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Determination of antiviral efficacy against lymphotropic herpesviruses utilizing flow cytometry

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Abstract

Epstein–Barr virus (EBV), human herpesvirus type 6 (HHV-6), and human herpesvirus type 8 (HHV-8) comprise a group of lymphotropic herpesviruses which are responsible for a wide range of diseases, including lymphoproliferative disorders and tumors. We have developed several flow cytometric assay (FACS) systems to evaluate antiviral efficacy against EBV, HHV-6 and HHV-8. Assays using either EBV or HHV-8, members of the gammaherpesvirus subfamily, have shown that while EBV responds well to acyclovir (ACV), HHV-8 was most sensitive to cidofovir (CDV). Since HHV-6 strains are divided into two sub-groups, A and B, we evaluated antiviral efficacy for strains from each group. The group A strain, HHV-6_{GS}, was inhibited by foscarnet (PFA), CDV and ganciclovir (GCV) in both Sup-T1 and HSB-2 cell lines. HHV-$6_{Z\text{-}29}$, a representative group B virus, was inhibited by GCV and CDV but not by PFA. Our findings indicate that flow cytometry can be utilized to efficiently evaluate new antiviral agents against lymphotropic herpesviruses and that the results are comparable to those obtained by other methods such as immunofluorescence.

Keywords: Flow cytometry; Herpesviruses; In vitro; Antivirals

1. Introduction

Epstein–Barr virus (EBV), human herpesvirus type 6 (HHV-6) and human herpesvirus type 8 (HHV-8) are members of a subset of lymphotropic human herpesviruses that also includes human herpesvirus 7. HHV-6 and HHV-8 are relatively newly described herpesviruses having been characterized in 1986 (Salahuddin et al., 1986) and 1994 (Chang et al., 1994), respectively. HHV-6 is a member of the β herpesvirus family based on its sequence homology with human cytomegalovirus, the prototype β herpesvirus, while EBV and HHV-8 are classified as γ herpesviruses based on their similarity to herpesvirus saimiri. The primary mode of transmission for these common human pathogens is via saliva and nasal secretions (Ernberg and Andersson, 1986; Jarrett et al., 1990; Koelle et al., 1997; Yao et al., 1989). Currently, there is no antiviral agent with proven clinical efficacy against lymphotropic herpesviruses.

Throughout the world, EBV infects over 90% of the population by adulthood (Stowe et al., 1998). EBV preferentially infects peripheral blood B-lymphocytes and oropharyngeal epithelium cells (Yao et al., 1989) and is associated with infectious mononucleosis, Burkitt's lymphoma, Hodgkin's disease and nasopharyngeal carcinoma in immunocompetent individuals (Henle et al., 1974; Klein et al., 1970; Reedman et al., 1974; Stowe et al., 1998). While treatment of acute infectious mononucleosis with acyclovir (ACV) has reduced the amount of virus shed in saliva, it did not alleviate or decrease the duration of clinical symptoms. Cessation of treatment with ACV was associated with an increase of viral shedding in the saliva back to levels detected prior to treatment (Andersson et al., 1986, Ernberg and Andersson, 1986; Tynell et al., 1996).

HHV-6 infects more than 90% of the population by 12 months of age (Kimberlin, 1998) and is subdivided into distinct A or B variants based on restriction endonuclease cleavage patterns (Aubin et al., 1991; Dominguez et al., 1999; Isegawa et al., 1999). The HHV-6A variant is isolated primarily from immunocompromised hosts while HHV-6B is responsible for the majority of childhood illnesses attributed to HHV-6 (Lusso, 1996). Primary infection with HHV-6 can result in *Exanthem subitum* or sixth disease, as well as rare cases of encephalitis, lymphadenopathy and seizure disorders (Braun et al., 1997; Kimberlin, 1998; Kondo et al., 1990; Yamanishi et al., 1988). HHV-6 infection becomes more significant clinically in immunocompromised hosts

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such as transplant recipients or HIV positive individuals where it can infect CD4+ cells making them more permissive to infection by HIV (Lusso et al., 1989; Lusso, 1996). In transplant recipients, HHV-6 infection has been shown to cause several complications including interstitial pneumonitis, prolonged viremia and has been associated with graft versus host disease (Singh and Carrigan, 1996). Due to the neurotropic nature of the virus, there has been speculation about the possible role of HHV-6 in the pathogenesis of multiple sclerosis (Ablashi et al., 1988; Challoner et al., 1995; Braun et al., 1997; Sola et al., 1993).

HHV-8 has been reported in 20–30% of the population with an increased prevalence in HIV-1-infected individuals (Chandran et al., 1998). HHV-8 has been detected in peripheral blood mononuclear cells, human endothelial cells and oropharyngeal mucosa (Gnann et al., 2000). This virus has been associated with body cavity-based lymphoma, lymphadenopathy, multicentric Castleman's disease, Kaposi's sarcoma (KS) and has the ability to transform primary human endothelial cells in vitro (Flore et al., 1998). It has been found in neoplastic endothelial and spindle cells present in the lesions of more than 90% of KS patients (Boshoff et al., 1995; Chatlynne et al., 1998). In HIV-infected individuals the presence of HHV-8 in peripheral blood lymphocytes is a risk factor for development of KS (Koelle et al., 1997; Lennette et al., 1996).

Currently, there are no proven effective antiviral therapies available for treatment of individuals infected with these viruses. The most widely used antiherpesvirus drug available at this time, ACV, is transiently effective against EBV but ineffective at inhibiting HHV-6 or HHV-8 replication in vitro. Ganciclovir (GCV), foscarnet (PFA) and cidofovir (CDV) have significant efficacy in vitro against HHV-6 and HHV-8 but prolonged use of these drugs in humans has been associated with severe side effects due to their toxicity (Gnann et al., 2000). The broad range and severity of clinical manifestations that are attributed to these lymphotropic herpesviruses illustrates the necessity for effective antiviral therapeutic agents.

In order to determine the antiviral efficacy against EBV, HHV-6 and HHV-8, our laboratory has previously utilized indirect immunofluorescence assays (IFA). We have concurrently evaluated potential antiviral compounds against EBV by enzyme-linked immunosorbent assays (ELISA) and in situ DNA hybridization assays. The purpose of these studies was to develop more efficient assays for the detection of antiviral compounds that are effective against lymphotropic herpesviruses. Flow cytometry has been utilized previously for the determination of antiviral efficacy against HHV-6 and HHV-8 (Manichanh et al., 2000; Reymen et al., 1995; Zoeteweij et al., 1999; Amjad et al., 2001) and we have utilized similar assay systems for evaluating antiviral activity against EBV, HHV-6 and HHV-8. These assays have proven to be advantageous by reducing the amount of labor involved as well as reducing the amount of subjectivity that is intrinsic to other methods.

2. Methods and materials

2.1. Virus and cell preparation

Daudi cells (American Type Culture Collection, Manassas, VA), a Burkitt's lymphoma derived B cell line latently infected with multiple copies of the EBV genome and H1 cells (provided by Dr. Y.-C. Cheng, Department of Pharmacology, Yale University School of Medicine, New Haven, CN), a subclone of human P3HR-1 cells, were cultured in RPMI-1640 media containing 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 μg/ml gentamycin. Superinfection of Daudi cells was achieved by incubation with EBV that was prepared from human P3HR-1 cell (American Type Culture Collection) culture supernatant as described previously (Lidin et al., 1993). As H1 cells continuously produce a high yield of EBV, cells were passaged to maintain logarithmic growth.

The HHV-6A variant, GS (HHV-6_{GS}), HSB-2 and Sup-T1 cells were obtained from the NIH AIDS Research and Reference Program (Rockville, MD). The cells were cultured in RPMI-1640 media supplemented with 10% heat-inactivated FBS and 2 mM L-glutamine. In addition, 100 U/ml penicillin and 100 μg/ml streptomycin were added to Sup-T1 cultures.

The HHV-6B variant, Z-29 (HHV-$6_{Z\text{-}29}$; American Type Culture Collection), was assayed in umbilical cord blood lymphocytes (CBLs) isolated from umbilical cord blood (University of Alabama, Birmingham Hospital) using histopaque (Sigma, St. Louis, MO) gradient separation. Cells were cultured in RPMI-1640 media containing 10% heat-inactivated FBS, 2 mM L-glutamine, 0.1 U/ml interleukin-2 (Sigma), 0.5 μg/ml *Phaseolus vulgaris* agglutinin protein (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone.

All HHV-6 virus pools were prepared by incubating uninfected cells with virus-infected cells at a 1:5 ratio until the cells were more than 60% infected as determined by IFA. Aliquots containing 2.5×10^6 cells were frozen in media containing 5% dimethylsulfoxide (DMSO; Sigma) and stored at −80 °C. Working dilutions for EBV and HHV-6 virus pools were determined by end point titration using IFA, and calculated according to the Reed–Muench method (Reed and Muench, 1938).

HHV-8, latently expressed in the primary effusion lymphoma derived BCBL-1 cell line (NIH AIDS Research and Reference Program, Rockville, MD) was induced into lytic HHV-8 expression by addition of 100 ng/ml phorbol 12-myristate 13-acetate (TPA; Renne et al., 1996). BCBL-1 cells were cultured in RPMI-1640 media containing 10% FBS, 2 mM L-glutamine, 10 μM β-mercaptoethanol and 30 μg/ml gentamycin.

2.2. Antiviral drugs

Six licensed antiviral compounds with well-documented efficacy against herpesviruses were evaluated. GCV, ACV,

penciclovir (PCV), CDV, and zidovudine (AZT) were purchased from the University of Alabama Hospital Pharmacy. PFA was procured from Sigma. A stock solution of 10 mg/ml PCV was prepared in DMSO and diluted to a final concentration of 1 mg/ml in minimal Eagle's medium (MEM) containing 2% FBS. The remaining compounds were prepared in MEM containing 2% FBS to a final stock concentration of 1 mg/ml.

2.3. Primary antibodies

The primary antibodies used for the indirect IFA and flow cytometric assay (FACS) were selected for their antigen specificity, low cross-reactivity with other herpesviruses and fluorescence intensity as monitored by flow cytometry. Monoclonal antibodies selected for use in the HHV-6 assay systems were screened for variant specificity and demonstrated no A or B variant cross-reactivity in the assay systems. Monoclonal antibody 8532 (Chemicon, Temecula, CA) targets HHV-6-induced early nuclear proteins and was used as a primary antibody in the HHV-6_{GS} assay systems at a 5 μg/ml concentration. Monoclonal antibody 8535 (Chemicon), which targets a B variant 101 kDa virion protein, was used as a primary antibody in the HHV-$6_{Z\text{-}29}$ assay system at a 5 μg/ml concentration. The HHV-8 monoclonal antibody KS8.1 (Bala Chandran, Department of Microbiology, Molecular Genetics and Immunology, University of Kansas) targets the HHV-8 viral envelope associated glycoprotein 8.1 expressed in the late lytic phase of HHV-8 replication (Zoeteweij et al., 1999) and was used at approximately 5 μg/ml. Monoclonal antibody to the EBV viral capsid antigen (VCA) glycoprotein 125 (Chemicon) was used at a concentration of 2.5 μg/ml for ELISA and 5 μg/ml for IFA and FACS.

2.4. Determination of cytotoxicity

The cytotoxicity of antiviral compounds was determined using Trypan blue exclusion. Serial 5-fold dilutions of antiviral compounds starting at either 50 or 100 μg/ml were prepared and incubated with 1×10^6 cells. The duration of the incubation period varied according to the cell line. Daudi cells were incubated at 37 °C for 3 days, HSB-2 cells for 4 days, BCBL-1 and H1 cells for 5 days, and CBL and Sup-T1 cells for 6 days. After incubation, cells were rinsed with PBS and pelleted by centrifugation at 1000 × *g* for 5 min. Cells were resuspended in 4 ml of 0.2% Trypan blue in PBS and spotted onto hemocytometer slides. The proportion of live cells for each drug dilution was determined, and the 50% cytotoxic concentration (CC_{50}) was extrapolated from the plot of drug concentration versus the concentration of live cells. The concentration required to inhibit cell proliferation by 50% (IC_{50}) was extrapolated from the plot of drug concentration versus the total number of cells. These plots were evaluated using the MacSynergy II, version 1.0 (University of Michigan, 1992 release) linear regression program.

2.5. Preparation of samples for determining antiviral efficacy against EBV and HHV-6

Antiviral efficacy against EBV and HHV-6 was assessed in several cell lines. Serial 5-fold dilutions of drug ranging from 50–0.08 μg/ml or 100–0.03 μg/ml were prepared fresh in media. Infection was initiated by incubating 10^6 cells for 1 h at 37 °C with sufficient virus to superinfect 10% of Daudi cells or infect 25% of the other cell lines. Cells were rinsed with 3 ml of media to remove unbound virus and pelleted by centrifugation at 1000 × *g* for 5 min prior to the addition of 4 ml of appropriately diluted drug. H1 cells, that are persistently infected, were incubated with the appropriate dilution of drug. Mock-infected and viral controls were prepared by adding 4 ml of drug-free media to uninfected and infected cells, respectively. After incubation for the appropriate times, cells were prepared for the various assays.

2.6. Preparation of samples for determining antiviral efficacy against HHV-8

Antiviral efficacy against HHV-8 was assessed using TPA-induced BCBL-1 cells. Serial 5-fold dilutions of drug starting at 100 μg/ml were prepared in media. Samples were prepared by incubating 1×10^6 TPA-induced BCBL-1 cells with the appropriate dilution of drug at 37 °C for 5 days. Mock-infected and virus controls were prepared by incubating 1×10^6 uninduced and TPA-induced cells, respectively, with 4 ml of drug-free media. Cells were fed with 2 ml of media 2 days after infection. After incubation, cells were prepared for FACS and IFA, as described below.

2.7. IFA staining

Infected or control cells were rinsed with PBS and resuspended to a final concentration of 1×10^6 cells/ml. Slides were prepared for IFA by spotting wells with 2×10^4 cells for each drug concentration and controls. Slides were dried, fixed in ice-cold acetone for 1 h, rinsed and stored at −20 °C before staining for IFA. Daudi cells were rehydrated then blocked in PBS containing 5% FBS and 4% normal goat serum (NGS). H1 cells were rehydrated, blocked and permeabilized in PBS containing 0.1% saponin and 1% FBS. All other cells were rehydrated and then blocked and permeabilized in PBS containing 5% FBS, 4% NGS and 0.5% DMSO for 30 min. Cells were then incubated in a hydration chamber at 37 °C for 1 h with 20 μl of primary antibody diluted in blocking solution. Following a rinse with PBS, cells were incubated at 37 °C for 1 h with 20 μl of 3 μg/ml FITC-conjugated goat anti-mouse IgG + IgM secondary antibody (Jackson ImmunoResearch, West Grove, PA). Cells were rinsed with PBS prior to staining with 0.1% Evans blue dye (Fisher, Fair Lawn, NJ) in PBS for 5 min. Slides were rinsed to remove any excess contrast dye and coverslips were mounted using a solution of 50% glycerol in PBS.

Cells were viewed at a magnification of 400× using a Nikon (Nikon, Melville, NY) fluorescence microscope. For each concentration of drug, 500 cells were counted and the percentage of antigen-positive cells was calculated. The drug concentration required to inhibit virus replication by 50% (EC_{50}) was determined by plotting drug concentration versus the percentage of antigen positive cells. These plots were evaluated as described above.

2.8. FACS staining

Daudi cells were rinsed and blocked in PBS containing 5% FBS and 4% NGS, while H1 cells were blocked and permeabilized in PBS containing 0.1% saponin and 1% FBS. HHV-6- and HHV-8-infected cells were rinsed with PBS and permeabilized overnight in 2 ml methanol at −80°C. These cells were blocked in PBS containing 5% FBS, 4% NGS and 0.5% DMSO for 30 min at 37 °C. Samples were incubated with 20 μl of primary antibody diluted in blocking solution for 1 h at 37 °C. Samples were rinsed twice with 4 ml of blocking solution and pelleted by centrifugation at 1000 × *g* for 5 min. Following the second rinse, 0.5 ml of 3 μg/ml FITC conjugated goat anti-mouse IgG + IgM (Jackson ImmunoResearch) was added and samples were incubated for 1 h at 37 °C. Cells were rinsed twice with PBS and pelleted by centrifugation before being resuspended and fixed in 0.5 ml of 2% paraformaldehyde.

Flow cytometry data was acquired using a Becton-Dickenson FacsCalibur instrument (Becton-Dickenson, Franklin Lakes, NJ) and data were analyzed using the WinMDI 2.7 data analysis program (Scripps Research Institute, La Jolla, CA). The resulting dot plots were gated to remove non-specific and background staining from further consideration and the M1 bar was set so that <1% of the cells in the negative control were included in the determination of percent positive cells. The EC_{50} value for each drug was extrapolated from the plot of drug concentration versus percentage of antigen positive cells as described above.

2.9. EBV in situ hybridization

Monitoring EBV DNA synthesis in the presence of antiviral compounds was performed using the Simply Sensitive Horseradish Peroxidase-AEC In Situ Detection System (Enzo Diagnostics, Farmingdale, NY). Slides were prepared as described above and staining and detection were performed according to the Manufacturer's instructions. Five hundred cells were counted and the percentage of positive cells was calculated for each concentration of drug. The EC_{50} value for each drug was extrapolated as described above.

2.10. EBV enzyme-linked immunosorbent assay (ELISA)

Daudi cells were rinsed with PBS and seeded into 96-well plates at 4×10^5 cells per well. Cells were fixed in a solution containing 5% acetic acid in ethanol for 20 min at room temperature and rinsed three times with PBS. Cells were then incubated for one hour at 37 °C with 2.5 μg/ml monoclonal antibody to EBV VCA (Chemicon), followed by a 30 min incubation with horseradish peroxidase labeled goat anti-mouse IgG1 (Southern Biotechnology, Birmingham, AL) for 30 min. Plates were rinsed with PBS containing 0.05% Tween 20 between incubations. Substrate containing *O*-phenylenediamine dihydrochloride, citrate buffer (pH 5.0) and hydrogen peroxide was added to each well. Plates were covered and gently shaken at room temperature for 10 min. The reaction was stopped by the addition of 3N sulfuric acid and plates were read immediately on a microplate reader (Bio-Tek Instruments, Winooski, VT) at 492 nm. The EC_{50} value for each drug was extrapolated from the linear regression plot of drug concentration versus average OD_{492}.

3. Results

The efficacy and toxicity values for ACV, GCV, PCV, CDV, and AZT were obtained from multiple assays against EBV, HHV-6, and HHV-8 utilizing both FACS and IFA. The EC_{50} values determined by FACS were found to be statistically similar to those obtained by IFA in all assay systems (Mann–Whitney Confidence Interval Test). All values fell within an acceptable range of assay variability and served to validate efficacy values obtained by both assay systems. Typical flow cytometry for GCV as an example are illustrated in Fig. 1. These histograms illustrate the pattern of staining obtained in negative (Fig. 1a) and virus (Fig. 1h) controls as well as the dose-dependent response of HHV-$6_{Z\text{-}29}$ to various concentrations of GCV (Fig. 1b–g).

In the EBV assays, ACV with an EC_{50} value of 1.3 μg/ml served as our positive control as no other antiviral tested significantly inhibited EBV replication (Table 1). Previous results in our laboratory have shown that EBV-infected Daudi and Raji cells had similar EC_{50} values for ACV. To obtain a higher infection rate for FACS analysis, H1 cells were also used. In situ hybridization assays and ELISA were also used in order to assess antiviral efficacy against EBV. Results obtained by in situ DNA hybridization and ELISA proved to be consistent with FACS and IFA results in EBV assays and were shown to be statistically similar (Kruskal–Wallis Test). All four assay systems proved to be reliable systems for evaluation of antiviral efficacy against EBV.

Assays for HHV-6_{GS} (A variant) were performed in both HSB-2 and Sup-T1 cell lines (Tables 2 and 3) and the susceptibility of HHV-6_{GS} to all the compounds was similar in both cell lines. Effective inhibitors of HHV-6_{GS} replication in both cell lines included PFA, GCV and CDV, with EC_{50} values less than 10.6 μg/ml, while PCV and AZT with EC_{50} values of >100 and >91.6 μg/ml, respectively, were ineffective. ACV was weakly active against HHV-6_{GS} in HSB-2 cells with an EC_{50} value of 27.2 μg/ml, but was inactive in Sup-T1 cells with an EC_{50} value of >59.5 μg/ml.

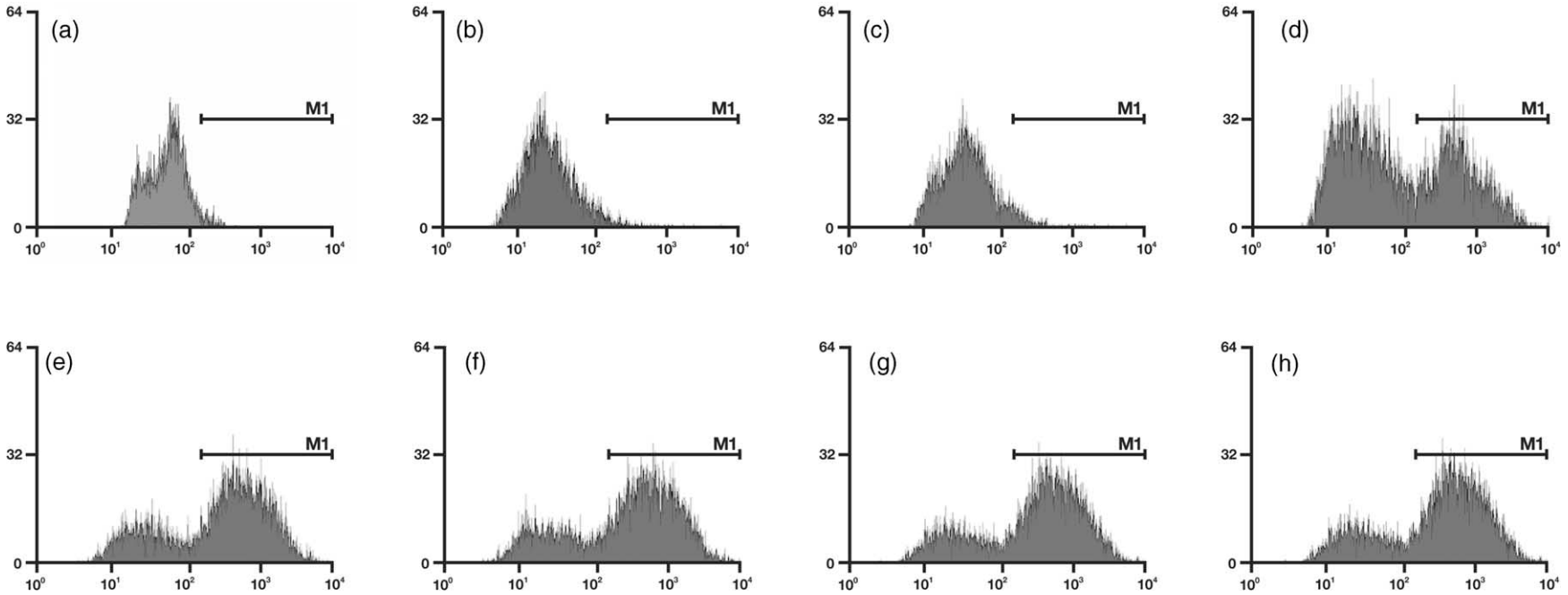

Fig. 1. Ganciclovir was assayed in HHV-$6_{Z\text{-}29}$-infected cord blood lymphocytes according to the protocol for determining antiviral efficacy against HHV-6 (see Section 2). Samples were stained and analyzed according to the staining protocol for flow cytometry (see Section 2). These histograms depict (a) uninfected cord blood lymphocytes, HHV-$6_{Z\text{-}29}$-infected cord blood lymphocytes that have been treated with (b) 50 μg/ml, (c) 10 μg/ml, (d) 2 μg/ml, (e) 0.4 μg/ml, (f) 0.8 μg/ml, (g) 0.016 μg/ml, or (h) 0 μg/ml of ganciclovir. In each histogram the *x*-axis denotes the intensity of FITC staining and the *y*-axis denotes the number of events. The M1 bar indicates the proportion of the cells considered to be positive for the HHV-6 antigen.

Table 1
Antiviral efficacy against EBV and cytotoxicity in Daudi cells

Drug	EC_{50} (μg/ml)[a]				Toxicity (μg/ml)[a]	
	Viral capsid antigen detection			DNA detection	CC_{50}	IC_{50}
	IFA	FACS	ELISA	In situ		
CDV	>39.3 ± 18.6	>50	>50	>28.1 ± 23.4	>50	>45.6 ± 7.7
GCV	>42.0 ± 13.9	>50	>50	>44.5 ± 9.5	>50	>30.4 ± 26.4
ACV	2.7 ± 1.3 (6)[b]	1.3 ± 1.1 (7)[b]	2.3 ± 0.5	4.5 ± 4.3	>50	>50
PFA	>50	>47.7 ± 3.9	>21.6 ± 24.6	>50	>50	>50
PCV	>33.0 ± 24.7	>50	45.1 ± 6.8	39.5 ± 8.0	>50	>50

[a] Values are expressed as the mean ± standard deviation of three replicate assays. Single assay values of >50 μg/ml are assigned a value of 50 μg/ml for the purpose of calculation and the mean value is designated with a '>' symbol. Similarly, single assay values of <0.08 μg/ml are assigned a value of 0.08 μg/ml for the purpose of calculation and the mean value is designated with a '<' symbol.
[b] Assays were performed in H1 cells.

Table 2
Antiviral efficacy against HHV-6(A)$_{GS}$ and cytotoxicity in HSB-2 cells

Drug	EC_{50} (μg/ml)[a]		Toxicity (μg/ml)[a]	
	IFA	FACS	CC_{50}	IC_{50}
CDV	12.8 ± 8.0 (4)	4.6 ± 3.5 (4)	39.6 ± 22.8 (4)	>82.7 ± 30.0
GCV	15.1 ± 3.0 (2)	10.6 ± 1.0 (2)	16.0 ± 3.6 (2)	79.1 ± 4.2 (2)
ACV	42.6 ± 52.1 (2)	27.2 ± 13.6 (2)	36.4 ± 26.2 (2)	>97.9 ± 3.0 (2)
PFA	8.8 ± 5.0 (7)	9.5 ± 5.7 (7)	>100 (6)	>100 (6)
PCV	53.7 ± 30.7	>100	77.3 ± 21.1	>76.7 ± 40.0

[a] Values are expressed as the mean of three replicate assays; the number of experiments is shown in parentheses if other than three replicates. See Table 1 for the definition of '<' or '>'.

Table 3
Antiviral efficacy against HHV-6(A)$_{GS}$ and cytotoxicity in Sup-T1 cells

Drug	EC_{50} (μg/ml)[a]		Toxicity (μg/ml)[a]	
	IFA	FACS	CC_{50}	IC_{50}
CDV	1.6 ± 1.7 (2)	2.0 ± 0.8	58.6 ± 21.4	85.6 ± 6.1 (2)
GCV	3.6 ± 2.6	7.6 ± 4.9 (13)	39.5 ±16.2 (12)	>80.0 ± 24.0 (7)
ACV	NT[b]	>59.5 ± 28.7 (7)	70.2 ± 39.0	>100
PFA	5.3 ± 4.1 (2)	6.3 ± 4.5 (4)	>85.2 ± 21 (2)	>100 (2)
PCV	NT[b]	>100	>90.8 ± 8.1	>99.8 ± 0.3
AZT	NT[b]	>91.6 ± 16.9 (4)	48.3 ± 28.9 (4)	>96.3 ± 7.0 (4)

[a] Values are expressed as the mean of three replicate assays; the number of experiments is shown in parentheses if other than three replicates. See Table 1 for the definition of '<' or '>'.
[b] Not tested.

Table 4
Antiviral efficacy against HHV-6(B)$_{Z\text{-}29}$ and cytotoxicity in CBL

Drug	EC_{50} (μg/ml)[a]		Toxicity (μg/ml)[a]	
	IFA	FACS	CC_{50}	IC_{50}
CDV	2.0 ± 1.2 (5)	1.4 ± 0.6 (5)	37.2 ± 23.8 (4)	>100 (5)
GCV	1.3 ± 0.9 (9)	2.1 ± 0.7 (9)	>59.5 ± 43.5 (9)	>88.6 ± 18.3 (9)
ACV	12.8 ± 1.1 (2)	16.9 ± 2.4 (2)	>92.5 ± 10.6 (2)	>79.3 ± 29.3 (2)
PFA	27.5 ± 23.3 (5)	35.9 ± 18.8 (5)	>79.7 ± 37.8 (5)	>100 (5)
PCV	37.4 ± 41.5 (2)	55 ± 58.4 (2)	30.8 ± 38.5 (2)	>100 (2)

[a] Values are expressed as the mean ± standard deviation. The number of replicate assays is shown in parentheses to the right of the value. See Table 1 for the definition of '<' or '>'.

Table 5
Antiviral efficacy against HHV-8 and cytotoxicity in BCBL-1 cells

Drug	EC_{50} (μg/ml)[a]		Toxicity (μg/ml)[a]	
	IFA	FACS	CC_{50}	IC_{50}
CDV	3.1 ± 1.6	3.9 ± 2.1	>89.2 ± 22.4 (9)	>68.0 ± 27.3 (9)
GCV	60.2 ± 42.0	53.7 ± 20.5	20.9 ± 17.8	>96.4 ± 6.2
ACV	53.7 ± 26.4	38.3 ± 17.3 (4)	>100	>100 (4)
PFA	72.9 ± 7.2	74.7 ± 15.0	>87.2 ± 22.2	>90.4 ± 16.7
PCV	74.8 ± 7.9 (2)	>82.0 ± 15.9	>86.9 ± 16.1	>100
AZT	>100	>100	32.2 ± 25.5	58.9 ± 14.7

[a] Values are expressed as the mean ± standard deviations for three replicate assays; the number of experiments is shown in parentheses if other than three replicates. See Table 1 for the definition of '<' or '>'.

The susceptibility of HHV-$6_{Z\text{-}29}$ (B variant) was similar to that of HHV-6_{GS} with the exception of PFA (Table 4). Antiviral compounds that effectively inhibited HHV-$6_{Z\text{-}29}$ replication included GCV and CDV, with EC_{50} values of 2.1 and 1.4 μg/ml, respectively. Moderate efficacy was demonstrated by ACV, with an EC_{50} value of 16.9 μg/ml, while PFA and PCV were ineffective against HHV-$6_{Z\text{-}29}$ in this assay system having EC_{50} values of 35.9 and 55.0 μg/ml, respectively. With the exception of PFA, the B variant of HHV-6 was slightly more sensitive to the antivirals tested than the A variant of HHV-6.

CDV was the only effective antiviral tested in the HHV-8 assay system having an EC_{50} value of 3.9 μg/ml (Table 5). No cytotoxicity of CDV was observed in BCBL-1 cells. All other antiviral drugs examined had little or no effect on HHV-8 replication in BCBL-1 cells.

4. Discussion

EBV, HHV-6, -7, and -8 are lymphotropic herpesviruses that have emerged as important targets for antiviral therapy. The wide variety and severity of clinical manifestations in humans pose a serious public health concern, particularly for immunocompromised patients. The lack of antiviral agents with efficacy against lymphotropic herpesviruses dictates the necessity for broad range screening of new compounds. We have developed flow cytometry-based assays in order to eliminate some of the time, cost and subjectivity that are intrinsic to other detection methods such as IFA. The purpose of our studies was to determine if FACS are sensitive, quantitative, and reproducible systems for evaluating antiviral agents.

Lymphotropic herpesviruses infect non-adherent cell lines that are particularly suited to analysis by flow cytometry. Selecting the proper lymphocytic cell lines was essential to the development of these assay systems. Primary human umbilical CBLs in an exponential growth phase are the ideal cells for assaying all the lymphotropic herpesviruses. Unfortunately, human CBLs have limited availability, are expensive to isolate and vary in growth characteristics. Continuous lymphocytic cell lines provide a reasonable option for culturing lymphotropic herpesviruses but the ability of these cell lines to metabolize antiviral compounds may differ from CBLs and all the viruses do not seem to grow in all cell lines. Our HHV-6_{GS} data has demonstrated the variability of continuous cell lines in their response to antiviral compounds. While most antiviral drugs responded similarly, we occasionally encountered drugs whose efficacy varied from one cell line to the next suggesting that the use of a single cell line for these types of assays may not result in a true reflection of a compound's activity against these viruses.

Once cell lines have been selected, screening antibodies for antigen specificity and fluorescence intensity are essential to the development of FACS systems. Both IFA and FACS rely on highly specific antibody–antigen interactions. We found that antibodies that are sufficient for immunofluorescence analysis do not necessarily provide good peak separation in the analysis of FACS data. Antibodies to late lytic antigens allowed us to monitor antiviral compounds that act either early or late in the replicative cycle. Implementation of these indirect fluorescence, flow cytometry-based assays has allowed us to evaluate currently licensed antiviral drugs for efficacy against EBV, HHV-6 and HHV-8.

Several factors influence the level of activity of an antiviral compound including choice of cell line, duration of incubation, concentration of drug and choice of detection methods. Although antiviral assays are commonly refed with multiple doses of drug during incubation, our efficacy values are based on assays where cells are initially exposed to antiviral compounds and fed, if required, with drug-free media. These results accurately reflect the antiviral and cytotoxic effects of a single administration of drug. The EC_{50} values obtained by our method are generally higher than those reported by other laboratories (Meerbach et al., 1998; Neyts and De Clercq, 1997; Reymen et al., 1995; Amjad et al., 2001), and this may be due to the differences in assay methodologies cited above. Using DNA hybridization and IFA on P3HR-1 cells that were TPA-induced and allowed to incubate with test compounds for 7 days, EBV was found to be susceptible to CDV, GCV, and ACV (Meerbach et al., 1998). Both CDV and GCV were shown to be active against HHV-8 using DNA hybridization assays performed on TPA-induced BCBL-1 cells (Neyts and De Clercq, 1997). In these assays, cells were allowed to incubate for 7 days and were fed with drug-containing media on day 4. In Reymen

et al. (1995), HHV-6_{GS} in HSB-2 cells was shown to be susceptible to CDV, GCV and PFA using CPE and FACS assays on cells that were incubated for 10 days and fed with drug-containing media on days 3 and 7. Although the results were similar to those observed in this paper, EC_{50} values were generally lower. These differences were likely due to the additional feedings with test compounds. In HHV-$6_{Z\text{-}29}$-infected MOLT-3 cells allowed to incubate for 7 days without additional feedings, the effects of GCV, ACV, PFA and PCV (Amjad et al., 2001) were similar to those observed in this paper confirming that differences in results like those observed above could be attributed to variations in assay methodologies. The efficacy values that we report are consistent across different detection methods, including FACS, IFA and in situ hybridization, and we are able to use these results to identify active antiviral compounds. This equates to previous work in which cytopathic effect inhibition, IFA, and DNA hybridization were compared to evaluate antiviral compounds against HHV-6 (Agut et al., 1989). Their work, as well as that presented in this paper, indicates that methods which measure accumulation of viral proteins are comparable to those which measure accumulation of viral DNA.

There are several established antiviral drugs with known efficacy against some herpesviruses but there remains a need to find more effective antiviral drugs against lymphotropic viruses. An accurate assessment of antiviral activity is dependent on the accuracy of the antiviral efficacy and cytotoxicity data. FACS provides a means of objective determination of antiviral efficacy and has proven to be a reproducible, reliable detection method. We have utilized FACS analysis in order to rapidly and consistently identify potential antiviral compounds for efficacy against HHV-6, HHV-8 and EBV and have found that results obtained using these assays are consistent with other established methods for screening antiviral compounds including IFA and in situ hybridization. Determination of antiviral efficacy by flow cytometry offers advantages over other screening methods by reducing the amount of labor, eliminating the subjectivity involved in other antiviral drug assay systems and providing a rapid and reliable method to screen potential antiviral compounds against lymphotropic herpesviruses.

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