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THE LIMITED *IN VITRO* LIFETIME OF HUMAN DIPLOID CELL STRAINS[1,2]

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PREVIOUS reports from this laboratory have emphasized the fact that serially cultured human diploid cell strains have a finite lifetime *in vitro* [2, 12, 14, 15]. After a period of active multiplication, generally less than one year, these cells demonstrate an increased generation time, gradual cessation of mitotic activity, accumulation of cellular debris and, ultimately, total degeneration of the culture. The limited *in vitro* multiplication of many kinds of cultured cells has been a common observation of cell culturists. Until recently [14], technical difficulties were invoked as an explanation for this event. This phenomenon in the course of *in vitro* cultivation of human fetal diploid cell strains, which we refer to as Phase III, has been shown to occur after 50 ± 10 serial passages *in vitro* using a 2:1 subcultivation ratio [14]. This event has now been confirmed in other laboratories [36, 41] and appears to be causally unrelated to conditions of cell culture, the media composition used, presence of mycoplasma or latent viruses, or the depletion of some non-replicating intracellular metabolic pool [14]. Consequently, we advanced the hypothesis that the finite lifetime of diploid cell strains *in vitro* may be an expression of aging or senescence at the cellular level. Experiments to be described extend the studies of this phenomenon and have further bearing on an interpretation based on a theory of senescence.

MATERIALS AND METHODS

Medium.—The medium used was Eagle's Basal Medium [7] supplemented with 10 per cent calf serum. Sufficient $NaHCO_3$ was added so that the medium, upon equilibration to 37°C, reached a pH of 7.4. For prevention of microbial (including

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mycoplasma) contamination, 50 μg/ml of Aureomycin (Lederle product no. 4691–96, intravenous) was used. The material, packaged in 500 mg amounts, was reconstituted, with agitation, in 50 ml of sterile distilled water at 37°C. Five ml aliquots of this stock concentrate were stored at −20°C. Each liter of warm medium was supplemented before use with 5 ml of stock concentrate. Mycoplasma determinations made over a three-year period on approximately 2000 cell cultures have revealed a total absence of mycoplasma where Aureomycin has been used. Subsequent growth of treated cultures in antibiotic-free medium for extended periods of time has also been proven negative for the presence of these microorganisms. Trypsin was prepared as previously described [14], pre-warmed to 37°C, and raised to pH 7.5 before use.

Human diploid cell strains.—Strains WI-26, WI-38 and WI-44 were used. WI-26 was derived from male fetal human lung and WI-38 and WI-44 from female human fetal lung. All embryos were obtained from surgical abortions and were of approximately three months' gestation.

Subcultivation of confluent cultures.—The method of subcultivation was a modification of that previously described [14]. The medium from confluent cell sheets was removed and pre-warmed (37°C) trypsin solution (Difco 1:250) was added to each culture for 1 min. All except 1 or 2 ml of the trypsin was then decanted and the bottle culture allowed to stand at room temperature for about 30 min. A small amount of Eagle's medium was added and splashed over the loosened cell sheet. The suspension was then vigorously aspirated with a narrow-bore 5 or 10 ml pipette to obtain discrete single cells. Sufficient additional medium was added for the total volume of the suspension to cover twice the surface area from which it was obtained. This is referred to as a 2:1 split ratio. Cultures were incubated at 37°C.

Initiation of the new strains, chromosome analysis, preservation in liquid nitrogen and reconstitution were performed as previously described [12, 14, 40]. These strains have characteristics similar to others previously reported [14]. Chromosome analyses have shown the human diploid cell strains WI-26, WI-38 and WI-44 to be normal or classic diploid [30]. Preliminary studies on the human cell strains of adult lung origin also indicate classic diploidy [24].

EXPERIMENTAL RESULTS

Reconstitution of frozen cells.—As previously shown, human fetal diploid cell strains, preserved at sub-zero temperatures and subsequently reconstituted, enter Phase III at a total number of passages (2:1 split ratio) of 50 ± 10 [12, 14, 15]. This compares favorably with the passage level at which Phase III occurs in the original passage series of the strain which had never been frozen [12, 14, 15]. Further experiments with human diploid cell strains WI-26 and WI-38 have confirmed and extended these results. As indicated in Fig. 1, the average passage level at which mitoses ceased (Phase III) in 20 ampules of WI-26, reconstituted from various passage levels and preserved for periods of time up to 19 months, was 47 passages. The

range was 38–60 passages. This compares favorably with 50 passages for the original unfrozen culture.

Seven hundred and fifty ampules of WI-38 were preserved at the eighth passage level. The average passage level at which Phase III occurred in 18 of these

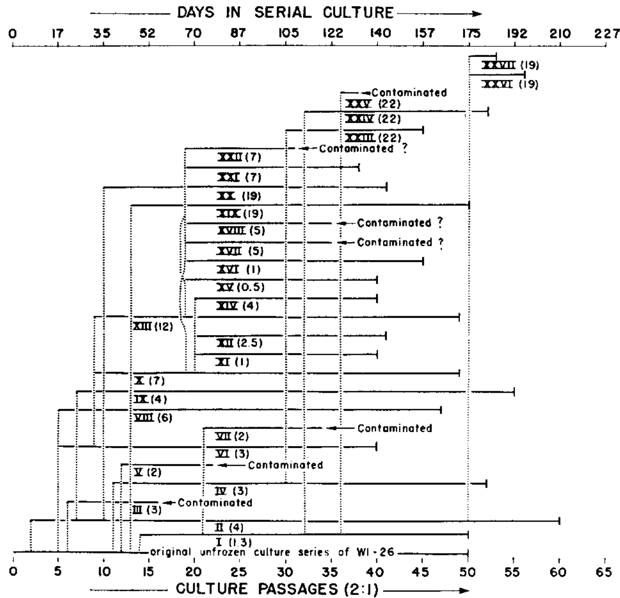

Fig. 1. Diagrammatic representation of the history of human diploid cell strain WI-26. Original unfrozen culture series represents the continuous subcultivation of the strain through 50 passages, during which time surplus cultures from each passage were stored in liquid nitrogen. Other series represented by the roman numerals were reconstituted at passage levels denoted by the origin of the vertical lines. The numbers in parentheses denote the number of months that the ampule giving rise to each series was stored. The average number of passages at which Phase III occurred is 47. The range is 38–60.

ampules preserved for periods of time up to 15 months was 47 passages. The range was 39 to 58 passages. These results are similar to the 48 passages obtained with the original unfrozen culture, as shown in Table I.

These results indicate that, regardless of the passage level at which a human fetal diploid cell strain is frozen, the total number of passages that can be expected at a 2:1 split ratio is about 50 ± 10, including those made prior to preservation. Therefore, it can be concluded that the onset of Phase III cannot be related to absolute calendar time but is related to the time during which the culture is actively proliferating.

This observation has been repeated with a number of different human

diploid cell strains of embryonic origin and confirmed in other laboratories [36, 41]. Furthermore, none of the approximately 200 laboratories that have received cultures of strains WI-26 and WI-38 has reported success in subculturing them indefinitely. All recipients, successfully cultivating these

TABLE I. *Passage levels at which Phase III occurred in thawed ampules of the 8th passage of WI-38.*

Culture	Passage level reached prior to cessation of mitoses[a] (Phase III)	Weeks preserved in liquid nitrogen	Culture	Passage level reached prior to cessation of mitoses[a] (Phase III)	Weeks preserved in liquid nitrogen
Parental (never frozen)	48	—	X	44	36
			XI	45	40
I	48	3	XII	47	40
II	48	8	XIII	47	40
III	42	10	XIV	49	50
IV	50	12	XV	47	50
V	43	20	XVI	39	59
VI	47	24	XVII	42	65
VII	53	30	XVIII	45	68
VIII	58	32	Average	47	
IX	47	32	Range	39–58	

[a] All passages done at a 2:1 split ratio. Figures include 8 doublings prior to preservation, except the parental culture.

strains, have reported that Phase III occurred at "about the 50th passage" when a 2:1 split ratio was used.

Split ratio effect on Phase III.—The measurement of Phase III as a function of the number of subcultivations may be, in effect, equivalent to a measurement of accumulated generations or cell doublings. No one has reported the successful cultivation of any human diploid cell strain in suspension culture, where it is possible to keep a cell population in continuous logarithmic growth, thereby permitting a more accurate determination of cell doublings.

Numerous attempts by us to grow human diploid cell strains in agitated fluid suspension have consistently failed under conditions allowing for the luxuriant growth of heteroploid cell lines [14].

The mechanical or enzymatic methods which must be used to subcultivate

cells grown as monolayers in static cultures repeatedly cause the cultures to pass through a "lag-log-lag" pattern. Thus, during the first 24 hr post-subcultivation little, if any, mitotic activity is observed. From 24 hr post-subcultivation (depending upon the inoculation density) there is a logarithmic

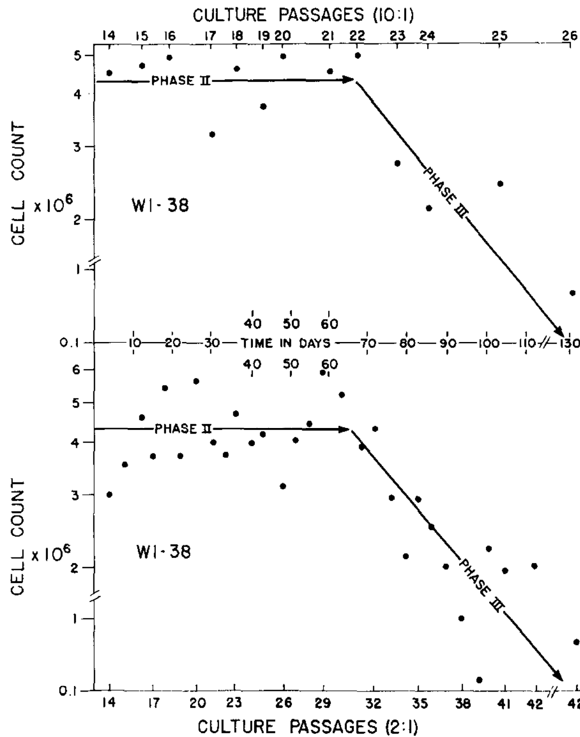

Fig. 2.—Cell counts determined at each passage of WI-38 for two different split ratios (10:1 and 2:1). Total cell counts are plotted as a function of time in days and actual passages. Although fewer actual passages occur when the culture is split 10:1, the total calendar time accruing is the same as that of the 2:1 split ratio in respect to commencement and termination of Phase III.

cell increase, followed by a lag associated with confluency of the monolayer culture.

In order to ascertain the correlation between commencement of Phase III and the total number of accrued doublings, rather than the total number of subcultivations (passages), sister cultures of strain WI-38, taken at the 14th passage, were subcultivated at two different split ratios, 2:1 and 10:1. At each subcultivation the cells in four to six parallel cultures were counted. Two persons manipulated these cultures independently so that the entire experiment was performed simultaneously and in duplicate. Each set of

cultures was grown on medium from two different sources to circumvent microbial contamination at any point in the five-month period of this experiment.

The human diploid cell strain WI-38 was thawed at the eighth passage, then at passage 14; cells from four separate confluent cultures were counted. Two cultures were then serially passaged by each of the two individuals, one culture at a 2:1 split ratio and the other at a 10:1 split ratio. All cultures were subcultivated when confluency was reached, at which times cell counts were made and averaged to accumulate the data plotted in Fig. 2. Those cultures which were split 2:1 were subcultivated when they reached confluency every three and four days alternately during Phase II (the period of active cell proliferation) and at increasingly longer intervals during Phase III. Cultures split 10:1 were subcultivated as soon as they became confluent. As can be observed in Fig. 2, the total calendar time accruing until Phase III commenced was similar for both split ratios. Phase III can be defined as the terminal period, during which, time intervals between population doublings are progressively greater. The accrued calendar time at which all cell mitoses ceased and culture degeneration began was also similar for both split ratios. From Fig. 2, a computation of the number of generations accruing for each split ratio before Phase III gives a total of 17 for the 2:1 split ratio and 27 for the 10:1 split ratio. Thus, for the 18 passages of the 2:1 split ratio accruing prior to Phase III, there was an average of 0.96 cell doublings per passage, whereas the eight passages of the 10:1 split ratio accruing during the same period yield an average of 3.33 cell doublings per passage. The figure 0.96 is very close to 1.00, which would, theoretically, be expected to be the number of doublings per passage of cultures split 2:1. The figure 3.33 is also very close to 3.25, the theoretical expectation of cell doublings per passage in cultures split 10:1.

It was assumed that regardless of the split ratio, the total number of cell doublings would be identical during the entire series of passages until Phase III. Contrary to expectation, the split ratio appears to affect the actinal number of generations. The greatest effect was observed when cell cultures were permitted to reach confluency more often during cultivation (2:1 split ratio) and the smallest effect under more efficient conditions where cell confluency occured least often. Variations in these patterns of efficiency have yielded the data shown in Table II. Clones were isolated, transferred to bottles and allowed to reach a density of 4×10^6 before the first 2:1 subcultivation was made. It is evident from the data in Table II, therefore, that the total number of doublings to be expected from a human diploid cell strain is reduced in

proportion to the number of times the culture is permitted to achieve confluency (lag period). Since mitotic activity lessens once these cultures become confluent [14], a greater, although finite, number of generations may be expected from a human diploid cell strain which has been cultivated almost

TABLE II. *Doublings of WI-38 as a function of split ratio.*

Treatment	Actual number of splits until Phase III	Number of doublings[a] until Phase III
All 2: 1 splits	42	40
2: 1 splits until passage 14, then 10: 1 splits	27	57
2: 1 splits until passage 9, cloned, then 2: 1 splits	37	57
2: 1 splits until passage 8, cloned, then 2: 1 splits to passage 15, recloned, then 2: 1 splits	20	63

[a] Based on 0.96 doublings per 2:1 split; 3.33 doublings per 10:1 split; and 22 doublings for a single cell to reach a density of 4×10^6 (confluent culture) when it was then split 2:1.

constantly in the "log phase" of growth. A 2:1 split ratio permits a considerably shorter overall period of time in log phase than does a culture that has been cloned, allowed to reach a maximum density and then recloned. This effect has also been described by Todaro, Wolman and Green [36], who, using different inoculation densities throughout the lifetime of a human diploid cell strain, observed that, generally, the total number of doublings at the high inoculation densities was reduced. This effect of realizing higher doubling potential as a result of more efficient conditions of growth remains to be clarified; but under the best conditions, eventual failure (Phase III) of the culture ensues.

The shape of the curves in Fig. 2 is of considerable importance in attempting to understand the mechanism of Phase III. It was desirable, therefore, to repeat the experiment with another human diploid cell strain and to accumulate cell counts from the earliest possible 2:1 passage until termination of Phase III. This was done starting with the fourth passage of strain WI-44 and the results are given in Fig. 3. The higher average cell counts obtained

with WI-44 as compared with WI-38 during Phase II resulted from the use of a slightly larger culture vessel. It is apparent from Fig. 3 that the shape of this curve is similar to those in Fig. 2. Such curves are similar to those obtained with the multiple-hit or multiple-target inactivation theory and an

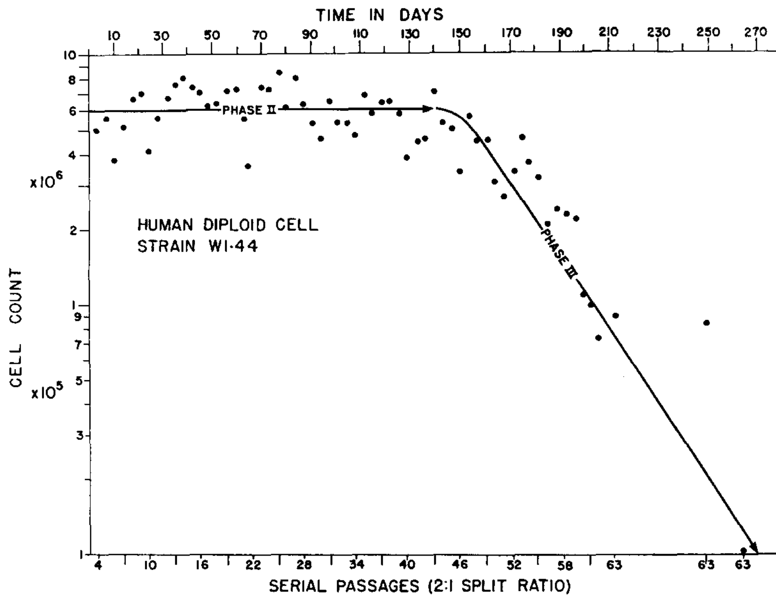

Fig. 3.—Cell counts determined at each passage of strain WI-44. This figure, like Fig. 2, results in a curve suggestive of multiple-hit or multiple-target inactivation phenomena as an explanation for the mechanism of the occurrence of Phase III. The initial plateau during Phase II, with no apparent loss of biological function as measured by constant doubling time, is followed by Phase III, where doubling time increases exponentially.

hypothesis of the mechanism of Phase III based on this phenomenon will be considered subsequently.

Cloning experiments.—Any satisfactory explanation of the finite lifetime of human diploid cell strains *in vitro* involves the question of whether each cell in the population is endowed with the "50 passage potential" or, alternatively, whether there exists a random distribution of passage potentials among individual cells composing the population which results in an average 50 passage potential for the entire population.

In order to investigate the possibility that cell progeny derived from different single cells of the same strain may show differences in the passage level at which they enter Phase III, three randomly selected clones were isolated from three Petri dish cultures of WI-38. This was done at passage

level two of the original unfrozen series. The three clones, designated C1, C2 and C3, were each transferred to a milk dilution bottle, incubated at 37°C in a CO_2 incubator, and allowed to reach confluency. They were then subcultivated semi-weekly at a 2:1 split ratio and the total number of passages was recorded starting with number one at this first bottle culture (first 2:1 split, post-cloning). As summarized in Table III, the three cloned cultures entered Phase III at passages 30, 27 and 26, respectively. These numbers do not include the two subcultures carried out before cloning and the cell *doublings* that occurred after cloning and prior to reaching confluency in the milk dilution bottle.

TABLE III. *Accrued doublings of three cloned populations of WI-38.*
Figures based on one doubling per 2:1 split.

Clone	Number of doublings before cloning		Calculated number of doublings for 1 cell to reach a density of 4×10^6		Number of doublings accruing after first 2:1 split, post-cloning		Total number of doublings
C1	2	+	22	+	30	=	54
C2	2	+	22	+	27	=	51
C3	2	+	22	+	26	=	50

A more accurate appraisal of the results should be based on a compilation of numbers of generations or, more precisely, average doublings of the population. The number of cell doublings necessary to reach a density of 4×10^6 (average cell content of a confluent culture in a milk dilution bottle) is 22. Thus the results tabulated in Table III include these data.

Unless the choice of these three clones was fortuitous, it appears that each *clonable* cell within the population is endowed with the same "50 doubling potential". Although the populations had only undergone a total of 32, 29 and 28 serial 2:1 subcultivations before entering Phase III, the added number of doublings accumulated during the cloning manipulation, in which a single cell was raised to a population of 4×10^6 cells, resulted in the expected value of a total of 50 doublings.

The results of this experiment simply serve to underline the fact that the number of generations expected from a human diploid cell strain is not strictly a function of the number of 2:1 serial subcultivations but rather of

the number of cell doublings, and that each clonable cell is endowed with the same doubling potential.

Furthermore, it would be predicted that it should not be possible to initiate a cloned population from, for example, cells of the 35th doubling or greater,

TABLE IV. *Occurrence of Phase III in mixed populations.*

Mixture	Passages of "oldest" component before mixing		Passages accruing after mixing		Total passages	Passages of "oldest" culture (unmixed control)
WI-26 VIII Passage 21 + WI-26 II Passage 45	45	+	29	=	74[a]	60 (WI-26 II)
WI-26 VIII Passage 21 + WI-26 IX Passage 11	21	+	42	=	63	46 (WI-26 VIII)
WI-26 IX Passage 11 + WI-26 II Passage 45	45	+	38	=	83[a]	60 (WI-26 II)
				Average ...	73.3[a]	55.7

[a] Values greatly in excess of any ever observed.

and expect that population to increase to 4×10^6 cells. This failure would be expected since the 35 doublings preceding cloning must be added to the 22 doublings necessary to raise a single cell to a density of 4×10^6. This range of 57 doublings would be near the outside limit, at which time Phase III would be expected to occur. Such is the case. Seven attempts to clone a fetal human diploid population at doublings greater than 30 have consistently failed to yield populations giving rise to as many as 4×10^6 cells. The clones that do result reach Phase III before any subcultivation is necessary.

Mixing populations of cells with different doubling potentials.—We had suggested [14] that the Phase III phenomenon was intracellularly determined. In that previously described experiment the male human diploid cell strain WI-1 at the 49th passage (Phase III) containing many metabolically active, non-dividing cells was mixed with a suspension of actively dividing female cells of strain WI-25 at passage 13 (Phase II). Metaphases from the mixed population, examined at 17 passages post-mixing, were found to consist entirely of female cells. The female cells of the mixed culture ultimately entered Phase III at about the same passage level as the unmixed control culture of WI-25. Thus it was concluded that "old" cells had no detectable

effect upon "young" cells or *vice versa*. This experiment also demonstrated the unlikelihood that latent micro-organisms (or media composition) could account for Phase III, since it is probable that the virus spectra of various human diploid cell strains are qualitatively similar [12, 14, 40]. Final proof

TABLE V. *Occurrence of Phase III in mixed populations.*

Mixture	Passages of "youngest" component before mixing		Passages accruing after mixing		Total passages	Passages of "youngest" culture (unmixed control)
WI-26 VIII Passage 21 + WI-26 II Passage 45	21	+	29	=	50	47 (WI-26 VIII)
WI-26 VIII Passage 21 + WI-26 IX Passage 11	11	+	42	=	53	55 (WI-26 IX)
WI-26 IX Passage 11 + WI-26 II Passage 45	11	+	38	=	49	55 (WI-26 IX)
				Average . . .	50.7	52.3

of these conclusions would, however, depend upon the outcome of an experiment in which cells from a given strain, approaching Phase III, were mixed with cells from the same strain reconstituted from frozen stock at an earlier passage (Phase II).

Cell cultures of human diploid cell strain WI-26 were reconstituted from frozen stock on different calendar dates at passage levels two (series II, Fig. 1), five (series VIII, Fig. 1) and seven (series IX, Fig. 1). When each had reached passage levels 45, 21 and 11, respectively, by semi-weekly subcultivations at a 2:1 split ratio, two cultures of each were counted and split 2:1. Since all experiments were performed in duplicate, one culture of each served as a control and the other two were mixed in equal numbers while in suspension (according to Table IV), planted and subsequently split 2:1, semiweekly, exactly as were the controls.

The purpose of this experiment, therefore, was to ascertain whether cells of the same strain from various widely separated passage levels would influence each other, as measured by the commencement of Phase III. If it were assumed that the "oldest" component of each mixed population survived (a possibility made implausible by the experiment previously described employing mixed male and female cells), Table IV would result. This ex-

planation of replacement by the "older" component results in passage levels of 74 and 83; levels not in keeping with any values ever obtained. Furthermore, these figures do not compare favorably with the controls.

Based on the supposition in Table V that the passage level at which Phase III occurred in the mixture was a function of the continuing multiplication of the "youngest" half of the mixed population after total loss of the "oldest" component, it is apparent that the observed values of total passages after mixing conform to expectations. The following conclusions can thus be drawn: Phase III in a human diploid cell population occurs at that time when the "youngest" cell component in a mixed population is expected to reach Phase III. The "older" cell component has no apparent effect upon the "younger" cells in such mixed populations. This experiment, incidentally, using a homogeneous cell system, again substantiates the previous conclusion [14]

TABLE VI. *A comparison of the passage levels at which Phase III occurred in human diploid cell strains of adult and fetal origin.*

All strains cultivated at a 2:1 split ratio. Fetal strains derived from donors of 3–4 months' gestation obtained by surgical abortion. Adult and fetal strains derived from both male and female tissue.

Fetal lung		Adult lung			
Strain	Passage level at which Phase III occurred (cell doublings)	Strain	Passage level at which Phase III occurred (cell doublings)	Age of donor	Cause of death
WI-1	51	WI-1000	29	87	Heart failure
WI-3	35	WI-1001	18	80	Cerebral vascular accident
WI-11	57	WI-1002	21	69	Bronchial pneumonia
WI-16	44	WI-1003	24	67	Dissecting aneuryism
WI-18	53	WI-1004	22	61	Renal failure
WI-19	50	WI-1005	16	58	Rheumatoid arthritis
WI-23	55	WI-1006	14	58	Pulmonary embolus
WI-24	39	WI-1007	20	26	Auto accident
WI-25	41				
WI-26	50				
WI-27	41				
WI-38	48				
WI-44	63				
Average	48		20		
Range	35–63		14–29		

that Phase III cannot be explained by the presence of a latent virus, mycoplasma or media composition.

Occurrence of Phase III in adult human diploid cell strains.—If, as has been determined, human diploid cell strains of fetal lung origin reach Phase III

TABLE VII. *A comparison of the passage levels at which Phase III occurred in parental and frozen substrains of adult lung human diploid cell strains.*

All strains cultivated at a 2:1 split ratio.

	Parental culture (never preserved)	*Substrain reconstituted from liquid nitrogen*		
Strain	Passage level at which Phase III occurred (cell doublings)	Preserved at passage	No. of weeks frozen	Passage level at which Phase III occurred (cell doublings)
WI-1000	29	6	6	23
WI-1004	22	6	5	20
WI-1003	24	8	2	20

at about 50 ± 10 passages, it would be of interest to compare the occurrence of Phase III in similar cultures derived from adult human lung. Eight diploid strains of adult human cells have been compared with our 13 fetal strains under identical cultivation conditions. A number of strains from both groups were cultivated simultaneously utilizing common sources of reagents and glassware. In addition, all strains were cultivated in duplicate by two different individuals.

All of the fetal lung strains were derived from fetuses of approximately three months' gestation. The adult strains were derived from lung tissue obtained at death at ages and from causes indicated in Table VI. The human adult diploid cell strains were morphologically indistinguishable from the fetal strains. Such properties as growth rate, nutritional requirements and others that were investigated indicated that no parameter other than the total number of doublings occurring during Phase II, could distinguish adult from fetal human diploid cell strains. A comparison of the number of doublings obtained prior to Phase III for the eight adult and 13 fetal human diploid cell strains is given in Table VI.

The average number of cell doublings accruing in adult lung strains prior to cessation of mitotic activity is 20 (range 14–29) which is significantly less

than the average number of 48 doublings (range 35–63) obtained with fetal lung strains.

There appears to be no exact correlation between the age of the donor and the doubling potential of the derived strain. If such a relation does, in fact, exist, it cannot be detected by the present crude methods of determining doubling potential. It is clear, however, that there is a significant difference between the doubling potentials of human diploid cell strains when derived from lung tissue of either fetal or adult origin.

It was also of interest to determine whether the doubling potential of adult strains preserved in liquid nitrogen is similar to that of the original unfrozen culture. The results of such a comparison, utilizing three different adult strains and one thawed ampule of each, are given in Table VII. The interpretation of these results is identical to that obtained with the fetal strains, in that the onset of Phase III is unrelated to absolute calendar time but is related to the total time during which the culture is actively proliferating. Furthermore, the passage potential of frozen adult strains is similar to that of the original unfrozen cultures from which they were derived. In both cases the passage potential is substantially less than that obtained with unfrozen or frozen strains derived from human fetal lung.

DISCUSSION

The finite lifetime of human diploid cell strains *in vitro* has been quantitatively examined and found to be related only indirectly to numbers of subcultivations at a particular split ratio. The effect is more precisely related to a finite number of cell doublings. Cloning experiments have led to the conclusion that the doubling potential is the same for each clonable cell in the population.

This event is not influenced by the presence of cells in the culture with a reduced doubling potential, as demonstrated by an experiment in which cells of the same strain were mixed at three widely separated passage levels. In all cases the total doublings accrued by the mixed cultures before extinction was a function of the doubling potential of the "youngest" component of the population. This coincided with the passage level at which Phase III occurred in the unmixed controls. It is possible that the Phase III phenomenon of cultured human diploid cell strains may be related to senescence *in vivo*. In this regard four points are relevant.

The cellular theory of aging.—A cellular theory of aging is generally considered unacceptable because of the apparent "immortality" of cell cultures

[4, 22, 23, 26]. This general belief is based on the "immortality" of those cell cultures (cell lines) now known to share many, if not all, of the characteristics associated with malignant cells [12, 14]. During the development of cell culture techniques from the beginning of this century until the early 1930's, it was apparent that, regardless of the vertebrate tissue of origin, cell populations derived *in vitro* could be kept in an active state of multiplication for a varied but finite period of time. This finite period of cell proliferation could not, generally, be extended much over a year. Variations in media composition, cultivation techniques, incubation temperature, and other parameters investigated by early workers failed to change this course of events. In fact, it was concluded that the primitive methodology used for *in vitro* cell cultivation was reason enough for the short term cultivation of cells *in vitro*. It is our contention that the finite lifetime of unaltered or diploid cell strains is an innate characteristic of the cells, unrelated to known techniques for optimum cell cultivation. One possible exception to this generalization was the highly popularized development from Carrel's laboratory wherein it was claimed that a population of cells derived from embryonic chick heart tissue was kept in serial cultivation for 34 years [25]. Since, even with more modern and sophisticated cell culture techniques, chick fibroblast cultures do not survive more than a year, there is serious doubt that the common interpretation of Carrel's experiment is valid. An alternative explanation of Carrel's experiment is that the method of preparation of the chick embryo extract, used as a source of nutrient for his culture and prepared daily under conditions permitting cell survival, contributed new, viable, embryonic cells to the chick heart strain at each subcultivation or feeding [9]. A consideration of the details of this experiment [9] indicates that waves of mitotic activity in his cultures were coincidental with the periodic addition of chick embryo extract. In any event, Carrel's experiment has never been confirmed.

It remained for Gey [10] in 1936 and Earle [8] in 1943 to demonstrate that cell populations derived from a number of mammalian tissues, including human tissue, could unequivocally be kept in a state of rapid multiplication for apparently indefinite periods of time. Since this pioneer work, cell populations with the extraordinary capacity of being able to multiply *in vitro* indefinitely have been spontaneously derived from at least 225 mammalian tissues [13]. A consideration of the characteristics of these populations, referred to by us previously as "cell lines" [14], has led to the conclusion that such cell lines, regardless of whether the tissue of origin is normal or cancerous, share properties with cancer cells. First, they are heteroploid, as are all transplantable solid tumors. Second, when inoculated into suitable

hosts, they form tumor masses. Third, less definitive tests, such as staining and microscopic examination, have indicated that cell lines share those properties that are usually descriptive of cancer cells. Conversely, cell strains are diploid and fail to exhibit such properties. The relationship that cell lines bear to cell strains is identical to the relationship that transplantable tumors bear to normal tissue. The former two systems are assumed to be *in vitro* expressions of the latter *in vivo* systems.

HETEROPLOID CELL LINES	:	TRANSPLANTABLE TUMORS	=	DIPLOID CELL STRAINS	:	NORMAL SOMATIC TISSUE
(*in vitro*)		(*in vivo*)		(*in vitro*)		(*in vivo*)
1. Heteroploid				1. Diploid		
2. Cancer cells (histological criteria)				2. Normal cells (histological criteria)		
3. Indefinite growth				3. Finite growth		

Thus the phenomenon of the alteration of a cell strain to a cell line [14] is important because, in its simplest terms, it can be regarded as oncogenesis *in vitro*. Spontaneous alterations do occur in human cell cultures but at a very low frequency and only a few photographs of this event have ever been published [11]. A set of precise environmental conditions under which alterations of human cells could take place were, until recently, unknown. The spontaneous alterations described in the literature [13] have arisen under many different kinds of cultural conditions. Reproducible conditions for inducing such alterations would be a most powerful tool for the study of the *in vitro* conversion of normal human diploid cells to cancer cells. Recently, it was discovered [18, 19, 32, 36] that the infection of primary cultures or human diploid cell strains, with the virus $S.V._{40}$ could provide these conditions.

Since heteroploid cell lines are known to possess qualities characteristic of cancer cells, the cellular theory of *in vivo* aging should be related to activities of *normal diploid cells in vitro* rather than to *heteroploid cell lines in vitro*. On this basis the cellular theory of aging must be reconsidered, since it has been shown [14, 36, 41] that normal human diploid cell strains *in vitro* are, in fact, "mortal". To our knowledge no one has thus far reported that cells having the karyotype of the tissue of origin have been able to multiply *in vitro* longer than the lifespan of the animal species from which the tissue was obtained. Cells which can be cultivated indefinitely *in vitro* (heteroploid cell lines) can only be compared with continuously cultivable cells *in vivo*, i.e., transplantable tumors. Likewise, diploid cells having a finite lifetime

in vitro can only be compared with normal cells *in vivo*, i.e., normal somatic cells.

The finite lifetime of cells in vivo.—The above relationship had led us [14] to consider an experiment designed to test the question as to the length of time normal tissue could be grown when transplanted from animal to animal in an attempt to escape from the normal senescence of the host. Recently, two series of experiments have been performed which bear upon this question.

A series of transplantation experiments devised by Krohn [20] appears to demonstrate that there *is* a finite period of cultivation of normal mammalian somatic cells *in vivo*. Using skin transplants from inbred strains of mice, Krohn attempted to determine whether skin tissue has an indefinite lifespan when transplanted from one host to another. His studies revealed that grafts from young donors remained in satisfactory condition for about 650 to 1000 days and after two to five transplantations. However, the grafts began to decrease in size at that time and between 850 and 1750 days the transplants had become "minute areas of skin which were unsuitable for further transplantation". In comparison with the longest recorded lifespan of 3½ years for any mouse [28], the maximum lifespan of skin transplants ranged from 4½ to 5 years. What is most important is that the skin transplants *did* demonstrate a finite period of cultivation *in vivo*, as do normal diploid mouse fibroblasts *in vitro* [16, 29, 37]. Transplanted normal mouse tissue does not exhibit the kind of immortality characteristic of transplantable mouse tumors, a number of which have been passaged for decades *in vivo* [38], as have heteroploid mouse cell lines *in vitro*.

Krohn [20] investigated time-chimeras by studying the viability of aged skin grafted onto young animals and observed that after initial growth the old grafts failed at an overall age far short of the normal lifespan of the host mouse. Thus, the results of these *in vivo* transplantation experiments with mouse tissue parallel directly the results reported here for human diploid cell strains *in vitro* derived from fetal and adult lung tissue. That is, the passage potential or expected number of cell doublings is generally related to the age of the donor. Any successes with indefinitely cultivable mouse cells that have been reported have always been the result of the diploid cell population altering to a heteroploid or to a near-diploid cell line. This event always occurs *in vitro* in far less time than the average life expectancy of the mouse.

A similar series of experiments performed by De Ome and his associates with transplanted mouse mammary tissue has resulted in similar findings [6]. Normal mammary tissue from adult C3Hf/Crgl ♀ mice was transplanted

into a group of three-week-old (virgin) C3Hf/Crgl ♀♀ mice who, when five months old served as donors for the next transplant generation. This process was repeated for 40 to 45 months when the outgrowths could no longer be found in the living animals and thus could not be further transplanted. This transplantation procedure has been repeated and the C3Hf normal tissue maintained by serial transplantation in animals less than five months of age was not recoverable after 40 to 45 months of transplantation. In addition, in two trials with normal mammary tissue from an adult C3H/Crgl ♀, which was serially transplanted using exactly the same method described above for the C3Hf/Crgl tissue, the normal tissue could no longer be recovered after 20 to 30 months. Finally, when normal mammary tissues from BALB/c/Crgl ♀♀ were used in a similar experiment, they could not be recovered after a period of only nine months. Similar serial transplantation experiments carried out with hyperplastic alveolar tissue rather than normal mammary tissue yielded, however, quite different results. Three such tissues which were serially transplanted for more than five years through more than 20 transplant generations have to date shown no reduction in growth rate and tumors derived from these three strains have been carried for many years without apparent loss of viability [6].

The implication of these two series of experiments may be, therefore, that the acquisition of potential for unlimited cellular division or the escape from senescent-like changes by mammalian somatic cells, even *in vivo*, can only be achieved by cells which have altered and assumed properties of cancer cells. This applies equally well to normal mammalian somatic cells growing *in vivo* or *in vitro*.

Chromosome anomalies associated with "old" cells in vivo and in vitro.—A number of studies [3, 14, 21, 34, 35] have shown that the karyotype of human fibroblast cells in long-term culture is very stable. More recently two reports have demonstrated that some aneuploidy and other aberrations do occur, but only in Phase III of the *in vitro* life of such cell populations [30, 41]. In the report of Saksela and Moorhead [30], aneuploidy was first detected at about the 40th passage in a number of sub-strains of both WI-26 and WI-38 as well as in very late passages of two other strains. In the work of Yoshida and Makino [41] there was no karyotype variation between the first and the 41st subcultures, but cells from the 45th to the 47th passages showed striking chromosome aberrations and their strain could not be subcultivated beyond the 48th passage. These metaphase studies only confirm an earlier study by Sax and Passano [31] in which it was shown that anaphase anomalies increased with age *in vitro* over a period of six months' subcultivation. Ab-

normally large interphase nuclei and bizarre nuclear shapes were also described by us in late passaged human diploid cell strains [14].

This direct correlation between age *in vitro* and the appearance of chromosome aberrations suggests that the chromosome damage itself may be responsible for the failure of the culture. Such an explanation would be similar to somatic mutation theories of aging already offered. It is equally plausible, however, that loss of proliferative ability and chromosome damage occur independently.

In this connection a relationship between spontaneous somatic cell aberrations *in vivo* and *natural* aging has been demonstrated by scoring anaphase anomalies in regenerating liver tissue of mice [5]. Strains of mice with different life expectancies revealed corresponding differences in the incidence of aberrations observed. Also, *within* each strain there was an age-correlated increase in anaphase and telophase aberrations scored following partial hepatectomy. Of even greater interest is the recent observation of Jacobs *et al.* [17] who have found in man that increased hypodiploid counts in peripheral blood leucocytes are correlated with the chronologic age of the donor. There exists, therefore, some *in vivo* evidence of age-associated chromosomal anomalies that may also be involved in the limitation upon the proliferation of human diploid cells *in vitro.*

Occurrence of Phase III as a function of donor age.—The experiments described which demonstrate a significantly decreased doubling potential for strains derived from human adult lung tissue appear to parallel the results of Krohn [20]; wherein, it was observed that the growth potential of skin transplants from older mouse donors was far less than that of skin transplants derived from young donors. The implications of these results are that normal tissue, when cultivated *in vitro* or transplanted *in vivo,* has a finite period of multiplication and, furthermore, that the age of the donor of such tissue, under either condition of cultivation, is directly related to the expected growth potential. In this connection, it was exactly 50 years ago that Carrel [1] observed that "fragments of connective tissue taken from an embryo, or from young adult and old animals and placed in normal adult chicken plasma grew at different rates". He concluded that "the velocity of the growth always varied in inverse ratio to the age of the animal from which the tissue had been extirpated".

MECHANISM: The mechanism of the Phase III phenomenon in cultured human diploid cell strains remains to be elucidated. When cell counts are made after each serial subcultivation of such strains and are plotted against time, the curves described in Figs. 2 and 3 are obtained.

The shape of these curves is similar to multiple-target or multiple-hit curves. Such survival curves are commonly obtained, for example, by plotting effects of irradiation on *E. coli* [39] or on human cells [27]. An initial threshold dose is required before an exponential form of the curve is established. Although it is not known whether background irradiation contributes to the Phase III phenomenon, the survival curves obtained (Figs. 2 and 3) are similar to such "dose-effect" curves and allow for hypotheses concerning mechanism:

(*a*) Each cell contains n targets, each of which must be inactivated. This is the multi-target concept.

(*b*) Each cell contains a single target which must sustain n hits before the target is inactivated. This is the multi-hit concept.

Ordinarily the extrapolation of the exponential portion of a multiple-target or multiple-hit curve to the ordinate gives a value for n which is the average number of targets struck per cell or the number of "hits" required to inactivate a single target. The crude method by which the exponential portion of the curves in Figs. 2 and 3 is obtained does not allow for an accurate extrapolation, since even slight inaccuracies in the slope of the exponential portion of the curve will result in a large error. However, it is apparent that the number of targets or number of hits on a single target would be large.

Such interpretations follow from the curves in Figs. 2 and 3 which describe an initial plateau with no apparent loss of potential to multiply as measured by the constant doubling time during Phase II, followed by an exponential loss of this function (exponential increase of doubling time in Phase III). Similar curves for other human diploid cell strains have also been described [36]. The plateau indicates that loss of function may require an accumulation of damage caused either by mechanisms (*a*) or (*b*) above.

Thus whatever the cell component(s) involved may be, the inactivation of which results in the Phase III phenomenon, the ultimate accumulation of non-dividing cells could be the result of accumulated heritable damage to some sensitive intracellular target(s). This accumulated damage may further result in what has now been described cytologically as an accumulation of aneuploid cells [30, 41] at Phase III.

We propose, therefore, that the basic step in the Phase III phenomenon is an accumulation of "hits" or errors in DNA replication which inactivates part of the genome. It is further assumed that the hits are random and that per unit period of time the probability that a part of the genome suffers such a hit is constant.

The mathematical expression of the type of multi-hit curve in Figs. 2 and 3

in which an initial threshold must be reached before an exponential form of the curve is established is similar to the mathematical model of *in vivo* aging postulated by Szilard [33] in which death occurs when the amount of genetic damage reaches a threshold level. The reduced doubling potential or earlier occurrence of Phase III in human adult diploid lung strains may imply that these cell populations grown *in vitro* had already acquired, *in vivo*, a significant number of hits. This may be analogous to the variation in inherited "fault loads" postulated by Szilard [33] to account for individual variations in time of death as a result of senescence.

Any satisfactory theory of senescence at the cellular level, including the somatic mutation theory, must include, as a corollary, an explanation for the apparent lack of senescent-like changes in transplantable tumors *in vivo* and in heteroploid cell lines *in vitro*. Lacking any evidence on this point, it could be argued that escape from the inevitability of aging by normal cells *in vivo* and diploid cell strains *in vitro* is only possible when such cells acquire, respectively, properties of transplantable tumors or heteroploid cell lines. One of the common denominators of these latter two systems is heteroploidy (usually modally distributed) which, when acquired by the cell may be the mechanism needed for it to circumvent the inevitability of death and thus escape from senescence.

SUMMARY

The time at which human diploid cell strains can be expected to cease dividing *in vitro* (Phase III) is not a function of the number of subcultivations but rather of the number of potential cell doublings. Each clonable cell within the population is endowed with the same doubling potential (50 $\pm$10). Cells of the same strain, but with different "doubling potentials", were mixed. Phase III in such mixed populations occurs at that time when the "youngest" cell component is expected to reach Phase III. The "older" component has no effect on the time at which Phase III is expected to take place in the "younger" component. An ancillary conclusion that Phase III cannot be explained by the presence of a latent virus, mycoplasma or media composition is confirmed. Human diploid cell strains derived from adult lung have a significantly lower doubling potential *in vitro* than do fetal strains. The Phase III phenomenon may be related to senescence *in vivo*. The cellular theory of aging must be related to normal cells *in vitro* and not to heteroploid cell lines. The former have a finite period of multiplication; the latter are indefinitely cultivable. *In vivo* experiments also indicate that transplanted normal tissue has a finite lifetime. Chromosome anomalies occurring in Phase III may be

related to such anomalies occurring in the cells of older animals, including man. The survival curves obtained with human diploid cell strains are comparable to "multiple-hit" or "multiple-target" curves obtained with other biological systems where an initial threshold dose is required before an exponential form of the curve is established. Whatever cell component(s) may be involved in the finite lifetime of human diploid cell strains, the ultimate accumulation of nondividing cells could be the result of accumulated damage to a single cellular target or to inactivation of many targets.

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