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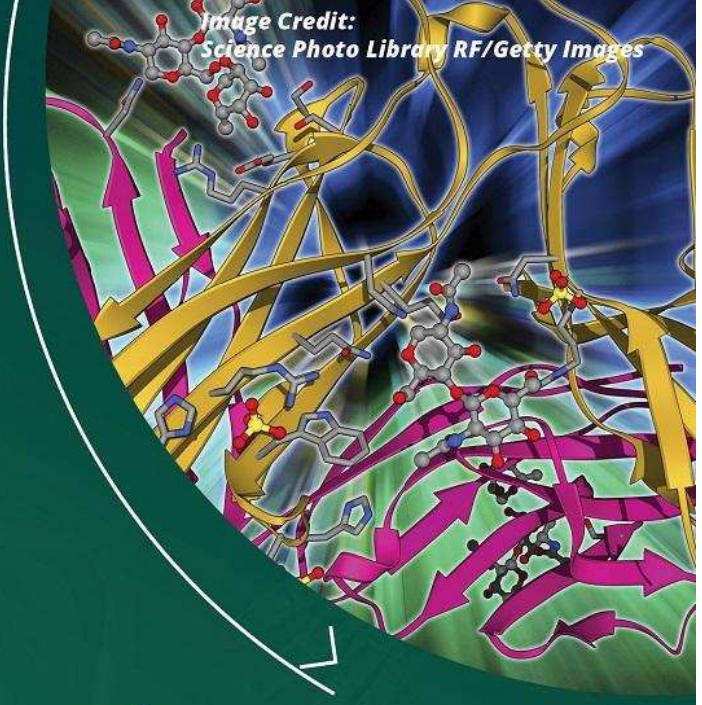

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A Quantitative Analysis of the Aging of Human Glial Cells in Culture

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The kinetics of aging of normal human diploid brain cells in culture have been determined using the miniclone technique in which cells are cloned in the presence of a large number of other cells. The miniclone technique records the behaviour of every viable cell in the sample, not merely those cells capable of forming visible clones. This technique permits the direct measurement of the reproductive potential of individual cells growing in bulk culture and of the dispersion of the sizes of colonies generated by dividing cells. The fraction of cells that are able to divide declines smoothly and continuously from the beginning of in vitro cultures of human glial cells. There is a broad distribution of colony sizes; even at the earliest passages there are significant numbers of small colonies. With increasing age of the culture there is a shift in the distribution, so that fewer large colonies and more small colonies occur. The distribution of intermitotic times is almost identical in young and middle-aged cultures. Our data seem to exclude quite positively any description in terms of a catastrophe or any abrupt change in the population. On the contrary, the decline in reproductive potential may be described adequately either as a linear change with time, or as predicted by the mortality theory of Shall and Stein (1979), in which the single constant, gamma, describes the change in reproductive potential over the entire lifetime.

Normal diploid fibroblasts can be grown in tissue culture and can be transferred from one confluent culture to a new dish in which the cells will again grow. However, it was observed some 25 years ago (Swim and Parker, 1957; Hayflick, 1965; Hayflick and Moorhead, 1961) that normal, diploid cells will continue cellular reproduction for only a limited number of passages in vitro. After a defined number of population doublings characteristic of species and cell type, the culture would cease to reproduce; these normal, diploid cultures are mortal. By contrast, aneuploid, transformed, and tumour cell cultures may be passaged apparently indefinitely; they seem to be immortal. These fundamental observations on cellular aging have been repeatedly and consistently confirmed (Macieira-Coelho, 1974; Macieira-Coelho et al., 1966; Martin, 1977; Martin et al., 1974; Pontén, 1973; Pontén and Westermark, 1980; Schneider and Smith, 1981).

The molecular basis of the mortality of normal, diploid cells and of the immortality of aneuploid, transformed, or tumour cells is unknown. Various hypotheses have been enunciated, and the subject has been very controversial. Of the variety of hypotheses propounded, two have been widely canvassed. One theory supposes that mortality is an ordered, genetically controlled differentiation phenomenon (Macieira-Coelho et al., 1966; Martin, 1977; Shall and Stein, 1979); the other theory suggests that a combination of autocatalytic error propagation in the translation of proteins and the standard experimental tissue culture procedures combine to kill off the cultures (Kirkwood and Holliday, 1975; Orgel, 1963, 1973).

We have investigated the mortality of normal human glial cells, which appear basically analogous in their proliferative behaviour (Blomquist et al., 1980; Pontén, 1973; Pontén and Westermark, 1980; Westermark, 1978) to the more frequently studied fibroblasts. In particular, we have asked whether a detailed study of the kinetics of the mortalization phenomenon could be used to exclude one or the other of the above hypotheses. In general terms, the differentiation hypothesis supposes that there is a smooth, continuous change in the proliferative behaviour of the culture from its inception (Shall and Stein, 1979); by contrast, the error theory specifically predicts a very high growth fraction in the population for a large part of its existence, followed by a catastrophic decline in reproductive activity near the very end of the lifespan of the culture.

Clearly, given the appropriate experimental approach, a kinetic analysis should be able to positively exclude one or the other of these general theories. We have used a miniclone technique (Westermark, 1978) to study the

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reproductive behaviour of individual normal cells in culture and have been able to demonstrate that the fraction of dividing cells declines smoothly and continuously in in vitro cultures of human glial cells. These results positively exclude error catastrophe hypotheses but are consistent with the notion that the mortality of normal, human diploid cells is a form of genetically regulated, terminal differentiation to reproductive sterility.

MATERIALS AND METHODS

Origin and early history of the cell strain used—787 CG

Brain tissue from the frontal lobe of a 38-year-old male was used within an hour of operation. Blood vessels and the remnants of meninges were removed. The tissue was finely minced with scissors in a small amount of tissue culture medium until a coarse "paste" was created. This was diluted with medium and portions of it were pipetted into 50-mm plastic tissue culture dishes that were then incubated, undisturbed, for 2 days at 37°C in a humidified CO_2/air mixture. The medium was then sucked off together with pieces of floating tissue. Then a regular routine of medium change twice a week was instituted. The medium was Eagle's MEM with 10% (v/v) newborn calf serum, which was a single pretested batch.

After 10 days a corona of glia-like cells that showed mitotic figures and migrated peripherally was visible around a small number of cell clumps which had settled. After an additional 11 days, that is, 3 weeks after explantation, the entire dish was covered by cells and subcultivation was begun.

We do not know how many population doublings occurred in the first 21 days of the primary culture. To compare the experimental data with theory, we assume that the cells that were viable grew with a generation time of 2.25 days (found at later passages), which results in some 9 population doublings during the first 21 days in primary culture.

Subsequent to the first passage, cells were transferred by 1:2 splits. A number of ampules each containing about 10^6 cells taken from the third passage were frozen. When recultured these cells showed unimpaired viability.

This cell strain—787 CG—has been extensively used for a variety of experiments and as a standard for the assay of growth factors (Pontén, 1973; Pontén and Westermark, 1980). The cells show strict density-dependent growth control with a terminal cell density of 6×10^4 cells per cm^2 during the early part of its life span.

Quantitative estimates of population doublings (PD)

Separate cultures were maintained for this purpose in 25-mm plastic dishes. At each passage two dishes were seeded with 5×10^5 cells and left until confluent cell layers had formed. The cells were removed by treatment with 0.25% trypsin, and the cell number in each dish was determined.

The cells from the two dishes were then combined. A portion of the cell suspension was used to seed two new dishes with 5×10^5 cells. Another portion was used for the analysis of growth potential by the palladium-island miniclone procedure. After passage 29, the total number of cells was insufficient for the above schedule. Subsequently, the number of cells seeded per dish for the estimation of population doublings was doubled. The time needed to reach saturation density was gradually prolonged until it reached about 10 days in the final passages.

Three experiments of identical design were carried out in succession with similar results. Only the first experiment, representative of the three, is described.

Analysis of growth potential by the palladium-island miniclone procedure

The palladium islands were prepared as described by Pontén and Stolt (1980) and consisted of either a 2×2 or a 1×2 array of sets of 100 circles, each circle with a surface area of 45,800 μm^2 or 18,000 μm^2, respectively.

At each passage a certain number of cells calculated to give a high proportion of islands inhabited by one cell only were seeded into two miniclone dishes. Until passage 25 this number was 8,000 cells in 2.5 ml of complete medium in each dish. Subsequently, 15,000 cells in 2.5 ml of complete medium were used. The increased number was used to compensate for a decreasing plating efficiency.

On the day after plating the number of cells attached to each specific palladium island was recorded. The medium was changed on day 4 and on day 7. The cells were fixed and stained on day 10 and the number of cells on each specific island was counted and recorded.

RESULTS

Figure 1 shows the appearance of miniclones that were used to accumulate the quantitative data described below. This figure shows qualitative representation of the results we have obtained. At early passages (Fig. 1A, B) there are many large colonies, but there are also a few small colonies with one to four cells. At later passages (Fig. 1C, D) there are no large colonies and only small colonies. The morphology of the cells in the late passage (Fig. 1D) conforms to previous descriptions; the cells are large and contain many granules.

The primary data that we have in the present experiments are the cell numbers measured at 1 and 10 days after plating on each of 1,200 individual palladium islands for each data point. The data from experiment 1 is reported as a representative example of the three experiments. From these data we derived two different types of information about the parameters that describe the growth of the cells. First, we derived the fraction of islands that, starting with one, two, or three cells, failed to divide even once during the 10-day course of the experiment. We have obtained such results at every passage over the complete lifespan of normal, untransformed human glial cells. Second, we observed the final colony size on each island that originally contained one, two, or three founders. Since at each passage we have a number of islands containing one, two, or three original cells, respectively, we can obtain both the mean and the dispersion of final colony sizes derived from one, two, or three starting cells.

In parallel with these experiments with the palladium islands, we have measured the extent of cell growth at each passage in standard plastic Petri dishes, and have computed from this the accumulated number of doublings that the cells have undergone.

Our data yielded at each passage the fraction of cells that do not divide. The fraction of single-founder colonies that did not reproduce during ten days of observa-

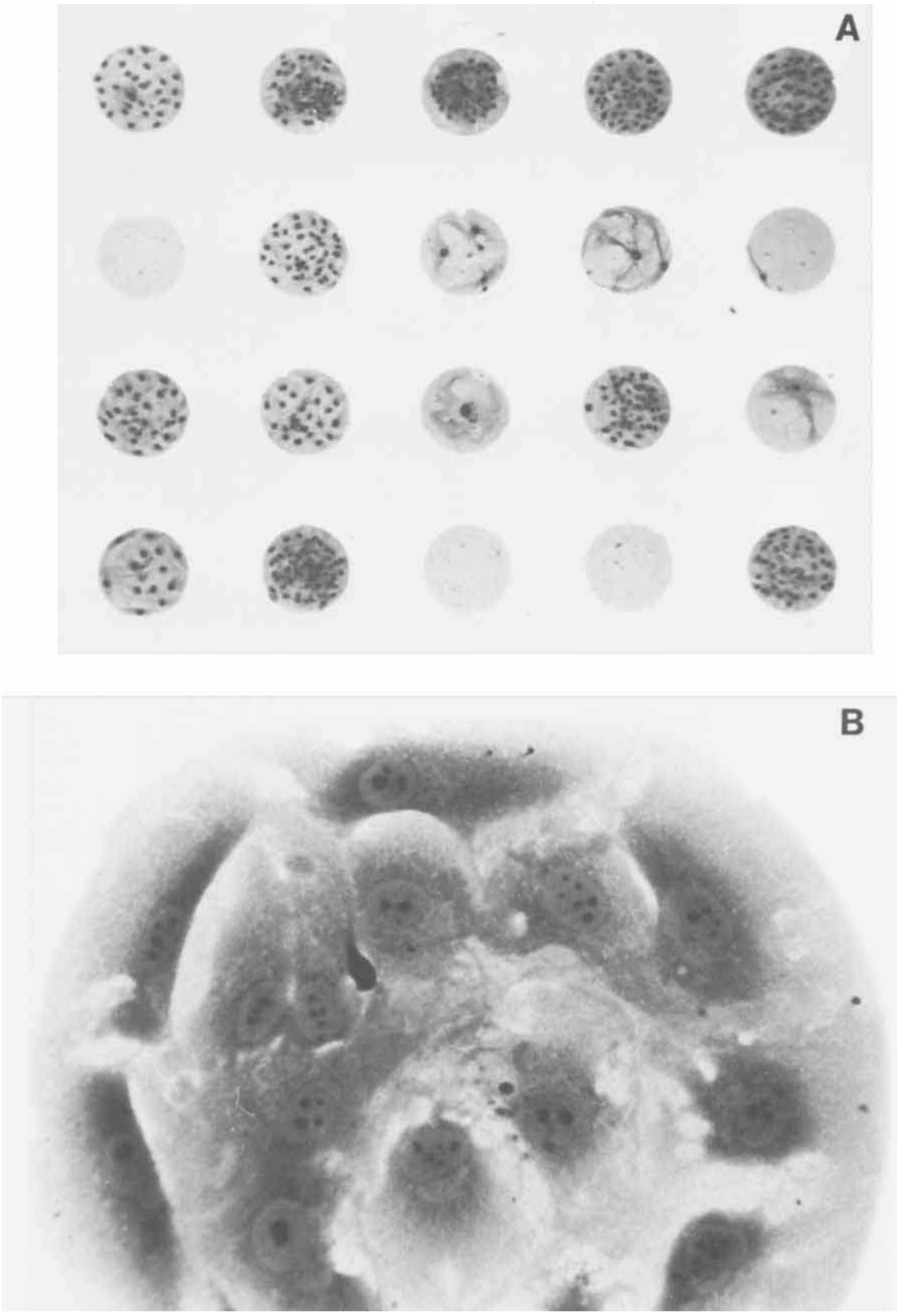

Fig. 1. Photographs of miniclone preparations, fixed and stained on the tenth day. A and B are from passage 11 (Day 4), and C and D are from passage 35 (Day 289). The actual diameter of each island in these four photographs is 242 μm.

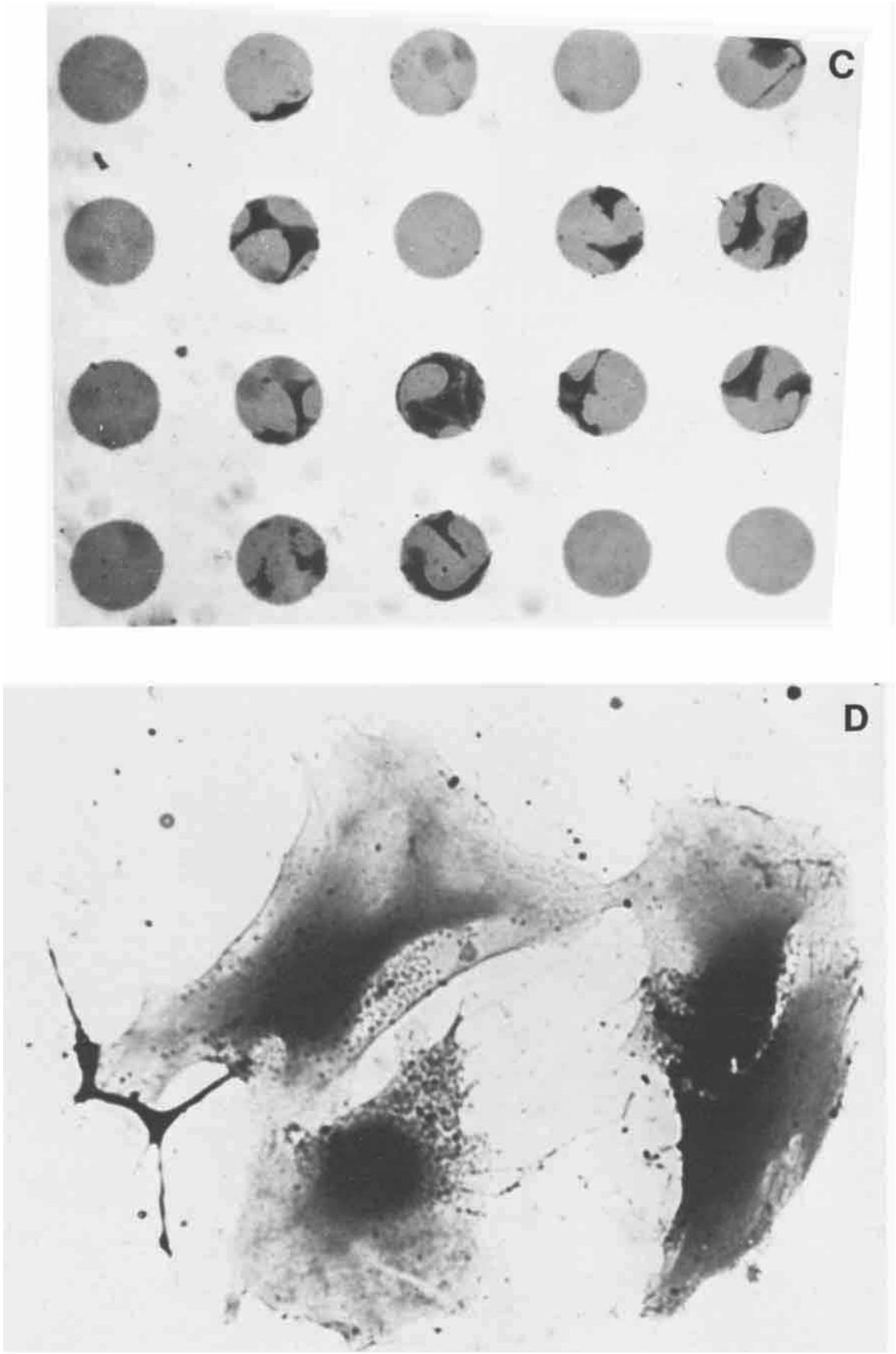

Figure 1C, D

tion are shown in Figure 2 as a function of the age of the culture. The fraction of nondividers is quite small at early times, but increases steadily from the beginning of the life of the culture reaching more than 0.60 after 300 days. This parameter of cell growth clearly displays a gradual change throughout the life of the culture. The solid line is the computed linear regression line; the two dotted lines show one standard error from the regression line. The parameters of the regression lines in Figure 2 and in Figures 3 and 4 are given in Table 1. In all three cases the P value for the correlation coefficient was < 0.001.

We also observed the fraction of colonies originating from either two or three founder cells (Figs. 3, 4) in which no cell divided. If the original two cells of a two-founder colony act independently of one another then the probability of *neither* dividing is equal to the square of the probability of an individual cell not dividing. Similarly, the probability of all three original cells in a three-founder colony not dividing is equal to the cube of the probability of an individual cell not dividing. Consequently, for noninteracting cells, the square root of the frequency of occurrence of nondividing, two-founder colonies, and the cube root of the frequency of occurrence of nondividing, three-founder colonies, should equal the frequency of occurrence of single-founder nondividers. The data for the two-founder and three-founder colonies are shown in Figure 3 and Figure 4, respectively. The regression lines for each of the three sets of data (Table 1) are indistinguishable, although there is more scatter in the two-founder and three-founder data, consistent with the smaller sample size (Table 1). All three sets of data show a gradual, smoothly increasing fraction of nondividers. The three sets of data are, of course, quite independent. Thus, it is clear that the probability of dividing gradually decreases as the culture ages, and is not affected by the presence or absence of neighbours.

TABLE 1. Parameters of computed linear regression lines of fraction of nondividers on one-, two-, or three-cell founder islands as a function of time through the life span of human glial cells

	One	Two	Three
Slope (%/day)	0.157	0.154	0.165
S.E.	0.014	0.027	0.039
Vertical intercept (%)	6.68	9.57	6.96
S.E.	2.53	5.46	8.12
Horizontal intercept (days)	−42	−62	−42
Correlation coefficient	0.899	0.772	0.688
Number of data points	31	24	22
Degrees of freedom	29	22	20
T factor	2.045	2.074	2.086

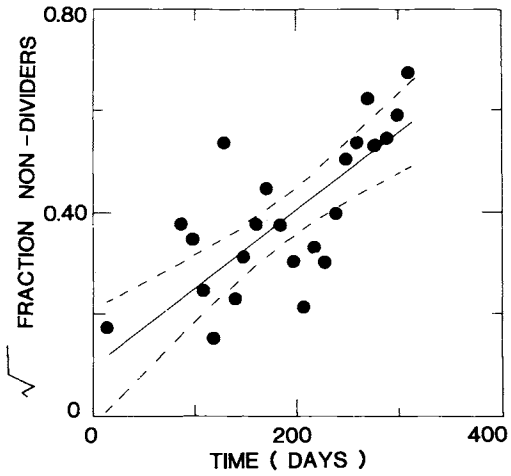

Fig. 3. Fraction of nondividing cells; islands with two founder cells only. The vertical axis (y) is the square root of the fraction of those islands on which neither cell divided. The regression line is y = 9.57 (± 5.46) + 0.154 (± 0.027) t. The dotted lines show one standard error.

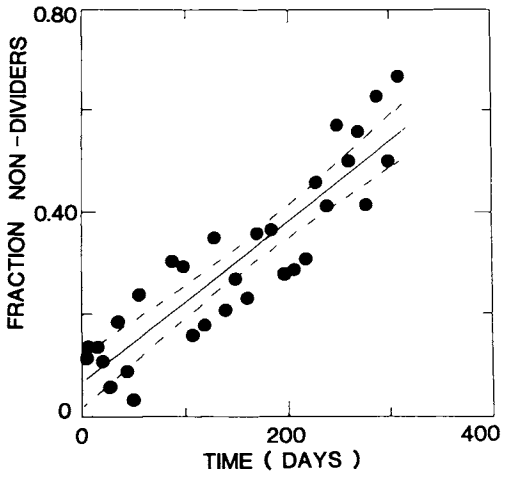

Fig. 2. Colony-forming efficiency as a function of passage at clonal density in the presence (●) or absence (○) of a 3T3 feeder layer. Cell populations were maintained and passaged in the presence of the feeder layer with periodic sampling for colony-forming efficiency in the presence or absence of the 3T3. In these experiments, the esophageal (rEs) cells (a) reached maximum colony-forming efficiency in 10 to 12 passages, while the epidermal (rEp) cells (b) required 17 to 20 passages.

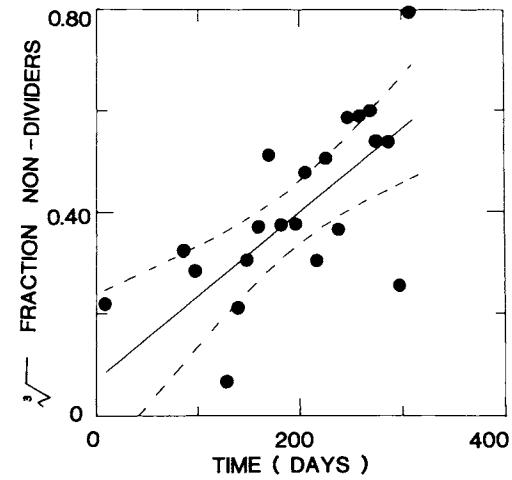

Fig. 4. Fraction of nondividing cells; islands with three founder cells only. The vertical axis (y) is the cube root of the fraction of those islands in which none of the three cells divided. The regression line is y = 6.96 (± 8.12) + 0.165 (± 0.039) t. The dotted lines show one standard error.

The dispersion of colony sizes

We then analysed the colony size dispersions of the colonies founded by single cells. The composite Figure 5 shows six representative histograms depicting the dispersion of colony sizes at different passages. In each case there is a broad distribution of colony size. Even at the earliest passages there are significant numbers of small colonies. With increasing age of the culture there is a shift in the pattern of the distribution, in that there are fewer large colonies and more small colonies. From the dispersion of colony sizes we derived the median colony size at each passage. Figure 6 shows the median colony size of colonies founded by single cells, plotted as a function of time. As the culture ages, the ability of single cells to proliferate decreases, and this decrease occurs steadily as a function of time, starting from an early passage. At the very earliest times, many single cells have the ability to produce colonies of 16 or more cells, showing that they go through at least four cycles of growth within the 10 days of the experiment. Already at early passages, however, some colonies formed by single cells consist of fewer than 16 cells. At a rather early stage in the history of the culture, indeed about one sixth of the way through the life of the culture, many individual cells can only reproduce two or three times, despite the fact that the culture as a whole will still expand a great deal from a diminishing proportion

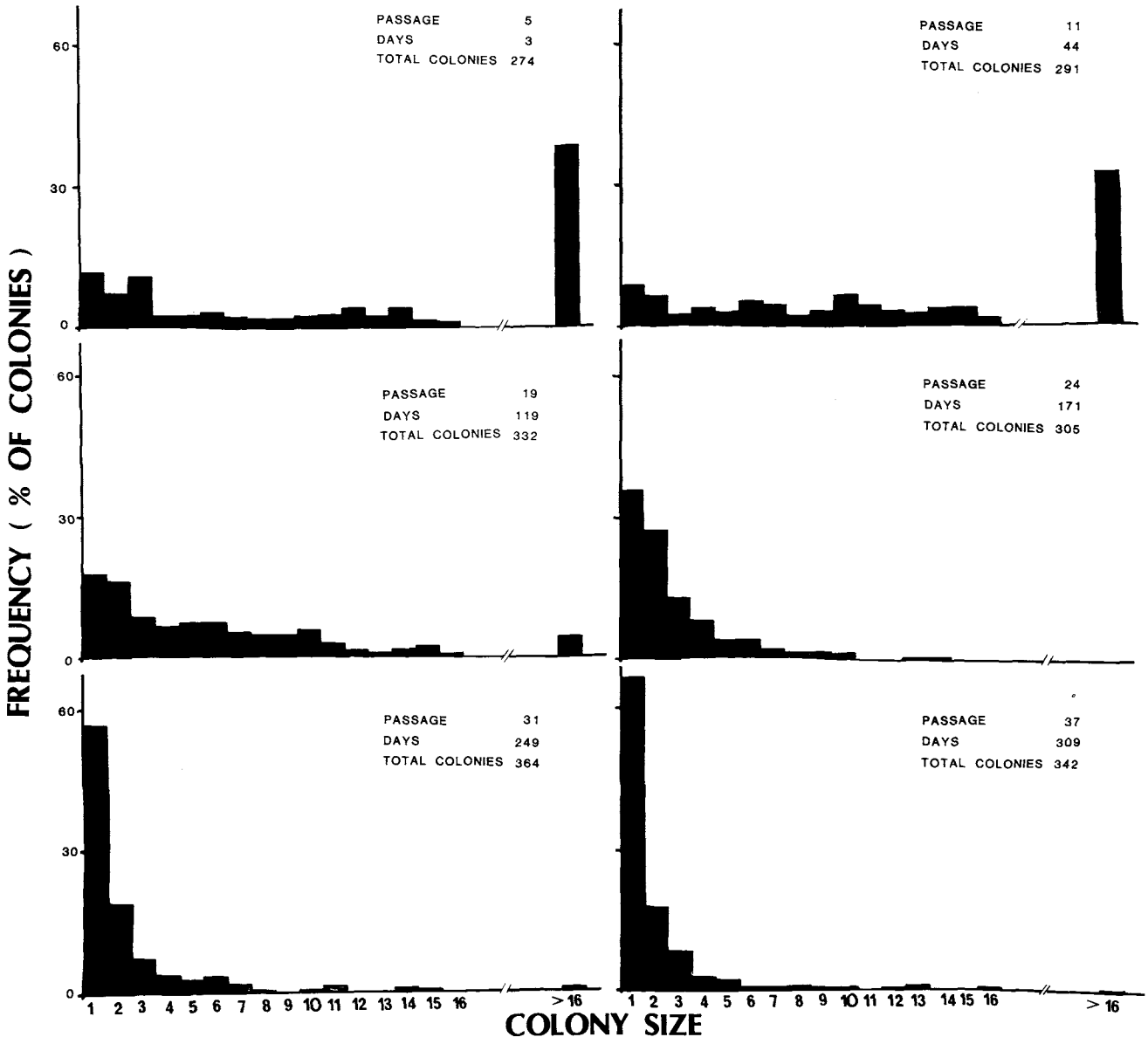

Fig. 5. Histograms of distribution of colony sizes after 10 days growth. Vertical axis is percentage of colonies in each size class as indicated on the horizontal axis. The time at which each assay was started and the number of colonies in the sample are indicated in each panel.

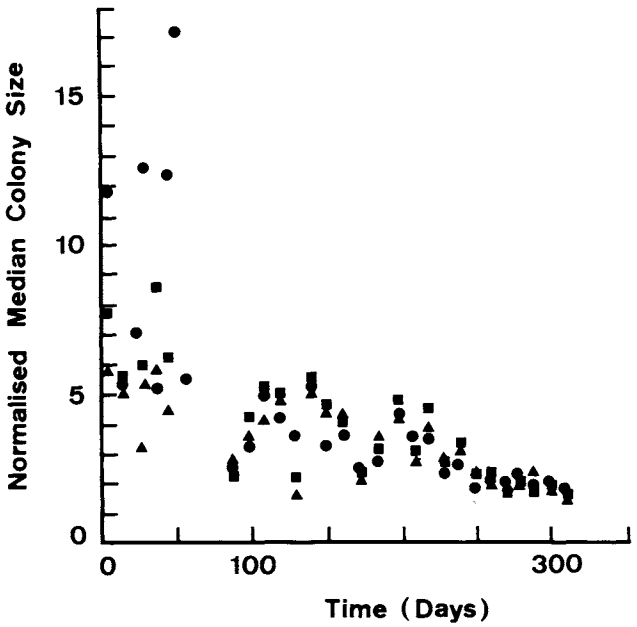

Fig. 6. Median colony sizes of all one-, two-, or three-founder islands normalized to single cell islands (see text for procedure). (●), Single-cell founders; (▲), two-cell founders; (■), three-cell founders.

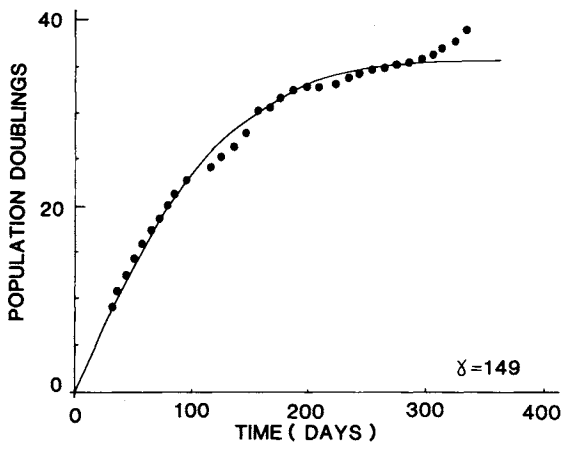

Fig. 7. Accumulated cell number of a culture of normal, untransformed, human glial cells. Vertical axis is accumulated population doublings; the horizontal axis is time in days. This shows the results of experiment 1. The solid line is the number of population doublings calculated from equation 1, with a gamma value of 150. The calculation is described in detail in Shall and Stein (1979).

of reproducing cells. At later passages, fewer and fewer individual cells can produce large colonies; and at the last passages hardly any single cell will build up to a 16-member colony, although the mass culture is still growing, albeit slowly.

To compare the data from two-founder colonies with one-founder colonies, we divided the two-founder colony size data by two. Similarly, we divided the three-founder colony size data by three. A similar growth behaviour to that of one-founder colonies is observed. It is striking that at middle and late times of the culture, the growth behaviour of colonies founded by one, two, or three cells is indistinguishable. This shows that all the cells are behaving independently in that single isolated cells show the same kinetic growth behaviour as do cells with neighbours. At early passages the two- and three-founder colonies fail to show their full reproductive potential, because the terminal density of each miniisland will be reached earlier as a function of the original number of founders.

Accumulated cell number

Figure 7 shows the accumulated number of population doublings as a function of time in culture for the glial cell cultures. The growth of these cultures shows a relatively fast doubling time of about 2 days per population doubling at the beginning of the culture. This rate decreases smoothly with time in culture until at about 35 to 40 doublings. There was no further growth of the culture in the last passage indicated. The growth that occurred at the end of the life span in this experiment was not apparent in two other parallel experiments with the same original culture. The curve drawn through the observed data is a theoretical computation for a model referred to in the Discussion. It seems to us unsatisfactory to describe the data as showing an initial logarithmic phase, followed by a phase of decreasing growth, and then by another phase of extinction, as has been suggested for some human fibroblast lines (Kirkwood and Holliday, 1975). Normal, untransformed, human glial cells seem to show a continuous loss of reproductive activity with time. The doubling time of these cultures is of course a composite of the generation time and the growth fraction.

The problem of cell mobility

Is there much mobility of cells in the culture of this experiment? Clearly, excessive movement of cells from one island to another will weaken any conclusions derived from this experiment. The degree of cell migration may be estimated by determining the number of islands that contained cells at the end but had no cells on them at the start of the experiment. The mean fraction of islands that were initially unoccupied but contained one or more cells on day 10 was 0.148 ± 0.145 (S.D., $N = 78$). These migrant cells might have come either from other islands or more likely from the palladium ring at the periphery of the dish, on which a large population of cells are growing. To obtain another measure of cell mobility we determined that the mean fraction of islands that were initially occupied by one cell, but that contained no cells on day 10 was 0.0643 ± 0.660 (S.D., $N = 113$). There was no change in the fraction of such migrant cells during culture life span. In seven of 113 experiments the fraction of such migrant cells was greater than 0.19; these seven cases were omitted from the overall analysis. Our measurements of colony size will be underestimated by migration of cells from the colony, but will be overestimated by migration into the colony. Both these rates of migration are small and of comparable size, hence our colony size measurements are likely to be fairly accurate. Of course, our estimates of migrants are over the whole 10 days; there will be fewer migrants at earlier times. The measures of migra-

tion refer to the whole population over 10 days, not just to the nondividers; therefore, the error contribution of migration to our estimates might be less than 5%.

Cell cycle duration measured by the miniclone technique

An estimate of the duration of the cell cycle for the population of dividing cells can be obtained using the miniclone technique. One counts the number of cells on each individual island twice each day and records the occasions at which an increase has occurred. With small numbers of cells on an island, unambiguous assignment of the interval between successive cell division can be made. Representative data from young (passage 11) and middle-aged (passage 20) cultures are shown in Figure 8, which shows the distribution of intermitotic times plotted semilogarithmically. The fraction of nondividing cells measured simultaneously in this same experiment was 0.18 in the younger culture and 0.32 in the older one. Yet, the figure shows clearly that the distribution of intermitotic times was almost identical in the cultures. The mean intermitotic time in the younger culture was 2.16 ± 0.13 days (S.E.M., N = 136) and 2.39 ± 0.13 days (S.E.M., N = 110) for the culture. These estimates of intermitotic time are significantly different from one another. If the increase in intermitotic time is assumed to continue at this rate through the entire life span, then at the last passage the generation time clearly could not contribute to the cessation increase in generation consistent with earlier reports (Macieira-Coelho et al., 1966; Absher et al., 1974).

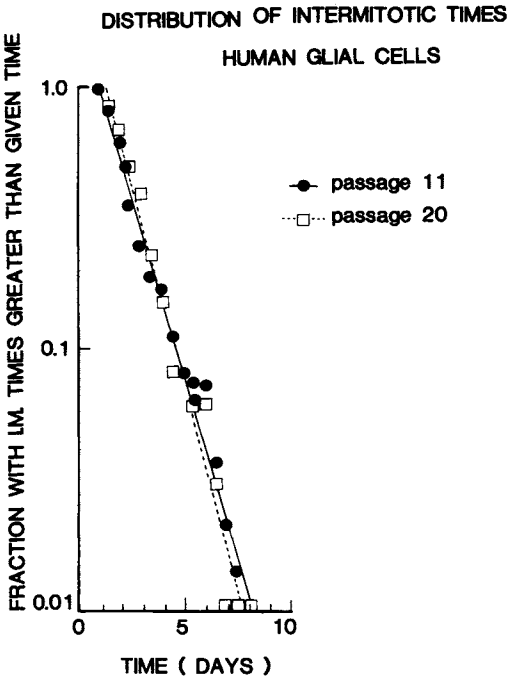

Fig. 8. Distribution of cell cycle times in two cultures of human glial cells. The vertical axis shows the logarithm of the fraction of cells with intermitotic times equal to or longer than the values shown on the horizontal axis.

DISCUSSION

The miniclone technique

The outstanding technical difficulty in analysing the behaviour of aging, normal cell cultures has been the measurement of the proliferative potential of individual cells. Smith and colleagues (Smith and Hayflick, 1974; Smith et al., 1978; Smith and Whitney, 1980) have attempted to provide the required data by measuring colony sizes achieved in cloning experiments. Merz and Ross (1969) determined the fraction of WI 38 cells capable of forming colonies in cloning conditions throughout the life span of the culture. Such a procedure may be open to the criticism that cells behave abnormally under the stringent conditions of cloning. Smith and Whitney (1980) have provided evidence that suggests that perhaps their cloning procedure provides a reasonable estimate of the reproductive potential of at least those individual cells that are capable of forming colonies. The results of Merz and Ross (1969) and of Smith and colleagues (Smith and Hayflick, 1974; Smith et al., 1978; Smith and Whitney, 1980) indicate that the fraction of nondividing cells in a population of normal human fibroblasts increases steadily with age. Nonetheless, this conclusion was not universally accepted (e.g., Macieira-Coelho, 1974; Holliday and Kirkwood, 1981; Kirkwood and Cremer, 1982). It is not possible to establish the pattern of increase of nondividers per generation from the data in the above papers.

A direct measurement of the reproductive potential of individual cells growing in bulk culture can now be made, however, using the miniclone technique (Westermark, 1978). In this technique cells are cloned, as it were, in the presence of a large number of other cells. Microscopic palladium islands are precipitated in the form of a grid on agarose; cells grow only on the palladium islands and not on the agarose. There is a broad ring of palladium at the periphery of the culture dish on which a large number of cells grow. The total number of cells in the dish approaches the number conventionally used in bulk cultures. The miniclones are thus provided with the same type of medium conditioning as would occur in a conventional bulk culture. By suitable choice of cell number, a large proportion of islands containing only one cell can be achieved. The growth of these individual cells can then be scored microscopically (Fig. 1), because each island on the grid is individually identifiable.

It has been established (Pontén and Stolt, 1980) that the cells in miniclones settle and multiply with the same efficiency as cells of a mass population; they are an unbiased sample of a bulk culture. In contrast to the conventional cloning technique where only those cells capable of forming visible colonies are scored, the miniclone technique records the behaviour of every viable cell in the sample. The distribution of colony sizes includes both single cells and small colonies as well as larger colonies.

It is useful to emphasize the large mass of primary data that is summarized in this paper. For each of three experiments, 30,000 to 38,000 individual palladium islands inhabited by one to over 16 cells were examined. The data points express, therefore, the average of a substantial number of observations.

We first consider to what extent the results described above are an accurate picture of the behaviour of these

aging cell populations. We then proceed to discuss possible interpretations of these data.

Every effort has been made to maintain a constant culture regime over the several months of the experiment. It could, perhaps, be suggested that the declining reproductive activity of the population shown in Figures 2 to 7 represents the effect of cumulative cell damage caused by inadequate culture procedures. However, the cells present at the first passage have been greatly diluted by the time of passage 30. At any point in time, very little of the contemporary cell material had been present in the culture for more than a few previous generations. Thus, if there is accumulated cell damage, it represents a more subtle phenomenon than is provided by this simple explanation. The phenomenon of a decline in reproductive activity has been noted many times in quite widely divergent biological material grown in different media and circumstances. This decline in reproductive activity is a widespread property of normal, untransformed cells in culture and is not simply an artifact of our nutritional, hormonal, or cell handling procedures.

Our most important observation is that the fraction of dividing cells and, therefore, the reproductive potential of the culture decline steadily from the very beginning of the culture (Figs. 2–4) confirming by a quite independent technique the conclusions of Merz and Ross (1969), Ryan (1979), and of Smith and co-workers (1978). It is not possible with these results to distinguish between several different patterns.

This observation has been very difficult to establish when conventional radioactive thymidine labeling has been used. This difficulty has arisen because there has been no satisfactory way of determining for how long the culture should be exposed to the radioactive thymidine in order to label all the cells capable of dividing. If the labeling period is too short, then one underestimates the fraction of cells capable of dividing. Therefore, it has been argued (Macieira-Coelho, 1974), that long periods of labeling must be used, especially for aged cells, to ensure that all competent cells have traversed at least one S phase. But a long labeling period allows those cells that do divide to go through more than one cell division; this may produce a gross overestimate of the true fraction of dividing cells in the culture. The large heterogeneity with respect to number of completed cell cycles per time unit disclosed here (Fig. 5) will probably make it quite impossible to arrive at a reliable estimate of the fraction of nondividers by thymidine labeling. Cristofalo and Sharf (1973) have measured the labeling indices of human diploid cell strains through their life span, but because of the potential difficulties outlined above independent evidence to confirm their conclusions is desirable. In the miniclone technique individual cells are observed for periods many times longer than a mean cell cycle, and the fraction of dividing cells is correctly estimated, irrespective of the total number of divisions achieved. Therefore, the miniclone technique is an alternative appropriate technical solution to this long-standing problem.

Our results are consistent with two previous observations that also suggested a continuous and early change in the properties of cells aging in culture. Aizawa and Mitsui (1979) found that a cell surface marker, measured by the adsorption of concanavalin A-coated red blood cells, increased as the cells (human diploid foetal lung fibroblasts) were passaged in culture, from passage 10 to passage 65. They observed a linear increase in the degree of binding with passage number. Ohno (1979) observed a strict relationship between the increasing concentration of serum needed to stimulate growth of diploid human lung fibroblasts and the time that these cells had been passaged in culture. This relationship was evident already when only 30% of the life span of the culture was reached. He noted that cultures at little more than 50% of their life span showed increasing requirements for serum. There was no evidence in this work for a sudden crisis in the proliferation of the cells. We, too, observe no evidence of a sudden change in behaviour late in the life span of the culture.

In his original demonstration of cellular aging in vitro, Hayflick (1965; Hayflick and Moorhead, 1961) measured the saturation density (i.e., the maximum number of cells that are present on a dish) of human fibroblasts at successive passages of the culture. He observed that the saturation density was constant for a considerable part of the life span, after which the number of cells harvested declined progressively. These observations have commonly been interpreted to mean that the rate of cell doubling in the culture (i.e., the reproductive potential) is constant for a long period and then declines. This interpretation is clearly inconsistent with our direct observation of the steady decrease of reproductive potential from the beginning of the culture (Figs. 2–6). Is there, then, an alternative interpretation of Hayflick's (1965) and Hayflick and Moorhead's (1961) data? The number of cells that can be present on a Petri dish is limited; therefore, when the reproductive potential is moderately high and enough time is provided, the cultures will reach this constraint and will cease reproducing before their full reproductive potential can be expressed. At later passages, however, the fraction of dividing cells is now so low that the limiting number of cells per dish is not reached, and only then does the declining reproductive potential become apparent. Hidden within the apparently unchanging behaviour of a bulk cell culture, there is a real fall-off in reproductive potential of the cells which an analysis of individual cell behaviour reveals (Figs. 2–6).

What might be the basis for the decline in reproductive potential that occurs in the culture as seen clearly in Figures 2–4? Is it due to an increase in the fraction of cells unable to divide, or does each cell merely take longer to divide? The data in Figure 8 settle this question clearly; there is only a marginal increase in the cell cycle duration at a stage when the fraction of cells unable to divide within 10 days has almost doubled. From Figure 8 we see that the 10-day assay period is sufficient to allow more than 99% of the cells to divide were they able to do so. If an increase in the duration of cell cycle were the only explanation for the increase in the fraction of nondividing cells in the two cultures depicted in Figure 8, a substantial fraction of the cells would have to have a cell cycle duration of greater than 10 days, i.e., more than a fourfold increase in cell cycle duration. The data in Figure 8 quite rule out this possibility. A small increase in cell cycle duration with cell aging has been reported by Macieira-Coelho et al. (1966), but is quite insufficient by itself to explain the decline in reproductive behaviour. Indeed, Bell et al. (1978) find no evidence

of an increase in average cell cycle time during aging in vitro. Our data provide compelling arguments for the hypothesis that the decline in reproductive potential during the aging of cultures is due to a continuous decline in the fraction of cells able to divide.

The decline in reproductive potential of the culture is seen also in the data on the colony size dispersions (Figs. 5, 6). The shift of the dispersion of colony sizes (Fig. 5) and of the median colony size (Fig. 6) toward smaller colonies clearly reflects the increasing fraction of cells unable to divide. Even at the earliest measurement (passage 5) there are cells that generate only small colonies (Fig. 5). When the culture is only one third of the way through its life span, already more than half the cells do not generate colonies that cover the whole island (Fig. 6).

Our data are therefore consistent with those of Smith and co-workers (Smith and Hayflick, 1974; Smith et al., 1978; Smith and Whitney, 1980), who measured the ability of single cells from diploid human lung fibroblasts to form macroscopic colonies at successive points in their life span, and with those of Merz and Ross (1969).

We saw that colonies that originally had two or three cells would be unable to display their full reproductive potential at early times in the life of the cultures. This is because the islands have a finite cell capacity, which seems to be about 20 to 30 cells in these experiments. The median colony size of highly reproductive colonies probably underestimates the real reproductive potential.

The kinetic description of aging cells in culture

The continuous decline in the fraction of nondividing cells seen in Figures 2–4 and the early loss in the ability of a fraction of the cells to form large colonies argue strongly against the suggestion that the decline in the doubling rate of the culture results from the accidental loss of "uncommitted" cells in the population, as suggested by Kirkwood and Holliday (1975). Our data simply show that there are no abrupt changes of the type required by the "commitment" theory of Kirkwood and Holliday (1975).

Can we develop a quantitative description of the smooth decline in reproductive ability of cells in culture? We have proposed (Shall and Stein, 1979) that in the reproducing cells in the population a process of mortalization (or more strictly reproductive sterility) occurs that irreversibly transfers affected cells out of the set of reproducing cells into a set of nonreproductive cells. We suggested that the probability of mortalization reflects an internal property of the cell and that this probability of mortalization increases smoothly from zero to unity during successive passages of the culture. A very simple formula relates the stochastic probability of mortalization, P_m, at any time, t, with time (cell generations) as follows:

$$P_m = \frac{t}{\gamma + t} \qquad (1)$$

where γ (gamma) is a constant characteristic of cell type, culture conditions, and history. Thus, it is possible to describe the entire dynamic reproductive history of the population by the single constant γ. At any time, t (generations), after the first passage, the probability of mortalization (reproductive sterility) is given by P_m and only a fraction, $1 - P_m$, of the newly born cells will divide again.

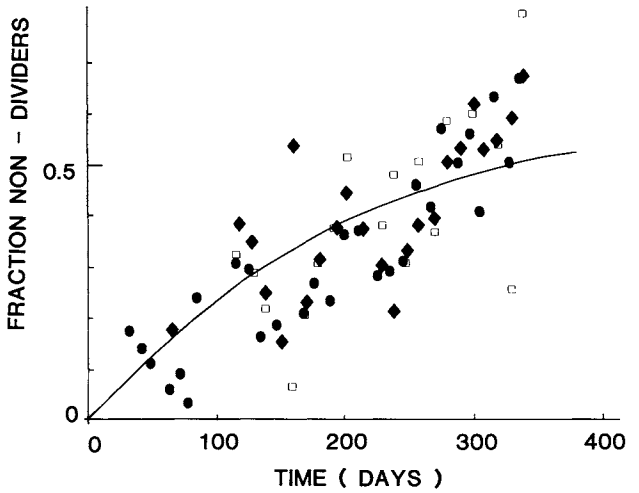

Fig. 9. Fraction of nondividing cells. ●, single cell founders; ♦, square root of two-cell founders; □, cube root of three-cell founders. The continuous line is the calculated P_m derived from the mortalization theory, with an assumed γ value = 150; for this calculation the horizontal axis is converted to generations using a generation time of 2–25 days; the vertical axis shows both fraction of nondividers and P_m (which is also a fraction).

TABLE 2. "Best fit" estimates of the mortalization equation $P_m = t/(\gamma + t)$ to the fraction of nondividers from one-, two-, or three-cell founder colonies of human glial cells[1]

	One	Two	Three	Combined
"Best fit" value of γ	164	140	143	149
Mean root residual differences (%)	0.089	0.106	0.130	0.107
S.E. (%)	5.3	5.4	6.8	3.4

[1]The "best fit" in each case was the γ value at which the mean root residual difference was at a minimum. The last column shows all the data combined in one set.

The data in Figures 2–4 of this paper are estimates of P_m as a function of time. This data may be fitted very satisfactorily by the P_m values (probability of reproductive sterility) derived from equation 1 (Shall and Stein, 1979) and a gamma value of 150. This is shown in Figure 9 where all the data of Figures 2–4 are plotted together with the probability of mortalization shown as the full line. Moreover, the data in Figure 5 are also qualitatively consistent with the results expected from equation 1. We can estimate the "best" values of gamma by fitting the data in Figures 2–4 to equation 1. The results are shown in Table 2. It is clear that equation 1 gives a reasonable description of the data. The same value of $\gamma = 150$ describes adequately the accumulated population doublings of the culture as a function of time (Fig. 7) bearing in mind that, particularly at early passages, the cultures were not able to express their full reproductive potential because of the lag period and a period of confluence.

We do not wish to argue here that the fit of the above kinetic model of equation 1 necessarily argues in favour

of the particular theoretical or molecular model previously proposed (Shall and Stein, 1979). Rather, we wish to conclude that the very good accord of the theoretical lines with the experimental data argues very strongly that the biologic phenomenon of senescence in cell culture of normal, untransformed, human glial cells can be described very simply as a continuous function of time. There is from the beginning a gradual, continuous change in the population leading to a progressive decline in reproductive activity. The observations seem to exclude a description in terms of a catastrophe or of any abrupt change in the population. They are consistent rather with the notion of gradual change in the population, generated by a genetically regulated developmental programme. A genetic programme is also suggested by the recent observation (Rohme, 1981) of a linear correlation over a large spectrum of mammalian species between the logarithm of the maximal life span in vivo (range 2 to 90 years) and fibroblast life span in vitro. Furthermore, red cell lifetime in peripheral blood correlated with fibroblast longevity in culture. These biological correlations imply a genetic programme relating the natural life span of species and cells in culture.

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