The Growth of some Tick-borne Arboviruses in Cell Cultures Derived from Tadpoles of the Common Frog, *Rana temporaria*

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**SUMMARY**

Primary cell cultures and a line consisting of fibroblast-type cells were obtained from tadpoles of the common frog, *Rana temporaria*. One cell line was taken through 21 subcultures during 5 months, when it was abandoned due to contamination by an anonymous *Mycobacterium* which was completely resistant to antibiotics. Cells from the primary cultures supported the growth of three tick-borne arboviruses without any cytopathic effect; Quaranfil, louping ill (two strains), and Langat. One of the virus strains, louping ill 369T2, was serially passaged in the cell cultures 11 times in 11 weeks, during which time there was considerable multiplication of virus.

**INTRODUCTION**

Arboviruses have been grown in a number of cell systems, primary cultures as well as established cell lines, mainly of avian or mammalian origin, and more recently in arthropod cells. Initial work on the use of tissues from poikilothermic animals for this purpose was carried out by Soret & Sanders (1954) who showed that Eastern equine encephalitis virus could be propagated in embryos of *Gambusia* (a viviparous teleost) maintained *in vitro*. Eastern and Venezuelan equine encephalitis viruses were grown by Officer (1964) in an established line of rainbow trout gonad (RTG) cells and Kroeker & Bird (1965) showed that a cell line from the goldfish, *Carassius auratus*, was capable of supporting the growth of Sindbis virus (a group A arbovirus) and some mammalian viruses.

Falcoff & Fauconnier (1965) grew Sindbis virus in kidney cell cultures of the tortoise, *Testudo graeca*, and Shindarov and his colleagues showed that this cell system was suitable for the cultivation of vesicular stomatitis virus (VSV) and some non-arboviruses (Shindarov & Sawow, 1964; Shindarov & Todorov, 1962; Shindarov, 1962a, b). Clark & Karzon (1967) grew VSV in an established cell line (TH-1) from the turtle heart. Cells from the slow worm, *Anguis fragilis*, were used by Somogyiova & Rehacek (1965) for the growth of tick-borne encephalitis virus, although cells from three different species of snakes did not support the growth of this virus.

Although amphibian cell lines have been established (Wolf & Quimby, 1964; Rafferty, 1965; Freed & Freed, 1970), these have not to our knowledge been used for the growth of arboviruses.

We used cells from tadpoles of the common frog, *Rana temporaria*, for studying the growth of three tick-borne arboviruses. The techniques of cell culture were based on those of Auclair (1961).

The culture of cells from poikilothermic animals offers a unique opportunity to study the
growth of viruses of homeothermic vertebrates (e.g. arboviruses) in cells of hosts far removed phylogenetically from the normal hosts and at temperatures below those of their normal hosts (Clark & Karzon, 1967).

METHODS

Cell cultures. These were either primary cultures or subcultured cells from tadpoles of the common frog, Rana temporaria, undergoing metamorphosis. The preparation and history of one of the cultures, Frog Tissue Culture 14 (FTC-14) is described. Six tadpole/frogs were washed in a 1/40 solution of Roccal (Bayer Products Company, Surbiton-upon-Thames, England) containing 1 % benzalkonium chloride, twice in sterile distilled water, and twice in phosphate-buffered saline (PBS), pH 7.2, containing 1000 units/ml. of penicillin, 100 µg./ml. of streptomycin and 2 µg./ml. of Fungizone (E. R. Squibb and Sons). The gut, skin and eyes were removed and the carcasses, after two washings in PBS, were kept in medium until all the dissections in PBS were completed. After three further washings in PBS, the tissues were cut up and put into 10 ml. of 0.05 % pronase (B grade, Calbiochem, England) and magnesium-free diluent (Varma & Pudney, 1967) and placed at 4 ° overnight. The following morning, the pronase was brought to room temperature and stirred on a magnetic stirrer for about 12 min. One ml. of foetal calf serum was then added to inactivate the pronase and the tissue clumps were broken down by pipetting. The cell suspension, after filtering through sterile gauze, was centrifuged at 800 rev./min. for 8 min. and the grey supernatant fluid discarded. The cells were washed in PBS and after further deposition the pellet was suspended in 12 ml. of medium and seeded as 1 ml. each into 12 Leighton tubes: in some experiments 1 oz. medical flat bottles were each seeded with 4 ml. The cultures were incubated at 28 ± 1 °. The composition of the medium was as follows: medium NCTC-109 (Burroughs Wellcome), 100 ml.; lactalbumin hydrolysate (Difco), 50 mg.; glutamine (Difco) 5 %, 0.6 ml.; NaHCO₃ 4.4 %, 5 ml.; glass-distilled water, 50 ml. Penicillin (1000 units/ml.) and streptomycin (100 µg./ml.) were added and the pH of the medium adjusted with 2 % KOH to 7.6. The medium was sterilized by filtration through sintered glass and, immediately before use, foetal calf serum (Flow Laboratories, Irvine, Scotland) was added to a final concentration of 10 %. In some experiments Difco NCTC-109 medium replaced Burroughs Wellcome NCTC-109 without difference in growth of the cultures.

Seeding density of the cultures varied but was approximately 800,000 cells/ml. The cells were attached to the glass and were beginning to spread by the following day. About 5 days after setting up the cultures, the surface of the tubes and bottles was covered with a monolayer of fibroblast-type cells (Fig. 1). Melanophores were still obvious but eventually disappeared. As the cultures aged the cell sheets began to roll up, particularly at the edges, and by about 4 weeks this was extensive.

FTC-14 was first subcultured on the 7th day; 0.05 % pronase solution was in contact with the cells in two of the tubes for 1 min., poured off and the film of pronase left in contact with the cells for 1 min. After the addition of foetal calf serum to inactivate the pronase, the cells were removed from the glass surface by pipetting and the pooled suspension was centrifuged. The cells were then resuspended in 4 ml. of medium and seeded in 1 ml. amounts into four Leighton tubes. The cells grew rapidly and medium replacements and subculturing were carried out according to the growth of the monolayer and the pH of the medium. This could be at intervals of as little as 3 days. At the third subculture, the pronase solution was used at a concentration of 0.025 % and fresh medium was added instead of calf serum, so that centrifugation to remove the serum was unnecessary: this was the standard procedure. A rubber policeman was used in some of the latter subcultures to ensure that all the cells
had been removed from the glass. Subcultures were made weekly and the cells split 1:2. Medium was usually changed at least once between subcultures.

Although FTC-14 was subcultured successfully it had to be abandoned at the twenty-first subculture because of contamination with an anonymous *Mycobacterium* found in fish and frogs. This agent was resistant to 28 different antibiotics and was non-pathogenic to mice on intracerebral inoculation. It did not appear to damage the cells and had been carried for some time in the cells before it was identified. Attempts to cure the contamination with kanamycin (Kannasyn, Bayer Products Co.) at up to 330 μg./ml., and to dilute it out by frequent medium changes—one on one occasion eight times in one day followed by two or three times a day for the following 5 days—were unsuccessful.

*Virus.* The virus strains used are defined in Table 1. The MOREDUN strain of louping ill virus was received from the Wellcome Research Laboratories at the 30th to 35th mouse brain passage and was used after seven further mouse brain passages.

*Virus inoculation and harvesting of tissue culture fluids.* With the exception of serial passages of virus, all infection experiments were made in cultures of primary cells. The 369t2 strain of louping ill virus was passaged serially in 11 successive subcultures.
For virus inoculation and harvesting the cell cultures in Leighton tubes or 1 oz. medical flat bottles were infected when cell monolayers had formed 5 to 7 days after seeding. In the first experiment (FTC-5) growth medium was used throughout the infection period: in the other experiments, the growth medium was removed before virus inoculation and replaced with maintenance medium of the same composition as the growth medium but with a reduced concentration (5%) of foetal calf serum. Bottle cultures were inoculated with 0.4 ml. of virus suspension and tube cultures with 0.1 ml. The virus was allowed to adsorb to the cells for 3 hr at 28°C. The medium with unadsorbed virus was removed and replaced with the appropriate amount of maintenance medium, 4 ml. for bottle cultures and 1 ml. for tube cultures. Uninoculated cultures with growth medium replaced by maintenance medium were kept as controls.

Table 1. Source and infectivity of virus strains

<table>
<thead>
<tr>
<th>Virus</th>
<th>Strain</th>
<th>Source*</th>
<th>Passage level</th>
<th>Infectivity (log. LD50/0.03 ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quaranfil</td>
<td>EGAR 1095</td>
<td>YARU</td>
<td>22; 23</td>
<td>5.5; 4.7</td>
</tr>
<tr>
<td>Louping ill</td>
<td>369T2</td>
<td>LSHTM</td>
<td>3; 4</td>
<td>8.2; 8.1</td>
</tr>
<tr>
<td></td>
<td>MOREDUN</td>
<td>WRL</td>
<td>37 to 42</td>
<td>7.5</td>
</tr>
<tr>
<td>Langat</td>
<td>TP64</td>
<td>LSHTM</td>
<td>10</td>
<td>7.8</td>
</tr>
</tbody>
</table>

* YARU, Yale Arbovirus Research Unit.
LSHTM, London School of Hygiene and Tropical Medicine.
WRL, Wellcome Research Laboratories.

Infected tissue culture fluids were sampled by removal of 0.5 ml. and replacement by 0.5 ml. of maintenance medium. This was done every one or two days up to the 15th or 20th day. When two bottles or tubes were infected with the same virus, the amounts harvested were pooled and 0.6 or 0.7 ml. sealed in glass ampoules and stored at -60°C for later titration. The remaining 0.4 or 0.3 ml. was mixed with an equal volume of heat-inactivated rabbit serum (Burroughs Wellcome, No. 1) and stored in sealed glass ampoules at -60°C. In the first experiment (FTC-5), heat-inactivated calf serum was used instead of rabbit serum for storage. When only one culture had been inoculated with the virus, the 0.5 ml. of medium harvested was mixed with 0.5 ml. of rabbit serum and stored in sealed ampoules at -60°C.

Serial passage of the 369T2 strain of louping ill virus in FTC-14. A bottle culture at the 10th passage was inoculated with 0.4 ml. of 10^-5 dilution of the virus. After 7 days the medium was removed and centrifuged to sediment any cell debris; the bottle culture was discarded. Of the clarified supernatant fluid 0.4 ml. was inoculated into a fresh bottle culture at the 11th subculture, which had been seeded 3 days previously; 2 ml. of the supernatant fluid were mixed with 2 ml. of rabbit serum in a bijou bottle and stored at -60°C for later titration. The virus was thus passed 11 times until the infected culture was discarded because of heavy contamination by an anonymous Mycobacterium.

Virus assay of tissue culture fluid. In a few cases, the culture medium was titrated before dilution with rabbit serum. Parallel titrations of medium stored at -60°C with and without rabbit serum showed that there was a slight loss in infectivity of about 0.5 log. in the absence of serum. Therefore only the infectivities of media mixed with rabbit serum and stored at -60°C will be quoted. For titration, serial tenfold dilutions were prepared in 0.75% bovine albumin in borate-buffered saline, pH 9.0 (BABS). Samples of 0.03 ml. of each dilution were inoculated into groups of five 3- to 4-week-old mice (TO strain). The LD50 was calculated using Thompson's moving averages (Thompson, 1947) and the infectivity expressed as log. (mouse LD50/0.03 ml. of inoculum).
RESULTS

Growth of virus

The three viruses (Quaranfil, louping ill and Langat, Table 1) were successfully propagated in tadpole cells, and demonstrated the ability of viruses from homeothermic animals to multiply in cells derived from a poikilothermic animal. Virus replication was clearly demonstrable in all cases, particularly when one considers the dilution of virus in the cultures after medium replacements following every harvesting. There was no cytopathic effect. Virus was not detectable after 7 days in medium inoculated with virus in the absence of cells.

![Graph showing growth of Quaranfil virus](image1)

Fig. 2. Growth of Quaranfil (EGAR 1095) virus in frog cells. ▲ — ▲, FTC-5; ● — ●, FTC-13; △—△, FTC-9.

![Graph showing growth of Langat virus](image2)

Fig. 3. Growth of Langat (TP64) virus in frog cells. ● — ●, FTC-13; ○ — ○, FTC-14.

Growth of Quaranfil virus (strain EGAR 1095)

Three experiments were done. One tube culture of FTC-5 was inoculated with approximately 1000 LD50 of virus, two bottle cultures of FTC-9 with approximately 600 LD50 each and two tube cultures of FTC-13 with approximately 150 LD50 each (Fig. 2). In all experiments the infectivity increased to a peak of 3 to 4 log. LD50/0.03 ml. on day 3 to 4 then fell sharply, except in FTC-5. Virus infectivity was still detectable in the culture medium on conclusion at up to day 20.
Growth of Langat virus (strain TP64)

Two tube cultures (FTC-13, FTC-14) were inoculated with approximately 2000 LD50 each of virus. The growth of the virus is shown in Fig. 3. In contrast to Quaranfil virus, higher maximum infectivities (4 to 5 log. LD50/0.03 ml.) were reached later at 10 to 15 days.

Growth of louping ill virus (strains MOREDUN and 369T2)

Two bottle cultures (FTC-9) were inoculated with approximately 4000 LD50 each of the MOREDUN strain, and 2 tube cultures (FTC-12, FTC-14) and 1 tube culture (FTC-13) with approximately 5000 LD50 each of the 369T2 strain. Although the pattern of virus growth (Fig. 4) for both strains was similar, the peak infectivity was lower in one experiment for the MOREDUN strain. The pattern of virus replication for both strains was similar to that for Langat virus. In the two experiments in which culture fluid of the 20th day was titrated the infectivities were still high (2.4 log. MOREDUN strain; 4.5 log. strain 369T2).

Fig. 4. Growth of two strains of louping ill virus in frog cells. △—△, strain MOREDUN (FTC-9); ▲—▲, strain 369T2 (FTC-12); ●—●, strain 369T2 (FTC-13); ○—○, strain 369T2 (FTC-14).

Table 2. Serial passage of louping ill virus (strain 369T2) in frog cells

<table>
<thead>
<tr>
<th>Passage no.</th>
<th>Infectivity of culture fluid: log. (mouse LD50/0.03 ml.)</th>
<th>Cumulative time of cultivation in days</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>5.2</td>
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</tr>
<tr>
<td>2</td>
<td>5.3</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>5.8</td>
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</tr>
<tr>
<td>5</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>11</td>
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</tbody>
</table>
Serial passage of the 369T2 strain of louping ill virus

Serial passage of virus was initiated by inoculating a bottle culture at the 10th subculture of cells with approximately $10^7$ LD 50 of virus. The virus infectivity at each passage and the cumulative time of cultivation in days are shown in Table 2. There was no regular trend in virus recovery at successive passages and infectivities ranged from 3.8 to 6.0 log. LD 50 / 0.03 ml. Since the medium was completely changed up to six times between harvests, the cumulative dilution of virus in the medium was considerable; even so, virus recovery remained high to the end of the experiment at the 11th passage of virus or 77 days in culture.

![Graph showing growth of viruses](image)

**DISCUSSION**

Both strains of louping ill virus tested produced the same pattern of growth, although the infectivity for the MOREDUN strain was lower in the one experiment. This could be a reflection of the high mouse passage history of this strain; the 369T2 strain of virus had undergone only three or four mouse brain passages.

The limited number of serial passages of louping ill virus (369T2) at 28° in cells from a poikilothermic animal did not appear to reduce its pathogenicity for mice or induce cytopathogenicity for these cells.

When the growth pattern of the three viruses in the same experiment (FTC-13) is compared (Fig. 5), the related group B viruses (Langat and louping ill) behaved similarly with a gradual rise in infectivity to a peak on the 10th day. Quaranfil virus (not group B) reached its peak infectivity earlier, on the 3rd to 5th day. The infectivities of the group B viruses were also higher in this experiment, and on the 10th day Langat virus-infected culture fluid showed an infectivity of 4.0 log., and that for louping ill virus 5.0 log.; the infectivity of culture fluid for Quaranfil virus had dropped to 1.4 log. In the other experiments the group B viruses also maintained higher infectivities for longer periods (Fig. 3, 4) than Quaranfil virus (Fig. 2). If other tick-borne viruses not in group B behave in the same manner, it would provide a biological differentiation between group B and other tick-borne viruses.
The authors wish to thank Professor D. S. Bertram, Director of the Department, for his continued interest in the work, Mr A. O. Langi for technical assistance, Dr J. S. Porterfield for supplying the strain of Quaranfil virus and Dr H. M. Darlow for identifying the Mycobacterium and for the antibiotic tests on it. Medium NCTC-109 was supplied by the Wellcome Research Laboratories and we are especially grateful to Mr D. J. Richmond of the Laboratories for responding promptly to our request for medium. The work was supported in part by a grant from the Medical Research Council of Great Britain.

REFERENCES


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