Biological Characteristics and Viral Spectrum of Serially Cultivated Fetal Rhesus Monkey Kidney Cells* (34040)

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Primary kidney tissue has been widely utilized for diagnostic virology and vaccine production because of its wide viral susceptibility and its ability to support good viral multiplication (1–11). Although laboratories and vaccine production houses use primary or secondary simian kidney tissue, there are significant drawbacks because of frequent contamination with simian virus (12–15). This ubiquitous contamination causes problems in diagnostic studies and dangers in vaccine production.

An ideal solution to the problem of monkey kidney cell production would be the development of a serially cultivated kidney tissue of wide viral spectrum, without adventitious agents, and with no malignant properties. Cells which reproduce *in vitro* are inexpensive and easy to handle. These cells can be screened in early passages for latent viruses and, if free of contaminants, continued in culture. This report summarized our experience with HL-8,[1] a serially cultivated, fetal rhesus monkey tissue culture.

Materials and Methods. Media. Growth media (GM) was Eagle's basal medium supplemented with 0.22% $NaHCO_3$ and 10% calf serum; 250 μg of penicillin, 250 mg of streptomycin, and 125 μ of nystatin were added per ml. Maintenance media (MM) differed only in that the serum was 2% newborn agamma calf.

Cultivation of primary cells. HL-8 originated from the kidney of a 125-day-gestation rhesus monkey embryo delivered by cesarean section (normal gestation for *Macaca mulatta* is 168 days). The sterile kidney was immediately processed by routine methods (16) with the following changes. Shorter step trypsinizations (0.25% Difco 1:250—10 min each) were used, and after the first trypsinization was discarded, the cell yield from the second through the sixth extraction was collected and stored at 4° to halt further trypsinization. The pooled extractions were washed twice in Hanks', diluted 1:400 in GM, and incubated for growth at 36°. After the first few passages, (2–3 months), cell cultures sheeted within 72 hr and were subcultivated approximately twice a week by standard methods (16) to give a 2:1 split or 100,000 cells/ml.

Preservation of cells. After trypsinization and centrifugation of a 48-hr-old culture, 2×10^6 cells were suspended in a 1-ml solution of 75% GM, 10% glycerol, and 15% agamma calf serum, and put into a 2-ml ampule. The ampules were slow frozen to —70° (17) and placed in liquid nitrogen.

For reconstitution, ampules were placed in a 37° waterbath, and, when thawed the cells (2×10^6) from one ampule were diluted with 25 ml of GM and placed in a 250-ml plastic flask. Contents of all thawed ampules grew, although it took longer to form a monolayer (4–5 days) with the first rejuvenated passage. Cells stored in liquid nitrogen for 9 months showed a 96% viability by trypan blue staining.

Chromosome analysis. Twenty-four to 48 hr after planting, cultures were treated with 0.3 μg/ml of colcemide for 5 to 6 hours at 37°. Cultures were then trypsinized, treated for 10 min with 0.075 *M* KCl hypotonic solution, and fixed in fresh cold 1:3 acetic acid–methyl alcohol. After several changes of fixative, air-dried slide preparations were made. A permanent aceto-orcein stain technique was

* Supported in part by the National Institute of Health Grants #FR00167 to the Wisconsin Regional Primate Center, #5-T1-HD-68-04, The Children's Bureau and the John A. Hartford Foundation, Inc.

[1] In previous publications this culture has been designated Fe-8.

used. Twenty to 40 consecutive, intact, metaphase plates were analyzed for each passage examined.

Virus isolation attempts. Original stool specimens, throat specimens, biopsy tissue, and other clinical material were studied in HL-8, adult rhesus monkey kidney (MK), human embryonic lung (WI-38), and human heteroploid (HEp-2) tissue cultures. Frequently, green monkey kidney (G), canine kidney (CK), primary human amnion (AM), and chick embryo (CD) were also used. Laboratory strains were similarly inoculated into HL-8 and the tissue (s) of choice for the virus.

Cultures were observed every other day for 3 weeks or until the tissue sheet showed CPE (cytopathic effect) or toxicity. Tubes not showing CPE were hemadsorbed with guinea pig (0.5%) and rhesus monkey (0.5%) erythrocytes every week. Negative tubes were passed once at 3 weeks. Tissue culture tubes with evidence of viral growth were passed three times before being titered in HL-8 and the tissue culture of choice. Enterovirus titers were held for 7 days, myxoviruses for 2 weeks.

Identification of viral isolates. Original specimens were identified as follows: enteroviruses pathogenic for TC by neutralization with type-specific rabbit antiserum, herpes virus by neutralization with guinea pig antiserum, and myxoviruses by neutralization or hemadsorption-inhibition with rabbit antiserum.

Results. Establishment of serial cultures and growth characteristics. The primary outgrowth of HL-8 tissue was epithelial-like, resembling primary adult rhesus MK. After two passages, growth slowed down and was spotty in distribution, but these islands of cells subsequently grew centrifugally and sheeted the flasks. The cells were then split twice weekly for the next 7 months. With all passages, the cells appeared epithelial-like in sparse culture and more long and narrow as the population in the flask increased (as illustrated in Fig. 1 A and B). The cells remained in a monolayer and did not pile up, presumably due to contact inhibition. The

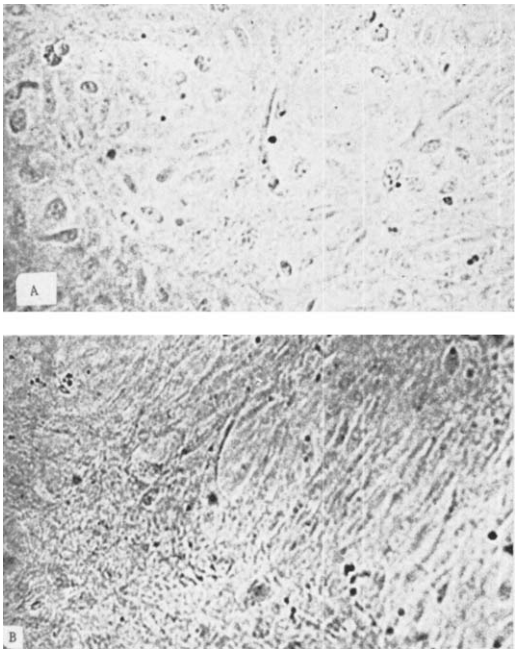

FIG. 1. HL-8 cells in a flask illustrating morphologic change in cell shapes with increase in population density. Note presence of contact inhibition. A. Cell culture at 3 days. B. Cell culture at 28 days.

cells died in two separate laboratories at passages 50 and 60 respectively after approximately 13 total months *in vitro*.

Absence of PPLO and latent viruses. Aliquots of alternate HL-8 passages were inoculated on G, WI-38, MK, and HEp-2 in an attempt to disclose latent viruses that might not show CPE or hemadsorption on HL-8; none were positive. Several common simian viruses, SV-5, SV-40, SV-11, SV-15, and measles were inoculated at levels of 100–1000 $TCID_{50}$ onto HL-8 and produced CPE. Viral cytopathology or hemadsorption was not observed in any uninoculated HL-8 cells during their lifespan, nor were inclusions seen in stained HL-8 tissue. Alternate passages were also tested for bacteria and mycoplasma.[2] One passage grew PPLO and this series of cells was destroyed.

Growth rate of HL-8. Growth rates, according to the method of Fernandes (18) (Fig. 2) were carried out at passage 43 and

[2] Courtesy of V. Allen, M. S.; Wisconsin State Laboratory of Hygiene.

TABLE I. Chromosome Analysis on HL-8 Passages.

Passage	Chromosome number						Total cells	Dicentrics
	≤40	41	42	43	≤44	Poly-ploids		
HL-8, pass. 24	8	5	9	8	1	2	33	0
HL-8, pass. 37	3	2	7	10	0	0	22	2
HL-8, pass. 49	8	0	11	14	0	1	34	0
HL-8, pass. 50	11	4	9	10	1	0	35	4
Total	30	11	36	42	2	3	124	6

44 (*in vitro* for 9 months) and on cells at generation 26 which had been frozen at passage 16 and kept in liquid nitrogen for 7 months before thawing. Each point is the average of viable cells from duplicate counts on four tubes as determined by trypan blue staining. A lag phase of 48 hr was present in the older cultures (passage 43 and 44) initially inoculated with 0.8×10^4 to 1.2×10^5 cells/ml/tube; the younger, rejuvenated passage (26 total generations) inoculated with 2×10^5 cells/ml/tube had a growth lag phase of 36 hr. Doubling of all three passages occurred in the next 36–48 hr.

Size of initial cell concentration as a function of population survival. HL-8 cells at passages 24 and 25 (*in vitro* 5 months) were studied to determine minimum inoculum size necessary for complete sheeting of 16 × 125 mm tissue culture tubes seeded with 1 ml of cell suspension. An inoculum of 100,000 cells/ml required 60 hr. As the initial cell concentration was decreased, the days necessary for sheeting increased. Concentrations below 2.5×10^4 cells/ml/tube often resulted only in island formation and less than 1.2×10^4 cells/ml/tube never sheeted.

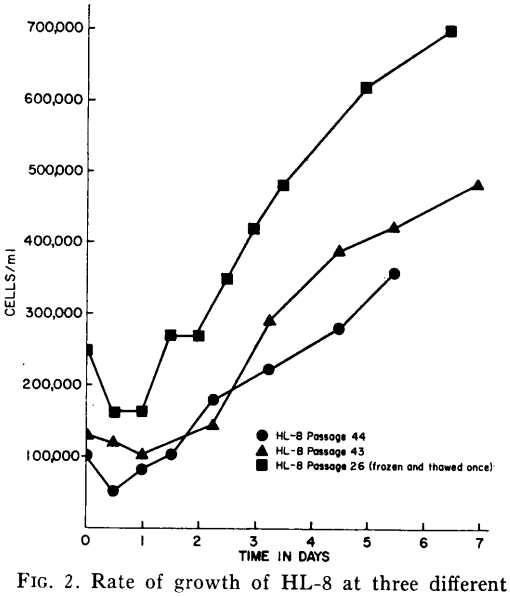

FIG. 2. Rate of growth of HL-8 at three different passages. Each point represents the average of duplicate counts on four separate tube cultures.

Results of chromosome studies. Chromosome studies were carried out on cells from passages 24, 37, 49, and 50 (Table I). In these passages, approximately 25% of cells were diploid, the remainder were aneuploid with a diploid mean chromosome number. There was no marked shift in this phenomenon between passages 24 and 50 and the degree of polyploidy was relatively small. A few dicentrics were found, but no other rearrangements were noted. In the 124 cells analyzed, 93 contained a minute chromosome, sometimes appearing as two closely-positioned dots. Because the fetal monkey had definite male internal and external genitalia, this tiny acrocentric must therefore be a small Y chromosome. Figure 3 illustrates a karyotype of 42 chromosomes, containing a Y.

Viral spectrum. HL-8 was compared with other tissue culture systems for its ability to propagate laboratory and wild strains. Sixty of 62 laboratory strains could be grown in HL-8 (Tables II and III). Only 40 laboratory strains grew in MK; thus six different tissues were necessary to recover 60 of 62 laboratory strains when HL-8 was not used. A total of 28 positive viral isolates were recovered from 42 clinical specimens (39

TABLE II. Ability to Propagate 31 Types of Enteroviruses in HL-8 as Compared with Other Tissue Culture Systems.[a]

Virus type (one strain of each type studied)	Titer after three passages	
	In HL-8	In tissue culture of choice[a]
Coxsackie A-9	$10^{-3.5}$	10^{-6} (WI-38)
Coxsackie B-1	10^{-7}	10^{-6}
Coxsackie B-2	10^{-5}	$10^{-4.5}$
Coxsackie B-3	10^{-5}	10^{-5}
Coxsackie B-4	10^{-6}	10^{-3}
Coxsackie B-5	10^{-5}	10^{-5}
Coxsackie B-6	10^{-5}	10^{-4}
Polio 1 (Brunhilde)	$10^{-5.5}$	$10^{-6.5}$
Polio 2 (Lansing)	10^{-6}	10^{-6}
Polio 3 (Leon)	$10^{-5.5}$	$10^{-6.5}$
Polio 1 (Sabin)	$10^{-5.5}$	$10^{-5.5}$
Polio 2 (Sabin)	$10^{-6.5}$	$10^{-6.5}$
Polio 3 (Sabin)	10^{-6}	10^{-6}
Echo 1	$10^{-5.5}$	10^{-7}
Echo 2	10^{-5}	10^{-6}
Echo 3	10^{-5}	10^{-6}
Echo 4	$10^{-1.5}$	$10^{-2.5}$
Echo 5	10^{-6}	10^{-6}
Echo 6	$10^{-4.5}$	10^{-6}
Echo 7	$10^{-4.5}$	10^{-6}
Echo 8	10^{-3}	10^{-6}
Echo 9	10^{-6}	10^{-7} (WI-38)
Echo 10	10^{-3}	10^{-3}
Echo 11	10^{-7}	10^{-7}
Echo 12	10^{-6}	10^{-6}
Echo 13	10^{-7}	10^{-6}
Echo 14	10^{-6}	10^{-4}
Echo 16	10^{-0}	10^{-4}
Echo 17	10^{-2}	$10^{-4.5}$
Echo 26	10^{-5}	10^{-6}
Echo 27	10^{-2}	10^{-5}

Total enteroviruses: 31

[a] MK alone unless otherwise stated. Abbreviations used as described in text.

were throat or stool swabs) (Table IV). Twenty-six of the 28 were recovered on HL-8. Thus, 86 of 90 viral strains could be recovered on HL-8. To equal this recovery rate required the combined use of six tissue culture systems. The second best single cell system for virus isolation was MK on which 64 of 90 viral strains grew.

OF the 50 enteroviruses studied, 48 grew

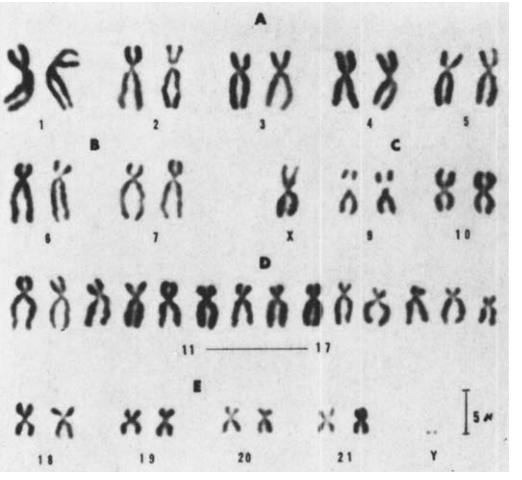

FIG. 3. Karyotype of HL-8 containing 42 chromosomes including a small acrocentric Y.

on HL-8 and 49 on MK. One Coxsackie A-16 grew in HL-8 only, while one Echo 9 and one Echo 17 grew in MK only (Table IV). Of the 45 enteroviruses passed and titered, high levels of virus multiplication, usually less than one log difference, were obtained in both HL-8 and MK tissue cultures. Neither tissue consistently gave the higher titer. Five Echo strains (one each of Echo 4, 8, 16, 17, and 27) showed decreasing rapidity of CPE and lowering of titer as judged by CPE with each HL-8 passage. Although the ability to produce CPE on HL-8 was lost, viable virus was present because HL-8 harvests transferred to MK grew in titers comparable to prepassage virus.

Of the 21 myxovirus strains tested, all grew in HL-8. However, it required the combined use of four other, different tissue systems to yield the same number of recoveries as obtained with HL-8 alone. Of the 10 myxoviruses passed and titered, results between the two systems were within 1 log except for parainfluenza 1 which gave extremely low titers in HL-8. All three reoviruses were easily passed in HL-8; adenoviruses 1, 2, 3, 5, and 7 were passed in HL-8. Of three vaccinia viruses which produced CPE in HL-8 only two of three grew in WI-38. Neither of the two herpes viruses which grew in WI-38 could be passed in HL-8. VSV produced CPE and a high titer on HL-8 was obtained. The

TABLE III. The Ability to Propagate Laboratory Strains of Selected Viruses in HL-8 as Compared with "Tissue Culture of Choice." Tissue Culture of Choice Always MK except for Substitutions Noted.

Virus type	Number of strains	Number of strains propagated in:			Number of strains titered	Titers after three passages in:		
		HL-8	MK	Other tissue of choice		HL-8	MK	Other tissue of choice
Measles—wild	3	3	0	3 (G, AM)	1	10^{-5}		10^{-5}(G) $10^{-4.5}$(AM)
Measles—vaccine	3	3	0	2 (G, AM, CK, CE)	2	$10^{-3.5}$		$10^{-3.5}$(G) $10^{-2.5}$(AM) $10^{-2.0}$(CK) $10^{-2.5}$(CE)
Influenza A_2	1	1	1		1	$10^{-1.5}$	10^{-4}	
Parainfluenza 1	2	2	2		2	10^{-2}	10^{-6}	
Parainfluenza 2	1	1	1		1	10^{-3}	10^{-3}	
Parainfluenza 3	1	1	1		1	10^{-5}	10^{-5}	
Mumps	1	1	1					
SV-5	1	1	1					
Respiratory syncytial	1	1	1					
Total myxoviruses isolated per number of attempts	14/14	14/14	8/14	5/6	8			
Adenovirus 1	1	1	0	1 (HEp-2)				
Adenovirus 2	3	3	0	3 (HEp-2)				
Adenovirus 3	1	1	0	1 (HEp-2)				
Adenovirus 5	1	1	0	1 (HEp-2)				
Adenovirus 6	1	1	0	1 (HEp-2)				
Adenovirus 7	1	1	0	1 (HEp-2)				
Reovirus 1	1	1	1					
Reovirus 2	1	1	1					
Reovirus 3	1	1	1					
Vaccinia	3	3	0	2 (WI-38)	1	10^{-4}		10^{-3} (WI-38)
Herpes simplex	2	0	0	2 (WI-38)				
VSV	1	1	0	1 (WI-38, HEp-2)	1	10^{-3}		10^{-6} (WI-38, HEp-2)
Total nonenteroviral laboratory strains isolated per number of attempts	31/31	29/31	11/31	18/20	10			

TABLE IV. Comparative Sensitivity of HL-8 and Other Tissue Cultures for Attempted Isolation of Viruses from 42 Unknown Clinical Specimens.

Virus type	Number of viral strains isolated	Number of strains isolated in:							Number of strains titered	Titer after three passages	
		HL-8	MK	WI-38	HEp-2	G	AM	CK		HL-8	Other tissue culture[a]
Coxsackie A-16	6	6	5	3					1	10^{-3}	10^{-2}
Coxsackie B-1	1	1	1	1					1	10^{-7}	10^{-6}
Coxsackie B-2	2	2	2	2					2	10^{-6}	10^{-5}
Coxsackie B-5	1	1	1	0							
Polio 1 (Sabin)	1	1	1	1					1	$10^{-6.5}$	10^{-7}
Polio 2 (Sabin)	2	2	2	2					1	$10^{-6.5}$	$10^{-6.5}$
Polio 3 (Sabin)	2	2	2	2					1	10^{-3}	10^{-4}
Echo 9	1	0	1	1							
Echo 17	2	1	2	0							
Echo 22	1	1	1	1							
Total enteroviruses	19	17	18	12					7		
Measles (avirulent)	1	1	0	0	0	0	0	1	1	$10^{-3.5}$	10^{-2} (CK)
Influenza B	2	2	2	0	0	0			1	10^{-5}	10^{-5}
Parainfluenza 2	1	1	1	1	1	0					
Parainfluenza 3	2	2	2	0	0						
Mumps	1	1	1	1							
Total myxoviruses	7	7	6	2	1	0	0	1	4		
Rubella	2	2	0	0	0	1			2	10^{-2}	10^{-2} (G)
Total positive isolates per number of isolation attempts	28/42	26/42	24/42	14/42	1/8	1/6	0/2	1/1			

[a] Titered on MK unless otherwise noted. Abbreviations as noted in text.

CPE on HL-8 closely resembled that seen on MK. Rubella grew but did not produce CPE.

Discussion. There has been no report in the literature of serially cultivated fetal rhesus kidney tissue. HL-8 originated from a 125-day gestation (normal 168 days) terminated intentionally by cesarean section. For diagnostic virology, HL-8 would be an excellent "kidney tissue of choice" because of the absence of simian virus contamination and its wide viral sensitivity. Eighty-six of 90 clinical isolates and laboratory strains grew on HL-8; it required six other tissues combined to equal this recovery rate. Enteroviral recovery rates and titers obtained were comparable on HL-8 and primary rhesus MK. All 21 clinical and laboratory myxovirus strains, including virulent and vaccine measles, could be isolated on HL-8. As with other serially cultivated cells, HL-8 was significantly inferior for propagation of influenza A_2 and parainfluenza 1. Rubella virus, adenovirus, reovirus, vaccinia virus, but not herpes virus strains grew and could be passed on HL-8.

Many attempts have been made to develop serially cultivated kidney cells of either human or simian origin but the majority have been unsuccessful. There have been only two published reports of diploid kidney strains, both of human fetal origin (3, 11). Kidney lines have been universally less sensitive to human viruses than primary rhesus MK (1, 2, 8, 9, 10). Although a green monkey line, BS-C-1, (10) and a diploid human fetal kidney strain, HFDK (11), are as sensitive as primary rhesus MK to enteroviruses, these and all other previous kidney lines are unsatisfactory for growth of influenza A_2, parainfluenza 1, 3, and 4, adenoviruses and herpes simplex virus (2, 8, 9, 10, 11).

Chromosomes of rhesus monkeys are difficult to identify and lack a standard nomenclature. Several workers (19, 20) found the diploid number, 42, present in the majority of nonpolyploid cells. Occasionally, cells with 41 chromosomes were found, but none with 40 or less (19). On cytogenetic analysis, HL-8 exhibited a consistent degree of aneuploidy with a diploid mean chromosome number in all passages. Schmidt and colleagues (10) noted this type of aneuploidy in a continuous green monkey kidney cell line, BS-C-1. Unlike HL-8, which died at passage 50 BS-C-1 developed immortality and by passage 75 had obvious neoplastic-like morphologic changes and a polyploid chromosomal configuration. With SV-40 transformation of human cells there is initial loss of contact inhibition and an early stage of hypodiploidy prior to gross heteroploidy, plus numerical and structural abnormalities such as dicentrics, fragments, minutes, rings, and marker chromosomes (21, 22). In HL-8 the degree of polyploidy was extremely small and dicentrics were the only rearrangements noted.

HL-8 tissue culture is inexpensive to use, easy to propagate, and holds up well under the conditions of freezing and thawing without changing its viral spectrum, morphology, growth characteristics, or gross chromosomal constitution. When establishment of a continuous cell line was attempted in two separate laboratories, HL-8 died at generation 50 and 60 respectively after 13 months *in vitro* on both occasions.

Summary. A serially cultivated tissue, designated HL-8, has been developed from fetal rhesus monkey kidney. A limited supply, frozen in liquid nitrogen, is available (ECD).[3] The tissue culture exhibits persistent aneuploidy with a mean diploid chromosome number. It displays contact inhibition, an epithelial-like appearance in sparse culture, and consistent morphology and viral susceptibility during an approximately 13-month lifespan *in vitro*. The viral spectrum is comparable to primary rhesus monkey kidney tissue culture. HL-8 was able to support the growth of enteroviruses as well as primary MK. Laboratory adenovirus and reovirus strains grew and could be passed on HL-8, as could vaccinia and rubella. Surprisingly, HL-8 was successful in the recovery of several myxoviruses including virulent and vaccine measles. As with other serially cultivated cells, HL-8 was significantly inferior for propagation of influenza A_2 and parainfluenza 1.

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Received Apr. 1, 1969, P.S.E.B.M., 1969, Vol. 131.