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Growth of Pathogenic Virus in a Large-Scale Tissue Culture System

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A model system is described for the mass propagation of Rift Valley fever (RVF) virus, utilizing large-volume fermentor units for suspension culture of tissue cells and the subsequent production of virus. Comparisons between laboratory- and fermentor-scale operations of tissue cell growth gave equivalent results. Cell viability dropped 24 to 30 hr postinfection with a subsequent virus yield between $10^{8.0}$ and $10^{9.0}$ mouse intracerebral median lethal doses per milliliter. Infecting volumes of tissue cell culture (20- or 40-liter working volumes) had no apparent effect on virus yields. Tissue cells grown under either oxidation-reduction potential- and *p*H-controlled or uncontrolled conditions showed little or no difference in their ability to produce RVF virus. We believe this tissue cell virus process to have potential application for large-scale production of vaccines for human or veterinary use or for the mass propagation of certain carcinogenic viruses for cancer research, once use of established lines for this purpose is accepted.

There is an increasing need for obtaining large quantities of different viruses, particularly carcinogenic viruses, for basic studies on chemical composition, transmission characteristics, and antigenic properties. There are few reports (1, 4–8, 10) on mass propagation of tissue cells without an accompanying product, namely, a virus for which the tissue cells are the growth medium.

There can be little doubt, however, that techniques for large-scale production of mammalian cells for virus vaccine production are being explored for human and animal use. Although not all viruses are adaptable for suspension cell culture or will grow in established cell lines, the use of large-volume fermentors for cultivation of tissue cells and subsequent growth of a highly infectious virus has been applied as a model system in this report. Once production of antigens in established lines is an acceptable procedure, the tissue cell virus process described here will have wide application in the area of human and animal vaccine production or production of certain carcinogenic viruses for cancer research, or both.

MATERIALS AND METHODS

Fermentors. The fermentors used in these studies were of the same design for both tissue cells and virus production. They were constructed by the New Brunswick Scientific Co., New Brunswick, N.J. as individual units consisting of two banks of four fermentors each of 316 LC stainless-steel 50-liter water-jacketed fermentors. Each fermentor was individually outfitted with a mechanically sealed agitator shaft with three turbine impellers operated at 200 rev/min. One bank of fermentors was used for production of tissue cells; the second bank was used exclusively for virus production. The fermentors have a length-to-diameter ratio of approximately three. Separate ports were provided for medium, inoculum, three electrode holders, and sampling adapters. Figure 1 illustrates the fermentor and its component parts. The control console for each fermentor consisted of a recorder for *p*H and oxidation-reduction potential (ORP), an agitator speed control, an indicator for liquid and foam levels, and a recorder-controller for temperature (Fig. 2). The gas sparger consisted of a single orifice immediately below the turbine-type impeller. Mixtures of either 95% N_2 and 5% CO_2 or 95% air and 5% CO_2 were used for sparging at a rate of 100 cc/min. An overlay of 95% N_2 and 5% CO_2 was maintained at a rate of 500 cc/min. (For increased biological safety, a negative head pressure of 9 to 10 inches of water was also maintained over the virus culture.) Sparging at the low rate of 100 cc/min and an overlay of gas resulted in little foaming and the elimination of the need for antifoam agents.

Tissue cell cultivation. An established mammalian line of Earl's L cell L-DR (1) was used for Rift Valley fever (RVF) virus production. Tissue culture inoculum for the 50-liter fermentors was produced from frozen seed stocks maintained in liquid nitrogen vapor (−175 C). Inoculum was initially built up

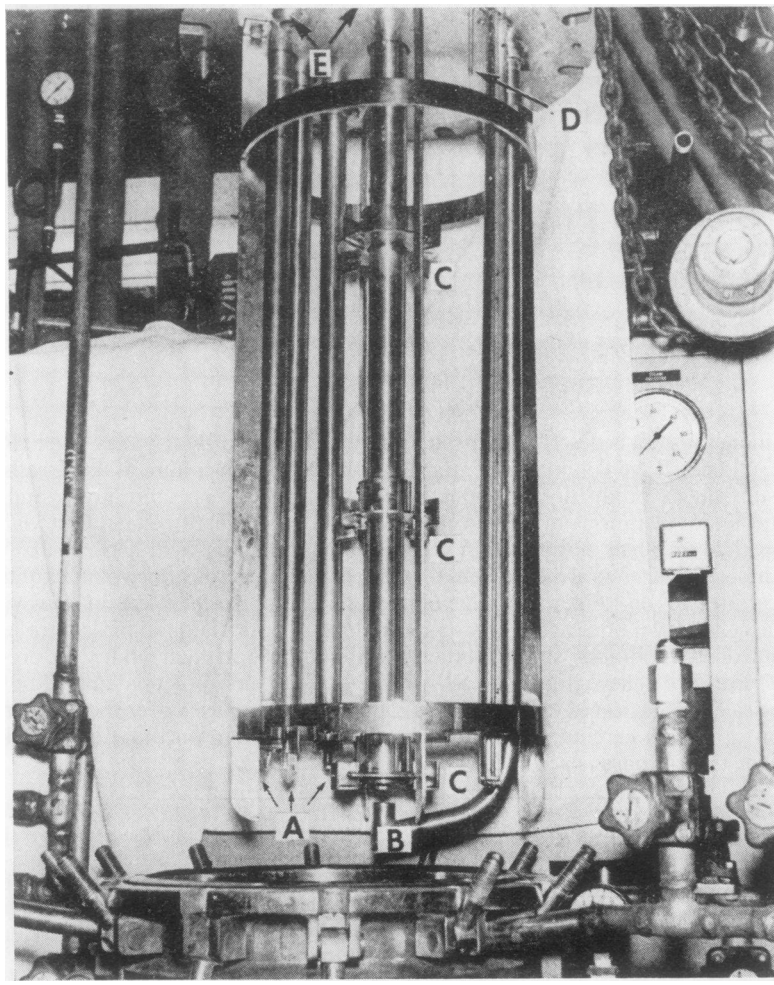

FIG. 1. *Fermentor head lifted to expose internal components. A, three electrode holders; B, sparger; C, three impellers; D, foam contact probe; E, two ports.*

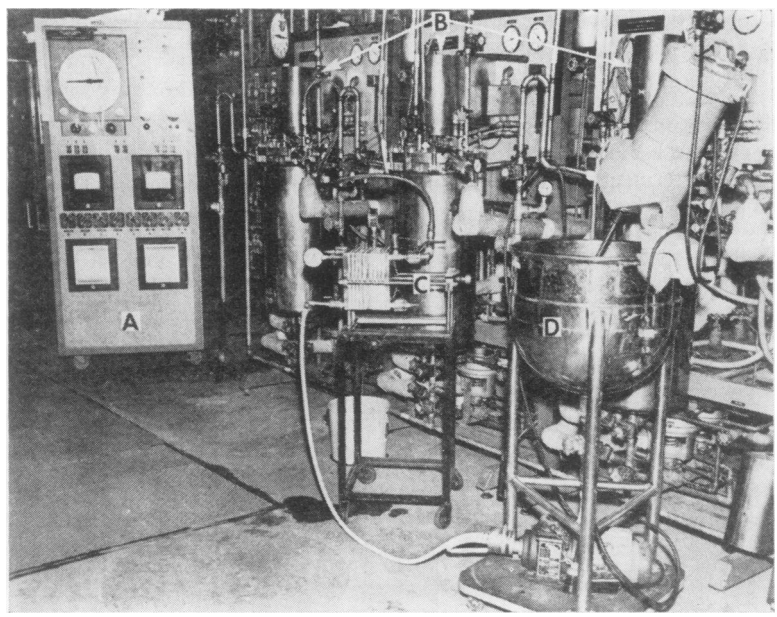

FIG. 2. *General arrangement of the pilot plant. A, control console for one fermentor; B, three fermentors; C, Seitz filter press; D, mixing kettles.*

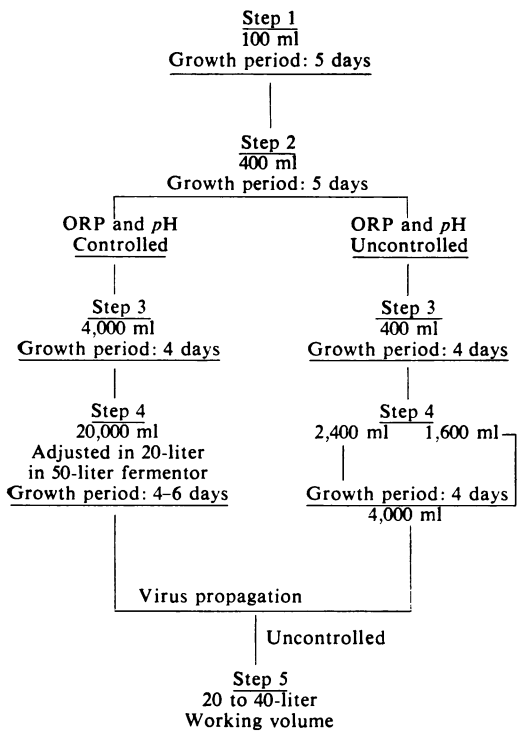

FIG. 3. *Production of tissue cells for Rift Valley fever infection in 50-liter New Brunswick fermentor. Step 1. One milliliter of frozen inoculum was diluted in 99 ml of medium in 250-ml shake flasks. Step 2. Forty milliliter quantities of inoculum from step 1 were added to 360 ml of medium in each of two 1,000-ml shake flasks. Step 3. Controlled pH and ORP: 400 ml of inoculum from step 2 was added to 3,600 ml of medium in 7-liter New Brunswick fermentor. Uncontrolled pH and ORP: 40 ml of inoculum from step 2 was added to 360 ml of medium in 1-liter shake flasks. Step 4. Controlled: each of two 50-liter fermentors was inoculated with sufficient cells from step 3 to provide a concentration of 1.0 to 1.5 × 10^5 cells/ml in 20-liter working volume. Uncontrolled: (A) 240 ml of inoculum from step 3 was added to 2,160 ml of medium in 6-liter shake flask; (B) 160 ml of inoculum from step 3 was added to 1,440 ml of medium in 4-liter shake flask. Step 5. Cells controlled: each of two 50-liter infection fermentors was inoculated with sufficient cells from step 4 to provide a concentration of 3.0 × 10^5 cells/ml in either 20- or 40-liter working volume. Cells uncontrolled: same as above.*

from frozen seed stock, through shake flask (9) and finally placed in an ORP- *p*H-controlled glass fermentor (7.5-liter volume; New Brunswick) or continued by transfer into uncontrolled large-volume shake flasks (4- and 6-liter volume). Each of these two methods supplied enough inoculum for two 20-liter or one 40-liter fermentor set. A diagrammatic sketch illustrating these two procedures for tissue cell production is presented in Fig. 3. In steps 3 and 4 the tissue cell buildup diverged in that cells were produced in either uncontrolled laboratory shake flasks or a fermentor system incorporating the use of *p*H and ORP electrodes for controlling growth environment. In each case cells for the propagation of virus (step 5) were maintained in uncontrolled (*p*H and ORP) environment for subsequent infection and growth of the virus. Specific details of the two procedures are as follows. (i) For controlled (ORP and *p*H) tissue cell growth, the tissue cells were propagated in suspension in 50-liter (20- and 40-liter working volume) New Brunswick fermentors. Cells were grown in Eagle's minimum essential medium as modified by Daniels et al. (1), supplemented with 10% bovine serum, 100 units of penicillin, and 100 units of streptomycin per ml. The growth medium was poised by stripping the medium of all oxygen by sparging with 95% N_2 and 5% CO_2 until a minimum ORP (−322 ± 23 min) was constant for at least 1.5 hr and then raising the ORP to +75 mv by sparging with 95% air and 5% CO_2. Medium batches with significantly different poising characteristics were considered suspect, and new medium was prepared. The *p*H of 7.0 was maintained by manual addition of acid or alkali and an ORP of +75 mv by the automatic addition of either 95% N_2 and 5% CO_2 or 95% air and 5% CO_2. Temperature was kept constant at 37 C. (ii) For uncontrolled tissue cell growth, Erlenmeyer flasks of varying sizes (Fig. 3) were outfitted as described by Weirether et al. (9) for production of tissue cells. Growth medium was the same as that described above with the exception of the of the poising procedure which was eliminated. The

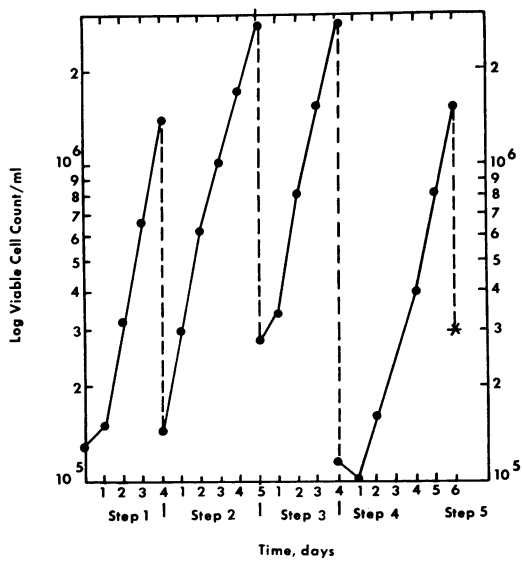

FIG. 4. *A typical stepwise buildup of L-DR tissue cells. The cell concentration and volume of medium used in each of the four steps to produce cells for the 50-liter virus infection vessel are given in Fig. 3. The asterisk indicates the cell concentration at the initiation of infection (step 5); for details see Fig. 3.*

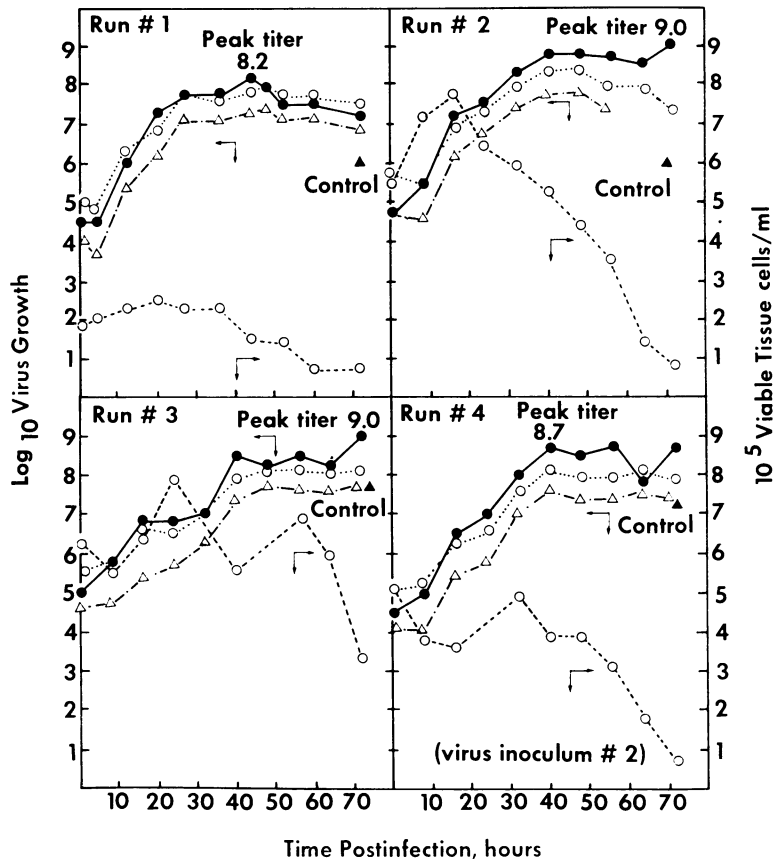

FIG. 5. *Rift Valley fever virus titer and viable cell count after infection of controlled growth of tissue cells in 50-liter New Brunswick fermentors (20-liter working volume). (●—), MICLD$_{50}$/ml; (△- -), PFU/ml; (○ ···), estimated MICLD$_{50}$/ml from PFU/ml; (○--), total viable tissue cells and (▲) control shake flask, 72-hr value.*

volume of culture medium was 40% of the flask's capacity. Flasks were shaken on the reciprocal shaker at 100 oscillations per min through 3-inch strokes. Incubation was at 37 C.

Virus inoculum. A viral suspension in the form of infectious lamb serum derived from the Van Wyk strain of pantropic RVF virus (V. R. Kaschula, D. V. S. Thesis, Univ. of Pretoria, Pretoria, South Africa) was used to inoculate the 50-liter fermentors. The seed used had undergone two lamb passages from the Van Wyk strain. It had a titer of $10^{10.0}$ to $10^{10.5}$ mouse intracerebral median lethal doses (MICLD$_{50}$) per ml.

Multiplicity of inoculum. The multiplicity of inoculum (MOI) is defined as the ratio of virus MICLD$_{50}$ to a single tissue cell and calculated by the following formula: volume of viral inoculum to be used = (A × B × C)/D, where A is the tissue cell count per ml, B is the volume of tissue cells, C is the MOI based on MICLD$_{50}$ desired, and D is the virus titer expressed as MICLD$_{50}$ per ml.

The fermentors in which virus was produced were inoculated with a MOI of 1.0 with a viable cell count of approximately 3 × 10^6 viable cells/ml.

Culture conditions for virus infectivity. Tissue cell growth medium used for the virus infectivity studies was identical to that used for growth of tissue cells. Medium was pumped directly into the infecting fermentors through a presterilized, stainless-steel, Seitz filter press (double-pass filtration, five primary plus four secondary pads). After poising and adjustment to 75 mv, the *p*H of medium was adjusted to 7.4 ± 0.1, and the system was calibrated to continuously monitor *p*H and ORP. Medium was cooled to 25 C, and fermentor-grown tissue cells were added to give a cell density of approximately 3 × 10^5 cells/ml. Tissue culture *p*H was again adjusted to 7.4 ± 0.1, and the temperature was increased to 37 C. To prevent loss of cell viability, temperature was increased gradually over a 4-to-6-hr period.

Virus inoculation and sampling. The calculated virus volume was routinely diluted 1:10 with growth medium and added manually to the fermentors.

The infectious process was allowed to proceed for 72 hr, and 10-ml samples were taken at 8-hr intervals. Each sample was titrated by the intracranial route in mice (MICLD$_{50}$) (3) and on tissue cell monolayers for plaque-forming units (PFU) (3)

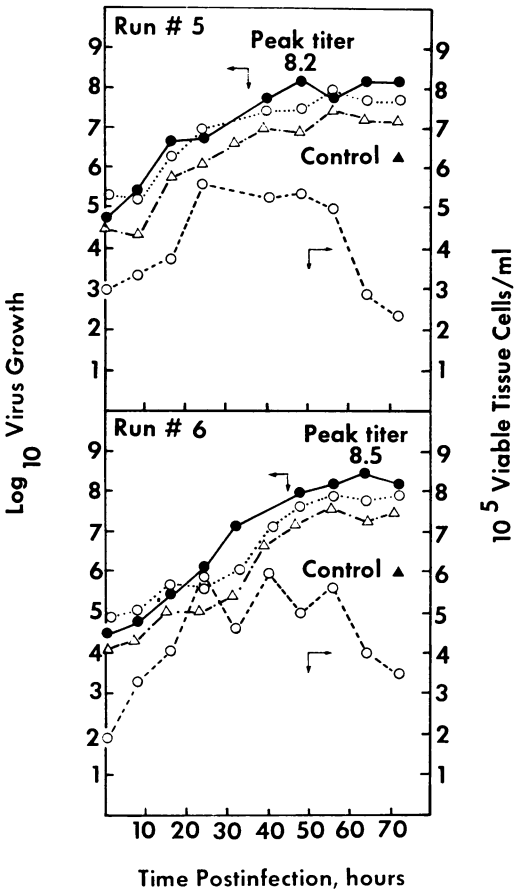

FIG. 6. *Rift Valley fever virus titer and viable cell count after infection of controlled growth of tissue cells in 50-liter New Brunswick fermentors (40-liter working volume).* (●—), *MICLD*$_{50}$*/ml;* (△-·-), *PFU/ml;* (○···), *estimated MICLD*$_{50}$*/ml from PFU/ml;* (○—), *total viable tissue cells; and* (▲), *control shake flask, 72-hr value.*

for a measure of virus growth. Both total and viable cell counts were determined on each sample. The Erythrosin B dye method was used to determine cell viability. For this purpose 0.5 ml of 0.5% water solution of Erythrosin B was mixed with 0.5 ml of the cell suspension and placed in a hemocytometer, and a direct cell count was made of both total and viable (nonstained) cells.

The estimated MICLD$_{50}$ using PFU was calculated by the method of Klein et al. (3).

RESULTS AND DISCUSSION

The actual tissue cell growth for each of the four steps beginning with the 1-ml frozen ampoule through the 50-liter fermentor is shown in Fig. 4. The tissue cell growth achieved during each step of the buildup was greater than a log increase in cell population. Slopes of the various growth curves were remarkably similar and highly predictable for scheduling the next step leading to the infection part of the process.

Under these two buildup systems, tissue cell growth peaks at a cell density of about 2.5×10^6 cells/ml; however, since cells grown beyond the logarithmic phase are unsuited for RVF virus infectivity, cells were transferred to the fermentor in which virus was produced when a growth of about 2.0×10^6 cells/ml was reached. Upon reaching the desired final cell density, the cells were harvested and diluted in the fermentor in which virus was produced as previously described.

The ability of the L-DR tissue cell to propagate RVF virus in suspension in 50-liter fermentors (20- and 40-liter working volumes) is demonstrated in Fig. 5 and 6. The cultures were initiated with concentrations of cells ranging from 2.00 to 6.20×10^5 viable cells/ml. It was of interest to observe the number of viable tissue cells as RVF virus infectivity progressed. For the first 24 to 30 hr postinfection, the viable cell count was maintained or slightly increased, then dropped precipitously to very low levels. This loss in viable cells probably was, at least in part, a result of cell rupture or lysing of the tissue cell induced by RVF virus infection. During this drop in cell viability, maximal RVF virus yields of $10^{8.0}$ to $10^{9.0}$ MICLD$_{50}$/ml were obtained. There appeared to be little difference in the optimal RVF virus yields in the 20- or 40-liter working volume cultures, or apparent time to death of the tissue cell. Titers, independent of tissue culture volume were shown to peak between the 48- and 72-hr postinfection period. The calculated relationship between MICLD$_{50}$ and PFU is shown in Fig. 5, 6, and 7 and indicates that either assay can be used to predict the amount of RVF virus produced. This would tend to confirm the utility of the procedure as stated in a previous publication (3). In all cases, the control culture flasks which contained virus-inoculated tissue culture from the fermentors titrated well below the fermentor culture at the 72-hr postinfection period, an observation of little meaning because virus growth could have peaked well before the 72-hr postinfection period.

It is readily apparent that viable cell count per milliliter is of little consequence for determining peak virus growth and subsequent harvest. Although, the measure of cell viability is limited in this respect, it does indicate virus infection and production are progressing.

The two growth systems used for buildup of tissue cell cultures had no real effect on the cell densities in the 50-liter growth fermentor. Cell densities of 2.0×10^6 to 2.5×10^6 viable cells/ml

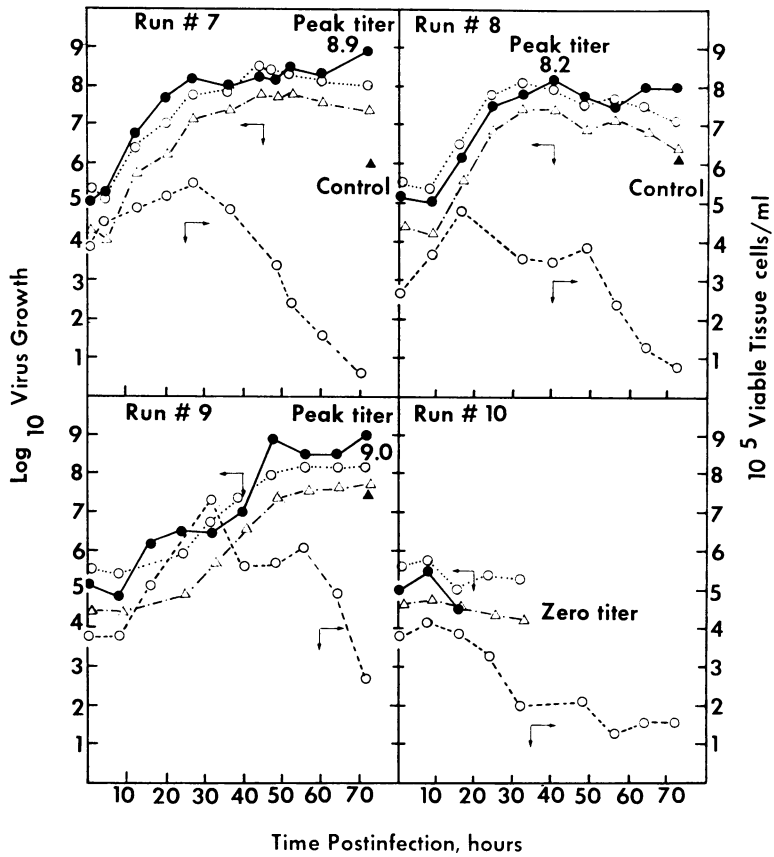

FIG. 7. *Rift Valley fever virus titer and viable cell count after infection of uncontrolled growth of tissue cells in 50-liter New Brunswick fermentors (20-liter working volume): (●—), $MICLD_{50}$/ml; (△-·-), PFU/ml; (○ ···), estimated $MICLD_{50}$/ml from PFU/ml; (○--), total viable tissue cells; and (▲), control shake flask, 72-hr value.*

were obtained after four to six days growth in the 50-liter fermentor independent of cell buildup systems. Likewise, the tissue cells from each of the two buildup systems were equally sensitive to RVF virus infection and subsequent growth (Fig. 5, 6, and 7).

Tissue cell virus culture became slightly acid (*p*H 6.9) after the 48-hr postinfection period. Once peak titer occurred, there was little loss in RVF virus infectivity or titer (Fig. 5, 6, and 7).

Run #4 (Fig. 5) used virus inoculum produced in run #2 (Fig. 5) after being stored for 7 days at 4 C. The virus titer was not affected in this single test. Another deviation from planned operations occurred in run #10 (Fig. 7), where tissue cells were in the stationary phase of growth and used as inoculum for step 5. In this case no increase in virus was observed, verifying laboratory observations that tissue cells in the logarithmic phase of growth were required for virus production.

The methods described earlier (2) to concentrate laboratory-produced virus from 50- to 100-fold worked equally well with virus produced in the 50-liter fermentors. Thus, it is possible to reduce 20 or 40 liters of virus to 400- to 1,000-ml volume by alum or methyl alcohol precipitation, thereby increasing virus titer by about two $\log_{10}$.

The problems of translating from the laboratory to pilot plant vessels at least to 50-liter size were minimal and led us to believe that the method used to produce RVF virus is readily adaptable for producing any type of virus that can be grown in an established cell line. Thus, the production of antigen (virus) for human or veterinary use and of certain carcinogenic viruses in any quantity appears quite feasible, once use of established lines for this purpose is accepted.

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