Establishment of Herpes Simplex Virus Latency in vitro with Cycloheximide

BY KIMIYASU SHIRAKI AND FRED RAPP*

Department of Microbiology, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033, U.S.A.

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SUMMARY

Human embryonic lung cells were infected with herpes simplex virus (HSV), treated with 10 μg/ml or more of cycloheximide for 24 h, incubated at 37 °C, and then shifted to 40.5 °C for various periods of time (0 to 40 days) without cycloheximide treatment. No infectious virus was detected after freezing and thawing of the cultures; however, infectious virus was recovered after temperature shift-down to 37 °C or superinfection with human cytomegalovirus (HCMV). The time course for formation of infectious centres after temperature shift-down was examined with and without HCMV superinfection during incubation at 40.5 °C. Two patterns of latently infected cells were identified: one pattern showed spontaneous reactivation of virus after temperature shift-down, and the second showed reactivation of HSV after superinfection with HCMV. The first pattern showed a rapid decrease in the number of infectious centres with time, whereas the second maintained a steady reactivation rate up to 40 days at 40.5 °C. The same tendency was observed for infectious centre formation at 37 °C with and without HCMV superinfection in the HSV latency system established with (E)-5-(2-bromovinyl)-2'-deoxyuridine and interferon treatment.

The growth of herpes simplex virus (HSV), which is strongly cytolysic in permissive cells, is inhibited both specifically and non-specifically by a variety of drugs during different stages of virus infection. The effects of cycloheximide on HSV-infected cells have been well studied (Honess & Roizman, 1974; Fenwick & McMenamin, 1984). The drug inhibits the translation of proteins and results in accumulation of immediate early mRNA, which encodes for the immediate early (α) proteins, when administered at the initial phase of infection (Rakusanova et al., 1971; Honess & Roizman, 1974; Marsden et al., 1976).

An in vitro HSV latency system established by treatment with inhibitors and maintenance at increased temperature after inhibitor removal has been reported (Colberg-Poley et al., 1979, 1981; Wigdahl et al., 1981, 1982). Reactivation of latent HSV was observed after superinfection with human cytomegalovirus (HCMV; Colberg-Poley et al., 1979, 1981) or reduction of the incubation temperature (Wigdahl et al., 1981). This paper describes the establishment of HSV latency in human embryonic lung (HEL) cells with cycloheximide. In addition, the state of infected cells at high temperature was examined by infectious centre formation at permissive temperature with or without HCMV superinfection, and the time course of reactivation of latent virus from different cell populations with or without HCMV superinfection was compared in the (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU)/interferon and the cycloheximide latency systems.

HEL and Vero cells were grown and maintained in Dulbecco's medium supplemented with 10% and 2% foetal calf serum, respectively. HSV type 1 (HSV-1; strain Patton) and HCMV (strain AD169) were grown in HEL cells. Infected cells were frozen and thawed three times followed by centrifugation at 1200 g for 10 min. The supernatants were frozen and stored at −70 °C. Mock antigen was made in the same way without virus infection. Confluent HEL cell cultures (25 cm² plastic flask containing 1 × 10⁶ to 2 × 10⁶ cells) were infected with 0.5...
p.f.u./cell of HSV-1 for 1 h at room temperature, washed three times with maintenance medium, and treated with maintenance medium containing 10 or 50 µg/ml cycloheximide for 24 h at 37 °C. The treated cultures then were washed three times with maintenance medium without cycloheximide, fed maintenance medium without cycloheximide, and incubated at 40.5 °C for various periods of time (0 to 40 days). Culture medium was changed every 3 days or twice weekly. Cultures to be tested for infectious virus were frozen and thawed three times and then centrifuged at 1200 g for 10 min. One ml of the supernatant was inoculated onto Vero cells in plastic Petri dishes (35 mm diam.) and allowed to adsorb for 2 h at room temperature. Virus-inoculated cultures were fed maintenance medium and observed for 10 days for the development of c.p.e. Infectious HSV was not detected in cultures (46 cultures) during incubation at 40.5 °C from 1 to 14 days.

Cultures treated with cycloheximide and incubated at 40.5 °C were shifted to 37 °C and culture medium was tested daily for infectious virus on Vero cells as described above. Infectious HSV was recovered from all cultures (a total of 63) incubated at 40.5 °C (1 to 14 days) after shift-down to 37 °C. The period before recovery of infectious virus after temperature shift-down to 37 °C ranged from 1 to 10 days. These results suggest that HSV remained in HEL cells in a non-infectious state that could be reactivated and, therefore, was considered latent.

Infectious centre assay of cultures incubated at 40.5 °C was performed to examine the state of infected cells with or without HCMV superinfection. Reactivation of latent HSV by temperature reduction (37 °C) also was quantified by infectious centre assay. The number of infectious centres after temperature shift-up to 40.5 °C was assessed at various periods of time (0 to 14 days) on Vero cells at 37 °C. Cultures that had been treated with cycloheximide and incubated at 40.5 °C were examined for formation of infectious centres. After mock superinfection or superinfection with HCMV (2.0 p.f.u./cell) for 1 h at room temperature, treated cultures were washed twice with Tris-buffered saline (pH 7.4; TBS) and once with Versene and trypsin, and dispersed cells were suspended in 2 ml maintenance medium. The cell suspension (0.2 ml) was then diluted in serial 10-fold dilutions with maintenance medium, inoculated onto Vero cells in plastic dishes (35 mm), and then overlaid with 1% methylcellulose nutrient medium. After incubation for 7 days at 37 °C, cultures were fixed with 5% neutral formalin and stained with methylene blue. For each treatment two cultures were assayed in parallel.

Latently infected HEL cells treated with BVdU and interferon and subsequently mock-superinfected or superinfected with HCMV (Wigdahl et al., 1982) were examined for infectious centres. Confluent HEL monolayers were pretreated with medium containing BVdU (10 µg/ml) and human leukocyte interferon (125 IU/ml) overnight at 37 °C, infected with HSV-1 (0.5 p.f.u./cell) and treated for 7 days with daily changes of medium containing inhibitors. The cultures were then washed twice with TBS, medium without inhibitors was added, and the cultures were incubated at 40.5 °C. Infectious centre assays were performed in the same manner as for cultures treated with cycloheximide.

The time course of infectious centre formation at 37 °C is shown in Fig. 1. Two patterns of latently infected cells were identified: one that showed spontaneous reactivation of virus after temperature shift-down and a second that showed reactivation of HSV after superinfection with HCMV (Fig. 1, Table 1). The first pattern showed a rapid decrease in the number of infectious centres with time; in contrast, the second pattern maintained a steady reactivation rate. The number of cells that reactivated virus after HCMV superinfection was maintained at 40.5 °C up to 40 days (Table 1). The profile of reactivation did not differ whether latency was established with 10 or with 50 µg/ml cycloheximide (Fig. 1).

The profile of reactivation for latency induced by BVdU and interferon was essentially the same as that induced by cycloheximide (data not shown). The dose–response data showed that the number of infectious centres reactivated by HCMV decreased significantly between 0.2 and 0.02 p.f.u./cell in both systems (Table 1).

In vitro HSV latency can be established after treatment with DNA virus inhibitors and maintenance at high temperature, as well as after cycloheximide treatment and increased incubation temperature. Latent HSV can be reactivated in at least two ways: by reducing the
Fig. 1. Time course of infectious centres after temperature shift up to 40.5 °C. The cultures were infected with HSV, treated with 10 μg/ml cycloheximide, mock-infected (○) or superinfected with HCMV (●), or treated with 50 μg/ml cycloheximide, mock-infected (△) or superinfected with HCMV (▲) and incubated at 40.5 °C without cycloheximide.

Table 1. Reactivation by superinfection with HCMV

<table>
<thead>
<tr>
<th>Superinfection (m.o.i.)</th>
<th>BVdU and interferon</th>
<th>Cycloheximide†</th>
<th>7 days</th>
<th>20 days</th>
<th>30 days</th>
<th>40 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>2.5 × 10¹</td>
<td>UN‡</td>
<td>UN</td>
<td>UN</td>
<td>UN</td>
<td>UN</td>
</tr>
<tr>
<td>HCMV 0.2</td>
<td>6.2 × 10³</td>
<td>3.18 × 10³</td>
<td>3.25 × 10³</td>
<td>3.3 × 10³</td>
<td>3.08 × 10³</td>
<td></td>
</tr>
<tr>
<td>0.02</td>
<td>6.9 × 10³</td>
<td>1.45 × 10³</td>
<td>ND§</td>
<td>1.7 × 10³</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>0.002</td>
<td>3.5 × 10¹</td>
<td>2.5 × 10¹</td>
<td>ND</td>
<td>2.25 × 10¹</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>0.0002</td>
<td>5.0 × 10¹</td>
<td>7.5 × 10⁶</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

* Results are expressed as the number of infectious centres per culture obtained from two duplicate cultures.
† Cultures were treated with 50 μg/ml cycloheximide after infection and were maintained at 40.5 °C for the times indicated.
‡ UN, Undetectable (<5 × 10⁰).
§ ND, Not determined.

incubation temperature to 37 °C or by superinfecting with HCMV. Reactivation of latent HSV at 37 °C quantified by infectious centre assay revealed that the number of cells that reactivated virus after temperature reduction decreased rapidly with time, whereas the number that reactivated virus after HCMV superinfection was steady. This suggests that the HSV-1 genome reactivated by HCMV superinfection but not by reduction of incubation temperature may represent a more stable form in these experimental systems.

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