Low Prevalence of Acyclovir-resistant Herpes Simplex Virus Isolates Among Saudi Patients

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Objective: To determine the prevalence of acyclovir-resistant isolates of herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2) among Saudi patients.

Setting: Molecular Virology and Infectious Diseases Laboratories of the Research Centre of King Faisal Specialist Hospital and Research Centre in Riyadh, Kingdom of Saudi Arabia.

Subjects: Specimens from Saudi patients attending various clinics in the Hospital were studied retrospectively.

Main outcome measures: The percentage of reduction in the number of plaques when various molar concentrations of ACV were added compared with controls without ACV was used to denote resistance. At ACV concentrations which inhibit susceptible strains, the lower the percentage of plaque reduction the higher the degree of resistance.

Results: At 5 µmol/l acyclovir concentration, 19 of 93 isolates of HSV-1 and 15 of 30 isolates of HSV-2 showed 76–100% plaque reduction. Two of the 30 HSV-2 isolates exhibited 28% and 59% plaque reduction and were from immunosuppressed patients. The prototype strains were fully susceptible at 1 µmol/l acyclovir concentration.

Conclusions: The prevalence of acyclovir-resistant herpes simplex virus strains is very low among Saudi patients. Resistant isolates can be expected from immunocompromised patients. Indiscriminate use of antivirals should be discouraged to minimize the emergence of resistant strains.

Keywords: Herpes simplex virus, HSV. Acyclovir. Resistance. Saudi Arabia. Plaque reduction assay. Susceptibility.
Acyclovir (ACV: 9-[2-hydroxyethoxymethyl] guanine) is an antiviral drug which has marked real progress in the clinical treatment of herpetic infections. It acts as a potent inhibitor of herpes simplex virus (HSV) and, to a lesser extent, varicella zoster virus replication. The mechanism of action involves the recognition of ACV as a substrate for the virus-encoded enzyme, thymidine kinase (TK). This enzyme phosphorylates ACV in a series of biochemical reactions, producing ACT-triphosphate which is a nucleotide analogue preventing viral DNA synthesis. As is the case with most antimicrobials, prolonged or indiscriminate use of ACV has resulted in the emergence of ACV-resistant isolates due to mutation in the TK gene or alteration in the DNA polymerase activity. Recent studies have documented the occurrence of ACV-resistant isolates of HSV, particularly after prolonged therapy in the immunocompromised patient. In this report we describe the prevalence of ACV-resistant isolates of HSV-1 and HSV-2 from Saudi patients at King Faisal Specialist Hospital and Research Centre, by using the plaque reduction assay method. Since there has not been a uniform understanding on the level of in vitro susceptibility as to what indicates a resistant strain, we are reporting our observation of resistance at various drug concentrations.

Materials and Methods

Cells and viruses

African green monkey kidney (Vero) cells were obtained from ATCC (Rockville, MD, USA) and used throughout this study. The prototype strains F and McIntire for HSV-1 and G and MS for HSV-2 (ATCC) were used as standards. The test specimens initially consisted of 69 and 30 clinical isolates of HSV-1 and HSV-2, respectively. All viruses were propagated in cell culture as described by Al-Ahdel et al. After plaque purification (see below), 20 of the 69 HSV-1 isolates showed an additional 24 variants as measured by the plaque size, making a total of 93 isolates for HSV-1. These variant HSV-1 isolates were further plaque-purified. All prototype strains and HSV isolates were confirmed as HSV-1 or HSV-2 by monoclonal fluorescent antibody test, and were all examined for their susceptibility or resistance to ACV.

Plaque assay

The plaque assay procedure was performed according to Siddiqui & Al-Ahdel. Briefly, 60 mm tissue culture dishes (Corning, NY, USA) were seeded with 5 ml medium containing 2.5 × 10⁶ Vero cells. Virus dilutions were made in ice-cold phosphate buffered saline containing 2% fetal bovine serum (PBS-FBS). Cell monolayers were washed with PBS-FBS and inoculated in triplicate with the virus dilutions in a volume of 0.2 ml. Dishes were incubated for 90 min at 36°C for virus adsorption, and the monolayers were rewashed with PBS-FBS; 5 ml of prewarmed agar overlay was added to each monolayer, and the dishes were incubated for up to 4 days before staining and counting the plaques. The agar overlay contained phenol red-free Eagle's minimum essential medium (Flow Laboratories, Irvine, Scotland) and 0.45% bacteriological agar (Sigma Chemical Co, St Louis, MO, USA), supplemented with vitamin mixture, 20 mmol/litre L-glutamine, 5.6 mmol/litre glucose, 30 mmol/litre magnesium chloride, 200 μg/ml
DEAE-dextran, 100 IU/ml penicillin, and 20 μg/ml gentamycin.

**Plaque reduction assay**

After enumeration of virus particles from the prototype and clinical isolates, dilutions were made which would give approximately 80 plaques in 0.2 ml inoculum. This volume is referred to as 'single plaquing dose', which was added to other cell monolayers as described in the plaque assay. In the plaque reduction assay, the overlay contained various micromolar concentrations of ACV (Zovirax®, Wellcome Foundation, England). Control dishes with the adsorbed virus were inoculated with the overlay containing no ACV. The Vero cell monolayers were tested with several doses of ACV to investigate any cytotoxic effect.

**Results**

All prototype strains of HSV showed over 97% reduction in plaque number at a 1 μmol/litre concentration of ACV. That is to say that they were fully sensitive to the drug at a concentration of 0.23 μg/ml. This finding is in agreement with published data regarding the sensitivity of prototype strains to ACV. Therefore, beyond this concentration, test isolates showed varying degree of resistance by our method. The results of our plaque reduction assays for susceptibility testing of HSV to ACV are summarized in Fig. 1 for HSV-1 and Fig. 2 for HSV-2. At a 0.1 μmol/litre concentration of ACV, all HSV isolates showed less than 25% reduction in the plaque number as compared to the controls in which there was no ACV added. As the drug concentration in the overlay increased, the number of isolates showing susceptibility also increased for both HSV-1 and HSV-2. At ACV concentrations of 0.5–2.0 μmol/litre, 74 isolates of HSV-1 and 13 isolates of HSV-2 showed a 76–100% reduction in plaque number. The plaque formation was inhibited in 19 isolates of HSV-1 and in 15 isolates of HSV-2 at a 5 μmol/litre concentration of ACV. The remaining two isolates of HSV-2 showed a slightly higher resistance, producing 26% and 59% reduction in plaque number at a 5 μmol/litre concentration of ACV. Compared to the prototype strains which were fully sensitive at 1.0 μmol/litre concentration, test isolates showed relative resistance to ACV in both types of HSV. For HSV-1, 38, 28 and 19 isolates showed 76–100% reduction in the plaque number at concentrations of 1.0, 2.0, and 5.0 μmol/litre, respectively. For HSV-2, 7, 6, and 15 isolates showed 76–100% reduction in the plaque number at concentrations of 1.0, 2.0, and 5.0 μmol/litre, respectively. From these data, it was obvious that the resistance was at a very low grade, but more notable in HSV-2 than in HSV-1.

**Discussion**

In this paper, we describe the prevalence of ACV-resistant strains among HSV isolates from Saudi patients with various diseases attending our tertiary care medical centre. The task of determining the ACV concentration at which an isolate is considered resistant to the drug is formidable, as such a concentration remains arbitrary. Coen & Schaffer defined resistant strains as those which can grow at a 10 μmol/litre (2.3 μg/ml) concentration of ACV. This concentration was agreed upon in a recent study by Englund et al. who reported that a value greater than 2.0 μg/ml
defines resistance. Since most laboratories would find a concentration greater than 1.0 μg/ml as clearly indicating resistance,13 we have considered that any of our isolates that can survive at an ACV concentration of 5.0 μmol/litre (1.15 μg/ml) would be defined as a resistant isolate. However, if resistance is determined by the plaque reduction assay, as is the case in this study, then the degree of resistance would depend on the percentage in the reduction of plaque number as compared to the control which contains no ACV. The lower the percentage of plaque reduction the higher the degree of resistance and the higher the percentage of plaque reduction the lower the degree of resistance.

Based on this consideration for our assay procedure, 19 (20.4%) of our HSV-1 isolates and 15 (50%) of our HSV-2 isolates can be considered relatively resistant to ACV. However, since these isolates showed only 75–99% reduction in plaque number, and most of them are in the range of 90–100%, they are thought of having a very low grade resistance. All of these isolates, except three were from immunocompetent patients. In the case of HSV-2, only two isolates showed 26–74% plaque reduction, and they are thought of exhibiting a medium grade resistance. It is not unusual to notice that HSV-2 isolates relatively exhibit more resistance than HSV-1. This phenomenon has been reported earlier by Collins18 and Nugier et al.,19 and our results showed that two isolates of HSV-2 exhibited a medium degree of resistance compared with none of HSV-1. These two isolates of HSV-2 were from patients undergoing heavy immunosuppression. It is natural to observe that at lower drug concentrations, the isolates were not susceptible, as there is not enough drug to ensure the inhibition of replication of all particles.

Our finding is considered encouraging, as the emergence of virus isolates that are resistant to this hitherto effective antiviral drug is reported worldwide. The resistant isolates, however, were isolated from severely immunocompromised patients, particularly patients who were infected with human immunodeficiency virus. Since patients with this illness are not prevalent among the Saudi population, it is not unusual to observe a low prevalence of ACV-resistant HSV isolates. Nonetheless, care must be taken to safeguard against the indiscriminate use of acyclovir since it is one of the few effective and relatively safe antiviral drugs. Experiments to define the mechanism of resistance at the gene level are in progress.

References


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