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Phenotypic and Genotypic Characterization of Acyclovir Resistant Herpes Simplex Virus.

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In vitro documented acyclovir (ACV)-resistant herpes simplex (HSV) has shown to be of little clinical consequence in patients with healthy immune systems. However, in patients with dysfunctional immune systems viral isolates with reduced sensitivity have been recovered. These lesions that were refractory to ACV therapy have posed significant challenges to the clinician for alternate therapies. From a surveillance program conducted for more than 20 years with over 5000 HSV clinical isolates evaluated for ACV susceptibility, only 170 were found to be ACV-resistant. HSV isolates were defined as resistant when EC_{50} values for ACV were shown by dye-uptake or plaque-reduction assays to be ≥ 3 µg/ml or ≥ 2 µg/ml, respectively. Phenotypic characterization by susceptibility testing against foscarnet, tandem plaque autoradiography with [^{125}I]iododeoxycytidine and [^{14}C]thymidine, and TK enzyme phosphorylation assays revealed that most isolates have TK-defective phenotypes. To address whether specific or consistent nucleotide changes within the HSV-TK gene are responsible for ACV-resistance, we are determining the DNA sequence of these isolates. Solid-phase ssDNA cycle sequencing is being done on the PCR-amplified HSV-TK gene products from homogenous ACV-resistant populations. To date we have sequenced and analyzed 51 ACV-resistant HSV-TK isolates. The interpretation of these results is limited until a larger database of wildtype TK sequences is available for comparison. It is of interest that approximately 40% of these resistant viruses have either a single-base deletion or insertion in homopolymeric regions of guanines or cytosines (5/15 of HSV1 and 15/36 of HSV2). These mutations can result in the expression of truncated HSV-TK proteins that are nonfunctional, producing TK-defective phenotypes that are ACV-resistant.

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GENOTYPIC CHARACTERIZATION OF VARIOUS HERPES SIMPLEX VIRUS TYPE 1 (HSV-1) CLONES RESISTANT TO ACYCLIC NUCLEOSIDE PHOSPHONATES

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We have identified mutations in the HSV-1 (KOS strain) DNA polymerase gene conferring resistance to 3-hydroxy-2-phosphonylmethoxypropyl (HPMP) derivatives of cytosine (HPMPC, cidofovir) and adenine (HPMPA), phosphonylmethoxyethyl (PME) derivatives of adenine (PMEA) and 2,6-diaminopurine (PMEDAP) and foscarnet (PFA). The nucleotide sequence of the DNA polymerase gene of two HPMPC^r, two HPMPA^r, one PFA^r, one PMEA^r and one PMEDAP^r plaque purified clones was performed after subcloning of the DNA polymerase gene. To determine the presence of specific mutations in various drug-resistant HSV-1 clones, direct polymerase chain reaction (PCR) sequencing was used. Viral DNA was prepared from each clone and amplified by a standard PCR protocol, using specific primers. The PCR products were then purified and sequenced by using sequenase. When several PMEA^r, PMEDAP^r and PFA^r clones were screened, the Ser to Asn change at position 724 occurred in all the clones analyzed. In contrast, an heterogeneous population was observed for the HPMPC^r strain. Thus, some of the HPMPC^r clones showed either the 573 Val → Met mutation or the 700 Arg → Met mutation. Interestingly, three of the HPMPC clones were negative for the mutations at positions 573 and 700. In order to identify the new mutations that occurred in these HPMPC^r clones, the subcloning and the analysis of the nucleotide sequence of the DNA polymerase gene of these clones is currently in progress.

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Proteolytic, Active-Site Mapping of Herpesvirus Thymidine Kinases Type 1 and 2 Photolabeled with Thymidine and Ganciclovir Photoaffinity Analogs. R Drake, T. Rechtin, S. Hume, and M. Black. Dept. of Biochemistry, Univ. of Arkansas for Med. Sci., Little Rock, AR 72205 and Darwin Molecular Corp., Bothell, WA 98021

Human herpesvirus thymidine kinases (HSV-TK) are primary targets of antiviral nucleoside drug treatments (GCV). Using a combination of azido-nucleotide photoaffinity probes, proteases and tricine-SDS-PAGE, a peptide region comprising the thymidine/GCV base binding site in HSV-1 and HSV-2 TKs was determined. Two photoprobes were utilized, a TMP analog, [^{32}P]5-azido-dUMP, and a GCV analog, [^{32}P]8-azido-GCVMP. The specificity of photoincorporation was demonstrated by saturation of photocrosslinking and inhibition by known HSV-TK substrates. Because the amino acid sequence of HSV-1 TK is known, endoprotease Lys-C, V8 protease, trypsin, or chymotrypsin were used to generate a map of photoincorporated peptides separated on tricine-SDS-gels. Analysis of the resulting peptides indicated the photoprobe was localized to one region comprising amino acids Ile^{112}-Tyr^{132} in HSV-1 TK (and homologous site in HSV-2 TK). Mapping analysis of site-specific HSV-1 TK mutants, C336Y and D162Q, yielded the same peptide. In support of recent X-ray crystallography studies for HSV-1 TK, these results demonstrate that the thymine base of thymidine and TMP and the guanine base of GCV bind at a similar shared site in HSV-1 TK, and in HSV-2 TK. This technique bypasses the problems of peptide purification and sequencing, and yields rapid results when the primary amino acid structure of the protein of interest is known. A similar approach could be used for any enzyme which is known to metabolize a nucleoside/nucleotide-derived drug.

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Induction of genetic damage and apoptosis by ganciclovir in Chinese Hamster Ovary cells transfected with the thymidine kinase (tk) gene of HSV-1

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CHO-9 cells transfected with the tk gene of HSV-1 and HSV-tk$^-$ neo$^+$ control cells of CHO-9 were checked for SCE-inducing and clastogenic activity as well as for reduction of plating efficiency and induction of apoptosis (TdT-assay and flow cytometry) following treatment with ganciclovir (GCV) for 25 h and a recovery period of variable duration. HSV-tk$^+$ transfectants were about 1000 times more sensitive with respect to genotoxic effects of GCV than the tk$^-$ controls. Thresholds of activity in tk$^+$ versus tk$^-$ cells for SCE induction were 1 nM / < 10 µM and for clastogenicity < 0.1 µM / 0.1 mM GCV, respectively. Plating efficiency approximated 0 % at 1 µM GCV in HSV-tk$^+$ cells and at > 0.1 mM in the neo control.

The apoptotic pathway was triggered at a 1 µM concentration of GCV in HSV-tk transfectants. Whether GCV induces apoptosis in tk$^-$ cells warrants further studies.

Previous experiments with non-transfected Chinese hamster V79-E cells (Thust et al., Antiviral Res. 31,105-113, 1996) suggested that GCV is internally incorporated into cellular DNA and that the adverse effects observed are due to repair processes at the GCV-containing template. This will be discussed.