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TECHNICAL MANUSCRIPT 583

GROWTH OF RIO BRAVO VIRUS IN CELL CULTURES

Alan R. Wilhelm
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FEBRUARY 1970

DEPARTMENT OF THE ARMY
Fort Detrick
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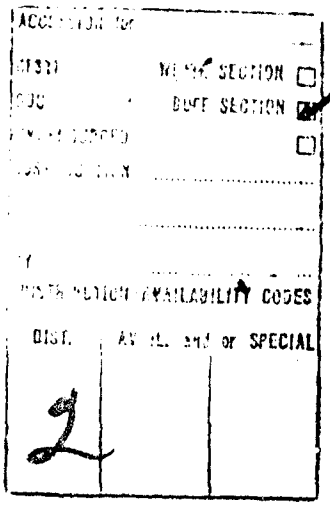

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GROWTH OF RIO BRAVO VIRUS IN CELL CULTURES

Alan R. Wilhelm

Peter J. Gerone

Virus & Rickettsia Division
BIOLOGICAL SCIENCES LABORATORIES

Project 1B562602AD01

February 1970

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

ABSTRACT

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-MK_2, 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-MK_2 cell monolayers was characterized by a latent period of about 12 hours followed by rapid virus production that peaked at 36 to 48 hours. Vero cell cultures can remain chronically infected with RBV for more than 100 days. They show evidence of cell destruction and their supernatant fluids contain virus at 10^4 to 10^5 $\log_{10}$ per ml.

I. INTRODUCTION*

Rio Bravo virus (RBV), formerly called U.S. bat salivary gland virus, has been isolated from the salivary glands of Mexican free-tailed bats by various investigators.[1-4] Although no arthropod vector has been reported for RBV, it is antigenically related to the St. Louis encephalitis complex of the group B arboviruses.[5,6] The virus is readily propagated in the brains of suckling or weanling mice. This paper presents data on the growth of RBV in various cell cultures.

II. MATERIALS AND METHODS

A. 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 $10^{8.8}$ weanling mouse intracerebral LD_{50} ($WMICLD_{50}$) per ml.

B. CELL CULTURES

Monolayer cultures of established cell lines, human embryo skin (HES), and primary chick embryo fibroblasts (CEF) in disposable plastic T-60 flasks** were used for screening experiments. After inoculation and adsorption at 37 C for 1 hour, the cell sheets were washed twice; then 30 ml of the appropriate maintenance medium was added (Table 1). Incubation was continued at 37 C. At daily intervals a portion of the maintenance medium was removed and assayed for virus content.

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* This report should not be used as a literature citation in material to be published in the open literature. Readers interested in referencing the information contained herein should contact the senior author to ascertain when and where it may appear in citable form.

** Falcon Plastics, Los Angeles, California.

TABLE 1. CELL CULTURE MAINTENANCE MEDIA

Cell System	Maintenance Medium
L, HeLa, FL amnion	Minimum essential medium (Eagle)[a] + 2% calf serum
LLC-MK_2	Basal medium (Eagle) (BME)[a] + 5% calf serum
Human embryo skin	BME + 2% calf serum
Vero, CEF	Hanks lactalbumin hydrolysate (HLH)[a] + 3% calf serum
BHK-21 (C13)	HLH + 10% tryptose phosphate broth + 0.4% glucose + 2% calf serum

a. Powdered Medium, Grand Island Biological Company, Grand Island, N.Y.

In single-step growth cycle experiments, cells were grown in 2-oz 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.

C. VIRUS ASSAY

Virus was assayed by intracerebral inoculation (0.05 ml) of weanling Swiss albino mice with 10-fold dilutions of virus. Animals were observed for a 14-day period and LD_{50} endpoints were calculated by the Karber method.[7]

III. RESULTS

A. 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:

1) Stability in cell-free medium at 37 C. RBV was diluted in three representative media to an approximate concentration of 10^7 $WMICLD_{50}$ 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 $\log_{10}$ of virus being inactivated every 6 hours (Fig. 1).

2) Effect of pH on virus stability. 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. It was difficult to maintain a constant pH with a bicarbonate buffer system, so the RBV was diluted in phosphate-buffered saline to a concentrated of 10^6 $WMICLD_{50}$ 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 inactivation. After 2 hours, samples from each buffer were assayed in weanling mice. As shown in Table 2, a marked reduction in infectious RBV was found in the buffers below pH 7.0.

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 ten times in BHK-21 cells. The maintenance media of three flasks were adjusted to pH 7.6 to 7.8 with addition of $NaHCO_3$ initially and at 24 and 40 hours after infection; the other three were not adjusted. Table 3 shows that an average of 100-fold more virus per ml of medium was recovered from the cultures maintained under high pH than from those in which the pH was not adjusted.

B. SCREENING OF CELL CULTURES

Eight cell culture systems were screened to determine (i) their sensitivity to infection with small doses of RBV and (ii) 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 they produced high titers of virus after infection with the highest dose,

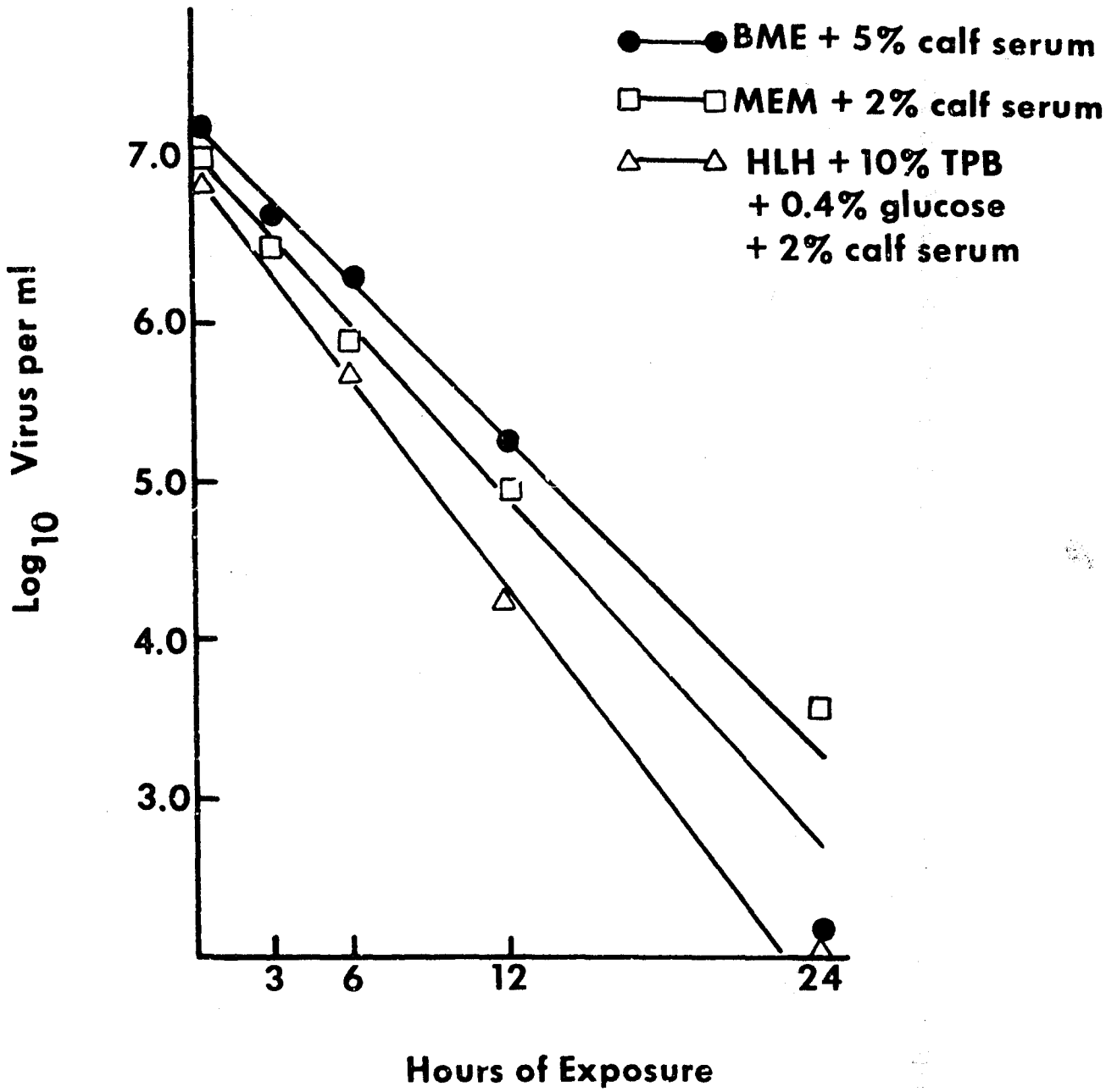

FIGURE 1. Stability of RBV in Cell-Free Media at 37 C.

TABLE 2. INFLUENCE OF pH ON STABILITY OF RBV IN PHOSPHATE BUFFER[a] CONTAINING 3% CALF SERUM

pH	Titer after 2 Hours at 37 C, $\log_{10}$	Titer Loss, $\log_{10}$
8.0	5.9	0.1
7.6	6.0	0
7.4	5.8	0.2
7.2	5.6	0.4
7.0	5.2	0.8
6.8	4.0	2.0
6.6	3.6	2.4

a. Each buffer contained $10^{6.0}$ $WMICLD_{50}$ before incubation.

TABLE 3. EFFECT OF pH ON YIELD OF RBV IN CELL CULTURE

Treatment	Initial pH of Medium	Final pH of Medium	Virus Titer[a]
None	7.5; 7.5; 7.4	6.6; 6.6; 6.6	6.0; 6.8; 6.6
pH adjusted at 24 and 40 hr	7.8; 7.9; 7.8	7.6; 7.6; 7.5	8.2; 9.2; 8.4

a. $\log_{10}$ $WMICLD_{50}$/ml.

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 10^4 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.

TABLE 4. YIELDS OF RBV IN VARIOUS CELL CULTURE SUPERNATANT FLUIDS

Cell System	MOI[a] = 2×10^{-1}		MOI = 2×10^{-3}		MOI = 2×10^{-5}	
	Titer[b]	Day	Titer	Day	Titer	Day
BHK-21 (C13)	7.5	2	7.5	5	7.9	6
L	7.1	3	<2.5		<2.5	
Vero	6.3	4	6.8	5	6.9	7
LLC-MK_2	5.8	2	6.3	4	3.6	5
HeLa	5.9	1	3.6	2	<2.5	
Human embryo skin	ND[c]		6.1	4	5.3	5
FL amnion	4.2	2	2.6	2	<2.5	
CEF	4.9	2	3.8	5	1.8	6

a. Multiplicity of infection, $WMICLD_{50}$ per cell.
b. Log_{10} $WMICLD_{50}$ per ml.
c. Not done.

C. 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-MK_2 cells were inoculated at a multiplicity of 20 $WMICLD_{50}$ per cell and sampled as previously described. In each case virus production was not apparent until 12 hours after infection (Fig. 2). Virus production peaked at 36 to 48 hours. 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.

D. PERSISTENT INFECTION OF VERO CELLS

We 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 cells showed cytopathic changes at any one time. Another culture was infected at a multiplicity of 0.002 $WMICLD_{50}$ per cell, and maintenance medium was changed weekly. Assays for virus at varying 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.

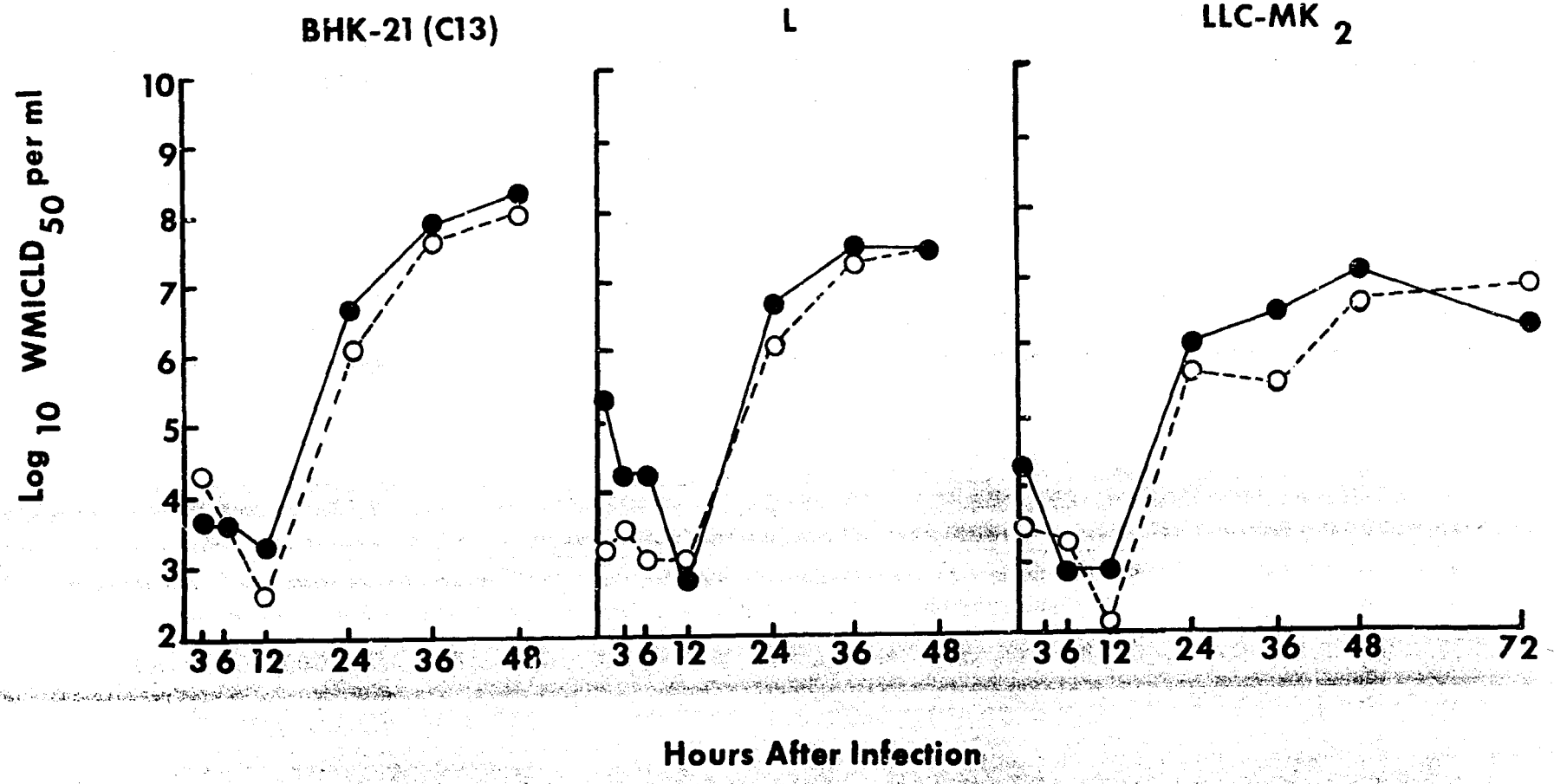

FIGURE 2.— Single-Growth Cycles of RBV in Cell Cultures.
Solid line = total virus, dotted line = supernatant virus.

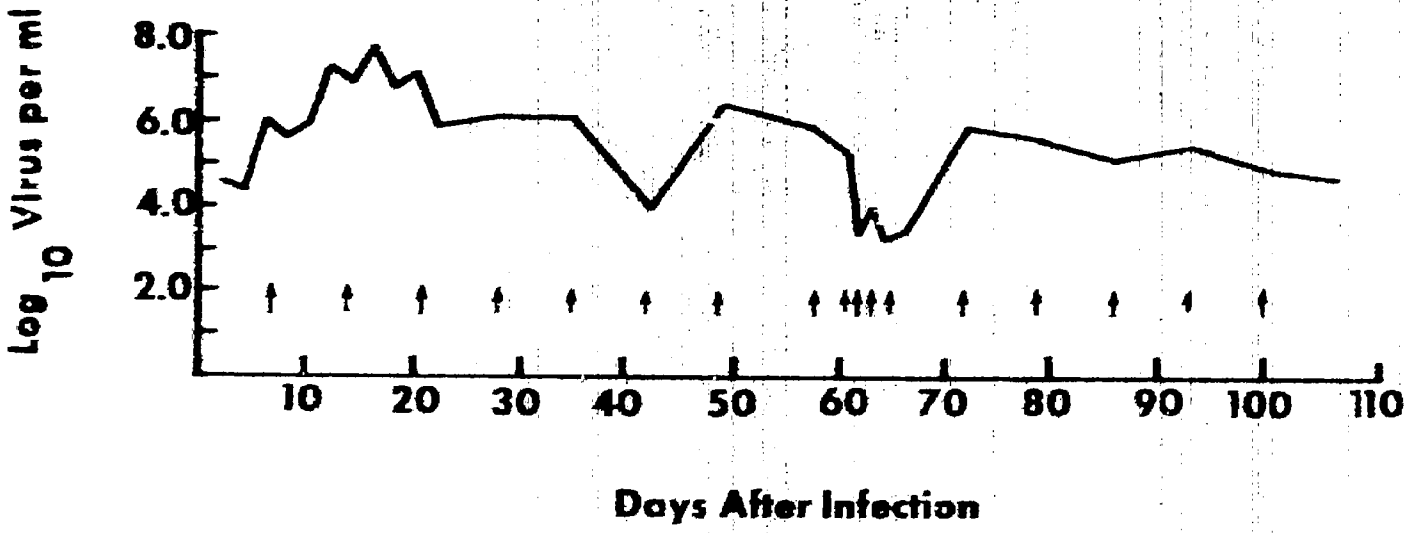

FIGURE 3. Persistent Production of HSV by Vero Cells.
(↑) = medium changed.

IV. DISCUSSION

Five cell lines produced substantial amounts of RBV when inoculated with the high dose (MOI = 0.2) 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 seven $\log_{10}$ of virus per ml was produced by cells infected with 20 and 0.2 $WMICLD_{50}$ per cell, but infection with a multiplicity of 0.002 $WMICLD_{50}$ 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 that cell line.

LLC-MK_2 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-MK_2 cells were sensitive at the lowest dose; the HeLa cells were not. Virus was serially passed in LLC-MK_2 cells with no change in peak titers during ten passages.

Single-cycle growth experiments in BHK-21, L, and LLC-MK_2 cells resulted in similar growth patterns. A latent period was observed for the first 12 hours. During that time the rate of decrease in the residual virus inoculum closely paralleled the inactivation curves of virus in cell-free medium, suggesting the inactivation of input virus in the medium or virus that had adsorbed, but had not penetrated the host cells.

After the initiation of virus release at about 12 hours, virus production peaked at 36 or 48 hours. Virus release appeared to follow the pattern of other enveloped viruses.[8] The total virus content of the medium and cells was always only slightly greater than that in the medium alone, so 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 both in maximum titers attained and in 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 those found for cells infected with 0.002 $WMICLD_{50}$ per cell. In the screening experiments (Table 4) this occurred on day 5. In the experiments reported in Figure 3, the peak did not occur until some days later. There was also considerable variation in peak titer between experiments. Sometimes, four to five $\log_{10}$ 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.[9,10] Most recently, Jarman et al.[10] 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 daily rather than at weekly intervals (Fig. 3).

Some persistent infections have been attributed to a balance between virus and interferon production. Desmyter et al.,[11] 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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Unclassified
Security Classification

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author)
Department of the Army
Fort Detrick, Frederick, Maryland, 21701

2a. REPORT SECURITY CLASSIFICATION
Unclassified

2b. GROUP

3. REPORT TITLE
GROWTH OF RIO BRAVO VIRUS IN CELL CULTURES

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)

5. AUTHOR(S) (First name, middle initial, last name)
Alan R. Wilhelm
Peter J. Gerone

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
February 1970	17	11

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
b. PROJECT NO. 1B562602AD01	Technical Manuscript 583
c. Task-Work Unit 01-007	9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
d. DD 1498 Agency Access. DA OL 0336	CMs 6646 SMUFD-AE-T 49589

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11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
Virus & Rickettsia Division	Department of the Army Fort Detrick, Frederick, Maryland, 21701

13. ABSTRACT

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14. Key Words

Rio Bravo virus
Cell cultures
Virus replication
Chronic infections

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