Interferon Susceptibility of Various Cell Lines Persistently Infected with Haemagglutinating Virus of Japan (HVJ)

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SUMMARY

Various cell lines persistently infected with para-influenza 1 virus, HVJ strain, were less susceptible to the antiviral action of interferon than the same cell lines when not infected with HVJ. When Vero cells persistently infected with a temperature-sensitive strain of HVJ were incubated at 38 °C, a non-permissive temperature, they became fully susceptible to interferon, whereas neither the haemadsorbing nor the cell-associated haemagglutinating activity of the virus was expressed. These findings suggest that the lowered interferon susceptibility of virus-carrier cells may be related to the maturation of virus in them.

It was found that the low susceptibility of virus-carrier cells to interferon is not due to blocked adsorption of interferon or to inability of the cells to respond to interferon. Studies with actinomycin D suggest that some step (or steps) before the synthesis of the messenger RNA for the antiviral protein is blocked.

INTRODUCTION

Low susceptibility to interferon has been reported in various virus-cell systems, i.e. para-influenza 3 virus-calf kidney cells (Hermodsson, 1963), Sendai virus-chick embryo cells (Valle & Cantell, 1965), Sendai virus-HeLa cells (Maeno et al. 1966), mumps virus-chick embryo cells (Frothingham, 1965), bovine diarrhoea virus-calf kidney cells (Diderholm & Dinter, 1966), tick-borne encephalitis virus-chick embryo cells (Libíková, 1965), tick-borne encephalitis virus-L cells (Vilček & Stanček, 1963) and so on. However, the mechanism involved in this anti-interferon effect of a virus is poorly understood.

In this present study, we have investigated this phenomenon, using various cell lines infected with a temperature-sensitive strain of HVJ (Nishiyama et al. 1976).

METHODS

Virus. The New Jersey strain of vesicular stomatitis virus (VSV), para-influenza 1 virus, HVJ strain (Sendai virus) and a temperature-sensitive strain of HVJ (HVJts) were used. HVJts was isolated from BHK-HVJ cells (Nishiyama et al. 1976).

Normal cell lines. The cells used in the present study were Vero (derived from green monkey kidney), BHK (derived from baby hamster kidney) and LLCMK2 (derived from Rhesus monkey kidney). These cells were cultured in glass bottles in Eagles’s minimum
essential medium (MEM) supplemented with 10% bovine serum, 10% tryptose phosphate broth and antibiotics. The maintenance medium contained no bovine serum.

**Virus carrier cells and the related cell.** The virus carrier cells used in this study were Vero-HVJts (Vero cells persistently infected with HVJts), BHK-HVJ (BHK cells persistently infected with HVJ) and LLCMK2-HVJ (LLCMK2 cells persistently infected with HVJ). The carrier cultures, BHK-HVJ and LLCMK2-HVJ, were derived by prolonged cultivation of cells surviving on HVJ infection. These were successively subcultured in growth medium at 35 °C. Persistently infected cell lines were established and have been maintained to date through 450 subcultures over a period of 7 years (BHK-HVJ cells) or 90 subcultures over a period of one year (LLCMK2-HVJ cells). In most passages, more than 80% of these cells could haemadsorb when grown to a confluent monolayer at 35 °C. Most of the virus products released from the cells were non-infectious. A temperature-sensitive phenomenon in virus maturation was found in BHK-HVJ cells (Nagata et al. 1972) and LLCMK2-HVJ cells (T. Yoshida, personal communication); the cells released much haemagglutinin into the medium at 32 °C, but only a little at 38 °C.

HT (hamster tumour) cells were derived from a tumour induced in a hamster by subcutaneous implantation of BHK-HVJ cells. These no longer contained HVJ antigen. However, when re-infected with a wild strain of HVJ, they supported growth of this virus to normal yields and more than 99% of the cells became positive for virus antigens, though there were minimal virus cytopathic effects. It therefore seems that HT cells were cured of HVJ infection through the action of immunological mechanisms during their growth in the hamster.

**Preparation of interferon.** Interferon was prepared from human peripheral leukocytes infected with Newcastle disease virus (NDV). To eliminate infectious virus, these interferon preparations were dialysed at pH 2 for 2 to 3 days. The interferon unit used in this study matched those defined by the human leukocyte research reference interferon preparation 69/19. Hamster interferon was prepared from the culture fluid of spleen cells co-cultivated with BHK-HVJ cells (Ito et al. 1974).

**Haemagglutination (HA) titration, and haemadsorption (Had) test.** HA titrations were carried out in plastic trays with 2.5% chicken erythrocytes as described previously (Nagata et al. 1972). Had tests were carried out with guinea pig red blood cells at 4 °C; the extent of Had was observed through a microscope.

**Immunofluorescent staining.** For immunofluorescent staining of intracellular antigens, cells were grown on coverslips in bottles, air-dried and fixed in acetone for 20 min at room temperature. The coverslips were covered with fluorescein-labelled rabbit anti-envelope protein (V) or anti-nucleocapsid (S) antisera and were incubated for 30 min at 37 °C. They were then washed three times, rinsed, mounted in buffered glycerol and viewed through an Olympus fluorescence microscope. The anti-envelope and anti-nucleocapsid antisera were made as described previously (Nagata et al. 1972).

**RESULTS**

**Susceptibility to the anti-viral action of interferon of cell lines persistently infected with HVJ and incubated at 35 °C**

Vero cells, either uninfected or persistently infected with HVJts (Vero-HVJts cells), were grown to confluence at 35 °C. The culture fluid was removed, and serial three-fold dilutions of a human interferon preparation were added. After 20 h of incubation at 35 °C, the culture fluid was removed, and the monolayers were challenged with VSV [about 50 plaque-
Interferon susceptibility of HVJ-infected cells

![Graph showing plaque reduction vs. amount of interferon](image)

**Fig. 1.** Susceptibility to the antiviral action of human interferon of Vero cells (●—●) and Vero-HVJts cells (○—○) incubated at 35°C.

**Table 1.** Interferon susceptibility of HVJ-infected cells*

<table>
<thead>
<tr>
<th>Cells</th>
<th>Interferon treatment</th>
<th>Reduction in plaques (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>BHK</td>
<td>124.3†</td>
<td>2</td>
</tr>
<tr>
<td>BHK-HVJ</td>
<td>127.7</td>
<td>106</td>
</tr>
<tr>
<td>HT</td>
<td>115.3</td>
<td>3</td>
</tr>
<tr>
<td>HT infected with HVJ</td>
<td>118.3</td>
<td>93.3</td>
</tr>
<tr>
<td>LLCMK₂</td>
<td>28</td>
<td>1</td>
</tr>
<tr>
<td>LLCMK₂-HVJ</td>
<td>47</td>
<td>53</td>
</tr>
</tbody>
</table>

*Confluent cell monolayers grown at 35°C were inoculated with 10 units/ml of interferon and after incubation for 20 h at 35°C, were infected with VSV. After 2 or 3 days of further incubation, the monolayers were stained with neutral red and plaques were counted.

† See Methods for details of cells.

‡ Plaque counts.

Forming units (p.f.u.). After 1 h of incubation at 35°C, the cultures were overlain with 1% agar in MEM. On day 3 after virus challenge, the monolayers were stained with neutral red and plaques were counted next day. Fig. 1 compares the effects of each amount of interferon in the two cell lines. At a concentration of 22 human interferon units/ml, there was complete inhibition of plaque formation in uninfected Vero cells, but no effect at all in Vero-HVJ₁₈ cells. Thus, Vero-HVJ₁₈ cells incubated at 35°C were considerably less susceptible than
uninfected Vero cells to the antiviral action of exogenous interferon. Essentially similar results were obtained in experiments with other cell lines persistently infected with HVJ (Table 1).

The interferon preparation had no detectable effect on growth of the endogenous HVJ in the cultures persistently infected with this virus (Vero-HVJts cells and BHK-HVJ cells-data not shown). Since the plaque-forming efficiency of VSV was almost the same irrespective of whether the cells were infected with HVJ or not (Table 1), the HVJ in carrier cultures apparently did not interfere with the expression of VSV infectivity.

**Interferon susceptibility of Vero-HVJts cells incubated at 38 or 32 °C**

Virus growth has been observed to be temperature-sensitive in a variety of cell-virus combinations (Nagata et al. 1972; Preble & Youngner, 1975). Vero-HVJts cells were established with a temperature-sensitive strain of HVJ and the characteristics of the virus carrier state in these cells depends on the incubation temperature. Therefore, the effects of the incubation temperature on their interferon susceptibility were examined.

Confluent Vero and Vero-HVJts cells grown at 35 °C were incubated in maintenance medium at 38 or 32 °C for 24 h and the cells incubated with various concentrations of human interferon at 38 or 32 °C, respectively. After 24 h, the culture fluid was removed and the cells were challenged with about 50 p.f.u. of VSV. After 3 days of further incubation at 38 or 32 °C, respectively, the cell monolayers were stained with neutral red and plaques were counted next day. The results are shown in Fig. 2. Vero cells incubated at either 38 or 32 °C were almost equally susceptible to the antiviral action of interferon. On the other hand,
Interferon susceptibility of HVJ-infected cells

Table 2. Effects of temperature shift from 32 to 38 °C and from 38 to 32 °C on the interferon susceptibility of Vero-HVJ18 cells

<table>
<thead>
<tr>
<th>Temperature</th>
<th>During pre-incubation with or without interferon (°C)</th>
<th>During VSV challenge (°C)</th>
<th>Interferon treatment</th>
<th>Reduction in plaques (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>38</td>
<td>38</td>
<td>-</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td>32</td>
<td>38</td>
<td>34†</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>38</td>
<td>32</td>
<td>35</td>
<td>15.5</td>
<td>56</td>
</tr>
<tr>
<td>32</td>
<td>32</td>
<td>30</td>
<td>31</td>
<td>0</td>
</tr>
</tbody>
</table>

* Confluent Vero-HVJ18 cells grown at 35 °C were incubated in maintenance medium at 38 or 32 °C for 24 h and then with medium with or without 10 units/ml of interferon and at the same temperature as before. After 20 h, the monolayers were infected with about 50 p.f.u. of VSV, kept 1 h at room temperature for virus adsorption and then incubated for 3 days with or without a temperature shift to 38 or 32 °C.
† Plaque counts.

Vero-HVJ18 cells were much less sensitive to the antiviral action of interferon at 32 °C, but almost fully sensitive at 38 °C. VSV growth was almost unaffected by the temperature shift, as is shown later (Table 2, 3). These results show that the interferon susceptibility of these virus carrier cells is suppressed at 32 °C but present at 38 °C.

Characteristics of Vero-HVJ18 cells incubated at 38 or 32 °C

Vero-HVJ18 cells were grown at 35 °C for 3 days. After confluent monolayers had formed, they were incubated in maintenance medium for 24 h at 38 or 32 °C. The presence of virus antigens in the cytoplasm of the cells was examined by fluorescent antibody staining. Nucleocapsid protein and envelope protein antigens were detected in the cytoplasm and the intensity of fluorescence was comparable when cells were incubated at either 38 or 32 °C. Cells incubated at 38 °C did not haemadsorb and formed no cell-associated haemagglutinin, while those incubated at 32 °C had distinct haemadsorption and haemagglutinating activity. A considerable amount of haemagglutinin was detected in the culture fluid at 32 °C, but none at 38 °C.

These characteristics of Vero-HVJ18 cells are identical with those of BHK-HVJ cells, as reported by Nagata et al. (1972).

Effects of temperature shift from 32 to 38 °C and from 38 to 32 °C on interferon susceptibility of Vero-HVJ18 cells

The foregoing observations led us to study the effects of temperature shift, both up and down, on the interferon susceptibility of virus-carrier cells. Confluent Vero-HVJ18 cells grown at 35 °C were therefore incubated in the maintenance medium at 38 or 32 °C for 24 h. The culture fluid was then replaced with medium with or without 10 units/ml of interferon. After incubation for 20 h at 38 or 32 °C, respectively, the monolayers were infected with about 50 p.f.u. of VSV, kept 1 h at room temperature for adsorption and then incubated with or without a temperature shift to 38 or 32 °C. After 3 days, the monolayers were stained with neutral red and counted for plaques on the following day. As shown in Table 2, irrespective of the temperature during pre-incubation with interferon, formation of VSV plaques at 38 °C was completely inhibited. However, formation of VSV plaques at 32 °C was partially inhibited when the monolayers had been pre-incubated with interferon at 38 °C, but not at all after pre-incubation with interferon at 32 °C. These results indicate that interferon can be adsorbed at either 32 or 38 °C. Also, the antiviral state in Vero-HVJ18
Table 3. Effects of actinomycin D on the interferon susceptibility of Vero-HVJts cells*

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Virus yield (p.f.u./o·i ml x 10^{-5})</th>
<th>Reduction in yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>During pre-incubation with or without interferon (°C)</td>
<td>During VSV challenge (°C)</td>
<td>Without act. D</td>
</tr>
<tr>
<td>38</td>
<td>38</td>
<td>140</td>
</tr>
<tr>
<td>32</td>
<td>38</td>
<td>75</td>
</tr>
<tr>
<td>32</td>
<td>32</td>
<td>240</td>
</tr>
</tbody>
</table>

* Vero-HVJts cells, pre-incubated for 20 h in medium with or without 10 units/ml of interferon (IF) were further incubated for 1 h in medium with or without 10 μg/ml of actinomycin D (act. D). The cells were then washed three times and infected with VSV (m.o.i. 10) and after 12 h, the culture fluids were harvested and assayed for virus.

Effects of actinomycin D on the interferon susceptibility of Vero-HVJts cells

In order to determine which step(s) in the development of the antiviral state triggered by interferon in Vero-HVJts cells is blocked at 32 °C, the effects of actinomycin D were studied. Vero-HVJts cells, pre-incubated for 20 h in medium with or without 10 units/ml of interferon, were further incubated for 1 h in medium with or without 10 μg/ml of actinomycin D. Subsequently, the cells were washed three times and infected with VSV (m.o.i. 10). After 12 h, the culture fluid was harvested and assayed for virus yield. The results are summarized in Table 3.

When Vero-HVJts cells were incubated with interferon at 38 °C and infected with VSV at either 38 or 32 °C, the virus yield was markedly reduced to less than 5% of the control value. Addition of actinomycin D did not appear to influence the antiviral state of these cells.

When Vero-HVJts cells were incubated with interferon at 32 °C and then infected with VSV at 32 °C, there was no significant antiviral action of interferon irrespective of actinomycin D treatment. However, when Vero-HVJts cells pre-incubated with interferon at 32 °C were infected with VSV at 38 °C, the virus yield was markedly reduced, indicating full development of the antiviral state. In contrast, when the cells were treated with actinomycin D for 1 h prior to the temperature shift-up, the development of the interferon-induced antiviral state was almost completely blocked, as indicated by only a small reduction in virus yield. Thus, subsequent to the temperature shift-up, the development of the antiviral state is highly sensitive to actinomycin D.

DISCUSSION

The present study has shown that various cell lines persistently infected with HVJ were less susceptible to the antiviral action of interferon than were uninfected control cells. However, the reduced interferon susceptibility of Vero-HVJts cells could be increased to the level of uninfected cells by a temperature shift-up to 38 °C.

The mechanism by which a non-cytocidal virus renders a cell unresponsive to the action of interferon is not well understood. One possible explanation is that the virus may inhibit
Interferon susceptibility of HVJ-infected cells

Cellular metabolic pathways for interferon to induce antiviral resistance. Alternatively, substances produced in the virus-infected cells may counteract the action of interferon (Vilček, 1969), for example, stimulon (Chany & Brailovsky, 1967) and/or anti-interferon (Ghendon, et al. 1966). However in our system, susceptibility to the antiviral action of interferon was restored shortly after a temperature shift-up to 38 °C, so that the reduced susceptibility can hardly have been due to a factor(s), apart from a virus component, blocking the action of interferon.

Carrier cultures of Vero-HVJts were established with a temperature-sensitive strain of HVJ isolated from BHK-HVJ cells (Nishiyama et al. 1976). This virus (HVJts) has been found to change a number of cell lines into the carrier state at both a permissive (32 °C) and a non-permissive (38 °C) temperature, while causing little or no cytopathic effect. The temperature sensitive step in the replication of HVJts is virus maturation: when infected BHK cells are incubated at 38 °C, production of infectious virus, haemagglutinin and neuraminidase is markedly reduced, but considerable amounts of virus nucleocapsid and envelope antigens can be detected in the cells (Nishiyama et al. 1976). However, no temperature-sensitive protein has yet been identified. A temperature-sensitive phenomenon of virus maturation has also been observed in various other carrier cultures established with HVJts. In the present study, Vero-HVJts cells incubated at 38 °C showed no haemadsorption or cell-associated haemagglutinating activity and had almost normal interferon susceptibility. We therefore infer that the reduced interferon susceptibility of virus carrier cells which we have observed in this study may be related to maturation of virus in them.

Which step(s) in the development of the antiviral state triggered by interferon is suppressed? In the interferon-treated cells, development of this state is thought to depend on the production of an antiviral protein (Taylor, 1964). When Vero-HVJts cells were incubated with interferon at 38 °C and infected with VSV at 32 °C, the virus yield was reduced to less than 5% of the control value whether or not the cells were treated with actinomycin D for 1 h before VSV infection. This indicates that the manifestations of the antiviral state are not influenced by the incubation temperature once the antiviral state has developed in the cells. In the present study, actinomycin D inhibited the development of the antiviral state when the temperature was shifted from 32 to 38 °C. Therefore, the step suppressed in Vero-HVJts cells incubated at 32 °C must occur before synthesis of the messenger RNA for the antiviral proteins.

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REFERENCES

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