Effect of Serum on the Yield of Lymphocytic Choriomeningitis Virus in Baby Hamster Kidney Cells

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The yields of the Armstrong and WE strains of lymphocytic choriomeningitis virus in baby hamster kidney (BHK) cells cultivated in either bovine, calf, fetal bovine, or horse serum were investigated. Lines of BHK cells were established in these sera. When the infected cell lines were observed by immunofluorescence, the percent fluorescing cells for a given virus strain did not vary. However, for both strains, the extracellular virus yields per cell were significantly greater in the fetal bovine-cell line than in the other serum-cell lines.

Components of normal sera exhibit diverse effects on the replication of viruses in cell culture. Some serum components act directly on the virus and appear to be similar to immunoglobulins (11). Other antiviral substances reduce infectivity but exhibit a function different from neutralizing antibody (8). Additional serum components inhibit virus yield by acting on the host cell rather than directly on the virus. Such serum inhibitors delay adsorption of virus to the cell, suppress release of virus at the end of the infectious cycle, and reduce plaque size (9). Specific antiserum to host cell antigens may produce the same effects (4). Furthermore, it would appear that continued cultivation of some cell lines in one species of serum may select a cell population which is inherently less susceptible to infection than comparable cells cultivated in another serum (10).

The effect of serum on the yield of lymphocytic choriomeningitis (LCM) virus in cell culture has not been reported. Because of the potential effect of the type of serum used in the culture medium, we have examined the replication of LCM virus in baby hamster kidney (BHK) cells grown and maintained in serum of different species.

MATERIALS AND METHODS

Cell culture. BHK-21, clone 13 (BHK-21/13) cells were grown in basal medium Eagle (BME) with Hanks balanced salt solution base, supplemented with 10% Trypsone phosphate broth and 10% serum. Maintenance medium consisted of BME with Earle's balanced salt solution base, supplemented with 2% serum. Maintenance medium was added in 1.5-ml quantities to roller tubes. For experimental purposes the BHK cells, which were cultured initially with fetal bovine serum, were grown and maintained in bovine, calf, fetal bovine, or horse serum. The sera were not heat-inactivated. To determine the number of cells in roller tubes (16 by 125 mm), monolayers were treated with trypsin solution, suspended in saline, stained with crystal violet, and counted with a hemocytometer. Cell cultures and virus stocks were tested periodically for Mycoplasma (2). Those found to be contaminated were discarded.

Calculation of cell population doubling time. A 1-ml amount of cell suspension in growth medium (2.9 X 10^4 cells/ml) was planted per roller tube. Every 6 hr, until a cell monolayer formed, the cells in five roller tubes were counted and averaged. The cell population doubling time was calculated from those averages (3).

LCM virus. The Armstrong strain (VR134) was obtained from the American Type Culture Collection. The WE strain was received from J. F. Winn of the Center for Disease Control. Stock suspensions of both viruses were harvested with cell culture fluid and were stored at -70 C.

Decional dilutions of virus made in Tryptose phosphate broth were titrated by intracranial inoculation (0.03 ml) of 4- to 6-week-old white Swiss mice. The LD_{50} titer was determined by the method of Reed and Muench. At the time the virus was harvested, the virus yields per cell were determined from the average number of cells in five control tubes inoculated with 0.2 ml of sterile Tryptose phosphate broth.

Immunofluorescence procedure. Anti-LCM virus antiserum was prepared in guinea pigs (1). Rabbit anti-guinea pig gamma globulin, conjugated with fluorescein isothiocyanate, was obtained from Nutritional Biochemical Corp. (Cleveland, Ohio).

Leighton tube cover slips (10.5 by 22 mm) containing BHK cells inoculated with LCM virus (2 X 10^{-4} mouse LD_{50}/cell) were rinsed in phosphate-buffered saline (PBS; pH 7.4), fixed with acetone for 10 min
at -20 C, and air-dried. After three 5-min rinsings in PBS and one rinse in distilled water, the cover slips were overlaid with anti-LCM immune serum for 30 min, followed by three additional 5-min rinsings in PBS and one rinse in distilled water. The cover slips were then overlaid with anti-guinea pig gamma globulin conjugate for 30 min, rinsed as before, and mounted on clean glass slides with a drop of 10% PBS in glycerol. Between 150 and 300 cells per cover slip were observed for the percentage of cells displaying specific fluorescence for LCM virus antigen.

Positive fluorescence was brilliant green in color and limited to the cytoplasm. Specific fluorescence was not observed in the following controls: (i) BHK cells stained by the above procedure, (ii) BHK cells inoculated with LCM virus and treated with anti-LCM antiserum, and (iii) BHK cells inoculated with LCM virus and treated with conjugated serum.

Virus yield per infected cell. Assuming that the cells containing LCM virus antigen were infected, the number of infected cells per Leighton tube was calculated by multiplying the percentage of fluorescent cells by the number of cells per Leighton tube. From this product, the relationship of the virus present in the maintenance medium to the number of infected cells was determined.

RESULTS

Establishment of BHK cell lines. The cell population doubling (CPD) time of the original BHK cell line, cultured in fetal bovine serum (BHK-FB line), was used as the criterion for the establishment of BHK cell lines in bovine, calf, and horse sera. The CPD times were 18 hr or longer after a single passage in these sera (Table 1). After three subcultures, the CPD times were reduced approximately to the CPD time of the BHK-FB line and did not decrease further after six subcultures in the same serum. Therefore, BHK cells subcultured three or more times in bovine, calf, and horse serum were designated, respectively, BHK-B, -C, and -H lines and were used in the following experiments.

Effect of the source of virus inoculum on the replication in BHK cells. LCM virus harvested from mouse brain and from a single passage in BHK cells was inoculated into roller tubes of BHK cells (2 × 10^2 mouse LD50/cell). The maintenance medium was changed every other day. At 96 hr after inoculation, the viruses were harvested and titered. The results of two experiments indicate that the replication of both the Armstrong and WE stains of LCM virus were the same regardless of the source of virus inoculum (Table 2).

LCM virus yield in BHK cell lines. After the BHK-B, -C, -FB, and -H lines formed a complete monolayer in roller tubes, the growth medium was replaced with maintenance medium, and five roller tubes of each BHK cell line were inoculated with 0.2 mL of LCM virus diluted in Tryptose phosphate broth (2 × 10^4 mouse LD50/cell). The maintenance medium was changed at 48, 72, 96, and 120 hr after inoculation. At 72, 96, and 120 hr, medium was pooled and the extracellular virus yields were determined. At the same time intervals, the cells in the control cultures inoculated with 0.2 mL of sterile Tryptose phosphate broth were counted. From these data, the virus yields per cell were calculated.

The mean values of the virus yields per 0.03 mL of culture fluid and the virus yields per cell with their respective standard deviations for three experiments are shown in Tables 3 and 4. A coefficient of variance test (6) was performed to compare the standard deviations for the virus yields per 0.03 mL of culture fluid to the virus yield per cell. The variability of the virus yields per cell was less than that of the virus yields per 0.03 mL of culture fluid at a 97.5% but not a 99% confidence interval.

In view of the consistency of the results expressed as the virus yield per cell, a t test analysis was performed on this data. At 96 hr after inocu-

### Table 1. Cell population doubling times for BHK cells cultured in bovine, calf, fetal bovine, or horse serum

| Culture serum     | Cell population doubling time (hr) |
|-------------------|-----------------------------------|
|                   | One subculture | Three to six subcultures |
| Fetal bovine      | NAa            | 12.0                   |
| Bovine            | 18.9           | 12.3                   |
| Calf              | 18.0           | 12.6                   |
| Horse             | 18.0           | 13.7                   |

* Not applicable, since the initial BHK cells were cultured in fetal bovine serum.

### Table 2. Effect of inoculum source on replication of lymphocytic choriomeningitis (LCM) virus in BHK cells

| Strain of virus | Source of Inoculum | Yield of virus from BHK cells* |
|-----------------|--------------------|-------------------------------|
|                 |                    | Exp 1 | Exp 2 |
| Armstrong       | Mouse brain        | 6.3   | 5.0   |
|                 | BHK cells         | 6.0   | 5.2   |
| WE              | Mouse brain        | 5.7   | 4.5   |
|                 | BHK cells         | 5.6   | 4.5   |

* Log10 mouse LD50 per 0.03 mL of culture fluid at 96 hr after inoculation.

b After a single passage in BHK cells, the LCM virus was reinoculated into BHK cells.
TABLE 3. Extracellular yield of the Armstrong strain of lymphocytic choriomeningitis (LCM) virus in lines of BHK cells

| Cell lines | LCM virus yielda |
|------------|------------------|
|            | 72 hrb | 96 hr | 120 hr |
|            | Mouse LD_{50} per 0.03 ml² | Mouse LD_{50} per cell | Mouse LD_{50} per 0.03 ml² | Mouse LD_{50} per cell | Mouse LD_{50} per 0.03 ml² | Mouse LD_{50} per cell |
| BHK-B      | 0.22 (±1.5) | 11.8 (±2.5) | 0.94 (±1.1) | 18.1 (±0.0) | 0.96 (±2.5) | 12.1 (±4.6) |
| BHK-C      | 0.43 (±0.3) | 16.8 (±0.0) | 6.46 (±19.6) | 35.4 (±2.7) | 0.24 (±0.3) | 23.7 (±4.6) |
| BHK-FB     | 0.82 (±0.6) | 18.1 (±0.0) | 9.50 (±14.6) | 50.0 (±3.2) | 0.25 (±0.9) | 18.1 (±1.9) |
| BHK-H      | 0.15 (±2.3) | 12.6 (±1.2) | 0.76 (±7.5) | 29.9 (±5.7) | 0.36 (±0.9) | 12.6 (±0.0) |

* Mean values for three experiments. The numbers in parentheses indicate standard deviations.

b Hours after inoculation of cell cultures with LCM virus.

c Cell lines were cultivated and maintained in bovine (B), calf (C), fetal bovine (FB), or horse (H) serum.

d Values expressed ×10⁴.

TABLE 4. Extracellular yield of the WE strain of lymphocytic choriomeningitis (LCM) virus in lines of BHK cells

| Cell lines | LCM virus yielda |
|------------|------------------|
|            | 72 hrb | 96 hr | 120 hr |
|            | Mouse LD_{50} per 0.03 ml² | Mouse LD_{50} per cell | Mouse LD_{50} per 0.03 ml² | Mouse LD_{50} per cell | Mouse LD_{50} per 0.03 ml² | Mouse LD_{50} per cell |
| BHK-B      | 1.00 (±15.1) | 9.0 (±4.8) | 1.20 (±0.8) | 16.8 (±0.0) | 0.65 (±0.0) | 12.1 (±2.3) |
| BHK-C      | 0.43 (±0.0) | 10.0 (±2.0) | 10.0 (±4.6) | 36.3 (±6.1) | 1.21 (±0.5) | 22.3 (±0.0) |
| BHK-FB     | 0.46 (±0.54) | 12.0 (±0.1) | 11.41 (±1.2) | 50.0 (±5.7) | 1.56 (±0.1) | 22.3 (±1.4) |
| BHK-H      | 0.46 (±0.9) | 11.2 (±0.3) | 1.46 (±0.2) | 25.1 (±0.0) | 0.14 (±1.2) | 12.1 (±1.3) |

* Mean values for three experiments. Numbers in parentheses indicate standard deviations.

b Hours after inoculation of cell cultures with LCM virus.

c Cell lines were cultivated and maintained in bovine (B), calf (C), fetal bovine (FB), or horse (H) serum.

d Values expressed ×10⁴.
formed. At 96 hr after infection, the extracellular virus yield per cell was determined and the percentage of cells displaying specific fluorescence for LCM virus at 96 hr after inoculation was determined.

The percentages of cells displaying specific fluorescence were similar in all cell lines inoculated with both the Armstrong and WE strains of LCM virus (Table 5). However, the virus yields per infected cell in the BHK-FB line were greater than the yields in the other BHK cell lines for both strains of virus. The cell lines inoculated with the Armstrong strain of LCM virus had more cells fluorescent than the cell lines inoculated with the WE strain.

**DISCUSSION**

Although the same multiplicity of infection was used in every experiment, the large standard deviations for the virus yield per 0.03 ml of culture medium indicated that an uncontrolled variable was operative within the experiment. When the virus yields were calculated per cell, a significant reduction in the standard deviation indicated that this variability resulted in part from a difference in the number of cells constituting the monolayer at the time the virus samples were taken. In view of this observation, calculation of the yield per cell rather than the yield per culture appears to offer an advantage in experimental design.

The virus yield per cell was based on the number of cells in the control culture tubes to avoid the hazard of counting infected cells. The consistency of results when the virus yield was determined per cell inferred that the control and infected cultures contained the same number of cells. By direct cell count, Lehmann-Grube (5) showed that cultures infected with LCM virus contained the same number of cells as the control cultures.

The maximum extracellular virus yields per cell were obtained in BHK cells cultivated in fetal bovine serum, followed by the BHK cells cultivated in calf serum. It is possible that the high gamma globulin content of horse and bovine sera compared to fetal bovine and calf sera may suppress the amount of virus produced. Sell (7) similarly correlated the high gamma globulin content of adult serum with the cytoxicity exhibited by the serum. Adult bovine serum, which contained 2% gamma globulin, was toxic for rabbit lymphocytes, whereas fetal bovine and calf serum, which both contained 0.1% gamma globulin, satisfactorily maintained the cells. Alternatively, an increase in virus yield may have resulted from selection of a virus that was resistant to inhibitors in fetal bovine serum when the virus stock was propagated in the BHK-FB culture. However, since the LCM virus harvested from mouse brain and from BHK-FB line did not differ in their replication in the BHK-FB line, it is unlikely that this mechanism would account for the higher virus yield in the BHK-FB line.

If interference of LCM virus adsorption were the reason for the lower virus yields as was shown with echovirus (4), one would expect more infected cells with the BHK-FB line. However, the per cent infected cells observed by immunofluorescence did not vary with the type of serum. Therefore, the adsorption of LCM virus does not seem to be blocked either by action of the serum on the virus or on the cell. Furthermore, cultivation of BHK cells in any of the sera apparently did not select a line of cells that was inherently more susceptible or resistant to infection with LCM virus.

Since the sera apparently did not affect virus adsorption, it is believed that the difference in the virus yields per infected cell may be due to the effect of sera on some cellular function. Either the replication or the release of virus may have been affected by the sera. In this regard, release of echovirus was inhibited with anticytotoxic anti-serum (4). Also, cellular release of poliovirus was inhibited with normal horse serum (8).

The purpose of this investigation was to determine whether the serum used in BHK cell culture had an effect on the yield of LCM virus yield. In light of the significant difference in the maximum LCM virus yield obtained with BHK cells cultivated in fetal bovine serum compared to BHK

| Cell lines | LCM virus yielda |
|------------|------------------|
|            | Armstrong strain | WE strain    |
| BHK-B      | 41 (48)          | 34 (37)      |
| BHK-C      | 50 (47)          | 51 (37)      |
| BHK-FB     | 82 (46)          | 102 (36)     |
| BHK-H      | 40 (50)          | 32 (39)      |

a Mouse LD50 per infected cell. Numbers in parentheses represent the percentage of cells displaying specific fluorescence for LCM virus at 96 hr after inoculation.

b Cell lines were grown and maintained in bovine (B), calf (C), fetal bovine (FB), or horse (H) serum.
cells cultivated in bovine, calf, or horse serum, it is recommended that fetal bovine serum be used when BHK cells are used to investigate LCM virus.

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