A-26

LF-10

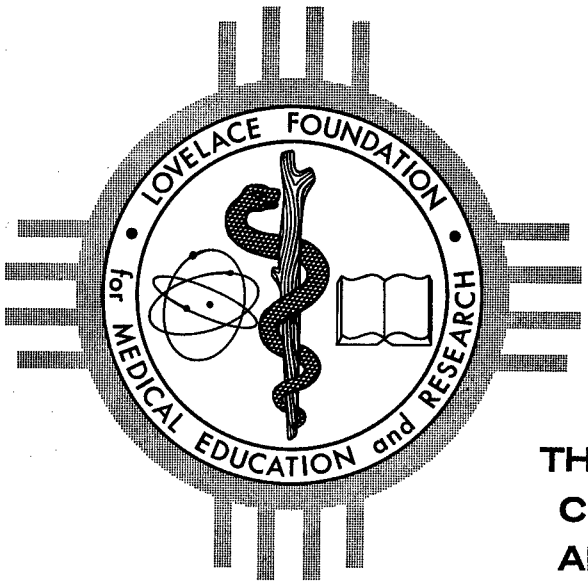
LF-10

LOVELACE FOUNDATION

for Medical Education and Research

AEC RESEARCH AND DEVELOPMENT REPORT

UNCLASSIFIED



Albuquerque, New Mexico

THE DEVELOPMENT OF A CONTINUOUS CELL LINE FROM NORMAL DOG LIVER AND ITS SUSCEPTIBILITY TO VIRUSES

by

FRANK F. PINDAK AND WILLIAM E. CLAPPER

August, 1963

ATOMIC ENERGY COMMISSION-
LOVELACE FOUNDATION
FISSION PRODUCT INHALATION PROJECT

LF-10
Biology and Medicine
TID-4500(20th Ed.)

THE DEVELOPMENT OF A CONTINUOUS CELL LINE FROM NORMAL DOG LIVER AND ITS SUSCEPTIBILITY TO VIRUSES

by

Frank F. Pindak and William E. Clapper

Submitted as a
Technical Progress Report
to
The Division of Biology and Medicine
United States Atomic Energy Commission
on
Contract No. AT(29-2)1013
August 1963

From the Section of Microbiology
Lovelace Foundation for Medical Education and Research
Albuquerque, New Mexico

ABSTRACT

A continuous line of cells was developed from a primary culture of new-born dog liver. The cells were inoculated with five types of Coxsackie B virus, three types of poliovirus, nine types of ECHO virus, and six miscellaneous viruses. Nineteen of these produced cytopathic effect without need of adaptation to the cells. One virus became adapted after four passages, one lost its ability to destroy the cells after the first passage and two failed to grow even after several blind passages.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to: Dr. Clayton S. White for his helpful suggestions in the preparation of the manuscript; Mr. Ambrosio Sanchez for technical assistance; Mrs. Beth Brown for typing the preliminary manuscript; and Mrs. Elsie Brehm for editorial aid and typing the final draft.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

THE DEVELOPMENT OF A CONTINUOUS CELL LINE FROM NORMAL DOG LIVER AND ITS SUSCEPTIBILITY TO VIRUSES

by

Frank F. Pindak and William E. Clapper

INTRODUCTION

According to Hayflick and Moorhead [1], the mounting number of cultured cells of animal origin can be divided into three basic categories: (a) cell lines which, under proper conditions, can be serially propagated indefinitely, (b) cell strains which, for at least a limited number of passages, retain certain characteristics of the tissue from which they have been developed, and (c) primary cells which must be cultivated in vitro directly from the specific tissue of the animal. To the virologist each of these kinds of cells offers certain advantages, which, unfortunately, are accompanied by some disadvantages. It is, therefore, still desirable to search for the kind of cell which will be readily propagated serially, maintain its characteristics, and, at the same time, will be susceptible to a wide range of viruses.

In designing experiments preparatory to a study of the effects of ionizing radiation on the health of beagles, it was considered desirable to study the microbial flora of the animals. Viruses presumably make up an important part of this flora and, under certain conditions, produce disease. Kidney tissue cultures of the same species from which the viral isolations are to be attempted have been most often used. Preliminary experiments were, therefore, initiated to gain experience in culturing canine kidney, as well as heart, lung, and liver. This paper reports the isolation of a continuous cell line derived from the liver of a new-born beagle. This dog, which died shortly after birth, was one of a

litter of four. The morphology and some of the growth characteristics, as well as their susceptibility to 23 viruses during the early passages of the cells were studied.

MATERIALS AND METHODS

1. Preparation of Primary Cells

A randomly selected portion of liver from the new-born beagle was cut with scissors into small pieces measuring approximately 10 mm^3. The liver tissue was removed within one hour after the death of the animal. Blood and liberated tissue fluid were removed by repeated rinses with sterile Earle's solution. The tissue fragments were then transferred to a sterile Erlenmeyer flask containing Earle's solution, penicillin, streptomycin, neomycin, and phenol red. The pH of the solution was adjusted to 7.4 with sodium bicarbonate. A magnetic bar enclosed in plastic was placed in the flask. The tissue suspension was agitated at room temperature by means of a magnetic stirrer. After 15 minutes, the supernatant was decanted and replaced with fresh Earle's solution. The agitation of the tissue was continued at room temperature for about five hours. Neither trypsin nor any other cell dispersant was used. Hourly thereafter, the fluid, turbid with cells, was collected and replaced with new fluid. The harvested cells were centrifuged in a Servall angle head centifuge, model SP, for five minutes at between 300 and 500 rpm. The cells were washed three times in Earle's solution and resuspended in the growth medium to a concentration of 300,000 cells per ml, as determined by direct count in a hemocytometer.

2. The Media

The growth medium consisted of medium 199 supplemented with 10% calf serum, both of which were obtained from Microbiological Associates. The serum was heat inactivated at 56°C for 30 minutes. The medium also contained 100 units/ml of penicillin, 100 µg/ml of streptomycin, 100 µg/ml of neomycin, and 100 units/ml of mycostatin. The mycostatin was omitted after one week. The maintenance medium, used when complete

sheets of cells were obtained, consisted of medium 199 and the same antibiotics as in the growth medium.

3. Culturing of the Cells

The cell suspension in the growth medium was distributed in 10-ml amounts in milk-dilution bottles and incubated at 37°C. The growth medium was replaced every 24 hours for the first three days. After this time it was replaced every other day.

4. Harvesting Procedure

When full sheets of cells were obtained, the medium was poured off, the cells were rinsed with phosphate buffered saline lacking the calcium and magnesium ions; three ml of a 1:5000 dilution of versene in the phosphate buffered saline was added to each bottle. The bottles were incubated at 37°C until the cells became detached from the glass, which took 15-20 minutes. The cells were separated by centrifugation, resuspended in the growth medium, and seeded into new bottles or into tubes.

5. Sources of Viruses Used

Viruses were received from the following sources: Coxsackie B-1 ET 637, B-2 ET 637, B-3 ET 637, B-5 ET 652, and B-6 ET 637 from Dr. H. A. Wenner of the University of Kansas Medical Center; poliovirus types 1, 2, and 3 from Dr. C. Moscovici of the University of Colorado Medical School; ECHO type 2 strain Cornelis VR-32, ECHO type 4 strain Pesascek VR-34, ECHO type 5 strain Noyce VR-35, ECHO type 7 strain Wallace VR-37, ECHO type 11 strain Gregory VR-41, ECHO type 14 strain Tow VR-44, and ECHO type 18 strain Metcalf VR-48 from the American Type Culture Collection, Washington, D. C.; Adenovirus type 4 St. V. from Dr. S. G. Dunlop, University of Colorado Medical School; measles virus from Lederle Laboratories, Pearl River, New York; Western equine encephalitis virus Fort Dodge Laboratories strain, Eastern equine encephalitis virus Maryland strain, infectious canine hepatitis virus MLV Baker strain, and canine distemper virus MLV Baker strain from Jensen-Salsbery Laboratories, Kansas City, Missouri.

RESULTS

1. Isolation of the Continuous (CP) Cell Line

The first day after the initiation of the culture, a moderate number of cells adhering to the glass was observed. Those cells which did not attach were discarded. Beginning with the third day, there was noticeable multiplication of the cells. After about two weeks, they had a fairly clear cytoplasm, contained one or two visible nuclei, and measured from 30 x 40 μ, to 20 x 50 μ, to 20 x 200 μ. The dimensions were determined from photographs of the living cells on the inner surface of the bottle (Fig. 1). Routine microscopic examination of one bottle at this time revealed a group of a few cells with morphology distinctly different from the remainder. These cells were polygonal, tightly packed, and smaller in size than the rest of the cells. During the following week, several more patches of such cells appeared. At this time, one fairly well isolated colony consisting of about two dozen cells was picked up on a dry, sterile cotton swab. The cells were rinsed into a new bottle of growth medium. They grew rather rapidly, covering the entire surface of the bottle in eight days. This sheet of cells was harvested with versene and the cells were divided into two new bottles. Subsequently, the harvesting of the cells was done on the average of about once every four to five days. Each time the cells from one bottle were inoculated into two to four new bottles. In order to distinguish these cells from others, they were given the designation "CP" cells. The remainder of the cell cultures, in which the above described change was not observed, failed to yield further growth after the second transfer.

2. Initial Testing of the Susceptibility of the Continuous Cell Line to Viruses

Inasmuch as the cells continued to grow at an undiminishing rate and became available in almost unlimited quantity, it was desirable to test their ability to support the growth of several viruses. For this purpose, the harvested cells were suspended in growth medium to contain 100,000 cells per ml and one-ml amounts were inoculated into glass tubes. The

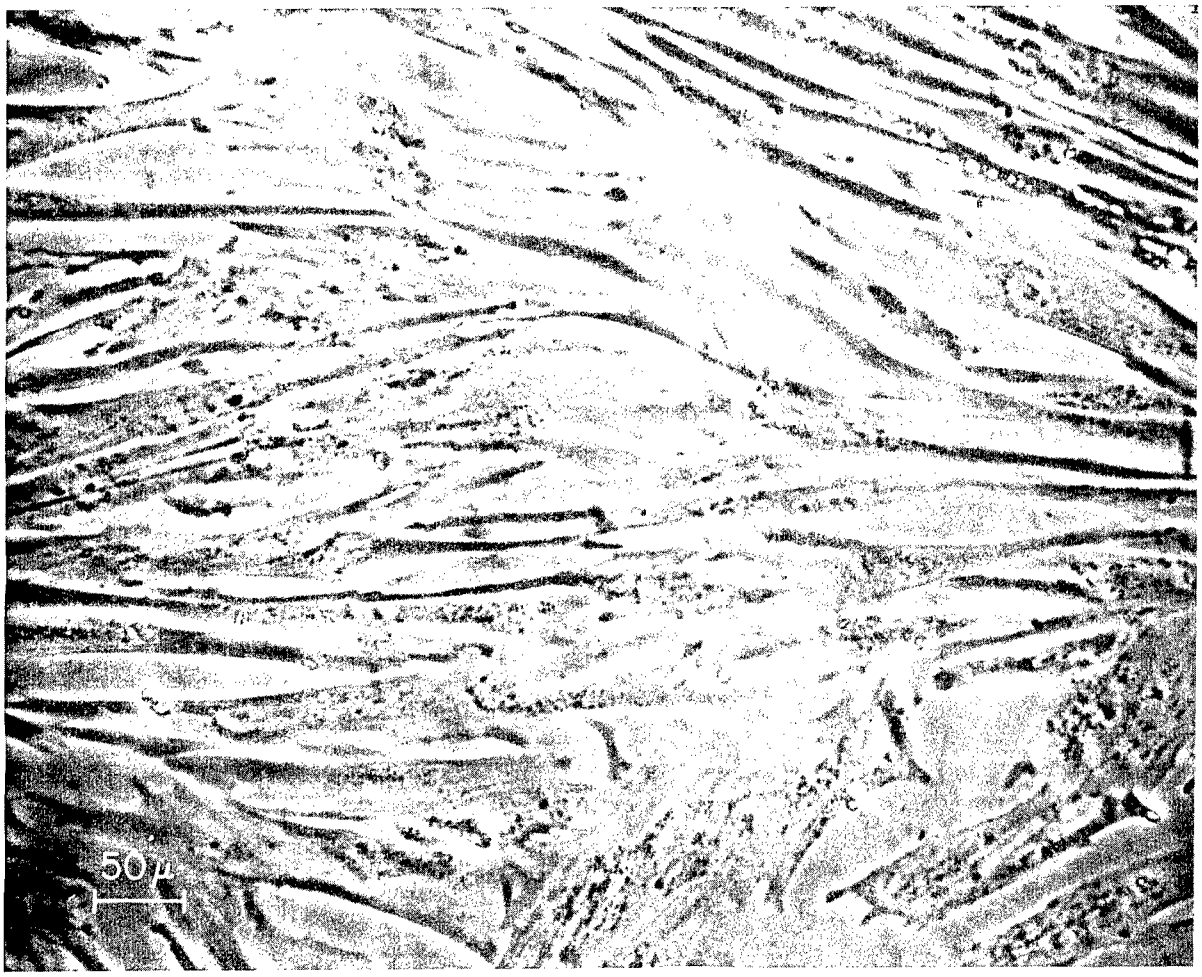


Fig. 1. Primary Dog Liver Cells. Living cells in 14 days old culture. Phase contrast.

cultures were incubated in a stationary position at 37°C. In three days, full sheets of cells were obtained. The growth medium was poured off. The tubes were rinsed twice with sterile Hanks' solution and one ml of maintenance medium was added to each tube. The cultures were inoculated with 0.1 ml of undiluted viruses, incubated at 37°C, and observed daily for signs of cytopathic effect (CPE) for six days. The viruses used for this purpose, as well as the results, are listed in Table 1. The majority of the 23 viruses used in this experiment completely destroyed the cells in three to five days. Subsequently, the Coxsackie B and the polio viruses were titred out and found to produce CPE in a dilution of 10^{-3} or greater. CP cells were therefore usable in neutralization studies on clinical specimens. Western equine encephalitis (WEE) and Eastern equine encephalitis (EEE) viruses were serially passed at least three additional times with complete destruction of the cells on each passage in not more than five days. Their representative titres were 10^{-3} and 10^{-4}, respectively. Adenovirus type 4 and measles virus were also passed successfully three times, but titrations of these viruses were not attempted at that time. The infectious canine hepatitis (ICH) virus caused a partial CPE in three to five days on the first passage. However, on the second and successive passages, no CPE was observed. The canine distemper virus failed to produce CPE even after four blind passages. This virus was one previously adapted to primary dog kidney tissue culture.

The initial results obtained with the ECHO viruses were of particular interest. Of the nine types tested, six (types 2, 6, 7, 9, 11, and 18) produced complete destruction of cells in three to five days. Type 5 destroyed about 75% of cells in six days. Types 4 and 14 did not produce any visible change in the cells during the six days of observation. These data indicated that the CP cells might be of use for propagation of at least some of the ECHO viruses. Since these viruses will not grow in HeLa cells unless first adapted to them, the advantages of such a possibility were obvious.

TABLE 1

SUSCEPTIBILITY OF CP CELLS TO 23 VIRUSES. FIRST PASSAGE OF VIRUS.

Virus	Source of inoculum	CPE on days after inoculation					
		1	2	3	4	5	6
Coxsackie B-1	Monkey kidney	+	2+	4+			
Coxsackie B-2	Monkey kidney	2+	3+	4+			
Coxsackie B-3	Monkey kidney	2+	3+	4+			
Coxsackie B-5	Monkey kidney	2+	4+				
Coxsackie B-6	Monkey kidney	+	2+	3+	4+		
Polio type 1	HeLa	+	2+	4+			
Polio type 2	HeLa	0	+	2+	4+		
Polio type 3	HeLa	+	2+	3+	4+		
ECHO type 2	Monkey kidney	0	0	0	3+	4+	
ECHO type 4	Monkey kidney	0	0	0	0	0	0*
ECHO type 5	Monkey kidney	0	0	0	+	2+	3+
ECHO type 6	Monkey kidney	+	3+	4+			
ECHO type 7	Monkey kidney	0	2+	3+	4+		
ECHO type 9	Monkey kidney	+	3+	4+			
ECHO type 11	Monkey kidney	2+	3+	4+			
ECHO type 14	Monkey kidney	0	0	0	0	0	0
ECHO type 18	Monkey kidney	0	0	3+	3+	4+	
Adenovirus type 4	HeLa	0	0	+	2+	3+	4+
Measles	HeLa	0	0	0	0	3+	3+
WEE	Chick embryo	0	+	+	2+	3+	3+
EEE	Chick embryo	2+	3+	4+			
ICH	Dog kidney	0	0	+	2+	3+	3+**
Distemper	Dog kidney	0	0	0	0	0	0
Controls		0	0	0	0	0	0

+, 2+, 3+, 4+ - denote degree of cell destruction: + = minimal but noticeable destruction; 4+ = complete destruction.

*Positive in 5th and successive passages.

**Negative in the 2nd and successive passages.

3. Serial Titrations of Selected ECHO and Coxsackie B-2 Viruses

Serial titrations were performed with six ECHO viruses and with a single Coxsackie B-2 virus in order to determine what titres might be obtained with each over several passages. Table 2 presents a typical example of the procedure used for this purpose. A fresh passage of the ECHO type 2 virus in monkey kidney (MK) cells was used as inoculum for the first passage of the virus in the CP cells. The tube with the highest dilution of the virus which still produced complete CPE (10^{-3}) was used as inoculum for the second passage. This step was repeated in the successive passages. This procedure was followed with ECHO types 5, 6, 7, 9, and 11 and with Coxsackie B-2. In the MK cells, each virus had a titre of at least 10^{-4}. No attempt was made to reach the endpoint of titres in these cells, since the primary purpose of the titrations was to confirm the viability of the viruses. After the virus completely destroyed the MK cells, the cultures were frozen at -20°C and subsequently thawed out (to preserve and then liberate virus) and the fluid containing virus was inoculated into the CP cells. It can be seen from Table 3 that all viruses tested did grow in the CP cells. Their titres were, for the most part, considerably lower than in the MK cells, but did not change significantly throughout the four passages. The lowest titre was shown by ECHO type 5. The remaining viruses grew each time to a concentration high enough to permit their CP cultures to be used for neutralization purposes. This, indeed, was attempted on a trial basis with satisfactory results.

4. Inoculation of CP Cells into Animals

Inasmuch as the CP cells continued to grow rapidly, the possibility that they might be cancerous was considered. Freshly harvested cells were washed in Earle's solution and inoculated intraperitoneally into six new-born mice, each animal receiving approximately 300,000 live cells. The animals were kept under observation for two months, then sacrificed and autopsied. Gross examination did not reveal any neoplastic growth. This experiment was repeated once more, with the same negative results. About 1,000,000 live cells were inoculated subcutaneously into

TABLE 2

SERIAL TITRATIONS SCHEME OF ECHO 2 VIRUS IN CP CELLS

Source of virus	Passage in MK cells		1st passage in CP cells		2nd passage in CP cells		3rd passage in CP cells		4th passage in CP cells	
	Virus dilution	CPE	Virus dilution	CPE	Virus dilution	CPE	Virus dilution	CPE	Virus dilution	CPE
MK	10^{-1}	4+	10^{-1}	4+	10^{-1}	4+	10^{-1}	4+	10^{-1}	4+
	10^{-2}	4+	10^{-2}	4+	10^{-2}	4+	10^{-2}	4+	10^{-2}	4+
	10^{-3}	4+	10^{-3}	4+	10^{-3}	4+	10^{-3}	4+	10^{-3}	4+
	10^{-4}	4+	10^{-4}	+	10^{-4}	4+	10^{-4}	4+	10^{-4}	4+
	10^{-5}	4+	10^{-5}	0	10^{-5}	3+	10^{-5}	4+	10^{-5}	2+
	10^{-6}	4+	10^{-6}	0	10^{-6}	0	10^{-6}	3+	10^{-6}	0

MK = virus culture in MK cells.

CPE = cytopathic effect of virus on cells: 0 = no cell destruction; + = minimal destruction of cells; 4+ = complete destruction of cells.

Arrows indicate the dilution of virus used for the following passage.

TABLE 3

RESULTS OF SERIAL TITRATIONS OF SEVEN VIRUSES IN CP CELLS

Virus	Source of inoculum	Titre in MK	Titres obtained in CP passages			
			1st	2nd	3rd	4th
ECHO type 2	MK	$>10^{-6}$	10^{-4}	10^{-5}	10^{-6}	10^{-5}
ECHO type 5	MK	$>10^{-4}$	10^{-1}	10^{-2}	10^{-1}	10^{-1}
ECHO type 6	MK	$>10^{-7}$	10^{-6}	10^{-6}	10^{-6}	10^{-6}
ECHO type 7	MK	$>10^{-6}$	10^{-3}	10^{-4}	10^{-4}	10^{-3}
ECHO type 9	MK	10^{-7}	10^{-6}	10^{-6}	10^{-6}	10^{-6}
ECHO type 11	MK	$>10^{-6}$	10^{-4}	10^{-4}	10^{-4}	10^{-4}
Coxsackie B-2	MK	10^{-5}	10^{-3}	10^{-4}	10^{-4}	10^{-4}

the ear of a normal adult rabbit. After one week the inoculation site had normal appearance. Later this animal was inoculated subcutaneously with two doses of live cells, each inoculum containing 2,000,000 cells, followed by two inoculations of 12,000,000 cells each. All inoculations were done at two-week intervals. Two weeks after the last injection, the animal was bled from the heart. The antiserum thus produced will be used for immunologic studies of the CP cells. Further information concerning the possible carcinogenicity of the cells might be obtained by their inoculation into dogs and hamsters.

5. Growth of CP Cells in Other Media

As previously mentioned, the CP cells were developed on medium 199 with 10% calf serum. It was of interest to determine whether this medium produced optimal growth. In one experiment, pooled human serum was substituted for the calf serum. In another, lactalbumin hydrolysate medium was used in place of medium 199, with calf serum and with pooled human serum. In all cases, the growth of the cells was noticeably inferior to that in the medium 199 with calf serum.

6. Present Status of the CP Cell Line

After about 50 serial passages, despite the routine use of penicillin, streptomycin, and neomycin in the growth medium, the stock culture of the cells showed contamination with a Gram negative bacillus which resulted in partial destruction of the cells. In an attempt to eliminate the contaminant, 100 μg/ml of kanamycin (Bristol Laboratories) was incorporated in the growth medium, which was changed daily for one week. At the end of this period, the cells appeared to have recovered from the infection, since it did not occur again when this antibiotic was withdrawn. However, soon thereafter it was noticed that the cells had a generally diminished susceptibility to viruses. Ten to 100 times higher inocula were thereafter needed to obtain complete cell destruction. At the present time the cells have been subcultured 134 times. Their growth rate is unchanged. They still appear to have the same shape and size

(average: 31 x 44 μ), but the cytoplasm contains irregular darker granules (Fig. 2). We do not know whether the present somewhat increased resistance to viral infection is due to an inherent change in the genetic properties of the cell or was caused by the kanamycin treatment, or by bacterial contamination, or whether the cells are latently infected with some microorganism. Perhaps it is an expression of the general aging process of the cells, related to those described by Hayflick and Moorhead [1]. These possibilities are being investigated.

DISCUSSION

The growth of viruses in tissue culture offers great advantages over other means of isolation and identification. However, there are certain drawbacks associated with such procedures. Many viruses will grow only in primary cells, which means a continuous supply of animals must be available to serve as donors of the particular organs from which the cells are obtained. In addition, some primary cells, notably MK cells, are frequently naturally contaminated with latent viruses. The cells of continuous lines are available in almost unlimited quantities and appear to be free of latent viruses, but usually are susceptible to a narrower spectrum of viruses than are the primary MK cells.

The reports on development of continuous tissue culture cell lines are so numerous that only the most significant ones can be cited. Attempts to establish new lines are still being made, since any one may have some desirable characteristics missing in the others. The earliest cell lines were obtained from human malignant tissues. Probably the first among these was derived from a human sarcoma as early as 1931 [2]. The establishment of the first really significant line, developed from a human carcinoma of the cervix and known as HeLa cells, was reported in 1952 [3]. Among the first non-cancerous, continuously propagated cells of human origin were those from liver, conjunctiva, kidney, and appendix, and reported by Chang in 1954 [4]. Hayflick and Moorhead [1] were successful in the cultivation of normal human diploid fibroblasts from fetuses in 1961.

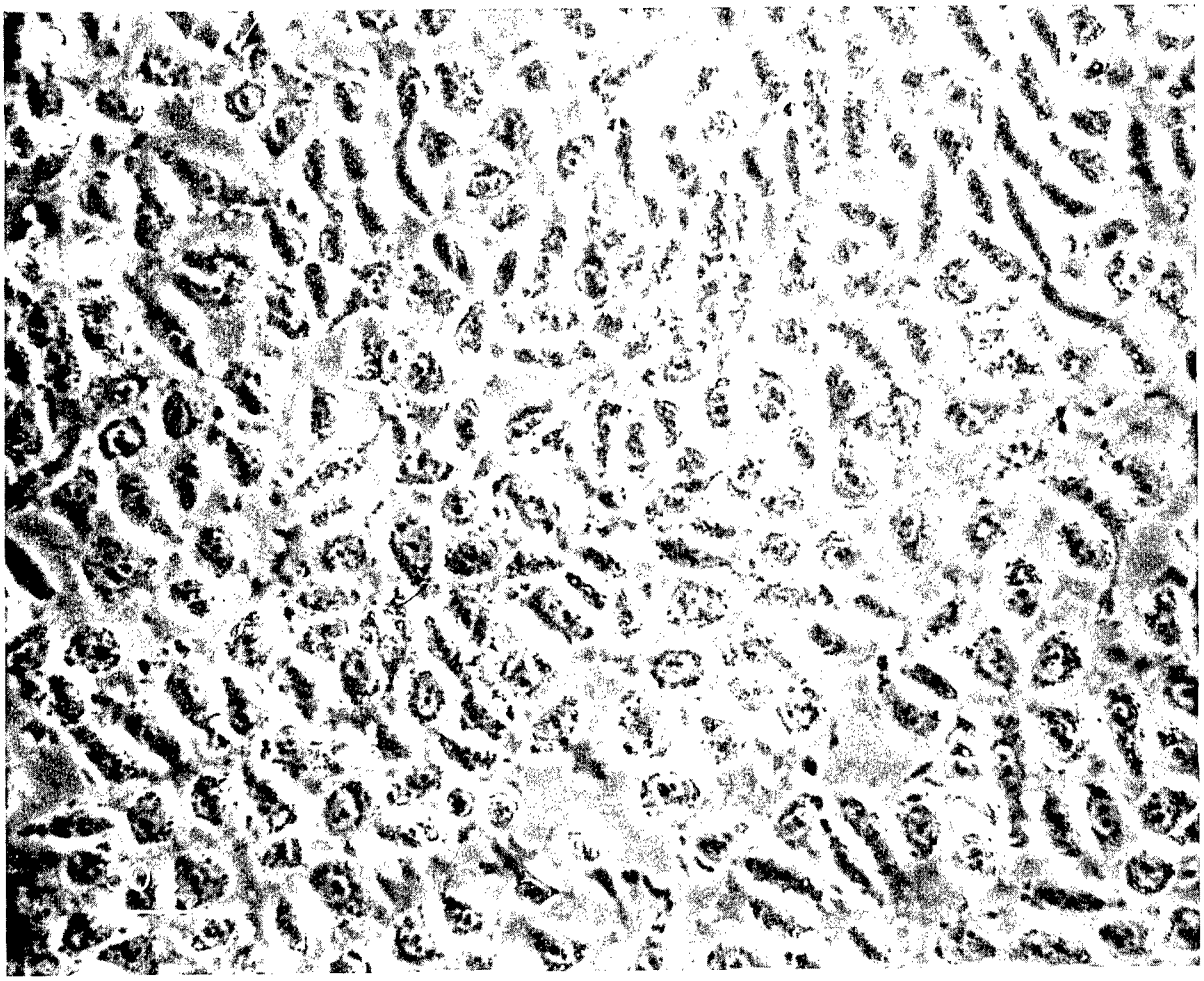


Fig. 2. Serially propagated CP cells developed from primary dog liver culture. Living cells in 130th passage. Phase contrast.

Gavrilov [5] reported the establishment of two continuous lines from adult sheep kidney. Hull, Cherry, and Tritch [6] cultivated two continuous lines from rhesus monkey kidney. Mackenzie, Mackenzie, and Reiss [7] were able to serially propagate cells derived from rabbit liver. Moore et al. [8] obtained a cell line from a malignant melanoma of the golden hamster, which retained its ability to synthesize melanin in vitro. DiPaolo [9] grew a cell line from Ehrlich ascites tumor cells of a mouse. Jensen, Force, and Unger [10] developed a continuous cell line from dog kidney. Wolf et al. [11], Wolf and Quimby [12], Freed [13], and Fauconnier and Pachopos [14] developed various continuous cell lines from cold blooded animals.

Each of the cell lines quoted above represents a contribution of its own merit. However, it is impossible to judge which of them may offer the greatest potential for studies of viruses because some lines, being used for other than virological purposes, were not reported to have been challenged with any viruses and others were used in connection with only a limited number of viral agents. In our studies of the CP cells, we were primarily interested in growing ECHO viruses. Attempts to grow them in continuous cell lines have been reported. Archetti, Weston, and Wenner [15] adapted 12 out of 13 ECHO virus types to HeLa cells. The unadaptable one was type 4. Schmidt et al. [16] propagated a total of 20 types in HeLa cells. They, too, were unable to adapt type 4, as well as types 15, 22, 23, and 27. Hull, Cherry, and Tritch [6] cultured six types in their serially propagated monkey kidney cells. The continuous lines of sheep kidney cells of Gavrilov [5] were found to propagate 11 types of ECHO viruses, along with all six types of Coxsackie B viruses and six types of adenoviruses. This author was unsuccessful in growing ECHO types 4, 10, 14, 16, 17, 18, and 27.

We have tested nine ECHO virus types for their ability to grow in the CP cells. Six of them were studied in detail, and were found to propagate in these cells without the prerequisite of adaptation. Attempts by others [5, 15, 16] to grow ECHO types 4, 14, and 18 were unsuccessful. Type 18 produced CPE in our cells, type 4 did so only after adaptation,

but type 14 did not. In a previous communication [17], we have reported four isolations of ECHO type 6 virus in the CP cells out of 16 rectal swabs from dogs. We believe that isolation of an ECHO virus in a continuous cell line has not been previously reported.

It is evident that a moderately wide range of viruses can be propagated in the CP cells. Although in most instances they are less sensitive than the MK cells, nevertheless they can be used in virus neutralization tests, thus diminishing the cost and work connected with the preparation of primary cells. Our limited experience shows that they might also be of use for primary isolation of viruses.

Although isolated from dog liver, the origin of the CP cells is not entirely clear. They were observed as morphologically distinct cells about two weeks after bottles were seeded with the cells obtained by disintegration of dog liver. It is impossible to say whether these cells were a progeny of only a single cell carried into the liver via the blood stream, or whether they, indeed originated from some component of the liver tissue. However, if they did develop from the liver cells, then it is likely that some mutation took place in a limited number of them during the first ten days of cultivation. While the majority of the cells in the culture resembled elongated fibroblasts, those from which the CP line was developed were smaller, polygonal, and more epithelioid in appearance. Growth differences between the two types were also striking. While these cells continued to propagate at an undiminished rate, the fibroblastoid type soon failed to reproduce and eventually died off, thus leaving a stable (although not necessarily homogenous) culture of the CP cells.

During the 50th and 60th passages, our stock cultures became contaminated with a bacterium. After elimination of the contaminant with kanamycin, the cells took on a somewhat granular appearance and their susceptibility to viruses decreased. It is possible that the bacterial infection, the kanamycin treatment, or both brought about the changes described above. On the other hand, one might explain these changes on the basis that we were dealing with a genuine strain of cells, which had

eventually "degenerated" into a line of different characteristics and that the change in the CP cells was analogous to that observed by Hayflick and Moorhead [1] in their human diploid fibroblasts. Their cultures were undergoing degenerative changes between the 41st and 50th passages, characterized by diminished growth rate, accumulation of debris, and loss of polarity. The degenerative process continued for one to three months, until the cultures were lost. Most of such changes were, indeed, seen in our cultures too, but they occurred as early as the third subcultivation of the "unchanged" fibroblastoid cells. However, while the original liver fibroblasts were undergoing a progressive degeneration until they completely disappeared, in the same bottle it was seen that the CP cells, with different morphology, proliferated at an unchanged pace until only these cells remained in the culture. When about six months later contamination in the CP cells occurred and their sensitivity to viruses decreased, the cells continued to maintain their original rate of growth. Today, after more than 130 subcultivations, they still are being harvested and subcultured every third to fourth day.

To date, a karyotypic analysis has not been done. This and some immunologic studies of the cells now in progress should further characterize them and help to determine their generic origin, as well as their relation to other cultured cells. Attempts to re-isolate these cells from primary dog liver, or to restore properties of the continuous line as they were in the early passages would seem to be worth further effort. Such cultures might be useful for in vitro studies of radiation effects on dog tissue, or as cells for the production of vaccines, as well as for the purposes suggested by the results reported in this paper.

SUMMARY

During routine attempts to grow cells from various organs in vitro, a continuous culture was obtained from a new-born beagle. Small pieces of liver tissue were agitated in Earle's solution by means of a magnetic stirrer at room temperature until a suspension of the cells was obtained. These were resuspended in the growth medium and seeded into milk-dilution bottles.

Initial growth of the cells adhering to glass was observed on the third day of cultivation. Two weeks later, a small number of them with distinctly different morphology was seen. One group of such cells was transferred into a new bottle. These continued to proliferate at a steady rate while the remaining ones, which did not manifest the same morphology, ceased to grow after the second subcultivation. The "changed" cells, called the CP cells, continued to grow and have been subcultivated over 130 times.

After 50 subcultivations, bacterial contamination was detected in stock cultures and eliminated. Subsequently, although the growth rate remained unaltered, the cells assumed a granular appearance and their susceptibility to viruses was diminished.

The CP cells supported the growth of viruses which normally proliferate in HeLa cells, as well as some others which do not, except after adaptation. Titres for several of these viruses approached those in MK cultures. Twenty-three viruses were studied in the CP cells. Nineteen of these grew without the need of adaptation. These included Coxsackie B types 1, 2, 3, 5, and 6, poliovirus types 1, 2, and 3, ECHO virus types 2, 5, 6, 7, 9, 11, and 18, adenovirus type 4, measles virus, WEE and EEE viruses. The ECHO type 4 virus grew only after adaptation. The ICH virus destroyed the cells only in the first passage. ECHO type 14 and canine distemper viruses failed to grow even after repeated blind passages.

REFERENCES

1. Hayflick, L. and Moorhead, P.S.: The serial cultivation of human diploid cell strains. Exp. Cell Res. 25: 585-621 (1961).

2. Gey, G.O., Bang, F.B., and Gey, M.K.: An evaluation of some comparative studies on cultured strains of normal and malignant cells of animals and man. Texas Repts. Biol. and Med. 12: 805-827 (1954).

3. Gey, G.O., Coffman, W.D., and Kubicek, M.T.: Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. Cancer Res. 12: 264-265 (1952).

4. Chang, R.S.: Continuous subcultivation of epithelial-like cells from normal human tissues. Proc. Soc. Exp. Biol. and Med. 87: 440-443 (1954).

5. Gavrilov, V.I.: Establishment of two continuous lines of adult sheep kidney cells. III. Cultivation of ECHO, Coxsackie, and adenoviruses. Vopr. Virusol. (Transl.) 6(2): 185-190 (1961).

6. Hull, R.N., Cherry, W.R., and Tritch, O.J.: 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. J. Exp. Med. 115: 903-918 (1962).

7. Mackenzie, C.G., Mackenzie, J.B., and Reiss, O.K.: Isolation and properties of a cell from liver characterized by lipid-rich particles. J. Cell.Biol. 14: 268-279 (1962).

8. Moore, G.E., Lehner, D.F., Kikuchi, Y., and Less, L.A.: Continuous culture of a melanotic cell line from the golden hamster. Science 137: 986-987 (1962).

9. DiPaolo, J.A.: Establishment of long-term cultures of Ehrlich ascites tumor cells. Proc. Soc. Exp. Biol. and Med. 109: 616-618 (1962).

10. Jensen, E.M., Force, E.E., and Unger, J.B.: Inhibitory effect of ammonium ions on influenza virus in tissue culture. Proc. Soc. Exp. Biol. and Med. 107: 447-451 (1961).

11. Wolf, K., Quimby, M.C., Ryle, E.A., and Dexter, R.P.: Preparation of monolayer cell cultures from tissues of some lower vertebrates. Science 132: 1890-1891 (1960).

12. Wolf, K. and Quimby, M.C.; Established eurythermic line of fish cells in vitro. Science 135: 1065-1066 (1962).

13. Freed, J.J.: Continuous cultivation of cells derived from haploid Rana pipiens embryos. Exp. Cell Res. 26: 327-333 (1962).

14. Fauconnier, B. and Pachopos, M.: Culture de cellules de rein de tortue Testudo graeca; sensibilite de ces cellules a differents virus. Ann. Inst. Pasteur 102(6): 661-669 (1962).

15. Archetti, I., Weston, J., and Wenner, H.A.: Adaptation of ECHO viruses in HeLa cells; their use in complement fixation. Proc. Soc. Exp. Biol. and Med. 95: 265-270 (1957).

16. Schmidt, N.J., Shinomoto, T.T., Dennis, J., Hagens, S.J., Fox, V.L., and Lennette, E.H.: Colorimetric test in HeLa cell system for assay of neutralizing antibodies to ECHO viruses. J. Lab. and Clin. Med. 59: 687-696 (1962).

17. Clapper, W.E. and Pindak, F.F.: The flora of healthy dogs. II. Isolation of enteroviruses from lower intestine. Lovelace Foundation Report No. LF-8 (1963).