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Sodium Butyrate: a Chemical Inducer of In Vivo Reactivation of Herpes Simplex Virus Type 1 in the Ocular Mouse Model[▽]

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Recent studies have explored the chromatin structures associated with the herpes simplex virus type 1 (HSV-1) genome during latency, particularly with regard to specific histone tail modifications such as acetylation and dimethylation. The objective of our present study was to develop a rapid systemic method of in vivo HSV-1 reactivation to further explore the changes that occur in the chromatin structures associated with HSV-1 at early time points after the initiation of HSV reactivation. We present a uniform, rapid, and reliable method of in vivo HSV-1 reactivation in mice that yields high reactivation frequencies (75 to 100%) by using sodium butyrate, a histone deacetylase inhibitor, and demonstrate that the reactivating virus can be detected at the original site of infection.

The herpes simplex virus type 1 (HSV-1) genome establishes latency in sensory neurons, where it exists as a circular episome associated with histones (6, 16, 21). During the latent phase of the HSV-1 cycle, only the latency-associated transcript (LAT) is abundantly transcribed (24, 25). While LATs have been implicated in numerous viral functions, including neuronal survival and antiapoptotic activities (18, 27), virulence (19, 27), the establishment of latency (20, 26), and reactivation (11, 14), the exact mechanisms by which LATs function are under investigation. Recent studies have begun to explore the chromatin structures associated with the HSV-1 genome during latency, particularly with regard to specific histone tail modifications such as acetylation and dimethylation (12, 13, 28). Chromatin immunoprecipitation studies of ganglia from mice with latent HSV-1 infections indicate that significant differences in the levels of enrichment with the acetyl histone H3 acetylated at tail residue lysines 9 (K9) and 14 (K14) exist between latently infected tissue and tissue subjected to (ex vivo) explant-induced HSV-1 reactivation (1). These differences are present at very early time points post-ganglionic explant and specifically indicate that explant-induced reactivation results in a rapid deacetylation of the LAT region, followed by a loss of LAT expression and a remodeling of the ICP0 promoter to an acetylated state (1). Further studies are addressing the aspect that the HSV-1 genome may possess insulated boundary regions, similar to those observed in cellular chromatin, that act to separate latent and lytic regions of the HSV-1 genome. These regions would be capable of recruiting modulatory enzymes, such as histone acetylases, histone deacetylases, and methyltransferases, to help maintain the structure of the latent HSV-1 genome (2). The association of specific histone modifications with the transcriptional status of HSV-1 genes during latency and lytic infection strongly suggests that chromatin modulatory enzymes such as histone deacetylases may play key roles in the regulation of HSV-1 gene expression (2).

There have been numerous reports examining effective methods of in vivo reactivation. Particularly common are those that involve hyperthermic stress or immunosuppression (3, 4, 9, 10, 29). Sawtell and Thompson reported in vivo reactivation frequencies of 60 to 75% using the hyperthermic stress model to induce HSV-1 reactivation in mice, in which the peak level of infectious virus was obtained at 24 h poststress (22, 23). While we have used this method in the past to induce HSV-1 reactivation (15), the objective of our present study was to develop a rapid systemic method of HSV-1 reactivation to further explore the changes that occur in the chromatin structures associated with HSV-1 at very early time points after reactivation. Specifically, we sought to develop a uniform, rapid, and reliable method of in vivo HSV-1 reactivation in mice that would yield very high reactivation frequencies when using a substance with the potential to alter the histone tail compositions of the chromatin structures associated with HSV-1.

Since many recent reports indicate that histone acetylases and histone deacetylases may play a major role in the HSV-1 transition from latency to reactivation (12, 13) and that the rapid deacetylation of the LAT region of the HSV-1 genome seems to be a key step in initiating explant-induced HSV-1 reactivation (1), the use of a chemical histone deacetylase inhibitor seemed to be a reasonable starting point. Sodium butyrate (NaB), a known histone deacetylase inhibitor, has induced HSV-1 reactivation in quiescently infected neuronal PC-12 cells (5). However, to our knowledge, there have been no reports that butyrate has ever been used in vivo to induce HSV-1 reactivation. In this study, we report that both single and multiple doses of NaB can be used to induce HSV-1 reactivation in mice with a frequency of 75 to 100%. Moreover, reactivating virus resulting from this butyrate induction could be detected in the eyes of the mice. To our knowledge, this is the first report of a paradigm of in vivo reactivation in mice

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TABLE 1. Results of the LD_{50} study for uninfected female BALB/c mice treated with NaB-PBS

Mouse group	NaB dose[a] (mg/kg/100 μl)	No. of deaths/no. of mice in group
1	5,000	6/6
2	2,500	6/6
3	2,000	6/6
4	1,600	4/6
5	1,400	3/6
6	1,200	1/6
7	600	0/6
8 (control)	0	0/6

[a] Mice were given one 100-μl intraperitoneal injection of the corresponding dose and monitored for 5 days posttreatment. All control mice were given an injection of PBS alone. Mice given doses of NaB of 2,000 mg/kg and higher died within 24 h of treatment.

that results in the recovery of virus at the initial site of infection.

Materials and methods. Four- to five-week-old female BALB/c mice (Charles River Laboratories Inc., Wilmington, MA) were used in all experiments in adherence to a protocol approved by the LSU Health Sciences Center Institutional Animal Care and Use Committee. Four viruses were used in the study: two wild-type high-reactivator HSV-1 strains (McKrae and 17syn+) and the corresponding low-reactivator LAT-null mutants, dLAT2903 (carrying a 2.9-kb deletion in the LAT region) and 17ΔPst (carrying a 202-bp deletion in the LAT core promoter region). Viral inoculation titers of 1.0×10^5 PFU/eye (17syn+ and 17ΔPst) and 5.0×10^4 PFU/eye (McKrae and dLAT2903) were placed on corneas scarified in a three-by-three crosshatch pattern. Slit-lamp examinations, swabbing, and infectious virus assays were performed as previously reported (15). Mice were considered to be latently infected at 28 days postinoculation when the slit-lamp examinations showed no signs of infection. The eyes of latently infected mice from all groups were swabbed prior to the onset of each experiment to confirm that all eyes were negative for the presence of infectious virus. In all multiple-dose studies, each experimental group was given a dose of NaB of 1,200 mg/kg of body weight in phosphate-buffered saline (PBS; final volume, 100 μl), followed by a 600-mg/kg dose (final volume, 100 μl) 24 h later. In all single-dose studies, each experimental group was given a 1,200-mg/kg dose of NaB in PBS (final volume, 100 μl). All control mice were given PBS alone (100 μl). All mice had their eyes swabbed daily and were sacrificed at 72 h posttreatment. Corneas were removed and placed in 24-well plates containing cell culture medium (Eagle's minimal essential medium supplemented with 20% fetal bovine serum), and the plates were incubated at 37°C (5% CO_2) for 48 h. The culture medium was then removed and assayed for infectious virus by examining for cytopathic effects (CPE) on primary rabbit kidney (PRK) cells. Trigeminal ganglia (TG) were removed and immediately homogenized in culture medium. The TG homogenate was centrifuged (2,000 rpm; Eppendorf 5402) to pellet the tissue, and the supernatant was collected and assayed for infectious virus on PRK cells. Samples were considered negative if no CPE were observed for 10 days. NaB concentrations in plasma were determined by using gas chromatography-mass spectrometry analysis at 1, 2, 4, and 24 h postinjection.

Prior to our induction experiments with mice, a small pilot study of oral NaB administration was done with a group of New Zealand White rabbits latently infected with HSV-1 McKrae. Twenty rabbits were separated into two groups (experimental and control), and either NaB-PBS or PBS alone was administered in the rabbits' water over the course of 7 days. Swab samples from the rabbits were collected daily, and our initial data showed that rabbits given NaB shed significantly more infectious virus (94 of 440 swabs, or 21.4%, were positive for infectious virus) than those given the control PBS (38 of 396 swabs, or 9.6%, were positive; $P < 0.01$), with no deaths occurring in either group. While it initially seemed logical to follow the same course of treatment in the mouse in vivo study, we determined that the nature of subsequent experiments designed to analyze potential changes in the chromatin profiles of the viral genome at very early (0.5- and 1-h) time points postinduction necessitated a uniform and rapid systemic method of drug delivery. Therefore, our first butyrate experiments with mice consisted of determining the 50% lethal dose (LD_{50}) of NaB for a naïve BALB/c mouse. Mice were separated into eight groups, each group comprising six mice. Mice in groups 1 through 7 received one intraperitoneal injection of NaB-PBS (100 μl), with NaB doses of 5,000, 2,500, 2,000, 1,600, 1,400, 1,200, and 600 mg/kg. Mice in the control group (group 8) received only PBS (100 μl). Mice were monitored for survival for 5 days after the NaB-PBS injection. Mice receiving doses of NaB of 2,000 mg/kg or higher died within 24 h of the NaB-PBS injection. The LD_{50} was identified when 50% mortality occurred in group 5, in which the mice were given the 1,400-mg/kg dose of NaB. One death occurred among the mice given the 1,200-mg/kg dose (17% mortality), and no deaths occurred

TABLE 2. Results of the two-dose NaB-PBS treatment regimen administered over a 48-h period[a]

Virus strain	Treatment on day: 1	Treatment on day: 2	No. of positive swabs/ total no. of swabs (%)	No. of positive TG/ total no. of TG (%)	No. of surviving mice/ total no. of mice (%)
McKrae	1,200-mg/kg dose of NaB in PBS	600-mg/kg dose of NaB in PBS	15/28 (54)	8/8 (100)	4/7 (57)
	PBS alone	PBS alone	0/24 (0)	0/8 (0)	4/4 (100)
dLAT2903	1,200-mg/kg dose of NaB in PBS	600-mg/kg dose of NaB in PBS	0/30 (0)	0/8 (0)	4/8 (50)
	PBS alone	PBS alone	0/24 (0)	0/8 (0)	4/4 (100)

[a] The regimen consisted of a 1,200-mg/kg dose of NaB in PBS given intraperitoneally on day 28 postinoculation, followed by a 600-mg/kg dose of NaB in PBS administered 24 h later, on day 29 postinoculation. Mice were assayed via eye swabbing for the presence of infectious virus in the eyes for three consecutive days. The swab data provided are cumulative for all swabs taken, but the presence of infectious virus was detected as early as 24 h posttreatment. All mice were sacrificed at 72 h after the first injection of NaB-PBS or PBS alone, and the TG were removed and homogenized. The supernatant was plated onto PRK cells and observed for CPE.

TABLE 3. Results of the single-dose NaB-PBS treatment regimen administered to mice with latent HSV-1 McKrae infection[a]

Mouse group	NaB dose (mg/kg/100 μl)	No. of positive swabs/total no. of swabs	No. of positive TG/total no. of TG	No. of deaths/ no. of mice in group
1	1,200	5/16	6/8	1/4
2	600	0/16	0/8	0/4
3	400	0/16	0/8	0/4
4	200	0/16	0/8	0/4
5 (control)	0	0/16	0/8	0/4

[a] Mice were given intraperitoneal doses of NaB ranging from 1,200 to 200 mg/kg in PBS. Eyes were swabbed prior to the onset of the experiment and at 72 h postinjection. The total number of eye swabs includes swabs taken before NaB-PBS treatment. At 72 h post-NaB-PBS administration, mice were sacrificed and their TG were harvested. TG were homogenized and pelleted, and the supernatant was plated onto PRK cells. The presence of infectious virus was indicated by CPE.

in the groups receiving doses lower than 1,200 mg/kg (Table 1). These data obtained for the BALB/c mice correspond to previously reported nonlethal doses of NaB-PBS given to transgenic mice (8). An analysis of plasma from mice treated with NaB-PBS showed a rapid decrease in NaB levels in plasma within 2 h of injection, and by 24 h post-NaB injection there was no detectable NaB in the plasma of these mice.

We determined that butyrate could be administered to mice to effectively induce the in vivo reactivation of the wild-type HSV-1 strain McKrae, typically a high-reactivator virus. The corresponding LAT-null McKrae mutant, dLAT2903, was used as a nonreactivating control. Initially, we began a two-dose NaB treatment regimen (1,200 mg/kg followed by 600 mg/kg 24 h later) to maximize potential viral reactivation. This two-dose regimen was administered to two experimental groups of mice latently infected with either McKrae or dLAT2903. Control mice, also latently infected with McKrae or dLAT2903, were given only PBS injections. Subsequent analyses of ocular tear film (15 positive swabs [54%] of 28 total) and TG (8 positive TG [100%] of 8 total) showed that butyrate induced reactivation in mice latently infected with HSV-1 strain McKrae as early as 24 h posttreatment (as indicated by the presence of infectious virus in 6 of 12 eye swabs done 24 h posttreatment) but not in mice latently infected with dLAT2903 (0 positive swabs of 30 total and 0 positive TG of 8 total) (Table 2). All control mice were negative for the presence of infectious virus both in ocular swabs and in TG. While the two-dose NaB-PBS treatment regimen gave us 100% reactivation in the mice assayed, we had an increase in mortality in both experimental groups only after the second dose of NaB-PBS (Table 2), a consequence that was unexpected based on the results of our LD_{50} study, our own gas chromatography-mass spectrometry analysis of plasma NaB levels, and the reported rates of NaB clearance from plasma, which indicate that by 24 h there are no detectable NaB levels in the blood (7, 17).

Given the unexpected mortalities associated with the two-dose NaB treatment regimen, we sought to determine an effective, nonlethal single dose of NaB-PBS that would yield results similar to those obtained with the two-dose regimen. Twenty mice latently infected with McKrae were separated into five groups of four mice each and were given one intraperitoneal injection of either PBS alone or a 200-, 400-, 600-, or 1,200-mg/kg dose of NaB in PBS. Ocular swabbing and infectious virus assays were performed as previously described. The most effective dose of butyrate administered was the 1,200-mg/kg dose, which resulted in the presence of infectious virus in 5 (31%) of 16 ocular swabs and in 6 (75%) of 8 TG from the surviving mice (Table 3) and yielded only one death. This level of mortality was consistent with that in the uninfected NaB-treated group (Table 1).

To determine the effectiveness of the 1,200-mg/kg dose of NaB and ensure that this method of HSV-1 reactivation was reproducible with different virus strains, we treated 10 mice latently infected with 17syn+ and 10 mice latently infected with 17ΔPst, the corresponding LAT-null low reactivator from the 17syn+ parent. Using the same methods of analysis, ocular swabbing, and infectious virus assays, we found that 21 (32%) of 65 ocular swabs were positive for the presence of infectious virus, with virus appearing as early as 24 h posttreatment. Of the TG assayed, 15 (75%) of 20 were positive for the presence of infectious virus by 72 h postinjection. Furthermore, among the corneas assayed, 15 (75%) of 20 were positive for infectious virus, indicating that virus persisted at the initial site of infection as late as 72 h posttreatment. It should be noted that no mouse in the 17syn+-infected group had negative results for both corneas and TG, indicating 100% reactivation in the mice latently infected with 17syn+ and treated with NaB-PBS. In contrast, among mice latently infected with 17ΔPst and treated with butyrate, 0 (0%) of 65 eye swabs, 0 (0%) of 20 corneas, and 0 (0%) of 20 TG were positive for infectious virus (Table 4). No deaths were observed among the mice latently infected with 17syn+ and treated with NaB.

The goal of this study was to develop a rapid and reliable method for in vivo HSV-1 reactivation in mice that could be used to further study epigenetic changes within the HSV-1-associated chromatin during latency and reactivation. To adequately show that consistent in vivo reactivation was

TABLE 4. Results of the single-dose NaB-PBS treatment regimen administered to mice with latent HSV-1 17syn+ and 17ΔPst infections[a]

Treatment	Virus strain	No. of positive eye swabs/ total no. of swabs (%)	No. of positive corneas/ total no. of corneas (%)	No. of positive TG/ total no. of TG (%)	No. of positive mice/ total no. of mice (%)
NaB-PBS	17syn+	21/65 (32)	15/20 (75)	15/20 (75)	10/10 (100)
	17ΔPst	0/65 (0)	0/20 (0)	0/20 (0)	0/10 (0)
PBS alone	17syn+	0/20 (0)	0/20 (0)	0/20 (0)	0/10 (0)
	17ΔPst	0/20 (0)	0/20 (0)	0/20 (0)	0/10 (0)

[a] Mice were given a 1,200-mg/kg intraperitoneal dose of NaB in PBS. Eyes were swabbed prior to the onset of the experiment and at 24, 48, and 72 h postinjection. The total number of eye swabs includes swabs taken before NaB-PBS treatment. The swab data provided are cumulative for all swabs done, but the presence of infectious virus was detected as early as 24 h posttreatment. At 72 h post-NaB-PBS administration, mice were sacrificed and their TG were harvested. TG were homogenized and pelleted, and the supernatant was plated onto PRK cells. The presence of infectious virus was indicated by CPE.

achieved, mouse TG and corneas were assayed for infectious virus at 72 h posttreatment, after the presence of infectious virus was detected in several eye swabs taken 24 h posttreatment. While no TG or corneas were assayed prior to 72 h posttreatment, the presence of infectious virus in the eye swabs taken 24 and 48 h posttreatment from mice latently infected with either McKrae or 17syn+ indicates that reactivation was occurring in the ganglia and the presence of infectious virus in the ganglia at these two time points posttreatment was likely. Conversely, no group of mice was observed for longer than 72 h posttreatment, and therefore, no data on the length of viral persistence in the eyes or ganglia can be provided at this time.

A single 1,200-mg/kg dose of NaB, administered intraperitoneally to mice, was used successfully to reactivate HSV-1 strains McKrae and 17syn+ in 75 to 100% of the mice latently infected with these strains. Studies are presently under way to further explore the specific mechanism of action of NaB in the mouse model with respect to cellular changes induced by NaB treatment in both high- and low-reactivator HSV-1 strains. These changes will be compared to changes induced by other methods of in vivo reactivation (e.g., heat stress and immunosuppression, etc.). Specifically, analyses of the enrichment of lytic regions of the HSV-1 genome with acetyl histone H3 with biotinylated K9 and K14 tail residues in latently infected untreated mice compared to latently infected butyrate-treated mice will be used to further assess the role of the hyperacetylation of lytic regions at early time points during reactivation. To date, we have obtained preliminary immunoprecipitation data showing that NaB initiates changes in the chromatin profiles in the ganglia of mice latently infected with 17syn+ as early as 1 h posttreatment. Specifically, the histone acetylation enrichment patterns associated with the LAT promoter, the LAT exon, and the ICP-4 promoter regions of latent ganglia are significantly altered in the ganglia of mice treated with NaB at 1 h post-butyrate treatment. This finding indicates that the acetylation of lytic promoters of the viral genome is occurring, and since NaB is a histone deacetylase inhibitor, the observations of the acetylation patterns in latently infected NaB-treated mice indicate that NaB probably acts as an in vivo histone deacetylase inhibitor. Furthermore, this deacetylase inhibition and subsequent acetylation of lytic promoters of the HSV-1 genome may be an early step in the initiation of reactivation. Nevertheless, the present study provides definitive evidence that NaB can be used efficiently to induce HSV-1 reactivation and that infectious virus can be recovered at the initial site of the viral infection (the eye) as early as 24 h posttreatment, as well as from the ganglia at 72 h posttreatment.

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