Characterization of Altered BHK Cells Resistant to HVJ (Sendai Virus) Infection

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SUMMARY

An altered baby hamster kidney cell culture which resists the c.p.e. of HVJ (haemagglutinating virus of Japan – the Sendai strain of parainfluenza 1 virus) has been obtained and characterized. These cells, designated BHK-R, were originally obtained by prolonged cultivation of cells surviving HVJ infection; they have been subcultured in the presence of HVJ. No infectious virus was recovered from BHK-R cells and no evidence for the presence of HVJ antigens in the cells was demonstrated by immunofluorescent staining. When BHK-R cells were inoculated with HVJ the growth of challenge virus was suppressed and no obvious cytopathic changes were detected, while these cells normally supported the replication of mumps, influenza, Newcastle disease, vesicular stomatitis or Sindbis viruses. BHK-R cells became susceptible to HVJ infection after serial subculture in growth medium free of HVJ. It was suggested that sialic acid residues present in the surface of BHK-R cell membranes and responsible for adsorption of HVJ were split off by the action of neuraminidase of virus particles, resulting in inhibition of the attachment of challenge virus of HVJ.

During the course of studies on the initial stage of the establishment of HVJ persistent infection, a baby hamster kidney (BHK) cell culture which showed a distinct resistance to superinfection with HVJ was obtained by inoculation of cells with HVJ at a high input m.o.i. and prolonged cultivation of survivor cells. It soon became evident that these cells were distinguishable from persistently-infected cells by the absence of virus and virus antigens. Characterization of the altered BHK cells is the subject of this report.

The Nagoya strain of HVJ, the Miyadera strain of Newcastle disease virus (NDV), the fowl plague strain and the NWS strain of influenza virus were propagated routinely by allantoic inoculation of 10-day-old embryonated eggs. The Miyake strain of mumps virus was propagated in LLCMK 2 cells. The New Jersey serotype of vesicular stomatitis virus (VSV) and Sindbis virus were propagated in BHK cells. VSV plaque-former phenotypically mixed with HVJ was obtained by the procedure described in a previous report (Kimura, 1973). A continuous cell line (BHK) derived from baby hamster kidney cells, an altered BHK cell culture resistant to HVJ infection (BHK-R), an HVJ carrier culture of BHK cells (BHK-HVJ) and the LLCMK 2 continuous line of monkey kidney cells were cultured in Eagle's minimal essential medium (MEM) containing 10% calf serum, 10% tryptose phosphate broth and 60 µg/ml kanamycin. Unless otherwise mentioned BHK-R cells were continually passaged in the presence of HVJ. Plaque titration of HVJ on LLCMK 2 cell monolayers was carried out by the addition of 3 µg/ml of trypsin in the agar overlay medium (Sugita et al. 1974). Mumps virus was assayed by plaque titration on LLCMK 2 cell monolayers, but in this case no trypsin was required for plaque formation. Haemagglutinin was titrated by the pattern method (Sever, 1962). Anti-HVJ virion and anti-HVJ nucleocapsid rabbit sera were prepared in rabbits according to the procedure of Compans & Choppin (1967) and Nagata et al. (1972). Fluorescent antibody staining was carried out by
the indirect method (Kimura et al. 1976a). Protein was determined by the method of Lowry et al. (1951). Cell-bound sialic acid was liberated by hydrolysis of cell homogenates with 0.05 M H$_2$SO$_4$ at 80 °C for 60 min (Svennerholm, 1958) and free sialic acid was assayed by the thiobarbituric acid method of Warren (1959).

BHK-R cells were obtained in the following way. Monolayer cultures of BHK cells were inoculated with HVJ at an input multiplicity of approx. 100 p.f.u./cell. After adsorption for 1 h, monolayers were incubated in maintenance medium at 36 °C. Almost all virus-infected cells degenerated and detached from culture bottles within the period of 48 h. The cell debris was removed and fresh growth medium together with about 10$^8$ p.f.u. of HVJ was added to the culture. One to two weeks later a few colonies of the surviving cells became visible. Thereafter the cells were subcultured in the continuous presence of approx. 10$^8$ p.f.u. of HVJ. The growth of the resistant cells was compared with that of normal BHK cells. Doubling times for cells of these cultures in MEM containing 10% calf serum were nearly equal and of the order of 20 h. The resistant cells appeared to be morphologically flatter and more slender than original BHK cells. Thus, the altered cells were designated BHK-R cells.

The growth characteristics of HVJ in BHK-R cells, in normal BHK cells and in an HVJ carrier culture of BHK-HVJ cells were examined. Both BHK-R and BHK-HVJ cells showed no obvious cytopathic changes, and no appreciable increase in challenge virus titres was detected. In contrast, normal BHK cells produced a large amount of progeny virus and degenerated.

Resistance to superinfection with the homologous virus is a general property of virus carrier cultures in a variety of different virus-cell systems. To clarify whether BHK-R cells have been actually infected with HVJ, attempts were made to isolate infectious virus from the cells. No infectious virus could be recovered from BHK-R cells at any incubation temperature, while large amounts of virus could be obtained from BHK-HVJ cells when incubated at 32 °C (Kimura et al. 1975). Next, BHK-R cells were tested for the presence of HVJ virus antigens by immunofluorescent staining. No fluorescence was detected in BHK-R cells after staining with anti-HVJ virion or anti-HVJ nucleocapsid serum. Further, no fluorescence could be seen even 16 h p.i. with HVJ. In contrast, BHK cells 16 h p.i. with HVJ and BHK-HVJ cells exhibited virus-specific fluorescence. Thus, it was unlikely that BHK-R cells were infected with HVJ transiently or persistently.

The process of adsorption and penetration of HVJ into BHK-R cells was investigated by the use of VSV plaque-former phenotypically mixed with HVJ. Original VSV produced plaques efficiently on both normal BHK and BHK-R cell monolayers. VSV plaque-forming virus with a coat of HVJ also produced plaques on BHK cells, while this type of VSV produced no plaques on BHK-R cell monolayers. These findings suggested that resistance of BHK-R cells to HVJ infection might occur at an early stage before the uncoating process. To examine whether HVJ can adsorb to BHK-R cells, a virus adsorption kinetics experiment was carried out. A large fraction of HVJ inoculated on to BHK-R cells remained unadsorbed. In BHK cell cultures the amount of free virus showed a steady decline with time of incubation.

To determine whether BHK-R cells could support the replication of other myxo- and paramyxoviruses, the growth of mumps virus was first tested (Table 1). Cell monolayers were infected with mumps virus at 1 p.f.u./cell and after a 1 h adsorption period cells were washed and incubated in maintenance medium. Replication of mumps virus proceeded equally well in both BHK and BHK-R cells. When influenza virus, NDV, VSV or Sindbis virus was used as the challenge virus no significant reduction of virus yield was found in BHK-R cells.
Short communications

Table 1. Susceptibility of BHK-R cells to heterologous enveloped virus infection

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Challenge virus</th>
<th>Virus titre*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BHK-R</td>
</tr>
<tr>
<td>1</td>
<td>HVJ</td>
<td>&lt; 2</td>
</tr>
<tr>
<td></td>
<td>Influenza virus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fowl plague strain</td>
<td>640</td>
</tr>
<tr>
<td></td>
<td>NWS strain</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>NDV</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>Mumps virus</td>
<td>4.4 x 10⁶</td>
</tr>
<tr>
<td></td>
<td>NDV</td>
<td>4.6 x 10⁶</td>
</tr>
<tr>
<td></td>
<td>VSV</td>
<td>5.5 x 10⁶</td>
</tr>
<tr>
<td></td>
<td>Sindbis virus</td>
<td>2.4 x 10⁶</td>
</tr>
</tbody>
</table>

* Titres determined 48 h after addition of challenge virus, expressed as HAU/0.05 ml in expt. 1 and as p.f.u./ml in expt. 2.

BHK-R cells were serially subcultured every 3 days in growth medium free of HVJ and at each passage responses of the cells to HVJ infection were examined. Cells were infected with HVJ at 10 p.f.u./cell. After incubation for 48 h culture fluids were assayed for virus growth at 38 °C (Table 2). When BHK-R cells were subcultured once in HVJ-free medium and then challenged with HVJ the cells remained morphologically unchanged. Further, no appreciable increase in virus yield was found, although very low titres of haemagglutinin (HA) and nucleocapsid and virion antigens were detected in the cells. BHK-R cells became gradually susceptible to HVJ infection by repeated subculture in HVJ-free medium and at the fourth subculture the growth of challenge virus in the cells corresponded to about half that in normal BHK cells. After five such passages the cells were completely restored to the original susceptible state. On the other hand, BHK-HVJ cells constantly showed a resistance to superinfection by the homologous virus; the growth of superinfecting HVJ was suppressed and no c.p.e. was detected. Moderate titres of cell-associated HA and virus complement-fixing (CF) antigens might be a manifestation of intracellular growth of the carried virus.

In considering the fact that myxo- and paramyxoviruses adsorb to specific receptors on cell surfaces which contain neuraminic acid (Hirst, 1943; Gottschalk, 1957), it was thought necessary to examine the sialic acid content of BHK-R and normal BHK cells. The sialic acid content of BHK-R cells was approximately half that of normal BHK cells. However, by repeated subcultivation in HVJ-free medium, the sialic acid content of the cells was virtually restored (Table 2).

When fetuin (2-1 mg/ml) was added to growth medium containing HVJ particles it gradually became difficult to maintain BHK-R cells refractory to cytolitic infection with HVJ, suggesting that the neuraminidase activity of the HVJ particles was blocked by fetuin and newly generated cellular receptors for HVJ were preserved from enzymic digestion.

In the present study BHK-R cells were completely resistant to c.p.e. when superinfected with HVJ. The resistance seemed to be due to inhibition of the attachment of challenge virus. The reduced capacity of cells for adsorption of HVJ was also demonstrated in cells previously inoculated with avirulent HVJ-pB (Kimura et al. 1976b). In the case of interference induced by HVJ-pB some protein synthesis coded for by the virus genome was required to establish and maintain the resistance. However, failures to demonstrate infectious virus or virus antigens in BHK-R cells suggested that during the cultivation of cells with large amounts of HVJ the virus did not enter the cells or cause any intracellular changes which could be involved in induction of resistance. The most plausible explanation for this type of resistance may be continual destruction of cellular receptors by the neuraminidase of extracellular virus particles added in the culture medium. Baluda (1959) reported on the externally-mediated modification of cell surfaces by u.v.-irradiated NDV,
Table 2. Reversible susceptibility of BHK-R cells to HVJ infection

<table>
<thead>
<tr>
<th>Cells</th>
<th>Sialic acid (µg)*</th>
<th>p.f.u./ml</th>
<th>HAU/0.05 ml†</th>
<th>Nucleocapsid</th>
<th>Envelope</th>
<th>c.p.e.‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHK-R 08</td>
<td>2.35</td>
<td>1.0 x 10⁶</td>
<td>&lt; 2</td>
<td>&lt; 2</td>
<td>&lt; 2</td>
<td>0</td>
</tr>
<tr>
<td>BHK-R 1</td>
<td>2.67</td>
<td>1.8 x 10⁶</td>
<td>&lt; 2</td>
<td>&lt; 2</td>
<td>&lt; 2</td>
<td>0</td>
</tr>
<tr>
<td>BHK-R 2</td>
<td>3.00</td>
<td>4.7 x 10⁴</td>
<td>8</td>
<td>16</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>BHK-R 3</td>
<td>3.08</td>
<td>3.8 x 10⁴</td>
<td>16</td>
<td>32</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>BHK-R 4</td>
<td>3.22</td>
<td>5.5 x 10⁴</td>
<td>64</td>
<td>32</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>BHK-R 5</td>
<td>3.41</td>
<td>2.2 x 10⁴</td>
<td>256</td>
<td>32</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>BHK-R 9</td>
<td>3.50</td>
<td>2.0 x 10⁴</td>
<td>256</td>
<td>64</td>
<td>32</td>
<td>4</td>
</tr>
<tr>
<td>BHK</td>
<td>4.00</td>
<td>1.5 x 10⁴</td>
<td>256</td>
<td>64</td>
<td>32</td>
<td>4</td>
</tr>
<tr>
<td>BHK-HVJ</td>
<td>1.95</td>
<td>5.4 x 10⁵</td>
<td>32</td>
<td>16</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

* Sialic acid content was calculated as µg/mg protein.
† Cell homogenates were prepared by 20 kHz sonic treatment of infected cells for 30 s in an ice bath.
‡ 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).
§ Passage number of cells in medium free of HVJ.

resulting in loss of the ability of cells to adsorb active virus. It is of interest in this connection that pre-treatment with receptor-destroying enzyme (RDE) protected mice against infection with influenza virus (Cairns, 1951). The finding that BHK-R cells were resistant only to HVJ infection and still remained susceptible to infection with other myxo- and paramyxoviruses, including mumps virus, indicated that the neuraminidase of HVJ particles destroyed specific receptors for HVJ only. Recently, it has been found that BHK-R cells are less sensitive to the antiviral action of interferon (IF) and that the sensitivity to IF action revives with an increase in sialic acid content of the cells (Y. Kimura et al. unpublished data). BHK-R cells would provide a useful system for analysis of the interaction between cell membranes and myxo- and paramyxoviruses.

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