Latency In vitro using Irradiated Herpes Simplex Virus

By YUKIHIRO NISHIYAMA AND FRED RAPP*

Department of Microbiology and Specialized Cancer Research Center, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033 U.S.A.

(Accepted 14 August 1980)

SUMMARY

Human embryonic fibroblasts infected with u.v.-irradiated herpes simplex virus type 2 (HSV-2, strain 186) and maintained at 40.5 °C did not yield detectable virus. Virus synthesis was induced by temperature shift-down to 36.5 °C. The induced virus grew very poorly and was inactivated very rapidly at 40.5 °C. Non-irradiated virus failed to establish latency at 40.5 °C in infected cells. Enhanced reactivation of HSV-2 was observed when latently infected cultures were superinfected with human cytomegalovirus (HCMV) or irradiated with a small dose of u.v. light at the time of temperature shift-down. HCMV did not enhance synthesis of HSV-2 during a normal growth cycle but did enhance synthesis of u.v.-irradiated HSV-2. These observations suggest that in this in vitro latency system, some HSV genomes damaged by u.v. irradiation were maintained in a non-replicating state without being destroyed or significantly repaired.

INTRODUCTION

Herpes simplex virus (HSV) can latently infect sensory ganglia of humans and experimental animals after productive acute infection (Stevens, 1975). Two hypotheses, termed static- and dynamic-state theories, have been proposed to explain virus–cell interactions during latency (Roizman, 1965); recent in vivo studies seem to favour the former theory (Stevens, 1975; Puga et al., 1978). However, the biochemical mechanisms involved in latency have remained unclear partly because of the lack of appropriate in vitro models. Most in vitro systems reported so far are characterized as carrier cultures in which there is continued virus production and the phenomenon of latency-reactivation cannot be observed (Hampar & Copeland, 1965; Nii, 1969; Robey et al., 1976; Nishiyama & Rapp, 1979). O’Neill and co-workers (1972, 1977) developed an HSV type 2 (HSV-2) latency model whereby infectious virus became undetectable and was reactivated only by temperature shift-down after prolonged treatment with cytosine arabinoside (ara-C) and elevated incubation temperatures. However, the mechanisms involved in this in vitro model remain unexplained due to the complicated procedure involved in the establishment of latency.

This report describes the establishment of an in vitro HSV-2 latency model using u.v.-irradiated virus. Reactivation of latent virus by superinfection with human cytomegalovirus (HCMV) or u.v. irradiation of the cultures was obtained. The observations suggest that HSV-2 genomes damaged by u.v. irradiation are maintained in a non-replicating state at 40.5 °C without being destroyed or repaired until reactivation occurs.

METHODS

Cells and viruses. Flow 5000 cells (human embryonic fibroblasts, purchased from Flow Laboratories, Rockville, Md., U.S.A.) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal calf serum, penicillin (100 units/ml) and streptomycin (100
μg/ml), and were in passages 15 to 22. Primary rabbit kidney (PRK) cells were prepared from weanling rabbit (21- to 28-day-old) kidneys and grown in 60 mm plastic culture dishes containing the medium described above. HSV-2 strain 186 (clone 3) isolated from a single plaque and HCMV strain AD169 were used throughout this study. Both viruses were propagated in Flow 5000 cells by inoculation at low multiplicities (0.01 to 0.1 p.f.u./cell). Infected cultures were harvested when almost all cells exhibited cytopathic effects (c.p.e.). After freezing and thawing three times and eliminating cell debris by centrifugation at 3000 rev/min for 10 min, viruses were stored at -80 °C.

Plaque assay. Virus assays were performed on confluent PRK cell monolayers in 60 mm plastic culture dishes infected with appropriate dilutions of virus. After a 1 h adsorption period, cells were overlaid with 5 ml 0.5% agarose in growth medium. Three to 4 days later, cultures were fixed with 5% formalin, stained with 0.7% crystal violet and plaques counted using a dissecting microscope at a x10 magnification. As described elsewhere (Nishiyama & Rapp, 1980), HCMV does not produce plaques in PRK cells and plaque production by non-irradiated HSV-2 is not influenced by the presence of HCMV.

U.v. irradiation. HSV suspensions were diluted 1 : 10 in tris-buffered saline and a 1 ml vol. in 60 mm plastic culture dishes was exposed to a General Electric G8T5 u.v. bulb for appropriate time intervals at a dose of 20 ergs/mm²/s as measured by a Black-Ray u.v. dosimeter model J-225; dishes were swirled constantly during irradiation. To irradiate cells, medium was removed, cells were washed with tris-buffered saline and then irradiated for 2 s at a dose of 20 ergs/mm²/s.

Establishment of HSV-2 latency. HSV-2 stocks were u.v. irradiated for 10 min at a dose of 20 ergs/mm²/s as described and confluent monolayers of Flow 5000 cells in 60 mm plastic culture dishes or in 25 cm² plastic culture bottles were exposed to irradiated virus at a multiplicity (before irradiation) of 0.3. Under these conditions, less than 100 plaques/culture formed when incubated at the permissive temperature (36.5 °C). After a 1 h adsorption period at 40.5 °C, cells were washed with tris-buffered saline and incubated with prewarmed growth medium at 40.5 °C. The medium was replaced with fresh prewarmed growth medium twice weekly.

RESULTS

Growth and inactivation of HSV-2 at 36·5 and 40·5 °C

One-step growth experiments were performed to determine the replicative capacity of HSV-2 (186) at 36·5 and 40·5 °C. Confluent monolayers of Flow 5000 cells were infected with HSV-2 at a multiplicity of 3 and incubated for 1 h at either temperature. After washing twice with tris-buffered saline, cells were incubated with growth medium at 36·5 or 40·5 °C. At various intervals after infection, the cultures were examined for c.p.e. and then frozen for virus assay. As shown in Fig. 1(a), virus growth was markedly suppressed at 40·5 °C compared to 36·5 °C and titres did not exceed 10⁴ p.f.u./ml. However, infected cells incubated at 40·5 °C developed the extensive c.p.e. evident in cells incubated at 36·5 °C (Fig. 1a), resulting in complete cell destruction.

Virus inactivation was also examined at each temperature. Viruses suspended in growth medium (pH 7·5) were incubated in a 5% CO₂ atmosphere under the same conditions used in the one-step growth experiments and samples were removed at various intervals and assayed for infectivity. Fig. 1(b) shows that HSV-2 was inactivated much more rapidly at 40·5 °C than at 36·5 °C. Virus infectivity (approx. 3 × 10⁶ p.f.u./ml) was almost completely destroyed at 40·5 °C within 48 h, whereas at 36·5 °C, more than 10⁴ p.f.u./ml was recovered 48 h after the initiation of incubation.
Establishment of HSV-2 latency and reactivation of latent virus

Confluent monolayers of Flow 5000 cells were infected with u.v.-irradiated HSV-2 at 0.3 p.f.u./cell (less than 100 p.f.u./culture when measured as infectious virus) and incubated at 36.5 or 40.5 °C. When cells were maintained at 36.5 °C, small plaque-like c.p.e. appeared 24 h post-infection (p.i.) and spread over the entire surface of the cultures by 72 h p.i. In contrast, neither c.p.e. nor infectious virus was detectable when cells were incubated at 40.5 °C. However, temperature shift-down from 40.5 to 36.5 °C caused the appearance of infectious virus and development of c.p.e. even when carried out more than 20 days after the initiation of latency (Fig. 2a). C.p.e. always appeared in plaque-like foci and then spread.
Table 1. Effect of superinfection with HCMV on replication of HSV-2

<table>
<thead>
<tr>
<th>Infection with HSV-2</th>
<th>Superinfection with HCMV*</th>
<th>Incubation temperature (°C)</th>
<th>Virus yield (p.f.u./ml) at h p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>Non-irradiated virus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(m.o.i. 3)</td>
<td></td>
<td>36.5</td>
<td>1.2 × 10⁶</td>
</tr>
<tr>
<td>+</td>
<td></td>
<td>36.5</td>
<td>1.0 × 10⁶</td>
</tr>
<tr>
<td>—</td>
<td></td>
<td>40.5</td>
<td>2.7 × 10³</td>
</tr>
<tr>
<td>+</td>
<td></td>
<td>40.5</td>
<td>1.0 × 10³</td>
</tr>
<tr>
<td>—</td>
<td></td>
<td>36.5</td>
<td>4.2 × 10³</td>
</tr>
<tr>
<td>Non-irradiated virus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(m.o.i. 5 × 10⁻⁵)‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
<td>36.5</td>
<td>1.8 × 10³</td>
</tr>
<tr>
<td>—</td>
<td></td>
<td>40.5</td>
<td>&lt;10</td>
</tr>
<tr>
<td>+</td>
<td></td>
<td>40.5</td>
<td>&lt;10</td>
</tr>
<tr>
<td>—</td>
<td></td>
<td>36.5</td>
<td>&lt;10</td>
</tr>
<tr>
<td>U.v.-irradiated virus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(m.o.i. 0-3)§</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
<td>36.5</td>
<td>&lt;10</td>
</tr>
<tr>
<td>—</td>
<td></td>
<td>40.5</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

* Flow 5000 cells were infected with u.v.-irradiated or non-irradiated HSV and then superinfected with HCMV at a multiplicity of 0.1.
† Not determined.
‡ Equal to 100 p.f.u./culture.
§ Virus suspensions were u.v. irradiated for 10 min at a dose of 20 ergs/mm²/s. Cells were infected with u.v.-irradiated virus at a multiplicity of 0.3 (<100 p.f.u./culture when measured as infectious virus).

Cultures superinfected with HCMV or irradiated with u.v. light at the time of temperature shift-down demonstrated enhanced reactivation of latent virus. However, reactivation was not observed at 40.5 °C even when cultures were treated with HCMV or u.v. irradiation (data not shown).

Further experiments were performed to determine whether infection of cells with non-irradiated HSV-2 would establish latency at 40.5 °C. When confluent monolayers of Flow 5000 cells were infected with HSV-2 at a low multiplicity (approx. 100 p.f.u./culture) and maintained at 40.5 °C, minute amounts of infectious virus were detectable for a few days p.i. and became undetectable by 5 days p.i.; virus did not reappear in cultures shifted-down from 40.5 to 36.5 °C at 7 days p.i. (Fig. 2b).

Effect of HCMV on replication of HSV-2

As described above, reactivation of latent HSV-2 after temperature shift-down was markedly enhanced by superinfection with HCMV. Previous reports from our laboratory had shown that HCMV enhances the reactivation of u.v.-irradiated HSV-2 in cells non-permissive for HCMV synthesis (Nishiyama & Rapp, 1980) and that HCMV induces the reappearance of HSV-2 from a quiescent state (Colberg-Poley et al., 1979b). Therefore, we examined the effects of HCMV infection on HSV-2 growth at 36.5 and 40.5 °C in Flow 5000 cells which are permissive for both HSV-2 and HCMV. Flow 5000 cells were infected with u.v.-irradiated or non-irradiated HSV-2. After a 1 h adsorption period, cells were superinfected with HCMV at a multiplicity of 0.1 and maintained at 36.5 or 40.5 °C. The results showed that HCMV did not enhance HSV-2 growth when cells were infected with non-irradiated HSV-2 and maintained at 40.5 °C. However, enhancement by HCMV was observed in cultures infected with u.v.-irradiated HSV-2 and maintained at 36.5 °C (Table 1). Further experiments (Fig. 3) confirmed this result. HCMV enhancement was most evident in cultures infected with HSV-2 irradiated for 15 min with u.v. light. These observations indicate that HCMV does not enhance HSV-2 growth but does enhance reactivation of u.v.-irradiated HSV-2.
Enhanced growth of u.v.-irradiated HSV-2 by superinfection with HCMV. Confluent monolayers of Flow 5000 cells were infected with non-irradiated virus at a low multiplicity (100 p.f.u./culture), or infected with u.v.-irradiated virus at 0.3 p.f.u./cell. After 1 h of HSV adsorption, 50% of the cultures were infected with HCMV at 0.1 p.f.u./cell, maintained at 36.5 °C and the cells harvested 48 h p.i. for infectivity assay. Virus suspensions were exposed to u.v. at a dose of 20 ergs/mm²/s as described in Methods. Virus titre is expressed as the ratio of HSV titres in HCMV-infected cells to uninfected cells. Four separate experiments are shown (○).

Table 2. Effect of u.v. irradiation on virus reactivation

<table>
<thead>
<tr>
<th>Time p.i. (days)*</th>
<th>Number of infectious centres/culture†</th>
<th>U.v. irradiation‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>22.0</td>
<td>19.5</td>
</tr>
<tr>
<td>5</td>
<td>17.0</td>
<td>18.5</td>
</tr>
<tr>
<td>10</td>
<td>7.5</td>
<td>14.5</td>
</tr>
<tr>
<td>15</td>
<td>6.5</td>
<td>19.0</td>
</tr>
<tr>
<td>20</td>
<td>4.5</td>
<td>12.5</td>
</tr>
</tbody>
</table>

* Confluent monolayers of Flow 5000 cells were infected with HSV-2 irradiated with u.v. for 10 min at a dose of 20 ergs/mm²/s at a multiplicity of 0.3 and maintained at 40.5 °C. At 5-day intervals the cultures were shifted down to 36.5 °C.
† Cells were covered with 0.25% agarose in growth medium and maintained at 36.5 °C for 7 days. Numbers are the average of duplicate cultures.
‡ Cells were exposed to u.v. at a dose of 40 ergs/mm² at the time of temperature shift down.

Effect of u.v. irradiation on virus reactivation

U.v. irradiation of latently infected cultures at temperature shift-down also enhanced synthesis of HSV-2. In these experiments, we noted that the number of small plaque-like foci which appeared after temperature shift-down was greater in u.v.-irradiated cultures than in non-irradiated cultures. The following experiment was carried out to determine whether irradiation of latently infected cultures would increase the number of infectious centres. Confluent monolayers of Flow 5000 cells were infected with u.v.-irradiated HSV-2 (10 min at 20 ergs/mm²/s) at a multiplicity of 0.3 and incubated for 1 h at 40.5 °C. After washing twice with tris-buffered saline, the cultures were maintained in growth medium at 40.5 °C. At 5-day intervals the cultures were shifted down to 36.5 °C. Cells were irradiated at a dose of 40 ergs/mm², then overlaid with 0.25% agarose in growth medium, maintained at 36.5 °C, and fixed 7 days after temperature shift-down. After staining, plaques were counted under a dissecting microscope. The results (Table 2) indicate that a small dose of u.v. irradiation increased the number of infectious centres reactivated following temperature shift-down.
DISCUSSION

When human cells in culture were exposed to HSV-2 irradiated with an appropriate dose of u.v. light and maintained at 40.5 °C, virus was not detectable but was induced after temperature shift-down to 36.5 °C. As shown in Fig. 1, HSV-2 grew very poorly and was rapidly inactivated at 40.5 °C; however, infected cells were destroyed at both 40.5 and 36.5 °C. It was also shown that infection of cells with non-irradiated virus failed to establish latent infection at 40.5 °C. Given these observations, it seems unlikely in this latent system that viruses are maintained in a dynamic state at 40.5 °C in which a small number of cells constantly produce infectious virus without destroying the equilibrium between virus and cell. The data seem to suggest that HSV genomes damaged by u.v. irradiation are maintained in a non-replicating state without being destroyed or repaired. This explanation of the mechanism(s) involved in our latency model is supported by the following observations. (i) Reactivation of latent viruses by temperature shift-down was markedly enhanced by superinfection with HCMV. We have shown previously that HCMV enhances the reactivation of u.v.-irradiated HSV-2 in HCMV-non-permissive cells (Nishiyama & Rapp, 1980). Furthermore, the results in Table 2 and Fig. 3 demonstrate that HCMV did not enhance replication of HSV-2 but enhanced the reactivation of u.v.-irradiated HSV-2 in Flow 5000 cells. (ii) U.v. irradiation of latently infected cultures increased the number of infectious centres after temperature shift-down, and u.v. irradiation of cells is known to enhance reactivation of u.v.-irradiated HSV (Bockstahler & Lytle, 1970; Lytle et al., 1974), although it has also been shown that cell irradiation inhibits HSV growth (Fenwick, 1977). It is noteworthy that both treatments, HCMV infection and u.v. irradiation, which enhance the reactivation of HSV in latent cultures, enhance the capacity of DNA repair (Lytle et al., 1974; Y. Nishiyama & F. Rapp, unpublished data).

At present, it is not known how HSV genomes damaged by u.v. irradiation survive in the cells without being destroyed or repaired. Since HSV-2 can express its c.p.e. at 40.5 °C, it is reasonable to assume that even u.v.-irradiated viruses are present in an uncoated state in the nuclei at 40.5 °C. However, if damaged virus DNA were completely repaired at 40.5 °C, it would express the cytopathic function, kill the cell and fail to establish latency. Recently, Biegeleisen and co-workers (1976, 1977) demonstrated that HSV DNA may be associated with host cell DNA during productive infection. Hirai (1979) also reported that HSV DNA is associated with cell DNA by alkali-labile bonds even in non-productive infection established in the presence of phosphonoacetic acid (PAA). Their observation might help to explain the physical state of HSV-2 DNA in latently infected cultures maintained at 40.5 °C.

O’Neill and co-workers (1972, 1977) and Colberg-Poley et al. (1979a, b) have reported the establishment of HSV-2 latency in vitro by prolonged treatment of cells with DNA inhibitors such as ara-C and PAA and elevated incubation temperatures. Although there are some significant differences between the two systems, it is possible that similar mechanisms are involved in the maintenance of latency since ara-C can produce chromosomal damage and can be incorporated into DNA (O’Neill & Rapp, 1971).

In our experiments we used human embryonic fibroblasts to establish latency. Since it has been shown that HSV is harboured in a latent state in neurons of sensory ganglia (Stevens, 1975), neuronal cells might be useful for study of latency. However, preliminary attempts to establish a latent system in neuroblastoma cells were unsuccessful, due partly to the requirement for long maintenance at high temperature. However, the virus–cell interaction described may prove useful in understanding the molecular mechanisms involved in HSV latency.

This investigation was supported by Public Health Service contract NO1 CP 53516 within the Virus Cancer Program of the National Cancer Institute and Public Health Service grant CA 18450 awarded by the National Cancer Institute.
REFERENCES


(Received 3 April 1980)