Conditions for Plaque Formation with a Phlebotomus (Sandfly) Fever Virus

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SUMMARY

Naples virus, a member of the Phlebotomus fever group of arboviruses, is able to multiply in cells derived from hamster embryos. It produces no cytopathic effect in these cells in a fluid medium and no plaques upon infection of confluent monolayers overlaid with a medium containing agar or the sodium salt of carboxymethylcellulose. Plaques are produced only when these cells are infected in suspension and plated to form monolayers under an overlay containing carboxymethylcellulose. A critical number of cells is required and the presence of carboxymethylcellulose is essential. The survival or death of a hamster embryo cell infected with this virus appears to depend upon the stage of growth of the cells at the time of infection and the environmental conditions.

INTRODUCTION

Some viruses produce a clear cytopathic effect (CPE) when susceptible cells are infected and incubated under fluid media. Others, notably the group B arboviruses, do not produce a CPE when susceptible cells are incubated under fluid media, but yield plaques when the same cells are incubated under an agar gel (Porterfield, 1959; Henderson & Taylor, 1959). The Naples strain of Phlebotomus (Sandfly) fever virus multiplies in hamster embryo cells without producing a CPE, when cultures are incubated under a fluid medium; it does not produce plaques when confluent monolayers are incubated under an agar gel or under an overlay containing the sodium salt of carboxymethylcellulose (CMC). Plaques appear only when hamster embryo cells are infected in suspension and seeded in Petri dishes so that they grow to form a monolayer under CMC (Salim, 1967a). This paper presents the conditions which are necessary for plaque formation by this virus in hamster embryo cells.

METHODS

Viruses. The Naples virus (prototype) was used after 58 intracerebral passages in 2- to 4-day-old suckling mice, without adaptation to tissue culture. Semliki Forest virus (KUMBA strain), which had undergone 42 intracerebral passages in suckling mice, was used for challenge.

Tissue culture. Primary and serially passaged hamster embryo cultures were used. Details of preparation and serial cultivation of these cells, the media and virus diluents were described by Salim (1967a).

Plaque production. The technique of plaque formation by infecting cells in suspension was described originally by Russell (1962) and modified by Salim (1966, 1967a).

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Briefly, about 5 to 7 x 10⁵ cells in 0.2 ml volumes of growth medium were distributed into small screw-capped bottles and 0.2 ml volumes of tenfold dilutions of virus were added. The mixtures were shaken at 37° for 30 min. and then 4 ml of growth medium was added to each bottle. The contents were mixed and poured into 45 mm. Petri dishes, which were incubated at 37° in a humidified atmosphere containing 4% CO₂ for 5 hr in order to allow the cells to attach to the glass. The medium was removed and the cells were overlaid with CMC and the dishes were incubated as before, for 8 days. The cells were then stained by the addition to the overlay of 1 ml of 1/5000 neutral red solution in growth medium, and the plaques were counted 6 to 8 hr later. Infection of confluent monolayers was made by the addition of 0.2 ml volumes of tenfold dilutions of virus which was allowed to adsorb at 37° for 2 hr. Unadsorbed virus was then removed and the cells were overlaid with CMC. Thereafter they were treated like the cells infected in suspension.

Giemsa staining. Cells were infected in suspension and seeded into Petri dishes containing circular coverslips. The cells were allowed to attach to the coverslips at 37° for 5 hr. The medium was then removed and the cells were overlaid with CMC. The dishes were incubated as described above. Coverslips were removed after 8 days of incubation, washed in saline, fixed in methanol and stained with 1/10 Giemsa stain for 30 min.

Infection of cells in suspension and incubation under a fluid medium. About 100,000 cells in 0.2 ml volumes of growth medium were distributed into test tubes and 0.2 ml volumes of tenfold dilutions of the virus were added. The cell + virus mixtures were shaken at 37° for 30 min. and 1 ml volumes of growth medium were added to the tubes. The contents were mixed and the tubes incubated at 37° in a stationary position and examined daily for CPE.

Virus titration in suckling mice. Suckling mice, 2 to 4 days old, were inoculated intracerebrally with tenfold dilutions of infected material (Salim, 1967a).

Resistance to infection with a challenge virus. About 50 p.f.u. of Semliki Forest virus were added to confluent monolayers which had been treated with infected and uninfected cells, as described later. The challenge virus was allowed to adsorb at 37° for 2 hr, unadsorbed virus was removed, and the cells were overlaid with CMC and incubated at 37° for 47 hr. The cells were stained with neutral red.

RESULTS

Plaque formation

When hamster embryo cells were infected in suspension with the Naples virus and overlaid with CMC they were able to grow and form monolayers in which plaques appeared on the 4th or 5th day after infection. The plaques increased in size on further incubation until they reached 2 to 3 mm. in diameter 8 days after infection. No plaques were produced when preformed confluent monolayers were infected with this virus and overlaid with CMC. This result was reproducible in these cells at all the passage levels tested so far (23 cell passages); the virus consistently failed to produce plaques on infection of confluent monolayers. The specificity of the plaques produced was confirmed serologically by a plaque reduction test (Salim, 1967b).
Plaque formation with Naples virus

Microscopic examination of plaques

When Giemsa-stained coverslip cultures were examined microscopically, degenerative CPE was seen in the cells where the plaques formed. Plaque formation was therefore the result of CPE production under these conditions.

Infection of cells in suspension and incubation in a fluid medium

No CPE was observed when cells were infected in suspension and incubated under fluid medium and examined daily for 12 days.

Table 1. Plaque formation with Naples virus in hamster embryo cells dispersed with trypsin or EDTA

<table>
<thead>
<tr>
<th>Virus dilutions</th>
<th>Cells dispersed with trypsin p.f.u./plate</th>
<th>Cells dispersed with EDTA p.f.u./plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-3}$</td>
<td>Confluent</td>
<td>Confluent</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>63</td>
<td>55</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Diluent only</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Titre</td>
<td>$3.2 \times 10^6$ p.f.u./ml</td>
<td>$2.8 \times 10^6$ p.f.u./ml</td>
</tr>
</tbody>
</table>

* Average number of plaque-forming units per plate.

The effect of dispersing cells with EDTA or by scraping on plaque formation

It was thought that trypsin, which was routinely used for dispersing cells, might have had some effect on the cells, making them more susceptible to the cytopathic effect of this virus under CMC overlay. Monolayers grown in 20 oz bottles were dispersed with 0.02% EDTA solution and the suspended cells were infected with 0.2 ml. volumes of tenfold dilutions of the virus and the infected cells were treated for plaque formation as described under Methods. As a control, the standard procedure of dispersing the cells with 0.25% trypsin (Difco 1:250) was included. Plaques were produced when the cell suspensions were obtained by either trypsin or EDTA (Table 1). In a similar experiment, suspensions were obtained by scraping monolayers with a rubber policeman and disaggregating the cells by pipetting. Uniform monolayers were not formed upon infection of cell suspensions obtained by this method, but plaques were produced in areas where good monolayers were formed.

The effect of the number of cells infected in suspension on plaque formation

On average, about $2 \times 10^6$ cells were obtained from a confluent monolayer in a 45 mm. Petri dish 3 or 4 days after seeding 5 to $7 \times 10^6$ cells. Suspensions usually contained 5 to $7 \times 10^6$ cells/culture. In order to see whether or not the cell number at the time of infection had any effect on plaque production in the two systems, samples of a suspension containing $2 \times 10^6$ cells and twofold dilutions of this suspension were infected with 0.2 ml. volumes of $10^{-3}$ dilution of virus (about 500 to 1000 p.f.u.). The number of plaques produced decreased as the number of cells in the suspension increased until no plaques were formed when $2 \times 10^6$ cells were used (Fig. 1). With $1.3 \times 10^6$ cells or less, confluent monolayers were not formed.
Treatment of monolayers with infected cells

Cells infected in suspension with Naples virus may be regarded as infective centres among a population of normal cells. Confluent monolayers were treated with infective centres. A suspension containing about $10^6$ cells in 1 ml. of growth medium was mixed with 1 ml. of $10^{-3}$ dilution of Naples virus containing about $5 \times 10^3$ p.f.u. The cell + virus mixture was shaken at $37^\circ$ for 30 min. One ml. of diluent was added to the same number of cells in 1 ml. growth medium and the cells were also shaken at $37^\circ$ for 30 min. They were washed three times with phosphate-buffered saline and suspended

Fig. 1. The effect on plaque formation of infecting different numbers of hamster embryo cells in suspension with the same dose (500 to 1000 p.f.u.) of Naples virus.

Table 2. Resistance to challenge virus infection induced by treatment of monolayers with infective centres

<table>
<thead>
<tr>
<th>Total number of cells added to monolayers</th>
<th>Estimated number of infective centres</th>
<th>SFV* p.f.u./plate†</th>
<th>Control SFV‡ p.f.u./plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>$2 \times 10^5$</td>
<td>1000</td>
<td>3</td>
<td>32</td>
</tr>
<tr>
<td>$2 \times 10^4$</td>
<td>100</td>
<td>9</td>
<td>36</td>
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<tr>
<td>$2 \times 10^3$</td>
<td>10</td>
<td>20</td>
<td>41</td>
</tr>
<tr>
<td>$2 \times 10^2$</td>
<td>1</td>
<td>28</td>
<td>47</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>45</td>
<td>43</td>
</tr>
</tbody>
</table>

* Semliki Forest virus (SFV) used to challenge cell monolayers treated with infective centres.
† Average number of plaque-forming units per culture.
‡ SFV used to infect monolayers treated with uninfected cells.
in 1 ml. of growth medium. Tenfold dilutions of these suspensions were made in growth medium and 0.2 ml. amounts of each dilution were added to confluent monolayers. A uniform distribution of the cells was obtained by swirling the Petri dishes several times. The cultures were then incubated at 37° for 2 hr to allow the cells to settle on the monolayers. Then, without removing the inocula, CMC overlay containing 5% newborn calf serum was carefully added to one set each of monolayers treated with infective centres and normal cells. Maintenance medium containing 5% newborn calf serum was added to another set each. Both sets of cultures were incubated at 37°.

![Graphs](image)

**Fig. 2.** Growth of Naples virus in preformed hamster embryo cell monolayers (○ --- ○) and in cells infected in suspension (●—●), as assayed by intracerebral inoculation of suckling mice. Each point represents total virus.

**Fig. 3.** Growth of hamster embryo cells at the fifth passage.

Monolayers overlaid with CMC were stained with neutral red 8 days after infection; monolayers incubated under the fluid medium were examined daily for CPE with an inverted microscope. No plaques were produced in cell monolayers treated with infective centres and overlaid with CMC, and no CPE was observed 8 days after treatment in those which were incubated under the fluid medium. On the 8th day the fluid was removed, the monolayers were washed three times with PBS and challenged with Semliki forest virus. Resistance to infection with the challenge virus was established in those monolayers treated with infective centres (Table 2).

**Multiplication of Naples virus in cells infected in suspension and in confluent monolayers**

Half ml. amounts of $10^{-3}$ dilution of virus containing about $4 \times 10^6$ mouse LD<sub>50</sub> were added to several monolayers. The virus was allowed to adsorb for 5 hr at 37°, because this was the time allowed for cells infected in suspension to stick to the glass while the virus was present. Unadsorbed virus was then removed, the cells were washed three times with phosphate-buffered saline, and maintenance medium was
added. At 5, 18, 48 and 72 hr after infection, cells from two monolayers were scraped in their maintenance medium, pooled and stored at \(-70^\circ\). For infection of cells in suspension, \(2\times10^6\) cells in 0.5 ml. volumes of growth medium were mixed with 0.5 ml. amounts of \(10^{-3}\) dilution of the virus. This cell number was equal to the number of cells in the monolayers infected previously with this virus. The cell + virus mixtures were shaken at 37° for 30 min., then 2 ml. volumes of growth medium were added, the contents mixed and poured into 45 mm. Petri dishes which were incubated at 37° for 48 hr. The infected medium was then removed, the cells were washed three times with phosphate-buffered saline, and the same amounts of maintenance medium were added as had been added to monolayers. Thereafter the cells were treated like monolayers.

To extract virus for assay, the cultures were frozen and thawed three times. Titration were made by intracerebral inoculation of suckling mice, a more sensitive method than the plaque assay (Salim, 1967a). The result is shown in Fig. 2. It can be seen that the growth of virus in confluent monolayers and in cells infected in suspension and allowed to grow was almost the same.

**The effect on plaque formation of infecting cells at different stages of their growth rate**

Petri dishes were seeded with 4 ml. of a suspension of cells containing about \(5\times10^6\) cells per ml. Cultures were trypsinized and cells counted daily. There were 3 stages of cell growth. (1) Lag phase during the first 18 hr after seeding: the cells attached to the glass, but did not multiply. (2) Growth phase from 18 to 72 hr after seeding: the cells multiplied actively with a mean generation time of about 20 hr. (3) Stationary phase 72 hr after seeding: cell cultures became confluent and cell multiplication slowed down until it ceased (Fig. 3).

In two experiments cells were infected at different stages of their growth cycle. Samples of \(7\times10^6\) cells were either infected in suspension with tenfold dilutions of virus and overlaid with CMC or seeded uninfected into Petri dishes in growth medium. Five, 18 and 72 hr after seeding, uninfected cells were examined with an inverted microscope to see the state of confluency of the cultures. Five hr after seeding, the cells were round in shape and only a few began to spread on the glass. At 18 hr after seeding, all the cells spread on the glass, covering about one-fifth of the area on the Petri dish, and 72 hr after seeding, confluent monolayers were obtained. After microscopic examination, the growth medium was removed from uninfected cells and tenfold dilutions of virus were added and allowed to adsorb at 37° for 2 hr. Unadsorbed virus was removed and CMC was added. All the cultures were incubated at 37° for 8 days. Plaques were produced in those cells which were infected in suspension and in cells infected 5 hr after seeding, but not in cells infected 18 and 72 hr after seeding.

**Discussion**

Naples virus behaves in hamster embryo cells in a way not so far described for viruses in susceptible cells. It is quite conceivable that some unknown viruses may behave similarly and may not be recognized by current tissue culture techniques.

I would like to thank Dr J. S. Porterfield and Dr H. G. Pereira for their valuable suggestions and discussions.
Plaque formation with Naples virus

REFERENCES


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