence (Genbank accession no. GU_734772). Boldface changes are published (ACV) resistance-associated aa mutations [9,19-26], underlined aa changes are novel polymorphisms not reported previously, and all other listed aa changes mutations are natural polymorphisms known not to affect ACV sensitivity of the respective HSV-1 strain [3,4,9-11,13,19-26]. Parenthetical values represent percentage of colonies per sample with the respective aa change. Fs and Stop, frameshift leading to a premature stop codon at the indicated aa position. Variants 2, 4, 15, and 23 were tested by mass spectrometry (see Figure 2).

and C336Y) TK polymorphisms were functionally confirmed [4, 9-11, 13, 19-26]. Notably, three newly identified TK polymorphisms (E210D, P268S and M322L) did not alter TK function (Table 2 and Figure 2).

Next, we tested the susceptibility of the TK variants to GCV, an alternative TK-dependent antiviral drug commonly used to treat herpetic eye diseases [27-30]. The inhibitory effect of GCV was assessed by measuring replication of an ACV/GCV cross-resistant HSV-1 strain in transfected cells expressing recombinant IOF-derived HSV-1 TK variants. Ganciclovir inhibited all 5 dominant intra-ocular ACV^S TK variants (patients UV2, UV3, UV5, UV7, UV9 and UV10) efficiently in a dose-response fashion. However, 3 of 4 dominant IOF-derived ACV^R HSV-1 TK variants obtained from patients UV1, UV4 and UV11 were cross-resistant to GCV (Table 2 and Figure 3).

Discussion

The plaque reduction assay is considered the gold standard to determine the ACV susceptibility profile of HSV-1 isolates [31]. The alternative and more rapid approach is to sequence the HSV-1 TK gene directly from clinical specimens. This, because ACV^R is largely attributed to a mutated viral TK gene and multiple ACV^R-associated TK polymorphisms have been documented [3, 4, 9-11, 13, 19-26]. The latter approach is of particular interest for diseases like HSV-1 encephalitis and uveitis where no infectious virus can be recovered from clinical specimens [14, 32, 33]. Besides its potential diagnostic value, the hypervariability of the HSV-1 TK gene provides insight into the genetic composition of HSV-1 isolates [12, 13].

We demonstrated that IOF samples from HSV-1 uveitis patients were commonly composed of a patient-specific HSV-1 quasispecies, consisting of one major HSV-1 TK variant and multiple genetically related minor TK variants (Figure 2). These data, along with the patients' positive HSV-1 serostatus, suggest that uveitis was due to reactivation of one endogenous HSV-1 strain from the patient's trigeminal ganglion (TG) [34]. The minor TK variants may have emerged spontaneously, due to ACV therapy or may have co-reactivated from the innervating TG. The analogous presence of multiple genetically related minor and one major TK variant in HSV-1 latently infected human TG supports the latter hypothesis [13]. The two genetically different HSV-1 TK clades in the IOF sample of patient UV11 suggest that the patient's eye was infected with two different strains (Table 3).

ACV^R-associated HSV-1 TK polymorphisms are predominantly located in 5 regions conserved among Herpesviridae (residues 83-88, 162-164, 216-222, and 284-289), the ATP- (residues 51-63) and nucleoside binding site (residues 168-176), and the highly conserved cysteine residue at position 336 of the TK protein [4, 11, 20, 23, 35]. Natural TK polymorphisms, referred to as ACV^S-associated mutations, are dispersed throughout the TK. Sequence analysis predicted that 4 (6%) and 37 (51%) of 72 unique IOF-derived TK variants had an ACV^S and ACV^R phenotype, respectively (Table 2 and Table 3) [13, 19, 20, 22-26]. We identified 48 novel TK protein polymorphisms resulting in 31 unique

intra-ocular TK variants with an indefinite ACV susceptibility profile. Four of these newly identified TK polymorphisms were located in the conserved regions (D162N, R216L, A218T and G285W) suggesting that these residues affect TK protein function. Two of these polymorphisms (i.e., D162N and R216L) are located at TK residues that with other amino acid changes (i.e., D162A, R216C and R216H) conferred ACV^R HSV-I [10, 36-38]. Additionally, three (i.e., A168V, L178P and A207T) and four (i.e., A243V, A243T, P268S and P274S) are located at TK residues that with other amino acid changes conferred an ACV^R (i.e., A168T, L178R and A207P) [7, 35, 39] or ACV^S HSV-I (i.e., A243S, P268T and P274T) [9, 22, 24, 35-37], respectively. Overall, HSV-I TK sequence comparisons with previously described TK genotypes with confirmed ACV susceptibility suggest an ACV^R and ACV^S phenotype of HSV-I viruses that contain 5 and 4 of the 48 novel HSV-I TK polymorphisms, respectively. Mass spectrometry analysis of a selected set of IOF-derived TK variants confirmed their predicted ACV susceptibility profile and provided novel insight into the functional role of the R41H and 3 novel TK polymorphisms (Figure 2). Three newly identified TK mutations E210D, P268S and M322L did not affect TK activity demonstrating that they are natural TK polymorphisms. The R41H polymorphism, expressed by the major TK variants of patients UV1 and UV4, has mainly been associated with ACV-sensitive HSV-I phenotypes [9, 35, 37, 38, 40]. In this study, however, we demonstrated that an R41H TK variant was unable to convert ACV into ACVmp, and the introduction of this HSV-I TK variant in an ACV/GCV-resistant HSV-I strain preserved the antiviral cross-resistant phenotype *in vitro*, suggesting that R41H is an ACV^R-associated polymorphism in the experimental setting of the current study (Figure 2 and Figure 3). The data contrast previous studies reporting on R41H as a natural TK polymorphism [9, 35, 37, 38, 40]. Except for the study by Sauerbrei and colleagues [40], previous studies on the R41H polymorphism have combined phenotypic ACV susceptibility and TK genotypic assays on whole virus stocks, but not virus clones [9, 35, 37, 38]. Because clinical isolates and low passage HSV-I stocks contain multiple virus strains [9, 41, 42], functional TK of ACV^S viruses in these virus pools may have compensated for R41H in functional assays to confer an overall ACV^S phenotype of the HSV-I isolate analyzed. Recently, the significance of R41H has been detailed by expressing the cognate recombinant TK protein and subsequent determination of the TK enzymatic activity by an enzyme-linked immunosorbent assay using the thymidine analogue bromodeoxyuridine (BrdU) as TK substrate [40]. The R41H-containing recombinant TK protein phosphorylated BrdU to values comparable to the positive control indicating that R41H is a natural polymorphism [40]. Although both BrdU and ACV are substrates for HSV-I TK, the chemical structures are different and thereby withhold extrapolation of the enzymatic activity of R41H-containing TK proteins obtained with BrdU to the guanosine analogue ACV as substrate. Further studies, including ACV susceptibility assays on both the respective HSV-I clones and recombinant TK proteins generated by marker rescue experiments, are warranted to complement the known set of ACV^S- and ACV^R-associated TK polymorphisms, including the R41H polymorphism, mandatory for a successful implementation of diagnostic HSV-I TK genotyping in the clinic.

The proportion of ACV^R viruses at the infection site determines the efficacy of ACV therapy [9, 42, 43]. We demonstrated that 4 of 10 (40%) HSV-I uveitis clinical isolates tested harbored a dominant ACV^R HSV-I variant, which is higher than expected for immunocompetent individuals [3, 6-8]. Three of 4 ACV^R HSV-I uveitis patients received no ACV

treatment (UV11), or only for 3 days (UV 1 and UV8), before IOF sampling suggesting primary ACV^R or reactivation of the corresponding latent ACV^R HSV-I [13]. Moreover, none of the patients received topical or systemic ACV treatment in the 2 years preceding sampling. Overall, the ACV susceptibility profile of intra-ocular HSV-I did not correlate with symptomatic ACV treatment during or preceding the uveitis episode sampled. Given the heterogeneous patient group with different uveitis entities, different numbers of disease episodes prior to moment of sampling, different antiviral treatment strategies and ways of application, no correlation between intra-ocular ACV^R HSV-I and disease outcome could be made. A future study on a larger and more homogenous group of ACV (non-)treated HSV-I uveitis patients is warranted to determine the association between ACV^R HSV-I and clinical outcome. Moreover, when ACV treatment fails or is poorly tolerated, other nucleoside analogues like GCV are optional to treat herpetic eye diseases [27-30]. GCV is also a HSV-I TK-dependent antiviral posing the risk of ACV/GCV-cross-resistance [4, 44]. Indeed, 3 of 4 dominant IOF-derived ACV^R TK variants were cross-resistant to GCV. Patient UV8, however, who had a dominant intra-ocular ACV^R/GCV^S TK variant would have benefitted from a switch to GCV treatment. Patients UV1, UV4 and UV11, with a dominant ACV/GCV-cross-resistant HSV-I TK variant, should have preferably been treated with TK-independent drugs like foscarnet [31].

To our knowledge, this is the first study reporting on the presence of HSV-I quasispecies and the relative high prevalence of ACV^R HSV-I in IOF samples of immunocompetent HSV-I uveitis patients. The data justify further studies on the prevalence and role of ACV- and GCV-resistant HSV-I in relation to clinical outcome of HSV-I uveitis. Mapping of ACV^R-associated HSV-I TK polymorphisms will aid in the development of a diagnostic HSV-I TK genotyping platform for a rationalized selection of the appropriate antiviral drugs to prevent the development of severe ocular HSV-I diseases.

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CHAPTER 7

Acyclovir refractory herpes simplex virus keratitis correlates with acyclovir prophylaxis

Monique van Velzen, David A.M.C. van de Vijver, Freek B. van Loenen,
Albert D.M.E. Osterhaus, Lies Remeijer, Georges M.G.M. Verjans

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ABSTRACT

Long-term acyclovir (ACV) prophylaxis, recommended to prevent recurrent herpes simplex virus type 1 (HSV-1) ocular disorders, may pose a risk for ACV-refractory disease due to ACV-resistance. We determined the effect of ACV prophylaxis on the prevalence of corneal acyclovir-resistant (ACV^R) HSV-1 and clinical consequences thereof in patients with recurrent HSV-1 keratitis (rHK). Frequencies of ACVR viruses were determined in 169 corneal HSV-1 isolates from 78 rHK patients with a history of stromal disease. The isolates' ACV susceptibility profiles were correlated with clinical parameters to identify risk factors predisposing to ACV^R rHK. Corneal HSV-1 isolates with >28% ACV^R viruses were defined as ACV^R isolates. Forty-four isolates (26%) were ACV-resistant. Multivariate analyses identified long-term ACV prophylaxis (≥ 12 months) (odds ratio [OR] 3.42, 95% confidence interval [CI] 1.32–8.87) and recurrence duration of ≥ 45 days (OR 2.23, 95% CI 1.02–4.87), indicative for ACV-refractory disease, as independent risk factors for ACV^R isolates. Moreover, a corneal ACV^R isolate was a risk factor for ACV-refractory disease (OR 2.28, 95% CI 1.06–4.89). The data suggest that long-term ACV prophylaxis predisposes to ACV-refractory disease due to the emergence of corneal ACV^R HSV-1. The data warrant ACV-susceptibility testing during follow-up of rHK patients.

Introduction

Herpes simplex virus type 1 (HSV-1) is a human alpha-herpesvirus that is endemic worldwide. The virus is typically acquired during early childhood via the orofacial route leading to the establishment of a life-long latent infection of neurons located within the trigeminal ganglia. Intermittent reactivations lead to virus shedding and occasionally recurrent disease [1]. HSV-1 causes a variety of diseases, ranging from mild herpes labialis to sight-threatening eye diseases [1]. Corneal HSV-1 infections, referred to as herpetic keratitis (HK), are a common infectious cause of visual impairment mainly due to its recurrent nature [2, 3]. Herpetic keratitis manifests as infectious epithelial keratitis (IEK) characterized by superficial viral replication or can infect the underlying corneal stroma and cause herpetic stromal keratitis (HSK) [2]. Recurrent HSK may result in corneal blindness [4].

The drug of choice to treat HSV-1 infections is acyclovir (ACV). Acyclovir is a pro-drug selectively converted by HSV-1 thymidine kinase (TK) to ACV monophosphate, allowing subsequent conversions by cellular kinases to its active form ACV triphosphate, that blocks viral replication [3]. The drug is generally well tolerated and the widespread use of ACV has resulted in a significant reduction of visual impairment of HSV-1-induced ocular diseases [1, 3]. However, ACV treatment may lead to the emergence of ACV-resistant (ACV^R) HSV-1. All clinical HSV-1 isolates contain ACV^R viruses and the frequency thereof determines the isolate's ACV susceptibility phenotype: ACV^R or ACV-sensitive (ACV^S) [5-7]. The prevalence of ACV^R HSV-1 is higher among immunocompromised patients (up to 30%) compared to healthy individuals (<1%), and may cause severe HSV-1 disease if not switched promptly to alternative antiviral therapies including ganciclovir and TK-independent drugs like foscarnet [8, 9].

In addition to symptomatic treatment, ACV prophylaxis is commonly prescribed to prevent recurrent herpetic eye diseases. The Herpetic Eye Disease Study group has reported that ACV prophylaxis, twice-daily oral 400 mg ACV for 12 months, significantly reduced the recurrence rate of both IEK and HSK in immunocompetent HK patients who had a disease episode within the preceding year [10, 11]. However, long-term ACV prophylaxis may pose a risk for ACV-refractory keratitis due to the selection for corneal ACV^R HSV-1 [5, 7, 12, 13]. In the current study, we determined the effect of ACV prophylaxis on the prevalence of ACV^R corneal HSV-1 and the clinical consequences thereof in recurrent herpetic keratitis (rHK) patients with a history of stromal disease.

Materials and Methods

Patients and clinical specimens

At the Rotterdam Eye Hospital (REH; Rotterdam, the Netherlands), between years 1981 and 2011, 169 corneal swabs from 78 immunocompetent rHK patients with a long history of stromal disease were obtained for diagnostic purposes and had been stored at -80°C. The median follow-up time at the REH of the included rHK patients was 17.6 years with an interquartile range (IQR) of 12.2–30.1 years. The virus was grown and

typed for HSV-I as described [5]. Herpetic keratitis was classified based on clinical criteria [2, 3]. Each enrolled patient had consented in future testing of their archived HSV-I isolates. The study was performed according to the tenets of the Declaration of Helsinki, approved by the local ethical committee and written informed consent for using the archived HSV-I isolates for the current study was obtained from the patient, or in case of death of the patient at start of the study, by the next of kin.

Study design

For all patients in this retrospective study, information on ACV treatment duration and dose were recorded. Scored parameters were age, gender, affected eye, sampling date, length of disease recurrence sampled, visual acuity (best spectacle-corrected vision), and clinical picture at presentation and at end of follow-up. Long-term ACV prophylaxis was defined as ≥ 12 months systemic ACV therapy, ranging from 200 to 4,000 mg ACV daily, which was prescribed to rHK patients with healed corneal lesions in response to a treatment for a prior disease recurrence [10]. The format of ACV prophylaxis provided to the patients was ACV or valacyclovir. Corneal isolate dates were stratified according to before and after the year 2000. Acyclovir prophylaxis was introduced as standard care for rHK patients at the REH in year 2000.

The start of an HSV-I keratitis recurrence was defined as the presentation of an isolated IEK characterized by a dendritic or geographic corneal ulcer or clinical symptoms related to HSK pathology including cell infiltrates in corneal stroma or anterior chamber and corneal stroma thinning, edema, vascularization or pseudoguttata [2, 14]. The end date of an isolated IEK was defined as the date of complete closure of corneal epithelium, when topical ACV treatment was discontinued. In case of an HSK-associated recurrence the end date of a recrudescence HSV-I epithelial defect was defined as the date of complete closure of the corneal epithelium combined with the resolution of the aforementioned HSK-associated clinical parameters observed at start of the disease recurrence. This combined with the return to both pre-recurrence topical steroid treatment (i.e. dexamethasone phosphate 0.1% or fluorometholone) and ACV prophylaxis. Whereas IEK lesions typically heal within 2 weeks on ACV treatment [15], HSV-I epithelial defects in corneas of patients with a history of stromal disease require combined treatment using both ACV and steroids. In the latter patient group resolution of corneal lesions generally take longer than 3-4 weeks (Remeijer, L; unpublished data). Consequently, we considered a disease recurrence duration of ≥ 45 days, being >3 -times the normal duration of an isolated IEK episode (i.e. 2 weeks), as ACV therapy refractory herpetic keratitis. The ACV susceptibility profile of the corneal isolates was unknown at time of ACV prescription and the authors were masked for the results of the ACV susceptibility assays during the assembly of the clinical data.

Acyclovir susceptibility assays

The percentage of ACV^R viruses in corneal HSV-I isolates was determined by the plating efficiency assay as described elsewhere [16, 17]. Briefly, serial 10-fold dilutions of corneal HSV-I isolates were inoculated onto monkey kidney cells (i.e. Vero cells) in culture medium consisting of Dulbecco's modified eagle medium supplemented with 5% heat-inactivated fetal bovine serum and antibiotics (all from Gibco). After adsorption at 37°C for 1 hour, the inoculum was removed and cells were overlaid with culture medium

containing 2% v/v methylcellulose (Sigma) supplemented with no ACV or 20 $\mu\text{mol/L}$ ACV (Roche) and cultured for 3 and 10 days at 37°C, respectively. The ACV concentration and culture period were chosen to ensure that only pre-existing ACV^R viruses formed plaques [5]. The percentage of ACV^R viruses in each isolate was calculated by dividing the number of virus plaques obtained in the presence of 20 $\mu\text{mol/L}$ ACV by the number of plaques obtained after culture in the absence of ACV [5, 7].

The overall ACV susceptibility phenotype of a selected set of corneal isolates was determined by an HSV-I specific real-time quantitative polymerase chain reaction (qPCR) assay as described elsewhere [12, 18]. In brief, Vero cells were infected with 100-fold diluted corneal HSV-I isolates. After adsorption at 37°C for 1 h, the inoculum was removed and cells were incubated, in triplicate, with different concentrations of ACV diluted in culture medium. At 24 h after inoculation, cells were lysed and the viral DNA load in the cell lysate was determined by an HSV-I specific qPCR as described [12, 18]. The median ACV inhibitory concentration (IC₅₀) of each isolate was defined as the concentration of ACV that reduced the number of viral copies by 50% compared with the control infected cells without ACV. Isolates were considered ACV^R at ≥ 1 $\mu\text{mol/L}$ [12, 18].

Statistical analysis

All statistical analyses were run using SPSS software (IBM SPSS Statistics 20). A one-phase exponential association approach was used to assess the correlation between data obtained by qPCR IC₅₀ and plating efficiency assays. The Mann-Whitney test was used to compare gender, visual acuity, follow-up time and recurrence rates between patient groups. Spearman's correlation tests were used to assess correlations between the percentage of ACV^R viruses in corneal HSV-I isolates, duration of disease recurrence, isolate date and the duration of ACV prophylaxis before corneal sampling. Generalized estimating equations were used to identify risk factors. All statistically significant covariates from the univariate analyses were included for multivariate testing. Wilcoxon signed ranks test was used to compare the ACV susceptibility phenotype of sequential isolates. All statistical tests were significant when the P-value was <0.05.

Results

To gain insight into the effect of long-term ACV prophylaxis on the prevalence of corneal ACV^R HSV-I, we determined the frequencies of ACV^R viruses in 169 corneal HSV-I isolates from 78 immunocompetent rHK patients. Disease severity at time of sampling varied from mild IEK to necrotizing stromal keratitis. Thirty-five of 78 patients (44.5%) were female, the overall median age at presentation was 59.3 years (IQR 45.2–70.1 years) and 43.6% of affected eyes were left eyes. For 70 patients, two (n=53) or at least three (n=17) sequential isolates obtained during a HK recurrence from the same cornea were available for ACV susceptibility testing. The mean time interval between sequential isolates was 4.8 years (range 0.1–22.3 years). Nineteen patients (24.4%) did not receive ACV prophylaxis in the 12 months prior to sequential sampling of their cornea and are hereafter referred to as ACV treatment-naïve rHK patients. In case of patients receiving ACV prophylaxis within 12 months prior to sampling, the mean time on therapy was

21.5 months (range 0.7–178.4 months). The median duration of disease recurrences and the median recurrence rate were 41 days (IQR 24–70 days) and 0.9 recurrences per year (IQR 0.6–1.2 recurrences/year), respectively. Sixty-nine patients received topical steroid therapy (93.2%), for 5 patients their steroid treatment regimen was unknown.

Prevalence of ACV-resistant HSV-I in corneal isolates from rHK patients

All clinical HSV-I isolates contain ACV^R viruses and the frequency thereof determines the overall ACV susceptibility phenotype of the corresponding isolate [5–7]. Moreover, the frequency of ACV^R viruses in sequential HSV-I isolates sampled during a disease recurrence from the same anatomical site provides insights into the emergence of ACV^R HSV-I due to selective pressure of ACV therapy. We determined the percentage of ACV^R viruses in all cornea isolates by the plating efficiency assay [16, 17]. The threshold of the percentage of ACV^R viruses resulting in an overall ACV^R phenotype of the corresponding isolate was determined by measuring the acyclovir IC₅₀ values of a subset of corneal HSV-I isolates (n=36) by an HSV-I specific qPCR-based assay. The IC₅₀ cut-off value for ACV^R isolates has previously been defined at >1 µmol/L acyclovir [12, 18]. The combined data demonstrated that isolates with >28% ACV^R viruses had an overall ACV^R phenotype (Figure 1A). In total, 44 of 169 (26%) corneal HSV-I isolates were defined as ACV-resistant. Uniform sequential ACV^R and ACV^S isolates were identified in 8 and 43 patients, respectively. Acyclovir susceptibility changed in time from ACV^S to ACV^R, or ACV^R to ACV^S, in 12 and 9 patients, respectively. Twenty-seven patients (34.6%) had an ACV^R HSV-I isolate at least once.

Identification of risk factors for corneal ACV-resistant HSV-I

Statistical analyses identified parameters that were not associated with corneal ACV^R HSV-I. These factors were inter-recurrence interval (P=0.56), recurrence rate (P=0.22), age (P=0.30) and gender (P=0.74). Patients were stratified based on clinical picture. Stromal involvement at time of corneal sampling was not significantly associated with corneal ACV^R HSV-I (P=0.17).

Several clinical parameters were associated with corneal ACV^R HSV-I (Figure 1 and Table 1). A weak correlation was observed between the duration of ACV prophylaxis before corneal sampling and the percentage of ACV^R viruses in the corresponding corneal HSV-I isolates (Figure 1B) and the duration of the sampled recurrence (Figure 1C). Additionally, the percentage of ACV^R viruses in corneal HSV-I isolates was weakly correlated with the duration of the disease recurrence sampled (Figure 1D) and the year at which the corresponding corneal HSV-I isolate was obtained (Figure 1E). Univariate analyses revealed the following predisposing risk factors: isolate date after the year 2000, duration disease recurrence for ≥45 days and long-term ACV prophylaxis (Table 1). Multivariate analyses identified both duration disease recurrence of ≥45 days (odds ratio [OR] 2.23, 95% confidence interval [CI] 1.02–4.87) and long-term ACV prophylaxis prior to corneal sampling (OR 3.42, 95% CI 1.32–8.87) as independent risk factors for an ACV^R phenotype of a corneal isolate (Table 1).

Next, we analyzed parameters associated with ACV refractory rHK, herein defined as duration disease recurrence of ≥45 days. Univariate analyses demonstrated that the isolate date after the year 2000, long-term ACV prophylaxis prior to corneal sampling and

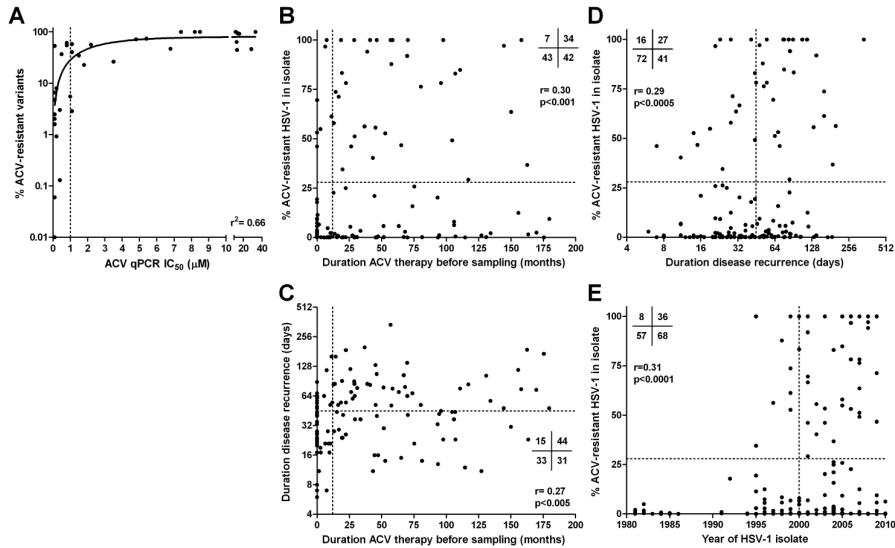


Figure 1. Correlations between acyclovir-resistant (ACV^R) corneal herpes simplex virus type 1 (HSV-1) and clinical parameters in patients with recurrent herpetic keratitis (rHK). A, A subset of corneal HSV-1 isolates ($n=36$) was subjected to an HSV-1 specific real-time quantitative PCR (qPCR), measuring the median inhibitory concentration of acyclovir (ACV) that reduced viral replication in vitro by 50% (ACV qPCR IC₅₀), and the plating efficiency assay that determines the percentage of ACV-resistant (ACV^R) variants in an HSV-1 isolate. The qPCR IC₅₀ cutoff value has been defined at $>1 \mu\text{mol/L}$ ACV [12, 18] and is indicated by the dotted line. The correlating results of both assays ($r^2=0.66$) demonstrated that HSV-1 isolates with $>28\%$ ACV^R viruses had an overall ACV^R phenotype. B and C, Correlation between duration of systemic ACV therapy before corneal sampling and (B) the percentage of ACV^R viruses in corneal HSV-1 isolates and (C) the duration of the disease recurrence sampled. D and E, Correlation between the percentage of ACV^R viruses in corneal HSV-1 isolates and (D) the duration of disease recurrence sampled and (E) the year at which the HSV-1 isolate was obtained. Dotted lines in panels B-E indicate the cut-off values applied to stratify the following parameters: 28% ACV^R viruses in a HSV-1 isolate (B, D and E), 12 months of ACV therapy before corneal sampling (B and C), disease duration of 45 days (C and D) and year 2000 at which the isolate was obtained (E). The inserts in panels B-E indicate the number of HSV-1 isolates grouped according to the quadrants marked by the dotted lines. The statistical analyses used were one-phase exponential association (A) and Spearman's correlation test (B-E).

a corneal ACV^R HSV-1 isolate were risk factors for ACV refractory rHK (Table 2). Multivariate analyses identified the ACV^R phenotype of a corneal HSV-1 isolate as an independent risk factor for ACV refractory rHK (OR 2.28, 95% CI 1.06-4.89) (Table 2). The data suggest a pathway of long-term ACV prophylaxis as a risk factor for corneal ACV^R HSV-1. Subsequent detection of ACV^R HSV-1 in rHK is a risk factor for ACV refractory disease.

Longitudinal effect of ACV prophylaxis on acyclovir-resistant corneal HSV-1

We determined the longitudinal effect of ACV prophylaxis on the ACV susceptibility phenotype of sequential corneal HSV-1 isolates for the individual rHK patients. The ACV susceptibility phenotype of sequential isolates did not change significantly in rHK patients who were either ACV treatment-naïve ($n=25$, $P=0.40$) or received ACV prop-

Table 1. Risk factors for acyclovir-resistant corneal HSV-I isolates of patients with recrudescence herpetic keratitis.

Variable	ACV ^S isolates	ACV ^R isolates	Univariate			Multivariate		
			OR	95% CI	P	OR	95% CI	P
Duration disease recurrence of ≥ 45 days	41 (33%)	27 (63%)	3.00	(1.53-5.91)	0.001	2.23	(1.02-4.87)	0.045
Isolate obtained after year 2000	59 (48%)	34 (79%)	4.16	(1.43-12.11)	0.009	2.69	(0.91-7.93)	0.073
Long-term ACV prophylaxis	31 (25%)	26 (61%)	4.38	(1.81-10.60)	0.001	3.42	(1.32-8.87)	0.012

Acyclovir (ACV) resistance of corneal HSV-I isolates was determined by the plating efficiency assay. Isolates containing $>28\%$ ACV-resistant viruses were considered ACV-resistant (ACV^R). ACV^S, ACV-sensitive isolates. Long-term ACV prophylaxis refers to systemic ACV use for ≥ 12 months before date of corneal sampling. Number and percentage of corneal HSV-I isolates with the indicated ACV susceptibility phenotype. Paired clinical parameters were available for 123 ACV^S and 43 ACV^R HSV-I isolates. Generalized estimating equations were used to determine odds ratios (OR). 95% confidence intervals (CI) and P-values are shown.

Table 2. Risk factors for a disease recurrence duration of ≥ 45 days.

Variable	Length		Univariate			Multivariate		
	<45 days	≥ 45 days	OR	95% CI	P	OR	95% CI	P
Long-term ACV prophylaxis	27 (30%)	30 (44%)	1.81	(0.93-3.55)	0.083	1.36	(0.66-2.77)	0.404
Isolate obtained after year 2000	45 (50%)	46 (68%)	2.09	(0.48-9.21)	0.049	1.70	(0.80-3.63)	0.170
ACV-resistant HSV-I isolate	16 (18%)	27 (40%)	3.00	(1.53-5.91)	0.001	2.28	(1.06-4.89)	0.034

Long-term acyclovir (ACV) prophylaxis refers to systemic ACV use for ≥ 12 months before date of corneal sampling. Acyclovir resistance of corneal HSV-I isolates was determined by the plating efficiency assay. Isolates containing $>28\%$ ACV-resistant viruses were considered ACV-resistant. Paired clinical and lab parameters were available for 90 and 68 corneal HSV-I isolates obtained from patients with the indicated disease recurrence duration. Generalized estimating equations were used to determine odds ratios (OR). 95% confidence intervals (CI) and P-values are shown.

hlysis during all sampling dates ($n=24$, $P=0.68$). Twenty-four patients (30.8%) had corneal HSV-I isolates obtained during alternating ACV treatment regimens. The prevalence of corneal ACV^R HSV-I did not change in patients from whom only the first isolate was obtained during ACV prophylaxis ($n=6$, $P=0.44$). Notably, patients who first were ACV treatment naïve and had ACV prophylaxis during consecutive sampling dates had an increased prevalence of corneal ACV^R HSV-I ($n=18$, $P=0.02$). The data suggest that ACV prophylaxis of rHK patients predisposes for corneal ACV^R HSV-I in time.

Discussion

Acyclovir prophylaxis significantly reduces the recurrence rate of herpetic keratitis [10]. This is of particular benefit to HK patients with a history of stromal involvement as they

are at increased risk to develop recurrent HSK that can lead to corneal blindness [11]. The current study suggests that long-term ACV prophylaxis is an important risk factor for ACV refractory rHK due to the emergence of corneal ACV^R HSV-I. Thirty-four of 76 (44.7%) corneal HSV-I isolates obtained during a breakthrough recurrence from rHK patients who received >12 months ACV prophylaxis were ACV^R (Figure 1B).

Previous reports on immunocompetent patients with non-ocular HSV-I infections have demonstrated that antiviral treatment was not associated with an increased risk for the emergence of ACV^R HSV-I [13, 19-21]. Nonetheless, ACV^R HSV-I isolates have been obtained after prolonged ACV treatment and appeared to correlate with treatment failure [22, 23]. Moreover, ACV resistance can revert when therapy is ceased [22]. The latter study described that ACV resistance in an immunocompetent individual developed in the setting of immunosuppressive drugs and sub-optimal ACV treatment [22]. The host's immune status, and in case of herpetic keratitis the infected organ, most likely attributes to the discrepancies found. In otherwise healthy individuals with non-ocular HSV-I infections, like herpes labialis and genital herpes, virus replication in infected tissues is largely controlled by local immune responses in which ACV therapy has a supportive role [19-21, 24, 25]. In contrast to the oral and genital mucosa, the cornea is an immunoprivileged site aimed to dampen local immune responses thereby preserving visual acuity [26]. Consequently, the role of a local immune response to control corneal infections is more restricted, resulting in a favorable environment for an ACV therapy-driven enrichment of corneal ACV^R HSV-I [26].

Asymptomatic shedding of infectious HSV-I and HSV-I DNA in tears of healthy individuals without a history of herpetic ocular diseases has been described [27-29]. A recent study, performed on tear samples collected twice-daily for 30 consecutive days, demonstrated HSV-I DNA in tears of 47 (94%) of 50 healthy study participants at least once [29]. The studies document that HSV-I reactivation more commonly leads to asymptomatic corneal HSV-I shedding compared to corneal disease [27-29]. Notably, valacyclovir treatment (500 mg daily) of healthy individuals did not reduce the asymptomatic HSV-I shedding rate and viral DNA load in tears during treatment for 30 days [30]. Corneal HSV-I isolates contain a mixed virus population, consisting of both ACV^S and ACV^R genetically related variants [5]. Consequently, ACV prophylaxis may lead to the enrichment of ACV^R HSV-I during asymptomatic corneal shedding and subsequently the development of ACV refractory rHK. The presence of latent ACV^R HSV-I in the cornea-innervating trigeminal ganglion poses a life-long risk of subclinical corneal shedding and symptomatic HK recurrences involving reactivated ACV^R HSV-I [5, 27-30].

The current study has two limitations. First, the study design did not allow verification of patient compliance. Several patients reported adverse effects of long-term ACV prophylaxis, mainly gastrointestinal complaints and drowsiness, which may have affected their adherence to therapy. Patients' non-compliance can influence the emergence of drug-resistant viruses during therapy [31, 32]. Second, all rHK patients included had extensive clinical records with frequent rHK episodes with stromal disease and are probably at increased risk for ACV resistance due to their extensive ACV treatment history. Indeed, we identified that about one-third of the patients had an ACV^R HSV-I corneal isolate at least once, which is higher than reported previously for HK patients [5, 12]. Further-

more, the inability to detect a correlation between the proportion of corneal ACV^R HSV-1 and the severity of disease at end of follow-up may also be due to the selection of severe rHK patients (data not shown). Future studies on a larger cohort of rHK patients, including patients with solely HSV-1 epithelial keratitis, are mandatory to address these issues.

In conclusion, the data presented argue for the rationalized use of long-term ACV prophylaxis for rHK patients. The associated risk of the development of ACV refractory disease due to the emergence of corneal ACV^R HSV-1 in time should be considered when choosing the appropriate antiviral treatment in rHK patients with a history of stromal disease, a patient group prone to long and extensive antiviral and steroid treatment regimens. Moreover, the unapparent cost-effectiveness of ACV prophylaxis to reduce ocular HSV recurrences implicates that therapeutic decisions for rHK must be made on a case-to-case basis [36]. When the integrity of the eye is not at risk, the combined data favor the use of discontinuous ACV prophylaxis, i.e. stop treatment after a maximum of 1 year [10,11], and more aggressive therapeutic intervention of a subsequent recurrence. Multi-drug therapy combining different mode of drug actions is common practice in the treatment of other viral infections [33, 34]. Combination therapy with anti-HSV drugs with discordant modes of action, such as systemic and topical ACV combined with topical foscarnet or trifluorothymidine, can be of significance [39]. This will potentially result in a shorter disease duration and may lower the risk of emergence of drug resistant viruses during therapy. Finally, antiviral drug sensitivity testing of corneal swabs is indicated for rHK patients with recurrent disease during ACV prophylaxis to guide the rationalized selection of the appropriate alternative antiviral agents.

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The background is a light gray with a textured, watercolor-like appearance. It features several overlapping circles of various sizes and shades of gray. A large, bright white circle is the central focus, surrounded by a thick, dark gray ring. Other circles of different diameters are scattered around, some with white outlines or centers, creating a complex, layered geometric pattern.

CHAPTER 8

Summarizing Discussion

Summarizing Discussion

Symptoms of herpes simplex virus type 1 (HSV-1) infections range from mild herpes labialis to sight-threatening ocular disease and life-threatening hepatitis or encephalitis in susceptible hosts. In immunocompetent individuals, virus replication in epithelial cells is effectively controlled. However, HSV-1 is never cleared from the body, as it establishes latency by infecting peripheral sensory neurons innervating the site of infection [1]. HSV-1 latency is characterized by limited viral gene transcription with no infectious virus being produced. Viral replication is monitored locally and in part controlled by a virus-specific memory T-cell response [2-4]. Nevertheless, intermittent viral reactivation does occur and virus is shed at the periphery. HSV-1 reactivation is commonly asymptomatic, but may cause symptoms that require specific antiviral treatment, especially in immunocompromised patients [1, 5, 6]. Antiviral treatment can also be given as prophylaxis to prevent recurrent HSV-1 infections [7-10]. Therapeutic or prophylactic antiviral treatment of HSV-1 infections is particularly warranted when ocular disease is involved [7, 11]. Recurrent ocular HSV-1 infections cause extensive morbidity and can lead to blindness [12, 13]. However, long-term antiviral treatment poses a risk of antiviral drug resistance and recurrent therapy-refractory disease [14]. The research described in this thesis was aimed to study the within host control of HSV-1 infections.

Immune control of HSV infections

During latency, HSV persists in sensory neurons innervating the site of primary infection. HSV-1 infections are commonly acquired via the orofacial route, resulting in a latent infection of the sensory neurons in the trigeminal ganglion (TG) [1]. Latently infected neurons express viral latency-associated transcripts (LAT), which appear to have anti-apoptotic and T-cell modulating functions [15-19]. The latter can be of importance, as T-cells have been found juxtaposed to HSV-1 latently infected neurons [2-4]. These T-cells, both CD4 and CD8 T-cells, have an effector memory phenotype but do not seem to destruct latently infected neurons [4, 20-23].

Most of the information on the role of T-cells during HSV-1 latency is obtained through the use of the experimental C57BL/6 mouse model [24, 25]. T-cells retained within the TG of latently infected mice produce interferon gamma (IFN- γ) and granzyme B and thereby inhibit viral replication in a non-cytolytic manner [26-28]. The majority of the CD8 T-cells in TG of latently infected mice are directed to HSV-1 antigens [24, 29]. T-cells recognize their cognate antigen-derived peptides bound to major histocompatibility complex (MHC) molecules. In humans, the MHC molecules are referred to as human leukocyte antigen (HLA) proteins and the HLA-encoding genes are highly polymorphic. The extensive polymorphisms in HLA class I and II loci facilitate a wide variety of antigens to be presented to T-cells. The HLA loci commonly used to presented peptides to human CD4 and CD8 T-cells are HLA-DP, -DQ and -DR, and HLA-A, -B and -C, respectively. For all MHC loci two different alleles may be expressed by antigen presenting cells. The inbred mice that were used for HSV-1 experimental infection studies are homozygous for all MHC loci. This restricted variability leads to a strong bias in the repertoire of virus-specific T-cells in experimentally infected mice. Despite the large DNA genome

of HSV-I encoding for 74-77 viral proteins [1], the majority of the HSV-I specific CD8 T-cells in TG of infected mice are directed towards a single epitope of glycoprotein B [29, 30]. Other HSV-I antigens recognized by TG-infiltrating T-cells in C57BL/6 mice are encoded by the early viral genes UL29 and UL39, but no immediate early viral proteins appear to be targeted [31, 32]. If TG-resident T-cells block HSV-I replication at an early stage, it is expected that they are specific for viral proteins expressed with immediate early and early kinetics.

Despite the extensive knowledge gained by studying HSV-I specific T-cells in mice, humans are the natural host and reservoir of HSV-I. This is of significance when studying pathogen-specific immunity since, in contrast to the mouse models, virus and host have co-evolved over millions of years. The first studies on HSV specific T-cells in humans focused on T-cells recovered from HSV-induced lesions in the eye and genital skin. It was shown that these tissue-infiltrating T-cells recognized diverse viral proteins, of which tegument proteins were major targets [33-38]. However, these studies focused on few HSV-I proteins. The repertoire of HSV-I specific T-cells was recently explored in great detail using an approach encompassing all open reading frames of the HSV-I genome. Proteins encoded by HSV-I genes UL39 and UL46 were dominant CD4 and CD8 T-cell targets in peripheral blood of humans and involved multiple distinct HLA alleles [39]. To boost TG-resident HSV-I specific T-cell immunity, HSV-I antigens that are recognized by TG-resident T-cells are rational candidates for incorporation in HSV-I subunit vaccines. The expression and accessibility of viral antigens in the latently infected TG are largely unknown. Therefore, in *Chapter 2*, we have identified and characterized the HSV-I proteins recognized by T-cells from latently infected human TG. Our results demonstrate that TG-resident HSV-I specific CD4 and CD8 T-cells recognize a limited number of viral proteins. HSV-I specific CD8 T-cells localize in close proximity to neuronal cell bodies, suggesting recognition and control of HSV-I replication in situ. The recognized HSV-I antigens did not cluster to a specific kinetic class, suggesting that the protein expression profile in latently infected human TG is unbiased. A recent study demonstrated that blood-derived HSV-I specific CD8 T-cells recognized 17 antigens on average per individual [39], whereas TG-derived CD8 T-cells recognized one to three HSV-I antigens per individual (*Chapter 2*). This discrepancy can result from the selection of appropriate reactivation-controlling T-cell clones that were selected and retained in the TG. Alternatively, they could reflect an unbiased selection of T-cell clones, representative from the blood-circulating T-cell repertoire. Blood and TG HSV-I specific T-cell repertoires should be studied in parallel to address this issue. The observations of T-cell specificities across all HSV-I kinetic gene expression classes can reflect discordant gene expression during latency. Recent literature suggests that the regulated temporal gene expression during productive infection may be disordered in latently infected murine ganglia [40]. Consequently, viral antigens expressed within latently infected TG can be of all kinetic classes. Future studies should address the antigen expression kinetics during the transition from latency to reactivation, and elucidation of the transfer mechanism of viral antigens within the TG will reveal the role of local antigen presentation to maintain the antiviral CD4 and CD8 T-cell response in situ. Through the identification of HSV-I antigen-specific CD4 and CD8 T-cells in latently infected human TG, we demonstrated that the TG is an immunocompetent organ maintaining local antiviral immunity in the face of constant viral reactivation (*Chapter 2*).

Local antigen presentation is crucial for activation, maintenance and modulation of an effective tissue-resident antiviral T-cell response. Given their neuron-interacting location, satellite glial cells (SGC) are likely to be key players in the neuron–T-cell cross-talk controlling HSV-I latency. SGC are known to provide mechanical and nutritional support to the neuronal cell bodies [41, 42]. *Chapter 3* describes the study on the phenotype and function of human TG-resident SGC in the context of antigen presentation. We demonstrated that human TG-resident SGC express markers specific for macrophages and dendritic cells, and TG-SGC are likely the key antigen presenting cell of sensory ganglia [43]. Moreover, human TG-SGC express proteins that can inhibit T-cell function. We propose that TG-SGC cross-present virus-derived antigens expressed in neurons to TG-infiltrating virus-specific T-cells (*Chapters 2 & 3*). In line with observations that HSV-I latently infected neurons encountered by T-cells are undamaged [3, 4], TG-SGC likely play a crucial role in the inhibition of cytolytic T-cell effector functions. Elucidation of the T-cell inhibitory mechanisms used in the peripheral nervous system will provide tools for the development of therapeutic intervention strategies to counteract undue cell damage associated with T-cell mediated chronic diseases of the nervous system, like multiple sclerosis. Therapeutic blockage of T-cell inhibitory mechanisms may enhance the local antiviral response [44, 45]. Moreover, the innate control that TG-SGC can exert on HSV-I replication in neurons is an important target of therapeutic intervention. Like macrophages and dendritic cells, TG-SGC may express innate antiviral cytokines such as type I interferons that limit viral replication in neurons. The stimulation of innate receptors on TG-SGC may create a micro-environment that blocks HSV-I reactivation and/or viral replication in neurons.

Vaccination remains the most cost-effective method to limit viral infections. Several issues should be taken into account when developing an HSV vaccine. First, stimulation of neurons, SGC or other resident cells will likely enhance the innate control of viral replication in sensory ganglia. This can be accomplished by addition of innate immunity stimulators to a vaccine formulation [46–49]. These can include adjuvants that bind pattern recognition receptors, such as Toll-like receptors, to boost the innate immune response and increase the host's ability to clear the virus [47–49]. Moreover, addition of adjuvants that skew the concurrent adaptive immune response towards a Th1 subtype may enhance the antiviral immune response [50, 51]. Second, specific targeting of SGC to enhance their antigen presenting capacities or T-cell modulating properties can boost the concurrent local adaptive immune response necessary to control HSV-I replication. Third, specific targeting of HSV-specific T-cells can boost the TG-specific immune response and decrease the frequency and load of peripheral virus shedding. Identification of HSV-I antigens recognized will aid in the design of a subunit vaccine capable of boosting the TG-resident T-cell population.

Vaccine development for HSV is focused on HSV-2, since it is associated with an increased risk of HIV acquisition [52–54]. Several HSV-2 vaccines that have been tested in humans aimed to reduce the frequency and severity of recurrences [53, 55, 56], and these studies focused on glycoproteins gD and gB as target antigens [55, 56]. Despite the fact that gD and gB are T-cell and B-cell antigenic targets in humans [33, 57, 58], vaccine design restricted to these antigens appeared to be too limited to have clinical effects. Vaccine-induced virus-specific antibodies did not prevent infection and did not

decrease the rate of recurrences [59-61], suggesting that vaccination with HSV antigens specifically targeted by T-cells would be necessary to enhance protection against infection or recurrences [39, 62]. Moreover, a vaccine covering both HSV-1 and HSV-2 would be desirable. HSV type-common T-cell epitopes have been described which are identical in amino acid sequence and could theoretically boost HSV-1 and HSV-2 specific T-cell immunity [39, 62-65]. To this end, virus-specific T-cell immunity in hosts co-infected with HSV-1 and HSV-2 should be studied in great detail. Candidate antigens for an HSV subunit vaccine should include proteins expressed during differential kinetic expression classes. As such, T-cells are induced or boosted that can interfere at multiple stages of the viral replication cycle. To block HSV replication at an early stage, immediate-early antigens such as ICP0 should be included in a subunit vaccine. Moreover, HSV proteins encoded by the genes UL39, and UL46 to UL48 have been shown to be common targets of HSV-specific T-cells and should therefore be included in subunit vaccines [33-36, 39]. Last, inclusion of glycoproteins in a subunit vaccine can boost virus-specific T-cells as well as B-cells producing neutralizing antibodies to these glycoproteins [33, 39, 57, 58]. If viral gene expression in latently infected ganglia is disordered [40], inclusion of T-cell antigens in subunit vaccines should encompass all kinetic expression classes.

Boosting immunity at sites of HSV latency is an approach to prevent reactivation and peripheral shedding. By extension, boosting immunity necessary to clear productive infections at peripheral sites can intervene with recurrent disease and transmission to susceptible persons. These goals are met in a vaccine that boosts TG-resident as well as mucosa-resident T-cell immunity and future studies should therefore address the role of mucosal immunity in the control of viral replication at the nerve ending/skin border. Local application of adjuvants boosting mucosal immunity can be an approach to limit viral replication at sites where frequent reactivations occur. One of the frequent concerns in the design of HSV vaccines is that the accomplishment of sterilizing immunity is not feasible for the following reasons. First, because HSV can easily access numerous epithelial cells at mucosal borders, HSV-specific antibodies in the mucosa will almost always be at insufficient levels and localization to neutralize virus prior to cell entry. Second, to efficiently limit viral replication and subsequent virus entry into mucosa-innervating nerve endings, virus-specific T-cells should be present at all mucosal sites where HSV can enter the body [53, 66]. Because these goals are unlikely to be met, current vaccine strategies aim at decreasing the latent viral load and the risk of reactivation in infected individuals [62, 67]. A lower ganglionic viral load will decrease the risk of peripheral shedding and transmission [4, 68, 69]. Blocking viral replication by virus-specific T-cells is therefore necessary at the level of the neuronal cell bodies in the ganglion as well as at nerve endings in the mucosa.

Current vaccine approaches are largely aiming at the identification of specific T-cell antigenic targets in HSV-seropositive individuals that experience asymptomatic recurrent viral shedding [67, 70, 71]. If such "asymptomatic" T-cells exist, their cognate viral antigens could be crucial subunit candidates for effective vaccines. Symptomatic and asymptomatic patients shed HSV-2 at similar rates [72, 73], suggesting that the number and nature of targeted antigens influence the rate of reactivation and clinical disease.

Lessons can be learned from the currently used vaccine against varicella-zoster virus (VZV), another member of the alpha-herpesviruses. A live-attenuated VZV vaccine was empirically introduced in children in 1974 [74]. The vaccine prevents varicella in children and reduces the incidence of zoster in the elderly [74, 75]. However, the vaccine establishes latency in the sensory ganglia of the host [76], posing the risk of reactivation and transmission [77-80]. For VZV, a clear correlation between ageing, waning T-cell mediated immunity to VZV, and the risk of developing herpes zoster has been established [81]. VZV-specific antibody levels are not correlated with the risk of developing herpes zoster [82]. Hence, boosting of VZV-specific T-cell immunity explains the success of the vaccine in reducing VZV-associated morbidity. Extrapolating these data to an HSV live-attenuated vaccine is likely to have similar success, because of the association of HSV-specific T-cells with latently infected neurons. Moreover, the detected diversity of the HSV-specific T-cell response in humans [39] implies that a whole virus based vaccine may be more suitable for vaccination than HSV subunit vaccines. From a kinetic protein expression point of view, a live-attenuated virus vaccine approach is most likely to mimic the immune response to a natural infection. However, current safety concerns associated with live-attenuated vaccines hamper the development of such a platform for HSV.

The longevity of vaccine efficacy is influenced by multiple factors. These include vaccine dose and the subject's health status and age [83]. Understanding how age and health status impact vaccine-induced immunity in humans appears to be crucial for the development of an effective HSV vaccine in individuals with viral reactivations in the presence of antiviral immunity [84]. Moreover, chronic virus infections have a devastating impact on the available T-cell repertoire [85, 86]. The T-cell repertoire in the chronically infected host will inflate due to repetitive antigenic stimulation, which is characterized by exhausted T-cells that will eventually be deleted [86]. Consequently, a limited number of virus-specific T-cell clones may persist in long-term infected hosts that may be difficult to boost through vaccination [86]. Despite the apparent increase of HSV reactivations during HIV progression and decreasing T-cell immunity [87, 88], advancing age and waning of virus-specific T-cell immunity are not associated with an increased HSV reactivation rate. Future studies should address questions on the quantity and quality of HSV-specific T-cells and how these can be boosted by vaccination. As such, it is expected that the greatest effect of a therapeutic HSV vaccine can be gained in otherwise healthy young adults who suffer from frequent symptomatic recurrences. Induction of complete and protective HSV-specific immunity in naïve subjects will be more complicated. The quantity and timing of viral antigens expressed during natural infection is difficult if not impossible to mimic with a non-replicating vaccine [39]. A live-attenuated HSV vaccine is likely to have the best protective effect in children between 1 to 12 years of age. Within one year of life, maternal anti-HSV antibodies have disappeared and the pediatric immune system has matured to cope with complex live-attenuated vaccines. The timing of vaccination would be based on experience with VZV vaccinations, which are common practice in the United States [89]. Induction of anti-HSV immunity through subunit vaccination in naïve individuals may limit primary infections and the latent HSV load [62].

In conclusion, defining targets for innate and adaptive immune control of HSV-I latency in humans will facilitate the development of a therapeutic vaccine. Vaccination goals primarily lie in boosting mucosal and ganglion-resident HSV-specific immunity thereby

minimizing the frequency of HSV reactivation, preferably for HSV-1 and HSV-2 simultaneously.

Prevention of infection can be accomplished by avoiding contact with infectious secretions from shedding individuals. In case of symptomatic disease, HSV infections can be treated with antiviral drugs to contain infection and limit viral transmission. Therapeutic or prophylactic treatment of HSV-1 infections is especially warranted for people at risk for life- and sight-threatening infections and for immunocompromised individuals. The latter group of patients suffers from more frequent and more severe clinical disease compared to immunocompetent persons. In contrast, oral shedding episodes in immunocompromised patients are cleared surprisingly rapid [87, 88, 90]. Shedding of HSV-1 and VZV, which are both latent in the same TG [4, 69], may be interrelated. Therefore, in *Chapter 4* we studied HSV-1 and VZV shedding in saliva of HIV-infected HSV/VZV-seropositive patients. We demonstrated that buccal shedding episodes were more frequent for HSV-1 than for VZV. The overall prevalence of VZV shedding was too low to draw conclusions on whether shedding of both viruses was interrelated. The increased frequency of HSV-1 shedding in HIV-infected patients could be explained by a higher ganglionic HSV-1 load, compared to VZV [4, 68, 69]. Moreover, HSV-1 and VZV express differential determinants of latency. HSV-1 expressed LAT and microRNAs, which are not shared by VZV, and these discrepancies could contribute to differential reactivation patterns between immunocompetent and immunocompromised individuals [19, 91]. Last, T-cells are considered pivotal to control HSV-1, but not VZV latency in human TG suggesting that different immune mechanisms are at play to control latency of both human alpha-herpesviruses [3, 4].

The research described in *Chapter 4* demonstrated that sequential shedding of HSV-1 in the individuals studied was due to the same virus strain, which can be resistant to the commonly used antiviral drug acyclovir (ACV). These data provide important information on the latency-reactivation cycle of HSV-1 and future studies should address the molecular mechanisms underlying the different shedding kinetics between HSV-1 and VZV. Despite observations indicating that a single neuron can be infected by both HSV-1 and VZV [69], control of the two viruses are most likely dissimilar. This is reflected by the differential presentation of recurrent disease. VZV reactivation, known as herpes zoster, typically occurs very infrequently or even just once in a life time [92], whereas HSV-1 DNA has been detected once every month in saliva of asymptomatic individuals [93]. Increased (a)symptomatic shedding may increase the latent HSV-1 burden in the innervating TG, thereby increasing the risk of viral transmission [94-96]. The host may exploit differential mechanisms to control a virus reactivating once in a life time, such as VZV, or a frequently reactivating virus, such as HSV. On the other hand, studies on the control of VZV latency may be beneficial for the knowledge on the control of HSV-1 latency. VZV latently infected neurons are not surrounded by clusters of T-cells [4], which phenomenon has also been observed for over 85% of HSV-1 latently infected neurons in human ganglia [97]. These HSV-1 LAT positive, T-cell negative neurons may control viral latency at levels similar to the control of VZV latency. HSV-1 latently infected neurons surrounded by T-cells likely express viral antigens beyond the expression of LAT [97]. The observation of HSV-1 specific neuron-interacting T-cell clusters may reflect the increased HSV-1 reactivation frequency compared to the situation with VZV. Future stu-

dies on host factors involved in the control of HSV and VZV latency should reveal these differential host control mechanisms.

Therapeutic interventions for HSV infections

In case of symptomatic disease, HSV infections can be treated with antiviral drugs to contain infection and limit viral transmission [1, 7, 8, 90]. Antiviral treatment of herpes labialis in immunocompetent hosts is rarely warranted. However, frequent and complicated HSV infections can effectively be treated by antiviral drugs. Two classes of anti-herpesvirus drugs have been developed. The first class are nucleoside analogues, which are pro-drugs requiring phosphorylation by viral thymidine kinase (TK). Incorporation of phosphorylated nucleoside analogues by viral DNA polymerase results in a block of viral replication. The prototypic drug in this class of antivirals is ACV, discovered due its enhanced specificity for viral TK protein rendering the drug specific and safe. The excellent toxicity profile has led to the widespread use of ACV in the treatment of severe epithelial and ocular HSV disease. In addition to its therapeutic use, ACV can be given as prophylaxis to prevent HSV recurrences [7, 8, 98]. The second class of antivirals used to treat HSV infections constitutes direct inhibitors of viral DNA polymerase, such as foscarnet (FOS) or cidofovir.

Long-term treatment with antiviral drugs has raised concerns related to the development of antiviral resistance. Resistance is most common in immunocompromised patients, illustrating the contribution of immunity to timely viral clearance [14, 99-101]. In line with its mechanism of action, specific mutations in the viral genes encoding TK and DNA polymerase have been associated with ACV resistance [102, 103]. Establishment of latency by an ACV-resistant (ACV^R) HSV-I could foster recurrent ACV refractory disease. Therefore, in *Chapter 5*, we have studied the presence of ACV^R HSV-I in latently infected TG of HSV-I seropositive immunocompetent individuals [104]. We demonstrated that HSV-I is present as a donor-specific quasispecies in latently infected TG. A major HSV-I TK variant was shared between the left and right TG of the same donor, strengthening the dogma that a single HSV-I strain colonizes the TG and persists as a perennial source of latent virus [105, 106]. In addition, multiple minor HSV-I TK variants were detected that were present unilaterally and were phylogenetically related to the cognate major TK variant. These findings suggest that the minor variants are the result of intrahost virus evolution and probably evolved from the major variant during sub-clinical reactivation within the TG. Several minor ACV resistance-associated HSV-I TK variants were detected in latently infected TG. The minor ACV^R TK variants could have emerged as a result of selective pressure by ACV treatment and/or could be the result of the HSV-I TK polymorphic nature. The HSV-I TK gene is the most polymorphic gene of the HSV-I genome [107]. This is caused by random mutations that occur in the TK gene during genome replication that preserve protein function. During virus replication, progeny viruses can emerge which display a heterogeneous mixture of functional TK variants. These viral quasispecies were detected in latently infected TG, but have also been described in HSV-I infected epithelial isolates and in HSV-I keratitis isolates [104, 108]. HSV-I variants with mutated, non-functional TK have limited pathogenicity and are im-

paired in their ability to reactivate from latency in experimentally infected animals [109, 110]. However, in humans, TK-negative ACV^R HSV-I may reactivate by itself, or functional TK from a co-reactivating ACV-sensitive HSV-I variant could complement in trans to allow spread to the innervated periphery, leading to recurrent disease [111, 112].

Anti-herpesvirus drugs have been of major value in the field of ophthalmology. In herpetic epithelial keratitis and stromal keratitis, ACV is applied to accelerate the elimination of replicating virus from the cornea [11, 113]. Corneal HSV-I infections are the leading cause of infectious blindness, due to its recurrent nature and the devastating consequences of corneal scarring and progressive loss of vision [114]. The prevalence of ACV resistance in herpetic keratitis is relatively high [115] and can be re-isolated from the same cornea during a subsequent recurrence [108]. These data, combined with the data presented in *Chapter 5*, demonstrate that ACV^R HSV-I is able to establish latency and reactivate causing recurrent ACV-refractory corneal infections in otherwise healthy individuals. The research described in *Chapter 6* extends on these studies and reports on the relatively high prevalence of ACV resistance in immunocompetent HSV-I uveitis patients. HSV-I uveitis is due to a cytopathic effect of the virus on resident cells followed by a chronic inflammatory response to the inciting virus [13]. We have demonstrated a relatively high prevalence of ACV^R HSV-I uveitis, which may have important clinical consequences. In addition, we showed that HSV-I uveitis isolates were composed of ACV sensitive and ACV^R HSV-I variants analogous to epithelial isolates and herpetic keratitis isolates [108]. Antiviral treatment can lead to the emergence of drug-resistant variants when ACV^R HSV-I quasispecies overgrow the sensitive variants during ACV therapy. Moreover, exposure of latent HSV-I to systemic ACV during therapeutic or prophylactic use can select for ACV^R HSV-I variants during subclinical reactivation occurring in the innervating latently infected TG. The unprecedented high prevalence of ACV resistance in the studied group of HSV-I uveitis patients was not associated with a worse clinical outcome and did not correlate with ACV treatment (*Chapter 6*). Studies on larger, more homogenous groups of herpes uveitis patients are warranted to determine an association between ACV^R HSV-I, ACV treatment and clinical outcome.

Systemic antiviral prophylaxis is associated with a decrease in the recurrence rate of HSV-I keratitis [7]. However, this beneficial effect of prophylactic antiviral medication was only observed during the period of antiviral administration. The subsequent observation period demonstrated similar recurrence rates, independent of previous placebo or antiviral use [7]. Previous studies identified no increased risk for the development of ACV resistance in patients who have been on prophylactic therapy versus therapy-naïve individuals [116, 117]. However, these studies described infections of the skin, which is not immunoprivileged and in which infiltrating immune cells contribute to rapid viral clearance. In contrast, ACV^R HSV-I isolates have been isolated from ocular and genital lesions after prolonged ACV treatment and correlated with treatment failure [108, 115, 118, 119]. The immunoprivileged cornea is avascular and tissue-resident cells express immunosuppressive factors [12]. As such, the cornea may provide the optimal environment for the ACV-driven enrichment of ACV^R HSV-I. The study described in *Chapter 7* aimed to identify risk factors for the development of ACV refractory HSV keratitis in patients with recurrent disease. To this end, ACV susceptibility test outcomes were correlated with clinical parameters. We demonstrated that long-term systemic ACV prophylaxis

was a risk factor for the development of ACV^R recurrent herpetic keratitis. Moreover, ACV^R corneal isolates were associated with longer recurrence duration. These data were acquired by studying recurrences of HSV-I keratitis patients recruited at the Rotterdam Eye Hospital (Rotterdam, the Netherlands), which is a referral hospital treating patients with complicated and severe ocular diseases. As such, patients with a disease history of severe recrudescence herpetic keratitis with stromal involvement were included for the study described in *Chapter 7*. Patients received extensive systemic ACV treatment explaining their increased risk to develop ACV resistance. In this study, the clinical picture was unrelated to the presence of ACV resistance. Previous work has demonstrated that ACV^R HSV-I keratitis was associated with a more severe clinical picture, identifying the need for ACV susceptibility screening to monitor resistance patterns of sequential corneal HSV-I isolates [108, 115]. The data demonstrate that the patient cohort described in *Chapter 7* was biased towards clinically more severe cases. The ACV resistance-associated increase in recurrence length suggests that therapy-refractory ocular HSV-I infections are more difficult to contain and antiviral susceptibility testing of the corneal HSV-I strains shed in this group of patients is warranted.

The patient cohort studied in *Chapter 7* experienced HSV-I recurrences despite antiviral therapy. Prophylactic ACV treatment regimen was empirically chosen based on studies by the Herpetic Eye Disease Study group [7]. It is not known whether higher doses, shorter prophylactic treatment period, or treatment with other antiviral agents would have differential effects on reducing the incidence of herpetic keratitis recurrences [7, 120]. Nevertheless, drug susceptibility monitoring in this vulnerable group of patients is especially warranted to limit therapy-refractory disease, and avert corneal morbidity. Antiviral drug testing of corneal HSV-I strains shed is warranted to select the appropriate antivirals, especially if the disease course is lengthened and before prophylaxis is initiated. The established association of numerous viral TK mutations with antiviral resistance of the respective HSV-I strain indicates that TK-based genotyping is a valuable diagnostic tool to assess antiviral drug sensitivity without the need for virus culture. To this end, viral TK mutations conferring antiviral resistance should be studied and tested in greater detail. Moreover, a database should be assembled describing all known HSV TK and DNA polymerase mutations and their identified functional impact.

Alternative antiviral drugs are available to treat ACV^R HSV-I infections. Ganciclovir was initially developed to treat cytomegalovirus infections, but has also excellent activity against HSV-I [121-123]. Despite the common mechanism of action between ACV and GCV, cross-resistance to both antiviral drugs can be limited [108, 124]. Moreover, TK-independent antivirals, such as trifluridine or FOS, are indicated when ACV and related antiviral drugs fail in clinical practice [14, 108, 125]. Synergistic use of a combination of anti-herpesvirus drugs can be highly effective at blocking viral replication. Combinatory drug therapy is applied in other chronic viral infections, and has been shown to limit the emergence of antiviral drug resistance during therapy [126, 127]. To this end, viral TK and DNA polymerase mutations associated with cross-resistance to multiple anti-herpesvirus drugs should be characterized. The resulting mutation-describing databases will have important clinical consequences for patients suffering from therapy-refractory disease, because they can help in switching to adequate antiviral therapy to limit clinical symptoms in a timely manner.

The data described in *Chapter 7* suggests that the efficacy of ACV or analogues as prophylaxis for herpetic keratitis patients should be re-evaluated. Although a reducing effect on the recurrence rate has been clearly demonstrated, the effect did not last after prophylaxis was ceased [7]. Moreover, antiviral pressure can foster the emergence of drug-resistant viruses. In addition, long-term antiviral use has been associated with side effects [7, 128, 129] and can lead to reduced patient compliance. These issues support symptomatic rather than prophylactic treatment for herpetic keratitis. Prophylaxis should be considered on a case-by-case basis, and is probably most warranted in patients with recurrent keratitis with stromal involvement, or in patients with lesions that affect the visual axis. Moreover, patients at risk for a recurrence, for example due to immunosuppressive therapy, can be considered for antiviral prophylaxis. For all patients, antiviral susceptibility testing before prophylaxis is indicated to select the appropriate antiviral therapy and to limit the emergence of drug resistance. Moreover, an increased disease length despite therapy is indicative for antiviral resistance and microbiologists and ophthalmologists should be aware of the clinical signs and determine the antiviral susceptibility of the corneal HSV-I strain shed. The costs of long-term antiviral therapy [130], together with incomplete protection because of compliance problems and the consequential emergence of drug-resistant HSV strains, suggest that an effective therapeutic vaccine may be a better approach to decrease HSV reactivations and limit recrudescence of ocular herpetic disease.

Current efforts towards control of HSV-I infections

Experimental animal models have been used extensively to study HSV-I infection, latency, reactivation and shedding. In particular the mouse model is preferred because of the availability of inbred and knockout strains, and research reagents to study antiviral immune responses. However, unlike humans, recurrent HSV-I shedding and disease does not occur in mice because HSV-I reactivation from latency is extremely rare in mice [131]. Therefore, the efficacy of therapeutic vaccines can not be reliably assessed in experimentally infected mice. This is exemplified by studies identifying antigenic HSV targets in experimental animals [132-134] that failed to demonstrate protective effects in subsequent human clinical trials [55, 56, 59, 60]. Mathematical modeling analyses have suggested that a vaccine with limited efficacy at preventing primary HSV infection can have a substantial effect on HSV epidemiology [135-137].

The pattern of viral shedding in rabbit eyes resembles that of humans most closely [138-140], and rabbits develop similar stromal eye disease upon experimental ocular HSV-I infection [141-143]. The development of transgenic rabbits expressing HLA antigens can enhance our understanding on the antiviral T-cell response in humans [144], but the lack of analogy with other components of the antiviral host response precludes the development of therapeutic vaccines for HSV-I disease in these “humanized rabbits”. In conclusion, the long co-evolution of HSV with humans limits the power of animal models to address important questions on HSV pathogenesis and control. One live-attenuated HSV vaccine has been tested in humans, which was associated with decreased self-reported recurrence rate compared with placebo [145]. The observation period in this study was relatively short, and viral load was not assessed.

Novel vaccination strategies under development commonly aim at HSV-2 because of its association with increased risk of HIV acquisition [52-54], but approaches may also apply for HSV-1 vaccine development. The recent change in HSV epidemiology towards increased genital HSV-1 compared to HSV-2 infections demonstrates that it is necessary to shift gears towards HSV-1 vaccine development [146, 147]. Targeting of latent viral genomes by cleavage enzymes are currently under investigation, an approach that theoretically could lead to eradication of the latent virus [148]. The genes encoding these cleavage enzymes can be delivered by HSV-based or adenovirus vectors [149, 150], but future studies are needed to study their true potential in vivo.

Gaps in the knowledge on the control of HSV infections in humans can be filled in by addressing the following topics. First, information on virus strain genotypes shed in tear fluid and saliva of the same individual will reveal the distribution of viruses throughout the three branches of the TG. Moreover, coinciding symptoms can provide information on the pathogenicity of certain virus genotypes in the same host. Second, identification of host determinants of protection will enhance our understanding of the control of HSV-1 infection. These factors can include genetic predispositions of the host and have been described in Toll-like receptor genes to confer increased severity or natural resistance to HSV infections [151, 152]. Moreover, it is important to identify the antigens recognized by HSV-specific T-cells that are uniquely present in individuals without symptomatic disease [67, 70, 71]. The recognized “asymptomatic” viral antigens are crucial candidates for an effective HSV subunit vaccine. Third, the symmetrical viral load in left and right TG is not associated with similar bilateral clinical disease. It is likely that viral replication at the periphery in the presence of symptoms results in increased sensitization of nerve endings. Consequently, these neurons may sustain increased susceptibility for reactivation-inducing triggers, explaining unilateral symptomatic reactivations. This hypothesis can apply for skin as well as for ocular HSV infections and may provide important clues on susceptibility of peripheral tissues for HSV-induced pathology. Fourth, although limited drug resistance has been associated with symptomatic treatment of HSV-1 infections, increasing information on correlates of resistance become evident. Long-term treatment especially in immunocompromised patients warrants drug resistance screening. More importantly, there is a need to develop novel anti-herpesvirus drugs with differential mechanisms of action which will be safe and effective against ACV- or multidrug-resistant HSV infections.

Conclusions

The research described in this thesis has provided novel insights into the control of HSV-1 infections, either exerted by host immunity or accomplished through antiviral treatment. We have demonstrated that CD4 and CD8 T-cells in latently infected human TG target HSV-1 proteins of all kinetic expression classes (*Chapter 2*). The antigens identified not only provide potential subunit vaccine candidates, they also provide novel insights into the regulation of viral protein expression and recognition at the site of latency in the human host. Moreover, we have identified the TG-resident SGC as local antigen presenting cells with potential T-cell modulatory properties (*Chapter 3*). As such, SGC can fulfill a crucial role in the cross-talk between latently infected neurons and virus-specific

T-cells. Both T-cells and SGC may represent important therapeutic targets to boost the ganglion-resident antiviral immune response and should be taken into account when developing an HSV subunit vaccine. Vaccination of naïve individuals may be important to limit subsequent HSV infection and the latent HSV burden.

Peripheral virus shedding and disease can result from insufficient control of virus replication in the TG. We have demonstrated that salivary shedding of HSV-I, not VZV, is common and presents as short episodes of genetically identical patient-specific HSV-I strains in HIV-infected individuals (*Chapter 4*). The data warrant additional studies on the differential mechanisms of shedding kinetics of HSV-I and VZV, especially in the immunocompromised. We have extended studies on the prevalence of ACV resistance in herpetic disease and demonstrated that ACV^R HSV-I is present in latently infected human TG, posing a risk of recurrent ACV refractory disease (*Chapter 5*). Moreover, we reported on the relatively high prevalence of ACV resistance in HSV-I uveitis, analogous to herpetic keratitis, necessitating additional studies on the role of ACV^R HSV-I infections on clinical outcome (*Chapter 6*). In line with these studies, we have evaluated risk factors for the development of ACV^R herpetic keratitis and demonstrated that ACV prophylaxis, indicated for herpetic keratitis patients with stromal involvement, is a risk factor for ACV^R HSV-I and an extended disease course (*Chapter 7*). These data indicate cautious use of ACV prophylaxis and antiviral drug sensitivity testing is indicated to select the appropriate antivirals. Moreover, the therapeutic potential of combination therapy should be considered to limit clinical disease and prevent the emergence of antiviral drug resistance.

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The background is a light gray with a large, central white circle. This white circle is surrounded by a thick, dark gray ring. Numerous other circles of various sizes and shades of gray are scattered across the page, some overlapping the central circle and others floating independently. The overall effect is a complex, layered geometric composition.

CHAPTER 9

Nederlandse Samenvatting

Nederlandse Samenvatting

Infecties met het virus herpes simplex virus type I (HSV-I) kunnen leiden tot milde ziekteverschijnselen, zoals een koortslip, zichtsbeperkende ziekteverschijnselen zoals veranderingen van het hoornvlies, netvlies of de iris, maar ook levensbedreigende ziekteverschijnselen zoals die veroorzaakt door infectie van de hersenvliezen. De meeste mensen worden op jonge leeftijd via de mond met dit virus geïnfecteerd. Het virus vermeerdert (repliceert) in de cellen van de huid, en wordt opgeruimd door het immuunsysteem. Tijdens deze huidinfectie zal HSV-I via de uiteinden van sensorische zenuwen het linker en rechter trigeminaal ganglion (TG) infecteren. In het TG schakelt de virusinfectie over op een fase die latentie wordt genoemd gedurende de resterende levensduur van de gastheer. Tijdens latentie vindt er geen virusrepliatie plaats, en virusactiviteit, dat wil zeggen aanmaak van virus RNA en eiwit, is beperkt. Het virus wordt in het TG onder controle gehouden door het afweersysteem, voornamelijk door virus-specifieke T-lymfocyten (T-cellen). Deze T-cellen vernietigen normaliter virus-geïnfecteerde cellen, zodat het virus snel wordt opgeruimd en verdere schade beperkt blijft. In het geval van T-cel gemedieerde controle van latent HSV-I, wordt virale replicatie onderdrukt zonder dat de latent geïnfecteerde neuronen worden vernietigd. Hierdoor wordt HSV-I nooit verwijderd uit het lichaam en draagt men het virus levenslang met zich mee.

Ondanks deze T-cel controle kan het virus met tussenpozen uit het stadium van latentie komen. Dit wordt reactivatie genoemd, en wordt gekenmerkt door virusrepliatie in de huid waar het virus oorspronkelijk het lichaam is binnengekomen. Reactivatie kan symptomen geven, maar blijft vaak onopgemerkt. Tijdens reactivatie kan het virus zich verspreiden via speeksel en andere lichaamsvloeistoffen en zo andere mensen infecteren. Afhankelijk van de ernst van de ziekte, zowel bij een eerste als een recidiverende HSV-I infectie, kan de patiënt worden behandeld met antivirale middelen, zoals acyclovir (ACV). Dit is vaak het geval bij patiënten met een verzwakt immuunsysteem. Daarnaast kan antivirale behandeling gegeven worden als profylaxe, waardoor reactivatie-geassocieerde symptomen achterwege kunnen blijven. Therapeutische en profylactische antivirale therapie zijn van groot belang ter behandeling van HSV-I oogenities, mede omdat deze infecties infecties kunnen leiden tot een tijdelijke of zelfs blijvende aantasting van het gezichtvermogen. Langdurige of suboptimale antivirale behandeling kan leiden tot resistentie van het virus tegen het gebruikte medicijn. Hiertoe moet HSV-I zijn genetische code veranderen (muteren). In het geval van ACV, een pro-drug die door het HSV-I eiwit thymidine kinase (TK) actief omgezet moet worden om virusrepliatie te kunnen remmen, muteert het virus zijn TK gen om ACV-resistent te worden.

Het onderzoek beschreven in dit proefschrift heeft de controle van HSV-I infecties bij de mens bestudeerd. Hierbij werd zowel de rol van het immuunsysteem in het TG, alsmede de rol van resistentie tegen ACV van HSV-I infecties nader onderzocht.

Het merendeel van de studies naar de rol van T-cellen in de controle van HSV-I infecties is uitgevoerd in experimentele muismodellen. Deze studies hebben geleid tot een beter inzicht in de kenmerken van HSV-I specifieke T-cellen (T-cel fenotype en functie) en de door de T-cellen herkende virale eiwitten (T-cel specificiteit). Extrapolatie naar de situatie in de mens blijft echter beperkt door een aantal factoren. Ten eerste, de hiervoor

gebruikte inteelt muizen zijn slechts in geringe mate representatief voor de mens met zijn grote genetische diversiteit. Genetische factoren hebben invloed op de virusinfectie en de daaropvolgende afweerreactie. Ten tweede, het merendeel van de ziekten veroorzaakt door HSV-1 is een gevolg van HSV-1 reactivatie en niet van een primaire infectie. Studies in muizen zijn doorgaans beperkt tot de bestudering van een primaire HSV-1 infectie. Ten derde, het is nog steeds onbekend of HSV-1 latentie in de muis overeenkomt met die bij de mens. De mens is de natuurlijke gastheer van HSV-1. Door miljoenen jaren co-evolutie zijn mens en HSV-1 aan elkaar aangepast, wat meest waarschijnlijk geleid heeft tot unieke virus/gastheer controle mechanismen. Hierdoor kunnen resultaten verkregen in muismodellen niet zomaar geëxtrapoleerd worden naar de mens. Aannemend dat T-cellen in HSV-1 latent geïnfecteerde TG van muizen een overeenkomstige controlerende rol hebben als in de mens, is het van belang dat de kenmerken en specificiteit van deze beschermende HSV-1 specifieke T-cellen in kaart gebracht worden. Tot op heden is nog geen HSV-1 vaccin op de markt. Idealiter zal dit vaccin er voor zorgen dat minder neuronen worden geïnfecteerd en dat minder vaak en in kleinere hoeveelheden of in het geheel geen infectieus HSV-1 uitgescheiden wordt tijdens reactivatie.

Hoofdstuk 2 beschrijft onderzoek naar HSV-1 eiwitten die in latent geïnfecteerde TG van mensen tot expressie wordt gebracht. Hiervoor werden de HSV-1 eiwit specificiteit bestudeerd van T-cellen gekweekt uit TG van mensen. Ondanks de meer dan 74 eiwitten die HSV-1 theoretisch tot expressie kan brengen, herkennen T-cellen uit humane TG slechts 1 tot 3 HSV-1 eiwitten. Deze eiwitten behoren niet tot een specifieke temporale of structurele klasse, wat suggereert dat er “at random” HSV-1 eiwitexpressie in latent geïnfecteerde TG plaatsvindt. Het stimuleren van T-cellen die betrokken zijn bij de instandhouding van latentie is een belangrijk doel voor de ontwikkeling van een (subunit) vaccin. Dit vaccin zal gericht moeten zijn op verminderen van reactivatie en het beperken van reactivatie-geassocieerde morbiditeit. Een ander celtype in het TG is de satelliet glia cel (SGC). Elk sensorisch neuron is omringd door meerdere SGC en kan zo een intermediaire rol vervullen in de dialoog tussen virus-specifieke T-cellen en latent geïnfecteerde neuronen. Het onderzoek in *Hoofdstuk 3* kent een nieuwe functie toe aan deze SGC. De resultaten tonen aan dat SGC meest waarschijnlijk afkomstig zijn uit het beenmerg, en niet zoals voorheen gedacht uit neuronale stamcellen. Daarnaast kunnen ze een rol als antigeen-presenterende cel in het TG vervullen en brengen ze specifieke eiwitten tot expressie die T-cellen kunnen remmen om geïnfecteerde cellen te doden. Stimulatie van dit celtype middels adjuvantia in vaccins zou de antivirale afweer kunnen stimuleren, zowel via activatie van virus-specifieke T-cellen als wel via onderdrukking van virale replicatie in latent geïnfecteerde neuronen.

HSV-1 reactivatie kan problematisch verlopen in patiënten met een verzwakt immuunsysteem, zoals personen geïnfecteerd met HIV. Infecties met HSV-1 in HIV patiënten kunnen ernstige weefselschade veroorzaken en hebben vaak een lange tijd nodig om zich te herstellen. Hierdoor is er een risico dat de infectie zich uitbreidt naar andere delen van het lichaam. Vaak is antivirale therapie in de vorm van zalf of pillen nodig om de symptomen te beperken. Bovendien kan antivirale therapie als profylaxe worden gebruikt om zo HSV-1 recidieven te voorkomen. Het risico op het ontwikkelen van antivirale resistentie is groot bij langdurig of suboptimaal gebruik van antivirale middelen, voornamelijk in patiënten met een verzwakt immuunsysteem. De studie beschreven in *Hoofdstuk 4* on-

derzocht de frequentie en hoeveelheid aan HSV-I DNA in speeksel van HIV patiënten. Ook is getracht een vergelijking met varicella-zoster virus (VZV) te maken, omdat dit verwante herpesvirus vaak in hetzelfde TG latent aanwezig is en de interactie tussen beide virussen reactivatie zou kunnen beïnvloeden. De episodes waarbinnen HSV-I DNA werd gevonden, zijn van korte duur, maar komen veel vaker voor dan episodes waarbij VZV DNA werd gevonden. Dit geeft aan dat het immuunsysteem deze twee verwante latente herpesvirussen in de TG waarschijnlijk via een ander mechanisme controleert. Daarnaast beschrijft *Hoofdstuk 4* dat patiënten herhaaldelijk hetzelfde HSV-I genotype uitscheiden in het speeksel. Soms kon uit het TK genotype opgemaakt worden dat het bijbehorende uitgescheiden HSV-I virus meest waarschijnlijk resistent was voor het ACV. Deze bevindingen kunnen belangrijke consequenties hebben voor immuungecompromiteerde patiënten en geven informatie over de reactivatiecyclus van HSV-I stammen in HIV patiënten. Resistentie tegen ACV kan spontaan optreden, maar risico erop is groter wanneer langdurige of suboptimale ACV therapie wordt gebruikt. Het onderzoek in *Hoofdstuk 5* beschrijft dat ACV-resistent HSV-I in latent geïnfecteerde TG van mensen aanwezig is. Omdat deze virussen mogelijk kunnen reactiveren, leidt dit tot een risico op ACV ongevoelige recidieven. Dit bemoeilijkt therapeutische mogelijkheden, omdat andere veelgebruikte anti-herpesvirus medicatie zoals ganciclovir en foscarnet eveneens afhankelijk zijn van het HSV-I TK eiwit. Kruisresistentie is hierdoor waarschijnlijk.

Acyclovir wordt veelvuldig gebruikt in de behandeling van HSV-I ooginfecties. HSV-I kan het hoornvlies infecteren en daaropvolgend een afweerreactie opwekken die onherstelbare schade aan het hoornvlies kan geven. Het recidiverende karakter van HSV-I hoornvliesinfecties kan leiden tot blijvende schade aan het hoornvlies en zelfs blindheid door littekenvorming en oedeem. Uveitis is een ontsteking van de iris en/of het netvlies die eveneens veroorzaakt kan worden door een HSV-I infectie. Behandeling van HSV-I uveitis patiënten bestaat uit antivirale therapie, zowel topicaal als systemisch, en ontstekingsremmers om de mogelijk schadelijke afweerreactie te onderdrukken. *Hoofdstuk 6* beschrijft het onderzoek naar de prevalentie van ACV-resistentie bij HSV-I uveitis patiënten. Een groot aantal van de bestudeerde patiënten hadden een ACV-resistente HSV-I stam in hun oog. Tevens waren deze virussen ook ongevoelig voor alternatieve antivirale therapie. Voor deze groep patiënten is het noodzakelijk een screeningsmethode aan te bieden, idealiter op grond van het virale genotype, die de gevoeligheid van HSV-I stammen uit het oog voor diverse antivirale middelen kan testen. Hiermee kan gericht antivirale therapie worden aangeboden om de HSV-I infectie in het oog efficiënt te onderdrukken. Daarnaast moet er worden geïnvesteerd in de ontwikkeling van alternatieve antivirale middelen die veilig zijn en geen kruisresistentie hebben met de bestaande middelen.

Omdat een eerdere hoornvliesinfectie met HSV-I een verhoogd risico geeft op een recidief, wordt aan deze patiënten vaak langdurige antivirale profylaxe voorgeschreven. Het onderzoek in *Hoofdstuk 7* bestudeerde de risicofactoren op ACV-resistentie in patiënten die antivirale profylaxe gebruiken vanwege een eerdere HSV-I hoornvlies infectie. Het gebruik van ACV geeft een verhoogt risico op resistentie en een ACV-resistente HSV-I hoornvlies infectie is geassocieerd met een langere duur van het recidief. Deze resultaten geven aanleiding voor een herziening van de voorschriften voor ACV profylaxe in patiënten met een eerdere HSV-I hoornvlies infectie, vooral bij patiënten die een ont-

steking van het middelste deel van het hoornvlies hebben doorgemaakt. Daarnaast is het van belang dat deze patiënten tijdens een recidief worden getest op de aanwezigheid van ACV-resistent HSV-I en zo nodig een ander antiviraal middel krijgen voorgeschreven.

Het onderzoek beschreven in dit proefschrift heeft geleid tot een beter inzicht in de controlemechanismen van HSV-I infecties in de mens. Enerzijds geeft het onderzoek aanleiding tot een meer doordachte ontwikkeling van HSV-I subunit vaccins, anderzijds is er belangrijke additionele informatie verkregen op het gebied van antivirale resistentie en de behandeling van patiënten met HSV-I infecties.

The background of the page is a light gray with a subtle, organic texture. It is decorated with numerous overlapping circles of various sizes and shades of gray. A large, prominent white circle is centered in the upper half of the page, surrounded by a dark gray ring. To the right of this central circle, the text is aligned. The word 'ADDENDA' is in a bold, black, sans-serif font. Below it, the list of contents is in a smaller, black, sans-serif font, with each item on a new line.

ADDENDA

Curriculum Vitae
PhD portfolio
List of publications
Dankwoord

Curriculum Vitae

Monique van Velzen was born on November 13, 1984 in Spijkenisse, the Netherlands. She finished high school at OSG De Ring van Putten in Spijkenisse in 2002 and immediately started her study Biomedical Sciences at Leiden University. Her bachelor internship at the department of Clinical Oncology, Leiden University Medical Center (LUMC), focused on HLA class I expression and apoptosis in colorectal cancer. During her first master internship, she studied candidate tumor suppressor genes in acute myeloid leukemia at the department of Toxicogenetics, LUMC, in Leiden. Her second master internship was performed at the department of Virology of the Erasmus Medical Center in Rotterdam, which focused on the immune control of herpes simplex virus type I latency. After graduating from Leiden University in 2007, she started as a PhD student at the same department under the supervision of prof.dr. Albert Osterhaus and dr. Georges Verjans. Her PhD project focused on the control of herpes simplex virus type I infections in humans, and has resulted in the present thesis. As of January 2013, Monique is working as a researcher at the department of Anesthesiology at the LUMC in Leiden.

PhD portfolio

Research group: Erasmus MC, department of Viroscience

Research school: Post-graduate Molecular Medicine

PhD period: 2008-2013

Promotor: Prof.dr. Albert DME Osterhaus

Co-promotor: Dr. Georges MGM Verjans

In-depth courses

- 2012 Course Research Integrity, provided by the postgraduate school Molecular Medicine, Erasmus MC, Rotterdam, the Netherlands
- Workshop on InDesign CS5, provided by the postgraduate school Molecular Medicine, Erasmus MC, Rotterdam, the Netherlands
- 2008 Course on Immunology, provided by the Leiden Institute for Immunology, LUMC, Leiden, the Netherlands
- Course in Virology, provided by the postgraduate school Molecular Medicine, Erasmus MC, Rotterdam, the Netherlands
- 2006-2012 Internal and external presentations at the department of Virology, Erasmus MC, Rotterdam, the Netherlands (twice a week)

Presentations

- 2012 “Acyclovir refractory herpes simplex keratitis correlates with acyclovir prophylaxis”, at the clinical department of Virology, Erasmus MC, Rotterdam, the Netherlands (oral)
- “HSV-I specific T-cells in human trigeminal ganglia recognize viral proteins of all kinetic classes”, at the International Herpesvirus Workshop, Calgary, Canada (oral)
- “Herpes simplex virus T-cell responses in latently infected human trigeminal ganglia”, at the T-cell consortium meeting, department of Pulmonary Medicine, Erasmus MC, Rotterdam, the Netherlands (oral)
- “Fine specificity and localization of herpes simplex virus specific T-cells in latently infected human trigeminal ganglia”, at Molecular Medicine day, Rotterdam, the Netherlands (poster)

- 2011 “Satellite glial cells in human trigeminal ganglia”, at the Netherlands Institute for Neurosciences, Amsterdam, the Netherlands (oral)
- “Satellite glial cells: microglia of the peripheral nervous system”, at the meeting on Research on Brain Tissue, Rotterdam, Erasmus MC, the Netherlands (oral)
- “Immune control of HSV-I latency in human trigeminal ganglia”, at the Colorado Alpha Herpes Virus Latency Symposium, Vail, United States (oral)
- “Acyclovir sensitive and resistant HSV-I strains co-exist in human trigeminal ganglia”, at the Molecular Medicine day, Rotterdam, the Netherlands (poster)
- 2010 “Acyclovir sensitive and resistant HSV-I strains co-exist in human trigeminal ganglia”, at the International Herpesvirus Workshop, Salt Lake City, United States (poster)
- 2009 “Neuron-interacting satellite glial cells in human trigeminal ganglia have antigen presenting cell properties”, at Biomedical Primate Research Center, Rijswijk, the Netherlands (oral)
- “Immune control of latent herpes simplex virus infections”, at the mini-symposium Brain-Body Immune Debate, department of Immunology, Erasmus MC, Rotterdam, the Netherlands (oral)
- “Neuron-interacting satellite glial cells in human trigeminal ganglia have an antigen presenting cell phenotype”, at the European Congress of Immunology, Berlin, Germany (poster)
- 2008 “Phenotype and function of satellite cells in human trigeminal ganglia”, at the Molecular Medicine day, Rotterdam, the Netherlands (poster)
- 2007 “Satellite cells in the human trigeminal ganglion: from form to function”, at the Netherlands Institute for Neurosciences, Amsterdam, the Netherlands (oral)
- “Phenotype and function of satellite cells in human trigeminal ganglia”, at the Molecular Medicine day, Rotterdam, the Netherlands (poster)

Attended conferences and symposia

- 2012 Molecular Medicine day, Rotterdam, the Netherlands
- PhD Day, Erasmus MC, Rotterdam, the Netherlands
- Symposium Multiple Sclerosis, Herpes Virus and Aging, at the
department of Immunology, Erasmus MC, Rotterdam, the
Netherlands
- International Herpesvirus Workshop, Calgary, Canada
- 2011 Molecular Medicine day, Rotterdam, the Netherlands
- Meeting Research on Brain Tissue, Erasmus MC, Rotterdam, the
Netherlands
- Colorado Alpha Herpes Virus Latency Symposium, Vail, United States
- 2010 Molecular Medicine day, Rotterdam, the Netherlands
- Symposium Advanced Immunology, VUmc, Amsterdam, the
Netherlands
- International Herpesvirus Workshop, Salt Lake City, United States
- 2009 Molecular Medicine day, Rotterdam, the Netherlands
- Symposium Brain-Body Immune Debate, at the department of
Immunology, Erasmus MC, Rotterdam, the Netherlands
- Virology meeting, Dakar, Senegal
- European Congress of Immunology, Berlin, Germany
- 2008 Molecular Medicine day, Rotterdam, the Netherlands
- 2007 Molecular Medicine day, Rotterdam, the Netherlands
- Symposium Post-Infectious Diseases, Erasmus MC, Rotterdam, the
Netherlands
- Virology meeting, Greece

Awards

- 2012 Early travel award International Herpesvirus Workshop, Calgary, Canada
- Award for best poster presentation, Molecular Medicine day, Rotterdam, the Netherlands

Teaching

- 2012 FACS introductory lecture, department of Viroscience, Erasmus MC, Rotterdam, the Netherlands
- 2009-2012 Rotations Master of Science students “Infection and Immunity”, Erasmus University, Rotterdam, the Netherlands
- 2009 Bachelor of Applied Science student, Hogeschool Rotterdam, Rotterdam, the Netherlands

List of publications

Speetjens FM, de Bruin EC, Morreau H, Zeestraten EC, Putter H, van Krieken JH, van Buuren MM, **van Velzen M**, Dekker-Ensink NG, van de Velde CJ, Kuppen PJ. Clinical impact of HLA class I expression in rectal cancer. 2008 *Cancer Immunol Immunother*; 57: 601-9.

van Velzen M, Laman JD, Kleinjan A, Poot A, Osterhaus ADME, Verjans GMGM. Neuron-interacting satellite glial cells in human trigeminal ganglia have an APC phenotype. 2009 *J Immunol*; 183: 2456-61.

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van Velzen M, Ouwendijk WJD, Selke S, Pas SD, van Loenen FB, Osterhaus ADME, Wald A, Verjans GMGM. Longitudinal study on oral shedding of herpes simplex virus type I and varicella-zoster virus in HIV patients. 2013 *J Med Virol*; in press.

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van Velzen M, van de Vijver DAMC, van Loenen FB, Osterhaus ADME, Remeijer L, Verjans GMGM. Acyclovir prophylaxis predisposes to antiviral resistant recurrent herpetic keratitis. 2013 *J Infect Dis*; in press.

van Velzen M, Jing L, Osterhaus ADME, Sette A, Koelle DM, Verjans GMGM. Local CD4 and CD8 T-cell reactivity to HSV-I antigens documents broad viral protein expression and immune competence in human ganglia. 2013, submitted.

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