Persistent Rubella Virus Infection in Hamster Lung Cells

By JEAN C. DOWNIE AND J. S. OXFORD

University of Sheffield, Virus Research Laboratory,
Lodge Moor Hospital, Sheffield 10

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SUMMARY

Cell cultures were established from the lungs of hamsters which had been previously infected intranasally with the JUDITH strain or the HPV77 vaccine strain of rubella virus. The hamster lung cells were cultured in vitro and produced infective rubella virus for at least 12 subcultures. The persistently infected cells had a lag phase of 48 hr compared to a lag phase of 24 hr in uninfected cells. Morphologically, the persistently infected cells were indistinguishable from uninfected cells. Treatment of the persistently infected cells with 25 μg./ml. amantadine, 0.15 μg./ml. actinomycin D or 250 μg./ml. hydrocortisone had no detectable effect on rubella virus yields. Infective cell counts indicated that between 5 and 50% of hamster lung cells were releasing infective rubella virus. The persistently infected cells were resistant to challenge with vaccinia virus and, to a lesser degree, with herpes simplex virus.

INTRODUCTION

Persistent or carrier-type virus infections produced under in vitro conditions have been described for many animal viruses. Such infections are characterized by an apparent balance between host cell destruction and continuous virus production; they differ from latent virus infections where the virus genome persists but the production of infective virus is uncommon and sporadic (reviewed by Andrewes, 1940; Gajdusek, 1965).

Persistent infection with rubella virus has been described in two laboratory animals, the hamster (Oxford & Schild, 1966) and the ferret (Fabiyi, Gitnick & Sever, 1967). This virus therefore provides a useful model for the study of persistent virus infection in vivo as well as in vitro. The present report describes the establishment and characters of cell cultures derived from the lungs of hamsters which were already persistently infected with rubella following intranasal instillation of the virus. Attempts were made to inhibit the continuous rubella virus multiplication in these cells by various methods.

METHODS

Viruses. The JUDITH strain of rubella virus (supplied by Professor K. McCarthy, Liverpool) had been propagated twice in cercopithecus monkey kidney cells and 47 times in Glaxo Laboratories RK 13 cells. It had a titre of $10^{5.2}$ TCD50/ml in RK 13 cells. The HPV77 vaccine strain (supplied by Dr H. M. Meyer, National Institutes of Health, Bethesda, Maryland, U.S.A.) had been subcultivated 77 times in grivet monkey kidney cells and once in RK 13 cells; the titre of the stock pool was $10^{4.5}$ TCD50/ml in RK 13 cells.
Herpes simplex virus (supplied by Dr A. D. Macrae, Colindale, London, N.W. 9) was subcultivated 3 times in RK 13 cells and twice in BHK 21 cells.

Vaccinia virus (supplied by Dr M. Wilson, Public Health Laboratory Service, Sheffield) was propagated 3 times in BHK 21 cells.

**Virus assays.** Rubella virus was assayed by end-point infectivity titrations using 4 RK 13 cell culture tubes per dilution. The cell cultures were examined for cytopathic effects after 10 days and tissue culture fluids subinoculated into a further set of cultures and again examined after 10 days. The 50% cytopathic end-points were determined by the Spearman–Karber method and expressed as TCD50/ml. (Dougherty, 1964).

**Experimental animals.** The hamsters (*Mesocricetus auratus*) used were from a randomly bred, closed colony. They were lightly anaesthetized with ether for intranasal inoculation.

**Establishment of persistently infected cell cultures.** Litters of hamsters 1 to 2 weeks of age were inoculated intranasally each with 0.05 ml./animal of rubella virus or a control inoculum of uninfected RK 13 cells. Animals from each litter were bled and killed 4 weeks after inoculation. The lungs from these animals were minced with scalpel blades and treated for 30 min. at 37° with Difco trypsin 1:250. The resulting cell suspension was inoculated into prescription bottles and the cultures were incubated at 36° to 37° until confluent monolayers were obtained.

The growth medium was Eagle’s basal medium with 10% inactivated calf serum, 10% tryptose phosphate broth (Difco) and 0.44 g./l. NaHCO3. The hamster lung cells were subcultured every 3 or 4 days by splitting 1:2 using trypsin to disperse the cells. A confluent monolayer in a 20 oz prescription bottle contained an average of 8 x 10^8 cells.

**Growth curves of hamster lung cells.** Tubes were seeded with 7 to 8 x 10^4 cells/ml. of the different hamster lung cell cultures at various passage levels. The cells were incubated at 36° to 37° and the growth medium changed on the 3rd and 5th days after seeding. At daily intervals after subculture six tubes were harvested to determine the cell count, the concentrations of extracellular virus and total virus and the number of infective cells. Three tubes were subjected to three cycles of freezing and thawing to release cell-bound virus for assay of total virus. The cell culture fluid of the remaining three tubes was collected, centrifuged at 150 g for 10 min., and the supernatant fluid used for assay of extracellular virus. The cells remaining in these three tubes were trypsinized, pooled and gently pipetted to give a suspension of single cells. For each cell suspension eight separate fields with a total of 300 to 500 cells were counted in a Fuchs–Rosenthal haemocytometer. The cells were then centrifuged at 56 g for 10 min., the trypsin was removed and the cells were resuspended in 0.2 ml. of human γ-globulin and incubated at room temperature for 1 hr. The cells were washed in three changes of Eagle’s basal medium to remove residual extracellular virus and γ-globulin and then titrated in RK 13 cells to determine the number of infective cells. The γ-globulin used had a titre of 1/800 against the JUDITH strain of rubella virus determined by a microplaque method (Taylor-Robinson et al. 1964).

**Test compounds.** Amantadine was kindly supplied by Dr A. Galbraith of Geigy (U.K.) Ltd. Ammonium acetate was a standard analytical reagent (British Drug Houses). The compounds were prepared as 10% stock solutions in deionized water and stored at 4°. Amantadine was used at a concentration of 25 μg./ml. and ammonium acetate at 250 μg./ml.
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Actinomycin D (Merck, Sharp and Dohme Ltd) was used at a concentration of 0.15 μg./ml. Hydrocortisone sodium succinate (Organon Laboratories Ltd) was prepared as a solution containing 50 mg./ml. and diluted immediately for use.

RESULTS

Characteristics of cell cultures established from lungs of rubella infected hamsters

Cell lines were established from the lungs of hamsters which had been inoculated intranasally 4 weeks previously with 100 TCD 50 of the JUDITH rubella strain, 1000 TCD 50 of the HPV77 rubella vaccine strain, or with uninfected RK13 cells. Rubella virus was recovered from both the JUDITH and the HPV77 hamster lung cell cultures from the first to the 10th or 12th subcultures (Table 1). Cellular release of both strains of rubella virus appeared to be continuous since virus was detected within 24 hr of each subculture and virus levels remained at 4 to 6 log. TCD 50/ml. throughout. There was no detectable difference between concentration of total virus and extracellular virus. The identity of the agent recovered from these cultures was confirmed as rubella virus by specific neutralization with rabbit and human antisera. In addition, the agent produced typical rubella virus cytopathic changes on RK13 cells, including microplaque formation. No cytopathic agent was recovered from the hamster lung cell culture lines originating from the hamsters inoculated initially with uninfected RK13 cells.

Table 1. Recovery of rubella virus from hamster lung cell cultures

<table>
<thead>
<tr>
<th>Strain of virus used to infect hamster</th>
<th>Log. TCD 50/ml. rubella virus at the indicated cell subculture level*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6</td>
</tr>
<tr>
<td>JUDITH</td>
<td>5.4</td>
</tr>
<tr>
<td>HPV77</td>
<td>5.5</td>
</tr>
</tbody>
</table>

* Cells at each subculture were frozen and thawed 4 days after trypsinization and after centrifugation (150 g/10 min.) the supernatant fluids were titrated for infective virus.

The rate of cell division was compared in the persistently infected and in the control hamster lung cell culture in early and late passages (Fig. 1). In cultures of uninfected cells there was a lag phase of 24 hr when no cell division was detected. This was followed by a rapid increase in cell division during the next 24 hr with the maximum number being reached after 110 hr. However, with the persistently infected cells the lag phase was more prolonged and no cell division was detected for the first 48 hr after seeding. However, the final number of cells in the persistently infected cultures after 110 hr was similar to that in control cultures. The increased lag phase in the persistently infected cells was noted in seven separate growth curve experiments where cell counts were compared to control cells growing in parallel. In both persistently infected cells and in control uninfected cells the number of cells staining with trypan blue was less than 1%. The growth rate of both infected and uninfected cells flagged after 8 to 12 subcultures, representing at least 24 generations of cells, and subcultivation could not be continued.

Coverslip preparations of the two persistently infected hamster lung cell lines and of the uninfected cells were fixed and stained with haematoxylin and eosin and examined.
microscopically. All the cells were typically fibroblastic in appearance and infected and uninfected cells could not be distinguished.

Influence of temperature, cell growth medium and virustatic compounds on rubella virus persistence in hamster lung cell cultures

Tube cultures of hamster lung cells persistently infected with the HPV77 rubella virus strain were incubated at 37°C with Eagle's basal medium containing either 10% or 2% inactivated calf serum. After incubation for 18 days at 36° to 37° with 2% calf serum the cell count was $10^4$/ml. and the total virus was $10^8$ TCD50/ml. In cell cultures incubated with 10% calf serum the cell count was $10^8$/$\text{ml.}$ and total virus was $10^4$ TCD50/ml. In addition, the rolling of cell cultures at 36° to 37° had no detectable effect on the growth of hamster lung cells persistently infected with the HPV77 strain of rubella or on the yield of virus from these cells. Nor was there a difference in the yield of rubella virus from cells incubated at 33° or 37° for 7 days (Table 2).

Treatment with amantadine (25 µg./ml.) ammonium acetate (250 µg./ml.) or hydrocortisone sodium succinate (250 µg./ml.) for 7 days had no detectable effect on the growth of the cells or on the release from them of infective rubella virus (Table 2). Actinomycin D (0.15 µg./ml.) had a slight inhibitory effect on cell growth rates but no detectable effect on the release of rubella virus from the cells.

Interference with superinfecting viruses

Different DNA- or RNA-containing viruses were titrated in tenfold dilution steps in hamster lung cells persistently infected with rubella and in uninfected hamster lung cells (Table 3). Interference was most marked with vaccinia virus; no cytopathic effects were noted in the cell cultures persistently infected with rubella virus when super-infected with 6.5 log. TCD 50/ml. of vaccinia virus. Little interference was noted
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with herpes simplex virus and adenovirus type 6. In contrast, no interference was detected in cultures superinfected with influenza virus A/TURKEY/ENGLAND/63.

Attempts were made to recover interferon from these persistently infected cells. The infected tissue culture fluids were removed and dialysed against glycine-HCl buffer at pH 2.0 overnight at 4°C and then redialysed against phosphate buffered saline pH 7.2 at 4°C for 24 hr. After centrifugation at 100,000 g for 1 hr the fluids were diluted 1/4 in Eagle’s medium and incubated with normal hamster lung cells for 24 hr at 37°C.

Table 2. Effect of various treatments on hamster lung cells persistently infected with rubella

<table>
<thead>
<tr>
<th>Strain of virus used to infect hamster</th>
<th>No additions</th>
<th>Actinomycin D, 0.15 µg/ml.</th>
<th>Amantadine, 25 µg/ml.</th>
<th>Hydrocortisone, 250 µg/ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>JUDITH</td>
<td>37°</td>
<td>37°</td>
<td>37°</td>
<td>37°</td>
</tr>
<tr>
<td>HPV77</td>
<td>2.2</td>
<td>1.9</td>
<td>Not tested</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
<td>5.0</td>
</tr>
</tbody>
</table>

* Appropriate medium was changed daily.

Table 3. Virus interference in hamster lung cultures persistently infected with rubella virus

<table>
<thead>
<tr>
<th>Challenge virus</th>
<th>Uninfected cells</th>
<th>Cells persistently infected with rubella</th>
<th>Reduction in titre (log. TCD50/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinia</td>
<td>6.5</td>
<td>&lt; 0.5</td>
<td>6.0</td>
</tr>
<tr>
<td>Herpes simplex</td>
<td>6.9</td>
<td>5.9</td>
<td>1.0</td>
</tr>
<tr>
<td>Adenovirus type 6</td>
<td>1.2</td>
<td>&lt; 0.5</td>
<td>0.7</td>
</tr>
<tr>
<td>Influenza A/TURKEY/ENGLAND/63</td>
<td>6.5</td>
<td>7.0</td>
<td>—</td>
</tr>
</tbody>
</table>

Table 4. Infective cell counts in persistently infected hamster lung cells

<table>
<thead>
<tr>
<th>Virus strain and passage number of hamster lung cells</th>
<th>No. of hamster cells inoculated per RK13 culture</th>
<th>No. of RK13 cultures with rubella C.P.E.</th>
<th>No. of hamster cells to infect 50% of RK cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV77 pass 6</td>
<td>10⁵±8</td>
<td>4/4</td>
<td>20</td>
</tr>
<tr>
<td>HPV77 pass 12</td>
<td>10⁵±8</td>
<td>4/4</td>
<td>2</td>
</tr>
<tr>
<td>JUDITH pass 13</td>
<td>10⁵±8</td>
<td>4/4</td>
<td>19</td>
</tr>
</tbody>
</table>

* No. of tube cultures with rubella cytopathic effects.

No. of tube cultures inoculated
No interference was detected when these cultures were challenged with 4 log. TCD$_{50}$/ml. of vaccinia virus.

Since the persistently infected cells were resistant to superinfection with vaccinia and herpes simplex viruses in the absence of detectable interferon it was necessary to determine whether the entire cell population was infected with rubella virus, so resulting in virus interference. Table 4 shows the results of three experiments to determine the number of infective cells in cultures persistently infected with the JUDITH and HPV 77 strains or rubella. The results of these and other similar experiments suggested that between 5 and 50% of hamster lung cells were releasing infective rubella virus. The variation in the estimates of the number of infective cells could not be correlated with the strain of virus used to infect the lung cells initially, or with the passage number of the cell culture.

**DISCUSSION**

Rubella virus has been shown to produce a persistent type of infection in human embryos infected *in utero* (Alford, Neva & Weller, 1964), in mammalian cells cultivated *in vitro* (Rawls & Melnick, 1966) and also in laboratory animals (Oxford & Schild, 1966; Fabiyi *et al*. 1967). Persistent infection with viruses other than rubella has been shown to result from infection of genetically resistant cells, from the presence of specific and non-specific virus inhibitors in the extracellular fluids, from interference and interferon or from other undetermined factors (reviewed by Baron, 1966). In the present study no interferon was detected in the hamster lung cells persistently infected with rubella. This negative finding does not necessarily exclude the possibility that interferon may play a role in the establishment of a virus carrier state in these cells. Intracellular interferon in amounts which could not be detected (Stancek, 1965) may still be effective or infection by complete or incomplete virus may induce components of the interferon system without inducing interferon production *per se* (Baron, 1966).

Of particular interest was the marked resistance of the rubella infected hamster cells to superinfection with vaccinia virus. In studies with rubella carrier cultures of human cells, Rawls & Melnick (1966) failed to demonstrate interferon production, although the cells resisted superinfection with vesicular stomatitis and herpes simplex viruses. Since 100% of cells in the human carrier cultures were releasing rubella virus, a virus interference mechanism was proposed to explain the cellular resistance to challenge with other viruses. However, in the present study infective cell count experiments suggested that less than 50% of the cells were infected with rubella virus. The explanation of resistance to vaccinia virus superinfection in the persistently infected hamster lung cells must therefore await further attempts to demonstrate possible low concentrations of interferon in the cultures.

Treatment of the persistently infected hamster lung cells with 25 µg./ml. of amantadine or 250 µg./ml. of ammonium acetate had no demonstrable effect on rubella virus production. These latter two compounds reduced the yield of rubella virus from cells acutely infected with rubella strains (Oxford & Schild, 1967) but only when they were present at the time of virus adsorption and penetration. Studies with rubella carrier cultures of human embryo lung cells (Rawls & Melnick, 1966) and with persistently infected monkey kidney cells (Maassab & Veronelli, 1966) have also failed to demonstrate any viral suppressive effect of amantadine.

Differences were noted between the growth rate of the persistently infected hamster
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lungs and control non-infected cells; this was particularly marked in later sub-
cultures of the cells where the lag phase of the infected cultures was prolonged. The
growth rate of persistently infected human embryo lung cells was also inhibited (Plotkin,
Boué & Boué, 1965) and a growth inhibiting substance has been isolated from such
cultures (Plotkin & Vaheri, 1967).

The relative ease with which lung cell cultures can be established from hamsters
persistently infected with rubella virus makes this a valuable system for more detailed
studies on the equilibrium between virus and cell. In particular, cell cultures established
from rubella infected hamsters which have been treated with virustatic drugs, gamma
globulin or interferon can be compared under controlled in vitro conditions.

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A short account of this work was presented at the 1st International Congress for

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