Growth of Rio Bravo Virus in Cell Cultures

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The growth of Rio Bravo virus (RBV) in eight cell culture systems was studied. Highest yields of virus were produced in BHK-21 (C13), L, and Vero cell lines, but L cells were resistant to low doses of virus. LLC-MK₂, HeLa, and human embryo skin cells produced moderate amounts of virus, but FL amnion and primary chick embryo fibroblasts supported little virus growth. Virus was rapidly inactivated by exposure to pH values below 7.0. Single-cycle growth in BHK-21, L, and LLC-MK₂ cell monolayers was characterized by a latent period of about 12 hr followed by rapid virus production that peaked at 36 to 48 hr. Vero cell cultures can remain chronically infected with RBV for more than 100 days. Such cultures show evidence of cell destruction, and their supernatant fluids contain virus at \(10^4\) to \(10^8\) log₁₀ per ml.

Rio Bravo virus (RBV), formerly called United States bat salivary gland virus, has been isolated from the salivary gland of Mexican free-tailed bats by various investigators (1, 2, 4, 9). Although no arthropod vector has been reported for RBV, it is antigenically related to the St. Louis encephalitis complex of the group B arboviruses (3, 11). The virus is readily propagated in the brains of suckling or weanling mice. In this paper, data are presented on the growth of RBV in various cell cultures.

MATERIALS AND METHODS

Virus. The HA-119 strain of RBV, which had been passed seven times intracerebrally in suckling mice, was used. The stock seed of 10% mouse brain suspension contained \(10^9\) weanling mouse intracerebral LD₉₀ (WMICLD₉₀) per ml.

Cell cultures. Monolayer cultures of established cell lines (LLC-MK₂, Vero, BHK-21, L, HeLa, FL amnion), human embryo skin (HES), and primary chick embryo fibroblasts (CEF) in disposable plastic T-60 flasks (Falcon Plastics, Los Angeles, Calif.) were used for screening experiments. After inoculation with 0.5 ml and adsorption at 37°C for 1 hr, the cell sheets were washed twice with 5 ml of medium, and then 30 ml of the appropriate maintenance medium (Table 1) was added. Incubation was continued at 37°C. At daily intervals, a portion of the maintenance medium was removed and assayed for virus content.

In single-step growth cycle experiments, cells were grown in 2-oz (ca. 60 ml) prescription bottles and inoculated as above. Three bottles were harvested at each interval. The media were pooled, and a sample was taken for assay of released virus. Calf serum to make a final concentration of 10% was then added to the pooled media. This was redistributed on the cell sheets, which were then rapidly frozen and thawed four times before a sample was drawn for assay of total virus.

Virus assay. Virus was assayed by the intracerebral inoculation of weanling Swiss albino mice with 0.03 ml of 10-fold dilutions. Mice were observed for deaths over a 14-day period. Fifty per cent end points were calculated by the Karber method (10).

RESULTS

Virus stability. To evaluate data on the virus content of cell culture fluids, it was desirable to know the effects of some physical factors of the culture environment on the stability of both the residual inoculated virus and the newly produced virus. Two parameters were tested. (i) To test stability in cell-free medium at 37°C, RBV was diluted in three representative media to an approximate concentration of \(10^7\) WMICLD₉₀ per ml and incubated at 37°C. At intervals, samples were removed and assayed in mice. The rate of inactivation was similar in all three media, with approximately one log₁₀ of virus being inactivated every 6 hr (Fig. 1).

(ii) Effect of pH on virus stability was tested. Because the decrease in the pH of culture media during cell metabolism may have a deleterious effect on released virus, the stability of RBV at various pH values was tested. Because it was difficult to maintain a constant pH with a bicarbonate buffer system, RBV was diluted in phosphate-buffered saline to a concentration of \(10^6\) WMICLD₉₀ per ml and incubated at 37°C. Calf serum was added to the buffers to a final concentration of 3% before final adjustment of pH to aid in stabilizing RBV against thermal in-
Table 1. Cell culture maintenance media

| Cell system               | Maintenance medium*          |
|---------------------------|-------------------------------|
| L, HeLa, FL amnion        | MEM + 2% calf serum          |
| LLC-MK2                   | BME + 5% calf serum          |
| Human embryo skin         | BME + 2% calf serum          |
| Vero, chick embryo fibroblasts | HLH + 3% calf serum      |
| BHK-21 (C13)              | HLH + 10% tryptose phosphate broth + 0.4% glucose + 2% calf serum |

* MEM, Eagle's minimum essential medium; BME, basal medium (Eagle); HLH, Hanks lactalbumin hydrolysate. All powdered media purchased from Grand Island Biological Co., Grand Island, N.Y.

activation. After 2 hr, samples from each buffer were assayed in weanling mice. A marked reduction in infectious RBV was found in the buffers below pH 7.0 (Table 2).

The deleterious effect of low pH on RBV was further demonstrated in the production of six cell culture seeds. Monolayers of BHK-21 cells were infected with RBV that had been passed 10 times in BHK-21 cells. The maintenance media of three flasks were adjusted to pH 7.6 to 7.8 with addition of NaHCO₃ initially and at 24 and 40 hr after infection; the other three were not adjusted. An average of 100-fold more virus per milliliter of medium was recovered from the cultures maintained under high pH than from those in which the pH was not adjusted (Table 3).

Screening of cell cultures. Eight cell culture systems were screened to determine their sensitivity to infection with small doses of RBV and their ability to produce RBV in high titer. Data in Table 4 show that the cells differed in these qualities. BHK-21 and Vero cells were very sensitive to RBV at low doses and produced equally high titers of virus, regardless of the infecting dose. The time required to reach maximum titer, however, increased with decreasing dose. L cells, on the other hand, although producing high titers of virus after infection with the highest dose, were repeatedly insusceptible to infection at the lower doses of virus. The CEF cultures were sensitive to RBV at the lowest dose, although virus production was barely detectable. Infection with 10⁴ times as much virus resulted in only slightly increased virus production. HeLa and FL amnion cells were relatively resistant to low doses of RBV. Other cells produced intermediate responses.

Single-cycle growth. The production of RBV during a single growth cycle in three different cell systems was studied. Monolayers of BHK-21, L, and LLC-MK₂ cells were inoculated at a multiplicity of 20 WMICLD₉₀ per cell and sampled as previously described. In each case, virus production was not apparent during the first 12 hr after infection (Fig. 2). Virus production peaked at 36 to 48 hr. The close agreement between the titers of released virus and total virus (released virus plus cell-associated virus) indicates a constant release of mature virus from the infected cells rather than storage within the cells.

Persistent infection of Vero cells. It was noticed in the screening experiments that infected Vero cell cultures were not totally destroyed during the 14-day holding period, but the individual cells became enlarged and more elongated than in normal cultures. Approximately 50% of these...
TABLE 3. Effect of pH on yield of Rio Bravo virus in cell culture

| Treatment                | Initial pH of medium | Final pH of medium | Virus titer<sup>a</sup> |
|--------------------------|----------------------|--------------------|-------------------------|
| None                     | 7.5; 7.5; 7.4        | 6.6; 6.6; 6.6      | 6.0; 6.8; 6.6            |
| pH adjusted 24 and 40 hr | 7.8; 7.9; 7.8        | 7.6; 7.6; 7.5      | 8.2; 9.2; 8.4            |

<sup>a</sup> Log<sub>10</sub> WMICLD<sub>50</sub>/ml.

TABLE 4. Yields of Rio Bravo virus in various cell cultures

| Cell system                 | MOI<sup>b</sup> = 2 × 10<sup>-3</sup> | MOI = 2 × 10<sup>-3</sup> | MOI = 2 × 10<sup>-3</sup> |
|-----------------------------|--------------------------------------|--------------------------|--------------------------|
|                             | Titers<sup>c</sup>    | Day | Titers    | Day | Titers    | Day |
| BHK-21 (C13)                | 7.5                    | 2   | 7.5       | 5   | 7.9       | 6   |
| L                           | 7.1                    | 3   | <2.5      | 5   | <2.5      | 7   |
| Vero                       | 6.3                    | 4   | 6.8       | 4   | 6.9       | 5   |
| LLC-MK<sub>2</sub>           | 5.8                    | 2   | 6.3       | 4   | 3.6       | 5   |
| HeLa                       | 5.9                    | 1   | 3.6       | 2   | <2.5      | 5   |
| Human embryo skin           | NC<sup>c</sup>        | 1   | 6.1       | 2   | 5.3       | 5   |
| FL amnion                  | 4.2                    | 2   | 2.6       | 2   | <2.5      | 5   |
| Chick embryo fibroblast     | 4.0                    | 2   | 3.8       | 5   | 1.8       | 6   |

<sup>a</sup> Multiplicity of infection; WMICLD<sub>50</sub> per cell.
<sup>b</sup> Log<sub>10</sub> WMICLD<sub>50</sub> per ml of supernatant fluid.
<sup>c</sup> Not done.

FIG. 2. Single-growth cycles of RBV in cell cultures. Total virus, solid line; supernatant virus, broken line.

cells showed cytopathic changes at any one time. Another culture was infected at a multiplicity of 0.002 WMICLD<sub>50</sub> per cell, and maintenance medium was changed weekly. Assays for virus performed at various intervals showed an initial increase in virus production followed by a plateau that was maintained up to 107 days after infection when the culture was discarded (Fig. 3). Between days 60 and 65, the medium was changed four times. The reduced virus titers of these samples suggest slow virus production by the cells, requiring a number of days before the concentrations of the plateau level were reached.

**DISCUSSION**

Five cell lines were found to produce substantial amounts of RBV when inoculated with the high dose (multiplicity of infection = 0.3) of virus. They differed markedly, however, in their sensitivity to infection by small multiplicities of virus. BHK-21 monolayers were equally productive, regardless of the infecting dose. Maximum virus titer in the single-cycle growth experiment was higher than those found in the screening experiments. However, more emphasis was placed on pH control in the former experiment, so the higher titer may be a reflection of decreased virus inactivation rather than of increased virus production.

The behavior of L cells is of interest in that a peak of more than 7 log<sub>10</sub> of virus per ml was produced by cells infected with 20 and 0.3 WMICLD<sub>50</sub> per cell, but infection with a multiplicity of 0.003 WMICLD<sub>50</sub> or less resulted in repeated failures to recover any virus from the cultures. Serial passage of RBV in L cells did not increase virus production in this cell line.

LLC-MK<sub>2</sub> and HeLa cells both produced
moderate amounts of virus when infected at higher multiplicities but differed in their sensitivities to lower multiplicities of RBV. The LLC-MK₂ cells were sensitive at the lowest dose; the HeLa cells were not. Virus was serially passed in LLC-MK₂ cells with no change in peak titers during 10 passages.

Single-cycle growth experiments in BHK-21, L, and LLC-MK₂ cells resulted in similar growth patterns. A latent period was observed for the first 12 hr. During this time, the rate of decrease in the residual virus inoculum closely paralleled the inactivation curves of virus in cell-free medium, suggesting that inactivation was responsible for the observed decrease.

After the initiation of virus release at about 12 hr, virus production reached a maximum at 36 or 48 hr. Virus release appeared to follow the pattern of other enveloped viruses (7). Since the total virus content of the medium and cells was always only slightly greater than that in the medium alone, it appears that there was little infectious virus stored within the cells and that virus was released soon after its maturation.

Virus production in Vero cells was variable in both maximum titers attained and the day of peak production. Usually, when maximum titer was reached, it remained at that level for a number of days. The variation in the first day of maximum titer can be seen by comparing that found for cells infected with 0.003 WMICLD₅₀ per cell. In the screening experiments (Table 4), this occurred on day 5. In the experiments reported in Fig. 3, the peak did not occur until some days later. There was also considerable variation in peak titer between experiments. Sometimes, 4 to 5 log₁₀ of virus per ml was the most that could be recovered from a culture. This variation may be a reflection of inapparent differences in the maintenance of cultures during virus growth or of a heterogeneous cell population.

The chronic infection of Vero cells by RBV appears to be unique. There have been few reports on cell cultures chronically infected with a group B arbovirus (6, 8). Most recently, Jarman et al. (8) reported the chronic infection of L-929 cells by West Nile virus, a group B virus closely related to RBV. Their descriptions of cell cultures are similar to these changes seen in Vero cells with RBV. Our Vero cultures continued to produce substantial amounts of RBV while undergoing marked, yet not complete, cytopathic changes. In preliminary studies, infected cells were subcultured three times at weekly intervals without noticeable alteration of cell growth or virus production. Virus release from cells must be relatively slow. Markedly less infectious virus was found in the culture medium when it was changed at daily rather than weekly intervals (Fig. 3).

Some persistent infections have been attributed to a balance between virus and interferon production. Desmyter et al. (5), however, have reported that Vero cells do not produce interferon. The factors responsible for the persistence of RBV in Vero cultures have not been elucidated.

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