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Short Communication

Comparison of antiviral assay methods using cell-free and cell-associated varicella-zoster virus

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Summary

Assay methods for varicella-zoster virus (VZV) susceptibility to acyclovir (ACV) of VZV were compared by using cell-free (CF) and cell-associated (CA) virus of 6 × plaque-purified VZV. The 50% effective doses (ED_{50}) of ACV, as required to reduce virus plaque formation by 50%, were about 8 times higher for CA virus than for CF virus. Also, the ED_{50} of 1-β-D-arabinofuranosyl-(*E*)-5-(2-bromovinyl)uracil (BVaraU) for CA-VZV was higher than for CF-VZV, and fresh clinical isolates of VZV gave higher ACV ED_{50} values than CF virus. CA virus prepared at various times after CF virus infection showed a gradual increase of the ACV ED_{50} with time, ranging from the ED_{50} for CF virus to that for CA virus.

Varicella-zoster virus; Acyclovir; BVaraU; Cell-free virus; Cell-associated virus

Varicella-zoster virus (VZV) is cell-associated (CA) and labile (Gershon et al., 1973). It is not easy to obtain cell-free (CF) virus (Brunell, 1967), but CF virus has been used for Oka varicella vaccine production. The ratio of the virus particle/infectivity of CF virus is approximately 10^{4-6}:1 (Shiraki and Takahashi, 1982), and CF virus is stable as long as it is stored properly. Numerous reports concerning the susceptibilities of VZV to antiherpetic drugs have been accumulated by using either CA virus or CF virus (Baba et al., 1987;

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Biron et al., 1980; Bryson et al., 1976; Cheng et al., 1983; Cole et al., 1986; Collins et al., 1982; Crumpacker et al., 1979; Elion et al., 1977; Field et al., 1983; Machida, 1986; Preblud et al., 1984; Shigeta et al., 1983; Shiraki et al., 1984, 1990). However, the susceptibilities of herpes simplex virus (HSV) and human cytomegalovirus (HCMV) have been generally evaluated by using CF virus and not CA virus. In this study, we compared the antiviral assay methods for VZV using CA virus and CF virus of a strain that had been 6 × plaque-purified.

Human embryonic lung (HEL) cells were grown and maintained in Eagle's minimal essential medium supplemented with 10% or 2% fetal bovine serum (FBS), respectively. The VZV strain used was the Kawaguchi strain which had been 6 × plaque-purified as CF virus (Shiraki et al., 1983, 1985). In addition, we also studied 5 fresh clinical isolates and the HF strain of HSV-1. The latter was grown in HeLa cells and assayed in Vero cells overlaid with nutrient methyl-cellulose medium.

Acyclovir (ACV) and 1-β-arabinofuranosyl-(*E*)-5-(2-bromovinyl)-uracil (BVaraU) were kindly supplied by Nippon Wellcome K.K., Osaka, Japan and Dr. H. Machida (Yamasa Shoyu Co. Ltd. Japan), respectively.

VZV-infected monolayers exhibiting about 50–70% cytopathic effect (CPE) were treated with 0.1% EDTA in phosphate-buffered saline (PBS) and suspended in SPGC medium (PBS containing 5% sucrose, 0.1% Na glutamate and 10% FBS). The cell suspension was treated for 40 s in an ultrasonic disrupter, or frozen and thawed 3 times with vigorous pipetting followed by centrifugation at 3000 rpm for 20 min. The supernatant was frozen at −70°C as the CF virus stock (Asano and Takahashi, 1978; Shiraki and Takahashi, 1982). CA virus was prepared from the cells infected with CF virus or from cells inoculated with infected cells at a ratio of one infected to ten uninfected cells. Infected cells were trypsinized and used as CA virus. For dimethyl sulfoxide (DMSO)-frozen CA virus, infected cells with 50–70% CPE were trypsinized, suspended in FBS containing 10% DMSO, and stored at −70°C. CF virus of HSV-1 was prepared from the HSV-1-infected cultures after 3 cycles of freezing and thawing followed by centrifugation. CA virus of HSV-1 was prepared from cells infected at 0.02 plaque forming units (PFU)/cell and harvested about 40 h later by trypsinization after washing with PBS.

To determine the appropriate method to assess antiviral susceptibility of VZV, the 6 × serially plaque-purified VZV was used and the deviation of the susceptibilities due to the heterogeneity of virus population was minimized. The virus was passaged 3 times by cell-to-cell infection, harvested as CF virus stock or CA virus stock, and stored at −70°C until use. Comparison of the susceptibilities between CF virus and CA virus was examined in parallel.

Susceptibilities to ACV was assessed by measuring the 50% effective dose or dose required for 50% plaque reduction (ED_{50}) (Biron and Elion, 1980; Crumpacker et al., 1979; Shiraki et al., 1983, 1990). Briefly, HEL cell monolayers in 60 mm plastic dishes in duplicate or triplicate were infected with 100 PFU of CF virus for 1 h and incubated in maintenance medium containing

TABLE 1

Comparison of drug susceptibilities between CF virus and CA virus

Three experiments were done independently.
[a] CF virus and DMSO-frozen CA virus were prepared as described in the text.
[b] CA virus was prepared from cells infected with approximately 0.01 PFU/cell of CF virus and harvested at 24 h after infection.

	ED_{50}			
	Expt. 1	Expt. 2	Expt. 3	Mean ± S.D.
VZV[a]/ACV (μM)				
CF virus	5.0	4.3	5.3	4.87 ± 0.42
CA virus	37.5	36	46	39.8 ± 4.4
VZV[b]/ACV (μM)				
CF virus	4.3	4.6	4.9	4.6 ± 0.3
CA virus	37	39	35	37.0 ± 1.6
VZV/BVaraU (nM)				
CF virus	4.55	3.33	4.47	4.12 ± 0.56
CA virus	15.91	18.94	25.00	19.95 ± 3.78
HSV-1/ACV (μM)				
CF virus	0.80	0.88	0.89	0.857 ± 0.040
CA virus	7.6	3.2	4.9	5.23 ± 1.81

ACV (1, 2, 5, 10, 20, 50, 100 μM) or BVaraU (1, 2, 5, 10, 20, 50 nM). CA virus preparations were assayed at several dilutions of infected cell suspensions, and a dilution exhibiting about a hundred plaques per dish was used for the determination of the ED_{50} values. Drug susceptibility of HSV-1 was assayed in Vero cell monolayer in a 60 mm plastic dish overlaid with nutrient methylcellulose medium containing ACV. Monolayers inoculated with CF virus or CA virus of VZV were incubated for four or two days, respectively. After appearance of the cytopathic effects, the cells were fixed with 5% neutral formaline and stained with methylene blue, and the number of plaques was counted with a dissecting microscope. The ED_{50} was determined graphically.

As shown in Table 1, CF virus gave similar antiviral susceptibilities but CA virus frozen with DMSO showed an about 8-fold higher ED_{50} than CF virus. To examine the effects of DMSO and preparation procedures of infected cells, the susceptibilities of CA virus freshly harvested at different times after CF virus infection were compared with that of CF virus. This also confirmed that CA virus showed higher ED_{50} values than CF virus independently of the preparation method of CA virus (including DMSO-frozen virus). Also, susceptibility of CF virus was not affected by the preparation method. BVaraU is a much more potent inhibitor of VZV replication than ACV, and, again, BVaraU showed a higher ED_{50} for CA virus than for CF virus. Also, acyclovir showed a higher ED_{50} for the CA virus of HSV-1 than for CF virus.

To examine the effect of the replication cycle of the CA virus on drug

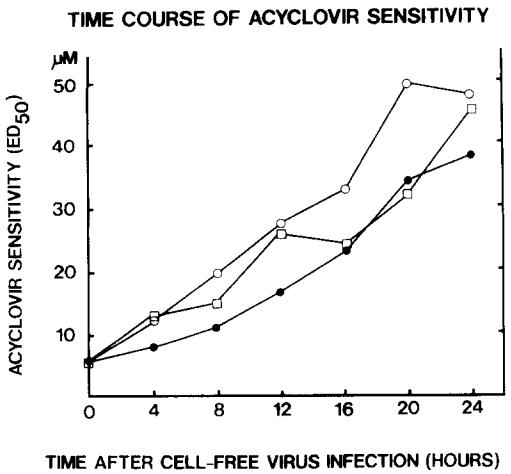

Fig. 1. Time course of susceptibility (ED_{50}) to ACV of CA virus prepared from cells infected with CF virus (VZV). CA virus was harvested at the indicated times after CF virus infection and ACV susceptibilities were determined in three independent experiments.

susceptibility, the cells were synchronized by infection with CF virus. Fig. 1 shows the time course of the ACV susceptibility of synchronized CA virus prepared from cells infected with CF virus. The ED_{50} of ACV for CA virus at 0 h was similar to that for CF virus (about 5 μM). Thereafter, the ED_{50} value for CA virus gradually increased to approximately 50 μM by 24 h after infection.

ACV susceptibilities of CA virus and CF virus were compared for 5 fresh clinical VZV isolates. With all 5 strains, CA virus needed 7–17 times higher ACV concentrations to inhibit plaque formation by 50% than did CF virus. These data (not shown) with fresh clinical isolates were similar to those obtained with a laboratory strain (as shown in Table 1).

VZV DNA synthesis begins within 6 h after infection as determined by DNA dot blot hybridization (Gelb, 1990) and spread of infection from cells infected with CF virus to neighboring cells is observed 14 h after infection (Yamanishi et al., 1980). The susceptibility of synchronized CA virus to ACV gradually increases with time after CF virus infection, as shown in Fig. 1. CA virus is usually prepared from infected cells which are not synchronized, and therefore might contain virus-infected cells at various stages of the virus replication cycle.

Although the differences in susceptibility to BVaraU were smaller than those observed for ACV, BVaraU showed a higher ED_{50} for CA virus than for CF virus. Similar observations were made with regard to the susceptibility of HSV-1 to ACV: the ED_{50} for CF virus was 6-fold lower than for CA virus (Table 1). A rough calculation has indicated that one CA virus, i.e. one infected cell, may contain at least 1000 infectious virus particles (Shiraki et al., 1982). This may explain why with CA virus higher ED_{50} values are obtained for the drugs than with CF virus. Thus, the higher ED_{50} values obtained with CA virus may be due to the higher multiplicity of infection.

We have extended our observations to fresh clinical isolates and confirmed the differences of the susceptibilities to ACV between CF and CA virus. The results obtained here with VZV and HSV led us to conclude that the use of CF virus is recommended for drug susceptibility assays of these viruses.

References

Baba, M., Konno, K., Shigeta, S. and De Clercq, E. (1987) In vitro activity of (S)-9-(3-hydroxy-2-phosphonylmethoxy)adenine against newly isolated clinical varicella-zoster virus strains. Eur. J. Clin. Microbiol. 6, 158–160.

Biron, K.K. and Elion, G.B. (1980) In vitro susceptibility of varicella-zoster virus to acyclovir. Antimicrob. Agents Chemother. 18, 443–447.

Bryson, Y.J. and Connor, J.D. (1976) In vitro susceptibility of varicella zoster virus to adenine arabinoside and hypoxanthine arabinoside. Antimicrob. Agents Chemother. 9, 540–543.

Brunell, P.A. (1967) Separation of infectious varicella-zoster virus from human embryonic lung fibroblasts. Virology, 31, 732–734.

Cheng, Y.-C., Huang, E.-S., Lin, J.-C., Mar, E.-C., Pagano, J.S., Dutschman, G.E. and Grill, S.P. (1983) Unique spectrum of activity of 9[(1,3 dihydroxy-2-propoxy)methyl]guanine against herpes viruses in vitro and its mode of action against herpes simplex virus type 1. Proc. Natl. Acad. Sci. USA 80, 2767–2770.

Cole, N.L. and Balfour Jr., H.H. (1986) Varicella-zoster virus does not become resistant to acyclovir during therapy. J. Infect. Dis. 153, 605–608.

Collins, P., Appleyard, G. and Oliver, N.M. (1982) Sensitivity of herpes virus isolates from acyclovir clinical trials. Am. T. Med. 73, 380–382.

Crumpacker, C.S., Schnipper, L.E., Zaia, J.A. and Levin, M.J. (1979) Growth inhibition by acycloguanosine of herpes viruses isolated from human infection. Antimicrob. Agents Chemother. 15, 642–645.

Elion, G.B., Furman, P.A., Fyfe, J.A., de Miranda, P., Beauchamp, L. and Schaeffer, H. (1977) Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl)guanine. Proc. Natl. Acad. Sci. USA 74, 5716–5720.

Field, A.K., Davies, M.E., DeWitt, C., Perry, H.C., Liou, R., Germershausen, J., Karkas, J.D., Ashton, W.T., Johnston, D.B.R. and Tolman, R.L. (1983) 9-{[2-hydroxy-1-(hydroxymethyl) ethoxy]methyl} guanine: a selective inhibitor of herpes group virus replication. Proc. Natl. Acad. Sci. USA 80, 4139–4143.

Gelb, L.D. (1990) Varicella-zoster virus. In: B.N. Fields and D.M. Knipe (Eds), Fields Virology, 2nd edn., pp. 2011–2054. Raven Press, New York.

Gershon, A.A., Cosio, L. and Brunell, P.A. (1973) Observations on the growth of varicella-zoster virus in human diploid cells. J. Gen. Virol. 18, 21–31.

Machida, H. (1986) Comparison of susceptibilities of varicella-zoster virus and herpes simplex viruses to nucleotide analogs. Antimicrob. Agent Chemother. 29, 524–526.

Preblud, S.R., Arbeter, A.M., Proctor, E.A., Starr, S.E. and Plotkin, S.A. (1984) Susceptibility of vaccine strains of varicella-zoster virus to antiviral compounds. Antimicrob. Agents Chemother. 25, 417–421.

Shigeta, S., Yokota, T., Iwabuchi, M., Baba, K., Konno, K., Ogata, M. and De Clercq, E. (1983) Comparative efficacy of antiherpes drugs against various strains of varicella-zoster virus. J. Infect. Dis. 147, 576–584.

Shiraki, K., Namazue, J., Okuno, T., Yamanishi, K. and Takahashi, M. (1990) Novel sensitivity of acyclovir resistant varicella-zoster virus to anti-herpetic drugs. Antiviral Chem. Chemother. 1, 373–376.

Shiraki, K., Ogino, T., Yamanishi, K. and Takahashi, M. (1983) Isolation of drug-resistant mutants of varicella-zoster virus: cross-resistance of acyclovir-resistant mutants with phosphonoacetic acid and bromodeoxyuridine. Biken J. 26, 17–23.

Shiraki, K., Ogino, T., Yamanishi, K. and Takahashi, M. (1985) Immunochemical characterization of pyrimidine kinase induced by varicella-zoster virus. J. Gen. Virol. 56, 421–430.

Shiraki, K. and Takahashi, M. (1982) Virus particles and glycoproteins excreted from cultured cells infected with varicella-zoster virus (VZV). J. Gen. Virol. 61, 271–275.

Shiraki, K., Yamanishi, K. and Takahashi, M. (1984) Susceptibility to acyclovir of Oka-strain varicella vaccine and vaccine-derived viruses isolated from immunocompromised patients. J. Infect. Dis. 150, 306–307.

Whitley, R.J., Ch'ien, L.T., Dolin, R., Galasso, G.J., Alford, C.A. and the collaborative study group. (1976) Adenine arabinoside therapy of herpes zoster in the immunosuppressed NIAID collaborative antiviral study. N. Engl. J. Med. 294, 1193–1199.

Yamanishi, K., Matsunaga, Y., Ogino, T., Takahashi, M. and Takamizawa, A. (1980) Virus replication and localization of varicella-zoster virus antigens in human embryonic fibroblast cells infected with cell-free virus. Infect. Immunol. 28, 536–540.