**Effect of Hexamethylene Bisacetamide and Cyclosporin A on Recovery of Herpes Simplex Virus Type 2 from the in Vitro Model of Latency in a Human Neuroblastoma Cell Line**

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**ABSTRACT**

The goal of the present work was to examine whether hexamethylene bisacetamide (HMBA) and cyclosporin A affect the recovery of herpes simplex virus type 2 (HSV-2) from an in vitro model of HSV-2 latency in human neuroblastoma cell line IMR-32. IMR-32 cells were infected with HSV-2 at a multiplicity of infection of 0.1 plaque-forming units/cell and were cultured at 40°C for 14 days, resulting in the establishment of a model of HSV-2 latency in IMR-32 cells. When the cultivation temperature was shifted down from 40 to 37°C, recovery of virus growth began to occur after an incubation period of 2 days. During the time of shift-down of the incubation temperature, the latently infected cells were further cultured at 37°C in the presence or absence of 5 mM HMBA or 0.5 μg/ml cyclosporin A, which does not affect stability of HSV-2 nor proliferation of IMR-32 cells. Consequently, the rate of HSV-2 recovery from the latently infected cells cultured in the presence of 5 mM HMBA was significantly increased, compared with the untreated controls. In addition, the DNA methylation level of the latently infected IMR-32 cells cultured in the presence of HMBA was significantly decreased when compared to the level in the untreated controls. On the other hand, the cultivation of the latently infected cells in the presence of 0.5 μg/ml cyclosporin A resulted in a significant decrease in the rate of HSV-2 recovery. These findings indicate that the recovery of HSV-2 from the model of latency in IMR-32 cells is enhanced by HMBA treatment, which induces a significant decrease of total genomic DNA methylation level, and is inhibited by cyclosporin A treatment.

**INTRODUCTION**

It has been shown that hexamethylene bisacetamide, a polar compound with a molecular weight of 200, which functions as a DNA-hypomethylating agent (1), induces in vitro morphological and functional differentiation of murine and human leukemia cells (2–9) as well as a variety of human solid tumor cell lines (10–14). While clinical trials of HMBA have been performed mainly in the United States (15–22), several investigators have reported that HMBA can produce therapeutic response in patients with cancer (21, 22). In the Phase I studies examined for the toxicities and pharmacology of HMBA, we have found neurotoxicity and renal insufficiency as well as cutaneous or oral herpes infections during HMBA treatment (19, 22), which might be due to the reactivation of herpes simplex virus from latently infected ganglia or the augmentation of viral replication in the sites of primary HSV infection. It is well known that neurons harbor latent HSV infection (23, 24).

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The abbreviations used are: HMBA, hexamethylene bisacetamide; HSV-2, herpes simplex virus type 2; IMR-32, human neuroblastoma cell line; MEM, minimal essential medium; MOI, multiplicity of infection; PFU, plaque-forming units.

On the other hand, it has been suggested that the DNA hypomethylating agents such as HMBA or 5-azacytidine enhance in vitro HSV reactivation from latently infected mouse neural and peripheral tissues (25–27). This may indicate a role for methylation in HSV latency and reactivation. Recently, we have established an in vitro model of latency of HSV type 2 in a human neuroblastoma cell line IMR-32, in which the IMR-32 cells can be latently infected with HSV-2 at 40°C and when the incubation temperature of infected cells at 40°C is reduced to 35°C, infectious virus can be recovered from the cultures after a lag period of 2–9 days (28). In general, it is well known that the HSV reactivation from latent HSV infection in the immunocompromised patients with cancer, or with renal or bone marrow transplantation, causes severe recurrent or primary HSV infection (29).

Cyclosporin A, a potent immunosuppressive drug, is commonly used as prophylaxis of graft versus host disease in man after allogeneic bone marrow transplantation for leukemia (30) and is utilized clinically to maintain heterotransplantation (31). Since cyclosporin A selectively inhibits T-lymphocytes (32), infections with HSV, cytomegalovirus, Epstein-Barr virus, and varicella zoster virus are a problem in bone marrow, renal, heart, and liver transplant patients treated with cyclosporin A. Although these infections are facilitated by T- and natural killer cell suppression (32–34), it has also been reported that cyclosporin A inhibits replication of HSV type 1 in vitro by inhibiting direct cell-cell transmission of virus (35). Thus we have taken much interest in whether HMBA or cyclosporin A affects HSV-2 recovery from the model of latency in IMR-32 cells. In this communication, we report that recovery of HSV-2 from the latently infected IMR-32 cells is enhanced by HMBA treatment, which induces a significant decrease of total genomic DNA methylation level, and is inhibited by cyclosporin A.

**MATERIALS AND METHODS**

**Culture and Media.** A human neuroblastoma cell line IMR-32 (36), and African green monkey kidney cell line Vero (37) were used. IMR-32 cells were grown in Eagle's MEM supplemented with 5% fetal calf serum, 5% newborn calf serum, 2 mM L-glutamine, and nonessential amino acids (Flow Laboratories, Rockville, MD) in the presence of 5% CO₂ in a temperature gradient incubator (Toyo Industrial Co., Tokyo, Japan) at 37 or 40°C, as described previously (28). Vero cell monolayers were prepared in MEM supplemented with 5% newborn calf serum and 2 mM L-glutamine in a 5% CO₂ incubator at 37°C. IMR-32 cells were purchased from Flow Laboratories.

**Virus and Virus Assay.** Herpes simplex virus type 2, strain UW-268 (38), was propagated at 37°C in Vero cell monolayers. Of 10 virus clones from the plaques of HSV-2 formed on Vero cell monolayers, one clone which showed most stable growth on Vero cells was used for this study. To prepare virus stocks, HSV-2 at a multiplicity of infection of 0.01 plaque-forming units/cell was adsorbed for 1 h at 37°C onto Vero cell monolayers and was then cultured for 48 h at 37°C, as described previously (28). Thereafter, the infected cells were harvested.
and the disrupted cells were prepared through 3 cycles of freezing and thawing. They were clarified by slow speed centrifugation and the supernatants were stored in a −80°C deep freezer until used. The virus stocks were not temperature sensitive at 39°C.

HSV-2 was assayed by plaque-forming ability on Vero cell monolayers, as described previously (28). Briefly, the confluent cell monolayers formed in 35-mm plastic Petri dishes (Falcon Labware, Oxnard, CA) after 2 days of incubation were infected with virus samples in serial 10-fold dilution. After an adsorption period of 60 min, the monolayers were overlaid with growth medium containing 0.7% (w/v) methylcellulose, 4000 cp (Wako Pure Chemical Industries, Ltd., Osaka, Japan). They were further incubated in a 5% CO₂ incubator for about 5 days at 37°C. After removal of methyl cellulose, the cells were fixed with methanol, stained with 1% (w/v) crystal violet dissolved in 20% methanol, and then the plaques formed were counted.

Immunofluorescent Antibody Staining Technique. Cells grown on coverslips (24 x 32 mm) in 60-mm plastic dishes (Falcon) were fixed with acetone at 4°C for 10 min and air dried. The fixed cells were incubated at 37°C for 1 h with 50 μl of a 50-fold dilution of rabbit antiserum to HSV-2 in Dulbecco’s phosphate-buffered saline (39). The cells were washed with Dulbecco’s phosphate-buffered saline for 30 min under mechanical shaking and stained with fluorescein isothiocyanate conjugated swine anti-rabbit IgG (Dakopatts, Copenhagen, Denmark). To remove nonreacted antibodies the cover slips were washed, mounted with buffered glycerol (pH 9.4), and examined under a Nikon fluorescence microscope (Nikon Co., Tokyo, Japan).

The rabbit antiserum to HSV-2 was prepared as follows. UV-inactivated stock virus pools emulsified with complete Freund’s adjuvant were inoculated i.m. into an albino rabbit once a week for a total of 5 inoculations. One week after the last immunization the blood was collected by cardiac puncture and the serum separated was used as the control.

RESULTS

Effect of HMBA or Cyclosporin A on Thermal Inactivation of HSV-2 at 37°C and Growth of IMR-32 Cells. To examine the effect of HMBA or cyclosporin A on growth of HSV-2 at 37°C, HSV-2 sample containing infectious virus of about 10⁴ PFU/ml was mixed with HMBA (2, 5, or 10 mM) or cyclosporin A (0.5, 1.0, or 5.0 μg/ml) in 2 ml of growth medium and was incubated at 37°C. Also, HSV-2 in growth medium alone was incubated at 37°C and its thermal inactivation was examined. At various time intervals, infectious virus in the incubation mixture was assayed by plaque formation on Vero cell monolayers. As a consequence, infectious virus of the specimens containing HMBA (2, 5, or 10 mM) or cyclosporin A (0.5, 1.0, or 5.0 μg/ml) disappeared in a rate similar to thermal inactivation of HSV-2 and became undetectable within 3 days after the initiation of incubation. This finding indicates that the stability of HSV-2 is not affected directly by HMBA or cyclosporin A under the present experimental condition.

IMR-32 cells were cultured for 10 days in the presence and absence of HMBA (2, 5, or 10 mM) or cyclosporin A (0.5, 1.0 or 5.0 μg/ml), and the effects of these chemicals on the growth of IMR-32 cells were examined. Consequently, the difference between untreated control and culture in the continued presence of HMBA (2 or 5 mM) with regard to the cell growth was not significant. However, the growth rate of IMR-32 cells cultured in the presence of 10 mM HMBA was significantly decreased when compared with the untreated control (P < 0.01; 2-way analysis of variance and Tukey studentized range test). On the other hand, the growth of the IMR-32 cells cultured in the presence of 0.5 or 1.0 μg/ml cyclosporin A was not significantly affected in comparison to that of the untreated control in the present experimental conditions, whereas the growth of IMR-32 cells was significantly inhibited by 5.0 μg/ml cyclosporin A (P < 0.01; 2-way analysis of variance and Tukey studentized range test). The doubling time for the untreated IMR-32 cells was 40.8 h.

In the cultivation of IMR-32 cells in the growth medium without HMBA or cyclosporin A, IMR-32 cells were spindle shaped or stellate and tended to grow in the form of cell clusters for a few days after the start of cultivation. On the other hand, about 5 days after treatment of IMR-32 cells with HMBA (2 or 5 mM) or cyclosporin A (0.5 or 1.0 μg/ml), cells began to form long cytoplasmic processes which seemed to be neurite structures. While the number and length of processes in the treated IMR-32 cells were gradually increased, the untreated IMR-32 cells formed neurites spontaneously and their cell morphology in culture became similar to that of treated IMR-32 cells about 10 days after the treatment with HMBA or cyclosporin A.

Effect of HMBA or Cyclosporin A on Growth of HSV-2 at 37°C. IMR-32 cells were seeded at a density of 2 x 10⁴ cells/35-mm plastic Petri dish and were cultured for 3 days in a 5% CO₂ incubator at 37°C. After formation of monolayered cell cultures, they were infected with HSV-2 at a MOI of 0.1 PFU/cell. After an adsorption of 60 min, infected cultures were washed 3 times with MEM and then incubated at 37°C in growth medium containing HMBA (2, 5, or 10 mM) or cyclosporin A (0.5, 1.0, or 5.0 μg/ml). Also, IMR-32 cells were pretreated with 5 mM HMBA for 24 h, infected with HSV-2 at a MOI of 0.1 PFU/cell, and further cultured in the continued presence of 5 mM HMBA. At regular intervals, infected cultures were harvested and cell homogenates obtained after 3 cycles of
freezing and thawing were assayed for infectious virus. Fig. 1 shows a representative result of the experiments. The difference between untreated control and culture in the presence of HMBA (2, 5, or 10 mM) or untreated control and HMBA-pretreated culture with regard to the production of HSV-2 was statistically significant at $P < 0.05$ each (2-way analysis of variance and Tukey studentized range test). On the other hand, the difference between untreated control and culture in the presence of cyclosporin A (0.5, 1.0, or 5.0 $\mu$g/ml) with regard to virus yield was not significant, although the difference between the pair of 0.5 and 5.0 $\mu$g/ml of cyclosporin A was significant at $P < 0.05$ (2-way analysis of variance and Tukey studentized range test).

Effect of HMBA or Cyclosporin A on Recovery of HSV-2 from in Vitro Model of Latency by Shift-down of Incubation Temperature. IMR-32 cells were plated onto 35-mm plastic Petri dishes at a density of $2 \times 10^5$ cells/dish and were cultured for 7 days in a 5% CO$_2$ incubator at 37°C. Then the monolayered cultures of IMR-32 cells were infected at 37°C with HSV-2 at a MOI of 0.1 PFU/cell. After an adsorption of 60 min, infected cultures were incubated for 14 days in a 5% CO$_2$ incubator at 40°C. The infectious virus titer became undetectable by day 7 after infection, as described previously (28). When the incubation temperature of infected cultures was reduced to 37°C, the culture medium was changed to fresh growth medium containing 5 mM HMBA or 0.5 $\mu$g/ml cyclosporin A, and then the cultures were maintained at 37°C. As shown in Table 1, the difference between untreated control and culture in the presence of 5 mM HMBA with regard to the rate of HSV-2 recovery for the time period ranging from day 5 to day 14, was statistically significant ($P < 0.05$ each on day 5 to day 14; Fisher’s exact probability test). In the latently infected IMR-32 cells cultured at 37°C in the presence of 0.5 $\mu$g/ml cyclosporin A, infectious virus could be first detected in only 1 culture on day 5 and thereafter the recovery of HSV-2 from other cultures was not found within the current experimental period. The difference between untreated control and culture in the presence of 0.5 $\mu$g/ml cyclosporin A with regard to the rate of HSV-2 recovery for the time period ranging from day 19 to day 26 was statistically significant ($P < 0.01$ each on day 19 to 26; Fisher’s exact probability test). Fig. 2 shows the relationship between percentage of recovery of HSV-2 and the time period after shift-down of the incubation temperature on the basis of the data obtained by Table 1.

DNA Methylation of IMR-32 Cells Treated with HMBA. We measured total genomic DNA methylation level in the latently infected IMR-32 cells cultured in the presence of 5 mM HMBA on the basis of the extent of methylation at the internal cytosine in the CCGG sequence determined by HpaII-MspI restriction endonuclease analysis. As shown in Table 2, the ratios of high-molecular-weight DNA to low-molecular-weight DNA after HpaII digestion of DNA from the latently infected IMR-32 cells treated with 5 mM HMBA for 1 day (0.775 ± 0.008, n = 3, $P < 0.05$; Mann-Whitney U test) or 3 days (0.710 ± 0.031, n = 3, $P < 0.05$; Mann-Whitney U test) were significantly decreased when compared to the levels in the untreated controls [0.822 ± 0.004 on day 1 (n = 3); 0.809 ± 0.001 on day 3 (n = 3)], whereas the difference between untreated control and culture in the presence of HMBA with regard to the ratios of high-molecular-weight DNA to low-molecular weight DNA after MspI digestion of the cell DNAs was not statistically significant.

DISCUSSION

This paper reports the enhancement by HMBA or suppression by cyclosporin A of the recovery of HSV-2 from the in vitro model of latency in human neuroblastoma cell line IMR-32, using the concentrations of HMBA or cyclosporin A in which the growth of IMR-32 cells and the stability of HSV-2 are not affected.
lymphoid cell line, cells in the two latent states harbor one or more copies of HSV-1 DNA per cell that is heavily methylated at CGGG sites, whereas producer cells contain 40–80 copies of viral DNA which appears to be nonmethylated (40). In addition, it has been shown that cellular and viral DNA hypomethylation is associated with induction of Epstein-Barr virus lytic cycle (41). Moreover, it has been reported that the regulation of Epstein-Barr virus nuclear antigen and latent membrane protein expression in cells latently infected with Epstein-Barr virus is related to the methylation in the specific regions of virus genome (42), and that methylation of integrated adenovirus type 12 DNA sequences in transformed cells is inversely correlated with viral gene expression (43). On the other hand, it has been reported that latent HSV-1 DNA is not extensively methylated in vivo, suggesting that extensive methylation of the viral genome is not a necessary condition for, or consequence of, maintaining the latent state in vivo (44). Although the methylation level of HSV-2 genome remains to be determined in the current study, it has been suggested that the recovery of HSV-2 may accompany the hypomethylation of total genomic DNA, which is induced by HMBA treatment of HSV-2 latency model in IMR-32 cells. In the current study, it has been demonstrated that the cultivation of the infected IMR-32 cells pretreated with or in the presence of HMBA results in the augmentation of HSV-2 replication as compared with the untreated control, whereas the HSV-2 titer in the HMBA-treated culture increased only moderately in the experiments on productive infection. On the contrary, it has been reported that methylation of HSV-1 DNA during active viral DNA synthesis is a prerequisite for infectious virus production (45). Also, it has been found that the cultivation of IMR-32 cells in the presence of 10 mM HMBA results in a significant reduction of the cell growth. It is well known that the cell DNA hypomethylation causes the alterations of cellular proliferation and differentiation (46, 47).

It has been suggested that HSV fuses directly with the cell plasma membrane and enters cells (48, 49). On the other hand, it has been reported that cyclosporin A stabilizes lipid bilayers and inhibits fusion of lipid (50, 51). Thus, the current finding that the cultivation of latently infected IMR-32 cells in the presence of 0.5 µg/ml cyclosporin A at 37°C results in a significant suppression of the recovery of HSV-2 may indicate that cyclosporin A inhibits cell-cell fusion induced by infected
cells. Moreover, it has been found that the cultivation of IMR-32 cells in the presence of 5.0 μg/ml cyclosporin A under the current experimental conditions results in a significant reduction of the cell growth. It has already been reported that cyclosporin A inhibits the proliferation of various cell types, including cells of hematopoietic and non-hematopoietic origin (52, 53).

Several investigators have indicated that some neuronal cell lines such as mouse neuroblastoma and rat neurotumor cells may offer unique advantages in the study of the virus-cell interaction in latently infected neurons (54–57). In general, it seems to be essential for establishment of the in vitro HSV latency models that the cytoidal potential of HSV is minimized by culturing infected cells in the presence of serum antibody, interferon, some chemical inhibitors, or a combination of these agents, in addition to control of the incubation temperature (58–61). Since the in vitro HSV latency model used in the current study has been established only by control of the incubation temperature, this system provides a useful model to investigate the mechanisms which may be involved in HSV latency and reactivation in vivo. However, it remains to be shown in the current study that the mechanism of inhibiting virus replication in this model system is similar to that occurring during viral latency such as expression of an endless episome of HSV DNA or HSV latency-associated transcripts (62, 63).

In conclusion, the clear indication from this study is that HMBA enhances recovery of HSV-2 from the latency model in IMR-32 cells, whereas HSV-2 recovery is inhibited by cyclosporin A treatment. In particular, when HMBA is used as a therapeutic agent for cancer patients, the current finding may indicate that some preventive methods such as administration of acyclovir (64) are needed for the inhibition of occurrence of recurrent HSV diseases.

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