Herpes Simplex Virus Latency in Cultured Human Cells Following Treatment with Cytosine Arabinoside

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SUMMARY

Treatment of human cell cultures infected with herpes simplex virus type 2 (HSV-2) with cytosine arabinoside resulted in the prevention of both virus cytopathology and synthesis of detectable infectious virus. Following removal of the inhibitor infectious HSV-2 reappeared and the cultures were destroyed. However, there was a delay of at least 5 to 6 days following inhibitor removal before infectious virus could be detected. The reappearance of infectious HSV-2 was paralleled by virus cytopathology. The period between the disappearance and reappearance of infectious virus is defined as the latent period. Cultures during this period were as sensitive as control cultures to superinfection with HSV-2 or vesicular stomatitis virus. Infectious centre assays performed with cultures during the latent period indicated that as many as 1 in 800 cells were capable of ultimately synthesizing infectious virus. Attempts to prevent the reappearance of infectious HSV-2 by treating infected cultures with cytosine arabinoside for up to 22 days were unsuccessful, thus indicating that HSV-2 can remain associated with the cells in a non-infectious form for an extended period without being degraded.

INTRODUCTION

Until the recent reports suggesting an association between herpes simplex virus (HSV) infection and cervical carcinoma (Rawls et al. 1968; Naib et al. 1969; Aurelian, Royston & Davis, 1970) the illness produced by this virus was considered to be relatively innocuous (Roizman, 1965). Only approximately 1 % of the individuals with primary infections were thought to suffer severe illness (Scott & Tokumaru, 1965).

Interest in this virus as an infectious agent has continued because of the observation that many individuals periodically suffer the discomfort of HSV-induced cold sores or fever blisters. Of greater interest, however, is that this disease tends to be recurrent, often over long periods. The recurrent lesions usually involve the same location in the oral cavity or other areas where the primary lesion occurred. Although infectious virus can usually be isolated from active lesions, it generally cannot be detected at locations where lesions have healed and is generally undetectable between recurrences (Roizman, 1965).

It has been argued that in periods when infection is inapparent, the virus is latent (Roizman, 1965; Scott & Tokumaru, 1965) and may be harboured in some unknown ‘virogenic’ cell where virus multiplication has been interrupted reversibly (Roizman, 1965). Following physical (Good & Campbell, 1948; Ellison, Carton & Rose, 1959), emotional (Warren,
Carpenter & Boak, 1940) or chemical stimulation (Schmidt & Rasmussen, 1960) the virus multiplies, resulting in herpetic eruptions or fever blisters.

Attempts to mimic HSV latency in vitro have not been entirely successful (Roizman, 1965). Although virus-carrier cultures have been established in which there is minimal cell destruction, infectious virus can usually be detected when the cells are disrupted (Roizman & Turner, 1960; Szanto, 1963; Hinze & Walker, 1961; Roizman, 1965).

The present report describes a human cell culture system in which cell destruction and virus multiplication following infection with HSV type 2 (HSV-2) is prevented by the DNA inhibitor, cytosine arabinoside (ara-C). Furthermore, it is shown that HSV-2 remains associated with these cells in a non-infectious state for various periods while retaining the potential to produce infectious progeny at a later time.

**METHODS**

**Cell cultures.** Human embryonic lung (HEL) cultures were grown in Eagle's medium enriched with 10% foetal bovine serum and containing 100 units of penicillin and 100 μg. streptomycin/ml. Only cells up to the 20th subcultivation were employed. Cultures of primary rabbit kidney cells were prepared as described by Rapp (1963) and propagated in Eagle's medium with 10% calf serum and the antibiotics.

**Viruses.** Herpes simplex virus type 2 (HSV-2), strain 316-D, was obtained from Dr W. E. Rawls, Baylor College of Medicine, Houston, Texas. This virus was passed twice and plaque-purified twice in rabbit kidney cells before one passage in HEL cells. Virus stocks were prepared by inoculating confluent monolayers of HEL cultures in 8 oz. bottles with HSV-2 at an input of 1 p.f.u./cell. Forty hr after infection the cultures were frozen and thawed twice and the fluids clarified by low-speed centrifugation. The virus was titrated in rabbit kidney monolayers in 60 mm. Petri dishes under a methylcellulose overlay (Rapp, 1963). Vesicular stomatitis virus (VSV, Indiana type) was propagated in HeLa cells and titrated on HEL monolayers under an agar overlay (O'Neill & Rapp, 1971 a).

**Infectious centre assays.** Replicate cultures treated with ara-C after infection with HSV-2 were trypsinized and washed once in growth medium. Approximately 2 × 10^6 cells from each of two replicate cultures were pooled and suspended in 2 ml. of growth medium. Serial dilutions of this cell suspension were made in medium. The cell suspensions from these dilutions were inoculated on to uninfected HEL cultures in 1 oz. prescription bottles growing as monolayers. The bottles were then inoculated with growth medium and fed twice weekly. Two HEL cultures were used for each dilution of cells. As a control, the remaining undiluted cell suspension was frozen and thawed 3 times and inoculated undiluted and at 1/10 on to replicate HEL monolayers. After adsorption for 1 hr the cultures were overlaid with medium containing 1% methylcellulose. Four days later the cultures were stained with crystal violet and examined for HSV plaques. In a number of experiments, a second methylcellulose overlay was added at 4 days post-inoculation and the cultures observed for c.p.e. for an additional 3 days.

**RESULTS**

Ara-C has been shown to be an effective inhibitor of DNA synthesis by interfering with the reduction of cytidine diphosphate (Chu & Fischer, 1962, 1965; Silagi, 1965). It may also be incorporated into cellular DNA (Chu & Fischer, 1965, 1968; Silagi, 1965). At concentrations as low as 10 μg./ml., ara-C has been shown to inhibit replication of HSV (Rapp, 1964) and
Fig. 1. Effect of cytosine arabinoside (ara-C) on the persistence of infectious HSV-2 in human embryonic lung cultures. Replicate confluent cultures were pretreated for 24 hr with ara-C at the concentrations shown and then inoculated with HSV-2 at an input multiplicity of 1 p.f.u./cell. Following virus adsorption the cultures were fed with medium containing ara-C which was changed daily. Replicate samples were harvested periodically and tested in rabbit kidney cells for the presence of infectious virus. Concentrations of ara-C in medium: ●, 0.1 μg./ml.; ○, 1.0 μg./ml.; △, 10 μg./ml.; ▽, 20 μg./ml.; □, control without ara-C.

synthesis of virus and cellular DNA in HSV infected cell cultures (Levitt & Becker, 1967; O’Neill & Rapp, 1971a). Virus-induced chromosome abnormalities were not prevented by this inhibitor (O’Neill & Rapp, 1971b). Because of its capacity to halt HSV-2 replication, ara-C was employed in these experiments in an effort to obtain non-productively infected HEL cultures.

**Effect of cytosine arabinoside on HSV-2 infection**

These experiments were made to determine the concentration of ara-C necessary to prevent completely the synthesis of infectious virus. We also determined the time required for the disappearance of infectious HSV-2 from infected HEL cultures when effective concentrations of ara-C were employed. Confluent, replicate HEL monolayers in 1 oz. prescription bottles were infected with HSV-2 at an input of 1 p.f.u./cell and the virus was allowed to adsorb at room temperature for 1 hr. The cultures were exposed for 24 hr before infection and after infection to growth medium containing ara-C at concentrations of 0.1, 1.0, 10 or 20 μg./ml. The ara-C medium was changed daily. Two replicate samples from cultures from each ara-C concentration were harvested periodically and assayed for the presence of
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Fig. 2. Course of reappearance of HSV-2 following removal of ara-C. Replicate confluent cultures of HEL were pretreated with ara-C (10 μg./ml.) for 24 hr. HSV-2, at an input multiplicity of approximately 1 p.f.u./cell, was then inoculated and allowed to adsorb for 1 hr. The cultures were then fed with fresh medium containing ara-C and refed daily with this inhibitor containing medium. At 7 days after infection all replicate cultures were washed once with tris buffer and refed with inhibitor free growth medium. The medium was changed twice weekly. All cultures were then examined daily (microscopically) for the presence of virus c.p.e. Replicate samples were harvested periodically and assayed in rabbit kidney cells for the presence of infectious virus.

The results show that ara-C at a concentration of 0.1 μg./ml. did not prevent the production of infectious virus (Fig. 1). At 4 days after infection these cultures were destroyed almost completely and assays for infectious virus were terminated. Ara-C at a concentration of 1 μg./ml. produced some inhibition of virus replication but did not prevent destruction of the cells. However, at concentrations of 10 and 20 μg./ml. there was almost total inhibition of virus synthesis, so that by 4 days after inoculation no infectious virus could be detected (< 10 p.f.u./ml.). The inability of these cultures to produce infectious HSV continued through 9 days post-infection (Fig. 1). Microscopic examination of these cultures showed that the cells appeared normal although partial toxicity was apparent about 7 days after infection.

Attempts to ‘cure’ HSV-2 infected HEL cultures

Preliminary experiments were performed in an attempt to ‘cure’ HSV-2 infected cultures by treatment with ara-C for periods up to 7 days. The results showed that when the inhibitor was removed and the cells fed with ara-C free growth medium, infectious virus could always be recovered. However, there was a significant delay before infectious virus could be detected (Fig. 2). Thus we determined the period of continuous ara-C treatment, if any, which would eliminate all infectious virus and prevent its return even after the inhibitor was removed. Confluent HEL cultures in 1 oz. bottles were pretreated with ara-C (10 μg./ml.) for 24 hr and then infected with HSV-2 at a multiplicity of 1 p.f.u./cell. Following adsorption for 1 hr the
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...cultures were fed with growth medium containing ara-C (10 μg./ml.). The medium was changed daily for the first 6 days but only twice weekly thereafter for cultures with ara-C treatment extending for 9 to 21 days. Replicate cultures were washed once with tris buffer and inoculated periodically with inhibitor free growth medium from 7 to 22 days after infection. At least five replicate cultures were employed for each of the 4 ara-C treatment periods. Following removal of the inhibitor all replicate cultures were examined carefully each day under the microscope for foci of HSV-2 c.p.e. The dates of the appearance of foci were recorded. Also beginning at 2 days after infection, and every second day thereafter, replicate cultures were assayed for infectious virus in rabbit kidney cells. At 4 days after infection, no infectious virus could be detected (< 10 p.f.u./ml.). The results from these experiments showed that prolonged treatment with ara-C for periods up to 22 days did not prevent subsequent return of virus c.p.e. (Table I). However (Table I), there was a significant delay between the time of removal of ara-C and the appearance of visible foci of virus c.p.e. This delay was usually 6 to 11 days, although single foci of c.p.e. could be detected occasionally as early as 5 days after ara-C removal (Table I). It was also noted that the appearance of this virus c.p.e. was usually isolated with only 1 or 2 such foci appearing in each of the replicate cultures. However, within 1 to 2 days after the appearance of these foci, c.p.e. became widespread. The identity of the virus was confirmed as HSV-2 in neutralization tests with rabbit anti-serum to HSV.

Establishment of latent HSV-2 infected cells

Experiments were designed to determine if HSV-infected cultures contained infectious virus during the period between reversal of ara-C treatment and appearance of HSV c.p.e. Approximately 50 replicate HEL cultures in 1 oz. bottles were pretreated for 24 hr with 10 μg./ml. of ara-C. HSV-2 at a multiplicity of 1 p.f.u./cell was then adsorbed to the cells for 1 hr. The cultures were inoculated with fresh medium containing ara-C (10 μg./ml.) and the medium with inhibitor was changed daily until the seventh day after infection. At this time all of the replicate cultures were washed once in tris buffer and then fed with growth medium without inhibitor. The medium was then changed twice weekly. Following removal of ara-C, all cultures were examined daily for the presence of virus c.p.e. At approximately 2-day intervals (see legend to Fig. 2) replicate samples were harvested and assayed for the presence of infectious virus on rabbit kidney cells. The results from these experiments showed that infectious HSV could not be detected (< 10 p.f.u./ml.) in replicate samples by 4 days after infection and remained undetectable for 6 days after removal of the ara-C (13 days after infection, see Fig. 2). At 13 days after infection nearly 30 % of the replicate cultures showed microscopic evidence of virus c.p.e. This figure increased to 65 % on day 14 and continued to increase until day 18, when all the remaining cultures showed evidence of virus c.p.e. (Fig. 2). The reappearance of infectious virus coincided with the appearance of virus c.p.e.

Table 1. Reappearance of herpes simplex virus (HSV-2) in human cell cultures treated with cytosine arabinoside (ara-C) for various periods

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<tr>
<th>Removal of ara-C (days after infection)</th>
<th>Time (days) before appearance of cytopathology</th>
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in replicate cultures and occurred in high yields since infectivities over 10^7 p.f.u./ml. were obtained in cultures harvested 48 hr after appearance of virus c.p.e. (Fig. 2).

The possibility remained that the observed inability to detect infectious virus during the latent period (days 4 to 13) was the direct result of a cytotoxic effect induced by ara-C. To test this possibility, replicate HEL cultures in the latent period were tested for their ability to support the synthesis of HSV-2 and VSV. Replicate HEL cultures were pretreated with ara-C and infected with HSV-2 as before. The cultures were fed daily for 6 days with medium containing inhibitor. On the seventh day after infection the cultures were washed once with tris-buffered saline and fed with medium without inhibitor. Two days after removal of ara-C from the medium, two replicate cultures were inoculated with either HSV-2 or VSV at a multiplicity of 1 p.f.u./cell. Following adsorption for 1 hr the cultures were fed with inhibitor-free growth medium and incubate at 37°C for 24 hr. Infectious virus was then harvested and assayed on rabbit kidney cells. As controls, uninfected HEL cultures were also treated with ara-C and, with untreated uninfected HEL cultures, were similarly infected with HSV-2 or VSV. The results (Table 2) show that the cultures containing non-infectious HSV supported the replication of both viruses as well as did the control cultures.

**Infectious centre assays**

Replicate HEL cultures were treated with ara-C and infected with HSV-2 as before. Two replicate cultures were harvested for infectious centre assays on days 4, 7, 9, 11 and 14 after infection. Samples taken on days 9, 11 and 14 were from cultures maintained on inhibitor-free medium since day 7. Cells from these cultures when inoculated on to fresh HEL cultures attached to the monolayer within a few hours after plating. The cultures were fed twice weekly with growth medium and examined daily for the presence of foci of HSV-c.p.e. C.p.e. usually became apparent between 3 to 5 days post-inoculation but the cultures were observed for at least 7 days. The results showed some variability in the number of cells synthesizing infectious virus (Table 3). The level varied from 1 in 800 cells at days 4 and 11 after infection to 1 in 20,000 cells at day 7 after infection. Infectious virus was not found in

| Table 2. Susceptibility of latent phase cells to superinfection with 1 p.f.u. of either herpes simplex virus type 2 (HSV-2) or vesicular stomatitis virus (VSV, Indiana type) |
|-----------------|-----------------|-----------------|
| Cell type       | HSV-2 (p.f.u./ml.) | VSV (p.f.u./ml.) |
| Latent phase cells | 6.4 x 10^6       | 8.1 x 10^5       |
| Ara-C treated cells | 4.2 x 10^6       | 1.0 x 10^5       |
| Untreated cells | 5.0 x 10^6       | 4.0 x 10^5       |

| Table 3. Infectious centre assays of cytosine arabinoside (ara-C) treated HEL cultures inoculated with 1 p.f.u. of herpes simplex virus type 2 per cell |
|-----------------|-----------------|-----------------|
| Time (days) after HSV-2 inoculation | Infectivity (p.f.u./ml.) | Fraction of cells yielding infectious virus |
| 4               | < 10            | 1:800           |
| 7               | < 10            | 1:20,000        |
| 9               | < 10            | 1:2400          |
| 11              | < 10            | 1:800           |
| 14              | 10^5            | 1:160           |
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control assays on days 4, 7, 9 and 11 after infection. On day 14, as expected, infectious virus was found in the control cultures. These results were reproducible and numerous experiments also showed similar variability in the number of infectious centres. Analysis of all available data indicates that an average of 1 in 4000 cells produced infectious virus during the latent period.

DISCUSSION

The results of these experiments show that cytosine arabinoside, at concentrations as low as 10 μg./ml., prevented the production of infectious herpes simplex virus in human cells for as long as 3 to 4 weeks. When this inhibitor was removed there was a delay of at least 5 to 6 days before infectious HSV-2 reappeared. During the period between the disappearance and subsequent reappearance of infectious virus, the HSV-2 was latent. Infectious centre assays performed on cells during this period showed that although infectious virus was not detected, some cells contained HSV-2 in a non-infectious form. Probably more cells contained the virus genome than were detected as the plating efficiency of the HEL cells on HEL cells was low. Three to 5 days following plating of these cells on to the permissive cells, infectious virus and virus c.p.e. appeared. Since HSV-2 remained associated with HEL cells in a non-infectious form for such an extended period, the results suggest that the virus genome is not readily degraded by the cell under these conditions.

The mechanism responsible for the delay in the reappearance of infectious HSV-2 is not known. One possibility, mentioned in Results, is the apparent toxic effect of ara-C on HEL cultures. This effect may impede normal biosynthetic events and thus prevent maturation of HSV-2. This appears unlikely since superinfection of these cells with HSV-2 or VSV for 24 hr resulted in the production of large amounts of infectious virus which were not distinct from those obtained in control cultures. However, since only a small percentage of cells in the culture appeared to be capable of producing infectious virus, the possibility that these cells were damaged reversibly cannot be excluded completely. Another possibility is that the mere washing of these cultures and inoculation with fresh medium is not sufficient to remove enough inhibitor to allow synthesis of virus DNA and completion of the HSV-2 replication. The elimination of ara-C from these cultures may occur over a period as prolonged as 6 days. The results of the superinfection experiments also appear to discount this possibility since residual ara-C would be expected to inhibit superinfection by HSV-2. The apparent non-participation of interferon in this system was established by failure to detect interferon in fluids harvested from replicate cultures during the latent phase.

Since it has been shown that both ara-C (Nichols & Heneen, 1964; O'Neill & Rapp, 1971b) and HSV (Hampar & Ellison, 1961; Stich, Hsu & Rapp, 1964; Rapp & Hsu, 1965; O'Neill & Miles, 1969) can produce chromosome damage and that ara-C can be incorporated into DNA, it is possible that the virus DNA is defective during the latent period. The time required for the reappearance of infectious virus may coincide with the effort of the cell to repair this damage. Still another possibility is that the virus DNA becomes associated with nuclear DNA and thus is protected from normal degradative processes. Resumption of normal nuclear DNA synthesis following removal of ara-C may allow for a dissociation between the nuclear and virus DNA. These possibilities are being investigated.

Although latency of HSV in vitro has not been described previously, several reports describe persistent in vitro infections with this virus. In most of these studies cultured cells were infected with HSV at low multiplicities and were propagated in medium containing anti-HSV serum (Roizman & Turner, 1960; Hinze & Walker, 1961; Szanto, 1963). Under these conditions extracellular spread of HSV was prevented and the cells continued to
multiply, sometimes for months (Hinze & Walker, 1961; Szanto, 1963). Persistent infection has also been described in L cells following exposure to a high input multiplicity of HSV (Nii, 1969). The chronic infection was established without use of virus-specific antibody in the medium. However, foci of HSV-like c.p.e. usually appeared and infectious HSV could be isolated from the cultured cells although not always from the culture fluids (Roizman, 1965; Nii, 1969). Thus, unlike our system, these cells were not, at any time, really free of infectious virus. Hampar & Burroughs (1969) reported persistent in vitro infection of Chinese hamster cells by HSV. In their system persistence depended upon a genetically determined cell–virus equilibrium in which continued passage of cells resulted in increased resistance to HSV infection. However, these persistently infected cultures did not appear to be free of infectious virus.

The persistence of infectious virus in previously reported in vitro carrier systems perhaps resembles recurrent HSV infections in man and is consistent with the ‘dynamic state’ hypothesis in which there is a constant but slow turnover of infected cells (Roizman, 1965). In the system reported here, it is shown that following the prevention of synthesis of detectable infectious virus, HSV remains stable and is not degraded by the host cell. Thus, although this system is somewhat artificial because of the use of ara-C, it fulfils the criteria defined for the ‘static state’ hypothesis (Roizman, 1965) in which the production of infectious virus is completed following specific stimulation of virogenic cells harbouring eclipsed HSV blocked during some early stage in its reproductive cycle. These results are also consistent with the many failures to detect infectious virus in the healed sites of HSV-induced lesions in the interim between recurrences (Roizman, 1965). Investigation of the fate of HSV-2 during the latent period and of the mechanisms regulating the reappearance of infectious virus in this system may clarify the problem of virus latency in man.

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