VARIATION IN THE LIFE-SPAN OF CLONES DERIVED FROM HUMAN DIPLOID CELL STRAINS

J. R. SMITH and L. HAYFLICK

From the Department of Medical Microbiology, Stanford University School of Medicine, Stanford, California 94305. Dr. Smith's present address is Veterans Administration Hospital, Martinez, California 94553.

ABSTRACT

The doubling potential of several hundred clones derived from WI-38 and WI-26 cell cultures has been determined. Clones were isolated at various population doubling levels (PDLs) during the finite in vitro life-span of the mass (uncloned) cultures. In all cases, there was a large variation in population doubling potential (or life-span) among the clones isolated from a single mass culture. When clones were isolated from mass cultures which had undergone eight or nine population doublings, only about 50% of the clones were capable of more than eight population doublings. This percentage was further reduced when clones were isolated from mass cultures at higher PDLs. Mass cultures appear to be composed of two subpopulation classes: one with a low population doubling potential, and the other with a higher population doubling potential. Nevertheless, the highest doubling potential observed in clones isolated from any single culture was about the same as the doubling potential of the mass culture from which single cells were taken.

INTRODUCTION

Since our suggestion in 1961 (9) that the limited in vitro life-span of normal human diploid cells (phase III phenomenon) might be a manifestation of aging at the cellular level, an increasing number of investigators have used these cells as a model system in the study of aging. Much of this effort has been directed toward finding and quantitating age-related changes in the biochemistry, immunology, or morphology of mass cultures (1-3, 10, 11, 14). One important consideration in studies of this kind is the possibility that the cells studied might represent a heterogeneous population composed of cells at different population doubling levels (PDLs).

If one is, indeed, studying variables whose values depend upon the remaining doubling potential of cells in mass cultures, a heterogeneity of doubling potential among the cells within a culture is likely to increase the difficulty of detecting changes which occur during the finite in vitro lifetime of cultured normal cells. We previously found that for three clones isolated from a mass culture after the second population doubling, the doubling potential of each clone was about the same as that of

the mass culture (8). We interpreted this to mean that the doubling potential of each clonable cell was about the same. This early interpretation has come under question in the light of our more recent findings.

Cultures of WI-38 cells have been found to be heterogeneous with respect to their ability to divide (15) and to synthesize DNA (4). The number of cells unable to synthesize DNA or divide increases exponentially with the age of the culture. Moreover, evidence from time-lapse cinemicrophotography shows that early in their in vitro life-span, normal human cells have a 3.5–18% probability of dying without dividing (16).

The study reported here was undertaken to determine the degree of heterogeneity in doubling potential within single mass (uncloned) cultures of WI-38 cells. The doubling potential was determined for many individual cells isolated from mass cultures at various PDLs during their in vitro life-span. Our findings have a significant bearing on delineating the mechanism of the phase III phenomenon and on aging at the cellular level.

MATERIALS AND METHODS

Cell Cultures

The human fetal diploid lung fibroblast strains used were WI-38 derived from a female fetus and WI-26 derived from a male fetus (8, 9). The methods used for manipulating these cells have been described previously (8, 9). Cell cultures were initiated from eighth (WI-38) or 19th (WI-26) PDL ampules of cells stored in liquid nitrogen since 1962.

Cloning Media

Several media were used to establish clones from isolated single cells. All media consisted of Eagle's basal medium (BME) with various additions and modifications.

CMI: Half fresh, half conditioned BME supplemented with 15% fetal calf serum, twice the usual concentration of vitamins and amino acids (15), and 0.4% methylcellulose (Methocel 12, Union Carbide Corp., Linde Div., New York).

CMII: BME supplemented with 30% calf serum, 28 mM HEPES (Sigma Chemical Co., St. Louis, Mo.) buffer, and 0.4% methylcellulose.

CMIII: BME supplemented with 30% calf serum.

Sufficient sodium bicarbonate was added to each medium to obtain a pH of 7.1 in a 5% CO_2 atmosphere. Since the serum used was found to be the most important and variable constituent, several lots were tested for their ability to promote clonal growth. Serum lots producing the highest plating efficiencies and largest clone size after 12 days were used.

Isolation of Single Cells

A modification of the method of Martin and Taun (13) was used for isolation of single cells from mass cultures. Glass chips were prepared by crushing no. 1 microscope cover glass (Van Lab, Van Waters & Rogers, Inc., San Francisco, Calif.) with a mortar and pestle. The crushed glass was passed through a series of sieves, and chips were selected which passed through 1-mm diameter pores but not through 0.5-mm diameter pores. The chips were washed thoroughly in running deionized water to remove the fine glass powder which tended to adhere to them and were subsequently sterilized by autoclaving. Approximately 1×10^4 cells were planted in 60-mm plastic Petri dishes (Falcon Plastics, Oxnard, Calif.) containing glass chips ranging in area from 1 to 0.25 mm^2. 5 ml of cloning medium was added to each dish which was then incubated overnight at 37°C in a humidified desiccator jar containing an atmosphere of 5% CO_2 in air. Dishes were then scanned using an inverted microscope placed inside a horizontal laminar flow hood. Using fine sterile forceps, glass chips found to contain only one cell were transferred to wells having a 1-cm^2 surface area (one chamber of a Lab-Tek 8 chambered slide, Lab Tek Products, Miles Laboratories, Inc., Miles Research Div., Kankakee, Ill.). Each chamber contained 0.25 ml of cloning medium which was equilibrated in a 5% CO_2 atmosphere to pH 7.1. In some cases it was necessary to scrape all except one cell from a glass chip with the tip of the forceps.

Growth of Clones

The cloning chambers, each containing a single cell, were incubated at 37°C in a 5% CO_2 atmosphere, and cell replication was monitored at weekly intervals. When a clone had covered most of the area of a glass chip, the cells were detached with trypsin, dispersed by aspiration in 0.25 ml of cloning medium, and the cells allowed to attach to the surface of the 1-cm^2 cloning chamber. When this area was covered, the clone was dispersed as described and transferred to a 25-cm^2 T flask (Falcon Plastics) containing 2 ml of cloning medium. In order to maintain the seeding density (cell/square centimeter) as high as possible at this transfer, the flasks were placed on end so that the cells attached over an area of approximately 5 cm^2. A clean sterile microscope cover slip cut to approximately 1×4 cm was placed in the bottom of the flask. This provided a better surface for cell growth than the end of the tissue culture flask. When the clones formed an almost confluent monolayer on the available area, they were again dispersed and allowed to attach to the 25-cm^2 surface of the flask in 5-ml cloning medium. Thereafter, the clones were treated as mass cultures and subcultivated weekly at a 1:4 split ratio in BME supplemented with 10% fetal calf serum. When cell

multiplication diminished (phase III) the cultures were subcultivated at a 1:2 split ratio at weekly or bimonthly intervals.

Estimation of Clonal Life-Span

The number of cells in each isolated clone was determined at weekly intervals until they reached a size sufficient to form a confluent monolayer in a 25-cm^2 tissue culture flask. When clones did not increase appreciably in cell number over a period of 2 wk, they were considered to have completed their in vitro life-span. The maximum size reached by each clone was noted and the number of population doublings undergone by the progeny of a single cell calculated (e.g., a clone size of 1,000 cells corresponds to 2^{10} cells or 10 population doublings). The number of cells per clone was determined in the following way:

(*a*) 0–50 cells per clone—direct count under an inverted microscope.

(*b*) 50–10^6 cells—estimated from the area covered by the clone and the cell density in the clone. The accuracy of this method was confirmed by direct count of cells in some clones whose size had been estimated. When the estimate of cell number was 200 or less, the actual number of cells was determined by counting cells in fixed and stained clones under a microscope at ×250 magnification. When the estimate was greater than 200, the cells were dispersed and counted in a Coulter counter (Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla.).

(*c*) It was found that about 20 population doublings were necessary (i.e., 10^6 cells) to obtain a confluent monolayer in a 25-cm^2 cell culture flask. After clones had reached that size, two population doublings were assumed for each subcultivation done at a 1:4 split ratio. One population doubling was assumed for each subcultivation done at a 1:2 split ratio. Good (6) has shown that only the initial inoculum in a series of flasks and the yield from the final flask in a series need be considered when calculating the number of population doublings. Therefore, this method of estimating the number of population doublings will not be in error by more than one or two population doublings.

RESULTS

Clones derived from mass cultures at a variety of PDLs were cultured until the population of cells could no longer double. The percentage of cells isolated from WI-38 at PDL 8 (50 clones established using CMI as medium) and PDL 9 (216 clones established using CMIII medium) which were able to undergo a specific number of population doublings was determined and is plotted in Fig. 1. About 85% of the cells isolated at PDL 8 and 9 were able to divide at least once. About 95% of the cells which were able to divide once gave rise to clones capable of doubling at least once more. The percentage of clones which contained progressively larger numbers of cells decreased linearly up to clone sizes equivalent to about eight population doublings, or 256 cells. Subsequently, the percentage of clones containing progressively

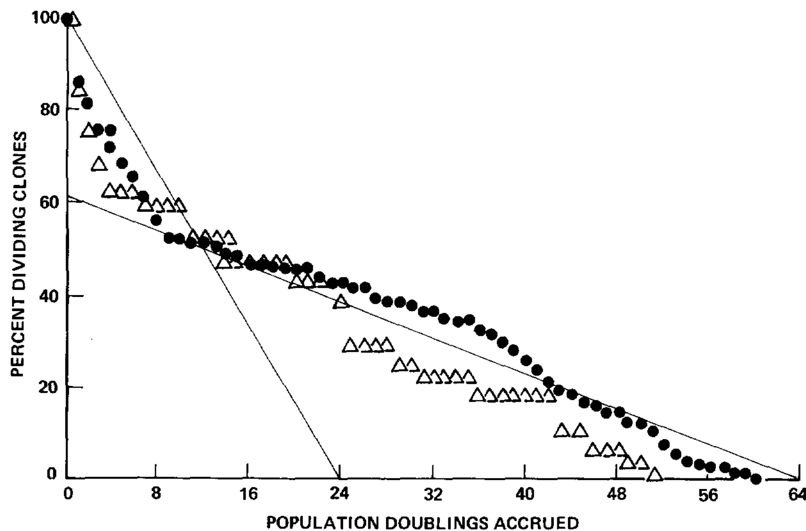

FIGURE 1 The percentage of isolated cells undergoing a specific number of population doublings. The two sets of clones were from different WI-38 subcultivation series. In one set, 216 clones were isolated at the ninth PDL and grown in CMIII (the mass culture stopped growing at PDL 53) ●; 50 clones isolated at the eighth PDL and grown in CMI (the mass culture reached phase III at the 45th PDL) Δ.

larger numbers of cells decreased at a much slower rate. In the case of cells isolated at PDL 9, about 5% of the clones were composed of cells capable of undergoing more than 53 population doublings after cloning. The mass culture from which the clones were derived was able to undergo 44 population doublings after the clones were isolated. However, almost 20% of the clones isolated from this culture were able to undergo more than 44 population doublings after cloning. The highest doubling potential observed among this set of clones was about 30% higher than that of the mass culture.

Several clones were isolated and established in CMII medium from a single subcultivation series at PDL 22, 28, 30, and 32. The percent of clones reaching particular PDLs is shown in Fig. 2. The data in Fig. 3 are derived from WI-26 cloned at PDL 30 and WI-38 cloned at PDL 32 and 41 using CMII medium. The clones were isolated at the same time, using the same lot of cloning medium. In all cases except one (see Fig. 2, clones isolated after 30 population doublings), the number of clones able to undergo a given number of population doublings was found to decrease rapidly when clone sizes were less than 2^8–2^{10} cells. In that one case, however, most of the clones consisting of two cells went on to yield clones containing at least 2^7 cells. Clones isolated from the same mass culture after only two additional population doublings give rise to a more typical distribution of percent growing clones vs. doubling potential (Fig. 2).

In all of these experiments no clones were found to be capable of population doublings greater than

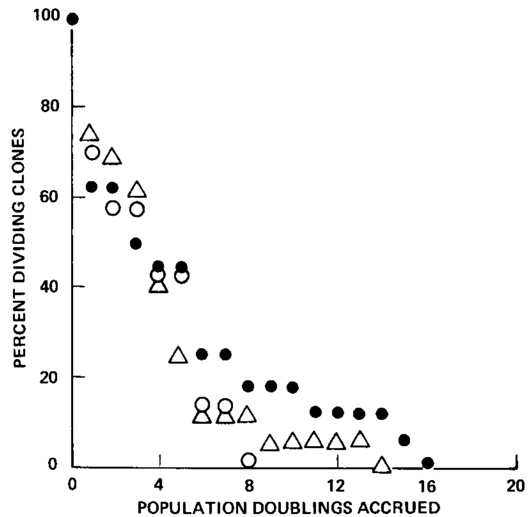

FIGURE 3 Ordinate and abscissa are the same as in Figs. 1 and 2. Sets of clones isolated from different subcultivation series at the same time. Each set consists of 16 clones. All clones were grown in CMII. WI-26 cells cloned at PDL 30. ○; WI-38 cells cloned at PDL 32, ●; WI-38 cells cloned at PDL 41, △. The mass cultures from which these clones were derived reached phase III after 49, 52, and 58 population doublings, respectively.

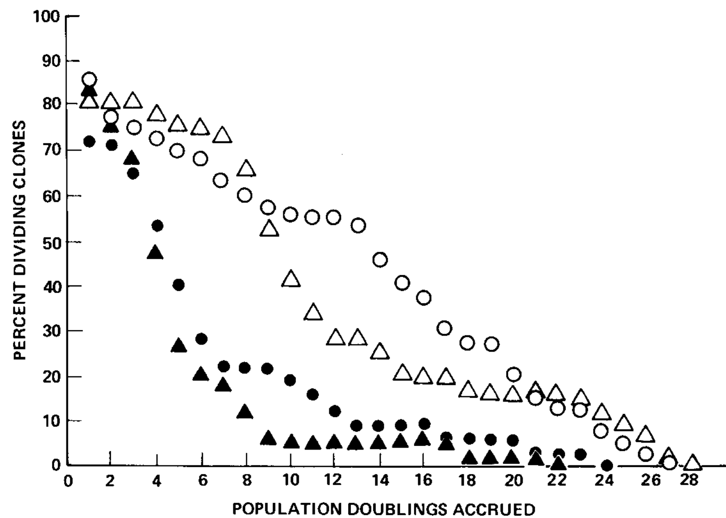

FIGURE 2 The percentage of isolated cells undergoing a specific number of population doublings. All sets of clones were isolated from the same WI-38 subcultivation series. Isolated at PDL 22, ○; 28, ●; 30, △; and 32, ▲ of the mass culture. All clones were grown in CMII. The mass culture reached phase III after PDL 53. Each set consists of from 30 to 50 clones.

30% of those undergone by the mass culture from which they were isolated (Table I). When the mass cultures had already undergone 20 or more population doublings before clones were isolated, the maximum life-span of the clones was very nearly the same as that of the mass culture.

The observation that about 50% of the cells isolated at PDL 9 are unable to give rise to clones larger than 2^8 cells suggests that about half the cells are less than eight population doublings away from the end of their in vitro life-span after only nine population doublings of the mass culture. It must be stressed, however, that the behavior of single isolated cells grown as clones may not accurately reflect the behavior of those same cells in mass culture.

DISCUSSION

The data presented here appear to contradict our earlier conclusions (8) which were based on data from three clones isolated from a mass culture after two population doublings. All three of those clones attained very nearly 50 population doublings. We concluded, therefore, that all clonable cells had a cumulative 50 PDL potential (8). However, those clones were selected from the largest clones present several days after cloning, so that clones with doubling potentials of less than about 15 were not considered. In retrospect, by selecting the most actively growing clones one would expect that those with close to the maximum growth potential might be chosen. If this assumption is correct, our earlier results are compatible with the results presented here.

TABLE I

Maximum Number of Population Doublings of Clones Derived from WI-38 and Compared to Mass Cultures from Which They Were Isolated

PDL* of mass culture at time of cloning	Maximum PDL of clones	Maximum PDL of mass culture
8	60	45
9	69	53
22	50	53
28	48	53
30	57	53
30‡	38	49
32	55	53
32	49	53
41	55	58

* Population doubling level.

‡ Strain WI-26, all others represented are strain WI-38.

Cloning efficiencies of from less than 1 to 50% (5, 7, 13, 17, 18) have been reported for human diploid fibroblasts, using a variety of methods and a variety of ways of determining cloning efficiencies. Hayakawa (7) reported that about 50% of inoculated cells form visible colonies after 10 days for cells cloned before the 10th serial subcultivation. Martin and Taun (13) reported that 8–40% of the cells which attached formed colonies of unspecified size. In our experiments, about 50% of the attached cells from mass cultures at the eighth or ninth PDL formed clones of 100 or more cells. As the PDL of the mass cultures increased, the percentage of clones consisting of 100 or more cells decreased. Thus, our cloning efficiencies are within the limits of previously published data.

It is not presently known whether the cell cohorts that stop dividing early in their in vitro life-span do so for the same reasons as those which fail near the end of their in vitro life-span (phase III). However, if the mechanism is similar, then even a very "young" mass culture (at the ninth PDL) contains about 50% "old" cells and that proportion increases as the mass culture undergoes more population doublings. This high degree of heterogeneity in the age of cells in mass culture presents some difficulty in interpreting age-related biochemical or morphological changes that have been reported in mass cultures of normal human diploid cells (1, 3). If some way could be found to separate the two subpopulations, this might reduce but would not entirely eliminate the difficulties. Since a large proportion of the cells in a young culture is capable of only a few additional population doublings, one might conclude that there is a large variation in the number of divisions which individual normal human fibroblasts can undergo, even though they are derived from the same tissue source. If that is so, the mechanism which establishes the finite in vitro life-span would not be simply the number of cell divisions. However, if one assumes random cell interdivision times, then at any PDL the mass culture would consist of cell cohorts which had undergone different numbers of divisions. A detailed mathematical study of this interpretation which assumes a constant number of potential cell divisions and random interdivision time has been made and predicts heterogeneity in clonal life-span similar to that reported here.[1]

It would be of interest to know how quickly the heterogeneity in cell doubling potential develops

[1] Good, P. I., and J. R. Smith. 1974. *Cell Tissue Kinet.* Submitted for publication.

among the cells in a clone. Information of this kind, taken together with the results presented here, would form a valuable basis on which to assess various hypotheses concerning the limited in vitro life-span of normal human diploid cells. Martin et al. (12) have reported variations in population doublings found during the early development of subclones of human skin fibroblasts. However, these clones were not followed to the end of their in vitro life-span, and the heterogeneity in doubling potential was not determined. The data presented here indicate a high degree of heterogeneity in doubling potential among individual cells composing a mass culture of human diploid cells.

In general, the clones isolated in this study appear to be composed of two subpopulations: one with a maximum doubling potential of about eight population doublings, and the other with a much greater doubling potential. Furthermore, the subpopulation of single cells having low doubling potential appears to increase with increasing PDLs of the mass culture at the time of cloning. About 50% of the clones isolated at PDL 8 or 9 of the mass culture were capable of only eight population doublings while 90% or more of the clones isolated at the 30th–40th PDL of the mass culture had a low doubling potential. It might be expected that all of the cells with low doubling potential would be eliminated from the mass culture within 10 population doublings. However, this is not the case. It appears that cells are recruited into the low doubling potential subpopulation as the mass culture undergoes more population doublings.

It is possible that the subpopulation of clones with low doubling potential is an artifact arising from the cloning process itself. Although this cannot be ruled out with certainty, it is unlikely since we used several different cloning media, all with essentially the same result. Furthermore, Martin et al. (12) have reported similar results for clones derived from human skin fibroblasts and cultured under different conditions.

The heterogeneity observed in clonal doubling potential of cells undergoing more than 15 or so population doublings is not due to the effect of small numbers of cells since these clones contain enough cells to behave like mass cultures. Nevertheless, a high degree of heterogeneity was observed even in clones capable of at least 15 or more population doublings.

We are grateful to Mrs. Aida Zerrudo for excellent technical assistance.

This work was supported in part by research grant HD 04004 from the National Institute of Child Health and Human Development, U. S. Public Health Service, National Institutes of Health.

Received for publication 5 October 1973, and in revised form 21 January 1974.

REFERENCES

1. Brautbar, C., R. Payne, and L. Hayflick. 1972. *Exp. Cell Res.* **75**:31.
2. Cristofalo, V. 1970. *In* Aging in Cell and Tissue Culture. E. Holeckova and V. Cristofalo, editors. Plenum Publishing Corporation, New York. 83.
3. Cristofalo, V. 1972. *Adv. Gerontol. Res.* **4**:45.
4. Cristofalo, V., and B. B. Sharf. 1973. *Exp. Cell Res.* **76**:419.
5. Freeman, A. E., T. G. Ward, and R. G. Wolford. 1964. *Proc. Soc. Exp. Biol. Med.* **116**:339.
6. Good, P. I. 1972. *Cell Tissue Kinet.* **5**:319.
7. Hayakawa, M. 1969. *Tohoku J. Exp. Med.* **98**:171.
8. Hayflick, L. 1965. *Exp. Cell Res.* **37**:614.
9. Hayflick, L., and P. S. Moorhead. 1961. *Exp. Cell Res.* **25**:585.
10. Holliday, R., and G. M. Tarrant. 1972. *Nature (Lond).* **238**:26.
11. Macieira-Coelho, A., J. Ponten, and L. Philipson. 1966. *Exp. Cell Res.* **42**:673.
12. Martin, G. M., C. A. Sprague, T. H. Norwood, and W. R. Pendergrass. 1974. *Am. J. Pathol.* In press.
13. Martin, G. M., and A. Taun. 1966. *Proc. Soc. Exp. Biol. Med.* **123**:138.
14. Mellman, W. J., R. T. Schimke, and L. Hayflick. 1972. *Exp. Cell Res.* **73**:399.
15. Merz, G. S., and J. D. Ross. 1969. *J. Cell. Physiol.* **74**:219.
16. Norrby, K., and J. Mellgren. 1971. *Pathol. Eur.* **6**:56.
17. Pious, D. A., R. N. Hamburger, and S. E. Mills. 1964. *Exp. Cell Res.* **33**:495.
18. Weinstein, D., and M. Lebowitz. 1967. *Exp. Cell Res.* **48**:176.