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THE SERIAL CULTIVATION OF HUMAN DIPLOID CELL STRAINS[1]

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ONLY limited success has been obtained in tieveloping strains of human cells that can be cultivateti 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 *uitro* 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 esclusion of their use for the protluction 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 he even more important if viruses played a role in human neoplasia. In general, if strains of human cells could he 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 he 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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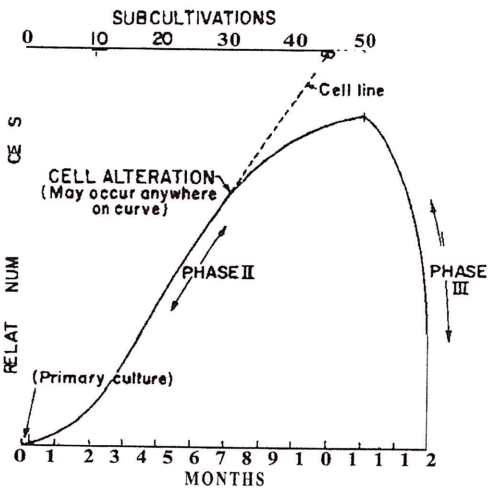
The results to be presented stress, incidentally, the need for a clarification of certain terms used by tissue culturists to describe a number of phenomena. Consideration of eleven important criteria show the term "cell line" to be inapplicable to the type of cells described in this report. Precedence 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], he 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 he 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.

Subcullivalion 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.

Preservalion 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 1.5–2.0 $\times 10^6$ 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 cm^2) 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.

Chromosorne 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.

Homotransplantalion of WZ-I 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 $\mu \times 15$ μ. 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.

titers of $10^{-7.2}$, indicating that with the passage of other viruses in the diploid strains, higher titers may be achieved. M'I-1 cell degeneration in the presence of Polio Strain Koprowski-Chat is illustrated in Fig. 18.

The growth of the CVS 24 strain of rabies fixed virus in the WI-1 strain was determined by mouse inoculations, since a variable cytopathogenic effect was observed. A complete cytopathogenic effect was observed with most of the viruses listed in Table III. A culture of WI-1 cells inoculated with this strain of rabies virus continued to replicate virus for periods up to one month after periodic complete medium changes as measured in *vivo*. The cultures continued to metabolize during this time. When the medium became acid every 4–5 days, the sheet was washed with PBS and refed. Intracerebral inoculations of 3 to 4-week-old Swiss mice with aliquots of spent medium taken 1 and 4 weeks post inoculation of the WI-1 culture and fluorescent antibody staining of cell sheets at $2\frac{1}{2}$ weeks showed *the* presence of rabies virus [25, 26]. Recent reports [10, 27] indicate similar growth of rabies virus in primary hamster kidney cells with no concomitant cytopathology. The experiments with this virus in WI-1 cells indicate that rabies virus can now be grown in nonneural human cells in uitro.

Plaque formation was also readily obtained with WI-1 cells inoculated with Koprowski-Chat poliovirus Type 1 as indicated in Fig. 19 and with Poliovirus strain Mahoney as indicated in Fig. 20. Characteristically smaller plaques were obtained with Chat than with Mahoney.

It is also of interest that Coxsackie A9 can be grown in passaged WI-1 cells since it has been reported that this virus can only be grown on primary primate cells [32]. The Salisbury strains [60], which are closely identified with the common cold, were also observed to give an unmistakable cytopathogenic effect in high passaged human kidney and lung strains. Varicella reacted similarly in high passaged human lung strains.

Implantation of diploid human cell strains into hamster cheek pouches.– Since it is known that heteroploid cell lines of malignant origin will form tumors which develop progressively when implanted into the hamster cheek

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Fig. 20.—Strain WI-1, 30th passage. Plaque formation in the presence of Poliovirus Type 1, strain Mahoney. 50 mm Petri dish.

Fig. 21.—Strain WI-1, 35th passage. Multilayer growth after prolonged incubation. Each cell plane is oriented in a different direction. May-Griinwald Giemsa stain. x 140.

Fig. 22.—Strain WI-1, 35th passage. Incubation for one month in a 50 mm Petri dish. Note membrane is beginning to curl away from the edges of the dish. May-Grhnwald Giemsa stain.

Fig. 23.—Strain WI-1, 39th passage. Membrane produced in a Blake bottle and curled up in the medium. × 12.

tissue cannot be subcultured successfully beyond about the 5th passage. This excludes those rare cases [44] in which alterations to heteroploidy occurred. This degenerative phenomenon has also been observed when certain members of the adenovirus group are unmasked in tonsil and adenoid tissue cultivated in *vitro* [52, 53].

The isolation and characterization of human diploid cell strains from fetal tissue make this type of cell available as a substrate for the production of live virus vaccines. Other than the economical advantages, such strains, in contrast to the heteroploid cell lines, exhibit those characteristics usually reserved for "normal" or "primary" cells (Table IV) and therefore make the consideration of their use in the production of human virus vaccines a distinct possibility.

TABLE VI. *Differential characteristics for human cell lines and cell strains.*

Character	Cell lines	Cell strains
1. Chromosome number	Heteroploid	Diploid
2. Sex chromatin	Not retained or variable	Retained
3. Histotypical differentiation	Sot retained	Partially retained
4. Growth in suspended culture	Generally successful	Unsuccessful
5. Pathological criteria for malignancy as determined on biopsies of cells inoculated into hanrsters or human terminal cancer patients	Positive	Negative
6. Limitation of cell multiplication (life of strain or lint.)	Unlimited	Limited
7. Virus spectrum compared to corresponding primary tissue	Often different	Same
8. Cell morphology compared to corresponding primary tissue	Characteristically different	Same
9. Acid production	Less than that produced by equal number of cell strain cells	More than that produced by equal number of cell line cells
10. Retention of Coxsackie A 9 receptor substance	Lost	Retained
11. Ease of establishment	Difficult (not predictable)	Usually successful

The question of the presence of latent viruses in any cellular material is one that can probably never be answered with absolute certainty; yet it is possible to perform exhaustive studies with techniques now available (e.g. irradiation) to rule out effectively the presence of latent viruses in one strain of diploid cells so that attention can be concentrated on the use of such a "clean" strain for the production of live human virus vaccines.

It would not be necessary to test large numbers of such strains for latent virus content. Even though these strains do degenerate as late as the 50th passage (strain WI-1), if all the surplus cells from each subcultivation were stored in the frozen state a potential yield of 20 metric tons of cells could be obtained from any single strain if its "passage potential" was even as low as 30 subcultivations. Clearly, the potential "senescence" of any diploid strain should not detract from its usefulness, since the potential cell yield is abundant, if not inexhaustible, for all practical purposes.

SUMMARY

The isolation and characterization of 25 strains of human diploid fibroblasts derived from fetuses are described. Routine tissue culture techniques were employed. Other than maintenance of the diploid karyotype, ten other criteria serve to distinguish these strains from heteroploid cell lines. These include retention of sex chromatin, histotypical differentiation, inadaptabilitv to suspended culture, non-malignant characteristics *in vivo,* finite limit of cultivation, similar virus spectrum to primary tissue, similar cell morphology to primary tissue, increased acid production comparetl to cell lines, retention of Coxsackie A9 receptor substance, and ease with which strains can he developed.

Survival of cell strains at −70°C with retention of all characteristics insures an almost unlimited supply of any strain regardless of the fact that they degenerate after about 50 subcultivations and one year in culture. A consideration of the cause of the eventual degeneration of these strains leads to the hypothesis that non-cumulative external factors are excluded and that the phenomenon is attributable to intrinsic factors which are expressed as senescence at the cellular level.

With these characteristics and their extremely broad virus spectrum, the use of diploid human cell strains for human virus vaccine production is suggested. In view of these observations a number of terms used by cell culturists are redefined.

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