GROWTH CHARACTERISTICS OF MONKEY KIDNEY CELL STRAINS LLC-MK_1, LLC-MK_2, AND LLC-MK_2(NCTC-3196) AND THEIR UTILITY IN VIRUS RESEARCH

By R. N. HULL, Ph.D., W. R. CHERRY, and O. J. TRITCH

(From the Lilly Research Laboratories, Indianapolis)

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Monolayer cultures of primary monkey kidney cells (pMK) prepared by the trypsin method (1) are used extensively in many laboratories. This popularity is due to the broad spectrum of viral agents which can be propagated in these cultures as well as the relative ease with which a large number of cultures can be prepared. These freshly trypsinized cells adhere to glass quite well and are less sensitive to toxic factors, infrequent medium changes, temperature fluctuation, and other adverse factors or conditions than are most continuous cell lines. These features also have encouraged the use of this type of tissue culture.

The use of primary cultures of monkey kidney cells, however, is not without problems and possible hazard. The procurement, cost, and housing of monkeys is a handicap, especially to the small laboratory, or to those who only occasionally have need for monkey kidney cultures. The use of any primary tissues always allows for the possible presence in the cultures of adventitious agents derived from the host, and this is especially true of cultures of monkey kidney cells (2–7). Among the many viruses known to occur in monkey kidney cells is the highly pathogenic B virus, first described by Sabin (8). According to a recent review by Soper (9), 15 human cases of B virus infection have been recorded, 13 of which resulted in death.

The development of continuous cell strains from virus-free monkey kidney tissue was instigated in our laboratory during the early phases of the production and testing of poliomyelitis vaccine. After many failures two cell strains, LLC-MK_1 and LLC-MK_2, were established in 1955. These were described in 1956 (10) and were listed in the Provisional Catalogue of Established Cell Strains compiled in 1956 by Dr. Leighton for the Tissue Culture Association. Since this time, a subline of LLC-MK_2 was reported by Evans *et al.* (11) which could be cultivated on the chemically defined medium NCTC-109 in the absence of added protein. A clonal line capable of growth on NCTC-109 also was obtained (12). Likewise, clonal lines of LLC-MK_1 and LLC-MK_2 were isolated in our laboratory utilizing the plating method of Puck (13). The development,

growth characteristics, and virus susceptibility of these monkey kidney strains are described in this report.

Materials and Methods

A. Development of Cell Strains.—

LLC-MK$_1$: Both kidneys from a single rhesus monkey were minced, pooled together, and trypsinized by the procedure described by Younger (14). These cells were planted on February 8, 1955 in Carrel D3.5 flasks with a medium consisting of 50 per cent Earle's balanced salt solution, 30 per cent horse serum, and 20 per cent filtered chick embryo extract. Later the cells were adapted to a medium consisting of medium 199 containing 1.0 per cent horse serum. The culture medium was routinely changed three times a week and generally subcultures were made once a week.

LLC-MK$_2$: The development of this strain followed essentially the same pattern as described for LLC-MK$_1$ except that it was derived from a pool of cells prepared by trypsinization of the kidneys from six rhesus monkeys. The date of origin of this strain was March 29, 1955. This strain was also adapted to medium 199 plus 1 per cent horse serum.

LLC-MK$_2$ (NCTC-3196): Strain LLC-MK$_2$ at its 91st passage level was adapted to growth on the chemically defined medium NCTC-109 (15) by Evans *et al.*, and was described in their publication (11). The only difference in the handling of this strain in our laboratory was the use of the trypsin technique for making subcultures rather than "the cellophane mopping procedure" employed by Evans *et al.*

LLC-MK$_1$C$_1$, LLC-MK$_1$C$_7$, LLC-MK$_2$C$_1$, and LLC-MK$_2$C$_2$ were obtained by plating out the two respective parent strains. These clonal lines were obtained following three consecutive platings and clone isolations in order to obtain a high degree of purity. These clonal strains were also adapted to growth on medium 199 with 1 per cent horse serum.

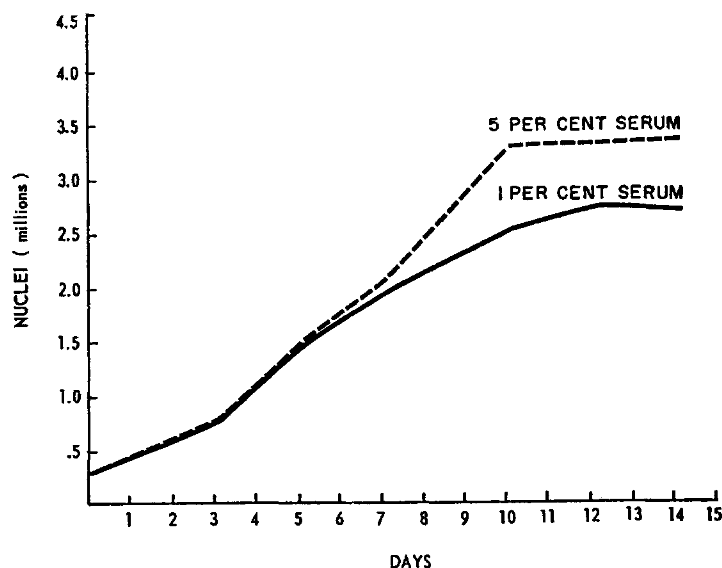
The viruses used in these studies were obtained from the laboratories indicated in the Tables. For the sensitivity studies, virus pools prepared in primary monkey kidney cells were employed, with the few exceptions noted in the Tables.

All quantitative studies involving cell growth were done by a modification of the method of Sanford *et al.* (16), utilizing T-15 flasks and nuclei counts. Because of the extreme adhesiveness of these cells to glass, a trypsin solution was used to remove the cells from the flask in place of citric acid, although the latter was still used to free the nuclei from the cytoplasm before counts were made.

RESULTS

Both LLC-MK$_1$ and LLC-MK$_2$ were carried in continuous cultivation for over 6 years with relative ease of handling. The cells adhered to glass very well even when held without fluid changes for 10 to 14 days during virus titrations. They were easily removed from the culture flasks, however, by trypsin treatment for preparation of cell suspensions. The 1 per cent horse serum medium was utilized in order to obtain maximum sensitivity to poliovirus and although more rapid growth of cells could be obtained with higher concentrations of horse serum, the rate of growth on 1 per cent horse serum was satisfactory to provide large numbers of cultures when needed. A typical growth curve comparing the growth of LLC-MK$_2$ on 1 and 5 per cent horse serum in T-15 flask cultures is seen in Text-fig. 1. A tenfold increase in the population was obtained in 10 days with 5 per cent horse serum medium while in the same period of time an

eightfold increase was obtained with 1 per cent horse serum medium. The population levels obtained appeared to be maximal for T-15 flask cultures of this strain on these media. Other quantitative studies in 16 ounce bottle cultures generally yielded similar rates of growth although rates as high as 20-fold in 8 days were seen. Quantitative studies of the growth of LLC-MK_1 on the 1 per cent horse serum medium revealed a similar rate of growth through the first 7 days, but a decline was observed beyond this point in comparison with strain LLC-MK_2. An incubation period of 15 days was necessary in order for



TEXT-FIG. 1. Comparison of growth of LLC-MK_2 on 1 and 5 per cent horse serum, in T-15 flask cultures.

LLC-MK_1 cells to equal the eightfold increase in 1 per cent serum medium obtained with strain LLC-MK_2 in 10 days.

The minimal cell inocula to provide tube cultures suitable for assay procedures within 3 days' incubation were determined. For both strains 50,000 cells/tube appeared to be the minimal inoculum which met the above requirements while as few as 12,500 cells/tube gave satisfactory cultures following 6 days' incubation. Similar studies were made to determine the minimal inocula which would produce a full cell sheet in 16 ounce bottle cultures in a 7 day period. For strain LLC-MK_2 the minimal inoculum/bottle was found to be 125,000 cells while LLC-MK_1 required 165,000 cells. While these inocula produced bottle cultures with apparently solid sheets of cells in 7 days, maximum populations were not obtained until 14 days of incubation. These 16 ounce

bottle cultures, with a floor area of 98 cm^2, yielded final cell populations of 16 to 25 million cells for LLC-MK_1 and from 22 to 34 million cells for LLC-MK_2. Thus, the combined results indicated that a 16 ounce bottle culture could provide sufficient cells of strain LLC-MK_1 to plant 300 to 400 tube cultures while a similar culture of strain LLC-MK_2 could provide the inocula for approximately 500 tube cultures. In actual practice, however, the bottle cultures were not held until maximal stationary populations were obtained but were used after 7 days of incubation for seed to plant tube cultures. At this time the cells were still actively multiplying and the cultures contained about 4 million cells/bottle. Approximately 100 tube cultures could be planted with the cells from one of these bottle cultures.

Many attempts were made to grow these strains in fluid culture, but as reported previously (17), none was successful. Strain LLC-MK_2 was maintained in a "stirrer culture" for 5 months in one experiment but a logarithmic growth phase was never obtained and the culture was eventually lost through contamination. This was repeated again a year later. Current experiments, however, show promise.

Preservation of strains LLC-MK_1 and LLC-MK_2 was possible by freezing in either liquid nitrogen or in dry ice. Although best results were obtained when the cells were frozen in the presence of 5 per cent horse serum plus 10 per cent glycerol; recovery of the strains after freezing in 1 per cent serum medium plus glycerol was satisfactory. The freeze storage of strain LLC-MK_2 (NCTC-3196) without added protein was not possible until recently. Two successful recoveries from liquid nitrogen storage have been made in our laboratory and Dr. V. J. Evans of the National Cancer Institute has obtained similar satisfactory results. These successful recoveries were made using 10 per cent filtered glycerol and gradual removal of the cells from glycerol after thawing.

Morphologically these cell strains resembled primary cultures of monkey kidney cells in their earlier passages but this similarity decreased with continued cultivation. From direct microscopic observation of numerous cultures, strain LLC-MK_2(NCTC-3196) looked more like primary cultures of monkey kidney cells than the other two strains as more elongated cells were seen. Cells of strain LLC-MK_1 were larger than the cells of either of the other two strains or of primary cultures. The cells of strain LLC-MK_2 were the smallest and this strain was further distinguished from the others by a higher frequency of giant cell formation. The relative sizes of the cells in strains LLC-MK_1 and LLC-MK_2 were probably the reason for the difference in the maximum populations obtainable with these two strains. Living unstained preparations of the three cell strains and of pMK cells are seen in Fig. 1.

Limited studies were made to determine chromosome number in these three cell strains. Although completely accurate counts were not obtained, it appeared that all three strains had counts in excess of the normal 42 pairs for

this species. LLC-MK_1 and LLC-MK_2 yielded counts ranging from 60 to 65 pairs while LLC-MK_2(NCTC-3196) had approximatlye 60 pairs. No attempt was made to prepare idiograms for chromosome analysis.

That these cell strains after many passages were in fact monkey cells was fairly well confirmed by their sensitivity to certain viruses (18). The CPE group II simian viruses were found to be highly specific for rhesus monkey kidney cells as no human or other animal cells were found which supported growth of these viruses. Included in the latter group were three species of monkeys other than rhesus and cynomolgus monkeys. Recently, Stulberg (19) reported that strain LLC-MK_2 was immunologically identical to pMK cells.

The possibility of malignant transformation was investigated by the inoculation of rhesus monkeys with cell strains LLC-MK_1 and LLC-MK_2. At the 143rd passage level, approximately 15 million cells of strain LLC-MK_1 were inoculated intracerebrally into one monkey and the same number of cells intramuscularly into each of two other monkeys. Following 13 months of observation, all three animals remained healthy and there was no evidence of tumor or even nodule formation. Three other monkeys received similar inoculations of strain LLC-MK_2 at the 130th passage level and they also remained negative throughout the 13 month period of observation. In addition, another monkey was treated with 50 mg of cortisone acetate on each of 3 days previous to intravenous inoculation with 119th passage level LLC-MK_2 cells. Two additional 50 mg doses of cortisone acetate were given on the 3rd and 4th days after inoculation. X-ray examination of the lungs made at 8, 36, and 78 days after the inoculation were all within normal limits and yielded no evidence of tumor formation. The animal was held under observation for a total of 16 months during which time it remained active and apparently in good health. The limited numbers of animals employed in these studies detracted from the significance of the results, but within these limits large doses of viable cells of both cell strains failed to produce tumors or other evidence of disease in these monkeys.

In a recent report, Eddy *et al.* (20) described the production of tumors in hamsters by the injection of extracts of primary rhesus monkey kidney cells. An attempt is in progress to determine if extracts of these three monkey kidney cell strains might also produce tumors when inoculated into hamsters following the same procedure reported by Eddy. A separate group of animals were also inoculated with S.$V._{40}$ to determine if this frequent virus contaminant in rhesus monkey kidney cells might be a tumor-producing virus in this species. The animals have been under observation for 6 months at the time of writing and no tumor formation has been noted except in those animals inoculated with S.$V._{40}$. Large tumors appeared at the site of inoculation 20 weeks following injection. (Since the original publication Eddy has also shown that S.$V._{40}$ was responsible for the tumors obtained in her studies) (26).

It was stated in the introduction that an attempt was made to obtain virus-

free cell strains from cultures of monkey kidney cells. To the best of our present knowledge based on all tests performed, these strains were found to be free from virus contamination. Of the recognized or classified simian viruses only $S.V._5$ and $S.V._{40}$ could conceivably have been present and missed through lack of a cytopathic effect either in the cell strains or in subcultures of them made in primary rhesus monkey kidney and other cell cultures. When $S.V._5$ is present either with or without a visible cytopathic effect, it can be detected by the hemadsorption technique. Hemadsorption studies were negative on stock cultures and also on pMK subcultures of the fluids from stock cultures. The new vacuolating agent, $S.V._{40}$, which is present in about 70 per cent of the kidneys from rhesus monkeys according to Sweet and Hilleman (7) was unknown at the time these cell strains

TABLE I

Comparative Titrations of Type I Poliovirus in Primary Monkey Kidney Cultures and in Three Continuous Monkey Kidney Cell Strains

Cell type	No. assays	Over-all av. titer	Selected pMK av. titer*	Assay extremes	
				Low	High
pMK	53	$10^{6.87}$	—	$10^{5.62}$	$10^{8.00}$
LLC-MK_1	32	$10^{6.87}$	$10^{7.09}$	$10^{5.82}$	$10^{7.55}$
LLC-MK_2	45	$10^{6.72}$	$10^{7.09}$	$10^{5.82}$	$10^{7.33}$
LLC-MK_2 (NCTC-3196)	14	$10^{7.38}$	$10^{7.58}$	$10^{6.62}$	$10^{8.00}$

* The figures in this column are the average titers obtained from titrations performed in pMK cells at the same time and with the same set of dilutions as those used in the indicated cell strain.

were first isolated. Recent tests utilizing rhesus testicular cell cultures and cercopithecus monkey kidney cultures, in both of which $S.V._{40}$ produces a cytopathic effect, revealed no evidence of the presence of this agent when subcultures were made to these two types of cells. Whether or not $S.V._{40}$ was present initially and lost through continued passage of the cells could not be determined.

All stock cultures maintained in our laboratory were checked from time to time for the presence of PPLO contaminants both by us and by others in our organization more familiar with these organisms. Several types of culture media were employed but in no incidence were PPLO's isolated.

In studying the virus sensitivity of these cell strains greatest attention was given to poliovirus since it was hoped that one of these cell strains could be used for the tissue culture safety testing of poliomyelitis vaccine. During a period from December 15, 1958 through December 21, 1959 an attempt was made to perform weekly comparative titrations of type I poliovirus in pMK, LLC-MK_1, and LLC-MK_2 cells. Titrations were done using tenfold dilutions of

the virus inoculated into 10 tube cultures/dilution with the same set of dilutions used for all three cell types. During the virus phase, medium 199 only was used to maintain the cultures of LLC-MK_1 and LLC-MK_2 and no fluid changes were made. Medium NCTC-109 was used with strain LLC-MK_2(NCTC-3196) throughout these tests. The average titer obtained in 53 assays done in pMK was $10^{6.87}$/0.5 ml; in 32 assays done in LLC-MK_1 the average was also $10^{6.87}$/0.5 ml, and in 45 assays in LLC-MK_2 the average titer was $10^{6.72}$/0.5 ml. LLC-MK_2-(NCTC-3196) was included in only the latter part of this study and 14 titrations done in this strain had an average titer of $10^{7.38}$/0.5 ml. Since all strains were not

TABLE II

Comparative Titrations of Type I Poliovirus in Primary Monkey Kidney Cultures and in Clonal Lines of Strain LLC-MK_1

Test date	pMK	LLC-MK_1C_1	LLC-MK_1C_7
Sept. 14, 1960	6.71*	7.00	6.75
Sept. 23, 1960	6.75	6.75	6.75
Oct. 17, 1960	7.44	6.75	7.25
Oct. 31, 1960	6.62	7.25	7.25
Nov. 7, 1960	6.82	6.96	6.95
Nov. 15, 1960	6.55	7.00	6.82
Nov. 23, 1960	7.38	6.82	6.50
Dec. 2, 1960	6.71	6.44	6.44
Jan. 10, 1961	7.09	7.28	6.55
Jan. 17, 1961	6.71	7.28	6.62
Average	6.87	6.95	6.88

* Log_{10}.

included in each week's study a more realistic comparison was made by determining the average of titers obtained in pMK cells on the same day that one of the other cell strains was included in the test. The average titers obtained in pMK cultures in direct comparison to the 32 LLC-MK_1 titrations was $10^{7.09}$/0.5 ml and it was also $10^{7.09}$/0.5 ml when compared to the 45 assays in LLC-MK_2. pMK titrations done in direct comparison with the 14 assays in LLC-MK_2-(NCTC-3196) averaged $10^{7.58}$/0.5 ml. These data are seen in Table I.

When these data were analyzed statistically it appeared that with a mean titer of $10^{7.00}$/0.5 ml in pMK that the mean titer obtainable in LLC-MK_1, LLC-MK_2, and LLC-MK_2(NCTC-3196) would be $10^{6.80}$, $10^{6.61}$, and $10^{6.80}$/0.5 ml, respectively. The reproducibility of the results in all four cell types at the 95 per cent confidence level was $\pm$0.684 logs. At an 80 per cent level, however, the standard deviation was only $\pm$ 0.2 logs. The week to week variations in titers were greater for pMK cultures than for any of the cell strains, but these

were within the range of the calculated experimental error at the 95 per cent confidence level.

More recently a series of 10 comparative titrations of type I poliovirus were done in pMK cells and in two clonal lines of LLC-MK_1, LLC-MK_1C_1, and LLC-MK_1C_7. These titrations were done as described above. The individual and average titers are seen in Table II. In these more limited studies the two clonal lines of LLC-MK_1 without question appeared to be equal in sensitivity to pMK cells.

Comparative titrations done with the two clones of LLC-MK_2, LLC-MK_2C_1, LLC-MK_2C_2, and pMK cells revealed both clones to be less sensitive than pMK cells by 0.5 to 1.0 log and also growth of the clonal lines was inferior to that obtained with the parent strain. Additional clones are being isolated.

During the time in which the sensitivity studies with the parent lines were in progress several factors were discovered which influenced their sensitivity to poliovirus. These are enumerated below:

1. Horse Serum.—When cells were grown in the presence of more than 1 per cent horse serum their sensitivity to poliovirus was reduced even though no serum was used during the virus assay. This effect of horse serum could not be rinsed away. When cultures were maintained on a medium of 5 per cent horse serum, it was found necessary to make at least three serial passages of the cell strain in 1 per cent horse serum medium in order to regain maximal virus sensitivity. Numerous studies on the effect of serum in the culture medium failed to show any definite advantage of lower levels or the absence of horse serum as long as medium 199 was employed. Calf serum was found to be equally, or more inhibitory, than was horse serum with the exception of "antibody-free" calf serum supplied by Microbiological Associates.

2. Trypsin Treatment.—The use of the trypsin procedure for preparing subcultures was found to produce more sensitive cultures than scraping, shaking, or other means of preparing cell suspensions. This was found to be especially true of strain LLC-MK_2 (NCTC-3196) which was grown in chemically defined medium.

3. Age of Cultures.—The bottle cultures from which assay tubes were prepared provided the most sensitive cultures when they were used at 7 days after planting and were in the early logarithmic growth phase. The tube cultures planted from these bottles were found to be most sensitive to poliovirus when they were planted sufficiently heavy (50,000 nuclei/tube) so as to be ready for use within 3 days after planting and without medium changes. Continued incubation and/or medium changes resulted in decreased sensitivity. This latter observation was not true, however, for strain LLC-MK_2 (NCTC-3196) grown on medium NCTC-109.

4. Length of Assay.—In pMK cultures peak poliovirus titers were nearly always obtained by the 7th day of incubation and not infrequently after the 3rd day. The development of peak titers in all the cell strains, however, was slower and 10 days of incubation were necessary to obtain final endpoints.

The ability of these cell strains to support the growth of many viruses other than poliovirus was studied. Of the classified simian viruses all could be cultivated in all three strains with the exceptions of $S.V._6$, $S.V._{29}$, $S.V._{36}$, and $S.V._{40}$. The failure of $S.V._6$ to grow was not surprising since this virus, although growing to titers of 10^5 to 10^6/0.5 ml in pMK cells could not be cultivated even in second generation monkey kidney cultures.

Other viruses studied for growth in these cell strains and the results obtained are listed in Table III. In these studies at least three serial passages of the viruses were made with appropriate dilutions at each passage so that the accumulated dilutions were well beyond the titers

of the original seed viruses. Some of the viruses, especially the adenoviruses and the influenza viruses, produced toxic effects in the first, or even the second passages, but no virus multiplication occurred except for adenovirus type 2 in strain LLC-MK_2. The latter was a surprising result but extensive retesting confirmed the original finding. Other than the two groups of

TABLE III

Growth of Miscellaneous Viruses in Monkey Kidney Cell Strains

Viruses	Source	Virus seed prep. in	LLC-MK_1	LLC-MK_2	LLC-MK_1 (NCTC-3196)
Coxsackie B-1, 2, 4, 5	Huebner	pMK*	+	+	+
Coxsackie A-9	Mogabgab	"	+	+	+
ECHO-1, 5, 14	Melnick	"	+	+	+
ECHO-7, 11	Sabin	"	+	+	+
ECHO-11 (Swayer)	Lipton	"	+	+	+
ECHO-12	Hammon	"	+	+	+
Adenoviruses-1, 3, 4, 5, 7	Huebner	"	0	0	0
Adenovirus-2	Huebner	"	0	+	0
Asian Influenza	N.I.H.	"	0	0	0
Influenza GL1739	N.I.H.	"	0	0	0
Influenza Swine	N.I.H.	"	0	0	0
Influenza Lee B	N.I.H.	"	0	0	0
Influenza PR301	N.I.H.	"	0	0	0
Influenza Mogabgab A	Mogabgab	"	0	0	0
Influenza Mogabgab B	Mogabgab	"	0	0	0
JH	Price	"	+	+	+
COE	Lennette	HeLa	0	0	0
HA Type 1	Channock	pMK	+	+	+
HA Type 2	Channock	"	0	0	0
CA	Channock	"	+	+	+
CCA	A.T.C.C.	Chang, Liver	+	+	+
HGP	Tyrrell	MKT-1‡	+	+	0
Measles-Edmonston	Enders	MKT-1	+	+	+
Mumps-PO	Henle	pMK	+	+	+
Vaccinia	Lilly Labs.	pRK§	+	+	+
B-virus	Lilly Labs.	pMK	+	+	+
Pseudorabies	Khoobyarian	RHF	+	+	+

* Primary monkey kidney.

‡ Second generation monkey kidney.

§ Primary rabbit kidney.

agents just mentioned, all the other viruses studied multiplied and produced a cytopathic effect in all three cell strains with the exception of COE and HA type 2. The growth of COE virus according to Lennette *et al.* (21), and confirmed in our laboratory, was limited to certain cell strains of human origin, while HA type 2 failed to grow in any cells other than pMK cultures. The Swayer strain of ECHO 11 virus was isolated by Steigman and Lipton (22) from a fatal disease resembling bulbospinal paralytic poliomyelitis. These authors reported that the virus could not be cultivated in a continuous monkey kidney cell strain which they had used,

but this was not one of those described in this paper. A sample of the isolate was very kindly made available to us by Dr. Murray Lipton.

A 1:10 dilution of the original material from Dr. Lipton was inoculated into four tube cultures each of strain LLC-MK_2 and pMK cells. A cytopathic effect occurred in all four tubes of the cell strain and in three of four pMK cultures. The virus was recovered from both sets of cultures. The virus isolated in the three pMK cultures was pooled and subcultured to additional pMK cultures and to the three cell strains. Virus growth occurred in all cultures except those of strain LLC-MK_1. After one additional passage in pMK cells the virus was again tested in the three cell strains and this time growth and a cytopathic effect was obtained in all three strains.

TABLE IV

Comparative Type I Poliovirus Productivity of Monkey Kidney Cell Strains and Primary Monkey Kidney Cultures

Cells	Exp. No.	Nuclei/Bottle	Titer/0.5 ml in homol. cell	Titer/0.5 ml in pMK	$TCID_{50}$, yield/cell
pMK	1	3,160,000	7.82*	7.82	615
"	2	3,400,000	7.33	7.33	192
LLC-MK_1	1	1,370,000	8.00	8.00	2187
"	2	4,380,000	7.28	7.50	132
LLC-MK_2	1	12,750,000	7.33	7.55	48
"	2	12,400,000	7.55	7.33	84
LLC-MK_2 (NCTC-3196)	1	8,000,000	6.82	6.71	24
" "	2	8,020,000	6.71	7.09	18

* Log_{10}.

The "Salisbury cold virus" HGP was successfully grown in strains LLC-MK_1 and LLC-MK_2 under the specific temperature and medium requirements described by the originators (23), but could not be cultivated in strain LLC-MK_2 (NCTC-3196) on unaltered medium NCTC-109 even at the lower temperature of incubation. Since this was a special strain adapted to growth on a specific chemically defined medium we did not attempt to obtain growth of the virus in this strain utilizing other types of culture media.

These monkey kidney cell strains were found to be especially useful in the cultivation of measles, mumps, JH and HGP viruses as more extensive cytopathic effects were obtained than these viruses usually produced in pMK cells. Sensitivity studies comparing the cell strains to pMK for the assay of Coxsackie and ECHO viruses were not done as extensively as described above for poliovirus. It should be emphasized that in all these studies, laboratory strains of the various viruses were employed and that it remained to be determined whether or not the cell strains could be used to equal advantage in the primary isolation of these viruses from clinical specimens. Several investigators interested in the latter procedure are utilizing one or more of the monkey kidney cell strains for this purpose.

In the preceding data the sensitivity of these cell strains to various viruses was described. Although the use of cell strains for virus production for vaccine preparation is currently prohibited (24) the potential of these cell strains for the production of virus was compared with that of pMK cultures in terms of poliovirus yield/cell and milliliter of culture fluid.

The results of two such experiments are seen in Table IV. Three replicate four ounce

bottle cultures were prepared of each cell strain plus pMK and at the time of virus inoculation into one bottle of each set, nuclei counts were made on the two sister cultures and the average between the two counts taken as the number of nuclei in the infected bottle. Nuclei counts on the two cultures were very close together which indicated the estimated number in the inoculated culture was probably a reliable figure. The cultures were inoculated with a ratio of 1.0 $TCID_{50}$ of virus/10 cells. When the inoculated cultures first showed complete cell destruction the fluids were harvested and titered both in the homologous cell type and in pMK cells. As noted in Table IV there was good agreement between the two titers obtained on the same virus harvest. Irrespective of yield/cell the cultures of strains LLC-MK_1 and LLC-MK_2 produced as high a total yield of virus as did the pMK cells, but in both experiments LLC-MK_2 (NCTC-3196) grown on chemically defined medium produced somewhat less virus than the other cultures. The yield/cell for this strain was also the least obtained. The yield/cell, as a whole, was somewhat confusing, but the impression was that with smaller populations this figure was higher than it was with larger populations. This could have been owing to a possible decrease in productivity of older cells in the larger populations and/or a higher productivity of younger cells. It appeared, however, that either strain LLC-MK_1 or LLC-MK_2 could be used to produce virus of equal titer to that obtained from pMK cultures in equal sized flasks and with the same volumes of medium.

Both LLC-MK_1 and LLC-MK_2 cell strains were found suitable for plaque assays of poliovirus and both could be utilized in the pH, or color, test for the assay of poliovirus antiserum. Strain LLC-MK_2 (NCTC-3196) was not studied in these two procedures.

DISCUSSION

The three strains of rhesus monkey kidney cells described in this report proved most valuable as a tool in virus studies and for the most part replaced the use of pMK cells in our laboratory. Although these cell strains were not susceptible to all the viruses which could be cultivated in pMK cultures they were sensitive to many and in some instances appeared to be more siutable than the primary cultures. These strains were especially useful for the maintenance of, or preparation of, pools of simian viruses as these were the only cell strains found to be susceptible to most of these agents. The use of the monkey kidney strains for the growth of simian viruses eliminated the constant hazard of passing these agents in primary cultures which could contain other simian viruses which would contaminate the strains. Of the 31 classified simian viruses, all but S.V.$_{6}$, S.V.$_{29}$, S.V.$_{36}$, and S.V.$_{40}$ were capable of growth in these three cell strains.

In addition to poliovirus and the other enteroviruses the monkey kidney strains were found to be of considerable value in the growth and assay of measles and mumps viruses. Often higher titers of these agents were obtained in LLC-MK_2 or LLC-MK_2 (NCTC-3196) than were obtained in primary cultures. The two "cold viruses" JH and HGP both produced more extensive cytopathic effects in LLC-MK_2 than in primary cultures which facilitated microscopic observation. CCA, a rather elusive agent in most cell cultures was readily grown in LLC-MK_2 (NCTC-3196) and with some difficulty in the other two strains.

On the negative side, none of the three monkey kidney cell strains were susceptible to HA type 2 virus which grew readily in primary cultures. No other cell strain was

found, however, which was sensitive to this virus. The failure of these strains to support growth of the adenoviruses, with the exception of type 2 virus in $LLC-MK_2$, was surprising, since in earlier passage levels several adenovirus types were successfully passed in both $LLC-MK_1$ and $LLC-MK_2$. Although not reported in the previous section, adenovirus type 2 which could not be grown in $LLC-MK_1$ did grow in the two clonal lines derived from $LLC-MK_1$. The influenza viruses used in this study were all old laboratory egg passage strains which had been adapted to growth in pMK cells except for the Mogabgab A and B strains prior to testing in the cell lines. As noted in the previous section none of these eight strains of influenza virus grew in the cell strains. Other strains, or fresh isolates from human cases, were not studied. COE virus also could not be grown in the monkey kidney cell strains but it also was not infectious for pMK cells. Herpes simplex virus was not listed in Table III since a monkey kidney-adapted strain was not available for study. Many attempts to adapt rabbit kidney-grown *Herpes simplex* virus to pMK cultures failed as did similar attempts to adapt it to the monkey kidney cell strains. Likewise it was not possible to recover this virus from skin lesions using either the primary or the continuous cell lines of monkey kidney. The virus was readily isolated, however, from the same specimens when inoculated into a rabbit kidney cell strain, $LLC-RK_1$ or into primary cultures of rabbit kidney cells. B virus and pseudorabies virus, both similar to *Herpes simplex* were quite virulent for the monkey kidney cell strains.

In the previous section the sensitivity of these strains to various viruses, and in particular poliovirus, was stressed. This was an important feature but also of importance was the ease of handling and stamina of the cultures. Both $LLC-MK_1$ and $LLC-MK_2$ cells held up well on medium 199 without added protein for 10 to 14 days during virus assays without intermediate fluid changes. There was little or no tendency of the monkey kidney strain towards rounding up or sloughing off of the glass as happened with most other cell strains unless medium changes were made every 3 to 4 days. Fluid changes during virus titrations are both dangerous to the operator and to the test results as well as time consuming. Strain $LLC-MK_2$(NCTC-3196) was especially suited to prolonged virus assays, as this strain, adapted to chemically defined medium, could be used with the complete growth medium throughout the test periods. The stability of these cultures under test conditions made them especially attractive as a possible substitute for pMK cells in the tissue culture safety testing of virus vaccines, since these tests require the holding of cultures for at least 2 weeks with medium changes and subcultures performed at 7 day intervals. Many cell strains would not hold up consistently under these conditions.

The failure of these cell strains to proliferate in fluid suspension cultures was unfortunate since this type of culture facilitated the preparation of large numbers of tube or bottle cultures for assay or virus production. Some encouragement was obtained that this difficulty might be overcome at least for strain $LLC-MK_2$. Unpublished results from another laboratory (25) indicated a degree of success in the growth of $LLC-MK_2$(NCTC-3196) in shaker cultures. The preparation of cell suspension by the trypsinization of bottle cultures provided sufficient cells for planting fairly large numbers of assay tubes and was done with less time and labor than involved in planting equal numbers of pMK cultures.

Various tests were performed, or are in progress, to determine if these cell strains

possessed any malignant or carcinogenic properties. It was found after numerous serial passages that none had the normal diploid chromosome number although strain LLC-MK_2 did retain the normal 42 pairs through 90 serial passages. It was not possible to produce tumors in rhesus monkeys with these strains under the conditions of the test procedure outlined in the previous section. Attempts to produce tumors in other species are being studied, both in our laboratory and in two outside laboratories. It is hoped by all these tests to determine whether or not any malignant transformations have occurred in these cells and if no such transformations can be demonstrated that these strains could be considered for use in the production of virus vaccines. The use of these cell strains for this purpose would eliminate many problems now facing the manufacturers of such vaccines where it is mandatory that primary tissue culture systems be employed.

These three strains of rhesus monkey kidney cells have been submitted to the Cell Collection Committee of the Tissue Culture Association as candidate strains for the cell bank being set up by the organization. Prior to this submission and until the cell bank is functioning the strains have been available only from our laboratory. The availability of these strains has been restricted to those investigators who agree, upon receipt, not to further distribute the cultures.

SUMMARY

The establishment of two strains of rhesus monkey kidney cells in continuous tissue culture, the development of a subline adapted to chemically defined medium, and the isolation of several clonal derivatives were described.

Growth characteristics, chromosome numbers, malignant potentiality, and freeze storage data are presented. The cells were studied for their sensitivity to a large number of viruses and were extensively compared with primary cultures of monkey kidney cells for sensitivity to poliovirus. The cell strains were not sensitive to all the viruses which could be grown in primary cultures of the same tissue but were susceptible to most of them. In some instances an advantage to the use of the cell strain for certain viruses was noted.

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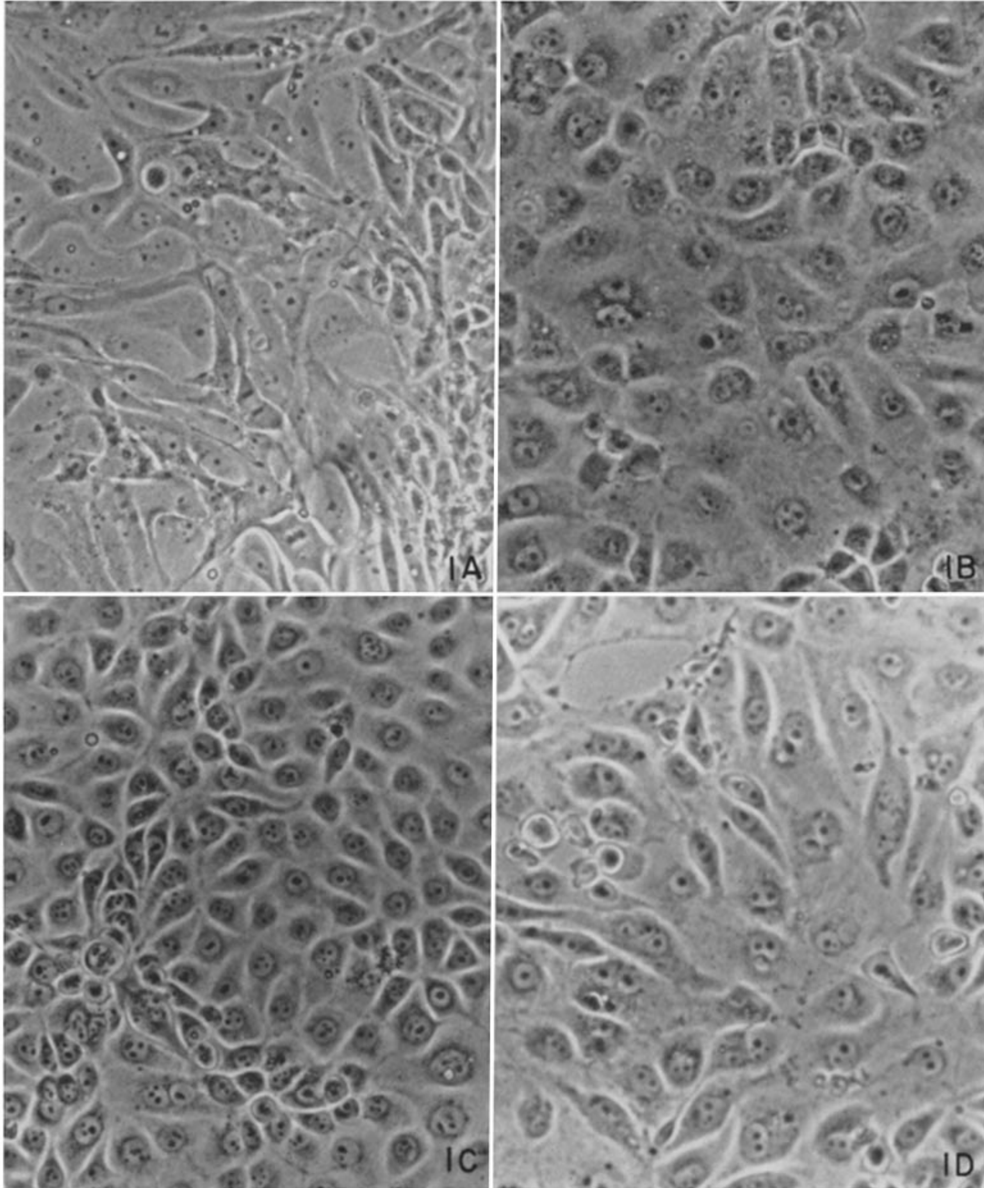
EXPLANATION OF PLATE 89

FIG. 1. Morphological comparison of monkey cell strains with primary monkey kidney cultures. *A*, primary cultures; *B*, LLC-MK_1; *C*, LLC-MK_2; *D*, LLC-MK_2 (NCTC-3196). × 90.



(Hull *et al.*: Kidney cell strains)