# Growth Characteristics and Viral Susceptibility of a Chimpanzee (*Pan troglodytes*) Lung Diploid Cell Line, SFRE:CL-1[1] (36365)

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Disadvantages encountered in the use of primary tissue cultures for diagnostic virology and vaccine production have been extensively discussed and generally acknowledged (1–4). Ubiquitous contamination by viruses and mycoplasma have caused concern regarding not only the maintenance and interpretation of assay procedures in these systems, but also the possibilities of neoplastic response to vaccines prepared therein (5–7). The unfortunate fact remains that primary cultures, particularly those derived from the kidney, are generally most susceptible to viral infection and the resulting expression of overt cytopathology. Established or continuous cell lines have virtually eliminated the problem of adventitious agents, but display a marked decline in viral susceptibility. Moreover, the presence of heteroploid chromosomal patterns impose a definite limitation to the value of these "transformed" lines as vaccine host systems (8–10). Diploid cells have, thereby, emerged as the generally accepted alternative to primary and heteroploid cultures.

With the continuing increase in number and diversity of viral isolates from both human and nonhuman primates, the need for greater selectivity among host systems has become apparent. This report describes the growth characteristics and sensitivity to varied human and simian viruses of a fetal chimpanzee lung cell line, SFRE:CL-1.

*Materials and Methods. SFRE:CL-1 cell cultivation.* Bilateral lung sections of a 9-week-old chimpanzee abortus were excised aseptically and prepared for tissue culture by routine trypsinization methods (11). The original cell yield was seeded into three 32 oz bottles. Upon achieving confluency, secondary and subsequent passages were subcultured by trypsinization at 1:2 split ratios twice per week.

Growth media consisted of Eagle's minimum essential medium in Hanks saline (HMEM), and 10% fetal bovine serum. Maintenance medium was Earle's buffered MEM (EMEM), with 2% fetal bovine serum. All media contained 100 units of penicillin, 1 unit of bacitracin, 100 $\mu$g of streptomycin and 100 $\mu$g of neomycin/ml.

*Storage of SFRE:CL-1 cells.* Successful preservation of SFRE:CL-1 cells at various passage levels was accomplished by the addition of dimethylsulfoxide to EMEM at a final concentration of 10% with increased concentration of fetal bovine serum to 20%. After trypsinization, cell pellets were washed once in EMEM and resuspended at a concentration of 2–5 $\times$ $10^6$ cells per ml in the above freezing medium. Sealed glass ampules were frozen gradually in insulated containers to $-90°$, whereupon they were removed to final storage in liquid nitrogen.

*Cell growth studies.* Suspensions of trypsinized SFRE:CL-1 cells were counted in quadruplicate on a hemacytometer and adjusted to $10^5$ cells/ml in HMEM, based on an average count. The erythrocin B dye exclusion technique was found not to significantly enhance the accuracy of replicate cell counts, and routine procedures were carried out using unstained material. Standard 16 $\times$ 125-mm tissue culture tubes were seeded with $10^5$ cells/ml each and placed at 37°. Triplicate or quadruplicate counts were performed daily. Growth medium was replaced with maintenance medium on the third day.

[1] W. H. O. Collaborating Laboratory on Comparative Medicine: Simian Viruses.

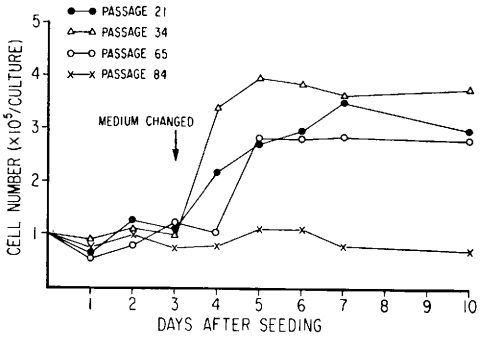


FIG. 1. Growth rate of four passage levels of SFRE:CL-1 cells after inoculation of $10^5$ cells/culture tube.

Studies to determine the time required for various cell concentrations to achieve confluency were performed in 2 oz bottles of 3 × 6.5-cm surface area.

*Virus susceptibility studies.* Viruses used in these studies were derived from the NIH–WHO Simian Virus Reference Center (12) located at this facility.

Undiluted virus stocks were inoculated 0.1 ml/tissue culture tube of SFRE:CL-1 cells, and were observed for cytopathology (CPE). Tubes were placed at −20° if 3–4+ CPE was observable prior to 14 days postinoculation, whereupon, all remaining tubes were frozen. After three freeze–thaw cycles at maximum CPE virus tissue culture fluids were repassaged twice in SFRE:CL-1 cells. Culture fluids from the third passage were simultaneously titrated in both SFRE:CL-1 and another cell of known susceptibility, usually that in which the original inoculum was grown. Virus titers are expressed as $\log_{10}$ $TCID_{50}$/0.. ml, according to the method of Reed and Muench (13).

*Karyology.* Chromosomal studies were performed through the courtesy of KAM Laboratories, Inc., Grandview, MO. A standard technique of treatment in hypotonic solution, fixation, flame spreading, and Giemsa staining was employed (14). The material examined was at passage 16, and had been recovered from frozen stock at passage 12.

*Results. Growth characteristics of SFRE: CL-1 cells.* SFRE:CL-1 line of fetal chimpanzee lung fibroblasts was initiated in this laboratory in May, 1969. The original

TABLE I. Susceptibility of SFRE:CL-1 Cells to Simian Viruses.

| Virus group and type | CPE | Titer after three SFRE:CL-1 passages: In SFRE:CL-1 | Titer after three SFRE:CL-1 passages: In other |
|---|---|---|---|
| Picornaviruses | | | |
| SV2 | — | 0 | 0 |
| SV16 | + | NT | NT |
| SV49 | + | 5.5 | 5.5 RMK[a] |
| 75s12[b] | + | 5.0 | 5.5 RMK |
| 111s12[b] | — | 0 | 0 BK |
| SA4 | + | 3.0 | 4.0 RMK |
| SV28 | + | 2.5 | 3.7 RMK |
| Reovirus | | | |
| SV12 | + | 6.0 | 5.0 RMK |
| Adenoviruses | | | |
| SA7 | — | 0 | 0 BK |
| SV1 | ± | 2.0 | 2.0 RMK |
| SV11 | — | 0 | 0 RMK |
| SV15 | + | 2.0 | 4.0 RMK |
| V340 | ± | 2.0 | 3.3 BK |
| CV23 | + | 5.0 | 5.5 WI38 |
| CV32 | + | 3.0 | 2.7 WI38 |
| CV33 | + | 4.7 | 5.5 WI38 |
| SqM1 | — | 0 | 5.5 LLC-MK4 |
| Herpesvirus A type | | | |
| SA8 | + | 4.0 | 3.7 BK |
| *H. tamarinus* | + | 2.5 | 3.3 BK |
| *H. papio* | + | 4.3 | 4.7 BK |
| Herpesvirus B type | | | |
| *H. saimiri* | — | 0 | NT |
| SA6 | + | 4.5 | 5.0 WI38 |
| Poxviruses | | | |
| Yaba-like | + | NT | NT |
| Monkeypox (Copenhagen) | + | 6.5 | 6.0 BK |
| Monkeypox (WRAIR) | + | 6.5 | 6.5 BK |
| Yaba | — | NT | NT |
| Myxoviruses | | | |
| SV5 | ± | 5.0[c] | 5.5[c] Vero |
| SV41 | ± | 5.0[c] | 6.3[c] Vero |
| Foamy 5 | — | NT | NT |
| Foamy 6 | — | NT | NT |
| SA10 | + | 4.0 | 3.0 BK |
| Papovavirus | | | |
| SV40 | — | NT | NT |

[a] RMK = rhesus monkey kidney, BK = baboon kidney.

[b] Unclassified chimpanzee enterovirus.

[c] Hemadsorption titer with guinea pig erythrocytes.

outgrowth remained in continuous culture for 14 months through 86 subpassages, using 1:2 splits twice weekly. At passage 80 the cells began to exhibit typical signs of decline (15), with marked reduction in growth potential and morphological alterations such as increased granularity. Frozen stocks from various passage levels were reestablished and were also capable of reaching levels in excess of 80 passages.

Growth rates of four different passage levels of SFRE:CL-1 were compared (Fig. 1). Passages 84 and 21 were derived through continuous culture from the primary outgrowth, whereas passages 65 and 34 came from stocks which had been frozen for periods in excess of nine months.

A lag phase of 3 to 4 days was observed in all but passage 84, the level at which SFRE:CL-1 cells had reached their maximum growth potential and became incapable of further doublings. In earlier passages, cell culture tubes reached their maximum population at 4 or 5 days and contained 3–4 $\times$ $10^5$ cells/vessel.

In studies pertaining to the influence of cell inoculum concentration on the time required to form complete monolayers, bottles inoculated with 4 $\times$ $10^5$ cells formed complete sheets in 2 days (Fig. 2). Concentrations of $10^4$ cells required 20 days to reach confluency, while lesser numbers formed islands which did not grow out.

*Viral sensitivity spectrum of SFRE:CL-1 cells.* Simian and human viruses of various types were inoculated and passaged in SFRE:CL-1 cells (Tables I and II). Cultures were observed microscopically for overt CPE; where indicated, third passage tissue culture fluids were titrated. SFRE:CL-1 passage levels used in this study ranged from 20 to 50.

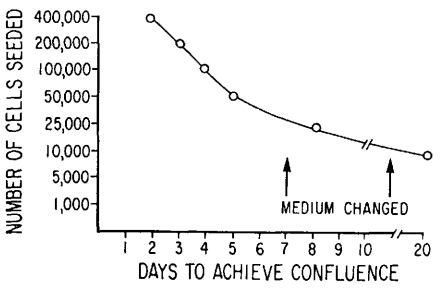


FIG. 2. Time required to obtain confluent monolayers (approximately 2 $\times$ $10^6$ cells) after seeding various concentrations of SFRE:CL-1 cells.

SFRE:CL-1 cells exhibited a wide susceptibility to enteroviruses, supporting growth of 21 of 28 of human origin. Those which did not replicate with CPE were limited to the coxsackievirus A group, known for its limited *in vitro* growth. Two of three simian and one of two chimpanzee enteroviruses grew readily, as did simian picornaviruses SA4 and SV28. Human reovirus types 1, 2 and 3, as

TABLE II. Susceptibility of SFRE:CL-1 Cells to Human Viruses.

| Group and type | CPE | Titer after three SFRE:CL-1 passages: In SFRE:CL-1 | Titer after three SFRE:CL-1 passages: In other |
|---|---|---|---|
| Picornaviruses | | | |
| Poliovirus 1 | + | 6.0 | 5.5 RMK |
| 2 | + | 5.5 | 5.0 RMK |
| 3 | + | 6.0 | 6.0 RMK |
| Echovirus 17 | + | 5.5 | 5.5 RMK |
| Rhinovirus | | | |
| Echovirus 28 | + | 4.0 | 4.0 WI38 |
| Reovirus | | | |
| Type 1 | + | 5.5 | 5.5 WI38 |
| Adenoviruses | | | |
| Type 1 | ± | 2.5 | 2.5 BK |
| 2 | + | 3.5 | 3.5 BK |
| 3 | + | NT | NT |
| 5 | + | 4.5 | 3.5 BK |
| 6 | + | 4.0 | 3.5 WI38 |
| 7 | + | 3.0 | 4.5 BK |
| 8 | — | 1.5 | 2.0 BK |
| Herpesviruses | | | |
| *H. hominis* I | + | 4.0 | 3.0 BK |
| *H. hominis* II | + | 4.5 | 4.3 Vero |
| Poxvirus | | | |
| Vaccinia | + | 5.5 | 5.5 BK |
| Myxoviruses | | | |
| Respiratory syncytial | + | 6.0 | NT |

[a] The following viruses were observed for typical cytopathic effect through two or more passages in SFRE:CL-1 cells: CPE-positive: echoviruses 1–6, 14–16, 21–23, coxsackieviruses A4, A9, B1, reoviruses 1 and 2. CPE-negative: coxsackieviruses A1, A2, A5, A6, A13, A19, A22, influenza Hong Kong A2, rubella, parainfluenza 1–3.

TABLE III. Comparative Titrations in High and Low Passage SFRE:CL-1 Cells.

| Virus | Low passage # | Titer | High passage # | Titer |
|---|---|---|---|---|
| Vaccinia | P22 | 5.5 | P61 | 6.0 |
| *Herpesvirus hominis* I | P22 | 5.0 | P61 | 5.5 |
| SA10 | P22 | 2.5 | P61 | 2.0 |
| Adenovirus 6 | P22 | 5.0 | P61 | 4.5 |
| SV12 | P22 | 5.5 | P61 | 5.5 |
| Echovirus 17 | P22 | 5.5 | P61 | 5.5 |
| Respiratory syncytial | P25 | 6.0 | P64 | 6.0 |
| SV49 | P26 | 5.5 | P64 | 5.5 |
| Echovirus 28 | P25 | 3.5 | P64 | 4.0 |

well as SV12, propagated well in SFRE:CL-1 cells with characteristic CPE. Titrations of third passage material of reovirus 1 and SV12 verified that SFRE:CL-1 cells were as sensitive to these agents as WI38 and primary rhesus kidney, respectively.

Based on cytopathology, 5 of 7 human and 3 chimpanzee adenoviruses and simian adenovirus SV15 replicated in the SFRE:CL-1 cells. Yields of infectious virus were generally low. Some virus was detected where a limited CPE was observed (adeno 1, SV1 and V340) but this may represent residual infectious virus from the inocula.

Both oral and genital strains of *Herpesvirus hominis* replicated successfully in SFRE:CL-1 cells. Of the simian type A herpesviruses tested, *H. papio* (Helmke, Rodriguez, Heberling, Kalter, in preparation), SA8 and *H. tamarinus* grew successfully. SA6, a simian cytomegalovirus, and *H. saimiri* were also inoculated, and only the former agent replicated with characteristic CPE. *H. saimiri*, isolated from New World species (16), has exhibited a limited *in vitro* infectivity spectrum.

All poxviruses tested replicated in SFRE:CL-1 cells with accompanying cytopathology. Yaba virus did not produce CPE during three passages in SFRE:CL-1 cells; Yaba-like agent, however, produced characteristic cytopathology.

Simian myxoviruses SV5 and SV41 grew in the absence of extensive CPE; evidence of successful replication was based on hemadsorption titrations employing guinea pig erythrocytes. Cercopithecus isolate SA10 was passaged and titrated on the basis of cytopathology and hemadsorption. Foamy viruses types 5 and 6 gave no evidence of growth in SFRE:CL-1 cells. With the exception of respiratory syncytial virus, human myxoviruses (parainfluenza 1–3, influenza A2, and rubella) failed to replicate in SFRE:CL-1 cells.

*Comparative viral titrations in high and low passage SFRE:CL-1 cells.* In order to evaluate whether the extraordinary longevity of SFRE:CL-1 cells presented an advantage in their usefulness in diagnostic virology, simultaneous titrations were performed on monolayers of high (60) and low (25) passage subcultures (Table III). When stocks of a variety of virus types were concurrently titrated for purposes of comparison of end points and appearance of cytopathogenicity, no significant difference between early and late passage levels was observable.

*Chromosome analysis.* Karyological analysis of SFRE:CL-1 passage 16 cells revealed a firm conformation to the normal diploid status for the chimpanzee (Table IV; Fig. 3). Of 50 spreads counted, 40 (80%) yielded a count of $2n = 48$.

The presence of paired X chromosomes in the karyotype as well as sex chromatin in stained coverslip preparations indicated that the fetus was female, a characteristic which was morphologically inevident due to the early time of expulsion.

*Discussion.* Observations over a 2 yr period have indicated that the SFRE:CL-1 cell strain would provide a useful addition to laboratories engaged in primate virology. The ease of growth, maintenance and preservation of SFRE:CL-1 cells has exceeded that displayed by other diploid strains used in this

TABLE IV. Karyological Analysis of SFRE:CL-1 Cells, Passage 16.

| | Chromosome number | | | | | Total cells counted |
|---|---|---|---|---|---|---|
| | 46 | 47 | 48 | 49 | 50 | |
| Number of cells | 1 | 3 | 40 | 5 | 1 | 50 |
| Percent | 2 | 6 | 80 | 10 | 2 | |

laboratory. Growth characteristics, as well as infectivity spectra, were apparently unaltered by retention as frozen stocks for periods in excess of 1 yr. Maintenance of confluent SFRE:CL-1 monolayers for periods of 3–4 weeks was consistently possible with the implementation of biweekly medium changes. Comparable durability was observed when SFRE:CL-1 cells were maintained under agar overlay, facilitating excellent plaque formation by a variety of viral agents.

Morphologically, SFRE:CL-1 cells are characterized by the paralleling, fibroblastic, whorling growth orientation seen in WI38 and certain other diploid cultures, particularly those derived from lung tissue. Moreover, cultures of SFRE:CL-1 cells have typically reacted less fastidiously than other fibroblasts towards toxins in different serum lots or on surfaces of culture vessels.

Like WI38 cells, SFRE:CL-1 supported the propagation of a wide variety of viruses. In addition, Jensen *et al.* (17) have recently reported successful replication in SFRE:CL-1 cells of a virus isolated from a rhesus monkey mammary carcinoma. It was noted with interest that SFRE:CL-1 cells tended to be more sensitive to human agents than to those of simian origin. This was most evident in studies with adenoviruses, wherein human serotypes were readily cultivated, while simian serotypes (chimpanzee excepted) were significantly less successful. A preference of simian viruses for growth in simian tissues, and human viruses for human tissues has previously been observed (18). That chimpanzee-derived SFRE:CL-1 cells would be preferentially selective to human agents is in keeping with the status of this primate species as man's nearest relative, immunologically closer than other simians. Accordingly, it is not surprising that the two species have been shown in several reports to share viral flora and susceptibilities (19–21).

Karyological analysis of SFRE:CL-1 cells at passage 16 revealed a diploid complement of 48 chromosomes in 80% of the cells counted. Undoubtedly, more exhaustive chromosomal studies would be warranted if SFRE:CL-1 cells are to be considered as a vaccine substrate. It would be of added interest to compare in detail the karyologies of several diploid lines at various passage levels, particularly as they enter phase III, the decline phase described by Hayflick (15).

It is curious that the *in vitro* longevity of the chimpanzee lung strain described herein exceeds that determined for most human strains by a factor of 2. This is somewhat in conflict with a suggestion of Hayflick that capacity for cell doublings *in vitro* may be a reflection of the *in vivo* lifespan of the species of origin (22). A possible source of discrepancy might be a dissimilarity in the relative age of fetuses employed for culture initiation and the degrees of differentation. The clinically estimated age of the chimpanzee fetus was 67 days; based on a gestation period of 227 ± 13 days (23), abortion occurred at 1/3 term or later. Fetal ages for WI38 and WI26 were reported as approximately 3 months (24), suggesting that the degree of fetal differentiation would be comparable.

SFRE:CL-1 cells have been offered not only as a diagnostic tool with the advantage of perhaps 25 more useful subpassages than human diploid fibroblasts, but more importantly as an alternate system for human or veterinary vaccine use (25). Production of human vaccines from human cells has evoked reticence from some workers that potential oncogenic factors might exist therein. Continuous observations of both living and stained preparations of SFRE:Cl-1 cells have yielded no morphological evidence of adventitious

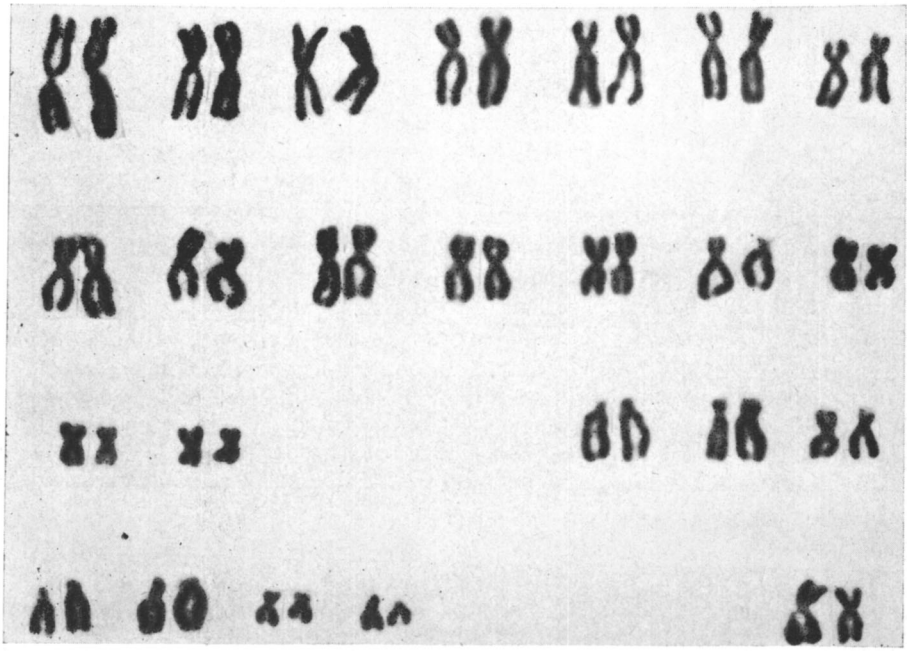

FIG. 3. Karyotype of passage 16 SFRE:CL-1 cells.

agents. Limited attempts to induce tumor formation with large quantities of SFRE:CL-1 cells in newborn hamsters have proven negative after several months.

Finally, the introduction of another primate cell line of genetically stable, though finite nature offers an implement by which to extend and complement studies on cell senescence through comparative ultrastructural, karyological and metabolic studies.

*Summary.* A cell line derived from a 9-week-old chimpanzee fetus has been established and grown for 2 hr through serial cultivation and recovery of frozen stocks. A normal female chimpanzee diploid karyotype of $2n = 48$ was observed, and inoculation of newborn hamsters with viable cells has yielded no tumors after several months. SFRE:CL-1 cells have shown a susceptibility to infection by a wide variety of human and simian viruses, usually with cytopathological manifestations. The original culture was carried through more than 80 subpassages prior to degeneration; frozen stocks have repeatedly progressed beyond 50 passages without apparent morphological alterations, reduction of growth potential, or loss of viral susceptibility. SFRE:CL-1 cells have consistently proven to be less fastidious in their growth and maintenance characteristics than human diploid fibroblasts (WI-38), which they resemble in their susceptibility spectrum and morphology. This cell line should provide a valuable tool not only for the isolation and propagation of primate viruses, but also for studies relative to cell senescence and vaccine production.

We wish to acknowledge the technical assistance of Mrs. Billie Stewart. This study was funded in part by U.S. Public Health Service grants RR00519, RR00278, RR00361 and RR00451 and contract NIH 69-93 and WHO grant Z2/181/27.

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Received Oct. 11, 1971. P.S.E.B.M., 1972, Vol. 139.