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Infection of human NT2 cells and differentiated NT-neurons with herpes simplex virus and replication-incompetent herpes simplex virus vectors

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The human embryonal carcinoma cell line NT2 differentiates irreversibly into postmitotic neuron-like cells following treatment with retinoic acid. These differentiated NT-neurons resemble central nervous system (CNS) neurons and are characterized by development of dendrites and axons and the expression of neuron-specific markers. Because of their unique biological characteristics, NT-neurons were investigated for their utility as a system for studying the replication of herpes simplex virus (HSV) in the neuron and for evaluating characteristics of HSV vectors designed for gene delivery to the neuron. Virus replication in differentiated NT-neurons was significantly reduced and delayed relative to replication in undifferentiated NT2 cells. Replication of thymidine-kinase (tk) deficient HSV was further impaired in NT-neurons, reflecting the behavior of tk-negative virus in primary neurons *in vitro* and ganglia *in vivo*. Furthermore, replication-incompetent HSV vectors were capable of infecting NT-neurons, expressing a foreign gene, and persisting in a recoverable state for at least 2 weeks following delivery. These results suggest that differentiated NT-neurons can provide a continuous source of human, post-mitotic neurons-like cells for the study of HSV biology and HSV vector development. *Journal of NeuroVirology* (2001) **7,** 43–51

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Introduction

Although herpes simplex virus 1 (HSV-1) replicates efficiently in many tissue culture cell lines, numerous viral genes are not essential for this process *in vitro* (for review, see Roizman and Sears, 1996). Of the more than 75 individual viral genes, nearly 40 are dispensable for growth in cultured cells. Because viruses deficient in so-called dispensable genes are rarely, if ever, isolated from human herpes simplex lesions, it is likely that the products of these viral genes are necessary for virus growth and persistence *in vivo*. Some genes which are dispensable for growth in culture may encode proteins which are necessary to evade detection by the host during infection, and others may encode proteins which are required for efficient virus replication in specific cell types or particular cellular environments. Examples of virus genes which have been shown to be required in a cell-specific fashion include the immediate-early gene encoding ICP22 and the late γ34.5 gene. Although not required for efficient virus replication in many cell culture systems, ICP22 is necessary for efficient replication of HSV in rodent and some human cell lines (Sears *et al*, 1985). Similarly, γ34.5 deficient viruses fail to replicate in primary human foreskin fibroblasts and human neuronal cell lines such as SK-N-SH, because of an inability to prevent shut-off of host cell protein synthesis (Chou and Roizman, 1992; Chou *et al*, 1994). Thus, some cell lines have been useful in elucidating the cell-specific roles and functions of individual HSV genes.

However, in spite of the obvious advantages to studying virus replication in cell culture, most cell lines have significant deficiencies for analyzing cell-specific HSV gene functions. This is particularly true for neuronal cell lines used to study virus-cell interactions and the behavior of HSV gene

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delivery vectors in neurons. For example, there are relatively few neuronal cell lines of human origin, and since they are dividing cells, they do not faithfully reflect key aspects of post-mitotic differentiated neurons. Primary neurons, although terminally differentiated, are not clonal in origin and can be obtained in significant amounts only from non-human sources.

Human derived NT2 cells are a relatively unexplored neuronal cell culture system for evaluation of HSV infection and replication. These cells are an embryonal carcinoma cell line derived from a human teratocarcinoma (Andrews *et al*, 1984). Unlike other neuronal cell lines, NT2 cells differentiate irreversibly into neuron-like cells in the presence of retinoic acid (Andrews, 1984). During differentiation, these cells develop dentrites and axons and express a variety of neuronal cell markers characteristic of central nervous system neurons (Pleasure *et al*, 1992; Younkin *et al*, 1993); following differentiation they maintain their non-dividing neuronal phenotype for at least several months in the absence of retinoic acid (Pleasure *et al*, 1992). Since they are post-mitotic, they can be transplanted into the central nervous system of rodents without causing tumors, and have been suggested as a potential treatment for nervous system diseases in humans (for review, see Trojanowski *et al*, 1997). Because of these unique biological characteristics, differentiated NT2 neurons appear to be a promising cell system for evaluating the interaction of HSV and the neuron as well as HSV vector-directed gene delivery to neurons.

In this report, the NT-neuron system is evaluated for its utility in studying both of these processes. Comparison of HSV replication in undifferentiated NT2 cells and in differentiated NT-neurons revealed that both wild-type and tk-deficient HSV replicate to high titers in NT2 cells. In contrast, virus replication in differentiated cells is reduced and delayed relative to NT2 cells. Interestingly, the replication of HSV tk mutants in NT-neurons is further inhibited compared to wild-type virus. In addition, infection of differentiated NT-neurons with replication-incompetent HSV vectors indicated that such vectors persist in these post-mitotic cells in a recoverable state for at least 2 weeks following infection. Taken together, the results suggest the utility of the NT-neuron system for analyzing HSV-neuron interactions and for evaluating the characteristics of HSV vectors in post-mitotic human neurons.

Results

Replication of HSV-1 in undifferentiated and differentiated NT2 cells

To determine the ability of HSV-1 to establish a productive infection in NT2 cells and differentiated NT-neurons, cells were infected with HSV-1 (F) and the resulting yield of virus was determined over the course of 5 days. Infected cultures were collected at each day post-infection, and the resulting virus was quantified by plaque assay on Vero cells. At a multiplicity of infection of 1.0, virus replication in differentiated NT-neurons was delayed, relative to virus replication in NT2 cells, and lower levels of virus were produced in NT-neurons compared to NT2 cells at each timepoint (Figure 1A). The yield of virus at day one post-infection was more than one log greater in NT2 cells, suggesting that virus replication was more efficient in the undifferentiated cells. At this multiplicity, most cells were infected at the initial time point, and virus yields peaked in both cell types at 3 days post-infection. At a multiplicity of infection of 0.01, the yield of virus in NT-neurons at day one post-infection again was

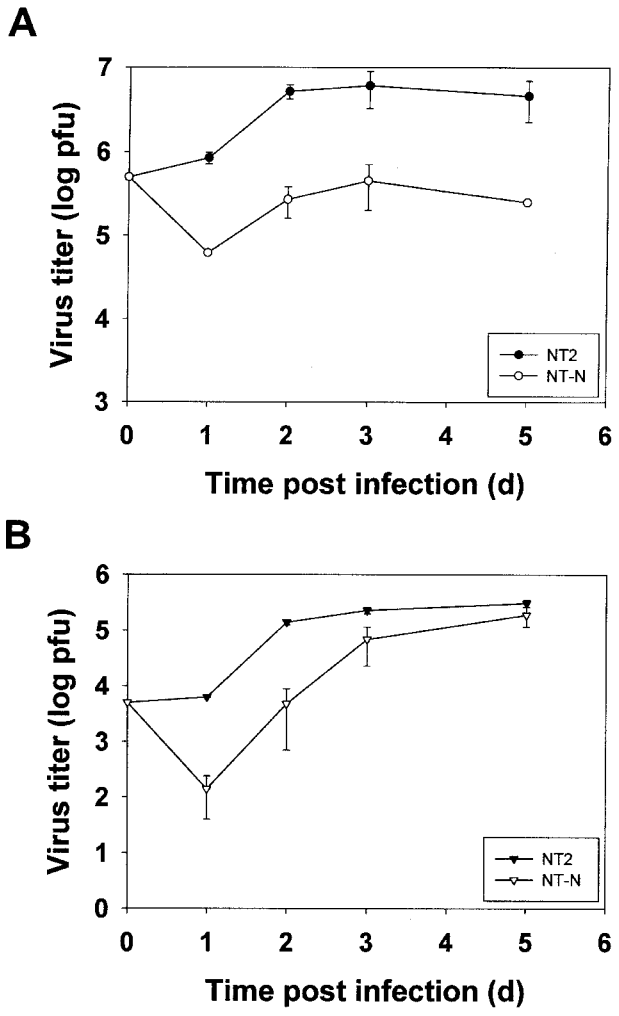


Figure 1 HSV infection of NT2 cells and NT-neurons. Cells (5×10^5) were infected with HSV-1 (F) at a multiplicity of 1.0 (**A**) or 0.01 (**B**). At the indicated day post-infection, cells were scraped into the media, disrupted by three successive cycles of freeze-thaw. Virus was quantified by plaque assay on Vero cells.

approximately 1.5 logs less than in NT2 cells (Figure 1B). At this multiplicity, the lower yield of virus in the NT-neuron culture resulted in a slower spread of virus than in the NT2 cells. By 5 days post-infection however, the total yield of virus in differentiated NT-neurons approached that in the NT2 cell culture. Histochemical staining of infected cells using an antibody to the HSV-1 immediate-early IPC4 protein indicated that similar numbers of NT2 cells and NT-neurons were infected at a given virus input and that all cells in each type of culture were susceptible to infection (data not shown). In addition, at both multiplicities, virus infection was lytic and all cells were eventually infected and destroyed.

The delay and reduction of virus replication in NT-neurons compared to undifferentiated NT2 cells could be due to impairment at any of several steps in the HSV-1 reproductive cycle. To examine the rate and magnitude of viral DNA replication in NT2 cells and differentiated NT-neurons, the two cell types were infected with HSV virus at a multiplicity of five and the levels of viral DNA determined at various times after infection (Figure 2). HSV-1 DNA replication was severely reduced in differentiated NT-neurons compared to NT2 cells; by 50 h post-infection, the level of viral DNA in NT-neurons was more than 10-fold lower than that in NT2 cells. Thus, synthesis of viral DNA correlates with virus replication in both cell types.

Since DNA replication depends upon the synthesis of viral immediate-early and early proteins, expression of immediate-early and early viral genes in infected NT2 cells and differentiated NT-neurons was compared. The mRNA levels of the immediate-early gene encoding ICP4 and the early gene encoding thymidine kinase were examined using an RNase protection assay (Figure 3). In NT2 cells, the ICP4 mRNA was easily detected by 2 h post-infection (Figure 3A). In contrast, there was a noticeable delay in the appearance of the ICP4 mRNA in NT-neurons, and lower amounts of detectable mRNA at each timepoint analyzed. Similarly, the early tk mRNA was detected earlier and in greater abundance in NT2 cells compared to NT-neurons (Figure 3B). Similar results were obtained when NT2 cells and NT-neurons were infected with a recombinant virus containing lacZ marker gene under control of the immediate-early ICP0 promoter. At 5 and 10 h post-infection, 12-fold and eightfold higher, respectively, β-galactosidase activity was measured in NT2 cells compared to NT-neurons (data not shown). These results indicate that replication of HSV-1 is restricted in differentiated NT-neurons relative to undifferentiated NT2 cells, and that the restriction appears at the earliest stages of infection.

Replication of thymidine kinase mutants of HSV-1 differentiated NT-neurons

Since thymidine kinase negative mutants of HSV-1 are severely impaired for replication in ganglia *in*

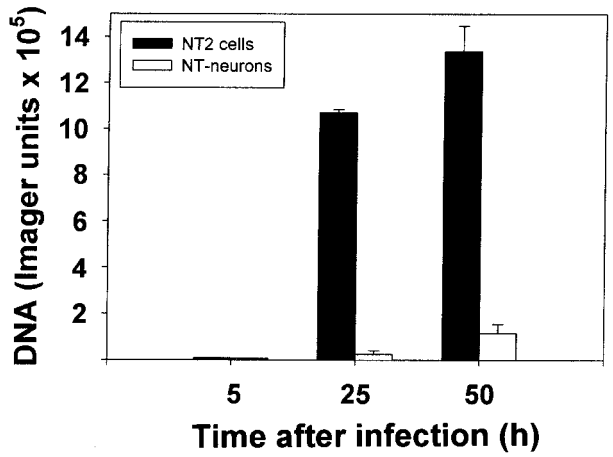


Figure 2 Replication of viral DNA in NT2 cells and NT-neurons. Cells (5×10^5) were infected with HSV-1 (F) at a multiplicity of five. At the indicated time post-infection, the media was removed and the total infected cell DNA was isolated and quantified by slot-blot hybridization.

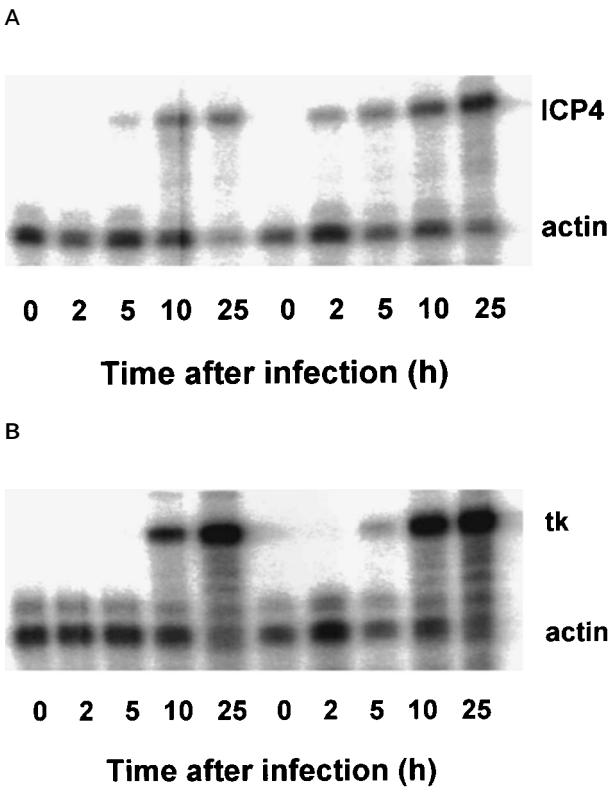


Figure 3 Expression of immediate-early and early mRNA in infected NT2 cells and NT-neurons. Cells (5×10^5) were infected with HSV-1 (F) at a multiplicity of five. At the indicated times after infection, RNA was isolated and analyzed by RNase protection using probes for β-actin and either the immediate-early ICP4 gene (**A**) or the early thymidine kinase gene (**B**).

vivo, it was of interest to determine whether such an impairment would be reflected in differentiated NT-neurons. Results from experiments using recombinant viruses containing a lacZ gene inserted into the thymidine kinase locus indicated that there was no detectable difference in the replication of such mutants in NT2 cells compared to wild-type HSV-1, but significantly delayed replication in differentiated NT-neurons (Figure 4). At a low multiplicity of infection, there was no evidence of virus reproduction until more than 3 days following infection, and even by 5 days post-infection, levels of virus in the infected cultures only returned to the level of the input virus inoculum. At the higher multiplicity of infection, levels of virus in the infected cultures remained more than a log lower than the input inoculum, but the level of detectable virus was similar from 1–5 days post-infection, suggesting a low level of virus replication. To eliminate any possible influence of the exogenous lacZ gene on replication, two additional viruses were constructed and used to analyze the replication of HSV-1 in differentiated NT-neurons. One virus, vTKS, has a 14-bp insert in the thymidine kinase and is thymidine-kinase deficient. The second virus, vTKS-R, has this mutation repaired to restore the wild-type sequence, and is thymidine kinase positive. Similar to the behavior of other thymidine kinases-deficient HSV-1 in NT-neurons, vTKS replicated to lower levels than either wild-type or the repaired virus (Table 1). At 2 days post-infection in NT-neurons, titers of vTKS were 40–50-fold lower than thymidine-kinase positive HSV-1. Reduced virus replication was mirrored by a concomitant reduction in viral DNA replication; by 50 h post-infection, the level of vTKS DNA replication was only 22% as much as that of HSV-1(F) (data not shown). Thus, disruption of the viral thymidine kinase gene results in a further restriction of HSV-1 replication in differentiated NT-neurons.

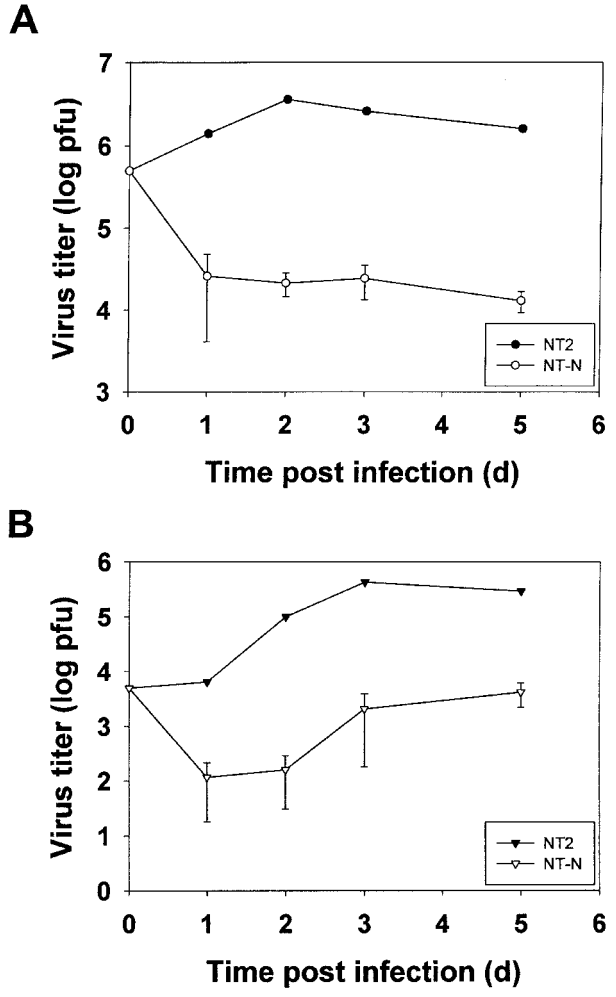


Figure 4 Replication of thymidine-kinase negative HSV-1 in NT-neurons. NT-neurons (5×10^5) were infected with vgCL3 at a multiplicity of 1.0 (**A**) or 0.01 (**B**). At the indicated day post-infection, cells were scraped into the media, and disrupted by three successive cycles of freeze-thaw. Virus was quantified by plaque assay on Vero cells.

Infection of differentiated NT-neurons with replication-incompetent HSV vectors

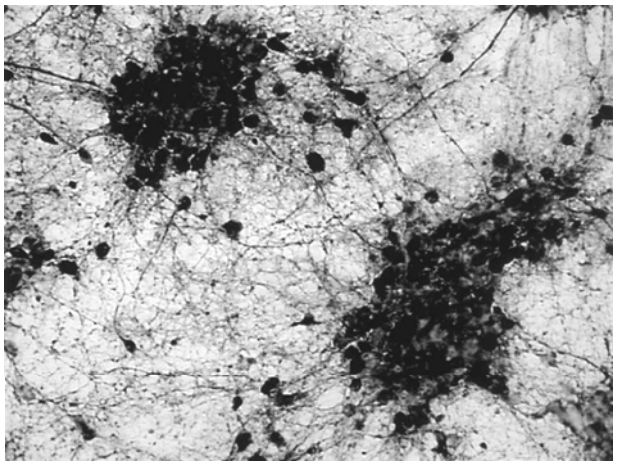
Since differentiated NT-neurons can be maintained in culture in a post-mitotic state for extended periods of time, it was of interest to determine whether these cells could be used to examine the persistence of HSV vectors after delivery. NT-neurons were infected with either of two replication-incompetent vectors, 14HΔ3 or d120/β-Gal. Both vectors are deficient for the immediate-early gene encoding ICP4. The vector 14HΔ3 also includes a mutation in the VP16 gene which reduces expression of the remaining immediate-early genes, and is therefore less cytotoxic than vectors containing a single mutation in the ICP4 gene. The vector d120/β-Gal has the lacZ gene under control of the immediate-early ICP0 promoter, and even though cytotoxic for many cell types, is useful for monitoring gene delivery since it expresses high levels of β-galactosidase upon infection. As observed previously during HSV infection of NT-neurons, all of the cells in culture were susceptible to HSV vector infection. Figure 5A shows d120/β-Gal infected NT-neurons at day 3

Table 1 Replication of HSV-1 in NT-neurons (2 days post-infection)

	Recovered Virus (log total pfu)[a]		
	HSV-1(F)	*vTKS*	*vTKS-R*
Input virus (log pfu)			
3.699	2.35±0.37	1.05±0.15	2.79±0.12
5.699	5.00±0.41	3.75±0.58	5.09±0.01

[a] 5×10^5 NT-neurons were infected with the indicated virus. Two days later, the cells and the media were collected and the recovered virus titrated in Vero cells.

A



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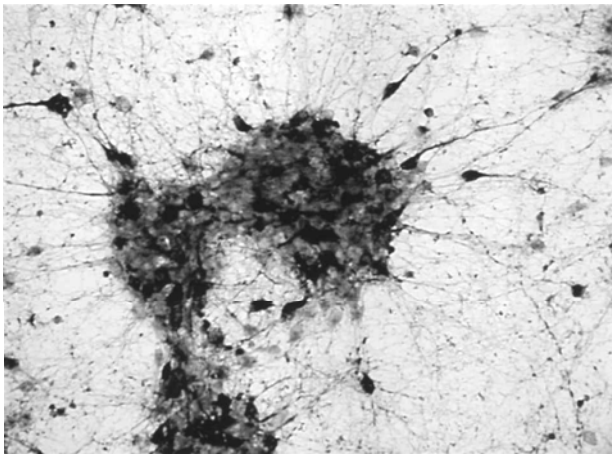

Figure 5 Expression of β-galactosidase in vd120/β-Gal infected NT-neurons. NT-neurons were infected with vd120/β-Gal at a multiplicity of approximately three. Cells were fixed and immunostained for β-galactosidase at day 3 (**A**) and 14 (**B**) post-infection. β-galactosidase positive cells appear black in these photographs. At this multiplicity of infection, almost every cell is infected with the vector as shown in **A**.

stained with an antibody to β-galactosidase; Figure 5B shows the β-galactosidase staining of NT-neurons at day 14 of the same experiment. β-galactosidase positive NT-neurons are apparent at both times post-infection (black cells), although fewer in number at day 14 than at day 3. To examine vector persistence in NT neurons, cells were infected with either replication-incompetent vector and then subsequently super-infected with a second replication-incompetent vector (deficient in the gene encoding the immediate-early protein ICP27). Both 14HΔ3 and d120/β-Gal vectors could be recovered from NT-neurons for at least 14 days (Table 2). There was reduced recovery of d120/β-Gal relative to 14HΔ3 which probably reflects the greater toxicity of the d120/β-Gal vector. Thus, the

Table 2 Recovery of HSV vector following superinfection of NT-neurons

	Recovered Virus (total pfu) after superinfection[a]	
	14HΔ3	*d120/β-Gal*
Days post-infection		
1	4.1×10^5	1.6×10^4
3	2.8×10^5	1.4×10^4
7	3.5×10^5	3.1×10^3
14	2.4×10^5	8.7×10^3

[a]5×10^5 NT-neurons were infected with the indicated virus. At the indicated day post-infection, the cells were superinfected with 5*dl*1.2 at a multiplicity of 10. At 24 h the cells were scraped into the media and the cell suspension was freeze-thawed three times. The cell lysate was plaqued on E5 cells. There was no recovered virus if cells were mock infected rather than vector infected or if vector-infected cells were mock-infected instead of superinfected with 5*dl*1.2

results suggest that differentiated NT-neurons provide an *in vitro* cell culture system for evaluating the behavior of HSV vectors in human neurons.

Discussion

A complete understanding of the interaction of herpes simplex virus with the neuron is hampered in part by the limited number of neuronal cell systems which possess characteristics of fully differentiated neurons. The human embryonal carcinoma cell line NT2 (Andrews *et al*, 1984) is unique in that it is capable of differentiating irreversibly into postmitotic neuron-like cells following treatment with retinoic acid (Andrews, 1984). The unique combination of phenotypic and morphological characteristics of NT-neurons that include its human origin, unlimited supply, and irreversible differentiated state, make it an attractive system for the evaluation of HSV infection and replication. Previous studies with NT-neurons are limited, but one report indicated that HSV-1 infects and spreads on monolayers of NT2 cells and NT-neurons (Kesari *et al*, 1995); a second report demonstrated that NT-neurons can be efficiently infected with an amplicon-based HSV vector (Fath *et al*, 2000).

The results presented in this report clearly show that HSV-1 replicated in both NT2 cells and differentiated NT-neurons. However, virus replication in differentiated NT-neurons was lower and delayed relative to that in NT2 cells. The initial virus burst at 1 day post-infection was 1 – 1.5 logs lower in NT-neurons than in undifferentiated NT2 cells. Although at a low multiplicity of infection, the total virus production in NT-neurons after 5 days of infection approached that measured in NT2 cells, the peak in virus yield in NT2 cells occurred approximately 2 days earlier. Thus, at 5 days post-

infection in NT2 cells, the measured virus yield reflects both synthesis from recently infected cells and gradual inactivation of virus synthesized earlier during infection. The restricted replication in NT-neurons correlated with reduced and delayed immediate-early and early gene expression and viral DNA replication. Taken together, these findings strongly suggest that HSV-1 immediate-early gene expression is inhibited in differentiated NT-neurons, relative to undifferentiated NT2 cells. Similarly, delayed and reduced immediate-early gene expression has been reported for HSV infection of primary rat peripheral neurons *in vitro* (Nichol *et al*, 1996). It has been suggested that immediate-early gene expression is inhibited in neurons *in vivo* and that this inhibition is necessary for the virus to establish latency in the neuron (Kemp *et al*, 1990; Kosz-Vnenchak *et al*, 1993; Hagmann *et al*, 1995). Thus, low level expression of immediate-early genes in NT-neurons may reflect limited immediate-early gene expression in neurons *in vivo*. Nevertheless, at the multiplicities of HSV infection used in the experiments described in this report, lytic infection was always observed. It will be of obvious interest to investigate whether conditions can be devised for development of an HSV latency model using NT-neurons. The development of *in vitro* latency models, such as nerve growth factor differentiated PC12 cells (Danaher *et al*, 1999) and primary rat neurons (Wilcox and Johnson, 1988), in which acyclovir is used to inhibit virus replication in the early stages of infection suggest that a similar model might be feasible with NT-neurons.

Significantly, replication of thymidine kinase mutants of HSV-1 was further restricted in NT-neurons relative to NT2 cells. This was demonstrated using several thymidine kinase mutants which differed in the nature of their tk disruption. In all experiments, there was evidence of low level virus replication over time, indicating that the block in virus replication was not absolute. This low level of virus replication was not due to the selective replication of virus in a subpopulation of NT-neurons, since histochemical staining of infected cells demonstrated that eventually all cells in the culture became infected. Similarly, it has been reported that replication of thymidine-kinase deficient HSV is reduced and delayed, although not eliminated, in primary rat neurons (Wilcox *et al*, 1992). HSV thymidine kinase, although dispensable for virus replication in most tissue culture systems, is thought to be necessary for efficient replication in post-mitotic neurons. Indeed, tk-deficient virus has been shown to be compromised for acute replication, as well as reactivation, in murine trigeminal ganglia (Coen *et al*, 1989; Jacobson *et al*, 1993). Nevertheless, restricted replication of tk-deficient HSV has not been reported for other neuronal cell lines. For example, in nerve growth factor differentiated PC12 cells, there was no difference in the replication of wild-type and tk-negative mutants (Rubenstein and Price, 1983). The difference in tk-negative HSV-1 replication in differentiated PC12 and differentiated NT-neurons is likely due to the irreversible differentiated state of NT-neurons.

We have not yet investigated the role of other HSV genes in HSV infection, and replication of NT-neurons. However, it has been reported that γ34.5 gene-deficient HSV-1 is unable to replicate in differentiated NT-neurons (Kesari *et al*, 1995). Added together, the observations regarding the behavior of HSV-1 in NT-neurons suggest that this neuronal culture system may be a valuable *in vitro* system for studying certain aspects of HSV-neuron interactions.

In addition to their utility as an *in vitro* system for studying the fundamentals of virus-host cell interactions, NT-neurons may be particularly useful for evaluation of HSV vectors before their use *in vivo*. Specifically, since post mitotic NT-neurons can be maintained in culture for extended periods of time, they provide a ready source of human neuron-like cells to study the level and duration of foreign gene expression and the potential toxicity of HSV vectors. The results presented here show that replication-incompetent HSV vectors can be recovered from vector-infected NT neurons for at least 2 weeks following vector delivery. The amount of 14HΔ3 vector recovered after super-infection was greater than the corresponding amount of d120/β-Gal vector recovered. However, for each vector, there was little change in the amount of recovered vector over the 2 week period, suggesting that the vector DNA was stable in the NT-neuron cultures over this time period. β-galactosidase staining of d120/β-Gal infected NT-neurons also indicated that the vector expressed protein was present during this time period, although there were fewer β-galactosidase positive cells at 14 days than at 3 days. Since NT-neurons can be maintained in their non-dividing, post-mitotic state for much longer than 2 weeks (Pleasure *et al*, 1992), such cultures may be particularly well suited for evaluation of vector gene expression and persistence.

In summary, the results presented here indicate that differentiated NT-neurons provide a continuous source of post-mitotic neuron-like cells which can be used to study at least some aspects of the interaction of HSV and the neuron and to evaluate features of HSV vectors which are designed for gene delivery to the neuron. The advantages of the NT-neuron system include their human origin, their renewable and homogenous nature, and their irreversible differentiation into post-mitotic neuron-like cells in the presence of retinoic acid. Their principal disadvantage is the time required for their preparation. While it is not possible to predict how faithfully all aspects of the interaction of HSV and the neuron will be reproduced in this or any other *in*

vitro system, NT-neurons should provide a useful tool for study of HSV biology and HSV vector development.

Materials and methods

Cells and viruses

NT2 cells (Andrews, 1984; Andrews *et al*, 1984) were obtained from Stratagene (La Jolla, CA, USA) and maintained essentially as described by the supplier. Briefly, undifferentiated NT2 cells were passaged at relatively high density ($>5\times10^6$/75 cm^2 flask) as recommended previously (Lee and Andrews, 1986) in Opti-MEM (Gibco/BRL Life Technologies, Gaithersburg, MD, USA) containing 10% fetal bovine serum. For differentiation, 5×10^6 cells (162 cm^2 flask) were treated for 5 weeks with retinoic acid (0.01 mM), then replated at a 1:5 dilution and treated with mitotic inhibitors as described by the supplier. After 10 days, the NT-neurons were mechanically dislodged by several sharp strikes to the flask. The cells were replated on flasks or slides treated with MATRIGEL (Collaborative Biomedical, Bedford, MA, USA) and cultured in DMEM containing fetal bovine serum without retinoic acid or mitotic inhibitors.

HSV-1 strain F and recombinant vgCL3 (Weir and Narayanan, 1990) were grown and titered on Vero cells. Recombinant virus vTKS was generated by co-transfection of the plasmid DNA pTKS and HSV-1 (F) genomic DNA into Vero cells using Lipofectamine (Gibco/BRL Life Technologies). Plasmid pTKS contains the HSV-1(F) thymidine kinase gene (3 kb *Bam*HI fragment) with a 14 bp oligonucleotide linker inserted at the unique *Sac*I site in the tk coding sequence (444 bp downstream from the start of translation). The virus produced after transfection was plaqued on Vero cells in the presence of 100 mM acycloguanosine (Sigma, St. Louis, MO, USA) to select for thymidine kinase negative viruses. Multiple individual plaques were selected and analyzed for the presence of the inserted restriction site by Southern blotting. The thymidine kinase repaired virus, vTKS-R, was constructed by co-transfection of plasmid DNA containing the authentic tk gene along with vTKS genomic DNA into Vero cells. Thymidine kinase-positive virus was isolated by plaque assay on 143tk$-$cells in the presence of HAT (hypoxanthine/aminopterin/thymidine) medium, and the repaired tk gene was verified by Southern blotting. The phenotype of vTKS and vTKS-R was verified by plaque titration of virus stocks on Vero cells in the presence and absence of acycloguanosine. The replication-incompetent HSV vectors d120/β-Gal and 14HΔ3 have been described previously (Johnson *et al*, 1994; Weir and Dacquel, 1995). Both vectors were grown and titered on the ICP4-complementing cell line E5 (DeLuca and Schaffer, 1987); 14HΔ3 was grown and titered in the presence of 5 mM hexamethylene bisacetamide (HMBA; Sigma) to partially complement the defect in plaque formation due to the VP16 mutation in this vector (McFarlane *et al*, 1992). The replication-incompetent virus 5*dl*1.2 was grown and titered on 3-3 cells as previously described (McCarthy *et al*, 1989).

Construction of plasmids

Routine cloning procedures were similar to those described by Ausubel *et al*. (1987). Probes for the RNase protection assays were constructed by PCR amplification of the DNA overlapping the transcriptional start site of each gene, using primers that were complementary to the viral DNA and containing unique restriction endonuclease recognition sites. PCR products were purified and cloned into the multiple cloning region of pGEM3 or pGEM4 (Promega, Madison, WI, USA). pICP4/GEM3 contains a 308 bp fragment which protects 193 bases of the mRNA; pTK/GEM4 contains a 181 bp fragment which protects 148 bases of the mRNA; pHu-βactin contains a 100 bp fragment of the coding region of human β-actin.

Viral DNA replication

To quantitate viral DNA replication, approximately 5×10^5 NT2 cells or NT-neurons were infected with virus at a multiplicity of five. The higher multiplicity, relative to virus replication experiments, was necessary due to the relatively low numbers of cells used for DNA isolation. At the indicated times post-infection, the cells were lysed with DNAzol (Gibco/BRL Life Technologies), and prepared according the manufacturer's instructions. Equivalent amounts of infected cell DNA were loaded in duplicate onto a slot-blot apparatus containing a Nytran membrane (Schleicher and Schuell, Keene, NH, USA), essentially as described by Ausubel *et al*, (1987). Samples were cross-linked with a UV Stratalinker (Stratagene, La Jolla, CA, USA), and hybridized to a ^{32}P-labeled probe from the glycoprotein C gene. Hybridized probe was quantitated using a Molecular Dynamics phosphorimager (Sunnyvale, CA, USA).

RNase protection asssays

RNA was prepared from approximately 5×10^5 cells at the indicated times after infection using TRIzol reagent (Gibco/BRL Life Technologies). As for the DNA isolation experiments, a multiplicity of five was used due to the numbers of cells used for each isolation. RNase protection assays were performed using a HybSpeed RPA kit (Ambion, Austin, TX, USA), with ^{32}P-labeled RNA probes prepared from the plasmids described above using a MAXIscript SP6/T7 kit (Ambion, Austin, TX, USA). Protected probes were electrophoresed on 6% polyacrylamide/urea gels (Novex, San Diego, CA, USA). Dried gels were analyzed with a phosphorimager.

Superinfection rescue assay
The HSV superinfection assay has been described previously (Weir *et al*, 1996) and is similar to that reported for detection of latent HSV in isolated ganglia (Coen *et al*, 1989; Leib *et al*, 1989). Briefly, for vector recovery, NT-neurons were superinfected with 5*dl*1.2 at a multiplicity of 10 on the indicated day following vector infection. At 24 h, cells were scraped into the media and the cell suspension freeze-thawed three times before plaque assay on E5 cells to determine the titer of ICP4-negative vector.

Immunostaining
Cells were grown on either 12-well plates or chamber slides that had been previously treated with MATRIGEL as described above, and infected with HSV-1 or HSV vectors. At the indicated times post-infection, cells were fixed with 5% glutaraldehyde and immunostained with monoclonal antibody to HSV-1 ICP4 (Advanced Biotechnologies, Inc., Columbia, MD, USA) or β-galactosidase (Promega Life Sciences, Madison, WI, USA). Immunoperoxidase staining was used for detection (VECTASTAIN ABC kit, Vector laboratories, Burlingame, CA, USA) with either Vector VIP (purple) or Vector SG (gray) as substrate.

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