The Role of Cyclic Nucleotide Mediators in Latency and Reactivation of HSV-1 Infected Neuroblastoma Cells

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Summary

The mechanisms that control herpes simplex virus type 1 latency and reactivation are still poorly understood. We developed an *in vitro* murine neuroblastoma cell HSV-infected, acyclovir suppressed model to study the influence of different cyclic nucleotide mediators on the latency and reactivation of HSV-1. A positive cDNA '*in situ*' hybridisation for HSV genome was used to prove the establishment of a viral-host cell nuclear relationship. An ABC-immunoperoxidase reaction to cell surface HSV mature glycoproteins was also performed to determine the time of viral reactivation with formation of mature virions. Supernates of cultured cells were placed on Vero cells for confirmation of reactivation by classic cytopathic effect. Theophylline (50 µg/ml) and dibutyryl-cAMP (0.1, 0.5, 1 mg/ml) produced the most pronounced response, accelerating HSV reactivation time by 150%. Epinephrine (10, 20 µg/ml) had an intermediate effect on accelerating viral reactivation; and verapamil (20, 50 µg/ml), theophylline and epinephrine at lower doses had a smaller effect. Carbamylcholine (10 µg/ml) prolonged the time to viral reactivation by 100%, 36 hours compared to control time of 18 hours. Insulin (0.1, 0.5, 1 mg/ml) also prolonged HSV 'latency' by six hours. Exogenous dibutyryl-cGMP and carbamylcholine at lower concentrations did not have an effect on viral reactivation. These findings suggest that there is a relationship between changes of intracellular concentrations of cyclic nucleotides and HSV latency and reactivation.

Latently infected trigeminal ganglia have been thought to be the source of virus for recurrent herpetic keratitis in humans, a major cause of corneal blindness in developed countries.¹ Current antiviral therapy has proved ineffective in preventing the establishment of latency. The molecular details of the relationship between virus and host cell DNA during the latent state have not been

definitely elucidated.^{2,3} An alternative therapeutic strategy to viral DNA elimination from latently infected ganglia might include efforts to indentify and manipulate factors involved in the control of mechanisms responsible for the induction of and reactivation from the latent state. A variety of physiological (fever, trauma, sunburn, menses, stress) and experimental (epinephrine, prostaglandins, ultra-

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violet light) stimuli can be associated with HSV-1 reactivation in patients and in experimental models respectively.⁴⁻¹⁰ These 'triggers' may influence, through the action of first order messengers such as catecholamines and/or arachidonic acid metabolites, cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP) concentrations within latently infected neurons. We have already shown in an *in vivo* and *in vitro* murine trigeminal ganglion latency model that the relative proportions of intracellular cAMP and cGMP may influence maintenance of (elevated cGMP with no change in cAMP levels) or reactivation from (elevated cAMP) latency.¹¹ In order to study further the relationship between intracellular neuronal cyclic nucleotide levels and HSV latency we studied the effects of putative mediators of intracellular cyclic nucleotide levels on an *in vitro* murine neuroblastoma cell HSV-infected, acyclovir-suppressed model using cDNA '*in situ*' hybridisation (viral DNA detection) and avidin-biotin immunoperoxidase (mature virion detection) techniques to determine the time of viral reactivation (positive cDNA '*in situ*' hybridisation, positive ABC immunoperoxidase) from 'latency' (positive cDNA '*in situ*' hybridisation, negative ABC immunoperoxidase) for each mediator. These findings may have implications for clinical ocular HSV recurrent infections.

Material and Methods

Cell lines: C-1300 murine neuroblastoma cells (American tissue Culture Collection, Rockville, MD) were cultured using Nutrient Mixture F-12 with L-glutamine media (Gibco Laboratories, Grand Island, NY) supplemented with 5% qualified fetal bovine serum (FBS) heated and inactivated at 60°C for one hour, 5,000 U/ml of penicillin, 5,000 mcg/ml of streptomycin (Gibco Labs), 250 mcg/ml of fungizone (Squibb Flow Labs, Inc., McLean VA), and 250 mg of gentamicin sulfate (Gibco Labs). Vero cells from monkey kidney (CCL81, ATCC) were cultured with Minimum Essential Medium (MEM) with Earle's salts (Gibco Labs), 5% FBS and penicillin-streptomycin-fungizone-gentamicin solution as above. 75 cm² sterile cell culture flasks (Becton-Dickinson Labware, Lincoln

Park, NJ) were used to grow and maintain the cell lines in an incubator at 37°C and 5% CO₂ during the experiments.

Mediators: Mediators that increase intracellular levels of cAMP: N6-2'-O-dibutyryladenosine 3',5' cyclic 'monophosphate sodium salt (purity = 97%) (Sigma, St Louis, MO), epinephrine (Elkins-Sinn, Inc., Cherry Hill, NJ), isoproterenol HCl (Winthrop-Beron Lab, New York, NY), theophylline (Elkins-Sinn, Inc.) and verapamil HCl (Knoll Pharmaceuticals, Whippany, NJ). Mediators that increase intracellular levels of cGMP: N2,2'-O-dibutyrylguanosine 3'5' cyclic monophosphate sodium salt (purity-96%) (Sigma), carbamylcholine (Alcon Inc., Humacao, Puerto Rico) and regular recombinant-DNA insulin (Eli Lilly and Co., Indianapolis, IN). Other mediators used: sodium cromoglycate (Fisons Corp., Bedford, MA), dexamethasone sodium phosphate (Elkins-Sinn, Inc.), cyclosporin A (Sandoz, Pharmaceutical Corp., East Hanover, NJ).¹²

Experiment design: Neuroblastoma cells were trypsinised and plated into 6-well, 9.62 cm² flat bottom tissue culture plates (Becton-Dickinson Labware) containing a 10.5 × 22 mm Thermanox tissue culture plastic coverslip (Miles Lab Inc., Naperville, IL). Each experiment was performed with its own set of controls and was replicated at least four times to demonstrate the reproducibility of the results.

HSV-1 infection of neuroblastoma cells: After three days of cell incubation of 37°C, 5% CO₂, the cultures were infected with 1 ml/well of HSV-1 KOS strain (titer = 2 × 10⁸ PFU/ml, from Dr David Knipe, Harvard Medical School, Boston, MA) diluted with FBS Nutrient Mixture F0-12 to an MOI of 2, for one hour. The viral suspension was then removed and the wells were washed and rinsed three times with FBS Nutrient Mixture F-12 followed by a post-infection incubation period of six hours with 5% FBS Nutrient Mixture F-12.

Suppression of HSV-1 with acyclovir: After the post-infection incubation period, the HSV-1 infected neuroblastoma cells were

treated with 10 µg/ml of acyclovir (ACV) (Burroughs-Welcome, Res. Triangle Park, NC) and 1 µg/ml of 7S-Nerve Growth Factor (7S-NGF) (Collaborative Research Inc., Bedford, MA) everyday for four days to induce suppression of HSV-1.¹³ At the end of day four, the cells were washed and rinsed three times with FBS Nutrient Mixture F-12 to remove the ACV/7S-NGF solution.

Time-course for detection of HSV-1 reactivation time: Cyclic nucleotide mediators at different doses were then added to the HSV-infected, ACV-suppressed neuroblastoma cells. Preliminary dose-response studies (unreported) were performed to determine the mediator toxicity limits. A time-course consisting of the removal of the supernate and a set of coverslips every two hours for cocultivation on Vero cells¹⁴ and immunohistochemical staining respectively was done. The time course was 48 to 72 hours depending on the type of mediator used.

Controls: These consisted of neuroblastoma cells on coverslip without HSV infection and without ACV/7S-NGF (positive-control); and a set of coverslips HSV-infected, ACV-suppressed, incubated with fresh culture media and nerve growth factor (1 µg/ml) alone, which were withdrawn along with the mediator coverslips during every time-course (model control).

cDNA 'in situ' hybridisation: Withdrawn coverslips were left to dry at room temperature for at least two days and then stained with an HSV cDNA probe assay (ENZO Diagnostics, Inc., New York, NY). Thermanox coverslips were cut into two equal pieces; one was used for the ABC immunoperoxidase stain; the other was rinsed with PBS (pH 7.2) and fixed with 100% acetone for 10 minutes. One drop of the DNA probe reagent was then applied to each Thermanox tissue culture coverslip and a glass coverslip was then placed to cover the preparation which was then placed onto a 92°C heating block for 3–4 minutes. After removing the coverslip from the block, hybridisation proceeded at room temperature for 20 minutes in a moisture chamber. The post-hybridisation reagent was

applied for 10 minutes after removal of the glass coverslip. The coverslips were rinsed with wash buffer for 10 seconds and the detection reagent (avidinbiotinylated horseradish peroxidase) was applied for 10 minutes. The coverslips were rinsed with wash buffer, counterstained with fast green FCF and mounted with gelvatol for light microscopy.

ABC immunoperoxidase staining: The other half of the coverslip was also rinsed in PBS (pH 7.2) and fixed with acetone as above. Coverslips were incubated with a 5% normal goat serum solutions for 30 minutes. A rabbit anti-HSV-1 antibody (IgG anti-HSV, Dako Corp., Santa Barbara, CA) was diluted to 1:100 with 1% BSA, filtered with a 0.2 µm microfilter (Gelman Sciences Inc., Ann Arbor, MI) and absorbed with neuroblastoma cells for 15 minutes on a rotating table. A further dilution to 1:1600 with 1% BSA and centrifugation with 2800 rpm for 10 minutes at 25°C was followed by adding it to the coverslips for 45 minutes (primary incubation) at room temperature in a moisture chamber. The coverslips were then rinsed three times with PBS (pH 7.2) for 10 minutes. H₂O₂ 0.3%, was added to each coverslip for 30 minutes. A rabbit ABC Vectastain kit (Vector Laboratories Inc., Burlingame, CA) was then used to follow the staining. The secondary incubation was performed after rinsing the coverslip; one drop of goat anti-rabbit IgG biotinylated antibody was applied for 45 minutes, followed by a rinse with PBS. The tertiary 45 minute incubation with the avidin-biotinylated peroxidase was performed before reacting the coverslips with the aminoethycarbazole substrate for 20 minutes. Coverslips were rinsed with acetate buffer (pH 5.0) and fixed with 4% formaline; 1% glacial acetic acid was applied before counterstaining with Gill's hematoxylin and mounting with gelvatol for light microscopy.

Cocultivation of supernates on Vero cells: All supernates from the time-course (mediator samples and controls) coverslips were filtered and placed on confluent monolayers of Vero cells and observed daily for 10 days, using an inverted light microscope for cytopathic effect (CPE).

Fig. 1. Positive cDNA 'in situ' hybridisation for HSV in HSV-1 infected neuroblastoma cells (40 $\times$)

Statistics: Differences in time for HSV-1 reactivation (positive ABC immunoperoxidase staining) for each mediator compared to control were analysed using the Analysis of Variance test ($p < 0.05$).

Results

A positive cDNA 'in situ' hybridisation for HSV was a consistent finding in all coverslips containing HSV-1 infected neuroblastoma

cells, showing the establishment of a viral-host cell genome relationship (Fig. 1). The percentage of cells showing a positive cDNA stain correlated directly with the MOI used (Table I). HSV-1 infected neuroblastoma cells treated with NGF and ACV also showed, consistently, cDNA positive cells for HSV DNA while, at the same time, manifesting no mature, late (gamma) HSV proteins. Supernatants from these cultures consistently failed to yield infectious virions when cultured on Vero cells; supernatants from such cultures not treated with experimental mediators were HSV negative for 18 hours after removal of ACV from the culture medium (desuppression).

The results show that the mean HSV reactivation time from an acyclovir-induced 'latency' in neuroblastoma cells was 18 hours (positive ABC immunoperoxidase reaction) after the withdrawal of the ACV-7S-NGF solution (Fig. 2). This finding was consistent throughout the different sets of experiments. Cultivation of supernates on vero cells produced CPE due to mature HSV. This tra-

Table 1. Percentage of neuroblastoma cells infected with HSV-1 as shown by a positive cDNA 'in situ' hybridisation for HSV using different multiplicities of infection (MOI)

MOI	cDNA positive cells (Mean count + SEM)*
1	13.04 + 1.712
2	21.40 + 2.831
4	43.12 + 2.796
5	45.32 + 5.103
6	52.54 + 4.995
8	49.38 + 3.692
10	73.58 + 4.441

*Cell count of 5 (450 $\times$) power fields of 2 coverslips per MOI using a 10 $\times$ 10 mm grid.

Fig. 2. Positive ABC-immunoperoxidase staining for anti-HSV-1 antibody in HSV-1 reactivation from an acyclovir-induced 'latency' in neuroblastoma cells (100 $\times$)

ditional assay, however, was clearly less sensitive and less precise than was the ABC assay for HSV gamma proteins. The mean time to HSV reactivation following withdrawal of ACV as determined by supernate HSV positivity was 24 hours (Table II).

Of the cyclic nucleotide mediators that increase the intracellular levels of cAMP (Table II), the phosphodiesterase inhibitor theophylline (50 μ g/ml) and dibutyryl-cAMP

(0.1, 0.5, 1 mg/ml) produced the most pronounced acceleration of the reactivation time of HSV-1, six hours compared to the controls' mean reactivation time of 18 hours ($p < 0.05$).

Epinephrine (10, 20 μ g/ml), an adrenergic agonist, accelerated the reactivation of HSV-1 by eight hours (mean time to reactivation, 10 hours; control reactivation time, 18 hours) ($p < 0.05$) (Fig. 3).

Lower concentrations of theophylline (10,

Table II. Effect of cyclic nucleotide mediators that increase intracellular levels of cAMP in viral reactivation of HSV-1 infected neuroblastoma cells

Mediator	Reactivation time (hours)		Dose
	+ABC	+CPE	
Dibutyryl-cAMP	6*	6	0.1, 0.5, 1 mg/ml
Theophylline	6*	12	50 μ g/ml
	12*	24	10, 20 μ g/ml
Epinephrine	10*	18	10, 20 μ g/ml
	12*	18	5 μ g/ml
Verapamil	12*	12	20, 50 μ g/ml
	18	18	10 μ g/ml
Isoproterenol	18	24	2.5, 5, 10 μ g/ml
Controls	18	24	

Significant values on the ABC immunoperoxidase staining using Analysis of Variance test ($P < 0.05$).

Fig. 3. Positive ABC-immunoperoxidase staining for anti-HSV-1 antibody in HSV-1 infected, acyclovir suppressed neuroblastoma cells after 12 hours with Epinephrine 20 µg/ml (16×).

20 µg/ml), epinephrine (5 µg/ml) and the calcium channel blocker, verapamil (20, 50 µg/ml) which has a demonstrated phosphodiesterase inhibitor activity,¹⁵ had a smaller effect, inducing HSV-1 reactivation six hours before the controls ($p < 0.05$).

Verapamil at lower dose (10 µg/ml) and isoproterenol (2.5, 5, 10 µg/ml), a beta adrenergic agonist, had no demonstrable effect on the induction of HSV-1 reactivation in this model.

Of the mediators that increase intracellular levels of cGMP by activation of the guanylate

cyclase system (Table III), the most stable synthetic metabolite of acetylcholine, carbamylcholine (10 µg/ml), a muscarinic cholinergic agonist, showed the most pronounced effect in prolonging the 'latency' of HSV-1 in neuroblastoma cells by doubling the time of reactivation compared to controls ($p < 0.05$) (Fig. 4).

Insulin (0.1, 0.5, 1 U/ml) also prolonged the reactivation time of HSV-1 by six hours compared to controls ($p < 0.05$) (Fig. 5).

Carbamylcholine at a lower dose (5 µg/ml)

Table III. Effect of cyclic nucleotide mediators that increase intracellular levels of cGMP in prolonging the latency of HSV-1 infected neuroblastoma cells

Mediator	Reactivation time (hours)		Dose
	+ABC	+CPE	
Dibutyl- <i>c</i> -GMP	18	24	0.1, 0.5, 1 mg/ml
Carbamylcholine	36*	48	10 µg/ml
	18	24	5 µg/ml
Insulin	24*	48	0.1, 0.5, 1 U/ml
Controls	18	24	

*Significant values on the ABC immunoperoxidase staining using Analysis of Variance test ($P < 0.05$).

Fig. 4. Negative ABC-immunoperoxidase staining for anti-HSV-1 antibody in HSV-1 infected, acyclovir suppressed neuroblastoma cells after 18 hours with Carbamylcholine 10 µg/ml (25×).

and dibutyryl-cGMP (0.1, 0.5, 1 mg/ml) showed no effect on prolonging the 'latency' of HSV-1 in this neuroblastoma cell model.

Cyclosporine at low doses (1, 2.5, 5 µg/ml) due to its high toxicity on neuroblastoma cells induced viral reactivation moderately compared to controls (Table IV).

Dexamethasone phosphate (20 µg/ml) and sodium cromoglycate (25 µg/ml) did not have an effect in inducing reactivation or prolonging the latent state of HSV-1.

Discussion

Recurrences of ocular HSV are related to the well known cycle of latent trigeminal ganglionic infection, ganglionic viral reactivation, and ocular virus shedding.^{4-10,12-15} Despite the recent advances into the nature of HSV latency, little is known about the mechanisms of HSV-1 latency and reactivation. Therefore there are no clinically reliable methods of preventing the establishment of latent infection of the trigeminal ganglion, of inhibiting ganglionic viral reactivation, or of preventing viral shedding. An alternative

strategy for preventing the cumulative permanent structural alterations of the eye which compromise vision is to manipulate the physiologic changes which control HSV latency and reactivation. If latency is controlled by the expression of certain genes not expressed during lytic infection, how can these genes be activated? If latency is controlled by the lack of expression of certain genes, how can these genes be blocked? If reactivation is the suppression of the mechanisms maintained during latency, how can this suppression be inhibited? It has been shown that regulation of cellular responsiveness to cAMP may be regulated at multiple biochemical levels that can clearly affect the macromolecular processes involved in gene expression, which in turn may lead to stimulation of cellular differentiation¹⁶ or proliferation.^{17,18} Similarly in HSV-1 infection, biochemical signals generated at the host plasma membrane by ligand receptor-coupled 'second messenger' (cAMP or cGMP) could foster the expression of specific genes fundamental in the initiation and maintenance of

Fig. 5. Negative ABC-immunoperoxidase staining for anti-HSV-1 antibody in HSV-1 infected, acyclovir suppressed neuroblastoma cells after 18 hours with Insulin 1 U/ml (16 $\times$).

HSV-1 latency or induction of reactivation. Recent studies on latency show that latency-associated transcripts (LATs), although not essential for establishment or reactivation of viral latency, may play a role in the efficiency of reactivation *in vitro*.¹⁹ It has been suggested that LATs expression may be inducible by extracellular signals through a pathway involving the cAMP dependent protein kinase.²⁰ We have previously shown that C-1300 (CCL147) neuroblastoma cells respond to different cyclic nucleotide mediators by altering the intracellular levels of cAMP and cGMP.¹¹ We have also shown in an *in vivo/in vitro* murine trigeminal ganglion latency model that first order messenger mediated

modulation of ganglionic cyclic nucleotide concentrations is associated with perturbations in the viral DNA-host DNA relationship. Elevation in intracellular cGMP concentrations is associated with retardation of HSV reactivation from latency, and elevation of intracellular cAMP levels is associated with provocation of HSV reactivation.¹¹ We now report, in an effort to develop a more sensitive and accurate method for measuring the time for reactivation, the results of cDNA 'in situ' hybridisation and ABC immunoperoxidase reaction during latency and reactivation in an *in vitro* murine neuroblastoma cell latency model. We find once again, in yet another latency model and using molecular

Table IV. Effect of other compounds in viral reactivation and latency in HSV-1 infected neuroblastoma cells

Mediator	Reactivation time (hours)		Dose
	+ABC	+CPE	
Cyclosporine	12	12	1, 2.5, 5 mg/ml
Dexamethasone	18	24	20 μ g/ml
Cromoglycate	18	24	25 μ g/ml
Controls	18	24	

techniques, cAMP enhancing mediators are capable of altering the viral-host cell latent relationship resulting in the accelerated induction of HSV reactivation and hence recurrent infection. On the other hand, substances which elevate cGMP in neural tissue maintain the viral-host cell latent relationship, retarding reactivation. Potential investigations on the prophylactic use of these substances open new perspectives for patients with HSV recurrent infections.

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