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STUDIES ON INTERACTIONS OF dTK⁻ HSV MUTANTS WITH NEURONS IN VITRO

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Summary. — Thymidine kinase negative (dTK⁻) mutants of herpes simplex virus type 1 (HSV-1) multiplied well in rat brain glioma cells. A proportion (<1%) of glioma cells survived the infection with HSV and were designated "survivor" glioma cells. Survivor cells of dTK⁻ mutant virus infection ceased to produce infectious virus after two passages and were highly resistant to both HSV-1 and HSV-2 but not to vesicular stomatitis virus (VSV). Flow cytometric studies indicated morphological differences between parental and survivor glioma cells, and HSV-1 specific antigens as well as DNA were detected in the survivor glioma cells, but only in early passages. Sensitivity to superinfection with HSV appears to correlate to loss of HSV-specific viral DNA in the survivor glioma cells. Survivor glioma cells after several subcultures lost their ability to resist superinfecting HSV, reverted morphologically to the appearance of parental glioma cells and also lost significant amount of HSV-1 specific DNA. These transient survivor glioma cells became persistently infected-virus producer cells upon HSV infection.

Key words: dTK⁻ mutants; HSV; neuronal cells; survivor cells; DNA hybridization; immunofluorescence

Introduction

Deoxythymidine kinase (dTK) is widespread in both prokaryotic and eukaryotic cells (Gentry *et al.*, 1983), and certain large DNA containing viruses also induce viral coded dTK in the cells they infect. Recently, the HSV-specific dTK enzyme has been exploited for the development of antiviral agents, and considerable success in this regard has been achieved (DeClercq, 1979). In the process it has been realized that HSV develops resistance to the drugs by acquiring mutations in two different loci; the dTK gene and the DNA polymerase gene (Coen and Schaffer, 1980; Schnipper and Crumpacker, 1980). Although the development of drug-resistance in HSV has been of great concern, the dTK⁻ mutants, at least, multiply poorly

Table 1. Growth of various drug-resistant HSV-1 mutants in glioma and BHK cells

Virus mutant	MOI	Titre (PFU/ml)*	
		Glioma	BHK
MMdU^r-2	3.0	1.5×10^6	6×10^8
MMdU^r-3	3.0	8.0×10^5	4×10^7
MMdU^r-12-3	0.1	1.7×10^6	5×10^8
MMdU^r-17	0.1	2.5×10^5	N.D.
MMdU^r-20	0.1	5.0×10^5	6×10^7
WT	0.1	1.5×10^5	5×10^8

* Glioma or BHK cells were infected with HSV-1 WT and dTK$^+$ or dTK$^-$ drug-resistant mutants. The cells were harvested 24 hr p. i. and titered in BHK cells. MMdU^r-20 is a drug-resistant mutant with and altered dTK.
N.D. = not determined.

and are unable to cause latent infections in vivo (Field and Wildy, 1978; Tenser and Dunstan, 1979). Because HSV is harbored in nervous tissue in a latent form and becomes reactivated under certain conditions, the interaction of HSV (especially dTK mutants) with neuronal cells would appear to be of some interest. Although several workers have studied the interaction of HSV with neuronal cells (Adler *et al.*, 1978; Doller *et al.*, 1979; Lanz and Zettlemoyer 1976; Levine *et al.*, 1980; and Vahlne and Lycke 1978), there have been very few in vitro studies on the interaction of dTK$^-$ mutants with neuronal cells.

We have recently shown that HSV WT interacts with rat brain glioma cells in a special way (Veerisetty *et al.*, 1985), and in the present communication we describe a similar interaction for HSV dTK$^-$ mutants. We also describe the conditions for isolation of virus-producer cells from survivor cells by superinfection with high doses of HSV. Further, we provide evidence for the loss of HSV-specific DNA in the survivor cells during subsequent generations.

Materials and Methods

Cells and viruses. Baby hamster kidney (BHK-21) and mouse L-cells were grown in minimum essential Eagle's medium (MEM) with Hank's basal salt solution and 5% bovine calf serum. 9L cells, a clone of a gliosarcoma induced by N-nitrosomethyl urea, were obtained from Barker (University of California, San Francisco, CA) and grown in MEM using 10% calf serum. All cells were maintained at 37 °C in 95% air-5% CO_2 at 100% humidity.

Stocks of HSV-1, strain 17 MP (Glasgow), its drug-resistant mutants MMdU^r-20, MMdU^r-2, MMdU^r-12-3, and MMdU^r-17, and HSV-2 were prepared by infecting BHK cell monolayers at 0.005—0.05 PFU/cell for 3 to 4 days, and plaque assays for HSV were made in BHK cells as described previously (Veerisetty and Gentry, 1981). Vesicular stomatitis virus (VSV-Indiana--sanjuan) was grown and titered as described by Hunt (1983).

Growth of WT HSV-1, dTK$^-$ HSV-1 mutants, HSV-2 and VSV in parental and survivor glioma and BHK cells. Cells were infected with the different viruses at indicated multiplicities of infection (MOIs; see Tables 1 and 2). After the plates were rocked for 2 hr at 37 °C the inoculum was

removed and the cells were washed 2—3 times with medium and then overlaid with 1 ml MEM. After 24 hr incubation at 37 °C the plates were sealed in a plastic bag and frozen at −70 °C until titrations were performed.

Karyotyping of cells. Cells were harvested by trypsinization in the maximum growth phase (24 hr post subculture). Mitotic activity was arrested in metaphase by the addition of 0.25 ml of 0.25 μg/ml colchicine to 4 ml cell suspension and incubation for 1 to 2 hr at 37 °C. Following centrifugation at 1000 rpm for 5 min to remove the growth media and mitotic poison, the cells were resuspended in 10 ml of a hypotonic solution 10 ml of 75 mM KCl for 10 minutes, and 1 ml of Carnoy's fixative (3/1 v/v absolute methanol/glacial acetic acid) was then added. The cells were collected by centrifugation at 1000 rev/min for 5 min and were resuspended in the above fixative for 20 minutes. This fixation-centrifugation step was repeated twice more, followed by a final resuspension of the cell button in fixative. This cell suspension was applied dropwise to cold, clean wet slides and dried at 60 °C on a hot plate. The slides were stained with Wright's stain, and observed microscopically. 30 consecutive mitoses were counted for each cell type and the mode number of chromosomes was obtained.

Detection of virus specific antigens and DNA. For antigen analysis preconjugated anti-HSV antiserum (with fluoresceinisothiocyanate) was used as described previously (Veerisetty *et al.*, 1985). Briefly, cells grown on coverslips were fixed in acetone, incubated with fluorescein conjugated antiserum, and after extensive washing were examined in the fluorescent microscope. For DNA analysis viral and cellular DNAs were extracted (see Veerisetty *et al.*, 1985 for details) and viral DNA was nick translated (Rigby *et al.*, 1977) with ^{32}P labeled nucleotides. Dot blot hybridization was carried out as described by Jeffreys and Flavell (1977).

Analysis of cells by flow cytometry. Flow cytometry was performed using an Ortho Model 50HH cytofluorograph interfaced to a Model 2150 computer (Orthodiagnostics, Westwood, MA). For our purpose measurements of the narrow angle forward scatter and 90° scatter components were made at 488 nm wavelength emitted by argon laser. Isometric displays of forward versus 90° light scatter and the mean values for light scatter were generated by analyzing 10^6 cells/sample.

Detection of infectious virus in survivor glioma cells. Persistently infected cells were trypsinized, collected by low speed centrifugation and resuspended in MEM (without serum). Log dilutions of cells were made and 0.1 ml of samples were added to duplicate wells of 90—95% confluent monolayer of BHK cells. After the plate was rocked for 1 hr at 37 °C, 1 ml fresh MEM (with 5% serum) was added to each well. Plaques were counted after 48 hr and the percentage cells containing infectious virus was calculated.

Table 2. Yield of infectious virus from cultures of survivor glioma cells infected with HSV-1, HSV-2 and VSV

Virus	Virus titer			
	MOI	9L	BHK 9L WT, P_7	9L3, P_4
HSV-1	10	1.5×10^7	2.0×10^3	2.5×10^3
	1	8.0×10^6	$<1.0 \times 10^3$	$<1.0 \times 10^3$
HSV-2	25	4.0×10^7	3.0×10^3	4.0×10^2
	2.5	2.0×10^9	$<1.0 \times 10^3$	$<4.0 \times 10^2$
VSV*	0.1	7×10^9	4.5×10^8	4.0×10^8
	1.0	N.D.	N.D.	N.D.

* VSV titers were not determined when the MOI was 1 or more because of extensive CPE.

Results

Growth of drug-resistant HSV mutants in glioma cells

To study the ability of dTK$^-$ HSV mutants to multiply in glioma cells we measured the infectious virus formed in 24 hr in these cells. HSV WT and a mutant (MMdU^r-20) with altered dTK (Veerisetty and Gentry, 1983) were also included in the experiment. Virus titers were obtained using BHK cells. The data (Table 1) indicate that the dTK$^-$ mutants multiplied to the same extent as WT virus or MMdU^r-20. This showed that dTK$^-$ HSV can multiply in neuronal cells in vitro.

Isolation of survivor glioma cells

In a previous communication (Veerisetty *et al.*, 1985) we described the isolation of 9L glioma cells that survived the initial HSV WT infection (designated "survivor" cells; Veerisetty *et al.*, 1985). By using similar methodology we successfully isolated survivor cells from glioma cells following infection with various drug-resistant HSV mutants. To date we have cell lines obtained by using the MMdU^r-HSV mutants-2, 3, 4, 12-3 and 17, which have been shown to be dTK$^-$ (Veerisetty and Gentry 1981).

Cocultivation of survivor glioma cells with BHK

We could not isolate survivor cells from BHK or 3T3 dTK$^-$ cells infected with HSV-1 (Veerisetty *et al.* 1985). Cocultivation of glioma and BHK cells was made as follows: Survivor glioma cells of wild type virus infection (9L WT, P_2) were challenged with HSV (>10 PFU/cell) and the cells were subcultured once. When the cells grew to confluency they were trypsinized and about 1×10^5 cells were seeded on to 50% confluent monolayer of BHK cells grown in 25 cm^2 flasks. After 72 hr a few (less than ten) virus plaques were seen, but further incubation destroyed most of the cells. The remaining cells were allowed to grow to confluency by replacing the media with fresh media every alternate day.

Such cells were designated as BHK 9L WT (because BHK cells were cocultivated with 9L WT survivor glioma cells). The cells were further passaged two more times and were karyotyped to test for lineage. The results suggested that the survivor cells were of 9L origin rather than BHK. Such survivor cells and parental 9L cells both contained an average of 65 chromosomes (2N = 42) indicative of aneuploidy (data not shown). This is not surprising because the parental cells were originally obtained by transformation with N-nitrosomethyl urea. BHK cells contained only 44 chromosomes as expected (2N = 44).

Detection of virus specific antigens in survivor glioma cells

HSV specific antiserum conjugated with fluoroisothiocyanate was reacted with parental and 9L3 P_4 (9L cells infected with HSV clone 3, passage 4) cells. Parental cells lytically infected with HSV were employed as a positive control. The results in Fig. 1 indicate that 9L3 P_4 cells contained some virus specific antigens as these cells showed bright fluorescence similar to that seen

in the positive control. However, no significant fluorescence could be seen in parental glioma cells (negative control).

Growth of HSV-1, HSV-2 and VSV in survivor glioma cells

Although survivor glioma cells failed to show cytopathic effect when superinfected with various strains of HSV, we wished to check for the possi-

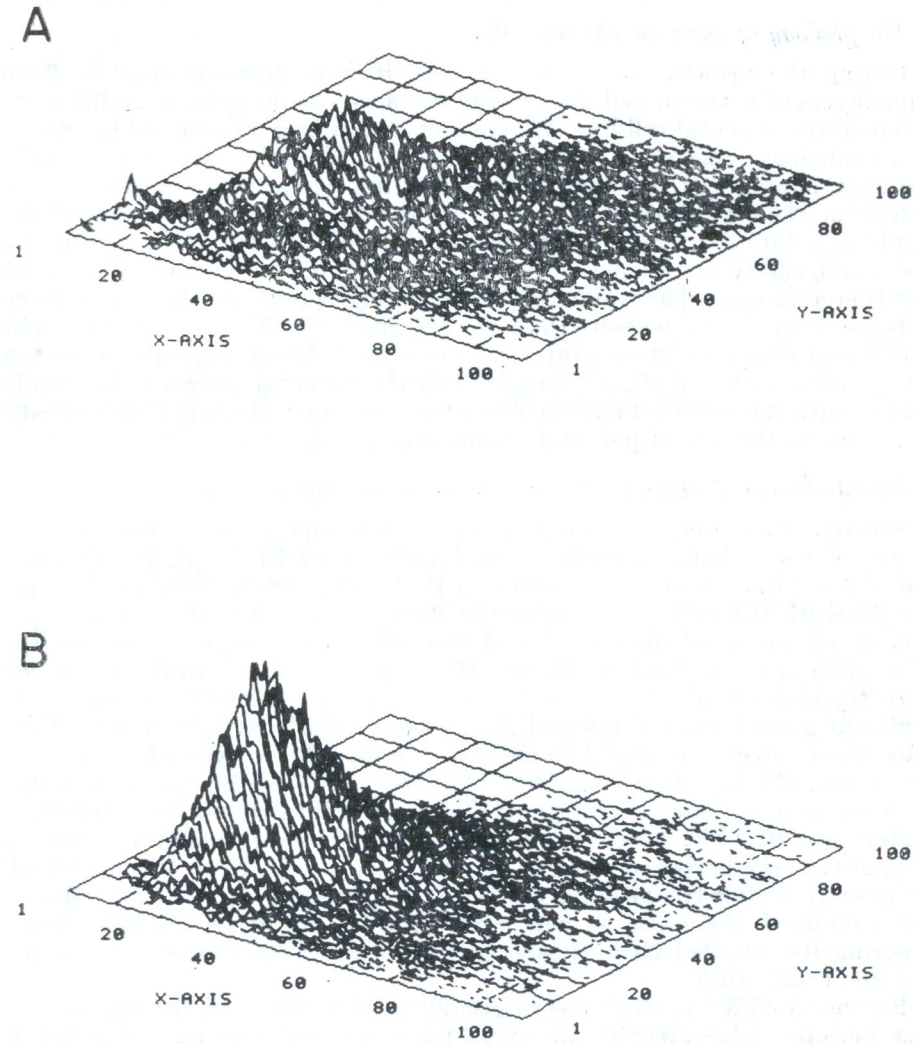

Fig. 2.
Isometric displays of the narrow angle forward light scatter (Y-axis) versus the 90° light scatter (X-axis) of survivor glioma cells (A) and parental glioma cells (B)

bility that there was some multiplication, and therefore determined the amount of infectious virus produced in parental and survivor cells 24 hr post infection (p.i.) HSV-1 and 2 and VSV were used at different MOIs. The results (Table 2) indicate that the survivor cells lines were permissive for VSV multiplication but not for either HSV-1 or HSV-2. In contrast the parental cells were permissive for the growth of all three viruses.

Morphology of survivor glioma cells

During the growth curve experiments, it was apparent that confluent monolayers of survivor cell lines never contained as many cells as did monolayers of the parental cell line. We suspected that this was related to changes in morphology, size in particular. Since the changes were not obvious by ordinary microscopy, we used light scattering by laser cytophotometry to determine the size differences of the various cells. It has been reported that combining data for forward light scatter and 90° provides an assessment of the homogeneity of cell populations (Buttke *et al.*, 1983), and this method was therefore used. The results presented in Fig. 2 indicate that the survivor cells are more heterogeneous than the parental cells. This information along with forward light scattering intensities (data not shown) suggested to us that the survivor cells are slightly larger than the parental glioma cells. Similar studies with the cells that lost their resistance to superinfecting HSV indicated a reversal to the size of parental glioma cells (data not shown).

Establishment of virus producer cells in survivor glioma cells

Survivor glioma cells were highly resistant to superinfection for a variable length of time when superinfected with HSV at 10 PFU/cell. For example, the 9L3 cell line became susceptible to HSV at about the 28th passage, and the BHK 9L WT cell line at about the 35th passage. In both cases a proportion of cells survived the infection at the indicated passage. These cells were allowed to grow, and when colonies of cell patches began to form, the cells were trypsinized and transferred to a new flask. Although the cells after confluent growth showed isolated plaques typical of HSV infection of glioma cells, these never progressed to destroy the monolayer even after extended incubation (72 hr). This suggested that the cells were in equilibrium with the virus as in a classical virus producing persistent infection. In an infectious center assay 9L3 P32-5 cells (at passage 5 of virus producer cells) showed that 36% of the cells harbored virus as against 0.1% for BHK 9L WT P39-4 cells (at passage 4 after the productive stage). At passage 9, only 15% of 9L3 cell line contained the virus as compared to 0.1% for BHK 9L WT. Similar experiments revealed that at passage 17 both cell lines ceased to produce any detectable HSV.

Because a dTK$^-$ mutant was originally used to establish the 9L3 cell line and because subsequently the virus producer cell line was obtained by superinfecting those cells with dTK$^-$ wild type virus, we checked to see if the virus produced in the producer cell lines was WT or the dTK$^-$ mutant. Virus in the supernatant of 9L3 P32-9 was used to infect confluent BHK cells for

24 hr and these cells were assayed for dTK activity as well as sensitivity of the virus to nucleoside analogs. A five fold increase in the dTK activity and a WT-like sensitivity to antivirals suggested that indeed the virus in the producer cells was WT. We did not test the possibility of the coexistence of WT and reactivated dTK⁻ mutants.

Detection of virus specific DNA in survivor glioma cells

We employed the dot blot hybridization technique to ascertain the presence of herpes viral specific DNA in various survivor glioma cells (Fig. 3). ^{32}P-labeled DNA probe was obtained by nick-translation of HSV DNA extracted from extracellular virus. Cellular DNAs were obtained from BHK, 9L glioma and survivor glioma cells (9L WT, P11; P24 and P28; BHK 9L WT, P7 and P47; and 9L3, P10 and P38). As shown in Fig. 3 DNAs from early passages of survivor cell lines (9L WT, P11; BHK 9L WT, P7 and 9L3, P10) hybridized significantly with the probe. Although DNAs from the later passages of the same cells (9L WT, P24; BHK 9L WT, P47 and 9L3, P38) failed to give significant hybridization, some of them showed slightly higher hybridization than the background given by the parental cell DNA. In a similar experiment we found significant hybridization of DNA of virus producer cell lines. In all these experiments, however, no quantitative studies were conducted for determining the fraction of the HSV genome present in these cells.

Discussion

The relative avirulence of deoxythymidine kinase negative (dTK⁻) mutants of HSV has been related to their failure to grow well in nervous tissues (Field and Wildy 1978; Tenser and Dunstan 1979; Tenser *et al.*, 1979). The interaction of such mutants with the nervous tissue cells in vitro, however, has not been studied thoroughly. Our studies indicate that both dTK⁻ and dTK⁺ viruses multiply well in glioma cells, though not as efficiently as in BHK cells (Table 1). It may be concluded that the glioma cells are semi-permissive for HSV infection. The most interesting feature of the cell-virus interaction was the survival of a proportion of cells with each HSV mutant infection and the resistance of the survivor cells to very high doses of super-infecting homologous virus (HSV) but not to heterologous virus (VSV) (Table 2).

Survivor cells of BHK and 3T3 cells apparently do not occur following initial infection with HSV at various M.O.I.s, or after cocultivation of virus producer glioma cells with BHK cells. Survivor cells from this type of experiment were found to be of 9L glioma origin by their karyotype. The presence of significant amounts of HSV specific DNA as well as viral antigens in the survivor glioma cella (Figs. 1 and 3) support the notion that certain virus specific antigens may be produced. Such cells differed from parental cells in their morphology and sensitivity to infection with HSV-type 1 and 2 (Table 2; Fig. 2). After several subcultures, however, these cells lost their

resistance to HSV infection, contained very little detectable HSV specific DNA (Fig. 3), and were indistinguishable from parental cells in morphology.

It has been suggested that defective interfering (DI) virus is nesessary to establish persistent infections (Huang and Baltimore, 1977; Holland and Villarreal, 1974; O'Callaghan *et al.*, 1981). In our system neither virus-like particles nor infectious virus could be detected (Veerisetty *et al.*, 1985). It can, however, be hypothesized that the viral DNA in glioma cells may have a role similar to DI particles though DI particles as such can not be demonstrated. Because the survivor glioma cells became susceptible to HSV superinfected after several subcultures and at the same time lost their viral specific DNA (Fig. 3) it is reasonable to conclude that presence of this viral DNA was essential for their resistance to superinfecting homologous virus (HSV-1 and 2).

The initial acquisition and concomitant loss of the foreign virus DNA by the glioma cells may have significant implications in latency and reactivation of HSV in vivo.

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References

Adler, R., Glorioso, J. C., and Levine, M. (1978): Infection by herpes simplex virus and cells of nervous system origin: characterization of a nonpermissive interaction. *J. gen. Virol.* **39**, 9–10.

Buttke, T. M., Mallett, G. S., and Cuchens, M. A. (1983): Positive selection of mouse B and T lymphocytes and analysis of isolated populations by Flow cytometry. *J. Immunol. Meth.* **58**, 193–207.

Coen, D. M., and Schaffer, P. A. (1980): Two distinct loci confer resistance to acyloguanosine in herpes simplex virus type 1. *Proc. natn. Acad. Sci. U.S.A.* **77**, 2265–2269.

Declercq, E. (1979): New trends in antiviral chemotherapy. *Arch. Int. Physiol. Biochim.* **87**, 353–395.

Doller, E., Acuker, J., and Weissbach, A. (1979): Persistence of herpes simplex virus type 1 in rat neurotumor cells. *J. Virol.* **29**, 43–50.

Field, H. J., and Wildy P. (1978): The pathogenicity of thymidine kinase deficient mutants of herpes simplex virus for mice. *J. Hyg. (Camb.)* **81**, 267–277.

Gentry, G. A., Allen, G. P., Holton, R., Nevins, R. B., McGowan, J. J., and Veerisetty, V. (1983): Thymidine salvage, mitochondria, and the evolution of the herpesviruses. *Intervirology* **19**, 67–76.

Holland, J. J., and Villarreal, L. P. (1974): Persistent noncytocidal vesicular stomatitis virus infections mediated by defective particles that suppress virion transcriptase. *Proc. natn. Acad. Sci. U.S.A.* **71**, 2956–2960.

Huang, A. S., and Baltimore, D. (1977): Defective interfering animal viruses, pp. 73–116. In H. Frankel-Conrat and R. R. Wagner (Ed.): *Comprehensive Virology*, vol. 10. Plenum Publishing Corp., N. Y.

Hunt, D. M. (1983): Vesicular stomatitis virus mutant with altered polyadenylic acid polymerace activity in vitro. *J. Virol.* **46**, 788–799.

Jeffreys, A. J., and Flavell, R. A. (1977): A physical map of the DNA regions flanking the rabbit β-globin gene. *Cell* **12**, 429–439.

Lancz, G. J., and Zettlemoyer, T. L. (1976): Restricted replication of herpes simplex virus in neural cells (39384). *Proc. Soc. exp. Biol. Med.* **152**, 302–306.

Levine, M., Goldin, A. L., and Glorioso, J. C. (1980): Persistence of herpes simplex virus genes in cells of neuronal origin. *J. Virol.* **35**, 203.

O'Callaghan, D. J., Gentry, G. A., and Randall, C. C. (1981): Equine herpes viruses. In H. Frankel-Conrat and R. R. Wagner (Ed.): *Comprehensive Virology II*, Plenum Publishing Corp., N. Y.

Rigby, P. W. S., Dieckman, M., Rhodes, C., and Berg, P. (1977): Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. *J. molec. Biol.* **113**, 237–251.

Schnipper, L. E., and Crumpacker, C. S. (1980): Resistance of herpes simplex virus to acycloguanosine: Role of viral thymidine kinase and DNA polymerase loci. *Proc. natn. Acad. Sci. U.S.A.* **77**, 2270–2273.

Tenser, R. B., and Dunstan, M. E. (1979): Herpes simplex virus thymidine kinase expression in infection of the trigeminal ganglion. *Virology* **99**, 417–423.

Tenser, R. B., Miller, R. L., and Rapp, F. (1979): Trigeminal ganglion infection by thymidine kinase-negative mutants of herpes simplex virus. *Science* **205**, 915–917.

Vahlne, A., and Lycke, E. (1978): Herpes simplex virus infection of in vitro cultured neuronal cells (mouse neuroblastoma C 1300 cells). *J. gen. Virol.* **39**, 312–332.

Veerisetty, V., and Gentry, G. A. (1981): 5-methoxymethyldeoxyurine-resistant mutants of herpes simplex virus-type 1. *Virology* **114**, 576–579.

Veerisetty, V., and Gentry, G. A. (1983): Alterations in substrate specificity and physiochemical properties of deoxythymidine kinase of a drug-resistant herpes simplex virus type 1 mutant. *J. Virol.* **46**, 901–908.

Veerisetty, V., Gentry, G. A., Veerisetty, I. K., Kapp, J. P., and Staczek, J. (1985): Herpes simplex virus type 1 and neuronal cells — a special case of persistence. *Intervirology* (in press).

Explanation of Figures (Plates I–II):

Fig. 1. Direct immunofluorescence staining for HSV-1 specific antigens. Cells grown on coverslips were fixed with acetone and reacted with anti HSV serum preconjugated with fluoresceinisothiocyanate. (I) Uninfected parental glioma cells. (II) HSV-1 infected glioma cells at > 10 pfu/cell (10 hr PI). (III) Survivor glioma (9L3, P_4) cells.

Fig. 3. Dot blot hybridization analysis of HSV-1 specific DNA. HSV-1 virion DNA was obtained from the purified extracellular virus and nick-translated using ^{32}P-labeled nucleotides. 30 μg of unlabeled cellular DNA (from different cell lines) and 0.33 and 0.066 μg of unlabeled HSV DNA (positive control; a and b respectively) were denatured, spotted onto nitrocellulose filter paper (50 μl aliquots) and probed with ^{32}P-labeled HSV DNA (sp. act. 10^6 c.p.m./μg) as described by Jeffreys and Flavell (1977).