

THE SERIAL CULTIVATION OF HUMAN DIPLOID CELL STRAINS¹

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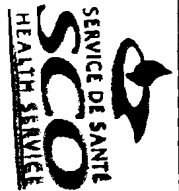
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ONLY limited success has been obtained in developing strains of human cells that can be cultivated for long periods of time *in vitro* and that still preserve the diploid chromosomal configuration [41, 47, 48, 58, 59]. Indeed, heteroploidy may be a necessary corollary or even the cause of the alteration of primary or diploid cells *in vitro* to the status of a cell line. Such changes in chromosome number appear to be independent of the type of primary tissue since they have been observed in cells derived from both normal and malignant tissue [4, 22, 23, 31].

These cell lines, of which over two hundred have been reported in the literature, have serious limitations for many kinds of biological studies. Chief among these is the exclusion of their use for the production of human virus vaccines. This limitation is based on the supposition that such heteroploid cell lines, whether of normal or malignant origin, share many of the properties of malignant cells [29, 30, 37]. This objection would be even more important if viruses played a role in human neoplasia. **In general, if strains of human cells could be kept continuously under conditions of rapid growth for extended periods of time with the retention of the diploid configuration these objections would not apply.**

Furthermore, diploid cell strains would parallel more closely the biology of cells *in vivo*. Although characterizations of heteroploid cell lines are often stated in terms of a modal chromosomal number, this should not obscure the fact that extensive pleiomorphism is present [21]. The cells comprising the modal class in heteroploid cell lines are found to be heterogeneous if chromosomal analysis is extended beyond a simple enumeration [49]. This genomic variability constitutes an important consideration in experiments using cell lines for the study of metabolic or other phenotypic, cell markers. The use of cloning as a means of reducing this variability in heteroploid cell lines is unfortunately limited by the rapid re-emergence of a range of chromosomal types among the progeny of the clone [5, 49, 50].

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The results to be presented stress, incidentally, the need for a clarification of certain terms used by tissue culturists to describe a number of phenomena. Consideration of eleven important criteria show the term "cell line" to be inapplicable to the type of cells described in this report. Precedence and usage confine the term "cell line" to only those cells that have been grown *in vitro* for extended periods of time (years). This period of time presumes potential "immortality" of the cells when serially cultivated *in vitro*. (The situation is analogous to transplantable tumors which are also apparently "immortal" in the sense that serial subcultivation in proper hosts guarantees the growth of the tumor for an indefinite period of time.) The diploid cell strains presently described are assumed to lack this characteristic of potential immortality. In addition, all mammalian cell lines examined to date vary from the diploid chromosome number. This fact alone should exclude the diploid cells from being termed "cell lines" and we have chosen to refer to them as "cell strains".

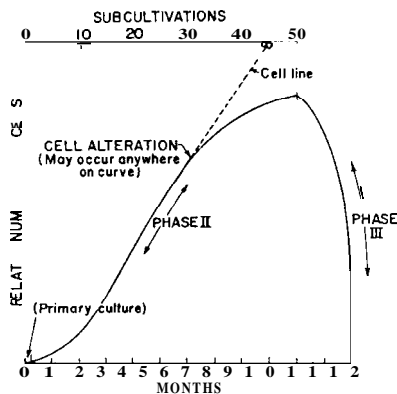
A *cell strain*, therefore, is a population of cells derived from animal tissue, subcultivated more than once *in vitro*, and lacking the property of indefinite serial passage while preserving the chromosomal karyotype characterizing the tissue of origin. Conversely, a *cell line* is a population of cells; derived from animal tissue and grown *in vitro* by serial subcultivations for indefinite periods of time with a departure from the chromosome number characterizing its source.

It is also possible, when observed, to include in the definition of a *cell line* the characteristic of cell "alteration" as described by Parker, Castor and McCulloch [44], and by Hayflick [17]. So such alterations have been found in the cell strains which are the subject of this report. It is proposed that the term "cell transformation", which has been used interchangeably with "cell alteration" [23], be excluded on grounds that the latter term has precedence and also that "transformation" has specific implications in the allied field of bacteriology which do not, as yet, apply to cell culture. The terms "established cell lines" and "stable cell lines" should likewise be avoided as the former is redundant and the latter implies that changes have ceased to take place or are no longer possible. The term *primary cells* should indicate those cells obtained from the original tissue that have been cultivated *in vitro* for the first time. If subsequent passages of these cells are made, it is assumed that such cells can properly be called a *cell strain* until they are either lost through further subcultivations or alter to the heteroploid state, in which case they could properly be referred to as a *cell line* (Text-Fig. 1).

It is the subject of this study to describe and characterize the development

of 25 strains of human cells derived from fetal tissue which retain true diploidy for extended periods of cultivation without alteration. Specific attention will be given to those cell characteristics which serve to distinguish cell lines from cell strains.

Text-Fig. 1.—Diagrammatic representation of the history of cell strains and the phenomenon of cell alteration. Phase I, or the primary culture, terminates with the formation of the first confluent sheet. Phase II is characterized by luxuriant growth necessitating many subcultivations. Cells in this phase are termed "cell strains". An alteration may occur at any time giving rise to a "cell line" whose potential life is infinite. Conversely, cell strains characteristically enter Phase III and are lost after a finite period of time.



MATERIALS AND METHODS

Media.—The growth medium (GM) used was Eagle's Medium in Earle's Balanced Salt Solution [8] supplemented with 10 per cent calf serum.¹ Twenty-five ml of 5.6 per cent NaHCO_3 , 10^5 units of penicillin and 10^5 μg of streptomycin, were added per liter. The final pH of the medium was 7.3, and before use it was brought to 37°C . Phosphate buffered saline (PBS) was prepared as described by Dulbecco and Vogt [7]. Difco trypsin (1:250) was prepared as a 0.25 per cent solution in PBS and supplemented after filtration with the antibiotics described above.

Isolation of primary cells.—Two methods of cell cultivation from primary tissue were employed in this study with identical qualitative results. The use of trypsin yielded far more cells initially than cultures prepared from fragmented or minced tissue. Since high cell yields were not required from the starting tissue, most cultures were started from fragmented or minced tissue. Such preparations gave fewer cells initially than could have been obtained from tissue treated with the enzyme preparation. Minced preparations were obtained by cutting the tissue in a Petri dish containing GM with paired scalpels or a scissors until the size of each piece approximated 1–4 mm^3 . Fragmented preparations were obtained by tearing apart the tissue with two pairs of forceps in a Petri dish containing GM until the pieces could no longer conveniently be grasped and shredded. The entire contents of the dish were emptied into one or more Pyrex Blake bottles (surface area 100 cm^2), depending on the size of the original starting tissue. The fragmented lungs, for example, from a three-month-old human fetus were usually placed in four Blake bottles. Treatment of tissue with trypsin was done, in general, according to the method of Fernandes [11].

¹ Obtained from Microbiological Associates, Inc., Bethesda, Maryland.

Initiation of cultures.—If the fetal tissue was viable when received, cells could be found in bottles planted by any one of the methods described after about three days of incubation at 36°C. When growth was first observed the cultures were refed. The spent medium and any fragments present were discarded. If additional bottles were required these fragments could be replanted in a new bottle. Fresh GM was added and as soon as the cells formed a confluent sheet the cultures were subcultivated. This normally occurred in about 10 days. Periodic feeding of the cultures was done when a sharp drop in the pH of the medium made it necessary.

In the beginning of this study attempts were made to minimize the period of time elapsing between the receipt of the fetus or fetal tissue and its cultivation *in vitro*. It was subsequently found that if either was viable upon receipt it could be kept for at least 5 days at room temperature, or 5°C, without apparent loss of viability. Minced tissue, kept in a minimal amount of GM has been found to be viable for periods of time up to 3 weeks, either at room temperature or 5°C.

Subcultivation of confluent cultures.—As soon as cell cultures were fully sheeted they were put on a strict schedule of subcultivations, which were done alternately every third and fourth day. The spent GM was discarded and trypsin solution was added to each bottle. After incubation at 37°C, or room temperature, for 15 min, the enzyme solution containing the dislodged cells was centrifuged for 10 min at 600 r.p.m. in an International Size 2 Model V Centrifuge. The trypsin solution was decanted after centrifugation and the cells were resuspended in a small amount of GM, aspirated with a 5 ml pipette, and evenly distributed to two Blake bottles. Sufficient fresh medium was added to each bottle to cover the surface adequately. This was called a 2:1 split. In the early part of this study split ratios of 3:1 were used with equal success. Incubation was carried out at 36°C.

Preservation of cells by freezing.—After trypsinization of a mature culture and resuspension of the centrifuged cells in a few ml of GM, the cell concentration was adjusted with GM to $1.5\text{--}2.0 \times 10^6$ cells per ml. Sterile glycerol was added to give a final concentration of 10 per cent and the suspension was dispensed in 2 ml portions in 5 ml ampules. The ampules were then sealed and held at 5°C overnight. The next day the ampules were placed directly at -70°C.

Recovery of frozen cells.—Ampules to be reconstituted were removed from the dry ice chest and placed quickly in a 37°C water bath. After the contents had thawed, the suspension was placed in a milk dilution bottle (surface area 40 cm²) and sufficient fresh GM added to cover the surface of the bottle adequately. After incubation at 36°C for one day the medium was completely changed. Periodic feedings of the culture were made until the cell sheet was confluent at which time the culture was manipulated as described above. Reconstituted cells frozen for up to one year invariably yielded viable cultures if these conditions were met. Although quantitative recovery of the frozen cells was not achieved, the fraction of the frozen population that did survive was always sufficient to recover the culture.

Chromosome analysis.—Thirteen of the strains were studied for purposes of chromosome analysis. Actively dividing cultures (usually 48 hr after seeding a Blake bottle) of these strains were sacrificed for chromosome studies of cells arrested in metaphase by colchicine treatment. Following 6 hr subjection to a concentration of 2×10^{-6} M colchicine in the medium, the cells were trypsinized free. Suspended cells were then processed for spreading on glass slides according to an air-drying technique [38]

each cell concentration selected. For control purposes, five hamsters were also inoculated with 10^5 HeLa cells per 0.1 ml.

Homotransplantation of WZ-1 cells into terminal cancer patients.—Two pools of strain WI-1 cells were used to inoculate six terminal cancer patients. The first pool consisted of those cells that had been grown serially for nine passages, held for 9 months at -70°C , restored, carried for an additional 21 passages, stored again at -70°C for 2 months, restored and subsequently carried for another seven passages (Series D in Text-Fig. 2). Thus this first pool of cells represented a total of 37 subcultivations. The second pool of WI-1 cells consisted of cells that had been grown serially for nine passages, held for 9 months at -70°C , restored, and subsequently carried for 36 more passages (Series B in Text-Fig. 2). Thus the second pool of cells represented a total of 45 serial subcultivations in vitro.

The cells of both pools were grown as indicated previously, harvested with trypsin, resuspended in PBS and adjusted to a concentration of 6×10^7 cells per ml. Cell counts were made in trypan blue and only viable cells were counted; dead cells constituted less than 3 per cent of the total harvest. One-half ml of this suspension was inoculated subcutaneously on the flexor surface of the forearm with a tuberculin syringe fitted with a No. 20 needle. The area was tattooed for subsequent identification when taking biopsies. The six patients used in this study were also skin tested with GM alone prior to cell inoculation in order to ascertain their sensitivity to calf protein. These patients had advanced incurable cancer and a very short life expectancy.

EXPERIMENTAL RESULTS

Establishment of diploid cultures.—In all cases where the original human fetal tissue was viable, indicated by cell growth in the first culture, the strains from the various organs were kept in serial cultivation as shown in Table I. When a particular fetus was found to yield non-viable cells from one organ, invariably cultures made from other organs were also found to be non-viable. It was found, therefore, that if a primary culture was obtained from tissue, the cell strain could be cultivated serially for periods of time up to 11 months. With the exception of strains WI-6 and WI-22 derived from heart tissue, all strains could be carried for at least 25 passages, extending over a period of 5 months. The maximum number of subcultivations obtained is exemplified by the WI-23 strain derived from lung, which lasted for 8 months during which 55 subcultivations were made. Without exception, all of the human strains were of the fibroblast cell type from about the 5th subcultivation. The kidney strains began as epithelial cell cultures with scattered nests of fibroblasts. The least successful cultures were those obtained from liver in which fully sheeted primary cultures were rarely obtained.

Although the subject of this report is confined to experiments involving human fetal cells, adult human cells have also been carried for similarly

extensive periods of time with retention of the diploid configuration. Other workers [41, 59] have reported similar results with adult human diploid cells.

Morphology of diploid human fibroblast cell strains.—Figs. 1 and 2 represent fibroblasts of the WI-1 strain of diploid human fetal lung cells after 35 subcultivations and 9 months in *vitro*. Characteristically these cells are extremely elongated fibroblasts averaging about $185 \mu \times 15 \mu$. The single nucleus contains from 1 to 4 nucleoli which vary from oval to branching bodies. Individual cells are markedly transparent with characteristically

TABLE I. History of human diploid cell strains.

Strain designation	Fetus no.	Tissue of origin	Months in serial cultivation ^a	No. of subcultivations
WI-1	1	Lung	11	51
WI-2		Skin and muscle	6 ^b	20 ^b
WI-3	2	Lung	5	35
WI-4	3	Kidney	6	29
WI-5		Muscle	7	33
WI-6	4	Heart	2.5	10
WI-7		Thymus and thyroid	5	25
WI-8	5	Skin	8.5	32
WI-9		Kidney	8.5	29
WI-10	6	Kidney	5 ^c	32 ^c
WI-11	7	Lung	5 ^c	30 ^c
WI-12	8	Skin and muscle	8	41
WI-13		Kidney	8	40
WI-14	9	Skin	8	43
WI-15	10	Kidney	7.5	28
WI-16	11	Lung	8	44
WI-17		Liver	5 ^b	24 ^b
WI-18	12	Lung	8	53
WI-19	13	Lung	8	50
WI-20	14	Skin and muscle	5 ^b	25 ^b
WI-21	15	Heart	5	26
WI-22	16	Heart	1	5
WI-23	17	Lung	8	55
WI-24	18	Lung	7	39
WI-25	19	Lung	6 ^c	38 ^c

^a Continuously passaged cells, never reconstituted from frozen stock (Series A).

^b Serial cultivation of strain lost through bacterial contamination but cells from previous passages stored at -70°C .

^c Still in culture as of February 28, 1961.

unobtainable. In this case where the WI-1 cells were lost after the 51st subcultivation and 11 months in continuous cultivation, it was possible to restore from frozen stock those cells that were derived from, for example, the 9th passage of this strain. These cells had been frozen for 9 months and the culture successfully restored. The WI-1 cells restored from frozen 9th passage cells and representing Series B of this strain were cultivated for an additional 41 passages over 5 months. Excess cells derived from each of the 41 passages of Series B were also frozen at -70°C and restoration achieved. The cells from Series B entered Phase III at the 41st passage after restoration of the 9th passage cells, or a total period of 50 passages. As indicated in Text-Fig. 2, restoration of other series of WI-1 cells from frozen stock yielded cultures that, regardless of the passage frozen, entered Phase III at total cumulative passages of 42 or better.

It is apparent, therefore, that by freezing cells at each subcultivation or every few subcultivations one could have cells available at any given time and in almost limitless numbers.¹ If it is assumed that the cell strains have a "passage potential" of 50 passages, as in the WI-1 strain, almost unlimited numbers of cells can be obtained by restoring cells frozen from surplus cultures at each passage and committing the excess cultures of this second series of cells to the freezer. This pattern can presumably be repeated until the theoretical progeny limit of 10^{22} cells has been achieved (Text-Fig. 2). Diploid cell strains restored from the frozen state still retain all those characteristics investigated for cell strains that have never been stored at -70°C , including their diploid karyotype.

Susceptibility of diploid human cell strains to viruses.—The WI-1 and WI-10 strains of diploid human fibroblasts were used, for the most part, in determining virus susceptibility. The virus titrations were made from the 17th to the 35th subcultivation of these cells. The qualitative results are tabulated in Table III. Tubes were prepared each with 5×10^4 cells per ml in GM. The tubes were refed 24 hr after planting and titrations were performed using three tubes for each tenfold dilution of virus. The final titrations yielded values consistently less than those obtained with the system in which the virus pool had originally been grown. WI-1 cells reconstituted from the frozen state did not vary in virus susceptibility. The end points of titrations made in WI-1 cells were read on the 6th day post inoculation. Subsequent production of pools of Polio Strain Koprowski-Chat in strain WI-1 yielded

¹ Assuming a 2:1 split ratio, the theoretical maximum cell yield for 50 generations is 10^{22} cells where 1×10^7 cells can be obtained from one Blake bottle. This total potential yield is equal to 2×10^7 metric tons of cells based on 5×10^8 cells equivalent to 1 g wet weight.

TABLE III. Virus susceptibility of human diploid cell strains.

Cell strain	Virus	Cytopathogenic effect
	Measles	+
	COE	÷
	Adenovirus Type 2	+
	Adenovirus Type 12	+
	Coxsackie A 9 ^a	+
	Coxsackie A 13	+
	ECHO 9	+
	Reovirus Type 1 ^a	+
	Poliovirus Koprowski-Chat Type 1 (attenuated)	+
	Poliovirus Strain TN Type 2 (attenuated)	+
WI-1	Poliovirus Koprowski-Fox Type 3 (attenuated)	+
(Passages	Poliovirus Type 1 (Mahoney)	+
20-35)	ECHO 21	+
	Varicella ^b	-
	Rabies Strain CVS	+ (see text)
	Mengo	+
	Endomyocarditis virus (EMC)	+
	Herpes simplex	+
	Vaccinia	+
	Yellow Fever, Strain 17D	+
	Influenza Type A, Strain jap	+
	Influenza Type B, Strain lee	
	Influenza Type C, Strain colindale	
	Coxsackie B 1	-
	Coxsackie A 1	-
	Western Equine Encephalitis	-
	Polyoma	--
	ECHO 11 ^a	+
	ECHO 20 ^a	+
	ECHO 22 ^a	+
	ECHO 28 ^a	+
	Respiratory Syncytial (Strain Long) ^a	+
	HE virus (Pett. strain) ^a	+
WI-10	Salisbury strain H.G.P. ^a	+
(Passages	Salisbury strain F.E.B. ^a	+
17-25)	Parainfluenza 1 ^{a, c}	--
	Parainfluenza 2 ^{a, c}	-
	Parainfluenza 3 ^{a, c}	+

^a Tested by Drs. Vincent V. Hamparian, Albert Ketler, and Maurice R. Hilleman of the Division of Virus and Tissue Culture Research, Merck Institute for Therapeutic Research, West Point, Pa.

^b Tested by Dr. Eugene Rosanoff, Wyeth Laboratories, Radnor, Pa.

^c Detected by hemadsorption.

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