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Mechanisms of Herpes Simplex Virus Type 1 Reactivation

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Primary cultures of trigeminal ganglion (TG) cells from herpes simplex virus type 1 (HSV-1) latently infected mice were used to study reactivation. Expression of HSV-1 latency-associated transcripts was noted in TG cell cultures. Infectious virus appeared in 75% of culture supernatants within 120 h after heat stress. Likewise, HSV-1 lytic-phase mRNA and proteins were detectable 24 h after heat stress. HSV-1 antigen first appeared in neurons after heat stress, indicating the neurons were the source of reactivation. The effect of heat stress duration on reactivation was determined. Reactivation occurred in 0, 40, or 67% of cultures after a 1-, 2-, or 3-h heat stress, respectively. However, 72-kDa heat shock protein expression was induced regardless of heat stress duration. Thus, reactivation was not a direct result of inducing the heat shock response. The capacities of several drugs to induce reactivation were also evaluated. While neither epinephrine, forskolin, nor a membrane-permeable cyclic AMP analog induced reactivation, dexamethasone did so in a dose-dependent manner. Furthermore, dexamethasone pretreatment enhanced the kinetics of heat stress-induced reactivation from TG cells. Collectively, the results indicate that TG cell cultures mimic important aspects of in vivo latency and reactivation. Therefore, this model may be useful for studying signalling pathways that lead to HSV-1 reactivation.

Despite two decades of research since sensory neurons were identified as the site of latent herpes simplex virus type 1 (HSV-1) infection (6), little is known about the mechanisms by which stress induces reactivation. While our knowledge of the HSV-1 genome and its gene products has increased tremendously, no new clinical treatments have been developed to block reactivation. Stressors such as epinephrine iontophoresis (19), cyclophosphamide and dexamethasone (Dex) administration (7), UV irradiation (20), and transient hyperthermia (35) induce reactivation in animal models. In no model, however, are the events that occur between stress and production of infectious HSV-1 known. Therefore, many unresolved questions remain. What are the reactivation-inducing signals that impinge upon latently infected neurons? Through what receptors are those signals recognized and transduced across the cell membrane? What intracellular effectors carry these signals to the nucleus? What changes in HSV-1 gene regulation constitute the event referred to as reactivation? Understanding these molecular mechanisms would greatly facilitate the rational development of reactivation-blocking drugs. Because such questions are difficult to address in vivo, an in vitro model which mimics the in vivo situation would provide an important tool for studying mechanisms of reactivation.

Culture systems in which HSV-1 reactivation occurs have been described, but most are poor models of in vivo latency and reactivation. For example, coculture of trigeminal ganglion (TG) explants with HSV-1 permissive cells is a useful assay for verifying latent infection (43) but is of limited value as a reactivation model because reactivation is induced regardless of treatment. Neonatal sensory neuron cultures are a good model for studying reactivation because HSV-1 latency is maintained until administration of a stressor (41, 48). However, this model differs substantially from adult ganglionic neurons in vivo. All of the cells in the neonatal cell cultures are neurons, and depending on the multiplicity of infection, up to 100% of these neurons can be latently infected. In contrast, 5 to 10% of cells in the ganglion are neurons, and only 5 to 10% of those become latently infected following HSV-1 ocular infection (14).

Moriya et al. (26) described a culture system which is potentially useful as an in vitro model of stress-induced reactivation. In the presence of an antiviral drug, primary cell cultures were established from latently infected mouse TGs. Latency was maintained following removal of the antiviral drug, and the investigators found that HSV-1 could be reactivated from TG cells by heat stress. Because critical points (e.g., demonstration that cytopathic effects were due to HSV-1) were not addressed in this study, the results were suggestive but not compelling.

In the present study, we confirm the results of Moriya et al. (26) and demonstrate that TG cell cultures mimic important aspects of in vivo latency and reactivation. Evidence is provided that (i) neurons remain viable in TG cell cultures, (ii) latency-associated transcription continues in culture, (iii) HSV-1 mRNA, protein, and virion synthesis are efficiently reactivated following heat stress, and (iv) latently infected neurons are the source from which HSV-1 reactivation occurs.

The capacities of cyclic AMP (cAMP), epinephrine, and Dex to induce HSV-1 reactivation from TG cell cultures were also studied. These compounds were chosen, in particular, because of their reactivation-inducing capacity in other herpesvirus models (19, 32, 41) and because of their relationship to the in vivo stress response. While epinephrine and cAMP had no effect on TG cells, Dex induced HSV-1 reactivation in a dose-dependent manner. Furthermore, Dex enhanced the kinetics of heat stress-induced reactivation. The results suggest a central role for glucocorticoid hormones in vivo in stress-induced HSV reactivation.

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TABLE 1. Oligonucleotide primers used in this study

Primer	Location in HSV-1	Sequence	Reference
G3PDH-a		GAATCTACTGGCGTCTTCACC	18
G3PDH-b		GTCATGAGCCCTTCCACGATGC	
ICP0-3′	←120987	TTCGACCAGGGCACCCTAGT	
LAT-a	120702→	GACAGCAAAAATCCCCTGAG	22
LAT-b	←120896	ACGAGGGAAAACAATAAGGG	
ICP27-a	114922→	TTTCTCCAGTGCTAGCTGAAGG	2
ICP27-b	←115204	TCAACTCGCAGACACGACTCG	
RR-a	88517→	ATGCCAGACCTGTTTTTCAA	15
RR-b	←88759	GTCTTTGAACATGACGAAGG	
TK-a	46622→	ATACCGACGATCTGCGACCT	22
TK-b	←46731	TTATTGCCGTCATAGCGCGG	

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MATERIALS AND METHODS

Animals and cells. Female 25- to 34-g ICR mice (Harlan-Sprague Dawley, Indianapolis, Ind.) were used in these experiments. The CV-1 African green monkey kidney cell line was obtained from the American Type Culture Collection (Rockville, Md.).

Infection of mice. Bilateral ocular infection of ICR mice (Harlan-Sprague Dawley) was performed as follows. Corneas were scarified with a 25-g needle, and tear film was blotted from the eyes with tissue. Mice were infected by placing 3 μl of medium containing 10^5 PFU of HSV-1 (McKrae strain) per ml on each eye. At the time of infection, mice were passively immunized with 0.1 ml of rabbit antiserum to HSV-1 by intraperitoneal injection to enhance survival. To verify primary infection, mouse eyes were swabbed 2 days postinoculation, and swabs were transferred to CV-1 monolayer cultures, which were observed for HSV-1-induced cytopathic effects.

Establishment of TG cell cultures. TG cell cultures were prepared according to a modified version of the protocol described by Moriya et al. (26). TG cells were cultured in minimum essential medium containing 10% fetal bovine serum (Gibco BRL, Gaithersburg, Md.), antibiotic-antimycotic solution (Sigma Chemical Co., St. Louis, Mo.), and 10 ng of nerve growth factor 2.5s (Collaborative Biomedical Products, Bedford, Mass.) per ml (TG medium). TGs were aseptically removed from latently infected mice (greater than 28 days postinoculation) and placed in TG medium on ice. TGs were pooled in 1.5 ml of calcium- and magnesium-free Hank's balanced saline solution containing collagenase type XI (1 mg/ml; Sigma) and collagenase type IV (1 mg/ml; Sigma) and incubated at 37°C for 75 to 90 min. To facilitate dissociation, ganglia were triturated every 25 min with a 1-ml serological pipette.

Next, 8.5 ml of TG medium was added to the dissociated cells, which were then pelleted by centrifugation (4°C, 5 min, 200 × *g*), and the collagenase-containing supernatant was discarded. The cells were rinsed twice more with TG medium and resuspended in 50 ml of TG medium containing 5 μg/ml of (E)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU; Sigma) per ml. The cells were distributed into two 24-well culture plates (1 ml of cell suspension per well) which had been thin coated with rat tail collagen type I (50 μg/ml; Collaborative Biomedical Products) and recombinant mouse laminin (2.5 μg/ml; Collaborative Biomedical Products) according to the vendor's directions. To promote cell adherence, culture plates were centrifuged for 3 min at 200 × *g*.

Cultures were incubated in a 37°C tissue culture incubator (5% CO_2, 95% humidity). Two days after culture establishment, 0.5 ml of TG medium was added to each culture well. Five days after culture establishment, BVDU was removed by replacing the culture medium. Cultures were incubated for 14 to 16 days before initiation of reactivation experiments. Culture medium was partially replaced every 5 to 7 days.

Heat stress and drug treatments. TG cells were heat stressed by placing culture plates in a 43°C tissue culture incubator (5% CO_2, 95% humidity) for 3 h.

In drug experiments, cultures were incubated with either forskolin for 1 h, chlorophenylthio (CPT)-cAMP for 1.5 h, epinephrine for 1.5 h, or Dex for the duration of the experiment. Five days after drug treatment, cultures were heat stressed to verify the presence of latent HSV-1.

The effect of forskolin on intracellular cAMP concentrations was determined 30 min after addition of forskolin to TG cells, using a cAMP enzyme-linked immunosorbent assay (PerSeptive Diagnostics, Cambridge, Mass.).

Monitoring of TG cultures for HSV-1 reactivation. Infectious HSV-1 was detected in cultures by transferring 100 μl of culture medium to CV-1 cell monolayers in 96-well plates and monitoring CV-1 cells for the appearance of cytopathic effect. Each TG culture well was sampled for infectious virus at 4, 8, and 12 days after plating and immediately before administration of a potential reactivation-inducing stimulus. Thereafter, wells were sampled every 24 h for 5 days to record the appearance of infectious virus.

Reverse transcription-PCR (RT-PCR). Total RNA was extracted directly from culture wells with Ultraspec RNA isolation reagent (Biotecx Inc., Houston, Tex.) according to the manufacturer's instructions. First-strand cDNA synthesis was performed on equivalent amounts of RNA from each sample, using an oligo$(dT)_{15}$ primer and avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, Wis.). Because the major species of latency-associated transcripts (LAT) is not polyadenylated (10), reverse transcription of LAT was achieved by using a LAT-specific primer, ICP0-3′. PCR was performed on equivalent amounts of cDNA; each reaction mixture contained 1× *Taq* buffer, 0.25 μM each PCR primer, 100 μM each deoxynucleoside triphosphate, and 2.5 U of *Taq* polymerase (Promega), and each reaction was performed in an MJ Research (Watertown, Mass.) thermal cycler with 35 cycles of 94°C (1 min 15 s) → 57°C (1 min 15 s) → 72°C (30 s). Oligonucleotide primers were obtained from LSU Medical Center Core Laboratories (New Orleans, La.) and are listed in Table 1. Densitometry of ethidium bromide-stained agarose gels was performed with an EagleEye II still video system (Stratagene, La Jolla, Calif.).

Immunocytochemical staining. Cell cultures were fixed with 10% formalin and treated for 10 min with 0.1 N sodium azide containing 0.3% H_2O_2 to inactivate endogenous peroxidases. HSV-1 antigen was identified by using a rabbit anti-HSV-1–horseradish peroxidase conjugate (DAKO Corporation, Carpinteria, Calif.). Neurons were labeled with rabbit anti-neuron-specific enolase (Zymed Laboratories Inc., San Francisco, Calif.), and the primary antibody was detected with a Vectastain ABC kit (Vector Laboratories Inc., Burlingame, Calif.). Antibody labeling was visualized with the substrate aminoethylcarbazole (Vector Laboratories).

Western blot (immunoblot) analysis. Following lysis of cultured cells in nondenaturing gel loading buffer, debris was pelleted by centrifugation. Aliquots of clarified supernatant were loaded on sodium dodecyl sulfate–7.5% polyacrylamide minigels. Separated proteins were electroblotted onto nitrocellulose (Bioblot NC; Corning Costar, Cambridge, Mass.) and fixed in methanol-Tris buffer. A mouse anti-72/73-kDa heat shock protein monoclonal antibody (Stressgen Biotechnologies Corp., Victoria, British Columbia, Canada) was used to detect 72-kDa heat shock protein HSP-72, which was visualized with the Western Light chemiluminescence detection system (Tropix Inc., Bedford, Mass.).

RESULTS

Neurons in TG cell cultures. Nerve fibers are the conduit by which HSV-1 enters the ganglion during acute infection, and neurons are the only known reservoir of viral DNA during latency (24, 30, 45). Therefore, we sought to verify that neurons survive ganglion dissociation and remain viable in TG cell cultures. Neurons were identified by light microscopy (Fig. 1A) on the basis of their large size and distinct nucleus. Immunocytochemical staining for neuron-specific enolase showed that these cells were in fact neurons (Fig. 1B). The number of neuron-specific enolase-positive cells per culture well was determined to be 410 ± 48 (mean ± standard deviation; $n = 5$ wells). Because each well received 0.4 ganglion equivalent of cells, approximately 1,000 neurons per ganglion survived dissociation and became adherent in culture.

Latency-associated transcription. During latent infection of the TG in vivo, HSV-1 LATs are detected exclusively in latently infected neurons by in situ hybridization (42, 44). Because TGs were taken from latently infected mice (greater than 28 days postinoculation), RT-PCR analysis was performed to determine if LAT expression persisted in TG cells following dissociation. LATs were detected in 10 of 10 cultures 14 days after establishment (Fig. 2). Presumably, latently infected neurons which survived dissociation are the source of LAT expression in TG cell cultures. However, in situ hybridization will be required to formally address this point.

Effect of BVDU in establishment of TG cell cultures. TG cells were initially cultured in the presence of an antiviral drug, BVDU (9), to prevent reactivation following ganglion dissociation. Contrary to previous results (26), reactivation did not always occur from TG cells established in the absence of antiviral drug. Reactivation occurred in 57% (16 of 28) of cultures established in the absence of BVDU. The lack of reactivation from the other TG cells was not due to an absence of

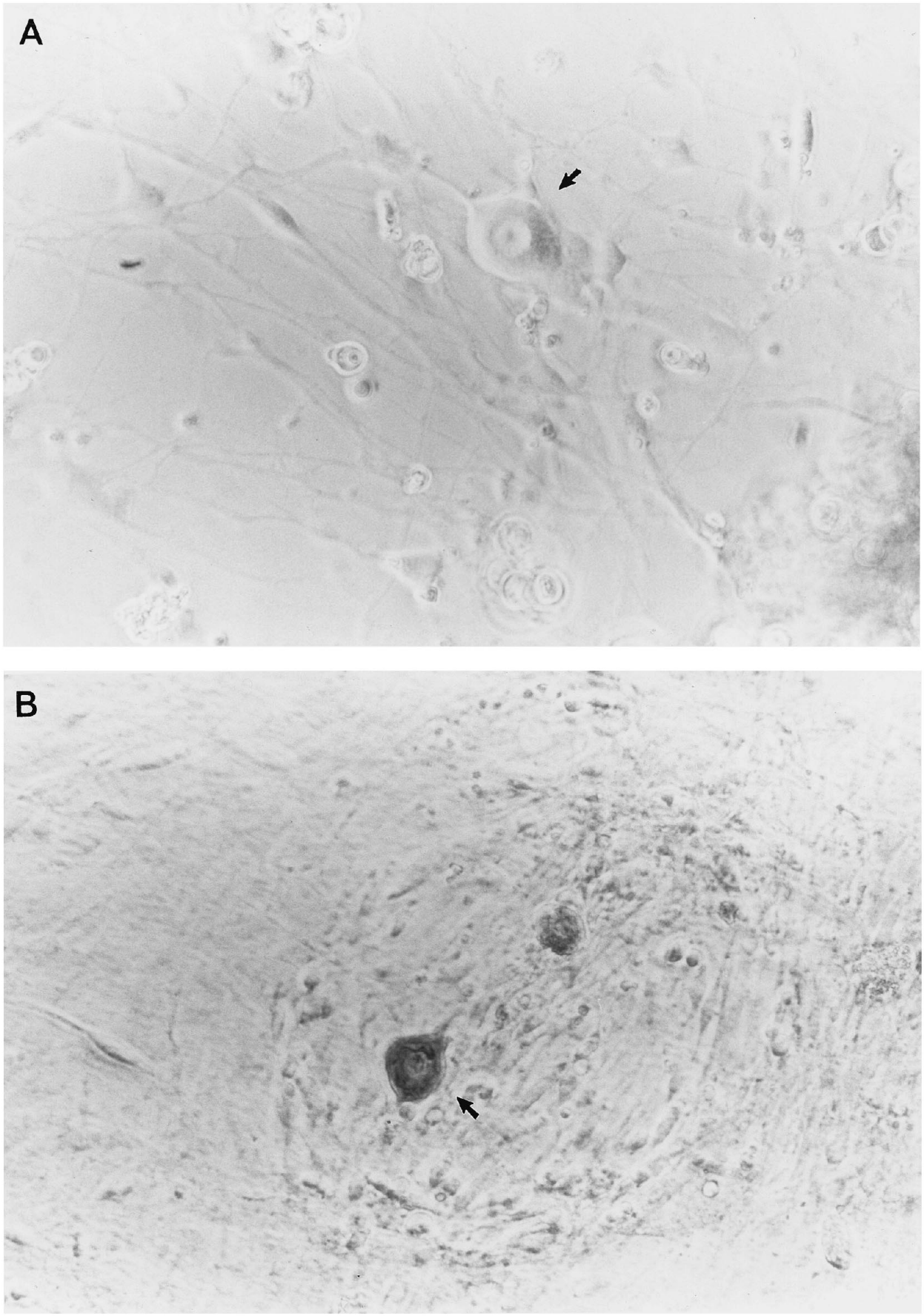

FIG. 1. Neurons in TG cell cultures. (A) Phase-contrast appearance of a neuron in culture (indicated by arrow) (magnification, ×40). Note regenerated neurite extending from left side of neuron. (B) Immunocytochemical staining reveals the presence of neuron-specific enolase (indicated by arrow) (magnification, ×20).

FIG. 2. Agarose gel analysis of LAT RT-PCR products (195 bp) amplified from cell cultures 1 to 10 (lanes 1 to 10). Each RNA sample was derived from the cells in a single culture well. No template (−) and HSV-1 DNA (+) served as negative and positive controls, respectively, in the PCR assay. ϕX174/*Hae*III markers are shown on the far left.

latent HSV-1; reactivation later occurred from 10 of the 12 remaining cultures following heat stress.

To determine the drug concentration which would consistently prevent HSV-1 reactivation, cultures were established in medium containing half-log dilutions of BVDU ranging from 0.5 to 50 μg/ml. Reactivation did not occur in cultures containing 1.6 μg or more of BVDU per ml (n = 4 per group).

RT-PCR analysis of HSV-1 gene expression was performed to verify the efficacy of 5 μg of BVDU per ml in preventing HSV-1 reactivation during culture establishment. Infected cell polypeptide 27 (ICP27) mRNA was present 24 h after culture establishment but was not detectable by 120 h (Fig. 3A). Therefore, HSV-1 immediate-early gene expression is only transiently upregulated in BVDU-treated cultures. In contrast, expression of HSV-1 ICP27 mRNA increased steadily after culture establishment in the absence of BVDU (Fig. 3A). While LAT expression was detected at all times in BVDU-treated cultures, LAT expression was downregulated in 3 of 4 untreated cultures during the 24- to 48-h time period when reactivation was first occurring (Fig. 3A). PCR of TG cell culture DNA verified that 5 μg of BVDU per ml effectively prevents HSV-1 DNA replication following culture establishment. Whereas amplification of HSV-1 DNA in untreated cultures was evident by 48 and 120 h after culture establishment, HSV-1 DNA levels remained constant in BVDU-treated cultures (Fig. 3B).

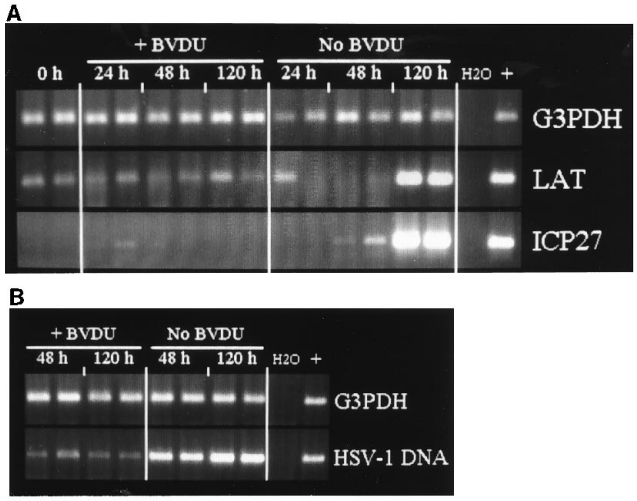

FIG. 3. BVDU treatment prevents HSV-1 reactivation. (A) Effect of BVDU on HSV-1 immediate-early gene expression, determined by RT-PCR analysis of RNA from duplicate samples of TG cells taken 0, 24, 48, and 120 h after establishment of TG cell cultures. The cells were all derived from the same pool of dissociated TGs. + BVDU, cultures that were BVDU treated; No BVDU, cultures that were untreated. RT-PCR was used to detect G3PDH, LAT, and ICP27 mRNAs. Each RNA sample was derived from the pooled cells of three culture wells. (B) Effect of BVDU on HSV-1 DNA replication. PCR was performed on equivalent amounts of DNA from TG cell cultures 48 and 120 h after culture establishment to detect the cellular G3PDH gene and HSV-1 DNA. HSV-1 ribonucleotide reductase primers were used to detect HSV-1 DNA. Each DNA sample was derived from the pooled cells of three culture wells. No template (H2O) and HSV-1 DNA (+) served as negative and positive controls, respectively, in each PCR assay.

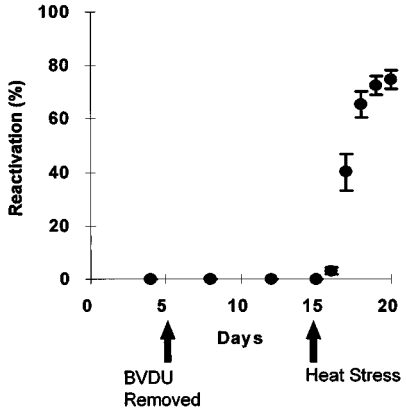

FIG. 4. Kinetics of heat stress-induced HSV-1 reactivation. Heat stress was administered 15 days after culture establishment. Reactivation is defined as the appearance of infectious virus in culture supernatants. Datum points represent the mean frequencies of reactivation (± standard errors of the means) observed in 11 experiments.

Heat stress-induced HSV-1 reactivation. Reactivation did not occur in any cultures following removal of BVDU from cells (5 days after culture establishment). However, heat stress rapidly induced HSV-1 reactivation (Fig. 4) from 74.7% ± 3.5% of TG cell cultures (n = 257 wells). This finding is consistent with the results of Moriya et al. (26). Of the cultures which eventually reactivated, HSV-1 was detectable in 88% of culture supernatants within 72 h after heat stress. In contrast, reactivation occurred from 0% of nonstressed controls (n = 52) over a 20-day culture period.

Heat stress induction of HSV-1 gene expression. In most experiments, reactivation was defined as the appearance of infectious virus in culture supernatants which caused cytopathic effect when virus was transferred to CV-1 cell monolayers. To confirm that heat stress triggered HSV-1 reactivation from TG cells, heat stress induction of ICP27 mRNA expression was verified by RT-PCR (Fig. 5). While ICP27 mRNA was not detectable before or 12 h after the initiation of heat stress, ICP27 mRNA was evident in TG cell cultures by 24 and 48 h after heat stress. In contrast, glyceraldehyde-3-phosphodehydrogenase (G3PDH) mRNA was uniformly present in all RNA samples. After normalization for G3PDH, densitometry indicated that the amount of ICP27 PCR product amplified from TG cell RNA samples increased 18-fold between 24 and 48 h after heat stress.

Immunocytochemical staining was used to determine the kinetics of HSV-1 protein induction following heat stress. Cultured cells did not express detectable HSV-1 protein prior to

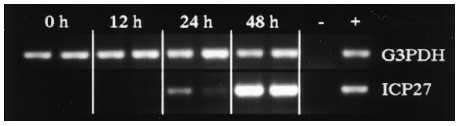

FIG. 5. Kinetics of ICP27 mRNA induction following heat stress. RT-PCR was used to detect G3PDH and HSV-1 ICP27 mRNAs in duplicate samples of TG cells prior to heat stress (0 h) and at 12, 24, and 48 h after initiation of heat stress. No template (−) and HSV-1 DNA (+) served as negative and positive controls, respectively, in each PCR assay. The samples shown are a subset of VEH PCR samples shown in Fig. 10.

heat stress. However, HSV-1 proteins were detected in TG cell cultures 22, 48, and 72 h after heat stress (Fig. 6A to C). 1HSV-1 antigen expression was limited to large, neuron-like cells 22 h after heat stress (Fig. 6D). Large foci of HSV-1 antigen-positive cells were seen 48 and 72 h after heat stress. On the basis of the discrete, circular pattern of staining, each HSV-1 antigen-positive cell cluster appeared to be the result of focal spread from a single reactivation event. The average numbers of reactivation events detected per culture well were 0.5 (n = 8 wells), 1.5 (n = 4 wells), and 1.75 (n = 4 wells) at 22, 48, and 72 h after heat stress, respectively. Therefore, it appears that only a fraction of neurons in which reactivation occurred contained detectable HSV-1 protein by 22 h after heat stress.

Effect of heat stress duration on HSV-1 reactivation. The effect of heat stress duration on TG cell cultures was studied to better characterize the mechanism by which heat stress induces HSV-1 reactivation. TG cells were heat stressed for 1, 2, and 3 h at 43°C and observed for HSV-1 reactivation (Fig. 7, 1st heat stress). Consistent with other experiments, HSV-1 reactivated from 67% (8 of 12) of TG cell cultures heat stressed for 3 h. However, 2 h of heat stress induced reactivation from only 40% of cultures (n = 28), and 0% of cultures (n = 28) reactivated following 1 h at 43°C. To verify the presence of reactivateable virus, the cultures previously treated for 1, 2, or 3 h were heat stressed a second time for 3 h (Fig. 7, 2nd heat stress). After a 3-h heat stress, HSV-1 reactivated from 61% of cultures previously heat stressed for 1 h. Likewise, the frequency of reactivation increased from 40 to 61% in those cultures previously heat stressed for 2 h. Interestingly, the frequency of reactivation increased from 67 to 92% in cultures that had been heat stressed for 3 h the first time. Twelve TG cell cultures that were not heat treated did not reactivate over the 25-day course of this experiment.

The results of others suggest that HSV-1 reactivation may be triggered by induction of the cellular heat shock response (26, 35). Expression of HSP-72 (often used as a marker of heat shock) was rapidly induced in TG cells following transfer to a 43°C incubator. While HSP-72 was not detected before or 1 h after transfer to 43°C, HSP-72 expression was evident 2 and 3 h after initiation of heat stress (Fig. 8A).

To account for the observed lack of reactivation after 1 h of heat stress, we thought that perhaps 1 h in a 43°C incubator was insufficient to induce a heat shock response. Therefore, the effect of heat stress duration on HSP-72 induction was determined as follows. Cultures were simultaneously placed at 43°C. One culture was returned to 37°C after 1 h, one culture was returned to 37°C after 2 h, and the other culture remained at 43°C for the entire 3 h. Protein samples were harvested from all cultures 3 h after initiation of heat stress, and levels of HSP-72 expression were compared by Western blot analysis. HSP-72 expression was induced to high levels in TG cells regardless of heat stress duration (Fig. 8B). Therefore, HSV-1 reactivation in TG cells is not a direct result of triggering a heat shock response.

Analysis of potential reactivation-inducing drugs. It has been reported that LAT facilitates reactivation (16), and cAMP has been proposed to contribute to reactivation by upregulating LAT transcription (21, 29). Smith et al. (41) found that either 500 μM CPT-cAMP or 50 μM forskolin (i.e., an activator of adenylyl cyclase) induced reactivation from neonatal neuron cultures. Likewise, epinephrine and glucocorticoids (e.g., Dex) have been used in animal models to induce reactivation of latent herpesvirus (19, 32, 46). Therefore, the reactivation-inducing potentials of cAMP, epinephrine, and Dex were studied in TG cell cultures to compare this system with other reactivation models.

While 0.5 μM forskolin did not elevate cAMP above basal levels (1.4 ± 1.7 pmol of cAMP per mg of protein [mean ± standard deviation]), cAMP levels were nearly 10-fold higher in TG cells 30 min after treatment with 50 μM forskolin (12 ± 2.6 pmol of cAMP per mg of protein; mean ± standard deviation). However, neither 50 μM forskolin nor 500 μM CPT-cAMP induced reactivation in TG cell cultures (Fig. 9). Epinephrine also had no effect in TG cell cultures. However, Dex induced reactivation in TG cells in a dose-dependent manner (Fig. 9). Following the 5-day period allowed for drug-induced reactivation, cultures were heat stressed to verify the presence of latent virus. Heat stress confirmed that in each treatment group, the majority of TG cell cultures contained reactivatable HSV-1 (i.e., 60 to 90% of cultures reactivated within 5 days after heat stress). As a control, some of the drug-treated cultures were not secondarily heat stressed; reactivation did not occur in these cultures.

Following heat stress, HSV-1 was detectable in culture supernatants of Dex-pretreated cells earlier than untreated controls. Likewise, comparison with results of previous experiments indicated that HSV-1 appeared significantly earlier in Dex-pretreated cultures (Table 2). Because of the known role of the glucocorticoid receptor as a transcriptional regulator (13, 31, 36), we postulated that Dex facilitated the induction of HSV-1 lytic-phase mRNA transcription following heat stress.

RT-PCR analysis of Dex- and vehicle-treated TG cells substantiated this hypothesis (Fig. 10). While viral mRNA was barely detectable in one of two vehicle-treated cultures 12 h after heat stress, viral transcripts for ICP27, thymidine kinase (TK), and ribonucleotide reductase (RR) were evident in two of two Dex-pretreated cultures 12 h after heat stress. Furthermore, detection of ICP27, TK, and RR mRNAs indicated that reactivation occurred in one of two DEX pretreated cultures that was not heat stressed. Because Dex was added just 15 h prior to extraction of RNA, the brief interval between stimulus and response suggests that Dex acted directly through neuronal glucocorticoid receptors to induce reactivation.

Despite the presence of ICP27, TK, and RR mRNAs, LAT was not detected in the Dex-treated culture that reactivated without being heat stressed. Interestingly, LAT was also not detected in vehicle-treated cultures 24 h after heat stress, despite the presence of lytic phase transcripts. On the basis of previous immunocytochemical staining experiments, RT-PCR was presumably detecting viral transcription in just one or two reactivation-positive cells. Therefore, the absence of LAT in these samples suggests a coordinated downregulation of LAT transcription during reactivation. Such an event has been proposed by Rock et al. (32) and could be mediated by ICP4, given the capacity of this HSV-1 regulatory protein to repress LAT transcription (1).

DISCUSSION

Establishment of TG cell cultures. The explantation of TG cells provides a reactivation-inducing stimulus to latently infected neurons during culture establishment. This is demonstrated by the upregulation of ICP27 mRNA expression in BVDU-treated and untreated cultures 24 to 48 h after culture establishment. Because the TG cells are plated as a single-cell suspension, however, the antiviral drug is effective immediately. Therefore, despite the induction of immediate-early gene expression, BVDU blocks HSV-1 DNA replication and prevents infectious virus production during culture establishment. The absence of ICP27 mRNA in BVDU-treated cells by 120 h after culture establishment indicates that the reactivation-inducing stimulus associated with explantation is tran-

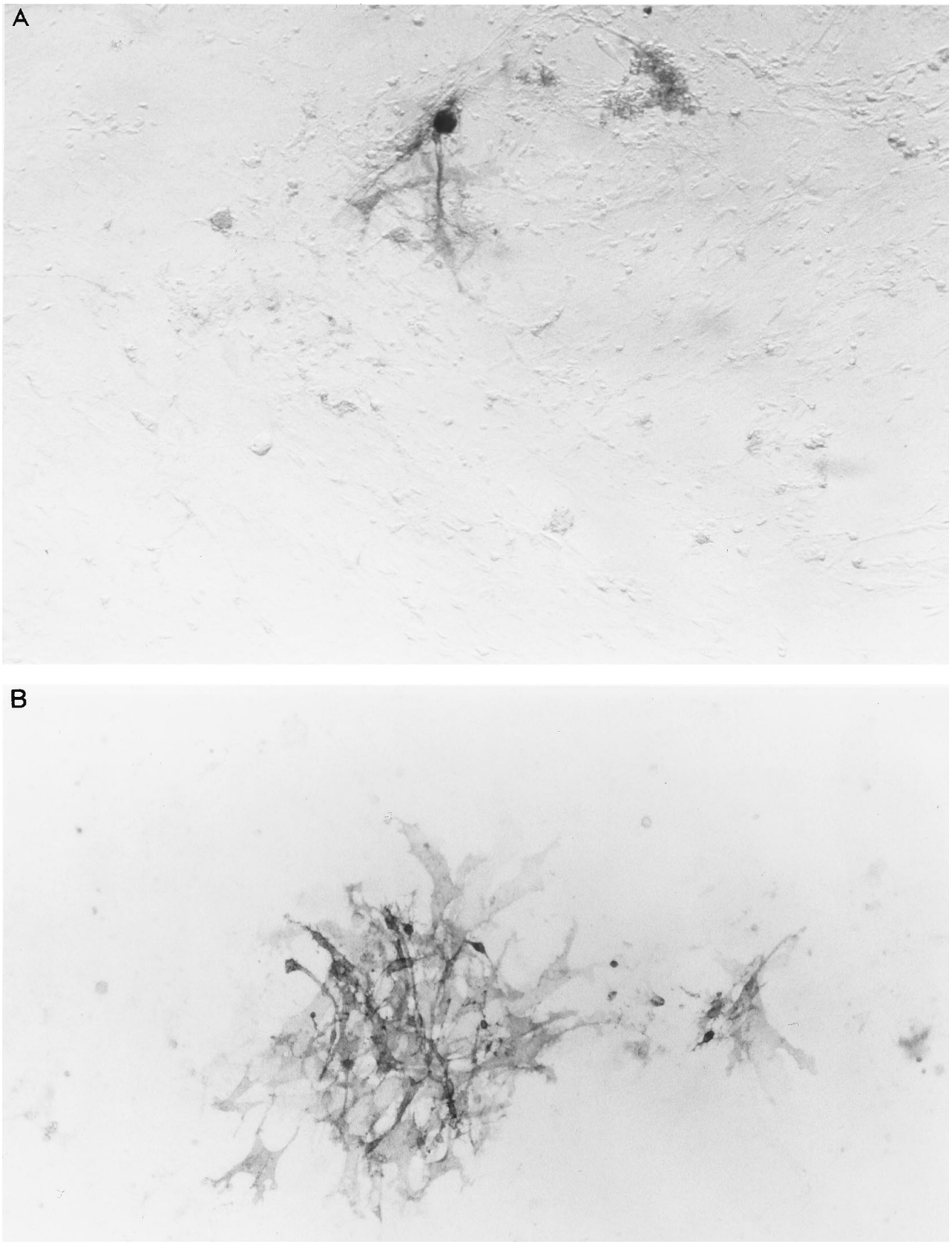

FIG. 6. Kinetics of HSV-1 protein induction following heat stress. TG cell cultures stained for HSV-1 antigen 22 h (A), 48 h (B), and 72 h (C) after initiation of heat stress (magnification, ×10). (D) A neuron stained for HSV-1 antigen 22 h after heat stress (magnification, ×40). All HSV-1 antigen-positive cells identified at this time point had a large nucleus and a prominent nucleolus, characteristic of neurons.

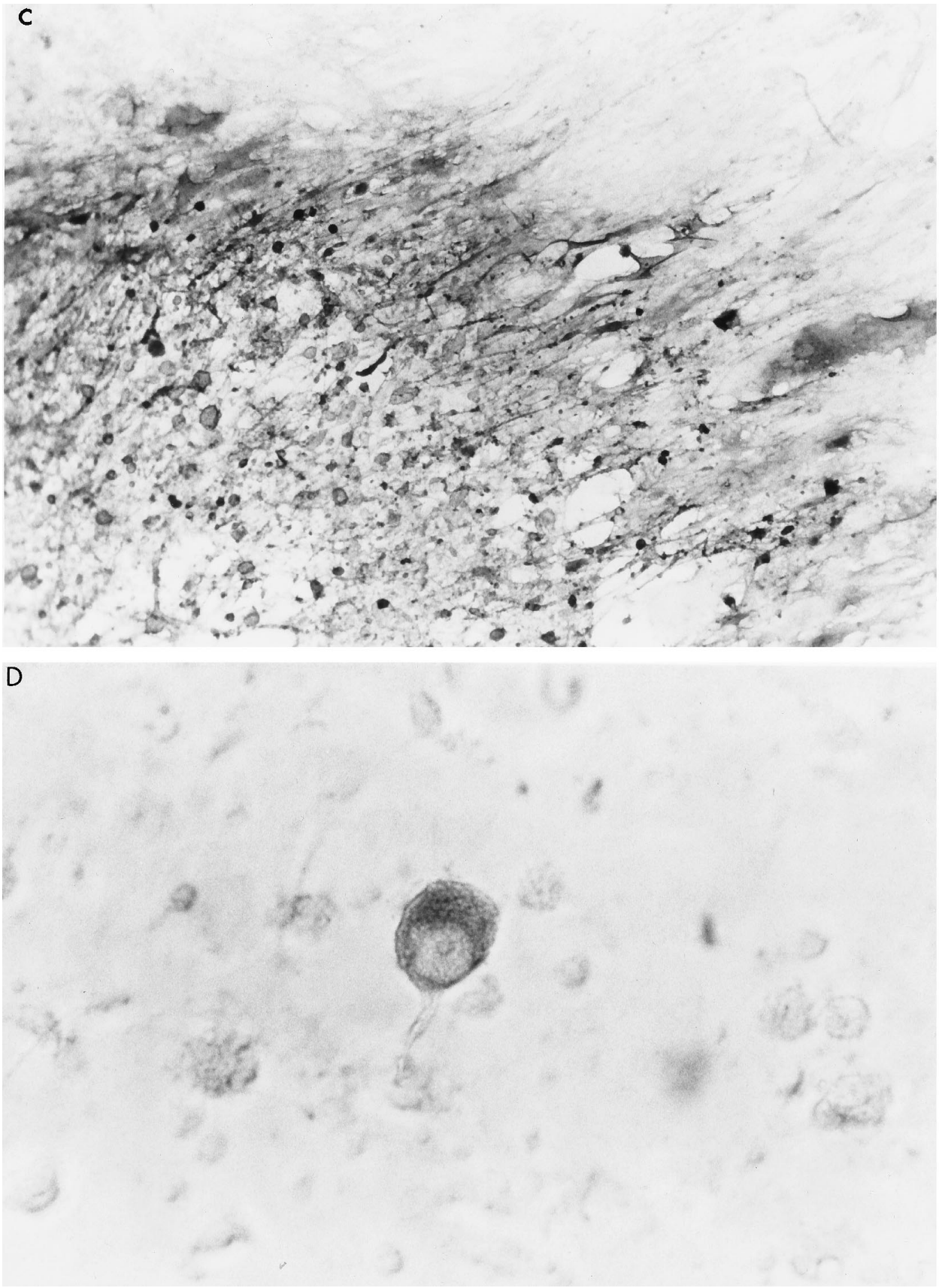

FIG. 6—*Continued.*

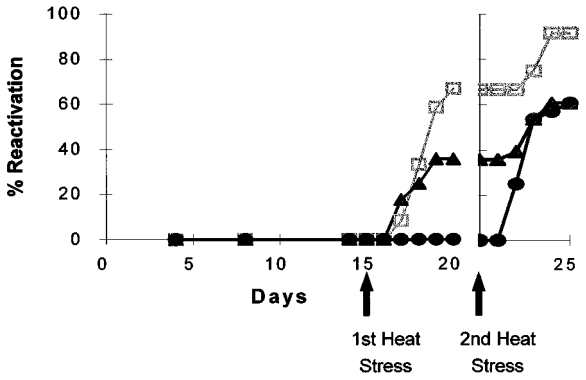

FIG. 7. Effect of heat stress duration on HSV-1 reactivation. Shown are percent reactivation following a 1-h (●), 2-h (▲), or 3-h (□) heat stress administered after 15 days in culture and percent reactivation following a second 3-h heat stress after 20 days in culture.

sient. Consistent with this hypothesis, throughout these studies reactivation was not observed following BVDU removal from TG cell cultures.

Reactivation from TG cells. In animal models, HSV-1 mRNA and antigen production in the ganglion is transient during stress-induced reactivation (2, 35). In light of evidence that T lymphocytes and cytokine expression persist in the TG after latency is established (4, 11, 40), the brevity of HSV-1 reactivation in vivo presumably reflects an efficient host immune response. In contrast, induction of reactivation in TG cell cultures is irreversible because lytic replication continues until all cells are destroyed.

Although HSV-1 remained largely undetected in culture medium until 48 h after heat stress, replication had initiated in neurons by 24 h after heat stress. The frequency of detecting HSV-1 in culture supernatants increased from 3 to 40% between 24 and 48 h after heat stress. HSV-1 antigen expression was restricted to neurons 22 h after heat stress but spread to plaque-like clusters of 50 to 500 cells by 48 h after heat stress. Likewise, RT-PCR indicated that the amount of ICP27 mRNA in TG cells increased substantially between 24 and 48 h after heat stress. Therefore, HSV-1 was not usually detected in culture medium until released from secondarily infected cells.

None of the treatments were 100% effective in inducing reactivation. In testing the effects of heat stress duration, 8 of 12 cultures reactivated after a first 3-h heat stress interval, but 3 of the remaining 4 cultures reactivated after a second 3-h heat stress. Likewise, while reactivation occurred in 16 of 28 cultures established in the absence of BVDU, 10 of the remaining 12 cultures reactivated following a secondary heat stress. Combined with the detection of LATs in 10 of 10 cultures, the results suggest that when cells are stressed, reactivation is initiated in only a fraction of latently infected neurons.

Mechanisms of HSV-1 reactivation. (i) Heat stress. While

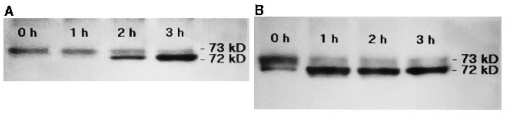

FIG. 8. Heat shock protein induction. (A) Kinetics of HSP-72 induction in TG cells 1, 2, and 3 h after transfer to a 43°C incubator (20 μg of protein per lane). (B) Effect of heat stress duration (1, 2, or 3 h) on heat shock protein induction by 3 h after transfer to 43°C incubator (35 μg of protein per lane).

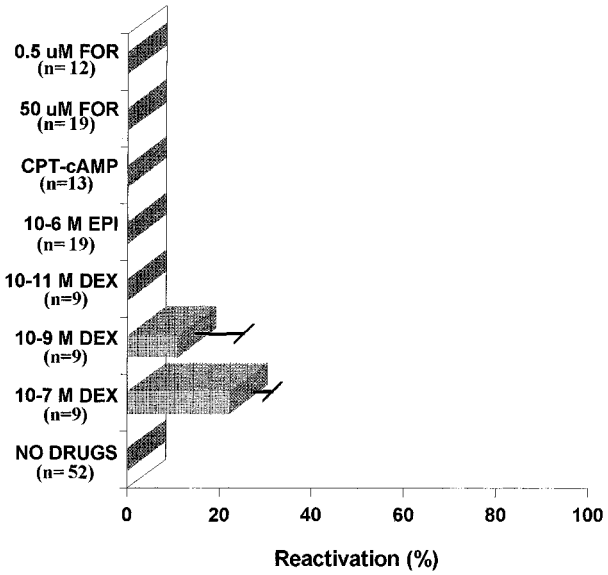

FIG. 9. Effects of drugs on reactivation. Frequency of drug-induced reactivation (± standard error of the mean) in TG cells treated with forskolin (FOR), CPT-cAMP, epinephrine (EPI), and Dex. Cultures were treated with drugs after 15 days in culture and observed for 120 h for reactivation.

HSP-72 expression was strongly induced in TG cells by 1 h of heat stress, reactivation occurred in 0 of 28 TG cell cultures incubated at 43°C for 1 h. Therefore, reactivation of HSV-1 gene expression is not a direct consequence of activating the signalling pathways which upregulate HSP-72 expression. However, heat stress induces HSP-72 expression more readily in glial cells than in neurons (34). Therefore, we cannot formally rule out the possibility that 1 h at 43°C induced HSP-72 expression in glial cells but was insufficient to activate the heat shock response in latently infected neurons.

(ii) Drugs. Stress stimulates epinephrine release from the sympathetic nervous system and glucocorticoid release from the adrenal glands. Given their roles as stress mediators, we hypothesized that these compounds serve as reactivation-inducing signals which act directly on latently infected neurons (i.e., ligand binding to neuronal adrenergic receptors or glucocorticoid receptors). In the case of epinephrine, this view is consistent with the finding that propranolol (β-adrenoceptor

TABLE 2. Enhancement by Dex of the rate of HSV-1 appearance following heat stress

Group	% reactivation[a] at indicated time poststress (mean ± SEM)		
	24 h	48 h	72 h
10^{-7} M Dex[b]	26 ± 11[c]	50 ± 17	100 ± 0
10^{-9} M Dex[b]	0 ± 0	90 ± 10[c]	100 ± 0
10^{-11} M Dex[b]	6 ± 6	89 ± 7[c]	100 ± 0
No Dex[b]	8 ± 8	48 ± 11	88 ± 13
Expected[d]	2 ± 4	46 ± 13	84 ± 6

[a] Considering only those cultures that eventually reactivated, results represent percentages of reactivated wells at each time point after heat stress.
[b] $n = 4$ experiments; four to five wells per experiment.
[c] $P < 0.05$ (determined by analysis of variance and Tukey's post comparing Dex treatment groups with expected).
[d] Pooled results of all other heat stress experiments ($n = 7$ experiments; 20 to 36 wells per experiment).

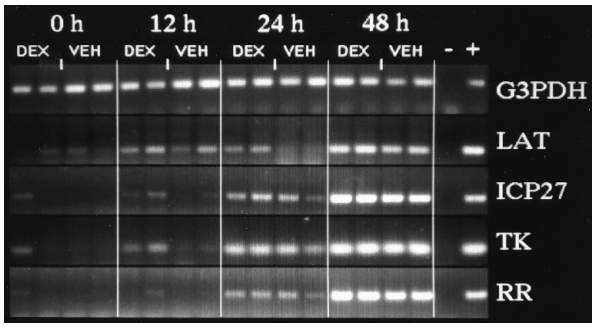

FIG. 10. Dex pretreatment enhances the rate of appearance of HSV-1 lytic-phase mRNA following heat stress. Shown is RT-PCR analysis of RNA from duplicate samples of TG cells prior to heat stress (0 h) and at 12, 24, and 48 h after initiation of heat stress. Dex, cultures which were treated with 10^{-7} M Dex 15 h before heat stress; VEH, cultures which were vehicle treated 15 h before heat stress. RT-PCR was used to detect G3PDH, LAT, ICP27, HSV-1 TK, and HSV-1 RR mRNAs. Each RNA sample was derived from the pooled cells of two culture wells. No template (−) and HSV-1 DNA (+) served as negative and positive controls, respectively, in each PCR assay.

antagonist) blocks reactivation from mice following hyperthermic stress (12).

Epinephrine did not induce reactivation of latent HSV-1 in TG cell cultures. Therefore, ocular iontophoresis of epinephrine may not act directly on neurons to induce HSV-1 reactivation in vivo. Rather, epinephrine iontophoresis may stimulate corneal cells, which in turn transmit a reactivation signal to the nerve endings of the ganglionic neurons. Denervation of the cornea has been shown to block epinephrine-induced reactivation of HSV-1 (33). Therefore, we cannot rule out the possibility that epinephrine fails to induce reactivation in vitro because the regenerated neurites of the neurons lack an essential component that is present in vivo. In contrast, because Dex and cAMP act on sites in the neuronal cell body, these results are more pertinent to the in vivo situation. Therefore, the inability of forskolin and CPT-cAMP to induce reactivation suggests that cAMP elevation is not a critical determinant of HSV-1 reactivation in ganglionic neurons.

Dex treatment induced HSV-1 reactivation in a fraction of TG cell cultures. Underwood and Weed (46) likewise found that glucocorticoids (i.e., prednisone) induced reactivation from a low but significant fraction (17%) of HSV-1 latently infected mice. Given these observations, one could hypothesize that while glucocorticoid receptors facilitate HSV-1 reactivation, other unidentified signals are necessary for efficient induction. Therefore, Dex may serve as more than an immunosuppressant when used in concert with cyclophosphamide and UV radiation to induce HSV-1 reactivation in animal models (7, 39). In contrast, Dex induces rapid reactivation of bovine herpesvirus 1 in nearly 100% of latently infected animals (8, 28, 32, 38, 47) and induces efficient reactivation of pseudorabies-virus in latently infected swine (25).

There is clinical precedence for herpes simplex and herpes zoster reactivation following systemic corticosteroid treatment (3, 5, 17, 23, 27, 37). The results of the present study suggest that glucocorticoids may contribute to disseminated herpetic disease not only by suppressing immune function but also by providing a reactivation trigger to latently infected ganglion.

Application of the in vitro reactivation model. Because dissociated TG cell cultures mimic key aspects of in vivo latency and reactivation, this paradigm offers a valuable tool for studying aspects of reactivation that are not readily addressed in vivo. For example, antisense inhibition could be used in cultures to identify viral and cellular genes that modulate reactivation. Likewise, reagents whose use is prohibitively expensive in vivo (e.g., recombinant proteins and monoclonal antibodies) can be used cost-effectively in vitro. Because the method used to detect reactivation does not require harvesting the cells, TG cell cultures are available for secondary manipulations after testing of potential reactivation-inducing stimuli. In contrast, detection of reactivation in vivo often requires sacrificing the host. Finally, TG cell cultures provide a means to identify treatments which act directly on ganglion cells to induce or block reactivation.

Inevitably with cell culture studies, one is left with the question, "How does this pertain to the in vivo situation?" At least with primary cell cultures, only the milieu is changed. Presumably in TG cultures, reactivation is induced from the same cells in which reactivation would occur in vivo. Therefore, we believe that this model can be used to identify molecular events associated with reactivation and generate hypotheses whose relevance can be determined in vivo. In this context, while the results of this study suggest a central role for glucocorticoid receptors in induction of HSV reactivation, additional in vivo studies are required to substantiate this hypothesis.

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