Mechanism of the Restricted Growth of Herpes Simplex Virus Type 2 in a Hamster Cell Line

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SUMMARY

A hamster cell line (HDC-22) developed in our laboratory was non-permissive for herpes simplex virus type 2 (HSV-2) but not for herpes simplex virus type 1 (HSV-1). The mechanism of resistance to productive infection by HSV-2 was investigated. Adsorption studies revealed that HSV-1 and HSV-2 adsorbed at similar rates to the HDC-22 cells. The HDC-22 cells, when infected with HSV-2, produced minimal amounts of virus-specific antigens which increased slightly after several days.

The amount of HSV-2 DNA produced in the HDC-22 cells was always less than when these cells were infected with HSV-1. Cellular DNA synthesis was severely depressed by infection with HSV-1 at a multiplicity of 1 while HSV-2 had no effect at this multiplicity. At a higher multiplicity (of 5), this effect was increased with HSV-1 and noticeable with HSV-2. These studies indicate that the block for infection of the HDC-22 cell line with HSV-2 is after virus adsorption and may involve synthesis of virus DNA.

INTRODUCTION

The replication of herpes simplex virus (HSV) has been extensively investigated (Roizman, Aurelian & Roane, 1963; Roizman & Roane, 1964; Roizman, Borman & Rousta, 1965). A few reports have dealt with the inability of HSV to replicate in certain cells (Aurelian & Roizman, 1964; Lowry, Bronson & Rams, 1971; Floyd et al. 1971) or under various environmental conditions (Farnham & Newton, 1959; Hoggan & Roizman, 1959; Crouch & Rapp, 1972).

In a previous publication (Docherty, O'Neill & Rapp, 1971 b) we described a hamster cell line, HDC-22, which was resistant to productive infection by HSV-2, but allowed replication of the closely related HSV-1. Comparative studies in a cell line (HDC-17) isolated from the same hamster embryos had revealed no resistance to HSV-1 or HSV-2.

Our continued studies with HSV have led us to investigate the mechanism of resistance to HSV-2 infection exhibited by the HDC-22 line. We have investigated adsorption of the virus to the cells, synthesis of virus antigen, and synthesis of virus DNA. The results suggest that the non-permissive condition of these cells for replication of HSV-2 may involve the synthesis of significant quantities of virus-specific DNA.
METHODS

Cells. The HDC-17 and HDC-22 cell lines were developed and isolated as previously described (Docherty et al. 1971b). The cells were passed at regular intervals and nourished with growth medium consisting of Eagle's basal medium, 10% foetal calf serum, 5% tryptose phosphate broth, penicillin 100 units/ml., streptomycin 100 μg./ml., fungizone 1 μg./ml., mycostatin 10 units/ml., and 0.075% or 0.23% NaHCO₃.

Virus. All herpes simplex virus strains used in this study were obtained from Dr William Rawls, Baylor College of Medicine, Houston, Texas. Virus stocks were prepared in primary rabbit kidney cells and stored at -65°C. All virus titrations were performed in primary rabbit kidney cell monolayers utilizing a 0.5% methylcellulose overlay (Docherty, Goldberg & Rapp, 1971a).

Virus growth studies. The HDC-17 or HDC-22 cells were dispersed with 0.25% trypsin, washed once in growth medium, and placed in 29.6 ml. prescription bottles at a concentration of 5 × 10⁶ cells/bottle with growth medium. The cells were permitted to attach to the glass surface for 3 hr at 37°C. At the end of this period, the growth medium was removed and the cells were infected with either HSV-1 or HSV-2 at a multiplicity of 1 p.f.u./cell. The virus was adsorbed to the cells for 1 hr at room temperature with intermittent manual rotation. The cells were then washed once with tris buffer (0.025 M-tris saline (pH 7.4)) before 5 ml. growth medium was added. At this time, the 0 hr sample was taken with other samples being harvested at 24 hr intervals thereafter. The samples were processed and titrated according to standard procedures as previously described (Docherty et al. 1971a) using the plaque assay in rabbit kidney cells.

Adsorption studies. The HDC-22 cells were dissociated with 0.25% trypsin and washed three times with growth medium. The cells were placed into 473 ml. prescription bottles with growth medium, and incubated at 37°C for 3 hr. At the end of this period, the cells were again harvested and washed three times with growth medium. The cells were counted in a haemocytometer and virus and cells adjusted to give a multiplicity of 0.01 to 0.02 p.f.u./cell. The proper concentrations of cells (6 to 7 × 10⁶) and virus (10⁶ p.f.u.) were placed in a final volume of 2 ml. of growth medium. Virus stability controls consisted of the virus in 2 ml. of growth medium. All tubes were placed in the 37°C water bath and were agitated at regular intervals. At the appropriate time, 0.1 ml. samples were removed from each tube and placed in 9.9 ml. blanks of growth medium to stop virus attachment. The samples were centrifuged at 450 g for 10 min. At the end of this period, unattached virus in the supernatant fluid was immediately measured by titration in rabbit kidney monolayers as previously described.

Antigen studies. To determine if the non-permissive cells were producing HSV antigens after infection, coverslips were placed in 60 mm. tissue culture dishes and seeded, with 2.5 × 10⁶ cells. The cells were permitted to attach to the coverslips for 3 hr at 37°C in a 5% CO₂ humid atmosphere. After cell attachment, the coverslips were rinsed once with tris buffer to remove unattached cells and placed in fresh dishes. The cells were then exposed to either HSV-1 or HSV-2 at a multiplicity of 1 p.f.u./cell. The virus was adsorbed to the cells for 1 hr at room temperature with frequent manual rotation. At the end of this period, the cells were rinsed once with tris buffer and growth medium was added to all plates.

Coverslips were harvested and processed in the following manner to prepare the cells for detection of HSV specific antigens. The coverslips were rinsed three times in tris buffer (37°C) and then placed at 37°C for 20 min. They were then placed in acetone for 3 min. at room temperature.
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The cells were exposed to rabbit anti-HSV serum prepared previously. The antisera remained on the cells for 30 min. at room temperature before the coverslips were rinsed three times in tris buffer. The coverslips were then exposed to horse anti-rabbit gamma globulin conjugated to fluorescein isothiocyanate previously adsorbed with mouse liver powder. The reaction was carried out for 30 min. at room temperature. The coverslips were then rinsed three more times with tris buffer and mounted on glass slides with elvanol (20 g. polyvinyl alcohol grade 51–05 E. I. Du Pont, Delaware in 80 ml. 0.15 M-NaCl 0.01 M-KH$_2$PO$_4$-Na$_2$HPO$_4$, 12 H$_2$O and 40 ml. glycerol). The slides were examined with a Zeiss photomicroscope equipped with an ultraviolet light source.

Rate of DNA synthesis

Incorporation of [$^3$H]-thymidine ([$^3$H]-TdR). The HDC-17 or HDC-22 cells were seeded in 29.6 ml. prescription bottles and infected with HSV-1 or HSV-2 at a multiplicity of 1 p.f.u./cell as described above. At the times indicated in Results, [$^3$H]-TdR was added to a final concentration of 2 µc/ml. (specific activity 20 c/m-mole; New England Nuclear). After 1 hr incubation, the cells were scraped off the glass surface, concentrated by centrifuging, washed once with 0.15 M-NaCl and resuspended in 1 ml. of 5 mm-tris-HCl buffer (pH 7.0) with 0.15 M-NaCl and 10 mm-EDTA. Samples were frozen and stored at −20° until tested. At that time cells were lysed by adding sodium dodecyl sulphate (SDS, 0.5%) and incubated with self-digested pronase (0.5 mg./ml.) for 30 min. at 37°. Then, 0.1 ml samples were spotted on circular filter papers (Whatman no. 3 mm) and washed in 5 % trichloroacetic acid, 95 % ethanol, and ether respectively. Radioactivity determinations were made by counting in toluene-omnifluor (New England Nuclear) in a Beckman LS-233 liquid scintillation counter. The results represent total (both virus and cellular) DNA.

Separation of cellular and virus DNA. The above studies were repeated infecting with HSV-1 and HSV-2 at 1 or 5 p.f.u./cell. These samples were digested 180 min. at 37° in Sarkosyl NL 30 (0.1 %, Geigy Industrial Chemicals) and pronase (1 mg./ml.). After digestion, 0.2 ml. samples were removed and mixed with 3.8 ml. of 0.1 x SSC (0.015 M-sodium citrate, 0.15 M-sodium chloride) – buffered caesium chloride ($\rho = 1.745$ g./cm$^3$). These samples were centrifuged in a Beckman L2-65B centrifuge (40° fixed angle rotor, 30,000 rev./min., 60 hr at 17°).

Fractions were collected from the bottom of the tube on filter paper disks and processed as before. At selected intervals, fractions were collected for measurement of refractive indices in a Bausch and Lomb refractometer.

RESULTS

Replication of HSV-1 and HSV-2 in HDC-17 and HDC-22 cell lines

A comparison of growth of HSV-1 and HSV-2 in 3 hr old cultures of HDC-17 and HDC-22 cells is graphically represented in Fig. 1. The data presented indicate that HSV-1 and HSV-2 both replicated in the HDC-17 cells. Furthermore, HSV-1 was able to replicate in HDC-22 cells. However, HSV-2 was unable to replicate in a normal fashion in HDC-22 cells. Virus production never attained a level greater than initial input virus throughout the 120 hr the cultures were monitored.
Fig. 1. The replication of herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) in young (3 hr) cultures of HDC-17 or HDC-22 cells. ■—■, HDC-17 + HSV-1; □—□, HDC-17 + HSV-2; ●—○, HDC-22 + HSV-1; ○—●, HDC-22 + HSV-2.

Fig. 2. The adsorption of herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) to HDC-22 cells. ●—●, HSV-1 control; ■—■, HSV-1 + HDC-22 cells; ○—○, HSV-2 control; □—□, HSV-2 + HDC-22 cells.
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Fig. 3. Formation of herpesvirus (HSV)-specific antigens in permissive and non-permissive cells. Cells were reacted with immunofluorescent reagents as described in Methods. (A) HDC–17 + HSV–1 24 hr post-infection; (B) HDC–17 + HSV–2 24 hr post-infection; (C) HDC–22 + HSV–1 24 hr post-infection; (D) HDC–22 + HSV–2 24 hr post-infection; (E) HDC–22 + HSV–2 48 hr post-infection; (F) HDC–22 + HSV–2 96 hr post-infection.

Adsorption of virus

To determine if HSV–1 and HSV–2 differed in ability to adsorb to the HDC–22 cells, quantitative uptake experiments were carried out. Fig. 2 presents data which suggest that HSV–1 and HSV–2 adsorb to the HDC–22 cells at similar rates. Adsorption patterns did not
reveal differences between HSV-1 and HSV-2 and more than 90% of both viruses attached after 30 min. exposure to the HDC-22 cells.

Antigen synthesis in HSV-infected HDC-17 or HDC-22 cells

Studies were initiated to determine whether virus-specific antigens were being produced in the HDC-22 cell line after infection with HSV-2. The HDC-17 or HDC-22 cells were infected with HSV-1 or HSV-2 as described in Methods. Cells on coverslips were harvested at appropriate intervals and reacted with specific immunofluorescent reagents. Fig. 3A represents the HDC-17 cells 24 hr after infection with HSV-1. The majority of the cells exhibited cytopathology and reacted with HSV-specific antisera. Similar results were seen at 24 hr when HDC-17 was infected with HSV-2 (Fig. 3 B).

When the HDC-22 cells were infected with HSV-1, the cells exhibited total cytopathology by 24 hr post-infection and reacted positively with HSV-specific antisera (Fig. 3 C). At 24 hr after infection with HSV-2, the HDC-22 cells showed little, if any, cytopathology or positive fluorescence (Fig. 3 D). By 48 hr post-infection, an occasional cell rounded and reacted with the HSV antisera (Fig. 3 E). At 96 hr, the number eliciting a positive response was not greatly increased but occasional areas of several positive cells could be seen (Fig. 3 F).

DNA synthesis in infected cells

(a) Incorporation of [³H]-TdR into DNA was measured by the incorporation of [³H]-TdR in HSV-1 or HSV-2 infected HDC-17 or HDC-22 cells over a period of 120 hr (1 hr exposure to [³H]-TdR every 24 hr for 120 hr). The rate of incorporation in uninfected cultures of HDC-17 is shown in Fig. 4a. Measurements from comparable cultures infected with HSV-1 or HSV-2 revealed that the rate of incorporation was severely depressed (Fig. 4a).

The normal incorporation of [³H]-TdR in the HDC-22 cells is shown in Fig. 4b. HDC-22 cells infected with HSV-1 also revealed a severely depressed incorporation of [³H]-TdR
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when compared to uninfected controls. However, the rate of incorporation of TdR in HSV-2 infected HDC-22 cells was not affected. In fact, the rate of incorporation in HSV-2 infected HDC-22 cells was very similar to that of uninfected HDC-22 cells.

(b) Cellular vs. virus DNA synthesis – Based on the results of the [3H]-TdR incorporation experiments, samples were selected and analysed by density gradient centrifugation. Radioactive distribution was determined for cellular and virus DNA and these results are presented in Fig. 5. Under the two permissive situations i.e. HDC-17 + HSV-2 (Fig. 5a) and HDC-22 + HSV-1 (Fig. 5b), the smaller distinct peak characteristic of herpes simplex virus DNA (Russell & Crawford, 1964; Plummer et al. 1970) was found in the 24 hr sample. The amount of cellular DNA seen is minimal. However, in the non-permissive system, HDC-22 + HSV-2 (Fig. 5c), a small virus peak is also seen at 24 hr. In contrast to the permissive systems, the amount of cellular DNA suppressed was not as great. In fact, the incorporation of [3H]-TdR into cellular DNA of the non-permissive system is approximately 2.5 × greater than the permissive systems at 24 hr. At 48, 72 and 120 hr (Figs. 5d, e, f), a very small persistent virus peak is seen but at a very low level. There is no obvious depression of incorporation of [3H]-TdR into cellular DNA synthesis which is of a high magnitude (note difference in scale of Fig. 5a–e and Fig. 5d–f).

The above studies were repeated with HDC-22 cells using a higher multiplicity of 5 p.f.u./cell. Results obtained with normal uninfected controls at 8 and 24 hr post-inoculation are represented by Fig. 6a and b. In the permissive system (Fig. 6c and d), HSV-1 severely depressed cellular DNA synthesis and maximum incorporation of [3H]-TdR was always into virus DNA. However, in the non-permissive system at 8 hr (Fig. 6e), HSV-2 also

Fig. 5. Synthesis of cellular and virus-specific DNA in HDC-17 and HDC-22 cells infected with herpes simplex virus type 1 (HSV–1) or type 2 (HSV–2) at a multiplicity p.f.u./cell of 1. (a) HDC-17 + HSV-2 24 hr post-infection; (b) HDC-22 + HSV–1 24 hr post-infection; (c) HDC-22 + HSV–2 24 hr post-infection; (d, e, f), HDC-22 + HSV–2 at 48, 72 and 120 hr post-infection respectively.
Fig. 6. Synthesis of cellular and virus-specific DNA in HDC-22 cells infected with herpes simplex virus type 1 (HSV-1) or type 2 (HSV-2) at a multiplicity of 5 p.f.u./cell. (a) HDC-22 cells 8 hr control; (b) HDC-22 cells 24 hr control; (c) HDC-22 + HSV-1 8 hr post-infection; (d) HDC-22 + HSV-1 24 hr post-infection; (e) HDC-22 + HSV-2 8 hr post-infection; (f) HDC-22 + HSV-2 24 hr post-infection.

depressed cellular DNA synthesis but not as dramatically as HSV-1. By 24 hr (Fig. 6f), a small virus DNA peak persisted in the presence of a larger cellular DNA peak. At both 8 and 24 hr in the non-permissive system (HSV-22 + HSV-2), most of the radioactivity was located in the cellular DNA fraction. These DNA studies emphasize relative differences between HSV-1 and HSV-2 replication in the HDC-22 cell line.

DISCUSSION

Attempts to define the reason for the relatively non-permissive nature of the hamster cell line HDC-22 to replication of HSV-2 were carried out. Adsorption studies indicated that HSV-2 attaches as efficiently as HSV-1, but minimal amounts of virus DNA and virus antigen were detected in the HSV-2 infected HDC-22 cells.

Aurelian & Roizman (1964) reported that abortive infection of dog kidney cells with the MP strain of HSV was not due to adsorption. Although unable to replicate in dog kidney cells, the virus was able to adsorb to them. We have observed a similar phenomenon between HSV-1 and HSV-2 in their adsorption pattern to HDC-22 cells. Both were able to adsorb at similar rates. Although our data suggest that adsorption is not the blocking site, it does not indicate whether the virus is penetrating or uncoating in a normal manner.

Previous communications by several authors (Roizman & Roane, 1964; Cohen, Vaughan & Lawrence, 1971) have shown that with increased HSV-DNA synthesis in productive infections, there is a decrease in cellular DNA synthesis. The results with our productive control systems (HDC-17 + HSV-1, HDC-17 + HSV-2 and HDC-22 + HSV-1) agree with this observation. However, in the non-permissive system (HDC-22 + HSV-2), very little
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Virus DNA was observed and virtually no inhibition of cellular DNA synthesis occurred after infection at a multiplicity of 1 p.f.u./cell, although some was seen when a higher multiplicity of infection was employed. This observation may be interpreted in two ways, (1) the replicative cycle of the virus did not progress to this point, or (2) the replicative cycle of the virus did progress to this point but was unable to successfully continue.

HSV-specific virus antigens can be detected as early as 4 hr after infection in a permissive system (Roizman, Spring & Roane, 1967; Spear & Roizman, 1968). Our 24 hr samples of HDC-17+HSV-1, HDC-17+HSV-2, and HDC-22+HSV-1 exhibited maximum cytopathology and were also positive for HSV-specific antigens. In contrast, the amount of antigen seen in the HDC-22 cells infected with HSV-2 was minimal. By 72 and 96 hr, only occasional single cells or groups of cells gave a positive reaction in the abortively infected cultures.

These results suggest that the block for HSV-2 replication in the HDC-22 cell line is after virus adsorption and may involve synthesis of virus DNA. However, Fig. 6 revealed a not insignificant quantity of virus DNA which may also be interpreted to suggest a block later in the replicative cycle of the virus. Whether this amount of virus DNA is formed by cells permissive for HSV-2 replication within the system is unknown.

The well-known virus inhibitor, interferon, could play a role in the observed resistance. However, several reports (Ho & Enders, 1959; Vilček, 1962; Schachter et al. 1970) have suggested that HSV is relatively resistant to interferon. One report (Oh, 1971) using an interferon inducer indicates that HSV-1 is more resistant to its effects than HSV-2. It is conceivable that a repressor-like (Cassingena & Tournier, 1968) substance specific for HSV-2 is produced by the HDC-22 cells which would account for the specific inhibition observed. A search for such a substance has been conducted in our laboratory but the results have been negative.

These studies suggest a basic difference in the replicative cycle of HSV-1 and HSV-2. Further work with the HDC-22 cell line should therefore contribute to a better understanding of this difference between these two related viruses.

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