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Growth of the IB-RS-2 Pig Kidney Cell Line in Suspension Culture and Its Susceptibility to Foot-and-Mouth Disease Virus

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The adaptation of the pig kidney cell line IB-RS-2, clone 60, to growth in suspension culture is described. When fully adapted, an approximate threefold increase in viable cells was obtained within 72 hr from initial cell concentrations of 5×10^5 per ml in culture volumes up to 1,500 ml. The monolayer cells (99th passage level) used to initiate the suspension cultures and the fully adapted suspension cells were shown to have an aneuploid chromosome karyotype, whereas earlier monolayer cultures (32nd passage level) had a pseudodiploid karyotype. Replicate virus titrations in monolayers prepared from suspension-adapted cells, IB-RS-2 monolayer cells, BHK monolayer cells, and in suckling mice showed that the suspension cells had retained sensitivity to foot-and-mouth disease virus. The geometric mean peak infectivity of seven strains of foot-and-mouth disease virus grown in IB-RS-2 suspension cells was $10^{8.2}$ plaque-forming units per ml, with a mean complement-fixing activity of approximately 135 complement-fixing units per ml. These preliminary results indicate that submerged cultures of these cells on an industrial scale may be useful for commercial foot-and-mouth disease vaccine production.

Capstick et al. (4) reported the adaptation of a cloned strain of hamster kidney cells (BHK-21, clone 13; reference 8) to growth in suspension culture and the susceptibility of these cultures to foot-and-mouth disease virus (FMDV). These cells have since been grown in deep suspension culture on an industrial scale in volumes of as high as 2,000 liters and are being used for virus production in the preparation of inactivated FMDV vaccines.

Telling and Elsworth (13) described the design and operation of an industrial type culture system for the submerged growth of BHK-21 cells in volumes of 30 liters. They also described a schedule of low-temperature storage and periodic revival of cell seeds which enabled cells of approximately the same passage level to be produced in successive months. With this system, no great changes in either BHK cell growth characteristics or susceptibility to virus have been observed.

The susceptibility of the IB-RS-2 pig kidney cell line and of clones derived from this cell line was reported by De Castro (5) and De Castro and Pisani (7). Monolayer cultures of clone 60 at passage level 30 were received at this Institute in 1967 and have been used (1, 11; J. Wilson, *personal communication*) for the assay of FMDV as an alternative to BHK and pig kidney secondary monolayer cultures.

This report describes the adaptation of the IB-RS-2 clone 60 cell line to growth in suspension culture and the susceptibility of the cultures to FMDV.

MATERIALS AND METHODS

Adaptation of IB-RS-2 cells to suspension culture. Suspension cultures in volumes of 250 ml were initiated in the cylindrical Pyrex glass vessels and laboratory apparatus described by Smith and Burrows (12). The growth medium used consisted of eight parts of Eagle's basal medium modified to contain twice the normal concentration of amino acids and vitamins, one part of a 2% (w/v) solution of Tryptose phosphate broth, one part Seitz (EK)-filtered adult bovine serum, and Pluronic Polyol F68 to a final concentration of 0.1%.

Monolayer cultures of the IB-RS-2 clone 60 cell line, passage level 99, were treated with a 0.01% ethylenediaminetetracetic acid (EDTA)- 0.01% trypsin mixture, and a cell suspension containing 5×10^5 viable cells per ml was prepared in the growth medium. Several small cylindrical vessels, seeded with 250 ml of cell suspension, were maintained at a temperature of 37 C and stirred at a speed of 300 rev/min. The initial *p*H of the medium was adjusted to 7.2 with 5% CO_2 in air mixture. Cell counts were made every 24 hr, and the number of viable cells was

enumerated by trypan blue permeability. Batch cultures were harvested every 4 to 6 days, and after centrifugation the cells were resuspended in fresh growth medium at a viable cell concentration of 5×10^5 per ml.

Chromosome analysis. Cell preparations for chromosome analysis were made by the addition of colchicine (final concentration 10^{-5} M) to actively multiplying cultures for 6 to 7 hr. The cells were then treated as described by Moorhead et al. (9) and made into permanent preparations. Suitably spread metaphase cells were selected under low-power objectives and subjected to detailed karyotype study under phase-contrast oil immersion optics. The modal chromosome number was determined on a minimum of 100 metaphase cells.

Cell size. Mean cell diameters of monolayer and suspension cultures were determined with the use of a graticule and also by projection microscopy. Diameters were recorded on viable cells when maximum cell population levels had been reached and active cell division had ceased. Mean cell diameters and confidence limits were calculated on 300 cells.

Virus strains. The virus strains used originated from infected cattle epithelium and had limited tissue culture passage histories. The virus was passaged in IB-RS-2 monolayers except where stated, and the supernatant fluid was frozen as stock virus at −70 C. The viruses used were: type 0, strain BFS 1860, 3rd IB-RS-2 passage; type 0, strain Pacheco, 3rd IB-RS-2 passage; type 0, strain Lausanne, 12th IB-RS-2 passage; type 0, strain Lausanne, 20th BHK passage; type A, strain 119, 14th IB-RS-2 passage; type A, strain Iraq 24/64, 4th IB-RS-2 passage; type C, strain Noville, 3rd IB-RS-2 passage; type SAT 1, strain SA 13/61, 5th IB-RS-2 passage.

Sensitivity of suspension cells to FMDV. To determine whether viral sensitivity of the cells had altered after adaptation to suspension culture, comparative titrations by the plaque technique were made on monolayers produced from IB-RS-2 monolayer and IB-RS-2 suspension passaged cells, BHK monolayer-passaged cells, and in suckling mice.

Growth of FMDV in suspension cells. The growth of several strains of FMDV in IB-RS-2 suspension cells was carried out in the cylindrical glass vessels. Virus was added to cell concentrations of 2×10^6 to 2.5×10^6 per ml to give an estimated virus to cell ratio of 0.1 to 1.0 in a final culture volume of 300 ml. Cultures were stirred at 350 rev/min at 36 C, and the *p*H was manually controlled at 7.2 to 7.3 with a CO_2 in air mixture. Samples were removed at intervals after infection for estimation of infectivity, complement-fixing antigen, viable cells, and culture *p*H.

Viruses were assayed by the plaque technique on 2-day-old monolayers of IB-RS-2 cells in 6-cm glass petri dishes and by the intraperitoneal inoculation of 5- to 7-day-old mice.

Complement-fixing antigen titers were determined by using a modification of the method of Bradish, Jowett, and Kirkham (2), as described by Capstick et al. (3).

RESULTS

Adaptation to suspension culture. To maintain a cell concentration of 5×10^5 per ml, it was necessary during the first 18 days in suspension (i.e., three batch cultures) to reduce the number of culture vessels.

Cell growth during the next 120 days was irregular and associated with considerable cell clumping and the appearance of large cytoplasmic vacuoles. To counteract cell clumping and to obtain a more accurate assessment of cell numbers at the end of a batch culture, the harvested cells were treated with the EDTA-trypsin mixture for 30 min before resuspension in fresh growth medium.

After 120 days in suspension culture, a progressive and marked decrease in cell clumping and the number of cytoplasmic vacuoles was observed. This continued until about the 180th day, when cell suspensions were virtually monodispersed. Since that time, consistent cell growth in successive batch cultures has been maintained and a regular average generation time of approximately 48 hr was established, indicating complete adaptation to suspension culture. Cell viability both at the start of a batch culture and at peak cell concentrations was between 96 and 98%.

The results of a typical growth cycle in 250-ml volumes are shown in Fig. 1, which illustrates that, from an initial cell seed of 5×10^5 cells

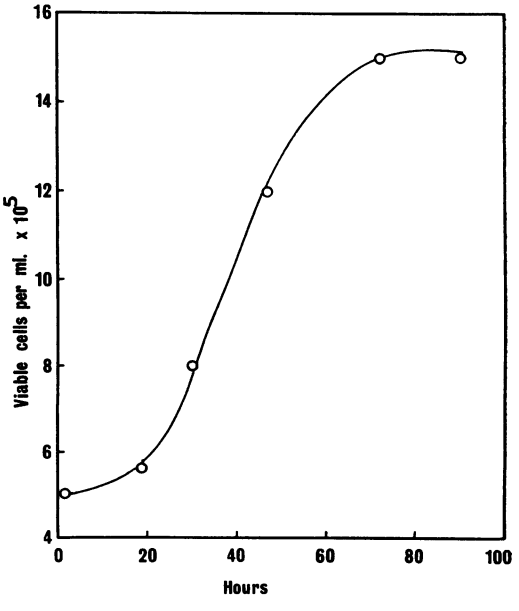

FIG. 1. *Typical growth curve of IB-RS-2 clone 60 suspension cells in 250-ml volumes.*

TABLE 1. *Chromosome numbers and cell diameters of IB-RS-2 clone 60 cells*

Passage history	Percentage cells with chromosome no.			Cell diameter (μm)	
	36	37	38	Mean	Range
32nd Monolayer	0	17	83	12.9 ± 1.8[a]	8–18
99th Monolayer	4	80	16	14.3 ± 2.0	9–22
99th Monolayer + 200 days in suspension	5	90	5	18.0 ± 1.1	9–27

[a] Standard error of mean.

TABLE 2. *Titration of foot-and-mouth disease virus in monolayer cultures produced from IB-RS-2 suspension and monolayer-passaged cells, BHK-21 monolayer-passaged cells, and in suckling mice*[a]

Virus strain	IB-RS-2 monolayer cells	IB-RS-2 suspension cells	BHK monolayer cells	Suckling mice
"C" Noville	6.25[b]	6.0[b]	5.8[b]	6.3[c]
"A" Iraq 24/64	7.0	6.8	6.8	7.2
SAT 1 13/61	7.0	6.7	6.2	6.2
"O" BFS 1860	6.5	6.7	6.3	7.2
"O" Lausanne (BHK-20)	7.7	7.5	7.4	5.8

[a] Results represent mean of three separate titrations.
[b] Log_{10} plaque-forming units per milliliter.
[c] Log_{10} median infective dose per milliliter.

per ml, a final concentration of 1.5×10^6 cells per ml was achieved in 72 hr.

Since adaptation to growth in suspension, successive batch cultures in volumes of 1.0 to 1.5 liters have been grown in bottles of about 4.5-liters capacity under conditions similar to those described by Telling and Elsworth (13) for the production of BHK suspension cell seed. The growth rate and cell concentrations obtained by using this culture system were similar to those obtained in 250-ml volumes in the small glass vessels.

Cells propagated under these cultural conditions have been transferred to 800-ml culture vessels fitted with automatic *p*H and temperature recording controlling equipment (3). From initial cell concentrations of 5×10^5 cells per ml, concentrations of 1.8×10^6 per ml were obtained in 48 hr with *p*H controlled at 7.4, temperature at 36.5 C, and a stirrer speed of 350 rev/min.

Chromosome analysis. Table 1 gives the frequency distribution of the numbers of chromosomes in metaphase cells prepared from IB-RS-2 clone 60 monolayer passage levels 32 and 99 and from fully adapted suspension cells. Eighty-three per cent of cells at the 32nd passage level possessed a pseudodiploid karyotype (2n = 38) which is in agreement with the findings of De Castro and Koseki (6). Cultures of the 99th monolayer passage level used to initiate the suspension cultures and cultures of the fully adapted suspension cells possessed an aneuploid

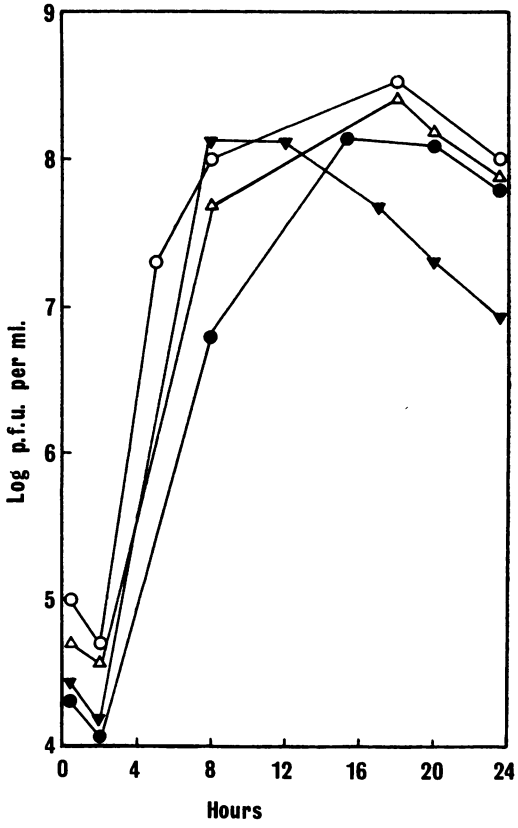

FIG. 2. *Growth of four strains of foot-and-mouth disease virus in IB-RS-2 suspension cultures. Symbols:* ○, *SAT1 13/61;* △, *A/Iraq 24/64;* ●, *"O" Lausanne 12th IB-RS-2 passage;* ▼, *"O" Pacheco.*

TABLE 3. *Peak infectivity and complement-fixing antigen titers obtained with FMDV strains grown in IB-RS-2 suspension cultures*

Virus strain	Infective antigen		Complement-fixing antigen
	IB-RS-2 monolayer	Suckling mice	
"O" Lausanne (IB-RS-2/12)	8.1[a]	8.8[b]	120[c]
"O" Pacheco	8.1	8.2	45
"O" BFS 1860	7.7	7.0	200
"A" 119	8.5	8.0	ND
"A" Iraq 24/64	8.6	8.6	163
"C" Noville	8.2	8.6	130
SAT 1 13/61	8.7	ND[d]	156

[a] Log_{10} plaque-forming units per milliliter.
[b] Log $_{10}$ median infective dose per milliliter.
[c] Complement-fixing units per milliliter.
[d] Not determined.

karyotype with a modal chromosome number of 37. This alteration in chromosome karyotype was due to the loss of a small acrocentric chromosome from group VIII [classification of Ruddle (10)].

Cell diameter. The mean cell diameters and diameter ranges are given in Table 1. The mean cell diameter of the fully adapted suspension cells is significantly greater ($P = 0.05$) than that of IB-RS-2 monolayer cells.

Sensitivity to FMDV. Five FMDV strains were assayed by the plaque technique and by inoculation in unweaned mice. Table 2 lists the results obtained, which show that the IB-RS-2 suspension-adapted cells have retained a high degree of sensitivity to the FMDV strains used.

Plaques formed on monolayers produced from suspension-passaged cells were similar in size to those formed by monolayer passaged cells but less clearly defined.

Growth of FMDV in suspension cells. Figure 2 shows typical virus growth curves obtained with four strains of FMDV, and Table 3 lists the peak extracellular infectivity titers and complement-fixing units obtained with seven strains of virus. Peak extracellular infective virus titers were reached between 8 and 24 hr after infection of the cells, and the peak complement-fixing activity occurred at least 24 hr later.

DISCUSSION

BHK suspension cells grown in large industrial-type cultures vessels are now widely used for the production of FMDV inactivated vaccines. It is also likely that other virus vaccines will be produced in a similar manner when suitable cell systems become available. The results of these studies indicate that the IB-RS-2 suspension cells could also be used for FMDV vaccine production.

The maximum cell concentrations of IB-RS-2 suspension cells, obtained in the 250-ml and 1- to 1.5-liter culture systems, were only slightly below those of BHK suspension cells grown under similar cultural conditions, although the time required to reach maximum yield was approximately 24 hr slower than BHK cells. However, it is extremely unlikely that optimal environmental conditions were being achieved in these cultures.

Apart from the initial adjustment to *p*H 7.2 at the start of a batch culture, no further *p*H control was made during the culture period, by the end of which the *p*H had usually fallen to 6.5 to 6.6. Telling and Stone (14) found that maximal cell concentrations were reached with BHK suspension cells under conditions of close automatic *p*H control and that more efficient utilization of the culture medium in terms of cell production occurred at specific *p*H values.

When IB-RS-2 suspension cells were grown in the 800-ml vessels with the *p*H controlled at 7.4, the time of maximum cell concentration was reduced to 48 hr and the cell yield increased to 1.8×10^6 cells per ml. This indicates that even higher cell yields may be obtained when optimal conditions of *p*H, stirrer speed, growth medium, and control of pO_2 have been achieved.

Preliminary experiments carried out by the Vaccine Research Department of this Institute indicate that successful growth of these cells can be achieved in a 5-liter vessel of the type described by Telling and Stone (14). It would seem reasonable to assume therefore that the IB-RS-2 suspension cells could be grown in an industrial-type culture apparatus.

The sensitivity of the IB-RS-2 suspension cells to the FMDV strains used was slightly higher than that observed in BHK cells (Table 2), and, although no direct comparisons were made, yields of infective virus and of complement-fixing antigen were at least as high as those normally obtained in the BHK cell suspension system. The possible value of the IB-RS-2 suspension cells for the production of FMDV antigen for vaccine preparation must await the results of animal experiments to relate the parameters of virus growth to that of immunizing ability. Preliminary vaccination experiments in guinea pigs have indicated that a satisfactory inactivated virus vaccine can be prepared from virus grown in IB-RS-2 suspension cells.

Long-term continuous growth of cells in suspension might lead to the emergence of a "changed" cell population or to a loss of de-

sirable characteristics. However, no alteration in cell morphology, growth characteristics, or viral susceptibility has been observed in the IB-RS-2 suspension cells over a period of 200 days since adaptation to suspension culture. At regular intervals during this period, cells have been stored at −70 C and at −196 C to investigate the possible effects of long-term storage at low temperature. Cells have been revived and restored to suspension culture after 4 months of storage at −70 C and have been found to be fully sensitive to FMDV.

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