Virus Susceptibility of a New Simian Cell Line of Fetal Origin

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The cultivation and characterization of a cell line derived from the foreskin of a fetal, rhesus monkey (rhfs2) are described. This cell line has proven satisfactory for isolation and assay of a variety of viral agents of human and simian origin. Virus titrations performed on foreskin cells yielded titers comparable to, or higher than, those obtained in rhesus monkey kidney cells (LLC-MK2). Replicate isolation attempts in our laboratory from simian clinical specimens have proven rhfs2 superior to LLC-MK2 for ease of detection and frequency of isolation.

The use of simian-derived cell cultures as a basic research tool in virus studies has long been recognized (5), but the inherent problem of endogenous viral contamination in tissues of simian origin is also well documented (1, 5, 10). Predisposing factors, such as source of animals, method of shipment, as well as quarantine and conditioning procedures, influence the degree of contamination by adventitious agents in subsequent cultures. Because restricted contact of animals with each other and with man seems to minimize the incidence of endogenous virus isolates (3), fetal tissue from laboratory-bred animals may be an optimal source of noncontaminated culture material. In this paper, we describe the establishment of three cell lines (rhfs1, rhfs2, and rhfs3) from individual fetal, rhesus monkeys which are a product of our controlled breeding program (11). These cell lines have been effectively employed in a number of seroepidemiological and virological studies (2; Fine et al., Nature [London], in press) in our laboratory. Although the LLC-MK2 line is not as sensitive as primary or secondary monkey kidney cells for isolation of viruses, they were used in comparison with the rhfs2 line in the studies described here, since they are routinely used in our laboratories for reasons of economy and convenience.

MATERIALS AND METHODS

Source of cell cultures. The foreskin tissue utilized in all cultures was derived from fetal, rhesus monkeys at 150 to 160 gestation days, delivered by cesarean section. The animals were bred and maintained in the closed primate breeding colony at Litton Bionetics, Inc., where monkeys housed under restricted containment have undergone extensive conditioning in efforts to establish a disease-free state. Details of colony management employed are described by Valerio et al. (11). LLC-MK2 cell cultures (4) were obtained commercially from Flow Laboratories, Inc., Rockville, Md.

Preparation of cell cultures. The tissue was processed by using a modification of the basic explan technique of Maitland, described by Paul (7). The tissue was washed with phosphate-buffered saline containing antibiotics, minced in a glass petri dish into pieces of approximately 1 mm³ in size, and transferred to a 30-ml flask (Falcon) by means of a capillary pipette. The pieces of tissue were allowed to adhere to the wall of the flask for 30 min at 37°C and gently overlaid with Eagle minimal essential medium (EMEM) containing 20% fetal calf serum (FCS) and antibiotics (penicillin, 100 U/ml; streptomycin, 100 µg/ml; fungizone, 2.5 µg/ml; and kanamycin, 200 µg/ml). Spent medium was replaced two to three times a week until 50% cell confluence was reached (usually within 4 to 6 weeks). The cultures were then passaged every 3 to 5 days using trypsin (0.25% MBA) to disperse the cells. After the fifth passage, the serum level was reduced to 10%. RPMI-1640 and medium 199 supplemented with 5 or 10% FCS were also found to be satisfactory growth and maintenance media. Three separate lines were established from foreskin tissue. These lines, screened at various passage levels, were found to be free of mycoplasma and endogenous virus. The methods employed are previously described (8). Attempts to stimulate latent viruses by using iododeoxyuridine and bromodeoxyuridine at concentrations of 40 µg/ml and 20 µg/ml, respectively, were unsuccessful. Cells stored in liquid nitrogen by using a standard freezing solution (EMEM with 7.5% dimethyl sulfoxide plus 15% FCS) were readily recovered 1 year later.

Viruses. Virus strains employed are listed in
Table 1.

**Virus titrations.** LLC-MK2 and rhfs2 cultures were seeded at $5 \times 10^4$ cells/ml in EMEM plus 10% FCS and antibiotics into 150 by 13-mm screw-cap culture tubes (1 ml/tube). Tenfold dilutions of virus were prepared in EMEM with FCS and antibiotics. Cell cultures were inoculated with 0.1 ml/tube, four tubes per virus dilution, and observed daily for 7 days for appearance of cytopathic effects (CPE). The titers of the viruses were computed according to the method of Reed and Muench (9).

**Virus isolation.** Washings from oral and anal swabs were inoculated onto LLC-MK2 and rhfs2 by the method previously described (6). Cultures were observed for CPE and passaged weekly or until CPE was demonstrated.

### Table 1. Virus spectrum of rhfs2 cell line

| Virus                                      | Onset of CPE (days postinoculation) |
|--------------------------------------------|-------------------------------------|
|                                            | rhfs2  | LLC-MK2 |
| Poliovirus, type I (Brunhilde)             | 1      | 1       |
| Echo 11                                    | 1      | 1       |
| Reo III (Abney)                            | 3      | 2       |
| Mumps (Enders)                             | 1      | 1       |
| Measles (Edmunston)                        | 7      | 7       |
| Rubella (M-33)                             | 3      | 3       |
| Foamy I (FV-21)                            | 7      | 7       |
| Foamy II (FV-34)                           | 3      | ND*     |
| Newcastle disease virus (Roakin)           | 2      | 2       |
| SV-5 (29682)                               | 3      | 3       |
| Vesicular stomatitis virus                 | 1      | 1       |
| Chikungunya (Bangani)                      | 1      | 1       |
| Mason-Pfizer monkey virus                  | 1      | —       |
| Feline syncytia-forming virus (F017)       | 6      | ND      |
| Herpesvirus hominis, type 1 (Mayo)         | —      | 2       |
| H. hominis, type 2 (KOS)                   | —      | ND      |
| H. tamarinus (MV-5-4-PSL)                  | 3      | 3       |
| H. saimiri                                 | —      | —       |
| H. simiae                                  | 2      | 2       |
| SA-8                                       | 4      | 6       |
| SV-11                                      | 6      | 6       |
| SV-15                                      | 3      | 4       |
| SV-17 (6630-1C)                            | 2      | —       |
| SV-31                                      | 3      | 3       |
| SV-33                                      | 6      | 6       |
| SV-37                                      | 3      | 3       |
| SV-40 (J436)                               | 4      | —       |
| Vaccinia                                   | 1      | 2       |
| Polyoma (11189)                            | —      | —       |

* ND, not determined.

**RESULTS**

**Virus susceptibility.** Replicate monolayer cultures of rhfs2 (Fig. 1) and LLC-MK2 (Fig. 2) cells were inoculated with each of the viruses listed in Table 1. These viruses were selected as
representing most of the major virus groups. The results demonstrated that the rhfs2 and LLC-MK2 cell lines were comparable in their susceptibility to the viruses employed. However, these and previous studies clearly demonstrated that the rhfs lines are highly efficient for detecting viruses from simians. We have had success in demonstrating CPE with the Mason-Pfizer monkey virus (M-PMV), SA-8 (3). Herpesvirus simiae, and H. tamarinus. Representative lots of M-PMV titering \( \geq 10^4 \) mean tissue culture infective dose units/ml consistently induce multinucleation of rhfs2 1 day postinoculation (pi). Herpesvirus isolates from several simian species produced extensive cellular degeneration of rhfs2 within 4 days pi. The onset of cytopathology was easily recognizable in the rhfs2.

Virus titrations. To show that the rhfs2 could serve as an assay tool for quantitative determinations such as infectivity, duplicate virus titrations were performed in rhfs2 and LLC-MK2. A common virus dilution scheme was employed to insure standardization of inocula. The results of this determination (Table 2) showed that equivalent titers were obtained in both rhfs2 and LLC-MK2 for poliovirus type I, simian virus 11 (SV-11), and vaccinia virus. Vesicular stomatitis virus, SV-33, SV-15, and mumps virus titered approximately 1 log higher in rhfs2. In addition, both SA-8 and SV-5 replicated in the rhfs2 but not in LLC-MK2.

Virus isolation. The rhfs2 cell line was employed during the course of a respiratory disease outbreak in one section of our simian colony in which the suspected etiologic agent was viral. Oral throat swabs were collected from 16 animals showing clinical signs; from these, 14 isolations were made. Several of these isolates were subsequently identified as adenovirus types SV-11 and SV-33. Since prior to this investigation LLC-MK2 was routinely employed in isolation attempts, these findings warranted a parallel study using both cell lines. Oral and anal swabs from 10 randomly chosen clinically asymptomatic animals were assayed in duplicate cultures of rhfs2 and LLC-MK2. Positive virus isolations were made on 4 (3 oral and 1 anal) of 10 animals by using LLC-MK2 and 5 (3 oral and 2 anal) of 10 animals by using the rhfs2 line. CPE associated with the oral isolates were characterized by areas of multinucleated and giant cells, whereas the CPE of the anal isolates were characterized by patches of rounded, refractile cells. In each case, an identical CPE was produced by serial transfer of fluids from the infected cultures to homologous noninfected cultures. No attempt was made to characterize the isolates further. Although these results show only a slightly higher isolation efficiency for the rhfs2, the ease of observation and sharp delineation between positive and negative cultures make it the more desirable cell line for isolation studies.

**DISCUSSION**

These results have demonstrated the rhfs2 line to be as susceptible as the long-established LLC-MK2 to some of the more common and easily propagated viruses, and superior as an isolation and assay tool for the more fastidious agents. In our laboratory, this cell line has proven invaluable as an indicator system for the M-PMV, a virus which previously had not demonstrated CPE in vitro (2).

The rhfs2 has maintained its original fibroblastic morphology through the 80th passage level. This feature has enabled us easily to maintain a long-term culture system for isolation studies. Furthermore, end-point determinations were facilitated because of the sharp contrast between infected and noninfected cultures.

These studies suggest that fetal tissue from a well-defined source may provide a realistic answer for eliminating endogenous viruses in primary and continuous cell cultures.

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