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Induction of Reactivation of Herpes Simplex Virus in Murine Sensory Ganglia In Vivo by Cadmium

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Herpes simplex viruses maintained in a latent state in sensory neurons in mice do not reactivate spontaneously, and therefore the factors or procedures which cause the virus to reactivate serve as a clue to the mechanisms by which the virus is maintained in a latent state. We report that cadmium sulfate induces latent virus to reactivate in 75 to 100% of mice tested. The following specific findings are reported. (i) The highest frequency of induction was observed after two to four daily administrations of 100 μg of cadmium sulfate. (ii) Zinc, copper, manganese, or nickel sulfate administered in equimolar amounts under the same regimen did not induce viral reactivation; however, zinc sulfate in molar ratios 25-fold greater than those of cadmium induced viral replication in 2 of 16 ganglia tested. (iii) Administration of zinc, nickel, or manganese prior to the cadmium sulfate reduced the incidence of ganglia containing infectious virus. (iv) Administration of cadmium daily during the first week after infection and at 2-day intervals to 13 days after infection resulted in the recovery from ganglia of infectious virus in titers 10- to 100-fold higher than those obtained from animals given saline. Moreover, infectious virus was recovered as late as 11 days after infection compared with 6 days in mice administered saline. (v) Administration of cadmium immediately after infection or repeatedly after establishment of latency did not exhaust the latent virus harbored by sensory neurons, inasmuch as the fraction of ganglia of mice administered cadmium and yielding infectious virus was similar to that observed in mice treated with saline. We conclude that induction of cadmium tolerance precludes reactivation of latent virus. If the induction of metallothionein genes was the sole factor required to cause reactivation of latent virus, it would have been expected that all metals which induce metallothioneins would also induce reactivation, which was not observed. The results therefore raise the possibility that in addition to inducing the metallothionein genes, cadmium inactivates the factors which maintain the virus in latent state.

The capacity of herpes simplex viruses 1 and 2 (HSV-1 and HSV-2) to remain latent in their hosts for the life of the host remains one of the most fascinating problems in the biology of these viruses. The available data reviewed elsewhere (10, 25, 38, 39, 47) are by no means sufficiently complete to render the phenomenon understandable at a molecular level. However, the following facts are relevant.

(i) Following primary infection, HSV-1 and HSV-2 infect nerve endings and are transported to the nuclei of sensory neurons, innervating the portal of entry. In the course of infection with wild-type viruses, some of the sensory neurons become productively infected and infectious virus progeny is readily demonstrable. In some sensory neurons, the virus is maintained in a latent state. The initial burst of viral replication observed during an interval of 3 to 7 days after infection is not essential for establishment of latency, and indeed, virus mutants from which essential α genes had been deleted are capable of establishing latency even though they are unable to replicate (23).

(ii) A single set of transcripts known collectively as the latency-associated transcripts or LATs have been detected in neurons harboring latent virus (50). Their role in the maintenance of the latent state has not been confirmed inasmuch as mutants which fail to express LATs are not defective in their capacity to establish latency in sensory neurons of experimental animal systems (3, 15, 18, 20, 21, 26, 43, 46).

(iii) Explantation of sensory ganglia results in reactivation of the latent virus from a fraction of infected sensory neurons. It has been reported that viruses rendered incapable of expressing LATs by deletion of appropriate DNA sequences reactivate less efficiently than viruses capable of expressing LATs (2, 15, 26, 46, 54). This finding is contrary to expectations, inasmuch as the abundant LATs accumulating in the sensory nerve nuclei are at least in part antisense and overlap the 3′ end of the α0 gene, and it has been suggested that the synthesis of LATs interferes with the expression of the α0 gene and the expression of α0 plays a critical role in viral reactivation. LATs have not been shown unambiguously to express proteins, and the experiments designed to elucidate the role of LATs by using deletion mutants do not differentiate between a necessary role in reactivation from latent state or a reduced capacity to replicate in rodent neuronal cells after reactivation.

(iv) HSV encodes transactivating and regulatory proteins for rapid and effective expression of viral genes in cells in culture as well as in a variety of organs in natural human and in experimental animal hosts (reviewed in reference 39). The fact that latent HSV reactivates spontaneously indicates that sensory neurons are at least transiently permissive. The key issue therefore is why viral genes other than those specifying LATs are not expressed in sensory neurons after infection and prior to the establishment of latency. The experimental procedures that have been used to address this question were both to force the expression of viral transactivating factors by placing the genes encoding them under promoters expressed in sensory neurons as described elsewhere (9, 41) and to define the conditions and factors which induce viral replication in sensory neurons.

We report that cadmium induces the replication of HSV in sensory neurons harboring latent virus. The implications of our results are discussed.

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MATERIALS AND METHODS

Cells and virus. Vero cells were obtained from the American Type Culture Collection. HSV-1 strain F [HSV-1(F)] is a limited-passage HSV-1 prototype strain described elsewhere (7). The virus was grown in HEp-2 cells and stocked at a titer of 4×10^9 PFU/ml.

Infection of mice. Four- to six-week-old female CBA/j mice (Charles River) were bilaterally inoculated with 1×10^6 to 4×10^6 PFU of HSV-1(F) per eye under sodium pentobarbital anesthesia (41, 42). Each cornea was scarified with a 30-gauge hypodermic needle prior to infection with 10 μl of virus diluted in medium 199V (mixture 199 supplemented with 3% newborn calf serum).

Metallation of mice. When indicated, mice were weighed individually prior to treatment to adjust dosage to body weight. Sulfated compounds of cadmium, zinc, nickel, copper, or manganese were prepared in sterile saline at concentrations which were equimolar to that of $CdSO_4$ at 2 mg/ml, except when otherwise indicated for $ZnSO_4$ solutions. Mortality in cadmium-treated mice was observed in one experiment only, as stated in the results. Mice were metallated by subcutaneous injection of metal sulfate solution into footpads. Each mouse typically received 50 μl (equal to 100 μg of $CdSO_4$), administered daily over a period of 3 days. Within 18 to 24 h of the final injection, mice were sacrificed under sodium pentobarbital anesthesia for removal of each trigeminal ganglion into 1.2 ml of Dulbecco's modified Eagle's medium supplemented with 5% newborn calf serum.

Assays for infectious and latent virus in ganglia. Trigeminal ganglia were harvested within 33 to 38 days after inoculation except as otherwise indicated. In assays for latent virus, ganglia were surgically removed from exsanguinated mice, incubated for 5 days in 5% CO_2 at 37°C, subsequently homogenized, and plated on Vero cell monolayers (one 25-cm^2 culture per ganglion). In assays for the presence of infectious virus, surgically removed ganglia were immediately quick-frozen in a dry ice-ethanol bath and either stored at −80°C or thawed, promptly homogenized, and plated on Vero cell monolayers. In both virus assays, the Vero cells monolayers were exposed to the homogenates with constant shaking for 2 h at 37°C. The homogenates were then removed, and each monolayer was incubated at 37°C in 6 ml of Dulbecco's modified Eagle medium supplemented with 5% newborn calf serum. The cultures were examined daily for 1 week and scored as positive if any viral plaques appeared.

For quantitation of viral plaques, monolayers were overlaid with mixture 199 supplemented with 3% newborn calf serum and 0.02% pooled human gamma globulin within 4 hours after the introduction of ganglion homogenates. The monolayers were methanol fixed and stained with Giemsa stain approximately 48 h after adsorption of homogenates, and the number of plaques was determined for each culture as previously described (37).

Statistical analyses. Statistical analyses were done by Fisher's exact test. The comparisons were done between groups of mice. In one study, comparisons were done both on groups of mice and groups of ganglia, since each of the two trigeminal ganglia were harvested and assayed separately.

RESULTS

Cadmium-induced reactivation of HSV-1 in trigeminal ganglia of mice. In this series of experiments, mice harboring latent HSV-1(F) were given daily footpad injections and then tested for the presence of infectious virus at daily intervals after one to six cadmium administrations. The trigeminal ganglia were bilaterally resected from four mice at 18 to 24 h after each cadmium administration, and each ganglion was individually freeze-thawed, homogenized, and assayed for the amount of infectious virus it contained. As shown in Table 1, infectious virus was readily detected 1 day after the second, third, and fourth cadmium administration. No infectious virus was detected in mice given saline or more than four cadmium injections. It is of interest that although virus was induced to multiply in 75 to 100% of the mice tested, the low yield of infectious virus was consistent with results of other reactivation studies (13, 34, 35, 40, 44), suggesting that only a small portion of the latent virus was induced to multiply by the administration of cadmium. These results were reproducible in other experiments.

TABLE 1. Effects of administration of cadmium to mice during the latent phase of infection on the recovery of infectious virus from mouse trigeminal ganglia[a]

No. of $CdSO_4$ injections	No. of positive mice/total no. tested	No. of positive ganglia/total no. tested	Avg titer (PFU/TG)
1	0/4	0/8	None
2	3/4	6/8	35
3	4/4	5/8	11
4	3/4	4/8	17
5	0/4	0/8	None
6	0/4	0/8	None

[a] Cadmium was administered from days 32 to 37 after inoculation. Mice were sacrificed within 18 to 24 h of the final injection. The average titer for each treatment group was determined by the geometric mean of individual ganglion titers. TG, trigeminal ganglia.

Other heavy metals are less efficient than cadmium in inducing the reactivation of latent virus. The objective of this series of experiments was to determine the capacity of other heavy metals to induce latent virus to multiply in trigeminal ganglia in vivo. The metal compounds were selected on the basis of their ability to induce heat shock (28), of their ability to affect metal-dependent regulatory factors (5), or for various levels of intrinsic toxicity (57). Metal sulfate solutions were prepared in sterile saline at concentrations which were equimolar to 2 mg of $CdSO_4$ per ml (8.83 mmol). As described above, 50 μl of solution was injected daily into the footpads of 10 mice in each experimental or control group. Within 18 to 24 h after the third injection, the trigeminal ganglia were harvested and separately assayed for infectious virus. As shown in Table 2, for the equal concentration of metals tested, infectious virus was recovered almost exclusively from cadmium-treated mice (geometric mean of viral titers = 55 PFU per ganglion). The exception was one positive ganglion (33 PFU) from a mouse treated with a nickel compound. In the cadmium-treated group, infectious virus was obtained from 85% of the ganglia and 100% of the mice. All ganglia from latently infected mice which received daily injections of zinc, copper, manganese, or saline tested negative for infectious virus.

Zinc is less effective than cadmium for inducing reactivation. Of the metals tested in the preceding experiment, only zinc is in the same transition group as cadmium and would therefore be more similar to the electrochemical properties of cadmium than the other metals tested. Zinc along with cadmium induces the metallothionein promoter. The objective of this series of experiments was to determine whether higher concentrations of zinc might be effective in inducing latent virus to multiply in trigeminal ganglia in vivo.

Sterile solutions of zinc sulfate were administered daily to

TABLE 2. Effects of administration of cadmium or other heavy metals to mice during the latent phase of infection on the incidence of recovery of infectious virus from trigeminal ganglia[a]

Administration (three injections)	No. of positive mice/total no. tested	No. of positive ganglia/total no. tested	Avg titer (PFU/TG)
Equimolar concn			
Saline	0/10	0/20	None
$CdSO_4$	10/10	17/20	55[b]
$MnSO_4$	0/10	0/20	None
$CuSO_4$	0/10	0/20	None
$NiSO_4$	1/10	1/20	33
$ZnSO_4$	0/10	0/20	None
Increasing concn			
$ZnSO_4$ (5×)	0/8	0/16	None
$ZnSO_4$ (12.5×)	0/8	0/16	None
$ZnSO_4$ (25×)	2/8	2/16	17

[a] Metal solutions were administered to cohorts in the equimolar section by the following schedule: Cu and Mn, 30 to 32 days p.i.; Cd and Zn, 31 to 33 days p.i.; and Ni and saline, 33 to 35 days p.i. Cohorts in the increasing concentration section were treated with 12.5× zinc from 32 to 34 days p.i. and with 5× or 25× zinc from 33 to 35 days p.i. Mice were sacrificed within 18 to 24 h of the final injection. Concentration of metal solutions listed in the equimolar section was 8.83 mmol; in the increasing concentration section, 44.15, 110.38, and 220.77 mmol were the concentrations of 5×, 12.5×, and 25×, respectively. The amount of metal solution administered was adjusted for the total body weight of each mouse. TG, trigeminal ganglia.

[b] The range ± 1 standard error is 10 to 308.

TABLE 3. Inhibitory effects of cadmium tolerance prior to administration of cadmium for the reactivation of latent virus in mouse trigeminal ganglia[a]

Pretreatment (one injection)	Administration (three injections)	No. of positive mice/total no. tested	No. of positive ganglia/total no. tested
Pentobarbital	$CdSO_4$	4/5	5/10
$MnSO_4$	$CdSO_4$	1/5	2/10
$NiSO_4$	$CdSO_4$	1/5	2/10
$ZnSO_4$	$CdSO_4$	1/5	1/10

[a] For pretreatment and for cadmium treatment, the concentration of each metal solution was 8.83 mmol. Pretreatment with metal solution was at 30 days p.i., and pretreatment with pentobarbital was at 42 days p.i. The first cadmium injection was administered 48 h after a single injection of the pretreatment solution. Mice were sacrificed within 18 to 24 h of the final cadmium injection. Comparison of results in Table 2 for mice treated with cadmium alone versus the results in this table obtained from all mice pretreated with metals yielded a P value of 0.00018 by the Fisher's exact test.

groups of mice in concentrations of 5, 12.5, or 25 times the effective cadmium molal concentration (20, 50, or 100 mg of Zn per kg of body weight, respectively), as indicated in Table 2. On the day after the third injection, ganglia were collected and assayed for infectious virus as described in Materials and Methods. As shown in Table 2, infectious virus was not recovered from any of the mice which received 5× or 12.5× concentrations of $ZnSO_4$. However, infectious virus was recovered from 13% of the ganglia or 25% of the mice which received a 25× concentration of $ZnSO_4$. We conclude from these experiments that zinc can reactivate latent virus, albeit less efficiently and at much higher concentrations than cadmium.

Pretreatment or delayed treatment reduces reactivation frequency. Earlier studies had shown that the survival rate of animals was increased when administration of a lethal dose of cadmium was preceded by treatment with cadmium (53) or zinc (59). The cadmium tolerance was maximal at 1 to 3 days (60) and was also attained by pretreatment with manganese (63) or phenobarbital (33, 64). Manganese and phenobarbital apparently protect by mechanisms which do not involve induction of Cd-Zn metallothioneins. In the next experiment, we tested the possibility that a preexisting tolerance to cadmium would inhibit its ability to reactivate virus. Latently infected mice (five mice per group) were given a single footpad injection of zinc, nickel, or manganese at concentrations which did not induce detectable levels of infectious virus. Another group of latently infected mice was anesthesized with pentobarbital (as described in Materials and Methods) but did not receive footpad injections. At 48 h after this pretreatment, all mice were given three daily footpad injections of cadmium sulfate and were sacrificed on the following day. Assay of the ganglion homogenates (Table 3) revealed that detection of infectious virus was drastically reduced by prior metallation (20% positive for ganglia and mice). When pentobarbital alone preceded cadmium treatment, there was only a slight decline in recovery of infectious virus (50% of ganglia positive and 80% of mice positive). Taken together, the results of these and preceding experiments are consistent with the hypothesis that cadmium induces the reactivation of latent virus by a mechanism which is not associated with a metallothionein-mediated response. Mice protected by pretreatment with factors which ameliorate cadmium toxicity are in any case much less likely to yield reactivated virus.

Scarification of cornea does not affect the induction of reactivation of latent virus by metal compounds. The rationale for the experiments described in this section was as follows. (i) Cadmium has been shown to induce elevated steady-state mRNA levels of several murine transcription factors, including both c-Jun (22) and c-Fos (8) components of the AP-1 complex, in addition to the primary heat shock and metal-regulatory transcription factors. In the studies described above, heavy metals other than cadmium which have been reported to induce heat shock and/or metal-responsive genes were relatively ineffective in reactivating latent virus.

(ii) Phorbol esters, which are known to activate a constellation of AP-1-responsive genes, have been reported to reactivate HSV-1 from nonproductively infected neuronal cell cultures (45). Explantation of trigeminal ganglia was reported to elevate the transcription of the genes specifying c-Jun, c-Fos, and Oct-1. The latter (also called OTF-1 or NFIII) is required for the induction of α genes by the viral α *trans*-inducing factor or α-TIF (also known as VP16; reviewed in reference 39). These cellular transcripts have been reported to accumulate within the explanted tissue concurrently with reactivation of latent virus (56). In the same study, transcription of the genes specifying Oct-1 and c-Jun (but not that specifying c-Fos) was detected in cells of the trigeminal ganglia within hours following corneal scarification.

To determine whether corneal scarification augments or inhibits the induction of reactivation of latent virus by cadmium, the procedure was done under anesthesia such that the maximum effects of scarification reported previously would coincide with the maximum effects of cadmium administration. With respect to cadmium, the maximum effects as measured by the incidence of infectious virus and the amounts of virus recovered in ganglia occurred on the second and third day postadministration of the cadmium salt (Table 1).

The experimental design employed in these studies was as follows. Mice in groups of six or seven were given daily injections of cadmium or saline for 3 or 4 days. Two groups were not treated further, whereas two groups were scarified as described in Materials and Methods after the second or third

TABLE 4. Null effects of corneal scarification when combined with administration of cadmium on the recovery of infectious virus from mouse trigeminal ganglia during the latent phase of infection[a]

No. and kind of injections	Corneal scarification	No. of positive mice/total no. tested	No. of positive ganglia/total no. tested
3, $CdSO_4$	No	6/6	9/12
3, $CdSO_4$	Yes	7/7	11/14
4, $CdSO_4$	No	3/6	5/12
4, $CdSO_4$	Yes	4/8	6/16
4, saline	Yes	2/7	2/14

[a] Corneal scarification was done under sodium pentobarbital anesthesia within 42 to 48 h prior to removal of ganglia. Mice were sacrificed at 33 days p.i., within 18 to 24 h after the final injection of cadmium or saline. Comparison of the results of three cadmium injections with scarification versus the saline control with scarification yielded P values of 0.02 for mice and 0.0018 for ganglia. Comparison of the results of three cadmium injections without scarification versus the saline control with scarification yielded P values of 0.03 for mice and 0.005 for ganglia. Comparison of administration of three cadmium injections with and without sacrification yielded P values of 1.19 for ganglia. All comparisons were done by the Fisher's exact test.

injection of cadmium. In each case, cadmium was administered for 1 more day after scarification. In addition, mice in one group were scarified but not treated with cadmium. Within 24 h after the final injection, the mice were sacrificed and the ganglia were promptly removed, homogenized, and assayed for infectious virus.

The results (Table 4) show the following. (i) As in the experiment listed in Table 1, mice receiving three injections of cadmium had a higher incidence of infectious virus than those receiving four injections. In each instance, scarification had no effect on the incidence of infectious virus in trigeminal ganglia of mice or on the number of trigeminal ganglia from which infectious virus was isolated. (ii) Scarification of the mice treated with saline in place of cadmium also resulted in the isolation of virus from two of seven mice or 2 of 14 trigeminal ganglia. We conclude that scarification did not contribute to the induction effected by cadmium and that while scarification alone was marginally effective in inducing latent virus, under the conditions tested it was much less effective than cadmium.

Effect of cadmium administration on the establishment of latent virus during the early stages of infection and on the recovery of latent virus from cadmium-treated mice. The objectives of this series of experiments were threefold. First, it has been repeatedly demonstrated that following corneal inoculation with wild-type HSV, infectious virus is readily detected in trigeminal ganglia for several days. Subsequently, infectious virus disappears and only latent or reactivated latent virus can be demonstrated in untreated healthy mice. It was questioned whether administration of cadmium affects this phase of infection of the trigeminal ganglia. Second, inasmuch as cadmium and/or corneal scarification induces the reactivation of latent virus, it was questioned whether administration of cadmium from the time of infection would preclude the establishment of latent virus. The third objective was to determine whether repeated administration of cadmium once latency has been established would result in a depletion of the pool of latent virus harbored in trigeminal ganglia of infected mice.

In the first series of experiments mice were anesthesized, scarified, inoculated, and injected daily for 2 weeks with either cadmium or saline beginning 2 weeks after inoculation. On each day of the first week and on every other day of the second week postinoculation (p.i.) of virus, trigeminal ganglia were resected from four cadmium-treated mice and four saline

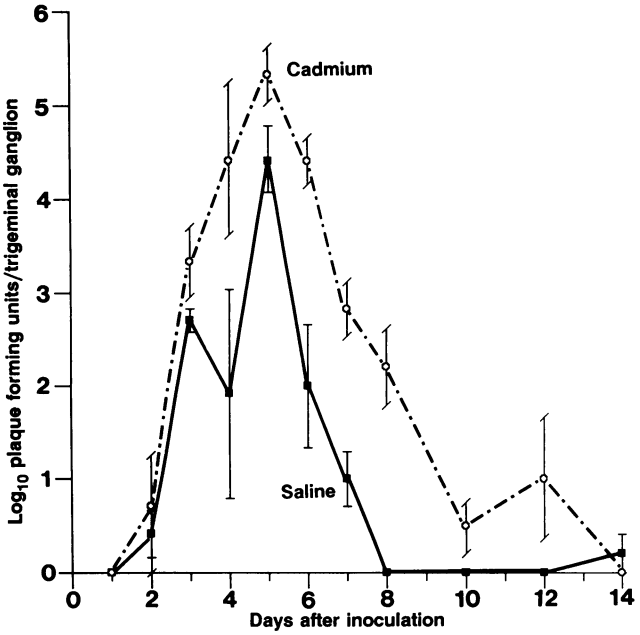

FIG. 1. Geometric mean titers of HSV-1(F) virus recovered from trigeminal ganglia of mice given cadmium sulfate (open circles, solid lines) or saline (filled squares, dashed lines) during acute-phase infection. Daily injection of cadmium or saline was begun at the time of inoculation and administered for the next 2 weeks. Each day p.i., a pair of mice were removed from both treatment groups and sacrificed within 18 to 24 h after the final injection. Each point represents the geometric mean of four ganglion titers obtained from a pair of cohorts.

control mice, separately homogenized, and assayed for the presence of infectious virus. As shown in Fig. 1, daily administration of cadmium resulted in a 10- to 100-fold increase in the geometric mean of viral titers compared with those obtained from saline controls. Duration of the acute phase (defined as the period in which infectious virus was continuously detected) was also extended from 7 days p.i. in the saline control group to 12 days p.i. in the cadmium-treated group. In the second series of experiments, cadmium was administered on days p.i. shown in Table 5, i.e., either during the acute stage of infection while infectious virus is present in sensory ganglia or during the latent phase. In this experiment, the ganglia were harvested and assayed for latent virus at 28 days after infection from mice treated with cadmium during the acute phase and at 42 or 62 days after infection from mice treated during the latent phase. As shown in Table 5, no demonstrable difference between cadmium- and saline-treated groups emerged.

DISCUSSION

Previous reports from several laboratories have shown that sensory ganglia from mice infected with wild-type HSV-1 or HSV-2 and not otherwise subjected to stress or medication rarely contain infectious virus. In contrast, spontaneously induced infectious virus has been demonstrated in rabbits, guinea pigs, and nonhuman primates (4, 11, 13, 14, 16, 19, 34–36, 40, 44, 49, 55; also reviewed in reference 38). Since in principle the sensory neurons become permissive on explantation of the sensory ganglia in culture, these results suggest that the viral functions are not expressed either because a specific cellular function is never expressed in the sensory neurons in vivo in the mouse or because mice express a strong repressor

TABLE 5. Effects of repeated administration of cadmium after virus inoculation on the recovery of latent virus from mouse trigeminal ganglia[a]

Administration (days p.i.)	No. of positive mice/total no. tested	No. of positive ganglia/total no. tested
Acute phase[b]		
$CdSO_4$ (−1, 2, 5, 8, and 11)	5/5	10/10
Saline (−1, 2, 5, 8, and 11)	9/10	16/20
Latent phase[c]		
$CdSO_4$ (20, 25, 30, and 35)	10/10	19/20
$CdSO_4$ (20, 25, 30, 35, 40, 45, 50, and 55)	9/10	17/20
Saline (20, 25, 30, 35, 40, 45, 50, and 55)	11/11	17/22

[a] A single injection of cadmium or saline was administered on each of the indicated days p.i. Mice treated during the acute phase were sacrificed at 28 days p.i. Mice treated during the latent phase were sacrificed 7 days after the final injection. All trigeminal ganglia were cultured for the presence of latent virus.

[b] Average weight of $CdSO_4$-treated mice at day of inoculation was 16.2 g. In this group of 10 mice, 5 died within 4 to 13 days p.i.

[c] Average weight of $CdSO_4$-treated mice at 55 days p.i. was 23.1 g. Note that in this group of 24 mice, 4 died of undetermined causes within 26 to 30 days p.i.

which effectively blocks viral gene expression. In recent years, a number of factors alone or in combination have been shown to induce viral replication in the mouse. These factors include local injury to the neurodermatome by freezing (34), by topical irritation (13, 16), by UV irradiation or local injection of prostaglandin E_2 (4), by ocular iontophoresis (11, 14, 62), or by neurectomy (6, 17, 32, 35, 36, 52, 58); systemic immunosuppression (11, 19, 34, 44, 55); intratracheal instillation of pneumococcus or mucin (49); and hyperthermia (40). To this list we have added cadmium sulfate. The singular feature of the results we report is that the incidence of infectious virus per total ganglia harboring latent virus or total infected mice is higher after cadmium administration than after any of the procedures reported to date.

Cadmium is a highly toxic agent known to accumulate in a variety of tissues including the peripheral nervous system, within which cadmium rapidly reaches highest levels in the sensory ganglia (1). Neuronal cells are particularly sensitive to cadmium toxicity, which impairs the function of calcium channels in the transmission of nerve impulses (12). In addition to its neurotoxic properties, cadmium variously affects different aspects of the murine immune response. Mice which were given $CdSO_4$ during the acute phase of HSV infection incurred a higher mortality rate than was observed in saline controls (Table 5). Administration of $CdCl_2$ at the time of inoculation with Japanese encephalitis virus was similarly reported to be associated with increased mortality (51). Although it is likely that cadmium interferes with immune clearance of replicating virus, this alone would not account for recovery of infectious HSV from latently infected ganglia, inasmuch as highly immunosuppressive regimens are relatively inefficacious for reactivation of HSV from mice (4, 11, 19, 34, 44, 48, 55).

One of the main host responses to intake of cadmium is the synthesis of metallothioneins which sequester the metal. The metallothionein genes are also induced by other heavy metals, including zinc. In our attempts to unravel the relationship between the induction of metallothionein genes and induction of virus, we noted that metallation by ions known to induce metallothionein genes does not cause reactivation of latent virus. The negative results obtained with zinc, manganese, and nickel sulfates are, however, significant in light of the observation that these metal ions induced a tolerance to cadmium, reflected in the loss of the capacity by cadmium to induce reactivation of latent virus. In the same vein, prolonged administration of cadmium (e.g., daily for 5 days, as shown in Table 1) may also establish tolerance and reduce the capacity of the metal to induce latent virus to replicate. However, only cadmium has the added property of effectively inducing viral reactivation, apparently by a mechanism which is not dependent upon induction of metallothionein or heat shock genes. One hypothesis which may explain these results is that cadmium directly affects the cellular machinery which maintains HSV in latent form.

A key observation relevant to the interpretation of the results presented in this article is that administration of cadmium at the time of infection increased the yield of infectious virus by a factor of 10 to 100. The significance of this observation stems from the following considerations. As indicated in the introduction, the observation that latent virus reactivates in neuronal cells placed in culture suggests that the neuronal cells are inherently able to sustain viral replication. Coupled with the observation that in many species virus reactivates spontaneously, it is conceivable that the permissivity of sensory neurons fluctuates on and off and that viruses entering cells in a transiently permissive state express their lytic functions and yield infectious progeny. The apparent effect of cadmium is to enhance permissivity, either by increasing the amount of virus yield per cell or by increasing the number of permissive cells. We lean towards the latter, since to date no treatment of cells has ever increased by 10- to 100-fold the yield of virus per cell. Although it is conceivable that cadmium induces, along with metallothionein genes, a transactivating factor which enables viral replication, the results are also consistent with the hypothesis that cadmium inactivates a factor which normally precludes viral replication.

The failure of repeated cadmium administration to either prevent the establishment of latency or to exhaust the viral reservoir by successive reactivation of latent virus was disappointing but not unexpected. First, it is noteworthy that the amounts of infectious virus recovered from sensory ganglia after cadmium administration were relatively low. Inasmuch as mice harboring latent virus after infection with wild-type virus are immune and cells expressing infectious virus would most likely be cleared rather rapidly, it is not known whether the amounts of infectious virus recovered represent virus present at the time of assay from neurons harboring virus induced randomly or from a subset of neurons supersensitive to cadmium. Previous studies have shown that sensory ganglia contain several populations of neurons and that they differ with respect to the incidence of latent virus (30, 31). It is conceivable that these subsets of neurons differ also with respect to their capacity to accumulate cadmium in concentrations sufficient to inactivate the factors which maintain the virus in a latent state.

It should be noted that in an earlier study, this laboratory attempted to preclude the establishment of latent virus or induce latent virus to replicate by treating mice infected with a recombinant virus containing a second copy of α-transinducing factor (α-TIF, also known as VP16) driven by a metallothionein promoter with cadmium (41). Although cadmium induced the expression of the chimeric gene and ganglia were shown to contain infectious virus, the procedures employed in those studies failed to preclude the establishment of latency or to

induce all latent virus. Since the object of those studies was to induce the virus to multiply, and since cadmium either as an inducer of the metallothionein promoter or of viral multiplication by other routes failed to induce all latent virus, the results presented in this paper leave unaltered the conclusion that failure of the virus to express all of its functions in latently infected cells is not due to the ineffectiveness or absence of α-TIF.

The key issue, that is, why HSV fails to express its functions in the sensory neuron in which it remains latent, remains largely unanswered. It has been postulated by a number of authors that the expression of viral functions is repressed by host factors (reviewed in reference 39). Operationally one approach to resolving this issue is to determine what factors confer permissivity upon the neuron harboring latent virus. The studies reported to date (e.g., deprivation of neuronal growth factor and the presence of transactivating factors with repressor functions) are all consistent with the hypothesis that the HSV genome is blocked from expressing its functions in latently infected cells (24, 27, 29, 39, 45, 61). The studies reported in this article are consistent with this hypothesis but do not provide the unambiguous evidence that this hypothesis requires.

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