Epstein-Barr Virus from P3HR-1 Cells Grown in Chemically Defined Medium

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A chemically defined medium, designated FCRC-1, supports the growth of P3HR-1 cells and concomitant replication of infectious Epstein-Barr virus.

Human lymphoblastoid cells are routinely propagated in a commercially available medium such as RPMI-1640 supplemented with 10 to 20% fetal calf serum (FCS). Since defining the nutritional requirements for virus replication can be accomplished best with a chemically characterized medium, these studies were initiated. Experiments with P3HR-1 cultures that produce Epstein-Barr virus (EBV) had indicated that modifying an existing chemically defined medium resulted in reduced cell growth but infectious virus yields equivalent to those generally obtained in a serum-containing medium. Initial medium modifications consisted of adding glutamine and increasing the inositol concentration. Glutamine was required even though the original medium supported the growth of a variety of mammalian cells in its absence. Whereas 900 mg/liter was optimal, omission of glutamine resulted in P3HR-1 cell death. The inositol concentration was raised to 50 mg/liter since it is normally included at high concentrations in other media such as the Roswell Park Memorial Institute (RPMI) series, McCoy 5a (modified), and Ham F-12. Attempts to demonstrate a definite requirement for increased concentrations of inositol gave equivocal results, but the higher amount was maintained until further studies could be made.

The medium, designated FCRC-1, was prepared essentially as previously described, with glutamine added to the autoclave-sterilized medium as a presterilized dry powder (procedure described in [5]) or as a filter-sterilized (0.22-μm membrane) liquid. The components of the complete medium are given in Table 1. The P3HR-1 cells were propagated in RPMI-1640 or FCRC-1 medium containing 10% FCS, washed once in FCRC-1 medium (serum free), inoculated into 25 ml of serum-free medium in 100-ml rubber-stoppered serum bottles, and incubated statically at 34 to 35 C with once daily hand agitation.

Duplicate cultures were removed daily, viable cell count and cell viability were determined, and the cells were assayed for the percentage of cells infected. Culture fluids were assayed for presence of infectious virus. The percentage of cells infected was determined by indirect immunofluorescence (4) with EBV-positive and EBV-negative human sera and goat anti-human immunoglobulin labeled with fluorescein isothiocyanate (Hyland Laboratories, Costa Mesa, Calif.). The EBV-positive serum was high in virus capsid antigen and low in early antigen. Acetone-fixed cell smears were prepared, and subsequent observation for virus capsid antigen was made using a Leitz microscope with an ultraviolet light source. The cell viability and viable cell count were determined by the trypan-blue dye exclusion technique and counting cells in a hemocytometer. The presence of infectious virus was determined by superinfecting 2.5 × 10^4 Raji cells (grown in RPMI-1640 + 10% FCS) with serial 10-fold dilutions of 0.2 ml of the virus samples (concentrated 100 ×) prepared from clarified supernatants of the cultures. Culture fluids were clarified by centrifugation of the cell cultures in the growth bottles at 500 × g for 10 min. Cells pelleted in the growth bottles were removed and suspended in 5 ml of phosphate-buffered saline, and 20-μl quantities were spotted for virus capsid antigen determinations. The resultant supernatant was removed and subsequently centrifuged in plastic tubes at 1,100 × g for further clarification. Virus was pelleted from the clarified fluid by centrifugation at 35,000 × g for 90 min.

The EBV-infected Raji cells were incubated at 34 to 35 C in T30 Falcon flasks for 4 days, centrifuged at 1,000 × g for 10 min, washed once in phosphate-buffered saline, resuspended in phosphate-buffered saline, spotted on Teflon-coated slides, air-dried, and acetone-fixed, and immunofluorescence procedures were applied. The infectivity titer was recorded as the reciprocal of the highest virus dilution giving >0.5% of
the cells positive immunofluorescence when examined under ultraviolet illumination for early antigen.

Figure 1 illustrates the data averaged from two experiments on EBV production from P3HR-1 cells grown in FCRC-1. A doubling in cell number occurred from 0 to 7 days (nearly two doublings can be measured if the period from 1 to 7 days is considered). We observed, however, that after inoculation into chemically defined media some of the washed cells adhered to the glass surface on initial settling and were not easily removed by agitating the cultures during the first 3 days of incubation. This initial attachment could account for the apparent drop in cell number during the first 3 days of incubation. Cell viability decreased throughout the 10-day period of observation, but the percentage of cells infected remained constant, approximating 10%. The titer of EBV reached a high of 10^7/0.2 ml on day 10, increasing from a low of <10^4-9/0.2 ml on day 1. Under similar growth conditions, but with RPMI-1640 medium containing 10% FCS, the maximal population would be about double the FCRC-1 count on day 7, there would be no cell adherence to the growth flask, and cell infection and infectious virus data would be comparable.

To our knowledge, FCRC-1 is the only chemically defined, serum-free medium tested to date that has allowed growth of P3HR-1 or any other lymphoblastoid cells. Although lymphoblastoid cells cannot yet be propagated continuously in a chemically defined medium, the FCRC-1 medium does permit several growth cycles and has some advantages over a serum-containing medium. The specific optimal medium constitu-

| Component          | Concn (mg/liter) | Component          | Concn (mg/liter) |
|--------------------|------------------|--------------------|------------------|
| Amino acids        |                  |                    |                  |
| L-Alanine          | 400              | NaCl               | 7,400            |
| L-Arginine-hydrochloride | 100          | KCl                | 400              |
| L-Asparagine       | 300              | CaCl_2 · 2H_2O     | 265              |
| L-Cysteine-hydrochloride | 75            | Ferric ammonium citrate | 3             |
| L-Glutamic acid    | 150              | MgCl_2 · 6H_2O     | 275              |
| L-Glutamine*       | 900              | NaH_2PO_4 · H_2O   | 300              |
| L-Histidine-hydrochloride | 60           | ZnSO_4 · 7H_2O     | 0.3              |
| L-Isoleucine       | 150              | Phenol red         | 10               |
| L-Leucine          | 300              | Methylcellulose, 15 centipoise | 500        |
| L-Lysine-hydrochloride | 300         | NaHCO_3*           | 2,500            |
| L-Methionine       | 60               | Vitamins*          |                  |
| L-Phenylalanine    | 120              | d-Biotin           | 1                |
| L-Proline          | 300              | Choline-chloride   | 50               |
| L-Serine           | 300              | Folic acid         | 1                |
| L-Threonine        | 135              | Nicotinamide       | 1                |
| L-Tryptophan       | 60               | Calcium pantothenate | 2             |
| L-Tyrosine         | 120              | Pyridoxal-hydrochloride | 1            |
| L-Valine           | 150              | Thiamine-hydrochloride | 1             |
| Carbon sources     |                  | i-Inositol         | 50               |
| Glucose            | 3,000            | Riboflavin         | 0.1              |
| Sodium pyruvate    | 110              | B_12               | 0.002            |

* Added aseptically as sterile dry powder or filter-sterilized solution.
* Added aseptically as a 5% solution.
* Added as a concentrated (100 ×) solution during medium preparation.

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FIG. 1. EBV from P3HR-1 cells grown in FCRC-1. VCC, Viable cell count; VI, virus infectivity; CI, cell infectivity; CV, cell viability.
ents required for cell growth and virus replication are more easily identified in the absence of serum, and such studies are in progress. A similar investigation by Hearn et al. (3) demonstrated an increase in titer of Venezuelan equine encephalomyelitis virus produced by L cells in a chemically defined medium by increasing the concentration of choline and by adding proline and serine. It would be reasonable to expect that virus obtained from growth in a defined medium would be relatively free of contaminant antigens of FCS. Our cultures require no washing prior to immunofluorescence studies. One additional advantage of FCRC-1 is the reduced cost of the serum-free medium, since FCS probably accounts for >75% of the total expense of any medium in which it is used.

The significance of the adherence of some of the cells to the bottle surface when first placed in chemically defined medium is not known, but it would be most interesting if population selection results from this simple procedure. Preliminary experiments have been inconclusive.

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