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Antiviral Research 40 (1998) 105–112

Antiviral Research

Solid phase ELISA for determination of the virus dose dependant sensitivity of human cytomegalovirus to antiviral drugs in vitro

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Received 10 July 1998; accepted 16 September 1998

Abstract

The main problems in determining the true in vivo susceptibility of human cytomegalovirus (CMV) to antiviral compounds are the influence of the size of the viral inoculum, the variation in the replication capacity of different CMV strains and the subjective evaluation of the inhibition of viral growth in the plaque assay. In this study, a specific assay was developed which reproducibly determines the sensitivity of primary isolates of CMV. The assay includes simultaneous virus titration and determination of the antiviral sensitivity. When individual virus doses were evaluated, the IC_{50} was generally dependent on the virus dose, except for resistant isolates, where the IC_{50} did not change irrespective of the dose of virus. The novel method of IC_{50} calculation takes into account all values derived from the linear part of the inhibition curve. This may better reflect the in vivo conditions, where the antiviral drug encounters different amounts of virus in different organs. Two human fibroblast-derived cell lines showed similar results.

Keywords: Antiviral sensitivity assay; Cytomegalovirus; ELISA; Antiviral drugs

1. Introduction

In some patients there is a lack of clinical response to antiviral treatment of cytomegalovirus (CMV) infection. This may be due to the presence

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0166-3542/98/$ - see front matter
PII S0166-3542(98)00054-0

of drug resistant virus, a high virus load in the patient, a lack of compliance, or insufficient level of drug at the site of infection. Resistance to ganciclovir has become a substantial problem in AIDS patients on long-term treatment for CMV retinitis (Field and Biron, 1994; Drew et al., 1996; Erice et al., 1997), and there is a risk that drug resistant CMV strains may become prevalent in society. Thus, the availability of rapid and adequate methods for evaluation of antiviral sensitivity is of importance.

The antiviral sensitivity of CMV to ganciclovir can at present be rapidly determined by analysis of mutations in the UL-97 gene (Sullivan et al., 1992), but not all resistant viruses exhibit these mutations (Chou et al., 1995). Resistance to foscarnet is uncommon, but when it occurs it has been described after treatment (Baldanti et al., 1996). Also, new antiviral compounds are being introduced, for which the mutations responsible are beginning to be described (Smith et al., 1997). The need for biological assays to determine antiviral resistance thus remains.

Several types of assays for the determination of drug sensitivity in CMV have been described (Gadler, 1983; Wahren et al., 1983; Dankner et al., 1990; Tatarowics et al., 1991; Drew et al., 1993; Lipson et al., 1993; Gerna et al., 1995). The plaque reduction assay (PRA) has been proposed as the reference method (Drew et al., 1993). In most of these methods, the viral isolate has first to be cultivated to relatively high titres, and a viral titration is necessary prior to sensitivity determination. Most of these assays lack a standardised measurement for evaluation of viral inhibition.

The aim of this study was to develop a reliable and objective method for measurement of the antiviral sensitivity of primary CMV isolates. The basis of the method is a solid phase antigen ELISA performed in microplates. This concept allows the determination of the IC_{50} for a given drug at many different dilutions of the virus isolate, thus enabling viral titration and determination of drug sensitivity to be performed simultaneously.

2. Materials and methods

2.1. Cell culture

Human foetal lung fibroblasts (HL, obtained from the Department of Virology, Sahlgrenska Hospital, Gothenburg), always below passage 18, were cultured in medium consisting of Eagle's minimal essential medium (GIBCO, Life Technologies, Sweden) supplemented with 2 mM L-glutamine, 2% foetal calf serum (FCS, Bodinco B.V., Holland), 10000 IU/l of penicillin and 10 mg/l of streptomycin. Maintenance medium consisted of Dulbecco's modified medium (MOD) (Life Technologies, Sweden) supplemented with 2 mM L–glutamine, 10% FCS and penicillin and streptomycin. Human foreskin fibroblasts (Hs27, CRL-1634, ATCC, Rockville, MD) below passage 35 were cultured in Dulbecco's MOD supplemented with 2 mM L–glutamine and 10% FCS without antibiotics.

2.2. Virus strains

CMV Towne (VR 977, ATCC, Rockville, MD), and clinical CMV isolates with different sensitivities to ganciclovir and foscarnet were used as cell-associated inoculum. The strains had been stored at −70°C, and passaged at least twice on human lung fibroblasts. One isolate, I4636, from a congenitally infected child and an isolate from a renal transplant recipient, AST105A, were sensitive to both ganciclovir and foscarnet in the plaque reduction assay performed at the Swedish Institute for Infectious Disease Control. Isolate AST106A was from a renal transplant patient and was resistant to GCV. Isolate AST13B, kindly donated by Dr Miner (USCF Mt Zion, San Francisco, CA) had been evaluated by plaque reduction assay at USCF Mt Zion to be resistant against both ganciclovir and foscarnet. These three virus strains and CMV Towne were used to compare the plaque reduction assay and the solid phase ELISA. A total of 14 isolates from nine renal transplant recipients (transplanted at Uppsala University Hospital, Sweden) who had not received antiviral drugs, were examined in the

solid phase ELISA. The 14 strains were also compared in the solid phase ELISA with HL and Hs27 cells. All virus strains were initially isolated on HL cells, except for AST13B which was isolated on Hs27 cells. CMV Towne was used as a control in all assays and the titer of the stock virus was determined by a plaque assay. A fresh aliquot of CMV Towne with a known content of infectious virus (plaque forming unit/ml; PFU/ml) was used in each experiment.

2.3. Antiviral compounds

Ganciclovir GCV, 9-(1,3-dihydroxy-2-propoxymethyl)guanine was obtained from Syntex Research (Palo Alto, CA), diluted in sterile distilled water and stored as a 10 mM stock solution at room temperature. Foscarnet (PFA; phosphonoformate) was obtained from Astra Arcus AB, Södertälje, Sweden as an 80 mM solution and stored at room temperature.

2.4. Solid phase ELISA method

Primary human lung fibroblasts (90×10^3 cells/well) were incubated in 96-well plates at 37°C in 5% CO_2 for 3–5 days until confluent, whereupon virus and antiviral compounds were added. The configuration of the microtiter plates comprised eight uninfected control wells, one virus-infected control well per virus dilution and eight virus dilutions to which five concentrations of antiviral compounds were added (for ganciclovir: 0.25, 1, 4, 16 and 32 μM; for foscarnet: 50, 100, 200, 400 and 800 μM for primary isolates and 25–400 μM for Towne). After removal of the growth medium, 200 μl of Dulbecco's MOD was added to the uninfected control wells and 200 μl virus inoculum was added to the remaining wells. The plates were centrifuged at $1500 \times g$ for 60 min at room temperature to facilitate virus adsorption. The fluid was aspirated from each well and 200 μl of fresh medium was added to the uninfected control wells and virus dilution control wells. Increasing concentrations of the antiviral compounds diluted in Dulbecco's MOD were added to the remaining wells. The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 7 days (3, 5 and 10 days were also investigated). The cytopathic effect was estimated microscopically, and the medium was removed from the wells. The plates were washed three times with cold saline and fixed for 5 min by addition of 200 μl of cold acetone/water (80:20, v/v). Before performing the solid phase ELISA the plates were allowed to dry at room temperature, and could thereafter also be stored in the refrigerator.

The wells were washed five times with 0.9% sodium chloride containing 0.05% Tween 20. A total of 100 μl monoclonal antibody (M7065, DAKO, Älvsjö, Sweden), directed to the lower matrix phosphoprotein pp65 (ppUL83), diluted in ELISA buffer (phosphate-buffered saline with 0.5% bovine serum albumin, 0.05% Tween 20, and 10% FCS) were added to each well and allowed to incubate for 60 min at 37°C. The wells were washed five times and 100 μl affinity-purified goat anti-mouse IgG (Bio-Rad Laboratories, Solna, Sweden) conjugated to peroxidase and diluted in ELISA buffer were added. After incubation for 60 min at 37°C, the wells were washed five times. A total of 100 μl substrate, orthophenylenediamine (Sigma, St. Louis, MO) at 1 mg/ml in phosphate-citrate buffer pH 5.0 containing 0.001% hydrogen peroxide, were added to each well and the colour reaction was developed at room temperature. The reaction was stopped after 30 min with 100 μl 2.5 M sulphuric acid and the absorbance was measured at 492 nm using an E-max spectrophotometer (Molecular Devices Corp. CA). The concentration of antiviral substance giving a 50% inhibition of viral growth (IC_{50}) after correction for the background absorption was calculated (see Section 3).

2.5. Plaque reduction assay (PRA)

The assay was performed as described by Drew et al. (Drew et al., 1993) with one modification, that agarose was used for the overlay. Human foreskin fibroblasts (Hs27, CRL-1634, ATCC, Rockville, MD) were grown in 24-well plates until confluent. The virus inoculum, diluted in Dulbecco's MOD and providing approximately 250–500 PFU (plaque forming units) per ml, was added in 0.5 ml aliquots to each well. Unadsorbed virus

was removed after 90 min of incubation at 37°C. An overlay of 1.5 ml 0.3% AgarPlaque PLUS agarose (AMS Biotechnology, Täby, Sweden) containing various concentrations of antiviral compounds or medium was added. Each concentration was tested in six-fold replicates. The cultures were incubated for approximately 7–10 days at 37°C in a 5% CO_2 incubator. When plaque formation was well-defined, the cells were fixed in 10% formaldehyde, the agarose was removed and the cells stained with methylene blue. Plaques were counted in an inverted microscope at low magnification. The IC_{50} for the plaque reduction assay was defined as the concentration of drug which resulted in a 50% reduction in the number of plaques relative to control wells.

2.6. Comparison between PRA and ELISA

HL cells were incubated with virus from an isolate and CMV Towne at different concentrations in duplicate ELISA plates. Each virus dilution was applied to six different wells in each plate. In one of the duplicate plates, each well was filled with agarose. After 7 days, the agarose was removed and the plaques stained as described in the plaque reduction assay. The other duplicate plate was used for antigen ELISA, as described in the ELISA method.

2.7. Statistical analysis

Mean and standard deviation were used for descriptive statistics. One-way analysis of variance was used to analyse the effects of viral dose on the IC_{50} values.

3. Results

3.1. Comparison between solid phase ELISA and plaque assay

There was a linear correlation between the absorbance in the solid phase ELISA and the number of plaques in the PRA (Fig. 1). A reduction in absorbance thus correlated to a reduction in PFU over the linear absorbance range. A 50% inhibition of virus replication was subsequently calculated as the concentration of the antiviral drug giving a 50% reduction in absorbance in the ELISA. The IC_{50} values of three clinical isolates, and the CMV Towne strain measured in both the standard plaque assay, and the solid phase ELISA using HL cells, are shown in Table 1. One isolate (AST13B) was evaluated as resistant (see below). Table 1 shows that the methods did not differ systematically from each other with respect to the IC_{50} values obtained.

3.2. Determination of the IC_{50} reference value in the solid phase ELISA

The IC_{50} values for susceptible strains decreased with decreasing size of the viral inoculum (Fig. 2a, c, d). In order to find a reference IC_{50} value for each virus isolate, the IC_{50} was calculated for dilutions of the virus where the optical density (OD) values for the untreated virus control were within the linear part of the absorbance curve. This was normally found between OD 0.7 and 3. Between two and four of the eight dilutions of virus used in the assay were normally evaluable by this criterion.

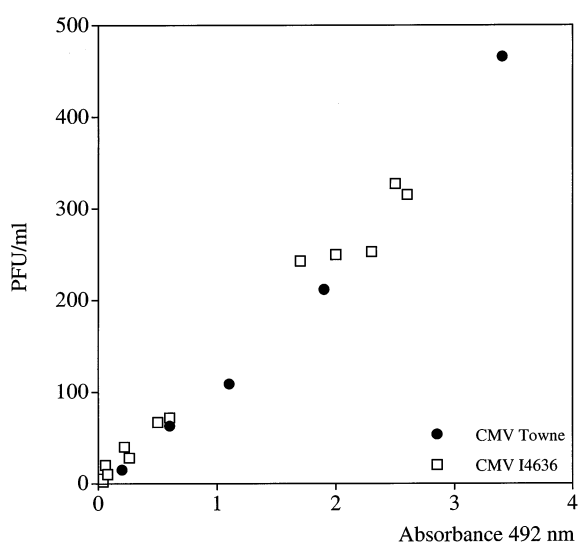

Fig. 1. Comparison between PFU in the plaque reduction assay, and the absorbance in the solid phase ELISA method after 7 days of cultivation of two different CMV strains. The mean of six determinations is shown.

Table 1
IC_{50} values obtained for CMV Towne and three clinical CMV isolates in the plaque reduction assay (PRA) compared with the viral sensitivity assay (ELISA)

Virus strain	IC_{50}, μM ganciclovir		IC_{50}, μM foscarnet	
	PRA	Solid phase ELISA	PRA	Solid phase ELISA
Towne	2.2; 4.6; 6[a]	2.4 ± 1.4[b]	65; 132; 129[a]	53 ± 25[b]
I4636	7.5	3.4	163	142
AST105A	2	3.8	160	165
AST13B	50	>32	500	>800

[a] Results of three separate examinations.
[b] Mean and standard deviation of 21 separate examinations.

A mean OD value for the evaluable doses of virus was calculated at each drug concentration as an arithmetic mean of the respective OD. This procedure was repeated for all antiviral drug concentrations with all selected doses of virus, giving a new reference curve. This new reference curve in turn was used to obtain the IC_{50} reference value. For resistant strains (e.g. Fig. 2b) it was not possible to calculate an IC_{50} value due to the very poor inhibitory effect of the antiviral drug, and the reference value was given as greater than the highest concentration used in the assay. When even very small virus doses showed no dose-response to the antiviral drug, the strains were evaluated to be resistant. The above calculations can be modified to incorporate viral concentrations inhibited by any antiviral drug, as long as data derived from the linear part of the inhibition curve are used.

3.3. Period of replication

In initial experiments, the plates were incubated for 3 days before fixation, in order to allow for a single CMV replicative cycle. Since large amounts of virus had to be used in order to detect antigen already after 3 days, this resulted in marked interassay variation, with a tendency towards elevated IC_{50} values compared to previously published results. Incubation for 7 days was found to give more reproducible results with IC_{50} values within acceptable ranges, and was chosen as the fixed endpoint of each experiment (data not shown).

3.4. Reproducibility of the assay, IC_{50} of isolates from untreated patients, and comparison between fibroblast cell lines

The mean and standard deviation when CMV Towne was examined 21 times on HL cells was 2.4 μM (S.D. 1.4) for ganciclovir and 53 μM (S.D. 25) for foscarnet, giving an interassay variation of 59 and 47%, respectively. A total of 14 CMV isolates from nine renal transplant recipients who were not treated with antiviral drugs were assayed using HL cells, and the mean IC_{50} for ganciclovir was 6.9 μM (S.D. 5.4) and for foscarnet 125 μM (S.D. 56). The corresponding IC_{50} values when Hs27 cells were used were 5.5 (S.D. 4.2) for ganciclovir, and 150 (S.D. 58) for foscarnet. The differences between these cell lines were not statistically significant, and for most isolates the variations between cell lines were minor (less than 2-fold). For three isolates 3–7-fold higher IC_{50} reference values were obtained in the HL assay, and for one isolate the value in the Hs27 assay was 3-fold higher than in the HL assay.

4. Discussion

The in vitro susceptibility of a virus isolate to antiviral compounds depends on the multiplicity of infection used in the assay, the number of replication cycles and the cell line used for replication (Harmenberg et al., 1980; Tatarowics et al., 1991). Thus it is necessary to standardise the

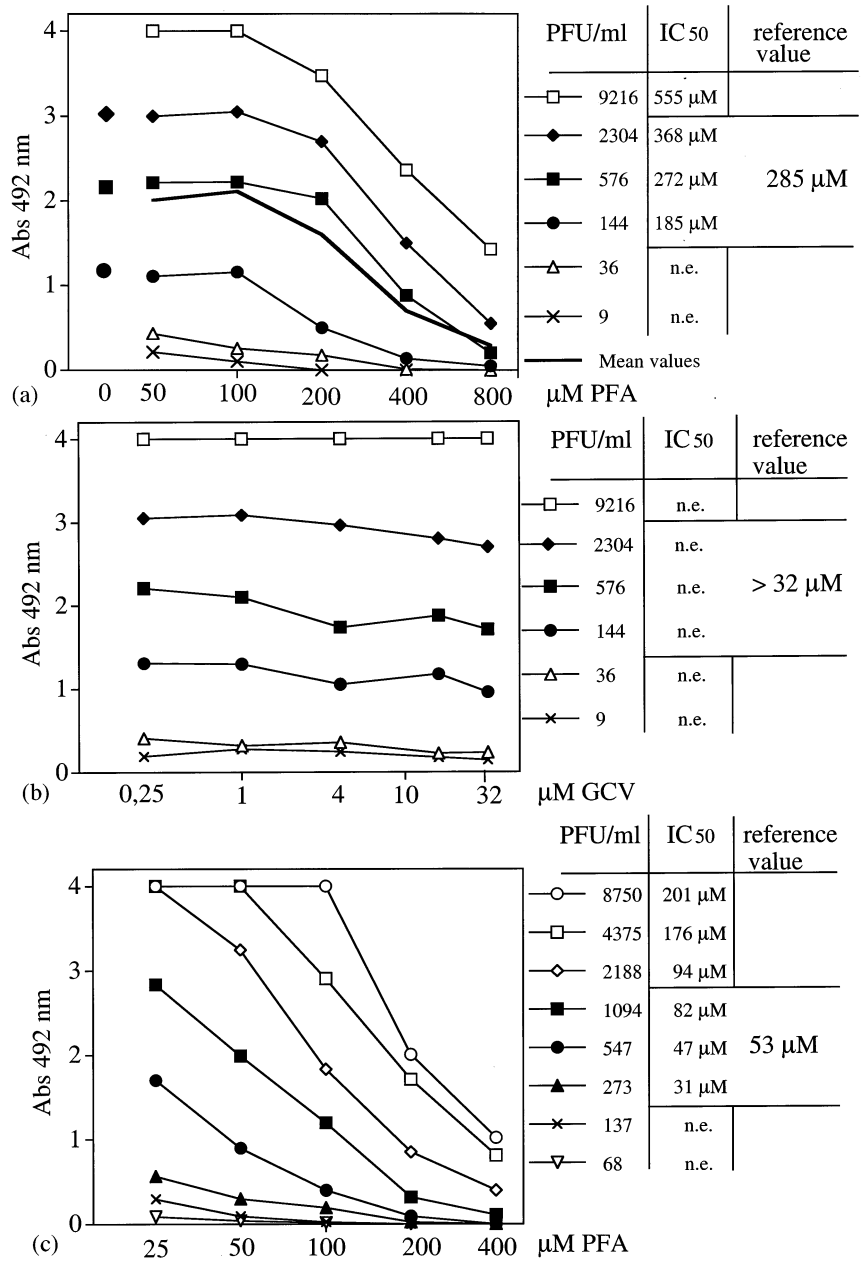

Fig. 2. Inhibition by foscarnet and ganciclovir, (a–b) of isolate AST106A from a renal transplant patient infected with CMV and (c–d) of the laboratory adapted control strain CMV Towne. A reference curve (line) indicates the calculated IC_{50} values obtained from three selected doses of virus (see text). ne, not evaluable.

length of incubation and the viral inoculum (through titration). The method presented in this paper is a development of previously published assays (Wahren et al., 1983; Tatarowics et al., 1991) and uses a solid phase antigen ELISA and multiple doses of virus which avoids the need for proceeding titration of an isolate. The use of absorbance provides objectivity in the evaluation

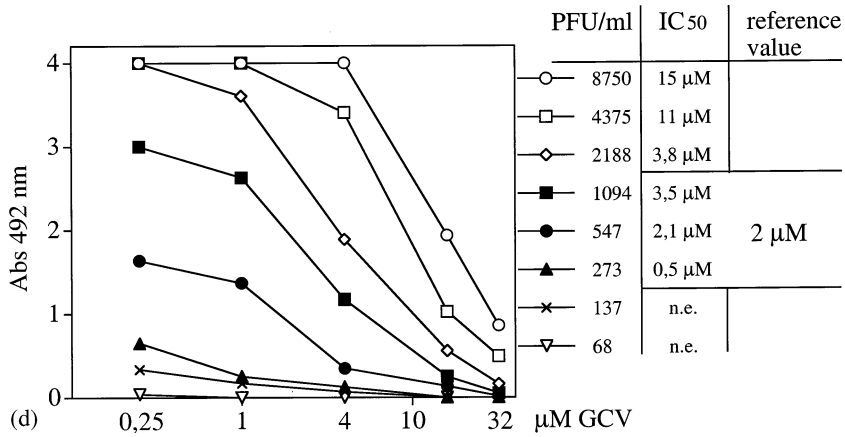

Fig. 2. (*Continued*)

of viral replication. A cultivation time of 7 days gave more reproducible results than 3 days, probably because the latter required too large a viral inoculum to produce ELISA results that could be evaluated in only 3 days. Nevertheless, there is a considerable time saving compared to plaque reduction assay. Furthermore, all reagents are commercially available, and the ELISA methodology is simple. The assay is also specific for CMV and discriminates adenoviruses.

In isolates from untreated patients, the mean IC_{50} values for ganciclovir were higher than previously published (Field and Biron, 1994; Gerna et al., 1995), while those of foscarnet were similar to previous data (Drew et al., 1993; Gerna et al., 1995). Higher IC_{50} values for ganciclovir but not foscarnet were also seen in a previous assay established in our laboratory (Wahren et al., 1983). The higher sensitivity of the ELISA compared to plaque formation could provide one explanation for the slightly elevated values. The quality and type of cells used are also important. For some isolates, considerably higher IC_{50} values were obtained for ganciclovir with HL cells compared to Hs27 cells. A reason for the variation in IC_{50} levels in different cells could be a reduced uptake or phosphorylation of ganciclovir. The activity of the cellular kinases responsible for phosphorylation of nucleoside analogues varies among different cell types and is critical for the antiviral activity of the drug (Gao et al., 1995; Palmer et al., 1996).

The exact number of passages that can be used must also be determined for each cell line. HL cells above the 18th passage were not used, since although the fibroblasts appeared intact up to passage 30, continuous controls showed that the cells began to lose their capacity to support CMV replication already by passage 18. In contrast, Hs27 cells gave identical expression of CMV antigen for passages up to 34. The decrease in CMV-specific OD seen at very high concentrations of antiviral drugs even when completely resistant viruses were examined (Fig. 2b) could be due to drug toxicity which may not have been visible under the microscope.

International reference panels, comprising CMV strains with known different sensitivities to antiviral drugs are required to validate different antiviral sensitivity assays and to determine comparable limits for degrees of sensitivity. In our assay, resistance is identified by the lack of response to the drug at any dose of virus, which may indicate an untreatable CMV isolate. The clinical outcome of antiviral therapy is determined by the balance between the viral load in the patient, the concentration and pharmacokinetics of the antiviral drugs at the site of infection, the immune status of the patient, and the degree of sensitivity and growth characteristics of the dominant viral strain in the patient. A good marker for the clinical outcome may be the infectious viral load at the specific site of infection (for example,

the retina). The assay described here enables estimation of the sensitivity for a specific dose of virus.

Thus, the method presented in this paper, using different doses of the virus isolate combined with a solid phase antigen ELISA to determine IC_{50} values of antiviral drugs, appears to be suitable for rapid characterization of the sensitivity of primary CMV isolates to antiviral drugs in various cell types, and can also be used as a model for antiviral sensitivity assays for other viruses.

Acknowledgements

This work was supported by a grant from the Swedish Medical Research Council (project MFR K97-06X-12180-01A).

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