Differential Susceptibility to Herpes Simplex Viruses of Hamster Cell Lines Established after Exposure to Chemically Inactivated Herpesvirus

By J. J. DOCHERTY, F. J. O’NEILL AND F. RAPP
Department of Microbiology, College of Medicine, The Milton S. Hershey Medical Centre of The Pennsylvania State University, Hershey, Pennsylvania 17033, U.S.A.

(Accepted 19 July 1971)

SUMMARY

The replication of herpes simplex virus type 1 and herpes simplex virus type 2 was studied in two isolated hamster cell lines. These lines developed after being treated with herpes simplex virus type 2 inactivated with 7,12-dimethylbenz(a)-anthracene. One cell line that developed, HDC-22, had a hypodiploid chromosome number and was missing a D-group chromosome. Growth studies revealed that this cell line did not support the replication of herpes simplex virus type 2 but did support the replication of herpes simplex virus type 1. The other cell line, HDC-17, had a normal karyotype and proved susceptible to both herpes simplex virus type 1 and herpes simplex virus type 2. These hamster cell lines afford an opportunity to study specific resistance to herpes simplex virus and the differences in the replicative cycles of type 1 and type 2 herpesviruses.

INTRODUCTION

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) have been shown to differ in many characteristics by several investigators (Pauls & Dowdle, 1967; Plummer et al. 1970; Nahmias et al. 1968; Figueroa & Rawls, 1969). Although both are often able to replicate in the same cell line, HSV-1 generally grows to a higher titre (Alford, Snider & Stubbs, 1967; Rawls et al. 1968).

Although quantitative differences in replication of HSV-1 and HSV-2 are well established, the growth of one virus type in a cell culture unable to replicate the second virus has not been reported. Recently, while studying several hamster cell lines developed in our laboratory, we noted that HSV-2 did not replicate in one of these lines. Another isolated hamster cell line which was derived from the same hamster embryos supported the replication of HSV-2. By contrast, HSV-1 replicated in a normal fashion in both cell lines. These lines developed and were isolated after primary hamster embryo cells were exposed to HSV-2 inactivated with 7,12-dimethylbenz(a)anthracene (DMBA).

This report describes the development of these hamster cell lines following exposure of hamster embryo cells to chemically inactivated HSV-2. Also presented are the unique characteristics of the cell line that resists productive infection by HSV-2 but supports the replication of HSV-1.
METHODS

Virus. The agents used in these studies were various strains of HSV-1 and HSV-2 obtained from Dr W. Rawls, Baylor College of Medicine, Houston, Texas. Stock virus was prepared by infecting primary rabbit kidney cultures grown in 8 oz. prescription bottles. Infected cultures were harvested 2 days after incubation at 37°C when the cultures exhibited greater than 75% cell destruction. The infected cultures were frozen and thawed twice, sonicated, and clarified by low-speed centrifugation. The clarified supernatant fluid was dispersed in 1 ml. amounts, quick frozen in a dry ice-alcohol bath, and stored at -65°C until used.

Inactivation of virus. The 316-D strain of HSV-2 was inactivated with 50 μg./ml. of DMBA as previously described (Docherty, Goldberg & Rapp, 1971). The DMBA was obtained from Eastman Organic Chemicals, Rochester, New York. The inactivated, as well as active, control virus was concentrated and partially purified by the method of Robinson, Pitkanen & Rubin (1965). Final centrifugation was performed in a Beckman L2-65B ultracentrifuge with an SW 50.1 rotor at 45,000 rev./min. for 2 hr. The virus-chemical band was collected by bottom puncture and used as described.

Virus growth studies. The growth of various strains of HSV-1 and HSV-2 was measured in the cell lines derived in this study. Cell cultures were grown in 1 oz prescription bottles or 16 x 150 mm tissue culture tubes. Various strains of HSV-1 and HSV-2 were inoculated on to the cell lines at a m.o.i. of 1. The virus was allowed to adsorb for 1 hr at room temperature with frequent rotation to uniformly distribute the virus inoculum. The cultures were rinsed once with tris buffer (pH 7.4) to remove unattached virus and overlaid with growth medium (Eagle's basal medium supplemented with 10% foetal calf serum, 10% tryptose phosphate broth, 100 units/ml. of penicillin, 100 μg. of streptomyacin/ml., 100 μg. kanamycin/ml., and 0.075% NaHCO3). The culture vessels were incubated at 37°C and sampled for virus production at various intervals after infection. The samples were harvested by the method described above. Virus titres were based on the formation of cytopathic foci in rabbit kidney monolayers as previously described (Docherty et al. 1971). The tests were carried out under a methylcellulose overlay. These values are expressed as p.f.u. per ml.

Cells. Primary hamster embryo cells were prepared according to the procedure described in detail by Duff & Rapp (1970). The animals were random-bred Syrian hamsters obtained from the Lakeview Hamster Colony, Newfield, New Jersey. The cells were used in the third tissue culture passage.

Clonal isolation. Cells were placed in 60 mm. plastic tissue culture dishes at a density sufficiently low to insure the development of only 10 to 30 clones in each plate. The location of these clones was determined by microscopic examination and their position marked. When a clone had developed sufficiently to be passed, a small glass cylinder (5 mm. in diameter) was rimmed with stopcock grease and placed over the clone. Trypsin, at a concentration of 0.25%, was introduced into the cylinder for 10 min. at room temperature. With the aid of a Pasteur pipette, the contents of the cylinder were withdrawn and placed in a small plastic tissue culture flask containing growth medium. The flasks were placed at 37°C and the cells permitted to grow to confluence.

Cytogenetic analysis. The chromosomes were prepared for analysis by a modification of the technique of Miles & O'Neill (1966). Actively growing cells in an 8 oz prescription bottle were arrested in metaphase by the addition of colcemid at a final concentration of 0.15 μg./ml. for 4 hr. The cells were released from the glass surface with 0.1% pronase and pelleted in a conical centrifuge tube by centrifugation at 500g. The cells were resuspended in 1 ml. of foetal bovine serum containing 100 units of heparin followed by the addition of 4 ml. of
double-distilled water. The cells were gently dispersed in this menstrum and permitted to
stand at room temperature for 15 min. The cells were then re-sedimented and the cell pack
gently covered with a fixative of 3 parts methanol and 1 part glacial acetic acid for 15 min.
The cells were then gently suspended in the fixative, sedimented, and resuspended in a small
amount of fresh fixative. Approximately 0.05 to 0.1 ml. of cell suspension was spread over a
wet microscope slide and allowed to dry in air. The resultant chromosome spreads were
stained with Giemsa blood stain and mounted in permount.

RESULTS

Development of cell lines

Herpes simplex virus normally undergoes a lytic cycle of infection in most cell systems. In
order to study other properties of the virus it is necessary to interrupt or alter this destructive
effect so that the infected cell system will be preserved. We attempted to overcome this prob-
lem by inactivating the 316-D strain of HSV-2 with DMBA and by removing as much residual
chemical as possible by ultracentrifugation. This chemically inactivated and partially purified
virus, as well as the chemical and untreated virus preparations, were inoculated on to log-
arithmically growing primary hamster cells and adsorbed to these cells for 1 hr at room
temperature with frequent rotation of the plates. The plates were then rinsed with tris buffer
(pH 7.4), and growth medium was added to all plates. Within 24 hr, the control cultures
that had received untreated virus were completely destroyed. Cells that received chemically
inactivated virus showed no cytopathic effects. Some toxic effects were observed in cultures
exposed directly to DMBA only. After 5 days, all surviving cultures had reached confluence
and were passed into large plastic tissue culture flasks. The medium was changed every 3 days.
By 12 days after infection, large multilayered cell clones appeared in bottles containing cells
treated with DMBA-inactivated HSV-2. The cells were passed into 60 mm. plastic tissue
culture dishes on day 14 at a dilution sufficiently high to insure development of individual
clones.

The clones reappeared within 9 days and were isolated as previously described. A total
of 37 clones were picked from cultures treated with inactivated virus and five clones were
selected from non-inoculated cultures. Ultimately, progeny from six of the original clones
survived from cultures treated with DMBA-inactivated HSV-2; these are presently in the
26th tissue culture passage. The two cell lines described in this report are HDC-17 which
is fibro-epithelial-like and is partially contact inhibited and HDC-22 which is epithelial-like
and is not contact inhibited. None of the control cells, untreated or DMBA-treated, have
survived. These control cells began to exhibit reduction in viability at passage number 9
and none survived passage number 15. The surviving clones are named by the treatment they
received and have the prefix HDC followed by their clone number (H-herpes, D-DMBA,
C-clone, and a number).

Chromosome analysis

When the cloned cells were in their 12th passage, chromosome analyses were carried out.
Four of the cell lines had a normal diploid pattern of chromosomes characteristic of the
Syrian hamster (Hsu & Benirschke, 1967). One line (HDC-22) was hypodiploid, missing a
D-group chromosome (Fig. 1, Table 1). The HDC-22 line has been monitored every 4th
passage since and has maintained a hypodiploid stemline with the missing D-group chromo-
some. The cell line HDC-17 was studied at passage level 12 and 24 and has remained diploid
with no outstanding chromosomal abnormalities.
Fig. 1. Karyotype of cell line HDC-22 (passage no. 12) showing 43 chromosomes with the missing D-group chromosome.
Differential susceptibility to HSV-1 and HSV-2 in cell lines HDC-17 and HDC-22

Since the cells were originally treated with inactivated HSV-2 strain 316-D, growth studies were initiated to determine if both HSV-1 and HSV-2 could replicate in HDC-22. Since HDC-17 appears to have a normal chromosome pattern this served as a ‘normal’ control for these growth studies. The cell lines HDC-17 and HDC-22 were infected with HSV-1 or HSV-2 as described in Methods. The growth of HSV-1 in the two cell lines is graphically represented in Fig. 2 over a 96 hr period. HSV-1 appears to replicate equally well in both cell lines. Maximum titres were achieved at 48 hr after inoculation. Later declines in titre are due to cell destruction and thermal inactivation of the virus. In contrast, the growth of HSV-2 differed markedly in the two cell lines (Fig. 3). Although HSV-2 was able to replicate in an apparent normal manner in the HDC-17 cells, the virus replicated poorly, if at

Table 1. Chromosome analysis of cell lines HDC-17 and HDC-22

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tissue culture passage no.</th>
<th>Total no. cells examined</th>
<th>Modal chromosome no.</th>
<th>Percentage of cells missing one or more D-group chromosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDC-17</td>
<td>12</td>
<td>50</td>
<td>44</td>
<td>4</td>
</tr>
<tr>
<td>HDC-17</td>
<td>24</td>
<td>50</td>
<td>44</td>
<td>6</td>
</tr>
<tr>
<td>HDC-22</td>
<td>12</td>
<td>50</td>
<td>43</td>
<td>92</td>
</tr>
<tr>
<td>HDC-22</td>
<td>16</td>
<td>50</td>
<td>43</td>
<td>92</td>
</tr>
<tr>
<td>HDC-22</td>
<td>20</td>
<td>50</td>
<td>43</td>
<td>86</td>
</tr>
<tr>
<td>HDC-22</td>
<td>24</td>
<td>50</td>
<td>43</td>
<td>86</td>
</tr>
</tbody>
</table>
all, in HDC-22 cells. After infection of the HDC-22 cell line with HSV-2, the titre dropped slightly at 24 hr with a slight amount of virus production at 48 hr. No further infectious virus was produced within the 120 hr that the cultures were monitored. These experiments were repeated with similar results.

To determine if this particular cell line was specifically resistant to HSV-2, the growth of several HSV-1 and HSV-2 strains was measured in this cell line. Again HDC-17 served as the normal control. Four strains of HSV-1 and four strains of HSV-2 were tested in these two cell lines. Fig. 4 presents data from the 48 hr, sample which we had previously shown to be the time of maximum virus yield in our system. Of the four HSV-1 strains tested, no significant growth differences in the two cell lines (HDC-17 or HDC-22) were observed. The four strains of HSV-2 that were tested in the two cell lines exhibited a different pattern. The HSV-2 strains were able to grow to a higher titre in HDC-17 than in HDC-22, although they never achieved the titres of HSV-1 even in the HDC-17 cells. In each instance, the titre of the HSV-2 in the HDC-17 cells exceeded that obtained in HDC-22 cells by more than one log. Other studies which corroborated these results were also carried out. When HSV-1 was placed on monolayers of HDC-17 or HDC-22 cells at a dilution sufficient to form only several foci, normal plaques developed. Similar results were observed when HSV-2 was placed on the HDC-17 cells. When HSV-2 was placed on HDC-22 cells, a few foci consisting of several rounded cells developed and an occasional microscopic plaque formed. Thus, little cytopathology was produced by HSV-2 on the resistant HDC-22 cells when compared to the cytopathology produced by HSV-1 and HSV-2 in the permissive systems.
DISCUSSION

Primary hamster cells were exposed to HSV-2 inactivated with DMBA. Following treatment with the inactivated virus, several clones were isolated and studied. One of these clones, HDC-22, was hypodiploid and resisted productive infection by HSV-2, but was susceptible to HSV-1.

It has been shown that HSV types 1 and 2 will cause a variety of chromosome abnormalities which include breaks, pulverization, and attenuation of secondary constrictions (O’Neill & Miles, 1969). Waubke, zur Hausen & Henle (1968) demonstrated that the capacity of HSV to induce chromosomal aberrations is four times less sensitive to inactivation by u.v. irradiation than its infectivity. Although DMBA reduced the infectivity of HSV-2, the ability of the virus to produce chromosome abnormalities may have been preserved.

Whether the missing D-group chromosome of the HDC-22 cells is in any way related to the effects of the inactive HSV-2 is presently unknown. Furthermore, the role that this missing chromosome may play in the resistance of the HDC-22 cells to HSV-2 infection is obscure. The chromosome could function in maintaining membrane stability. An alteration at this level may affect virus attachment. We have done preliminary studies in this area and find that both HSV-1 and HSV-2 attach equally well to the HDC-22 cells. Therefore, the block must be internal at the level of macromolecular control.

If a portion of the HSV-2 genome was present in the HDC-22 cells it may be responsible for the observed resistance to HSV-2 infection. It has been demonstrated that virus-transformed cells are often resistant to superinfection by the same virus (Black, 1968; Rapp & Trulock, 1970). The specificity of this inhibition in our cell line (HDC-22) is very apparent, as the cells replicate the very closely related HSV-1. The mechanism of resistance of these cells is unknown at this time. A direct interference in the normal replication cycle of the virus caused by the presence of the HSV-2 genome in the cell line is possible. It is also possible that this genome, if present, may produce an inhibitor that prevents virus replication. Such a repressor has been identified in the lambda phage system. Ptashne (1967) has shown that a repressor system is established by the phage which allows maintenance of the lysogenic state in bacteria. The DNA of superinfecting phage can enter the cell but is unable to produce progeny due to the presence of the repressor. Whether a repressor substance exists in mammalian cells transformed by an oncogenic virus is as yet undecided (Rapp & Trulock, 1970; Jensen & Koprowski, 1969; Cassingena & Tournier, 1968). Obviously, since HSV-1 was able to replicate in the HDC-22 cells, any repressor substance found in the HDC-22 cells would have to be highly specific for HSV-2. Preliminary experiments have failed to detect a soluble repressor in the HDC-22 cells capable of inhibiting replication of HSV-2.

If these cells are carrying virus genetic material it is possible that they may also contain virus-specific markers. Until now immunofluorescent studies have failed to reveal any markers specific for HSV-2. Further studies are currently underway to determine if the cells contain HSV specific nucleic acids. The cells have also been inoculated into hamsters to determine any oncogenic potential they may possess. Hopefully, these as well as other studies will aid in elucidating the mechanism of resistance exhibited by the HDC-22 cell line. In any event, the HDC-22 and HDC-17 cell lines should prove useful in characterizing other HSV isolates and in furthering the understanding of differences in the replication of HSV-1 and HSV-2.
Supported by Contract No. 70–2024 within the Special Virus-Cancer Program of the National Cancer Institute, NIH, PHS and Public Health Service Post-doctoral Fellowship Award 5 FO2 CA4438 to F. J. O’Neill.

We are grateful to Mrs Sharon Faulkner for her excellent technical assistance.

The cells described in this study, HDC-17 and HDC-22, were examined and found to be free of PPLO contamination by Dr L. Hayflick, Stanford University, Palo Alto, California.

REFERENCES


(Received 18 June 1971)