Inhibitory Effect of Herpes Simplex Virus Type 1 on Type 2 Virus Replication

By DANA ZELENÁ, J. ROUBAL AND V. VONKA

Department of Experimental Virology, Institute of Sera and Vaccines, 101 03 Prague, Czechoslovakia

(Accepted 14 July 1976)

Summary

Simultaneous infection with herpes simplex type 1 and type 2 viruses of chick embryo fibroblasts (CEF), which are only permissive for type 2 virus, or rabbit embryo fibroblasts (REF), which are permissive for both virus types, resulted in a marked reduction of type 2 virus production. This effect was dependent on the m.o.i. of type 1, being expressed at a high rather than a low m.o.i. The rate of interference decreased with the prolongation of the interval between infection with type 2 and type 1 viruses. No evidence suggestive of interferon involvement was obtained. Partial inactivation of type 2 virus by ultraviolet irradiation enhanced the inhibitory effect of type 1 virus. On the other hand, u.v. irradiation of type 1 virus resulted in a progressive loss of inhibitory activity. The results of the present experiments suggest that a type 1 genome function is responsible for the interfering effect, and that an early step in the growth of type 2 virus is sensitive to the particular type 1 virus product involved.

Introduction

The two antigenic types of herpes simplex virus (HSV) differ in a number of biological characters, among others in the capability of growing in chick embryo fibroblasts (CEF; Figueroa & Rawls, 1969). Although type 1 viruses capable of growth in CEF have been described (Waterson, 1958; Taniguchi & Yoshin0, 1963), the great majority of type 1 viruses do not replicate in these cells. On the other hand, CEF do support the replication of type 2 strains (Lowry, Melnick & Rawls, 1971). In tissue cultures derived from other animal species such type-dependent differences have not been described thus far.

In the present work we followed the formation of infectious virus progeny capable of replicating in CEF after co-infection of chick or rabbit cells with type 1 and type 2 viruses. The data presented indicate that in either cell system, type 1 infection resulted in a decreased yield of infectious type 2 virus.

Methods

Cells. Chick embryo fibroblasts (CEF) released by trypsinization of 10-day-old embryos were grown and maintained in Parker’s 199 medium supplemented with 10% of calf serum (CS), 5% of tryptose phosphate broth (TPB; Oxoid) and 0.045% of NaHCO3. The first passages of CEF, i.e. secondary cultures, were used throughout the present experiments.
REF cells derived from rabbit embryo tissues and HEF cells derived from hamster embryo tissues have been described in previous papers from this laboratory (Roubal & Vonka, 1973; Kutinová, Vonka & Brouček, 1973). 

**Plaque technique.** Infected REF cells were overlaid with Parker's medium enriched with growth promoting proteins of CS (Michl, 1961), 1% Difco Bacto agar, 0.15% NaHCO$_3$ and antibiotics. For the CEF cells, Parker's medium supplemented with 10% of CS, 5% of TPB, 1% agar, 0.15% NaHCO$_3$ and antibiotics was employed.

**Viruses.** HSV type 1, strain Kupka, isolated from a patient suffering from herpes labialis was kindly supplied by Dr R. Benda (Military Institute of Hygiene, Epidemiology and Microbiology, Praha). HSV type 2, strain 196, was obtained through the courtesy of Dr J. L. Melnick (Baylor College, Houston, Texas). Vesicular stomatitis virus (VSV), Indiana strain, was kindly provided by Dr L. Hronovský (Military Institute of Hygiene, Epidemiology and Microbiology, Praha).

Both HSV strains were propagated at a m.o.i. less than 1 p.f.u./cell. They were u.v. irradiated as described elsewhere (Roubal & Vonka, 1973). VSV stocks were prepared in REF cells by the same procedure as for HSV.

**Virus titration.** Virus stocks were serially diluted in tenfold steps and 0.1 ml amounts of each virus dilution were inoculated in 3 or 4 parallel tube cultures. The infected cultures were incubated at 37 °C for 7 days. Titres were determined according to Kärber (1931). Titration by the plaque technique in Müller bottles (surface area 15 cm$^2$) was performed as described elsewhere (Roubal & Vonka, 1973).

**Single-virus-growth experiments in CEF cells.** Tube cultures of confluent CEF cells were infected with HSV-1 and HSV-2 at a m.o.i. of about 3.5 p.f.u./cell. Virus was allowed to adsorb for 1 h at 37 °C. The cultures were then washed twice with medium, and 2.0 ml of fresh medium were added to each tube. Immediately after this, five parallel cultures were placed at −70 °C to determine the infectious virus content at time 0. The remaining cultures were incubated at 37 °C. At intervals three or four parallel cultures were withdrawn and placed at −70 °C. Prior to virus titration the cultures underwent three cycles of freezing and thawing. Parallel cultures were pooled and the fluids were clarified by low speed centrifugation (2000 g for 20 min). Infectious virus content was determined by titration in tube cultures from REF cells.

**Co-infection experiments.** Six parallel tube cultures of confluent CEF or REF cells were each infected with 0.1 ml of type 1 and 0.1 ml of type 2 virus suspension. The m.o.i. used in the separate experiments are indicated in the Results section. Control cultures were infected with one virus only and concomitantly 0.1 ml of medium was added. The cultures were treated as indicated in the preceding paragraph. The viruses were harvested after 24 h incubation. The amount of virus capable of growing in CEF was determined by plaque titration.

**Interferon assay.** The samples examined were prepared from CEF and REF cells 18 h after infection with HSV-1, strain Kupka, at a m.o.i. of 5 p.f.u./cell. After three cycles of freezing and thawing the suspensions were clarified by low-speed centrifugation and the supernatant fluids were treated by the procedure used by Cortada de la Peña & De Lustig (1974). In brief, the preparations were adjusted to pH 2.0 with 1 M-HCl and left at 4 °C for 48 h. They were then neutralized with 1 N-NaOH, centrifuged for 2 h at 100000 g and filtered through Millipore HAWP filters.

All samples were tested in tube cultures of CEF, REF and HEF cells. One ml amounts of each sample, undiluted and diluted in medium in twofold steps, were added to the cultures. After an 18-h incubation in a roller at 37 °C, the materials were infected either
Interference between HSV-1 and HSV-2

Fig. 1. Virus growth in CEF cells: •—•, HSV-2; ○—○, HSV-1; m.o.i. for HSV-1 and HSV-2 were about 3.5 p.f.u./cell, as determined by titration in REF cells.

with VSV or HSV type 2. In the first assay, 1 ml of medium containing 100 TCD50 of VSV was added to each culture. Cultures infected with the same amount of VSV which had been untreated or pre-treated with materials prepared from uninfected cells served as controls. The cultures were placed at 37 °C and were checked for cytopathic changes after a 48-h incubation. Their full development was considered evidence of the absence of interferon. In the second assay, the treated cells were infected with HSV type 2 at a m.o.i. of 1 p.f.u./cell. After 20 h of cultivation at 37 °C the viruses were harvested and titrated in REF tube cultures. The virus yields in the pre-treated and control cultures were compared. The absence of a difference in virus titres was considered to be evidence of the absence of interferon. CEF interferon kindly supplied by Dr H. Libíková (Institute of Virology, Bratislava) and rabbit interferon provided by Dr I. Tábořský (Faculty of Medicine, Brno), treated as mentioned above, were used as positive controls. Under the described experimental conditions, the first preparation prevented the development of c.p.e. induced by VSV and reduced markedly the HSV-2 production at the dilution 1:64 and 1:32, respectively. The rabbit preparation was effective against VSV and HSV-2 at the dilution 1:16.

RESULTS

Growth of type 1 and type 2 viruses in CEF cells

The results of growth experiments with HSV type 1 and type 2 viruses in CEF cells are shown in Fig. 1. It can be seen that HSV-2, strain '196', multiplied in CEF reaching the maximum titre at 18 to 22 h post infection (p.i.). On the other hand, CEF did not support the replication of HSV-1, strain Kupka. Although the type 2 virus replication in CEF was not too efficient, the differences between the maximum type 2 virus titres and corresponding
Table 1. Influence of HSV type 1 presence on HSV type 2 production in CEF and REF cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>HSV-2 irradiation*</th>
<th>Expt. no.</th>
<th>HSV-2 production (p.f.u. × 10^-6/ml)</th>
<th>Relative HSV-2 production in presence of HSV-1†</th>
<th>Mean</th>
<th>%</th>
<th>%‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEF</td>
<td>None</td>
<td>I</td>
<td>2.3</td>
<td>0.3</td>
<td>13.0</td>
<td>63</td>
<td>14.2 (± 2.9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>5.7</td>
<td>0.36</td>
<td>6.3</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>III</td>
<td>43.0</td>
<td>8.3</td>
<td>49.3</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>IV</td>
<td>52.0</td>
<td>9.5</td>
<td>18.2</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60 s</td>
<td>I</td>
<td>1.7</td>
<td>0.14</td>
<td>8.2</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>4.5</td>
<td>0.97</td>
<td>4.5</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>III</td>
<td>10.0</td>
<td>9.87</td>
<td>9.7</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>IV</td>
<td>2.5</td>
<td>0.16</td>
<td>6.5</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>120 s</td>
<td>I</td>
<td>0.48</td>
<td>0.02</td>
<td>4.2</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>2.0</td>
<td>0.07</td>
<td>3.7</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>III</td>
<td>2.6</td>
<td>0.07</td>
<td>2.6</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>IV</td>
<td>2.1</td>
<td>0.03</td>
<td>1.5</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>REF</td>
<td>None</td>
<td>I</td>
<td>38.0</td>
<td>3.0</td>
<td>12.6</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>180.0</td>
<td>21.0</td>
<td>16.9</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>III</td>
<td>160.0</td>
<td>27.0</td>
<td>16.9</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>IV</td>
<td>210.0</td>
<td>36.0</td>
<td>17.2</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60 s</td>
<td>I</td>
<td>6.5</td>
<td>0.2</td>
<td>3.1</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>25.0</td>
<td>2.5</td>
<td>10.0</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>III</td>
<td>31.0</td>
<td>2.0</td>
<td>6.5</td>
<td>64</td>
<td></td>
</tr>
</tbody>
</table>

* The pre-irradiation HSV-2 titre was 1.3 × 10^6 p.f.u./ml. The survival of the 60 s irradiated virus was 3.1 × 10^-2 in REF and 7.0 × 10^-3 in CEF cells. The survival of 120 s irradiated virus was 3.7 × 10^-4 in REF and 2.1 × 10^-3 in CEF cells. The m.o.i. of HSV-2, based upon pre-irradiation virus titre, was about 0.7 p.f.u./cell. The approximate m.o.i. of HSV-1 virus was 3.5 p.f.u./cell.  
† As determined by titration in CEF cells.  
‡ Standard mean errors are given in parentheses.

Type 1 virus contents, which most probably represented the residual inoculum infectivity, were 3 log₁₀ or more in repeated experiments. In agreement with this, type 1 did not induce plaque formation in CEF monolayers, while type 2 formed large clear plaques.

Co-infection experiments

CEF cells were infected concomitantly with both type 1 and type 2 viruses. The m.o.i. used is indicated in the legends to the tables and figures. Cultures infected with HSV-2 only served as controls. The titres of virus progenies in CEF cells determined 24 h p.i. are shown in Table 1. In all four experiments the production of virus growing in CEF (i.e. type 2 virus) was reduced in the presence of HSV-1, on an average amounting to 14% of the type 2 virus production observed in control cultures. The inhibitory effect was increased when using type 2 virus partially inactivated by u.v., more so when the virus was irradiated for 120 s than when it was inactivated for 60 s. Type 1 also interfered with type 2 replication in REF cells, which are permissive for both virus types. The results are shown in the lower part of Table 1. It can be seen that the presence of type 1 virus decreased the production of type 2 virus at the same rate in both REF and CEF cells. Also, the inhibitory effect was similarly enhanced in both cell types when the type 2 virus had been partially inactivated by u.v. light.
Interference between HSV-1 and HSV-2

Fig. 2. Relationship between HSV-2 production in CEF cells and m.o.i. of HSV-1. M.o.i. for HSV-2 was 0.6 p.f.u./cell as determined by titration in REF cells.

We also followed the dependence between the degree of inhibition and the m.o.i. of both viruses. In the first experiment, infection of CEF cells with type 2 virus was constant and the m.o.i. of type 1 varied. Conversely, in the second experiment the type 1 m.o.i. remained constant but the type 2 m.o.i. varied. The results of the first test are shown in Fig. 2. Maximum inhibition occurred at the highest m.o.i. tested (6.6 p.f.u./cell) of type 1. However, at a m.o.i. of only 0.11 p.f.u./cell the amount of virus growing in CEF was reduced by 60% in comparison with virus production in control cultures. On the other hand, the varying of type 2 m.o.i. from 4 × 10^-4 to 4.0 p.f.u./cell, while type 1 m.o.i. was constant, had a negligible effect on the rate of interference (results not shown).

In the subsequent experiments we tried to determine the relationship between the interference effect and the time of addition of type 1 virus after infection with type 2 virus. CEF cells were infected with type 2 virus and then superinfected with type 1 virus at different intervals. Twenty-four h after infection with type 2 virus the contents of viruses capable of growing in CEF cells were determined (Fig. 3). It can be seen that the maximum inhibition of type 2 virus production (i.e. minimum production of virus growing in CEF) was observed when cells were infected simultaneously with both viruses. With prolongation of the interval between infection and superinfection the inhibitory effect of type 1 virus gradually decreased. The effect of HSV-1 became negligible when the interval reached 6 h. It is possible to conclude from these data that type 1 virus was only capable of interfering with type 2 production early after infection with type 2.

We also investigated whether an interferon-like substance could be involved in the interference described. Extracts from HSV type 1-infected CEF and REF cells were tested for the presence of interferon, as described in Methods. None of the extracts prevented the development of c.p.e. induced by VSV in CEF, REF and HEF cells, or reduced the pro-
Fig. 3. Relationship between the time of type 1 virus addition and type 2 virus production in CEF cells. M.o.i. for HSV-1 was 3.3, for HSV-2, 0.7 p.f.u./cell.

production of HSV type 2 virus in these cells. Thus no evidence on the presence of interferon in the system was obtained.

To check whether an expression of type 1 virus genetic information was needed for the interference effect, CEF cells were co-infected with untreated type 2 virus and type 1 virus which had been irradiated with different u.v. doses. It is shown in Fig. 4 that the maximum interference effect was achieved when using untreated type 1 virus (6% of control virus production) and that the inhibitory effect of type 1 virus decreased when the u.v. dose was increased. When using 300 s irradiated type 1 virus, the production of virus capable of multiplying in CEF was reduced by only 50% when compared with control cultures infected with type 2 virus only.

**DISCUSSION**

In agreement with earlier observations (Figueroa & Rawls, 1969; Lowry et al. 1971), the type 1 Kupka virus did not replicate in CEF cells, while type 2 '196' virus grew well in this system. This difference was utilized for monitoring the effects of simultaneous infection with these two viruses on the production of type 2 virus. In both CEF and REF cells, type 1 infection resulted in a marked decrease in the production of virus capable of growing in CEF cells. This virus is referred to as type 2 virus, although we realize that a small amount of recombinant viruses might have been present in the virus populations (Timbury & Subak-Sharpe, 1973).
**Interference between HSV-1 and HSV-2**

Several experiments were performed to obtain more information on this interference. The findings can be summarized as follows. First, the rate of interference was dependent on the m.o.i., more so on the m.o.i. of the interfering type 1 virus than of the interfered with type 2 virus under the present experimental conditions. Interestingly enough, the interference was expressed even when the m.o.i. of type 1 virus was as low as 0.1 p.f.u./cell. This might either be due to the capability of the non-infectious particles to induce the phenomenon or to the involvement of a diffusible factor produced in the type 1 infected cells and influencing non-infected ones. Second, the inhibition effect of type 1 virus decreased with prolonging the interval between type 2 infection and type 1 superinfection. This indicates that an early step in the type 2 virus growth cycle was sensitive to the interfering action of type 1 virus. Third, the interfering capacity of type 1 was markedly decreased by u.v. irradiation, suggesting that a type 1 genome function was responsible for the phenomenon. It has been shown that the irradiation of HSV-1 results in a delay in the synthesis of virus-specific products (Ross, Wildy & Cameron, 1971). Thus, the delayed synthesis of the particular type 1 virus product involved could diminish the expression of the interference effect, imitating the effect of late type 1 virus addition. Fourth, partial inactivation of type 2 virus resulted in an enhancement of the interference. This observation might again be explained by the delay in the replication of HSV caused by u.v. irradiation (Ross, Cameron & Wildy, 1972; Roubal & Vonka, 1973). It is reasonable to presume that this
delay resulted in the prolonging of the period during which type 2 virus growth was sensitive to the effect of the particular type 1 virus product involved in the interference.

It seems unlikely that the observed interference was mediated by interferon. Herpesviruses are generally considered to be poor inducers of interferon and not very sensitive to its action (Lockart, 1973), although induction of interferon by HSV and its action on the virus have been reported (Waddell, Sigel & Wryk, 1963; Fruitstone, Waddell & Sigel, 1964; Libiková, 1973; Hooks, 1975; Rasmussen & Farley, 1975). Our results failed to demonstrate the presence of detectable amounts of interferon-like substances in either CEF or REF cultures infected with type 1 virus.

Interference with HSV not mediated by interferon was described by Roizman (1965): the co-infection of dog kidney cells with HSV MPdk− incapable of growing in these cells, and the MPdk+sp mutant, for which the dog kidney cells were permissive, resulted in a decrease of MPdk+sp virus production. It was assumed that this effect was due to the formation of non-functional heteropolymers between the polypeptides specified by the dk+ virus and the non-functional products of the dk− virus. It does not seem probable that a similar type of interference was operative in the present system, because the same effects as in CEF cells were also observed in REF cells, permissive for both type 1 and type 2 viruses.

Another type of apparently interferon-independent interference has been described recently by Bronson et al. (1973) and by Frenkel et al. (1975), who reported that the defective HSV particles produced by undiluted passages blocked the replication of infectious HSV. It is not likely that this would be the present case, since both virus stocks were prepared by passing the virus at a low m.o.i.

The mechanism causing the relative resistance of HSV-transformed cells to HSV replication as reported by Doller, Duff & Rapp (1973), Kutinová et al. (1973) and by Kimura et al. (1975) may, but need not, be related to those involved in the phenomenon presently reported.

The nature of the interference described in this report is not understood. One can only speculate, at this stage, about the mode of action of the responsible type 1 virus product in co-infected cells. It has been demonstrated that the regulation of HSV-1 polypeptide production is mediated by turning off the synthesis of the earlier products by the later groups of polypeptides (Honess & Roizman, 1974). Since differences between type 1 and type 2 viruses have been reported for both the transcriptional programmes (Roizman & Frenkel, 1973) and the synthesis of virus polypeptides (Powell & Courtney, 1975), it seems possible that some type 1 virus product(s) appearing in a sequence, or at a time, which is incorrect for the type 2 virus cycle, might prevent the expression of some function(s) essential for the growth of this virus.

REFERENCES


Interference between HSV-1 and HSV-2


(Received 22 March 1976)