Homologous Interference Induced by a Temperature-sensitive Mutant Derived from an HVJ (Sendai Virus) Carrier Culture

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SUMMARY
Homologous interference between a temperature-sensitive small plaque mutant (HVJ-pB) derived from an HVJ (haemagglutinating virus of Japan – the Sendai strain of parainfluenza 1 virus) carrier culture of BHK cells and the original wild-type virus (HVJ-W) has been investigated. Prior infection of LLCMK2, HeLa, BHK or mouse L cells with HVJ-pB, both at permissive and non-permissive temperatures, for 24 h resulted in a reduced yield of superinfecting HVJ-W, reflecting a smaller number of cells capable of producing the superinfecting virus. However, HVJ-pB did not interfere with the replication of vesicular stomatitis virus, Sindbis virus or Newcastle disease virus. Interference in this system seems to be due to inhibition of the attachment of superinfecting HVJ-W as a result of intracellular mechanisms operating at a late stage in the replication of the interfering virus. There is also blocking or destruction of cellular receptors by extracellular particles of the interfering virus. Protein synthesis coded for by the complete virus genome is required to establish and maintain the interference, and treatment with actinomycin D has no effect on the interference phenomenon.

INTRODUCTION
Almost all persistently virus-infected cell cultures studied show a distinct resistance to superinfection by the homologous virus. The BHK line of baby hamster kidney cells can be persistently infected with wild-type HVJ (HVJ-W), and no cytopathic change was detected when these cells were superinfected with the same virus (Nagata et al. 1972).

Recently, infectious virus was isolated successfully from BHK-HVJ cells by lowering the incubation temperature, and the strain of virus produced was designated HVJ-pB (Kimura et al. 1975). HVJ-pB was found to differ significantly from HVJ-W in its capacity to replicate at 38 °C and in its cytocidal activity (Kimura et al. 1975). These distinguishing characteristics of HVJ-pB are useful for an analysis of the phenomenon of homologous interference.

Interference phenomena during virus multiplication may be classified broadly into virus-
attachment interference and intracellular interference. The former is caused by blocking or destruction of cellular receptors. The latter involves interferon-mediated interference, or production of proteins coded by interfering virus, or competition for enzymes, substrates and replication sites. This paper describes and characterizes the homologous interference induced by a temperature-sensitive (ts) mutant of HVJ.

METHODS

Viruses and cell cultures. The isolation and characterization of HVJ-pB has been described by Kimura et al. 1975. The Nagoya strain of HVJ (HVJ-W), HVJ-pB and the Miyadera strain of Newcastle disease virus (NDV) were propagated routinely by allantoic inoculation of 10-day-old embryonated eggs with 0.2 ml of \(10^{-6}\) diluted seed virus. The New Jersey strain of vesicular stomatitis virus (VSV) and Sindbis virus were propagated in primary chicken embryo cells.

The LLCMK2 continuous line of monkey kidney cells, baby hamster kidney (BHK) cells, HeLa cells and mouse L cells were cultured in Eagle's minimal essential medium (MEM) containing 10% calf serum, 10% tryptose phosphate broth and 60 \(\mu\)g/ml kanamycin.

Infectivity titrations. Plaque titration of HVJ on LLCMK2 cell monolayers was carried out as described previously (Sugita, Maru & Sato, 1974). Before inoculation, HVJ virions grown in tissue culture were treated with trypsin at a final concentration of 5 \(\mu\)g/ml for 30 min at room temperature in order to enhance their infectivity for LLCMK2 cells (Homm, 1971). Incorporation of trypsin (3 \(\mu\)g/ml) in the agar overlay medium is essential for plaque formation by HVJ, and this characteristic distinguishes HVJ from some other viruses such as VSV, Sindbis virus and NDV.

Haemagglutination (HA) tests. Haemagglutinin was titrated by the pattern method described previously (Sever, 1962; Kimura et al. 1975).

Linear sucrose gradient centrifugation. One ml of virus suspension was applied on to 26 ml of a 10 to 40% (w/w) linear sucrose gradient in 5 \(\times\) \(10^{-3}\) M-tris-(hydroxymethyl)-aminomethane-hydrochloride, 1 \(\times\) \(10^{-3}\) M-ethylenediaminetetra-acetic acid, pH 7.4 (tris-EDTA buffer), and centrifuged in a Beckman SW 27 rotor at 20000 rev/min for 45 min. The gradient was fractionated by collecting drops through the bottom of the tube. Each fraction was dialysed against tris-EDTA buffer at 4°C overnight and tested for biological activity.

Ultraviolet (u.v.) irradiation of virus. Ten ml of virus suspension diluted tenfold in phosphate-buffered saline, pH 7.2, was exposed in a 93 mm Petri dish to a 10 W Toshiba germicidal lamp at a distance of 10 cm at 4°C with continuous and gentle stirring using a magnetic stirrer. At intervals, samples were removed and assayed for infectivity and for their ability to interfere with virus growth.

Infectious centre assays. Monolayer cultures of LLCMK2 cells infected with HVJ-pB at an input m.o.i. of 10 p.f.u./cell were superinfected 24 h later with HVJ-W at the same input m.o.i. After adsorption for 2 h the cultures were washed three times and further incubated for 20 min in maintenance medium containing 100-fold diluted anti-HVJ antiserum. They were then washed thoroughly to remove the remaining antibody, trypsinized and washed once in maintenance medium. Serial dilutions of the cell suspension were made in medium; these were inoculated on to uninfected LLCMK2 cell monolayers and incubated at 38°C to allow HVJ-W plaques to form.
Homologous interference by HVJ

Fig. 1. Growth curves for the ts mutant, HVJ-pB, and the wild-type virus, HVJ-W, in LLCMK2 cells at 32°C and 38°C. Infectivity was assayed at 32°C for HVJ-pB and at 38°C for HVJ-W, respectively. (a) HVJ-W; (b) HVJ-pB. --- ---, 32°C; -- --, 38°C. The extent of c.p.e. was scored as 0, 1, 2, 3 or 4 (degeneration involving, respectively, 0, < 25%, 25 to 50%, 50 to 75% or 75 to 100% of the cells).

RESULTS

Growth of HVJ-pB and HVJ-W in LLCMK2 cells at 32°C and 38°C

Monolayer cultures of LLCMK2 cells grown in 50 mm Petri dishes were infected with HVJ-pB or HVJ-W at an input multiplicity of approx. 1 p.f.u./cell. After incubation for 1 h at 32°C or 38°C, monolayers were washed five times and incubated in 4 ml of maintenance medium at the corresponding temperature. At various times after infection, culture fluids were harvested and assayed for virus infectivity. Replication of HVJ-W proceeded equally well at both 32°C and 38°C (Fig. 1a). The growth curve of HVJ-pB at 32°C was very similar to those of HVJ-W, but at 38°C, its growth was markedly restrained (Fig. 1b). HVJ-pB produced no obvious c.p.e. on LLCMK2 cells at either temperature.

Plaque formation by HVJ-pB and HVJ-W

HVJ-pB produced plaques on LLCMK2 cell monolayers at 32°C, but not at 38°C, while HVJ-W produced plaques equally well at both temperatures (Table 1). Plaques produced by HVJ-pB were round but noticeably smaller than those produced by HVJ-W (Fig. 2). Thus, in terms of plaque-forming ability at 38°C and plaque size, HVJ-pB was readily distinguished from HVJ-W.

Demonstration of homologous interference

LLCMK2 cells were infected at 32°C or 38°C with 10 p.f.u./cell of HVJ-pB. After adsorption for 1 h, cells were washed three times and incubated in maintenance medium for 24 h at the appropriate temperature. At this time, the culture fluids were removed and cells were superinfected with HVJ-W at a multiplicity of 1 p.f.u./cell. After a 1 h adsorption period,
Table 1. Plaque formation on LLCMK2 cell monolayers by wild-type HVJ and its ts derivative, HVJ-pB

<table>
<thead>
<tr>
<th>Incubation temperature (°C)</th>
<th>HVJ-pB</th>
<th>HVJ-W</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p.f.u./ml</td>
<td>Plaque diam. (mm)*</td>
</tr>
<tr>
<td>32°</td>
<td>1.5 × 10⁸</td>
<td>0.5 ± 0.3 †</td>
</tr>
<tr>
<td>38°</td>
<td>&lt; 10</td>
<td></td>
</tr>
</tbody>
</table>

* The mean diam. and standard deviations were calculated from 97 plaques of HVJ-pB, and 70 and 113 plaques of HVJ-W at 32° and 38°C, respectively.
† Standard deviation.

Fig. 2. Plaques produced on LLCMK2 cell monolayers at 32°C by HVJ-pB and HVJ-W. Cell cultures were incubated for 10 days after inoculation of virus. (a), HVJ-pB; (b), HVJ-W.

Table 2. Demonstration of homologous but not heterologous interference by HVJ-pB

<table>
<thead>
<tr>
<th>Challenge virus</th>
<th>Incubated at 32°C</th>
<th>Incubated at 38°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control cells</td>
<td>Infected with HVJ-pB†</td>
</tr>
<tr>
<td>HVJ-W</td>
<td>3.2 × 10⁶</td>
<td>3.0 × 10⁵</td>
</tr>
<tr>
<td>VSV</td>
<td>3.1 × 10⁷</td>
<td>2.4 × 10⁷</td>
</tr>
<tr>
<td>Sindbis virus</td>
<td>1.0 × 10⁴</td>
<td>1.0 × 10⁵</td>
</tr>
<tr>
<td>NDV</td>
<td>9.5 × 10⁵</td>
<td>5.0 × 10⁵</td>
</tr>
</tbody>
</table>

* Titres determined 48 h after addition of challenge virus.
† LLCMK2 cells were infected with HVJ-pB at an m.o.i. of 10 p.f.u./cell for 24 h at the temperature shown prior to superinfection with challenge virus.
‡ Not tested.

The cells were washed thoroughly to remove the unadsorbed superinfecting HVJ-W and incubated in maintenance medium for a further 48 h at the same temperature as before. The culture fluids were then harvested and the growth of HVJ-W was determined by plaque assay at 38°C (Table 2). Cells which had been previously infected with HVJ-pB showed no obvious cytopathic change after superinfection with HVJ-W and the growth of HVJ-W in these cells was suppressed at either temperature. In contrast, control cells which had received a mock infection produced a large amount of progeny HVJ-W virus and degenerated. Infectious centre assays showed that the reduction in HVJ-W virus yield was due to a reduc-
Homologous interference by HVJ

Fig. 3. Development of interference in relation to the time interval between HVJ-pB infection and superinfection with HVJ-W. Interfering activity is expressed as the log difference in HVJ-W virus yield in control cells and in cells previously infected with HVJ-pB.

Interference in the number of cells capable of producing superinfecting HVJ-W. Homologous interference by HVJ-pB was induced both at permissive and non-permissive temperatures. Similar interference with the growth of superinfecting HVJ-W was also found in HeLa cells, BHK cells and mouse L cells.

When VSV, Sindbis virus or NDV was used as the superinfecting virus, no interference was found in HVJ-pB infected cells (Table 2). However, it should be noted that fusion from within (Bratt & Gallaher, 1969) caused by NDV infection was suppressed in HVJ-pB infected cells.

Time course of establishment of interference

In order to clarify the relationship between establishment of interference and intracellular growth of interfering HVJ-pB, cells were challenged with HVJ-W (1 p.f.u./cell) at various times after HVJ-pB infection (10 p.f.u./cell) at 32°C. Culture fluids were harvested at 48 h after infection with HVJ-W and assayed for virus growth at 38°C (Fig. 3). HVJ-pB did not induce any homologous interference when superinfecting virus was added simultaneously. However, when HVJ-pB was inoculated 2 h before HVJ-W, interference became evident, and at longer time intervals it became more pronounced.

Determination of the interfering dose

To determine the virus dose necessary for the induction of interference in a given cell, cultures were infected with various doses of HVJ-pB, and after 24 h incubation at 32° or 38°C, cells were superinfected with HVJ-W (1 p.f.u./cell). Culture fluids were harvested after a further 48 h incubation at the corresponding temperature. The degree of interference correlated with the amount of interfering HVJ-pB inoculated (Table 3). This suggests that a single p.f.u. of HVJ-pB is sufficient to make a cell resistant to superinfection with HVJ-W, according to calculation by Poisson distribution. It should be noted that no c.p.e. was induced by superinfection with HVJ-W in the cells previously infected with HVJ-
Table 3. Determination of the amount of HVJ-pB necessary to induce homologous interference

Growth of HVJ-W and c.p.e. produced

<table>
<thead>
<tr>
<th>Input m.o.i. of HVJ-pB (p.f.u./cell)</th>
<th>Incubated at 32°C</th>
<th>Incubated at 38°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p.f.u./ml</td>
<td>H.A.U./0.05 ml</td>
</tr>
<tr>
<td>100</td>
<td>5.4 × 10⁶</td>
<td>64</td>
</tr>
<tr>
<td>24</td>
<td>4.6 × 10⁵</td>
<td>64</td>
</tr>
<tr>
<td>6</td>
<td>3.0 × 10⁵</td>
<td>64</td>
</tr>
<tr>
<td>1.5</td>
<td>2.3 × 10⁵</td>
<td>64</td>
</tr>
<tr>
<td>0.4</td>
<td>3.0 × 10⁴</td>
<td>128</td>
</tr>
<tr>
<td>0.1</td>
<td>5.8 × 10⁴</td>
<td>128</td>
</tr>
<tr>
<td>0.03</td>
<td>9.4 × 10⁴</td>
<td>128</td>
</tr>
<tr>
<td>0 (control cells)</td>
<td>7.9 × 10⁴</td>
<td>128</td>
</tr>
</tbody>
</table>

* c.p.e. was scored as described in Fig. 1.

Fig. 4. Interference by u.v.-irradiated HVJ-pB: effect of u.v. dose on the interfering activity and infectivity of HVJ-pB. Superinfection with HVJ-W was carried out 24 h after HVJ-pB infection. The residual fraction of interfering activity is shown as the log difference in the titre of HVJ-W grown in cells previously infected with HVJ-pB exposed to the indicated dose of u.v. light and grown in cells previously infected with intact HVJ-pB. ○, interfering activity; ●, infectivity.

pB at either the permissive or the non-permissive temperature. The results also suggest that at permissive temperatures, the growth of interfering HVJ-pB was not suppressed by superinfection with HVJ-W under the present experimental conditions: haemagglutinin was produced in good yield in HVJ-pB infected cells at 32°C in spite of the inhibition of HVJ-W replication, while at 38°C, there was no production of haemagglutinin because of the temperature-sensitivity of HVJ-pB and interference with HVJ-W by HVJ-pB.

Interference by u.v.-irradiated HVJ-pB

Samples of HVJ-pB exposed to different doses of u.v.-irradiation were assayed for infectivity and for their ability to interfere with the replication of superinfecting HVJ-W
Homologous interference by HVJ

Table 4. Interference by u.v.-inactivated HVJ-pB: effect of time of superinfection with HVJ-W

<table>
<thead>
<tr>
<th>Time of superinfection (h)</th>
<th>Growth of HVJ-W (p.f.u./ml)</th>
<th>Incubated at 32°C</th>
<th>Incubated at 38°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native</td>
<td>U.v.-inactivated</td>
<td>Native</td>
</tr>
<tr>
<td>Control cells (no HVJ-pB)</td>
<td>5.8 × 10⁷</td>
<td>1.4 × 10⁷</td>
<td>4.6 × 10⁷</td>
</tr>
<tr>
<td>Simultaneously</td>
<td>4.6 × 10⁷</td>
<td>2.0 × 10⁶</td>
<td>9.5 × 10⁷</td>
</tr>
<tr>
<td>1</td>
<td>7.3 × 10⁵</td>
<td>N.T.*</td>
<td>2.0 × 10⁴</td>
</tr>
<tr>
<td>2</td>
<td>2.4 × 10⁶</td>
<td>5.4 × 10⁴</td>
<td>9.1 × 10⁴</td>
</tr>
<tr>
<td>24</td>
<td>1.6 × 10⁴</td>
<td>2.8 × 10⁷</td>
<td>5.4 × 10⁴</td>
</tr>
</tbody>
</table>

* Not tested.

(Fig. 4). Cells were exposed to u.v.-irradiated HVJ-pB at 32°C for 24 h before superinfection with HVJ-W, and culture fluids were harvested 48 h after superinfection and assayed for infectivity at 38°C. An increased dose of u.v.-irradiation resulted in a decrease in both the infectivity and interfering ability of HVJ-pB. The rates of inactivation of infectivity and of interfering activity seem to coincide, suggesting that the interfering particle is the infectious HVJ-pB virion itself.

In the next experiment, the infectivity of HVJ-pB virions was completely inactivated by a large dose of u.v.-irradiation. Cells were exposed to these virions and after various times of incubation superinfected with HVJ-W. U.v.-inactivated interfering HVJ-pB induced interference for only a short period, and could not maintain the infected cells in a state refractory to superinfection with HVJ-W for more than 1 h after infection (Table 4). This early stage interference is possibly due to blocking or destruction of cellular receptors by u.v.-inactivated HVJ-pB virions.

Sedimentation analysis of HVJ-pB

A crude stock preparation of HVJ-pB was concentrated by centrifugation. A linear sucrose gradient centrifugation and fractionation was carried out as described in Methods. Haemagglutinating and infectivity titrations were performed on each fraction and interfering activity was determined by infecting LLCMK2 cells with each of the fractions and challenging 24 h later with HVJ-W virus. After a further 48 h incubation culture fluids were harvested and titrated by plaque assay at 38°C. The fractions containing the highest levels of infective HVJ-pB caused the greatest reduction in virus yields of superinfecting HVJ-W, and fractions containing incomplete haemagglutinating (HA) particles induced no interference under the present experimental conditions (Fig. 5). Thus the interfering activity of HVJ-pB co-sediments with its infectivity, as might be anticipated from the u.v.-inactivation results.

Effect of antimetabolites on establishment of interference

In order to analyse whether the interference observed was due to interferon-mediated interference, actinomycin D (5 μg/ml) was added to the maintenance medium throughout the period of infection, starting 2 h before inoculation with interfering HVJ-pB. Superinfection with HVJ-W was carried out in the presence of actinomycin D 24 h after infection with HVJ-pB. Culture fluids were harvested after a further 48 h incubation and assayed for infectivity at 38°C. As shown in Table 5, the interference was resistant to the action of
actinomycin D, suggesting that interferon is probably not involved in this system. This is consistent with the observation that no interferon was detected in HVJ-pB infected cell culture fluids.

On the other hand, when cells were treated with 100 μg/ml puromycin during the 24 h period of infection with HVJ-pB, the degree of interference was about 10 times lower than in the absence of the drug, suggesting that protein synthesis may be required to establish and maintain the interference.

Fig. 5. Sucrose gradient centrifugation of HVJ-pB. The infectivity of HVJ-pB in each fraction was assayed at 32°C. To measure interfering activity, LLCMK2 cells were infected with 0.1 ml of each fraction, and 24 h later were superinfected with HVJ-W. After further 48 h incubation, culture fluids were harvested and titrated by plaque assay at 38°C. O—O, infectious virus titre of HVJ-pB; □—□, HA of HVJ-pB; ●—●, amounts of superinfecting HVJ-W formed.
Homologous interference by HVJ

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Treatment*</th>
<th>Control</th>
<th>After HVJ-pB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Actinomycin D 5 μg/ml</td>
<td>3×10⁷</td>
<td>1×10⁴</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>9×10⁶</td>
<td>2×10⁴</td>
</tr>
<tr>
<td>2</td>
<td>Puromycin 100 μg/ml</td>
<td>3.5×10⁸</td>
<td>1×10⁶</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>5.7×10⁸</td>
<td>1×10⁶</td>
</tr>
</tbody>
</table>

* Cells were treated with actinomycin D throughout, i.e. during infection with both HVJ-pB for 24 h and HVJ-W for 48 h, or were treated with puromycin only during the 24 h period of infection with HVJ-pB. Controls were untreated.

**Table 5. Effect of treatment of HVJ-pB infected cells with antimetabolites on establishment of interference**

**Fig. 6. Adsorption of HVJ-W to cells previously infected with HVJ-pB.** LLCMK2 cells were infected with HVJ-pB at input m.o.i. of 10 p.f.u./cell, incubated for 24 h, and then superinfected with HVJ-W at 0.1 p.f.u./cell. At the indicated times after superinfection, unadsorbed virus was titrated at 38°C. ○—○, HVJ-pB infected cells; ●—●, mock-infected control cells.

**Adsorption of superinfecting HVJ-W**

A virus adsorption kinetics experiment was carried out to examine whether superinfecting HVJ-W can adsorb to HVJ-pB infected cells. A constant amount of HVJ-W was inoculated on to cell monolayers and at various times after incubation unadsorbed virus was titrated at 38°C. Far more unadsorbed virus was recovered from the cells pre-infected with HVJ-pB than from previously uninfected control cells (Fig. 6).

**DISCUSSION**

A ts mutant derived from a BHK-HVJ carrier cell culture induced homologous interference with the replication of wild-type virus of HVJ both at permissive and non-permissive temperatures.
The HVJ-pB virus used in the present study had been passaged in eggs at high dilution and its interference activity co-sedimented with the infectivity in sucrose gradient centrifugation, suggesting that defective interfering particles (Huang & Baltimore, 1970) do not play a major role in the present interference phenomenon. Incomplete Sendai virus particles could block virus replication even after attachment and penetration of standard virus and the mechanism of this homologous interference was interpreted as competition for enzymes specified by standard virus (Portner & Kingsbury, 1971).

A large amount of u.v.-inactivated HVJ-pB could also induce interference, but the cell remained refractory for only a short period, and 2 h after infection they became sensitive to superinfection with HVJ-W. In this type of interference, it seems likely that cellular receptors are destroyed by the enzymic activity of the HVJ-pB particles (Baluda, 1959), but rapidly regenerate. In contrast, the intensity of interference induced by intact virions of HVJ-pB increased with time, and inhibition of attachment of superinfecting HVJ-W was found in cells 24 h after infection with HVJ-pB. This suggests that the surface membrane of HVJ-pB infected cells may be functionally altered so that they are unable to interact normally with homologous virus. The newly synthesized protein(s) coded for by the complete virus genome and required for induction and maintenance of interference (see Table 5) may be involved in the alteration of the cell membrane. Although it is yet unknown whether the virus protein(s) involved is structural or non-structural, it seems unlikely to be the haemagglutinin or neuraminidase: the previous characterization of HVJ-pB indicated that virus envelope precursor proteins without any haemagglutinating or neuraminidase activity are produced at non-permissive temperatures (Kimura et al. 1975). It is of interest in this connection that cells persistently or acutely infected with HVJ possess on their surface a specific non-virion antigen induced by the infection (Kimura et al. 1976). The relationship between this antigenic change of the cell membrane and the functional alteration induced by HVJ infection remains to be clarified.

Many kinds of virus mutants have been isolated from a variety of persistently infected cell cultures of different virus-cell systems, and there may be some characteristics common to these mutants such as the formation of small plaques, low virulence, and temperature-sensitivity (Simizu & Takayama, 1969, 1971; Preble & Youngner, 1972; Haspel et al. 1973; Shenk, Koshelnyk & Stollar, 1974). These data and the present finding that HVJ-pB induces homologous interference (and also readily establishes persistent infections in several cell systems – Kimura et al. 1975) are of interest with regard to the establishment of chronic infections. Slightly- or non-cytocidal mutants contained in virus preparations may make certain cells refractory to secondary infection with virulent virus, so that thereafter the cells survive and multiply together with the interfering virus. Thus, homologous interference can be considered as one of the mechanisms of establishment of persistent infection.

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