Effects of 2,2′-O-Cyclocytidine and Acyclovir on Latent Herpes Simplex Virus in Trigeminal Ganglia of Mice

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The effects of 2,2′-O-cyclocytidine (CC) and acyclovir (ACV) on latent herpes simplex virus (HSV) in trigeminal ganglia were studied in an in vitro model using reactivation of HSV type 1 (HSV-1) as a model. It was shown that both CC (10 μg/ml) and ACV (2.5 μg/ml) significantly inhibited the reactivation of the latent HSV-1 in infected ganglia. The effect of CC (25 μg/ml), which was as good as that of ACV (10 μg/ml), did not last as long as that of ACV after removal of the drugs. The latent state of HSV-1 in vitro was dependent on the continuous presence of either drug. Even though the latent HSV-1 could not be eliminated completely from the trigeminal ganglia by discontinuous administration of either drug, its titers were markedly reduced. The combination of CC and ACV had a synergistic effect on preventing the reactivation of the latent HSV-1 in vitro.

Latently infected ganglia have been thought to be the source of virus for recurrent herpetic diseases in humans (8). At present, antiviral chemotherapy is generally recognized as the most promising way to eliminate or control latent herpes simplex virus (HSV) infection of ganglia. Unfortunately, although the prophylactic administration of the available drugs can prevent the establishment of latent HSV infection, there is no drug that has any effect on already established latent HSV infection (2, 7, 10, 11, 13). The efficacy of acyclovir (ACV) in preventing the in vitro reactivation of latent HSV has already been confirmed (6, 7, 9), but so far nothing has been reported about the effectiveness of 2,2′-O-cyclocytidine (CC), which has been used in the People’s Republic of China for the treatment of HSV ocular diseases for more than 10 years and which has an antiviral effect that has been confirmed in different types of HSV keratitis (3). In this study we investigated the effects of CC and ACV on latent HSV infection in trigeminal ganglia (TG) in combination or singly, and made a preliminary exploration of the possibility of eliminating or controlling latent HSV infection by discontinuous treatment with CC and ACV.

MATERIALS AND METHODS

Virus. HSV type 1 (HSV-1) strain SM44 was obtained from the Institute of Virology, China National Centre for Preventive Medicine, and was propagated on African green monkey kidney cells (Vero cells) to yield a titer of 10^6.5 50% tissue culture infective doses (TCID_{50}) per milliliter. Eagle’s minimal essential medium was used.

Mice. Inbred strain 615 mice (age, 5 weeks; weight, 15 to 18 g) were maintained for at least 1 week before the experiment.

Drugs. CC and 9-(2-hydroxyethoxymethyl)guanine (ACV) used in this study were supplied by the Shanghai No. 12 Pharmaceutical Factory and the Huabei Institute of Medicine Industry, respectively. One percent of CC solution and ACV suspension were prepared and stored at −10°C until used.

Inoculation of HSV-1. The scarified ears of mice were inoculated with a 10^3 μl suspension of HSV-1 (10^3.5 TCID_{50}/ml, i.e., 10 times the 50% lethal dose), as described previously (10).

Determination of virus titer in TG. At 3 to 8 weeks after the mice were inoculated with HSV-1, they were killed, and the TG were removed and placed in explant cultures in the absence or presence of drug. At various time intervals the TG were washed and homogenized. The titer of the reactivated virus in the homogenates was determined by a TCID_{50} assay in Vero cells.

RESULTS

Effect of CC and ACV on in vitro reactivation of latent HSV-1 in TG. The concentrations of CC and ACV used were 5, 10, 25, and 50 μg/ml and 1, 2.5, 5, and 10 μg/ml, respectively. The TG were randomly divided into groups of 12 and incubated in medium containing different concentrations of either CC and ACV for 4 days.

One group of TG was treated with both drugs.

The results (Fig. 1) showed that 10 μg of CC per ml and 2.5 μg of ACV per ml significantly inhibited the in vitro reactivation of latent HSV-1 in TG. The greatest inhibitory effects were obtained with 25 μg of CC per ml and 10 μg of ACV per ml. In both doses, the virus titers were 3 log TCID_{50} lower than those of the controls.

Duration of the effects of CC and ACV on the inhibition of the in vitro reactivation of latent HSV-1. The TG were randomly divided into six groups and put into the medium containing CC (25 μg/ml) or ACV (10 μg/ml) and incubated for 4 days. At that time the TG were placed into fresh drug-free medium and incubated for another 3 to 4 days. The virus titers were determined, and the results are presented in Table 1.

The virus titer in TG cultured in 25 μg of CC per ml for 4 days was 10^4.23 TCID_{50} on the day 1 after removal of the drug. This was significantly different from that of the control (P < 0.001), but the inhibitory effect was reduced by 50% as compared with the effect on day zero (Fig. 1). On day 2 after removal of the drug, the virus titer was not significantly different from that of the control (P > 0.05). The virus titers in TG cultured in medium containing 10 μg of ACV per ml for 4 days were 3.33 and 3.51 log TCID_{50} lower than those of the controls on days 1 and 2 after removal of the drug, respectively. Both inhibitory effects were similar on day zero. On day 3, the virus titer was 1.27 log TCID_{50} lower than that of the controls; and the inhibitory effect was only

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60% on days 0, 1, and 2. On day 4, the virus titer was not different from that of the controls ($P > 0.05$), which suggests that the effect of ACV on the reactivation of latent HSV could be maintained for 3 days after removal of the drug.

**Effect of CC and ACV on the reactivated ganglionic HSV-1 in vitro.** To initiate active replication of latent HSV, TG were cultured in drug-free medium for 3 days and then recultured in medium containing 25 μg of CC per ml or 10 μg of ACV per ml and cultured for an additional 4 days. At that time the TG were washed three times with fresh drug-free medium and incubated in drug-free medium. The virus titers were determined on days 0, 1, and 2 after removal of the drug.

The results of this experiment demonstrate that the virus titer required for reactivation of TG cultured in drug-free medium for 3 days and then in 25 μg of CC per ml for 4 days was $10^{3.5}$ TCID$_{50}$ on day zero after removal of the drug. This titer was 1 log TCID$_{50}$ lower than that of the controls ($P < 0.005$). On day 1 after removal of the drug, the virus titer was not significantly different from that of the controls ($P > 0.1$).

In the group treated with ACV, the virus titers were reduced by 2.77 and 2.44 log TCID$_{50}$ on days 0 and 1 after removal of the drug, respectively ($P < 0.001$). On the second day, the virus titer was not significantly different from that of the controls ($P > 0.20$). These results suggest that both the extent and duration of viral inhibition by ACV were superior to those by CC.

**Effects of discontinuous CC and ACV treatment on the in vitro reactivation of HSV-1 from latently infected TG.** Forty-eight TG were randomly divided into four groups of 12 each. Two groups were cultured in medium containing 25 μg of CC per ml or 10 μg of ACV per ml for 3 days and then transferred to drug-free medium for another 3 days. This cycle from drug-containing to drug-free medium was done three times. The other two groups were cultured in drug-free medium for 3 and 18 days, respectively. The virus titers were determined at the intervals indicated.

The results show that at the end of 18 days of incubation in drug-free medium, the virus titer was $10^{3.5}$ TCID$_{50}$, which was not significantly different from that in TG cultured for 3 days in the absence of drug ($P > 0.20$). From this, it was suggested that the loss of virus from the cultures was not caused by inactivation by the same culture medium during prolonged incubation periods (18 days). In the groups treated with CC and ACV, the virus titers were $10^{3.5}$ and $10^{4.0}$ TCID$_{50}$ and 3 and 2.5 log TCID$_{50}$ lower than those of the controls, respectively ($P < 0.001$) (Table 2).

**Effect of the combination of CC and ACV on the in vitro reactivation of latent HSV-1 in TG.** Concentrations of CC (5 μg/ml) and ACV (1 μg/ml) that exerted a threshold effect on viral growth (Fig. 1) were used. TG cultured in drug-containing medium for 4 days and virus titers were determined immediately after removal of the drug.

The results (Table 3) show that the virus titer in TG cultured in drug-containing medium was $10^{4.0}$ TCID$_{50}$, or 2.76 log TCID$_{50}$ lower than that of the controls ($P < 0.001$). This effect approximated that of 25 μg of CC per ml or 10 μg of ACV per ml alone. CC (5 μg/ml) or ACV (1 μg/ml) administered alone, however, had no effect on the reactivation of HSV-1. This suggests that the combination of CC and ACV has a synergistic effect on the in vitro reactivation of latent HSV-1 in TG (Table 3).

**DISCUSSION**

In this study we investigated the effects of CC and ACV on latently infected TG of mice maintained in explant culture.

### TABLE 1. Duration of effects of CC and ACV on the in vitro reactivation of latent HSV-1 in TG

<table>
<thead>
<tr>
<th>Group</th>
<th>Antiviral drug in medium (days)</th>
<th>Virus titer (log TCID$_{50}$ ± SD) on the following days after removal of drug from medium$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Control (no drug)</td>
<td>4</td>
<td>5.00 ± 0.44</td>
</tr>
<tr>
<td>CC (25 μg/ml)</td>
<td>4</td>
<td>3.23 ± 0.48</td>
</tr>
<tr>
<td>ACV (10 μg/ml)</td>
<td>4</td>
<td>1.67 ± 0.36</td>
</tr>
</tbody>
</table>

$^a$ Each value represents the mean for six TG. P values are the significant level of difference between drug treatment group and drug-free control group on the same day.
Corneas of mice were infected with HSV-1. At 3 to 8 weeks after inoculation, the virus entered a latent state. The TG of mice that survived were removed for explant culture. The positive rate of isolation of virus from TG was about 80%. The isolated virus was identified as the SM44 strain of HSV-1 by using HSV characteristic antisera.

Our results (Fig. 1) show that CC and ACV can effectively prevent the in vitro reactivation of latent HSV-1. The greatest effect was obtained with 10 μg of ACV per ml, which was similar to the results of 5 to 10 μg/ml obtained by others (7). The effect of 25 μg of CC per ml was equivalent to that of 10 μg of ACV per ml. However, after removal of the drug, the effect of CC did not last as long as that of ACV, as the inhibitory effect of ACV could be maintained for 3 days, whereas CC was effective only for 1 day.

The latent HSV-1 were not completely eliminated by treatment with reactivated virus, but its titer was greatly reduced. The generally accepted explanation is that the reactivation of all latent HSV-1 is not a simultaneous process in vitro (10). This could leave at least part of the total viral reservoir to serve as the source for future infectious virus once the drug is removed from the medium. The exact state of the viral genome during latency is not yet known. It is clear that a rational approach to therapy of latent HSV-1 infection cannot be made until this question is resolved.

Pavan-Langston et al. (10) and Kaufman et al. (5) have suggested that the frequent switching of an explant culture of latently infected ganglia from drug-free to drug-containing medium leads to a significant decrease in the proportion of ganglia containing virus that can be reactivated and that the recurrence of herpetic eye diseases is prevented or controlled in the subclinical state. However, the results presented in Table 3 show that after a three-cycle treatment, in which TG were alternately cultured in the presence and absence of drug, the virus titer was not reduced to zero.

The combination of drugs often has been used in the treatment of HSV-1 infection. This procedure may increase the effect of each drug and reduce the toxicity and the incidence of resistant strains. Results of experiments by Janz and Wigand (4) and Varnell et al. (12) confirm that the combination of antiviral drugs show additive or synergistic effects in varying degrees in vitro and in animals. The effect of the CC-ACV combination has not been previously studied. The initial results (Table 3) show that the combination of CC-ACV may have a synergistic effect in vitro. It is known that the mechanism of activity of CC is different from that of ACV (1), although both results in the inhibition of DNA synthesis. Since ACV is phosphorylated by a virus-specific thymidine kinase, it has a high degree of selectivity. It also possesses low toxicity. However, its clinical use is greatly limited because of its poor water solubility. Although CC will inhibit both viral and host DNA synthesis, it is readily soluble in water and has no cross-resistance with IDU and PAA (1). The combination of CC-ACV might prove to be a good therapy.

TABLE 2. Effect of discontinuous CC and ACV treatment on reactivation of HSV-1

<table>
<thead>
<tr>
<th>Group</th>
<th>Condition</th>
<th>Virus titer (log TCID&lt;sub&gt;50&lt;/sub&gt; ± SD)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3 days</td>
<td>3.83 ± 0.48 (P &gt; 0.20)</td>
</tr>
<tr>
<td>Control</td>
<td>18 days</td>
<td>3.50 ± 0.41 (P &gt; 0.20)</td>
</tr>
<tr>
<td>CC</td>
<td>1 3-day cycle</td>
<td>4.00 ± 0.54 (P &gt; 0.05)</td>
</tr>
<tr>
<td>ACV</td>
<td>1 3-day cycle</td>
<td>3.23 ± 0.48 (P &lt; 0.01)</td>
</tr>
<tr>
<td>ACV</td>
<td>3 3-day cycles</td>
<td>1.00 ± 0.44 (P &lt; 0.001)</td>
</tr>
</tbody>
</table>

* Each value represents the mean for 13 TG.

TABLE 3. Effect of combination of CC and ACV on reactivation of HSV-1

<table>
<thead>
<tr>
<th>Group</th>
<th>Virus titer (log TCID&lt;sub&gt;50&lt;/sub&gt; ± SD)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.76 ± 0.36 (P &lt; 0.001)</td>
</tr>
<tr>
<td>CC-ACV</td>
<td>2.00 ± 0.52 (P &lt; 0.001)</td>
</tr>
<tr>
<td>Control</td>
<td>4.87 ± 0.50 (P &gt; 0.05)</td>
</tr>
<tr>
<td>CC (5 μg/ml)</td>
<td>4.40 ± 0.51 (P &gt; 0.05)</td>
</tr>
<tr>
<td>Control</td>
<td>3.62 ± 0.45 (P &gt; 0.05)</td>
</tr>
<tr>
<td>ACV (1 μg/ml)</td>
<td>3.71 ± 0.44 (P &gt; 0.05)</td>
</tr>
</tbody>
</table>

* Each value represents the mean for 13 TG.

LITERATURE CITED