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A dual reporter cell assay for identifying serotype and drug susceptibility of herpes simplex virus

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ABSTRACT

A dual reporter cell assay (DRCA) that allows real-time detection of herpes simplex virus (HSV) infection was developed. This was achieved by stable transfection of cells with an expression cassette that contains the dual reporter genes, secreted alkaline phosphatase (SEAP) and enhanced green fluorescent protein (EGFP), under the control of an HSV early gene promoter. Baby hamster kidney (BHK) and Chinese hamster ovary (CHO) cell lines were used as parental cell lines because the former is permissive for both HSV serotypes, HSV-1 and HSV-2, whereas the latter is susceptible to infection only by HSV-2. The DRCA permitted differential detection of HSV-1 and HSV-2 by observation of EGFP-positive cells, as substantiated by screening a total of 35 samples. The BHK-based cell line is sensitive to a viral titer as low as a single plaque-forming unit with a robust assay window as measured by a chemiluminescent assay. Evaluations of the DRCA with representative acyclovir-sensitive and acyclovir-resistant HSV strains demonstrated that their drug susceptibilities were accurately determined by a 48-h format. In summary, this novel DRCA is a useful means for serotyping of HSV in real time as well as a rapid screening method for determining anti-HSV susceptibilities.

Herpes simplex viruses types 1 and 2 (HSV-1 and HSV-2, collectively HSV)[3] belong to the α subfamily in the Herpesviridae family [1]. HSVs cause a wide spectrum of clinical illnesses in immunocompromised individuals, especially newborns, transplant recipients, and patients with acquired immunodeficiency syndrome (AIDS) [1]. HSV-1 can cause both oral and genital infections, whereas HSV-2 is transmitted almost exclusively sexually and is mainly responsible for genital herpes. The recurrence rate and shedding of HSV is significantly lower in HSV-1 than in HSV-2 [2,3]. Therefore, correct serotyping of HSV specimens is an important matter of clinical concern. Chemotherapeutic compounds for HSV diseases have been clinically available with acyclovir (ACV), a guanosine analog that inhibits viral genome replication, being the most common one. However, long-term or frequent treatment with an anti-HSV drug, especially for immunocompromised patients, has been associated with the emergence of resistant HSV strains and progression of diseases [1,4,5]. Therefore, it is essential to accurately determine the susceptibility to an anti-herpetic compound in use.

Rapid and sensitive immunological and polymerase chain reaction (PCR)-based assays for detection of HSV have been developed [6,7]. Nevertheless, virus culture remains the only method to detect infectious virus particles and facilitates the analysis of clinical relevant phenotypes such as antiviral susceptibility [7,8]. Among assays to measure antiviral susceptibility, the plaque reduction assay (PRA) has been generally considered as the reference standard. However, the prevalent use of culture-based methods, including the PRA, is often hampered by a labor-intensive procedure, a lengthy turnaround time, and a subjective way to observe the cytopathic effect (CPE) or plaque formation. The development of alternative cell-based assays for simple, rapid, robust, and reliable measurement of infectious HSV and its drug resistance remains a high priority.

A commercial enzyme-linked virus-inducible system (ELVIS, Diagnostic Hybrids) for rapid detection of HSV has been reported. The system uses a genetically engineered host cell line that contains a chimeric gene sequence constructed on an *Escherichia coli lacZ* reporter gene driven by the promoter from the HSV-1 UL39 gene [9]. When cells are infected with either HSV-1 or HSV-2, transactivating viral proteins cause the expression of *lacZ* and the subsequent accumulation of intracellular β-galactosidase. A

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[3] *Abbreviations used:* HSV, herpes simplex virus; ACV, acyclovir; PCR, polymerase chain reaction; PRA, plaque reduction assay; CPE, cytopathic effect; ELVIS, enzyme-linked virus-inducible system; GFP, green fluorescent protein; SEAP, secreted alkaline phosphatase; EGFP, enhanced GFP; IC_{50}, 50% inhibitory concentration; BHK, baby hamster kidney; CHO, Chinese hamster ovary; IRES, internal ribosomal entry site; CMV, cytomegalovirus; MEM, minimum essential medium; FBS, fetal bovine serum; VZV, varicella-zoster virus; p.i., postinfection; PFU, plaque-forming units; mRNA, messenger RNA; DRCA, dual reporter cell assay; S/N, signal-to-noise ratio.

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histochemical stain then allows detection of HSV-infected cells that appear blue. ELVIS has been used to identify HSV-1 or HSV-2 in clinical specimens with the positive results gained 16–20 h post-infection [10–12]. The system was modified to include monoclonal antibody typing reagents to permit serotyping of HSV stocks [13,14]. The application was then extended to determine antiviral susceptibility [15,16]. However, the detection and quantitation of *lacZ* gene expression require cell fixation, exogenous substrates, and cell lysate preparation, procedures that cause gross cell damage unsuited for further study.

To continuously monitor HSV infections in the infected cells, two other reporter genes, green fluorescent protein (GFP) and secreted alkaline phosphatase (SEAP) genes, were taken advantage of to establish alternative reporter systems. GFP, originally identified from jellyfish *Aequorea victoria,* features the ability to efficiently emit internal fluorophore. A reporter cell system using the enhanced GFP (EGFP) gene, a GFP derivative with a strong red-shifted excitation [17], under the control of the HSV-2 ICP10 promoter was addressed [18–21]. Real-time observation of the EGFP signal can be achieved as early as 6 h after infection, with a progressive increase in intensity at later time points [21]. As for the SEAP reporter gene, it is a highly stable secreted enzyme that only requires supernatants from the cultured cells for detection, thereby making lysate preparation unnecessary and automation amenable [22,23]. A reporter cell line that uses the SEAP gene was also documented, highlighting the extremely sensitive detection of HSV over a broad linear range as measured by a chemiluminescence-based assay [24,25]. Moreover, the SEAP-based reporter cell line was adapted for rapid and accurate determination of the 50% inhibitory concentration (IC_{50}) of ACV [25].

Both HSV-1 and HSV-2 complete the full multiplication cycle in a variety of cell types in culture, including the baby hamster kidney (BHK) cells frequently used for cultivation of HSV. On the other hand, the Chinese hamster ovary (CHO) cell line was shown to permit the entry of HSV-2 but not of HSV-1 [26–29]. In this study, we generated a bicistronic construct that bears the EGFP and SEAP reporter genes, both of whose expressions are driven by the HSV-inducible ICP10 promoter. Stable cell lines based on transfection of the bicistronic construct into BHK and CHO cells were developed, by which real-time detection of the EGFP signal and sensitive measurement of the SEAP activity on HSV infection could be achieved. Moreover, the reporter expression profiles of this pair of stable lines were instrumental in differential serotyping, with the reporter proteins inducibly expressed in the BHK-based cells infected by either HSV-1 or HSV-2 and those of the CHO-based cells infected by HSV-2 but not by HSV-1. Furthermore, the dual reporter cell line was formatted to a simple, rapid, and robust screening assay for the determination of ACV susceptibility.

Materials and methods

Plasmid construction

The SEAP coding segment was obtained by PCR using pSEAP2–Basic plasmid (Clontech) as a template with the following primer pair: forward, 5′- GACGAATTCGCCCACCATGCTGCTGCTGC-3′; reverse, 5′-CTACTCGAGTCATGTCTGCTCGAAGCGGCCG-3′. The 5′ and 3′ primers were engineered with the *Eco*RI and *Xho*I restriction sites underlined, respectively, for cloning. The PCR product was subsequently digested and directionally cloned into the *Eco*RI and *Sal*I sites of pIRES2–EGFP (Clontech), a plasmid that harbors the EGFP reporter gene controlled by the internal ribosomal entry site (IRES) for bicistronic expression. The resulting plasmid was designated pCMV–SEAP–EGFP. A DNA fragment bearing the HSV-2 ICP10 promoter was obtained by PCR using pICP10–EGFP plasmid [21] as a template with the following set of primers: forward, 5′-GCG$_{ATTAAT}$GTCGACAGGCTGTACCGCTGG-3′; reverse, 5′-CTAGATCTGTCGACAGGACAGCACCGGCC-3′. The ICP10 fragment (with the engineered *Ase*I and *Bgl*II sites underlined, respectively) was cloned into the corresponding sites in pCMV–SEAP–EGFP to substitute the cytomegalovirus (CMV) IE promoter. The resultant construct was referred to as pICP10–SEAP–EGFP, and the authenticity was confirmed by automated DNA sequencing.

Cells and viruses

Baby hamster kidney (BHK-21, ATCC CCL-64.1, henceforth referred to as BHK), Chinese hamster ovary (CHO-K1, ATCC CCL-171, henceforth referred to as CHO), and Vero cell lines were propagated in minimum essential medium (MEM, Gibco–BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Laboratory strains of HSV-1 (KOS), HSV-2 (186), human CMV (AD169), and varicella-zoster virus (VZV, Ellen) were used. Clinical isolates, including HSV, adenovirus, influenza A, and coxackievirus A16, were collected from routine isolations in the Division of Clinical Virology, Veteran General Hospital–Taipei, unless otherwise indicated. The ACV-resistant HSV strains were described previously [30]. The HSV-1 and HSV-2 samples were further identified by a type-specific immunofluorescence assay (K6106, Dako) and then propagated with Vero cells. All of the HSV strains were titrated by a plaque formation assay [31].

Generation of HSV-inducible cell lines

The dual reporter cell lines BHK-SE and CHO-SE, based on the parental cells BHK and CHO cells, respectively, were established as reported previously [13,30]. Briefly, pICP10–SEAP–EGFP plasmid was transfected into BHK or CHO cells using Lipofectamine (Gibco–BRL) according to the manufacturer's instructions. Next, 48 h after transfection, the transfected cells were grown in MEM containing neomycin at 0.8 mg/ml until all of the mock-transfected cells were dead (~2 weeks). Stable clones with neomycin resistance were obtained by trypsinization followed by plating at limiting dilution onto 96-well tissue culture dishes. Each clone was grown and maintained in MEM containing 0.3 mg/ml neomycin.

Fluorescence microscopy

The imaging process was performed under an inverted fluorescence microscope (Nikon TE200) equipped with the EGFP filter set. Images were acquired with an interlined charge-coupled device camera (DXM 1200F, Nikon) controlled by ACT-1 software (version 2.62, Nikon). Images were viewed and processed with Adobe Photoshop 6.0 (Adobe Systems).

SEAP assays

SEAP activity was measured by a highly sensitive, chemiluminescence- based assay using the substrate CSPD (disodium 3-(4-meth oxyspiro[1,2-dioxetane-3,2′-(5′-chloro)-tricyclo[3,3,1,1^{3,7}]-decan]-4-yl) phenyl phosphate) in the Great EscAPe SEAP Chemiluminescence Kit (Clontech) as described previously [25]. The chemiluminescent signal was detected by a luminometer (Lumat LB953).

Antiviral susceptibility testing

Dual reporter cell assay

Fully 80–90% confluent BHK-SE cells were prepared in 24-well tissue culture plates. Cells were inoculated with a virus suspension of 100 μl. After a 1-h adsorption, the inoculum was aspirated and 500 μl of medium (MEM containing 2% FBS) alone or medium with ACV (Sigma) at 2 μg/ml was added to each well. Control groups