Persistent Herpes Simplex Virus Infection In Vitro with Cycles of Cell Destruction and Regrowth

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ABSTRACT

HAMPAR, Berge (National Institute of Dental Research, Bethesda, Md.), and Mary Lou Copeland. Persistent herpes simplex virus infection in vitro with cycles of cell destruction and regrowth. J. Bacteriol. 90:205–212. 1965.—The susceptibility of two Chinese hamster cell lines to herpes simplex virus (HSV) was studied from the time of their initiation through successive subcultures. The cells' susceptibility to the cytocidal effects of HSV decreased as the number of cell passages increased. During the early cell passages, the decrease in cell susceptibility to HSV was characterized by an increased time after infection for complete cell destruction to occur, with a concomitant increase in the period when virus could be recovered from supernatant fluids. This was followed by a number of cell passages during which persistent HSV infections were established. The persistent infections were characterized by (i) cycles of virus synthesis and cell destruction followed by regrowth of the cells, (ii) initiation and maintenance of cell growth in the absence of antibody, (iii) the cells' ability to be passaged while still maintaining their cycling patterns, (iv) a relationship between virus synthesis and cell proliferation, and (v) inability of long-term treatment with antibody to "cure" the persistent infections. The unique characteristics of this HSV infection were compared with other persistent in vitro viral infections.

In our original studies (Hammar and Ellison, 1961), we stated that Chinese hamster cell lines other than the MCH line were not susceptible to infection with herpes simplex virus (HSV). Subsequently, other workers showed that Chinese hamster cell lines were indeed susceptible to infection by this virus (Mazzone and Yerganian, 1963; Stich, Hsu, and Rapp, 1964). Since we had used established cell lines in our studies, it was of interest to resolve this apparent discrepancy by following the susceptibility of two lines of Chinese hamster cells to HSV from the time of their initiation as cell cultures through subsequent passages in the laboratory. Our findings show that the susceptibility of these cells to the cytocidal effects of HSV infection gradually decreased as the number of cell passages increased; the decreased susceptibility was paralleled by alterations in the cells' morphology and growth characteristics. Ultimately, HSV infection gave rise to persistent infections in the absence of viral antibody. These infections were characterized by cycles of cell destruction, with virus synthesis followed by regrowth of the cells.

MATERIALS AND METHODS

Cell line. Two lines of Chinese hamster cells were used. Line FEL was derived by George Yerganian and supplied to us as first-passage material. This line is presently in its 63rd passage. Line MAL was derived in this laboratory from the lungs of an adult male animal and is presently in its 75th passage. Since both cell lines gave similar results, the discussions to follow are primarily limited to the MAL line. The distinguishing morphological characteristics of the sex chromosomes in the Chinese hamster (Yerganian, 1959) were utilized to eliminate the possibility of cross-contamination. The only other cell line maintained during the course of these experiments was a line of HeLa cells used for virus propagation. All cell lines were grown as monolayers in plastic petri dishes at 37°C in a humidified atmosphere of 3% CO2, with medium previously described (Hampar and Ellison, 1961, 1964). Stock cultures were passaged weekly with trypsin (0.25%) to disperse the cells. All cultures, control and infected, were routinely fed three times per week.

Virus. The SAE strain of HSV (Hampar and Ellison, 1961) was used throughout these experiments. This strain of HSV has been passaged over 90 times in HeLa cells and produces a typical non-proliferative type of cytopathic effect (CPE; Gray, Tokumaru, and McNair Scott, 1958). Virus was titered by use of the tube dilution method in HeLa cells, and, after 7 days, the TCID50 was calculated by the method of Reed and Muench (1938).

Infection of hamster cells. Hamster cells grown in 60-mm plastic petri dishes (ca. 6 × 10⁴ cells
per plate) were inoculated with 0.5 ml of appropriate virus dilutions. At least two and usually three cultures were used for each virus dilution tested. Cells were incubated at 37°C for 2 hr, after which the original inoculum was removed, and the cell sheets were washed five times with Hanks' balanced salt solution (BSS). Heat-inactivated herpes-immune rabbit serum (0.2 ml) plus 1.8 ml of medium was added to each culture, followed by incubation for an additional 30 min. The cell sheets were again washed five times with BSS and fed with 4 ml of medium. The original virus inoculum and final wash was titered. In no case did the remaining virus titer exceed 10^7 TCID₅₀ per ml.

Virus titrations were performed at least three times per week, usually at the same time cells were to be fed. The entire supernatant fluid was removed, and a 0.5-ml sample was used for titration of virus. Cells were then fed with 4 ml of fresh medium. When titrations were to be performed on a day when the cells were not scheduled to be fed, only 0.5 ml of the supernatant fluid was removed and replaced with 0.5 ml of fresh medium. In this way, all cultures, control and infected, were fed on the same day with the same batch of medium.

**Determination of virus CPE.** Cells were usually observed daily for evidence of virus-induced CPE. The degree of CPE was recorded as ranging from 0 to 5, indicating 0, 20, 40, 60, 80, and 100% of the attached cells, respectively. When no viable cells were evident by careful microscopic examination, the CPE was recorded as +5. These cultures were subsequently maintained and observed for at least 7 days to insure that no cells remained. Though this type of data necessarily depended upon subjective analysis, it should be borne in mind that the CPE was recorded 7 days before the titer of infective virus was known. Further, the severity of CPE and relationship to virus titer were very reproducible, both in separate experiments and in parallel cultures of the same experiment.

**RESULTS**

*Decrease in cell susceptibility to the cytocidal effects of HSV during early cell passages.* Both the FEL and MAL cell lines, as originally isolated and during their initial passages, were fibroblastic in appearance. The karyotypes during the first 10 passages in culture remained diploid, but by the 15th passage both lines were classified as aneuploid (near diploid) and have remained so during subsequent passages. Between their 26th and 30th subcultures, both cell lines underwent alterations in their cell morphology from typical fibroblast to epithelial-like cells. These changes were accompanied by a decrease in cell doubling time from 16 to 14 hr, and an increase in cell density after 1 to 2 weeks in culture from 6 to 8 x 10⁶ cells per 60-mm plate to approximately 4 x 10⁷ cells per plate. The fact that the changes in cell morphology of MAL cells occurred 2 months prior to similar changes in FEL cells would eliminate the medium used as the causative factor.

The effects of HSV infection during the first 30 passages of these cells was studied. Typical results are shown in Fig. 1, where the passage numbers of the cells used and the titer of the original virus inocula are indicated. A definite change occurred in the cells' reaction to HSV infection as the number of subcultures increased. During the early passages (Fig. 1A), cell destruction and virus synthesis occurred relatively rapidly. As the number of cell passages increased beyond 10, the time required for complete cell destruction after virus infection increased, with a concomitant increase in the period of virus synthesis (Fig. 1B). Additional studies were undertaken to determine the effect of varying the size of the initial viral inoculum. The results indicated that the gradual decrease in cell susceptibility to the cytocidal effects of HSV was independent of the virus-cell ratio used over a wide range (10^-4 to 10). The type of CPE produced was dependent upon the number of cells present at the time of infection. With more than 10⁷ cells per plate, the areas of CPE were not clear, but the cell sheet persisted intact with the loci of infection expanding to contiguous cells. This was followed in a few days by the appearance of distant loci of infection until ultimately the entire cell sheet, though still intact, had a mottled appearance. The cell sheet finally dislodged from the petri dish as a single unit. In contrast, with less than 10⁷ cells per plate, CPE was characterized by clear plaquelike areas of cell destruction which ultimately involved the entire cell sheet. Apparently, virus transfer occurred by both the cell-to-cell and extracellular routes (Stoker, 1958).

*Establishment and characteristics of persistently infected cultures.* The findings described thus far were typical for all cultures tested (over 30) during the MAL cells' initial 32 passages in culture. In contrast, all cultures of MAL cells tested subsequent to their 35th passage gave rise to persistent infections when inoculated with a virus-cell ratio of one or less. These infections were characterized by cycles of virus synthesis with concomitant cell destruction followed by regrowth of the cells. Typical results are shown in Fig. 2 where parallel cultures of 45th-passage MAL cells were treated in various ways. Figure 2A shows the effects after infection of cells with 10⁵ TCD₅₀ of HSV. The cells entered their cycling pattern rapidly, with no delay in either CPE or virus synthesis. During the first 100 days of
Fig. 1. (A) Virus synthesis (solid and broken lines) after infection of 8th-passage MAL cells with 10^6, 10^4, and 10^2 TCID_{50} of HSV. CPE (not shown) in these cultures paralleled the increase in virus titers, and complete cell destruction was evident 1 to 2 days after maximal virus titers were attained. (B) Virus synthesis (solid line) and CPE (bars) after infection of 26th-passage MAL cells with 10^4 TCID_{50} of HSV.

persistent infection, there were six-plus cycles averaging approximately 16 days. Figure 3 shows the morphological changes observed from days 69 to 102 in these cultures. The photographs were taken of the same area on one plate on the days indicated. The description which follows is typical of all persistently infected cultures studied to date. An almost intact cell sheet with minimal CPE was present at the beginning of the cycle (Fig. 3A). When the CPE reached a maximum (Fig. 3C), very few viable cells remained. These cells grew out until, during the first part of the next cycle, an almost full cell sheet was again present (Fig. 3E). CPE began again, and, at its maximum, only one viable cell (Fig. 3F) was evident in this part of the petri dish. Figure 3G shows the area of regrowth present at the beginning of the next cycle. A number of areas on different plates have been similarly studied, and, from these we have made the following conclusions regarding the cycling patterns. First, there was a definite relationship between virus synthesis and cell proliferation; i.e., cell proliferation in each cycle occurred prior to, or parallel with the increase in virus titer. The fall in virus titer seen during the second half of each cycle was probably due to inactivation of virus already present. Second, the time for each cycle was relatively stable (ca. 16 days in Fig. 2A). Third, the rise in virus titer and increased CPE (first part of cycle) required about twice the time as the decrease in virus titer and CPE. This is not surprising, since the decrease in CPE was due to dislodgement of infected cells, which occurred relatively rapidly. Fourth, relatively few cells were required for the maintenance of persistently infected cultures. Less than 10^6 cells were present during the period of maximal CPE, and an average of 2 x 10^4 to 3 x 10^4 cells were present after regrowth during each cycle. Finally, persistent infections also occurred when cells were infected with hamster cell-adapted virus (virus passaged at least 15 times in MAL cells).

Studies were also carried out to determine the effect of various virus-cell ratios on the cells' ability to initiate persistent infections. When 45th-passage MAL cells (parallel cultures to those described in Fig. 2) were inoculated with 10^6 TCID_{50} of HSV (virus-cell ratio, 10), CPE and virus synthesis increased rapidly, followed by a fall in titer up to the 15th day. The cells recovered and have been maintained for over 5 months with no further evidence of intra- or extracellular virus present. In no case has the infection of cells, even with high virus-cell ratios (>20), resulted in destruction of the entire cell
Figure 2. Virus synthesis (lines) and CPE (bars) in persistent infections of 45th-passage MAL cells. No observations for CPE were made from day 45 to 54. (A) Persistent infection with an original virus inoculum of $10^4$ TCID$_{50}$. These cultures were passaged on day 62. (B) Persistent infection with an original virus inoculum of $10^5$ TCID$_{50}$. These cultures were passaged on day 62. (C) Same cultures as in (B), except that here the cultures were passaged eight times (arrows) during their first 100 days of infection.

At least a few cells always survived infection and proliferated to replenish the cell sheet. When these cells were tested by reinfection with HSV, they showed at least a 10-fold decrease in susceptibility, as measured by their ability to form a persistent infection, their ability to adsorb virus, and their ability to produce plaques. Parallel cultures of 45th-passage cells were also tested with an original inoculum of $10^6$ TCID$_{50}$ of HSV (Fig. 2B). From Fig. 2A and 2B, it can be seen that the maximal virus titers reached during each cycle were lower with the smaller inoculum. This occurred although the severity of CPE, after the first one or two cycles, was judged to be the same in all cultures. Table 1 shows the results of similar experiments with 55th-passage MAL cells. All of these cultures became persistently infected. The time of first virus appearance and maximal virus titers during the first cycle are shown. Persistent infections were initiated when cells were infected with virus-cell ratios from $2 \times 10^{-8}$ to 20. This is in contrast to the results described with 45th-passage cells where only one cycle occurred when a virus-cell ratio of 10 was used. From similar experiments with different passage cells, the following conclusions were made. First, the maximal virus-cell ratio capable of initiating a persistent infection increased as the number of cell passages increased. This was apparently due to continual changes in the susceptibility of MAL cells with time in passage to HSV. Second, the minimal virus-cell ratio capable of initiating an overt infection resulted in a persistently infected culture. Third, the time required for the appearance of virus and CPE varied with the titer of the original inoculum, e.g., the smaller the original virus-cell ratio used, the longer the period required for virus and CPE to appear. This relationship of size of virus inoculum to time of virus synthesis has been described by others with HSV (McNair Scott et al., 1953; Wheeler, 1960).

The ability of these cultures to maintain persistent infections after passage of the cells was also studied. Cells from infected cultures were dispersed by treatment with trypsin (0.25%), followed by centrifugation and suspension in growth medium. At least 10$^4$ cells were seeded in each of a number of 60-mm petri dishes, with a final volume of 4 ml of medium. We have thus far maintained over 250 cultures which have been passaged at weekly or longer intervals. In no case has such a culture failed to survive and maintain its persistent infection. Figure 2C shows the effects after a number of cell passages of persistently infected 45th-passage cells. These cultures were derived from the cultures described in Fig. 2B. As seen in Fig. 2C, the cycling pattern was maintained after passage of the cells. When cells were passaged during the first part of the cycle (increasing virus titers), they maintained the rise in titer during the next few days. Similarly, CPE proceeded normally after an apparent
FIG. 3. CPE during cycles of persistently infected MAL cells (see Fig. 2A). The photographs (A through G) were taken of the same area on the days indicated in the graph (H). Live cells. X 80.
but misleading decline. For instance, after the 4th passage, the virus titer continued to rise, whereas CPE appeared to decrease on the day after passage (Fig. 2C). This apparent decline was due to the inability of cells showing CPE to attach subsequent to passage, and they remained suspended in the supernatant fluid. The cells which were still viable and able to attach subsequently showed typical CPE. Conversely, when cells were passed during the period of fall in virus titer, the decrease in CPE subsequent to passage was more rapid than in nonpassaged cultures, again due to the inability of cells already showing CPE to attach.

A number of methods were employed to insure that the virus produced either during the initial 32 passages or subsequent to the onset of persistent infections was still HSV. Three types of herpes antiserum were used to demonstrate neutralization of virus. First, immune rabbit serum was produced by injection of HeLa-adapted HSV. Second, immune rabbit sera prepared against a number of strains of HSV other than the SAE strain were kindly supplied by Warren K. Ashe. These virus strains were grown in rabbit kidney cells and had not been passaged in HeLa or Chinese hamster cells (Ashe and Scherp, 1963). Third, concentrated human γ-globulin used for injection (Lederle Laboratories, Pearl River, N.Y.) was employed. In all cases, neutralization of virus obtained from the supernatant fluids of infected Chinese hamster cell cultures was attained. Infected cells were also studied microscopically, and again the virus was identified as HSV by the appearance of typical virus-induced cellular changes characterized by chromatin margination and intranuclear inclusionlike bodies. Similarly, electron microscopic examination of infected Chinese hamster cells showed numerous virulike particles of typical size and morphology of HSV in the infected cultures.

Finally, although the effects of antisemum on persistently infected cultures will be described in detail elsewhere, we can state that prolonged exposure (over 8 months) to antisemum did not "cure" the persistent infections even when cultures were passaged. Subsequent to the removal of antisemum, CPE appeared, followed by the onset of typical cycling patterns.

We have thus far maintained these cultures for more than 10 months.

**Discussion**

Although other persistent HSV infections in tissue culture have been reported (Wheeler and Canby, 1959; Fernández, 1960; Hinze and Walker, 1961; Szanto, 1963; Coleman and Jawetz, 1961; Glasgow and Habel, 1963), the Chinese hamster system is presently unique in its ability to maintain such an infection in the absence of any known supportive measures. The most common type previously reported required antibody in the medium for the maintenance of low extracellular virus titers. Coleman and Jawetz (1961) reported a persistent HSV infection in the absence of antisemum, but their system required a low level of cellular metabolic activity (31°C) and a small viral inoculum. Glasgow and Habel (1963) described a persistent HSV infection superimposed on a mouse embryo line previously infected with polyoma virus. The maintenance of the double infection was dependent upon the presence of the polyoma virus, which stimulated the production of interferon to levels capable of inhibiting cell destruction due to the HSV. Although we are not aware of the presence of another virus in our system, we cannot exclude the possibility that a second virus may be present which is either indigenous to these cells or has been accidently added during the course of these experiments. If such is the case, then the presence of this virus does not readily result in obvious cellular destruction in either Chinese hamster or HeLa cells.

A number of persistent infections with viruses other than HSV have been described and reviewed (Walker, Hanson, and Evans, 1958; Ginsberg, 1958; Henle, 1963), but we are aware of only one other report in which Chinese hamster cells were employed (Ford, Boguszewski, and Auersperg, 1961), in this case with polyoma virus. Furthermore, cycling patterns have been reported in only two cases. Bang and Gey (1952)

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**Table 1. Time of appearance of virus and time of maximal virus titer during first cycle after inoculation of cultures with different virus-cell ratios**

<table>
<thead>
<tr>
<th>Titer of viral inoculum (PFU) *</th>
<th>Virus-cell ratio (PFU/MAL cell)</th>
<th>Time of first virus appearance in supernatant fluid (days)</th>
<th>Time of maximal virus titer during first cycle (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$9 \times 10^7$</td>
<td>20</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>$9 \times 10^4$</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>$9 \times 10^4$</td>
<td>$2 \times 10^{-1}$</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>$9 \times 10^3$</td>
<td>$2 \times 10^{-5}$</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>$9 \times 10^2$</td>
<td>$2 \times 10^{-6}$</td>
<td>26</td>
<td>30</td>
</tr>
</tbody>
</table>

* Each plate was seeded with $4.5 \times 10^8$ 55th-passage MAL cells.
† Plaque-forming units, based on titration in HeLa cells.
‡ Days after inoculation of virus.
reported cycles of cell destruction and regrowth in a persistent infection of malignant rat cells with eastern equine encephalomyelitis virus, and Henle, Hinze, and Henle (1963) reported similar findings in persistently polyoma-infected L cells.

Although it may seem premature at this point to discuss the mechanism responsible for these persistent infections, we can say that probably more than one factor is involved. We do know that cells partially resistant to HSV are required. The partial resistance of these cells probably accounts for their ability to initiate persistent infections over a wide range of virus-cell ratios, but the maintenance of these cultures will probably be found to involve variations in cell susceptibility and virus-producing ability during the course of each cycle. Interferon or an interferonlike substance, which we know to be produced in this system (Hampar, unpublished data), could give transitory resistance, but the fact that media changes were made three times a week (Glasgow and Habel, 1962), and many cell divisions were necessary prior to the release of new virus, tends to rule this out as the sole factor. A number of possibilities have been considered by Henle, et al. (1963) in their system, but no conclusive conclusions have yet been established to account for the persistence of these cultures.

It is evident from these results that some Chinese hamster cell lines show a decrease in their susceptibility to the cytocidal effects of HSV with increased time in passage. We have thus far studied three cell lines in detail. The MAL and FEL lines are presently able to form persistent infections. The MCH line, which arose from an established line of Chinese hamster cells, was initially able to form a short-term carrier state with HSV (Hampar and Ellison, 1961), followed by a period during which viral effects were only evident when cell-virus mixtures were shaken (Hampar and Ellison, 1963). Ultimately these cells completely lost their susceptibility to HSV (Hampar, unpublished data). Whether this phenomenon occurs with all Chinese hamster lines or is dependent on the conditions we have employed remains to be seen. These results may, however, explain the discrepancy in our original report (Hampar and Ellison, 1961) regarding the lack of susceptibility of Chinese hamster cells to HSV, and the subsequent studies by other investigators who showed that Chinese hamster cells are susceptible to this virus (Mazzone and Yerganian, 1963; Stich et al., 1964). The results reported here indicate that the decrease in cell susceptibility to the cytocidal effects of HSV occurred independently of the change in cell morphology. Whether persistent infections are dependent on the presence of epithelial-like cells is not known.

Finally, it is of interest to compare the cycling pattern in this system with recurrent herpes simplex in man. A cyclical nature (periodic activation) of virus synthesis in the human disease has been considered by Andrews (1958), and the maintenance of antibody levels throughout life has been interpreted by Burnet and Williams (1939) as indicative of "a constant or intermittent antigenic stimulus. . . ." Further work is obviously required to determine whether or not an in vitro system such as described here parallels the events occurring in the human disease.

Addendum in Proof

Rapp and Hsu (Virology 25:491, 1965) recently reported a difference in the sensitivity of three diploid Chinese hamster cell lines to HSV. These lines are comparable to the early cell passages of the lines we employed (prior to aneploidy). One of the three lines studied by these authors (Don) was relatively insensitive to HSV infection, which indicates either a lack of susceptibility of some newly derived (diploid) Chinese hamster cell lines or that the decrease in susceptibility to HSV noted in our studies was not dependent on the cells' conversion to aneploidy. Without further information as to the history of time in passage of their cell lines, this question cannot be answered.

Literature Cited


Ginsberg, H. S. 1958. The significance of the


