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Fifty Percent Tissue Culture Infective Dose Assay for Determining the Titer of Infectious Human Herpesvirus 8

Sagar V. Nadgir,[a] Heather R. Hensler,[a]* Emilee R. Knowlton,[a] Charles R. Rinaldo,[a,b] Giovanna Rappocciolo,[a] Frank J. Jenkins[a,b,c]

Department of Infectious Diseases and Microbiology, Graduate School of Public Health,[a] Department of Pathology, School of Medicine,[b] and University of Pittsburgh Cancer Institute,[c] University of Pittsburgh, Pittsburgh, Pennsylvania, USA

We have developed a human herpesvirus 8 (HHV-8) 50% tissue culture infective dose ($TCID_{50}$) assay using the T1H6-DC-SIGN cell line. Infection of T1H6-DC-SIGN cells with HHV-8 induces expression of β-galactosidase, which was used to determine $TCID_{50}$ levels. Validation of $TCID_{50}$ values was performed by immunofluorescence assay of HHV-8 infection of immature dendritic cells at various $TCID_{50}$ doses.

Human herpesvirus 8 (HHV-8), also termed Kaposi's sarcoma-associated herpesvirus, is the etiologic agent of all forms of Kaposi's sarcoma as well as pleural effusion lymphomas and several forms of multicentric Castleman's disease (1–3). HHV-8 has been detected in several biological samples, including semen, saliva, blood, and tissues (4–11). The detection of HHV-8 in these samples has relied almost exclusively on PCR-based methods to amplify virion DNA. These approaches do not, however, determine levels of infectious virus. Further, as HHV-8 does not cause a cytopathic effect in cell cultures, standard plaque assays cannot be used to determine the titer of infectious virus. While some studies have relied on immunofluorescence-based assays to detect proteins of infectious virus (12–14), this approach suffers from a lack of quantitation. As a result, there is no accurate method for determining infectious titers of wild-type HHV-8 in biological samples or cell culture, hampering the study of this clinically important virus. The purpose of the present study was to develop a rapid and sensitive method for determining levels of infectious, wild-type HHV-8.

This assay utilizes T1H6 cells, a 293T cell line containing the β-galactosidase gene under the control of the HHV-8 T1.1 promoter (15). The 293T cell line is a human embryonic kidney cell line that contains the E6 gene of adenovirus and SV40 T antigen (16). The HHV-8 T1.1 promoter contains a replication and transcription activator (RTA) response element (RRE). The RTA protein (encoded by the HHV-8 open reading frame 50 [ORF50] gene) is sufficient and necessary for reactivation of latent HHV-8 (17) and is actively transcribed during a primary infection. Binding of the RTA protein to the RRE in the T1.1 promoter results in increased transcription of the β-galactosidase gene. HHV-8 infection of the T1H6 cells results in the production of the RTA protein by the infecting virus, which in turn induces expression of β-galactosidase. It has been shown previously that efficient infection of the T1H6 cell line with HHV-8 requires treatment of the cells with Polybrene to allow for receptor-independent infection (15). As a result, parental T1H6 cells are not suitable for measuring natural HHV-8 infectivity.

We have previously reported that HHV-8 utilizes DC-SIGN as a cellular receptor on immature DC and activated macrophages and B cells (18, 19). To utilize this cellular receptor for titration of infectious HHV-8, T1H6 cells were stably transfected with pcDNA3-DC-SIGN, which expresses DC-SIGN under the control of the cytomegalovirus (CMV) immediate early promoter (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from S. Pohlman, F. Baribaud, F. Kirchhoff, and R. W. Doms), creating the T1H6-DC-SIGN cell line. Flow analyses using anti-DC-SIGN antibody revealed that a majority of the T1H6-DC-SIGN cells (~70%) and less than 1.5% of the parental T1H6 cells expressed DC-SIGN on the surface of the cells (data not shown).

To demonstrate that the T1.1/β-galactosidase reporter gene (contained in the T1H6-DC-SIGN cells) responds to expression of the RTA protein, a plasmid containing the ORF50 cDNA under the control of the strong CMV promoter was transfected using Lipofectamine 2000 (Life Technologies, Grand Island, NY) onto T1H6-DC-SIGN cells plated in triplicate, and β-galactosidase activity was measured 48 h posttransfection using the β-Gal chemiluminescence detection kit II (Clontech, Mountain View, CA) according to the manufacturer's instructions. β-Galactosidase activity was expressed in a dose response manner to increasing amounts of ORF50 plasmid DNA (data not shown).

To compare induction of β-galactosidase activity by HHV-8 infection on T1H6-DC-SIGN cells, T1H6-DC-SIGN cells were infected with HHV-8, and β-galactosidase activity was monitored at 24 and 48 h postinfection (hpi). HHV-8 was purified from tetradecanoyl phorbol acetate (TPA)-induced BCBL-1 cells as previously described (18, 19). There was a significant increase ($P < 0.05$) in β-galactosidase expression in T1H6-DC-SIGN-infected cells at 48 hpi compared to that in uninfected cells. Based on these results, all future experiments used 48 h postplating or postinfection as the experimental endpoint.

To determine HHV-8 $TCID_{50}$ values, T1H6-DC-SIGN cells were plated in black Corning CellBind 96-well plates (Corning, Tewksbury, MA) at 8×10^4 cells/well in replicates of 6 wells. Tenfold dilutions of HHV-8 samples were prepared, and 15 μl was inoculated into each well. Forty-eight hpi, cells were harvested using 100 μl of CellStripper (CellGro, Tewksbury, MA). The con-

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Address correspondence to Frank J. Jenkins, fjenkins@pitt.edu.
* Present address: Heather R. Hensler, ProImmune, Inc., Oxford, United Kingdom.

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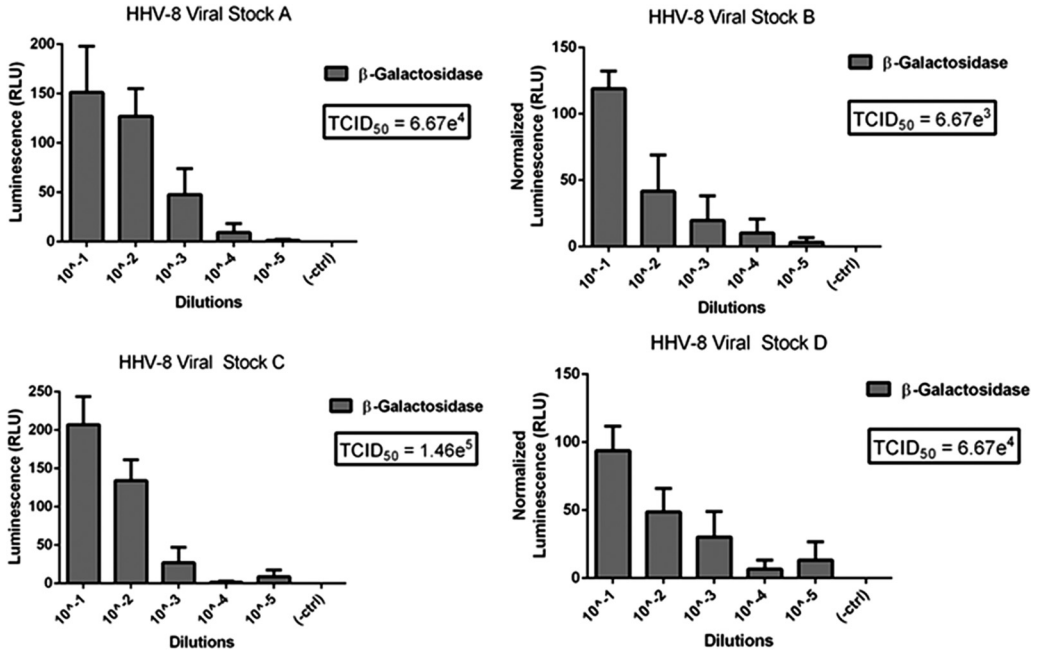

FIG 1 $TCID_{50}$ determinations of 4 separate HHV-8 viral preparations. Each graph shows the β-galactosidase levels with decreasing amounts of HHV-8 and the resulting $TCID_{50}$ values.

tents of each well were collected, and the cell pellet was harvested by centrifugation for 5 min at 13,000 rpm, 4°C. Each pellet was washed 3 times with 100 μl of ice-cold 1× phosphate-buffered saline (PBS). Pellets were resuspended in 100 μl of cold potassium phosphate-dithiothreitol (DTT) lysis buffer (100 mM potassium phosphate, pH 7.8; 1 mM DTT) to prevent oxidation of β-galactosidase. The solution was subjected to 3 freeze-thaw cycles using dry ice-ethanol and a 37°C water incubator. Cell debris was removed by centrifugation at 13,000 rpm for 10 min at 4°C, and lysates were carefully collected. A total of 15 μl of each lysate was plated in individual wells, in replicates of 6. Detection of β-galactosidase was determined using the β-Gal chemiluminescence detection kit II (Clontech, Mountain View, CA) according to the manufacturer's instructions with the following changes. For each well, 117.6 μl of reaction buffer and 2.4 μl of substrate were added in the dark. The substrate and buffer mixture were mixed with the cell lysates, and the reaction mixture was incubated for 1 h at room temperature in the dark. Chemiluminescence was measured using a BioTek Synergy II chemiluminescence reader (BioTek, Winooski, VT), with the data recorded in 10-s integrals at a sensitivity setting of 250. To distinguish a positive infection from a negative infection for $TCID_{50}$ measurements, the chemiluminescence readings of the uninfected cell lysates (background average chemiluminescence), plus twice the background standard deviation, was subtracted from each test value. If the resulting value was greater than 0, the well was considered to be infected. If the resulting value was ≤0, the well was deemed uninfected.

As shown in Fig. 1, β-galactosidase levels demonstrated a dose response with increasing dilutions of each HHV-8 viral preparation. The results from each well (positive or negative infection) were used in the Reed-Muench calculation (20) to determine the HHV-8 $TCID_{50}$ for each sample. The calculation used was as follows: $TCID_{50}/ml = 10^{(PD - a)}/b$, where PD = proportional distance, a = log dilution greater than 50% infected, and b = inoculum volume (ml). PD = % of wells infected over 50% − 50%)/(% of wells infected over 50%) − (% of wells infected less than 50%). Figure 1 shows the $TCID_{50}$ values of four separate HHV-8 viral preparations.

HHV-8 $TCID_{50}$ values were validated using immature monocyte-derived dendritic cells (iMDDC). Peripheral blood mononuclear cells (PBMCs) obtained from healthy blood donors were isolated with lymphocyte separation medium (Mediatech, Manassas, VA). Monocytes were isolated by plastic adherence and treated with 10 ml of IMDM medium (Iscove's modification of Dulbecco's modified Eagle medium; Mediatech, Manassas, VA) containing 10% fetal calf serum (FCS), 1,000 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF), and 1,000 U/ml interleukin 4 (IL-4; Schering Plough, Kenilworth, NJ). Media containing the cytokines were refreshed on day 4 of culture, and the cells (8×10^4/well) were plated on day 5 for experimentation. iMDDC were plated in replicates of 4 in a 96-well plate. The cells were infected with HHV-8 using either 1 or 2 $TCID_{50}$ amounts. Two different HHV-8 viral preparations were used in these experiments. At 48 hpi, cells were washed, fixed, and stained for expression of viral ORF59, a processivity factor for HHV-8 DNA polymerase (21), using a mouse-anti-ORF59 antibody (ABI, Columbia, MD) followed by goat-anti-mouse IgG-fluorescein isothiocyanate (FITC) (Santa Cruz Biotechnology, Dallas, TX). Images of the infections using both HHV-8 virus preparations were blinded, and the number of infected cells was manually counted by three individuals unrelated to this project. Further, this experiment was performed with iMDDC derived from the monocytes of two separate donors. As shown in Table 1, infection of cells with 1 or 2 $TCID_{50}$ HHV-8 values resulted in the expected percentage of infected cells using different virus preparations and iMDDC derived from different donors. Since MDDC do not sup-

TABLE 1 Validation of $TCID_{50}$ values[a]

Monocyte donor	HHV-8 viral preparation	$TCID_{50}$ level	% infected cells			
			Count 1	Count 2	Count 3	Mean ± SD
1	1	1	60	63	46	56 ± 9
	1	2	99	93	85	92 ± 7
	2	1	52	60	45	52 ± 8
	2	2	100	94	90	95 ± 5
2	1	1	43	61	42	49 ± 11
	1	2	100	100	95	98 ± 3
	2	1	48	59	48	52 ± 6
	2	2	89	93	94	92 ± 3

[a] Validation of $TCID_{50}$ values. Monocyte-derived immature DC from 2 separate donors were infected with 1 or 2 $TCID_{50}$ levels of HHV-8 from 2 separate viral preparations. The cells were stained for expression of the HHV-8 ORF59 protein at 48 hpi. Images of the stained cells were blinded, and the percentage of infected cells in each experiment was counted by three separate individuals. Results are presented as the means of the % infected cells ± SD.

port lytic replication of HHV-8 (19), we confirmed that this $TCID_{50}$ assay could quantify HHV-8 lytic replication in CD40L–IL-4-activated, peripheral blood B lymphocytes (18) (data not shown).

The ability to calculate infectious HHV-8 virus titers allows for the determination of specific infectivity, the ratio between $TCID_{50}$/ml and encapsidated (DNase-treated) viral copies. Specific infectivity demonstrates the ratio of infectious to total viral particles in a particular sample. As shown in Table 2, the specific infectivity of two separate HHV-8 preparations ranged from 3.5×10^{-5} to 1.27×10^{-6}, demonstrating that the majority of the HHV-8 virus particles were noninfectious.

HHV-8 DNA has been detected in a variety of biological samples, including saliva, blood, semen, and various tissues (22–26). These locations are associated with documented transmission of the virus from individual to individual (9, 10, 25, 26). There is currently no method to accurately determine the titer of infectious HHV-8 in these samples or in *in vitro*-generated samples. The latter is especially important for studies designed to synchronize cell culture infections and for assessment and reproducibility of *in vitro* experiments. It is also important to be able to determine viral loads in different biological samples in studies designed to study efficiency of transmission. An assay that can measure infectious virus titers can be used to determine neutralizing antibody titers which may be important in studies on HHV-8 immunity and development of Kaposi's sarcoma. Current methods to determine virus titers consist of measuring the amount of encapsidated viral particles by quantitative PCR of DNase-resistant viral DNA in various samples or detecting infected cells by antibody-based techniques, such as immunofluorescence (8, 12, 14). The former suffers from the inability to distinguish infectious from noninfectious particles, while the latter suffers from loss of sensitivity.

The T1H6-DC-SIGN cells allowed the development of a $TCID_{50}$ assay for the determination of infectious HHV-8 titers in various biological samples. We have determined the infectious titers of several different viral preparations and validated these levels by infecting immature dendritic cells at 1 and 2 $TCID_{50}$ values. Importantly, our results demonstrate that viral stocks prepared by polyethylene glycol (PEG) precipitation and centrifugation through sucrose cushions results in a significant number of noninfectious virus, as evidenced by specific infectivity values

TABLE 2 Specific activity of HHV-8 viral preparations[a]

HHV-8 sample	$TCID_{50}$/ml	No. of DNA copies/ml	Specific infectivity
A	6.11×10^2	4.80×10^8	1.27×10^{-6}
B	6.11×10^5	1.74×10^9	3.51×10^{-5}

[a] Specific activity of HHV-8 viral preparations. The $TCID_{50}$ values of two separate HHV-8 viral preparations were determined as described in the text. Total virus DNA copies were determined by quantitative PCR. Specific infectivity was calculated by dividing $TCID_{50}$ values by total DNA copies.

showing 1 infectious virus in 100,000 to 1 million virus particles. As a result, an experiment in which the virus titer is determined by quantitative PCR of DNase-resistant virus particles and the calculated multiplicity of infection (MOI) is 10 may actually be using an MOI of 0.0001 to 0.00001 (based on a specific infectivity of 1×10^{-5} to 1×10^{-6}).

The T1H6-DC-SIGN assay represents the first *in vitro* assay for determining infectious titers of wild-type, nonrecombinant HHV-8. This assay should prove to be very useful for cell culture experiments, including synchronized infections, determination of anti-HHV-8-neutralizing antibody levels, and determining levels of infectious virus in biological samples.

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