COLORADO TICK FEVER VIRUS IN CELL CULTURE
I. CELL-TYPE SUSCEPTIBILITY AND INTERACTION WITH L CELLS

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ABSTRACT

TRENT, DENNIS W. (University of Oklahoma School of Medicine, Oklahoma City) AND L. VERNON SCOTT. Colorado tick fever virus in cell culture. I. Cell-type susceptibility and interaction with L cells. J. Bacteriol. 88:702-708. 1964.—Colorado tick fever (CTF) virus was serially propagated in monolayer cultures of L and FL cells. Early passages of virus in FL cells yielded viral titters 10^4-fold greater than did the corresponding L-cell passages. During L-cell passage number 4, there was a 10^6-fold increase in the amount of infectious virus produced as compared with virus cultured earlier in this cell line. Viruses from L-cell passages 8 and 12 were identified with specific immune serum to be CTF viruses which were antigenically similar, if not identical, to the mouse-adapted virus. Parallel titrations of mouse-, L cell-, and FL cell-adapted viruses were performed in mice and replicate monolayers of L, FL, HeLa, KB, chick embryo, and cotton rat kidney cells. Cytopathic effects and viral replication were noted in all cultures except HeLa and cotton rat kidney. Cultures of L, FL, and chick embryo cells were as sensitive to infection as were suckling or weanling mice. KB cells were the least susceptible of those cell types examined. In L-cell cultures, 90% of the input virus was adsorbed to the cells during the first 30 min of incubation. The latent period lasted 10 to 12 hr, and was followed by rapid viral synthesis for the next 10 to 24 hr, depending upon the multiplicity of infection. Curves describing exponential increase in cell-associated and cell-released virus were separated by 4 hr. When the maximal total virus titers were reached, 80 to 90% of the virus was released from the cell.

Colorado tick fever (CTF) virus was initially characterized by Florio, Stewart, and Mugrage (1946), and was subsequently adapted by Koprowski and Cox (1946, 1947a, b) to replicate in the mouse and developing chick embryo. Monolayer cultures of KB cells were used for the propagation and in vitro neutralization studies (Pickens and Luoto, 1958; Gerloff and Eklund, 1959). Deig and Watkins (1963) recently described a plaque method for assay of mouse brain-adapted virus in hamster fibroblast cells. This paper is a report on the comparative in vitro sensitivity to infection by CTF virus of several cell types. The kinetics of viral adsorption and synthesis, as well as a description of the resulting cytopathic effects in L cells, are presented.

MATERIALS AND METHODS

Cells and growth media. Cultures of L-929, HeLa, KB, and FL cells (Microbiological Associates, Inc., Bethesda, Md.) were grown in minimal essential medium (Eagle, 1959) supplemented with 10% calf serum (MEM-C10). Viable cell counts were made with 0.1% trypan blue. Roller and stationary tube cultures of L cells were initiated with 5 × 10^5 viable cells in 1.0-ml volumes. The resulting monolayers contained 9 × 10^5 cells at 48 hr and 1.4 × 10^6 cells at 72 hr after seeding. Confluent monolayers of L cells 72 hr old were used for virus growth and adsorption experiments. Monolayers of serially propagated cell types used for viral assay were initiated with 2.0 × 10^5 viable cells in 0.5-ml volume, and were infected within 24 hr. Roller tube cultures of cotton rat kidney and chick embryo cells used for viral titrations were prepared and grown in a modified lactalbumin medium (Lennette et al., 1961). All growth media contained penicillin (100 units per ml) and streptomycin (100 μg/ml).

Virus. The Florio strain of CTF virus mouse passage 71 was obtained from Leo Thomas, Rocky Mountain Laboratory, Hamilton, Mont. After three serial propagations in suckling mice, a 10% suspension of infected mouse brain was pre-
pared in phosphate-buffered saline (PBS) (Dulbecco and Vogt, 1954a) containing 50% calf serum. This suspension was centrifuged at 3,000 × g to remove tissue debris, and the supernatant fluid was stored in sealed vials at −65 C.

Cell-adapted virus was prepared in roller tube cultures of L or FL cells nourished by MEM-C10 which contained double concentrations of amino acids and vitamins. Monolayers of L or FL cells containing 1.6 × 10^4 viable cells were inoculated with 1,000 TCID50 of virus in 0.2-ml volume, and were allowed to adsorb for 2 hr at 25 C. The cells were washed three times with PBS, growth medium was added, and the cultures were incubated for 50 hr on a roller drum. Heat-inactivated calf serum was added to the infected cultures to effect a final concentration of 50%. The cells and medium were then subjected to one freeze-thaw cycle, and the suspensions were pooled and sealed in ampules which were stored at −65 C.

**Viral assay.** Stock or experimental virus suspensions were diluted in PBS containing 10% calf serum. Each viral dilution was inoculated into five cell cultures or six mice. Suckling mice were inoculated with 0.05 ml by the intraperitoneal route, and weanling mice were inoculated with 0.03 ml intracerebrally. End points were calculated according to the method of Reed and Muench (1938).

**Viral neutralization.** Lyophilized mouse anti-serum which had a K value of approximately 2.45 min⁻¹ was obtained from Leo Thomas. This serum was rehydrated, and 0.5-ml volumes of serum were mixed with equal volumes of tenfold dilutions of either mouse-adapted or L cell-adapted CTF viruses. The virus-serum mixtures were incubated for 1 hr at room temperature. Five suckling mice were inoculated with 0.05 ml of each dilution of virus-serum mixtures. The titers of the mouse-adapted and L cell-adapted viruses in the presence of nonimmune pooled calf serum were similarly determined.

**Viral adsorption.** In a typical experiment, confluent monolayers of L cells in roller tubes were inoculated with 0.2 ml of viral suspension which contained 20 TCID50 of virus per cell (adsorption mixtures). Tubes without cells (control mixtures) were inoculated with a similar amount of virus to serve as a control of inactivation of this labile virus. Adsorption mixtures and control mixtures were incubated at 25 C in stationary racks. At 15-min intervals, five tubes were collected from each group, and the free virus was collected in PBS containing 50% calf serum. Three washes from each mixture per time-interval were pooled and stored at −65 C until assayed. The differences between the amounts of free virus recovered from the mixtures with and without monolayers were considered as the amount of adsorbed virus.

**RESULTS**

**Cytotoxicity and multiplication of virus.** Monolayer cultures of L and FL cells were initially infected with 0.2 ml of a 10% mouse-brain virus suspension. At intervals of 12 to 18 hr, the infected and noninfected cultures were examined microscopically to note morphological changes (Fig. 1). Figure 1a represents a typical noninfected monolayer of L cells. Cytopathic changes became evident approximately 40 hr after infection (Fig. 1b). Infected L cells were rounded with vacuolated and granular cytoplasmas. The monolayer lesion at 48 hr after infection appeared to be composed of aggregates of rounded, opaque, and anucleated cells (Fig. 1c). The infected cells had undergone further necrosis 72 hr after inoculation, and had become detached to produce a plaque (Fig. 1d). The cytopathic changes which occurred in FL cells were similar.

During the first three passages of virus in L cells, titers of 10^7.7 to 10^9.4 TCID50 of CTF virus were detectable (Table 1). The yield of virus after the fourth passage in L cells was 10^9.8 TCID50 greater than after the first, second, and third passages. Subsequent propagations of this adapted CTF virus in L cells repeatedly yielded virus titers of 10^5 TCID50 or greater.

Monolayers of infected FL cells supported viral replication and gave titers of 10^4.3 to 10^4.8 TCID50 during the initial and subsequent passages (Table 2). Cytopathic changes regularly appeared 40 to 48 hr after infection with cell-adapted virus. The amount of virus produced and the time required for the appearance of cytopathic changes were essentially the same during the next eight serial propagations. Virus which replicated in FL cells was as virulent for mice as was the mouse-adapted virus from which it was derived.

**Identification of virus.** In neutralization tests which were done to identify the virus propagated in L-cell cultures, the antisera prepared against the mouse-brain virus neutralized 99.9% of the homologous virus and 99.7 and 98.5% of the...
viruses from L-cell passages 8 and 12, respectively.

**Sensitivity of different cell types.** The sensitivity to CTF virus infection of six different in vitro cultivated cell types was compared (Table 3).

Parallel titrations of mouse-brain (passage 74), L cell (passage 8), and FL cell (passage 6)-adapted viruses were done on replicate monolayers of FL, L, HeLa, KB, chick embryo, and cotton rat kidney cells. These same viral suspensions were also assayed in weanling and suckling mice. Cell cultures were examined microscopically through 10 days for morphological changes and virus production. Mice were observed for deaths for 14 days.

Cultures of FL, L, and chick embryo cells were as sensitive to infection as were suckling or weanling mice. FL cells were the most susceptible and KB cells the least sensitive to infection of those cell types which produced detectable virus. Cytopathic changes, described previously, developed in FL, L, and chick embryo cell cultures within 48 hr after infection by either cell culture or mouse-adapted viruses. The morphological response of KB cells to infection appeared 12 to 18 hr later. Neither mouse-brain nor cell-adapted viruses replicated or produced cytopathic changes in cultures of HeLa or cotton rat kidney cells.

**Viral adsorption.** The data plotted in Fig. 2 are the combined results of three adsorption experiments. The most rapid adsorption of the virus occurred during the first 60 min of incuba-

**FIG. 1.** Cytonecrotic changes in L cells after infection with Colorado tick fever virus; (a) uninfected culture (200X); (b, c, d) infected cultures at 40, 48, and 72 hr, respectively (100X).
tion. Subsequently, the rate declined markedly. It can be seen that 90% of the virus was adsorbed during the first 15 min of incubation. After 2 hr of incubation, 1.0% of the virus remained unadsorbed.

*Growth curves.* Confluent monolayers of L cells were inoculated with a multiplicity of either 0.2 or 40.0 TCID₅₀ in 0.2 ml. The cultures were incubated for 2 hr at 25°C for adsorption, and then were washed three times to remove unadsorbed virus. Growth medium was added, and the cultures were incubated at 36°C on a roller drum. Samples were removed at 4-hr intervals, diluted in PBS containing 50% calf serum, and refrigerated at −65°C. Subsequently, they were assayed for total viral content, cell-associated virus (CAV), and cell-released virus (CRV).

### Table 1. Multiplication of Colorado tick fever virus in L-929 cell cultures

<table>
<thead>
<tr>
<th>Passage no.</th>
<th>Cumulative dilution of the original inoculum</th>
<th>TCID₅₀ per 0.2 ml in L cells</th>
<th>LD₅₀ per 0.05 ml in suckling mice</th>
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<td>OMP-3</td>
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<tr>
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<td>6.4</td>
<td>6.8b</td>
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<td>L-8</td>
<td>10⁻¹⁰</td>
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</table>

a Oklahoma mouse passage 3; 10% suspension.
b Titer expressed as positive log₁₀ of dilution which would infect 50% of cultures or mice.
c L-cell passage number 1.
d Data not available.

### Table 2. Multiplication of Colorado tick fever virus in FL cell cultures

<table>
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<tr>
<th>Passage no.</th>
<th>Cumulative dilution of the original inoculum</th>
<th>TCID₅₀ per 0.2 ml in FL cells</th>
<th>LD₅₀ per 0.05 ml in suckling mice</th>
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</table>

a Oklahoma mouse passage 3; 10% suspension.
b Titer expressed as positive log₁₀ of dilution which would infect 50% of cultures or mice.
c FL cell passage 1.
d Data not available.
The viral growth cycle in cells infected with a low viral input (Fig. 3A) had a 12-hr latent period which was followed by rapid growth. The rate of replication was logarithmic from the 12th to the 36th hr, at which time the total virus titer reached $10^{4.4}$ TCID$_{50}$ per 0.2 ml. CAV increased in a linear manner for 10 hr after the latent period, and reached a maximum of $10^{4.3}$ TCID$_{50}$ per 0.2 ml 60 hr after infection. CRV increased logarithmically from the 14th to the 34th hr, and then remained relatively constant for a period of 25 hr. Near the 80th hr after infection, the titer of CRV increased rapidly and reached $10^{4.2}$ TCID$_{50}$ per 0.2 ml at 102 hr after infection.

The growth-curve plot of total virus (Fig. 3B), resulting from a viral input multiplicity of 40 TCID$_{50}$, demonstrated a 10-hr latent period which was followed by a 10-hr period of exponential rate of replication. The maximal total virus titer attained 36 hr after infection was $10^{5.8}$ TCID$_{50}$ of virus per 0.2 ml. The linear increase in CAV began 10 hr after infection and continued for 12 hr. A logarithmic rate of increase in CRV began at the 14th hr and continued until the 40th hr after infection.

**DISCUSSION**

The experiments described showed that CTF virus may be propagated in several cell culture systems. Monolayers of L, FL, and chick embryo cells are susceptible to CTF virus infection and are more sensitive than are HeLa and KB cells. The cell types susceptible to infection by this virus are also susceptible to many of the arboviruses (Banta, 1957; Henderson, 1961; Scherer and Syvertson, 1954).

In early passages of CTF virus in FL cells, the maximal viral titers obtained were approximately 1,000-fold greater than in the corresponding passages in L cells. After the third passage in L cells, a significant increase in the amount of infectious virus was observed; however, the titers remained ten times lower than those obtained in FL cell cultures. Pickens and Luoto (1958) described a similar "adaption" phenomenon in their studies with CTF virus in cultures of KB cells. Western and Eastern encephalitis viruses required several passages in L-cell cultures before the production of cytonecrosis and before viral titers became stabilized (Eiring and Scherer, 1961). Although serial passage of the Asibi strain of yellow fever virus by Hardy (1963a, b) in HeLa cells resulted in a virus with modified biological characteristics, its infectivity was neutralized at the same index as was the early passage material. Another arbovirus, Venezuelan encephalitis virus, was reported by Hardy and Brown (1961) to replicate in L cells and to yield high titers without an "adaption" process. In the case of CTF virus, it is not known whether the adaption process corresponds to the selection of a pre-existing mutant from the unadapted parent population, or to the induction and selection of a mutant during serial passage of the virus in cell culture. It should be noted that, after adaption, yellow fever virus (Hardy, 1963b) was antigenically similar to the seed viruses from which it was derived. Data from the neutralization tests reported herein indicate that the L cell-propagated CTF virus closely resembles the mouse-brain virus in antigenic structure.

Adsorption of CTF virus to L cells proceeded at the most rapid rate during the first 60 min after inoculation. In the first 30 min, approximately 99% of the input virus was adsorbed to the cells and was not recoverable. The kinetics of CTF virus adsorption, as determined by free virus, are similar to those reported for Western equine encephalitis virus (Dulbecco and Vogt, 1954a). The adsorption of dengue virus to KB cells, determined by the number of plaques on the adsorption plate, is slower (Schulze and Schlesinger, 1963).
The growth of CTF virus in L cells is characterized by a latent period of 10 to 12 hr followed by a period of rapid viral synthesis (Fig. 3A, B). The latent period of this virus, however, is similar to that of dengue virus (Schulze, 1964). The lag phase of these two viruses is unusually long in comparison with some other arboviruses, e.g., Western encephalitis (Dulbecco and Vogt, 1954b) and Eastern encephalitis (Wagner, 1963) viruses. The reasons for these differences in the lag phase of growth are unknown. Isaacs (1959) pointed out that, in general, viruses containing ribonucleic acid (RNA) have a shorter latent period than do viruses containing deoxyribonucleic acid. Group-B arboviruses (Colter, 1958) and the virus of CTF contain RNA (Trent and Scott, 1964), but replicate much slower than do most small RNA viruses. Preliminary biochemical studies of the CTF virus-infected L cells indicate that syntheses of viral-specific protein and RNA begin 2 hr after infection and continue for 6 hr before the appearance of complete virus.

In a single-cycle growth experiment (Fig. 3A), peak titers of virus were attained 35 hr after infection, and correspond to an average value of \(10^6.4\) TCID\(_{50}\) per 0.2 ml or approximately 125 TCID\(_{50}\) per cell. The above value must represent an average minimal yield of virus per cell, because the actual number of cells producing virus in a culture is unknown. Release of virus from infected cells began 2 hr after the first CAV was detectable. At the time maximal viral titers were attained, 80 to 90\% of the total virus (CRV plus CAV) had been released from the cells. The increase in CRV which occurred late in the low multiplicity growth cycle (Fig. 3A) may represent virus released from cells which were infected after the first growth cycle.

**Acknowledgments**

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**Literature Cited**


KOPROWSKI, H., AND H. R. COX. 1946. Adaptation of Colorado tick fever virus to mouse and


