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HETEROGENEITY OF THE KINETICS OF PROLIFERATION WITHIN HUMAN SKIN FIBROBLASTIC CELL POPULATIONS

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SUMMARY

Human adult fibroblasts from the papillary layer of the skin showed increased survival *in vitro* when compared to reticular fibroblasts from the same biopsy. Fibroblasts of the papillary layer were also analysed *in vitro* to study the kinetics of proliferation between subcultivation and resting phase. It was found that the growth fraction and maximum cell densities vary as a function of the initial inoculum. The results are compatible with the presence of two cell populations with different growth potentials. The heterogeneity of these populations has to be taken into account when studying cellular aging.

INTRODUCTION

Human fibroblasts are used in a wide range of studies related to the pathology of the donor. However, it has been recently claimed that fibroblastic cells obtained from adult skin differ concerning their doubling potential, depending on the depth of the tissue layer from which they originate (Harper & Grove, 1979). This creates obvious pitfalls for the comparison of fibroblast-like cells originating from different donors. For these reasons, we have undertaken an analysis of the division potential of adult skin fibroblast cultures originating from different layers of the same biopsy and an analysis of cell proliferation of fibroblasts derived from one single skin layer, for comparison with previous results obtained using embryonic cells (Macieira-Coelho, Pontén & Philipson, 1966; Macieira-Coelho, 1967*a*).

We confirm the difference found in the division potential of fibroblastic cultures derived from different skin layers. We also find a heterogeneity even within the same population derived from a single tissue layer in regard to rate of growth and maximum cell density, which is apparent when one varies the initial inoculum at subcultivation in cells of some donors; these growth characteristics are different from those previously described in normal embryonic cells (Macieira-Coelho, 1967*a*).

MATERIALS AND METHODS

A human embryonic skin fibroblastic population, 964-S, was obtained from Dr J. Pontén (University of Uppsala, Sweden). Thoracic biopsies from normal skin obtained at the time of surgery were dissected into different layers, and handled for cell culture according to the method of Harper & Grove (1979).

The initial outgrowth around each explant derived from the upper-most region (epidermis and papillary layer) consisted of epithelial cells. Migration and sustained proliferation of fibroblastic cells occurred and confluency was reached within 30 days of explantation.

Outgrowth around each explant derived from the dermis seemed to consist of fibroblasts only. It was initially delayed and confluency was reached within 50–60 days of implantation.

When the cells from the primary cultures reached confluency, they were trypsinized and subcultivated at a 1:2 split ratio. The same split ratio was used to maintain the cultures until their extinction. Phase-II cultures were subcultivated every 6/7 days; this subculture interval increased progressively to 11 days in senescent cells. Cells were fed with Eagle's minimum essential medium supplemented with 10% foetal calf serum and 16 μg/ml gentamycin. The nutrient medium was left unchanged between each subcultivation. The culture protocol used is that described and recommended by Hayflick & Moorhead (1961).

Comparison between growth fraction and cell density

Only cultures derived from superficial skin regions were used in these experiments. Post-confluent cultures were trypsinized, cells were suspended at different concentrations in 3 ml culture medium and seeded into 30 mm plastic Petri dishes. Special care was taken to ensure equal and uniform cell density at the beginning of the experiment in all culture dishes belonging to the same group.

Each day after subcultivation, two cultures were trypsinized and counted with an electronic Coulter counter until no further increase in cell number could be observed without changing the medium. The counts in duplicate dishes differed by less than 10% throughout the whole experiment. [^{3}H]thymidine at a final concentration of 0·1 μCi/ml (sp. act., 2 μCi/mmol) was added to duplicate cultures each day after subcultivation and 24 h later the cells were prepared for autoradiography (Macieira-Coelho, 1967*a*). For the determination of the percentage of labelled cells, 2000 interphases were counted on each duplicate sample; the 95% confidence interval was the mean $\pm$ 10%. When the cell counts reached a plateau, a wound was created by scratching the monolayer with a pipette. Then [^{3}H]thymidine was added and the cultures were fixed 24 h later to count the percentage of cells synthesizing DNA. Cells were scored within the empty space left by wounding and along an edge 20 μm deep in duplicate slides. Cells were also scored in random fields far from the wound and in identical cultures without a wound in random fields across the monolayer.

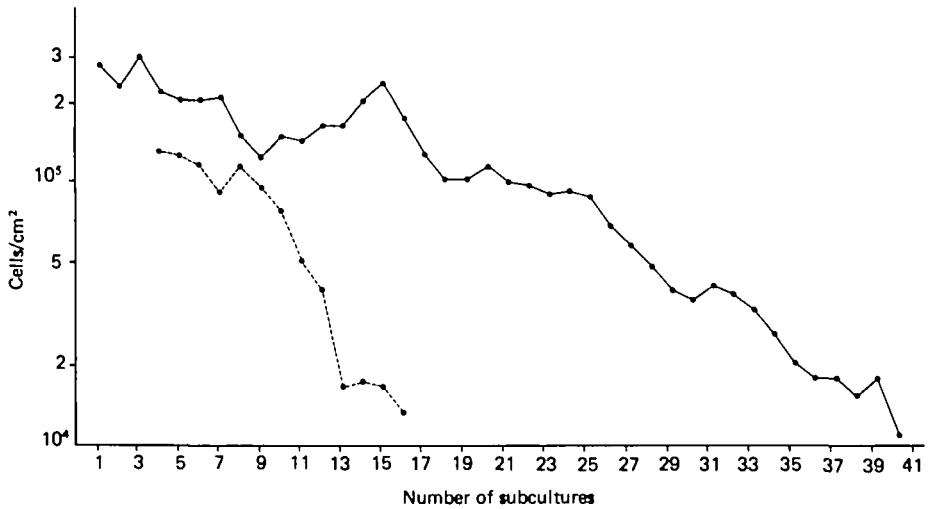

Fig. 1. Comparison between the survival of papillary (●—●); uppermost region) and reticular (● - - - ●; lowermost) fibroblasts (NMSI) derived from the thoracic skin of a 24-year-old patient operated on for plastic surgery.

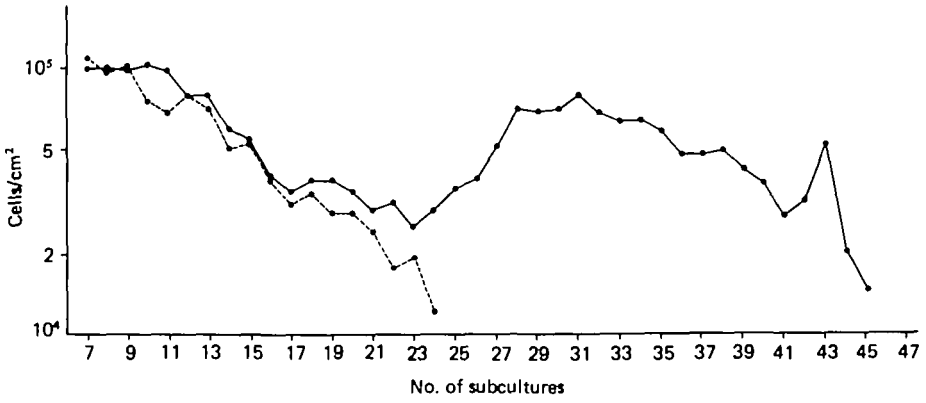

Fig. 2. Comparison between survival of papillary (●—●) and reticular (● - - - ●) fibroblasts derived from a 69-year-old patient with a mammary carcinoma (CM_5).

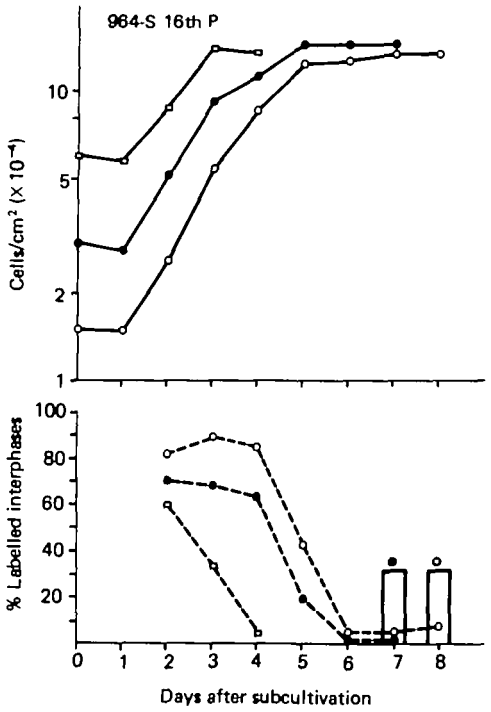

Fig. 3. Cell counts (——) plotted semi-logarithmically and percentage of labelled interphases (- - -) plotted arithmetically on different days after subcultivation. The bars indicate the percentage of labelled interphases along the edge of a wound obtained by scraping with a pipette at the time when the cells formed a confluent sheet. 964-S is a normal human embryonic skin fibroblastic cell line.

RESULTS

The analysis of survival curves of two skin fibroblast lines NMS1 and CM5 (Figs. 1, 2) shows in both instances that cultures derived from the uppermost region (epidermis and papillary layer) display significantly longer lifespans as compared with cultures derived from the lowermost region (reticular layer of dermis) of the same biopsy.

Fig. 3 shows the growth and DNA synthesis curves of human embryonic skin fibroblasts after plating the cells at different densities; in spite of different inocula the cells reach the same final density. It is well known that the latter, usually called the saturation density, is due to the interaction of several mechanisms that limit cell division under crowding conditions. This density, however, can be overcome, to a certain extent, in normal cells by repeated changes of medium (Todaro, Lazar & Green, 1966; Garcia-Giralt, Berumen & Macieira-Coelho, 1970) or by medium perfusion

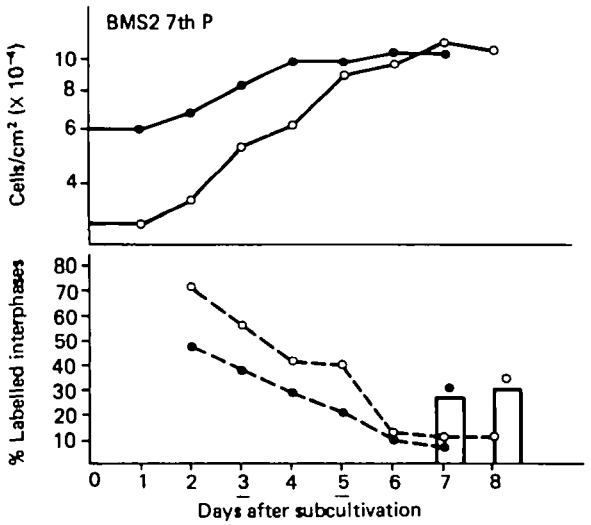

Fig. 4. Cell counts (—) plotted semi-logarithmically and percentage of labelled interphases (– – –) plotted arithmetically on different days after subcultivation. The bars indicate the percentage of labelled interphases along the edge of a wound obtained by scraping with a pipette at the time when the cells formed a confluent sheet. BMS2 papillary fibroblasts were derived from a 41-year-old patient with a nodular sclerocystic dystrophy.

(Kruse & Miedema, 1965). On the contrary, in some transformed cells (Temin, 1966; Macieira-Coelho, 1967*b*) identical maximum densities are reached whether the nutrient medium is renewed or not. For this reason, we did not change the medium between subcultivation and resting phase, to allow the expression of different growth potentials. The DNA synthesis curves of 964-S cells under these conditions show that the percentage of cells synthesizing DNA during the second day after subcultivation is inversely related to the inoculum size; it then decreases to low levels when the

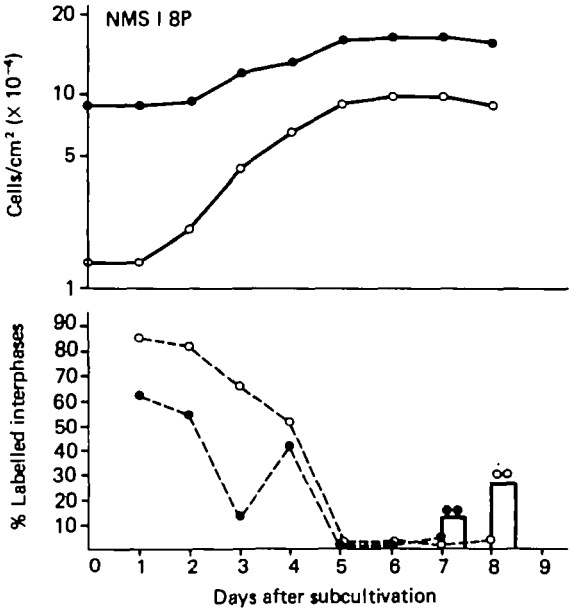

Fig. 5. Cell counts (—) plotted semi-logarithmically and percentage of labelled interphases (- - -) plotted arithmetically on different days after subcultivation. The bars indicate the percentage of labelled interphases along the edge of a wound obtained by scraping with a pipette when the cells formed a confluent sheet. NMS1 papillary fibroblasts were derived from a 41-year-old patient operated on for plastic surgery.

growth curve reaches a plateau. The nutrient medium, however, was not depleted at this stage since 30% of the cells could enter division along the wound.

Figs. 4–8 show the growth and DNA synthesis curves of human postnatal papillary fibroblast cultures after plating the cells at two different densities. BMS2 cells (Fig. 4) behaved as the embryonic skin fibroblasts, since they reached the same final density in spite of the size of the initial inocula. On the contrary, in NMS1, BMS5, CM5 and CM19 cultures the final saturation density increased in the groups seeded with a higher inoculum (Figs. 5–8). The DNA synthesis curves show that in BMS2 and NMS1 cells, as in embryonic skin fibroblasts, the percentage of labelled interphases during the second day after subcultivation is inversely related to the initial density.

On the other hand, the fraction of cells synthesizing DNA during the second day after the different inocula in BMS5 and CM5 cultures (Figs. 6, 7), and is directly related to the initial density in CM19 cultures (Fig. 8). In the latter, however, the maximum number of cells entering *S*-phase was identical in both groups, although it was reached only on the fourth day after subcultivation, after seeding with the lower inoculum.

In the case of experiments with medium depletion, BMS2, NMS1 and BMS5 behaved like 964-S since a significant number of cells entered DNA synthesis along the wound.

These data suggest that in these cell lines medium depletion does not play an important role in the determination of the final density. On the other hand, such a

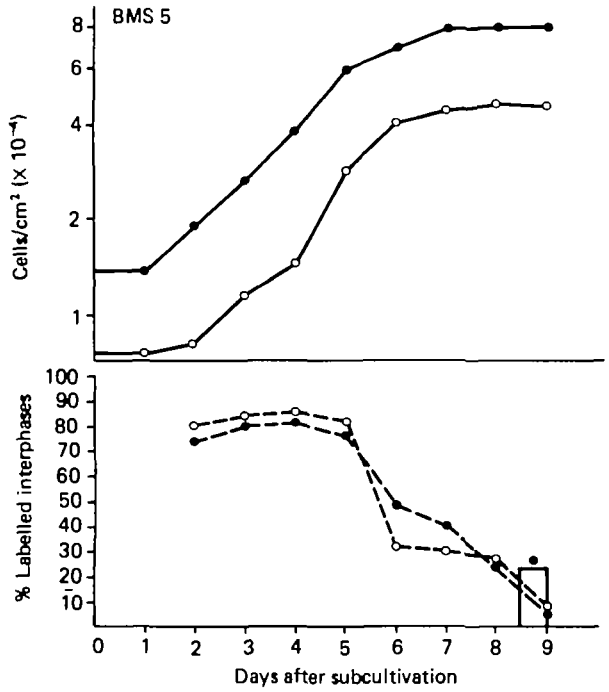

Fig. 6. Cell counts (—) plotted semi-logarithmically and percentage of labelled interphases (– – –) plotted arithmetically on different days after subcultivation. The bars indicate the percentage of labelled interphases along the edge of a wound obtained by scraping with a pipette at the time when the cells formed a confluent sheet. BMS5 papillary fibroblasts were derived from a 30-year-old patient with a benign microcystic lesion.

conclusion cannot be drawn from the experiments with CM5 and CM19 cultures since only a slight increase of cells entering *S*-phase could be obtained along the wounds. CM5 resting cultures had 2% labelled cells across the monolayer and 5% along the wound in the cultures initially seeded at lower density (Fig. 7). In CM19 resting cultures seeded at the higher inoculum, 3% of cells across the monolayer were labelled and 9% along the wound; cultures initially seeded at the lower inoculum showed 1·5% of labelled interphases across the monolayer and 4% along the wound (Fig. 8). These results show that medium depletion at resting phase does not take place to the same extent in different skin fibroblastic lines.

To see if higher inocula can cause an increase in the final cell densities, we performed the following experiments. At different passage levels, postconfluent 964-S and CM19 cultures routinely maintained with 1:2 splits ratios were divided into two groups; one group was carried on with 1:2 splits ratio and the other with 1:1 split ratio. In 964-S cultures, there was no increase of the final saturation densities when they were seeded at 1:1 ratio. On the contrary, in CM19 cultures (Fig. 8) multiple subcultivations at 1:1 split ratio caused a progressive increase in cell counts until final saturation densities were reached, which are significantly higher than those exhibited by parallel cultures maintained at a 1:2 split ratio.

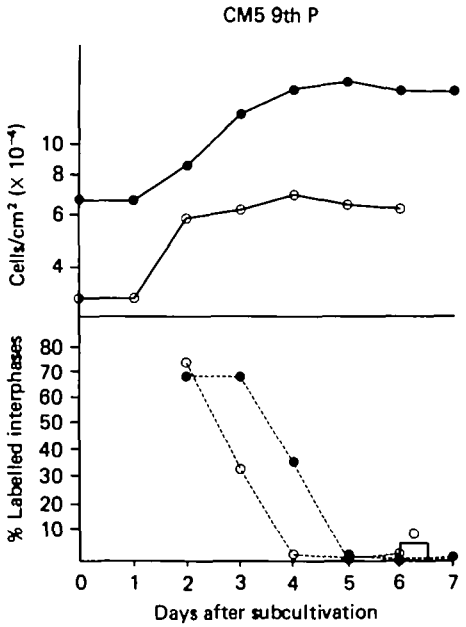

Fig. 7. Cell counts (—) plotted semi-logarithmically and percentage of labelled interphases (– – –) plotted arithmetically on different days after subcultivation. The bars indicate the percentage of labelled interphases along the edge of a wound obtained by scraping with a pipette at the time when the cells formed a confluent sheet. CM5 papillar fibroblasts were derived from a 69-year-old patient with a mammary carcinoma.

The maximum cell densities expressed by the cultures maintained with 1:1 split ratios at different passage levels decreased with the age of the cultures. To see if reaching a higher saturation density is due to the selection of a subpopulation with higher growth potential, we have performed the following experiments (Table 1). Identical postconfluent CM19 cultures (20th passage) with a density of $1{\cdot}4 \times 10^5$ cells/cm^2 were subcultivated at a 1:1 split ratio (i.e. $1{\cdot}4 \times 10^5$ cells/cm^2) and at a 1:2 split ratio (i.e. $0{\cdot}7 \times 10^5$ cells/cm^2. At confluency, the cell density was 2×10^5 cells/cm^2 in the 1:1 group (43% increase) and $1{\cdot}4 \times 10^5$ cells/cm^2 in the 1:2 group (100% increase). Cells from each group were subsequently replated at the same inoculum ($0{\cdot}3 \times 10^5$ cells/cm^2) in the presence of [^{3}H]thymidine. Then the cultures were prepared for autoradiography at different times in order to follow the appearance of labelled interphases after subcultivation.

Table 1 shows that the rate of entrance into *S*-phase is significantly faster in the cultures derived from the 1:1 group. These changes in the rates of thymidine incorporation after only a single round of growth at both densities could, however, be explained by physiological differences in the two populations resulting from the prior growth conditions. To check this hypothesis, we repeated the experiment three passages later. This time, however, serial subcultures at 1:1 split ratios were carried out until a stable increase (100%) in the saturation density levels was achieved (4th

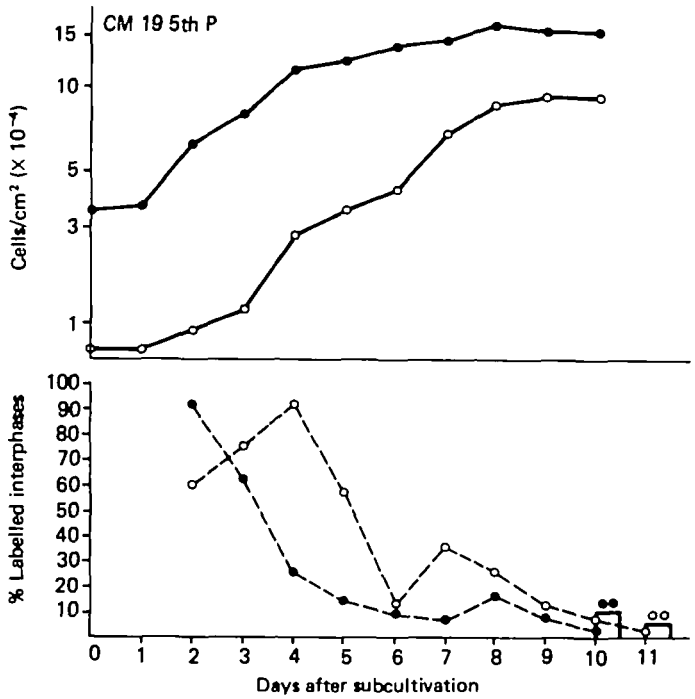

Fig. 8. Cell counts (—) plotted semi-logarithmically and percentage of labelled interphases (– – –) plotted arithmetically on different days after subcultivation. The bars indicate the percentage of labelled interphases along the edge of a wound obtained by scraping with a pipette at the time when the cells formed a confluent sheet. CM19 papillary fibroblasts were derived from a 36-year-old patient with a mammary carcinoma.

1:1 split serial subcultivations in Fig. 9). Then these cultures were replated at low density ($0{\cdot}3 \times 10^5$ cells/cm^2) and compared with the cultures maintained in parallel at a 1:2 ratio, which had performed the same number of doublings. These experiments were planned in order to perform the labelling assays with cultures that had spent a similar length of time at confluence. Table 2 shows that cultures derived from the 1:1 group had a faster rate of entrance into S-phase.

Finally, to exclude the possibility that these results could be merely due to the fact that the confluent cultures from which the inocula were derived were committed to a quiescent pre-*S*-phase condition from which they probably emerge at different rates we performed the following experiments on the same cultures. Both groups were seeded repeatedly at the same low density ($1{\cdot}2 \times 10^4$ cells/cm^2). After each passage and at identical intervals, the final cell number was checked and expressed as number of population doublings (p.d.) performed. Ninety days later, cultures derived from the 1:1 group had performed 13·3 p.d., while cultures derived from the 1:2 group had performed only 7·2 p.d. This shows that cultures derived from the 1:1 group achieve and maintain over a prolonged length of time an increased growth potential.

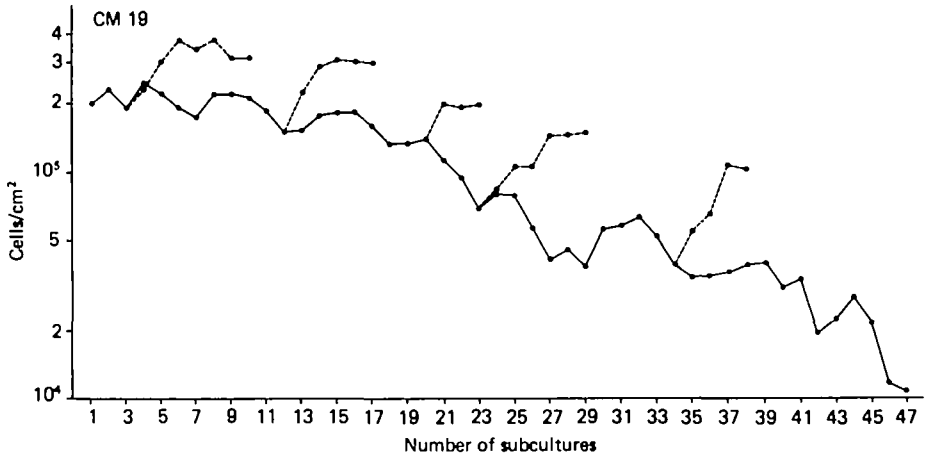

Fig. 9. Maximum cell densities before subcultivation of CM19 cells maintained at 1:2 (●—●) and 1:1 (○---○) split ratios.

Table 1. *Time of appearance of labelled interphases at different times after subcultivation in CM19 cultures subcultivated once at different densities and then replated at the same inoculum*

Time of labelling (h)	Percentage of labelled interphases	
	1:2-derived cultures	1:1-derived cultures
15	0·6	6
19	7	24
23	13	39
27	23	48
31	34	55

Results are based on the analysis of 2000 cells in each of two samples. [^{3}H]thymidine was added at the time of cell seeding and the cultures were fixed at the indicated hours.

Table 2. *Time of appearance of labelled interphases at different times after subcultivation in CM19 cultures repeatedly subcultivated at different cell densities and then seeded at the same inoculum*

Time of labelling	Percentage of labelled interphases	
	1:2-derived cultures	1:1-derived cultures
15	0·2	3
19	0·4	9·5
23	1·4	25·1
27	3·6	36·3
31	25	50

Results are based on the analysis of 2000 cells in each of two samples. [^{3}H]thymidine was added at the time of cell seeding and the cultures were fixed at the indicated hours.

DISCUSSION

Our data show that adult fibroblast cultures derived from the uppermost region displays a significantly longer survival time than that of fibroblastic cultures derived from the lowermost region of the same skin specimen, thus confirming the results of Harper & Grove (1979). Moreover, we have also observed a heterogeneity of the kinetics of proliferation, even with cultures derived from the most superficial layer.

The division cycle of phase-II human fibroblasts during the logarithmic and post-logarithmic growth phases (Macieira-Coelho *et al.* 1966) and the influence of cell density on their growth kinetics have been previously characterized in foetal lung fibroblastic cultures (Macieira-Coelho, 1967*a*). These cells enter resting phase at a density specific for each cell line, which is independent of the inoculum size and declines with aging *in vitro*. The percentage of cells synthesizing DNA during the second day after subcultivation is inversely related to the seeding density. Here we show that human embryonic skin fibroblasts behave in the same way. Postnatal fibroblasts, however, display different kinetics of proliferation. This is expressed as a variation of the maximum cell density (Figs. 5–8) and of the initial percentage of cells synthesizing DNA. A possible explanation for this phenomenon is the existence, in what are generally assumed to be homogeneous populations, of at least two cell subsets with different cpacities for reaching high cell densities. Plating the cells at a high inoculum could inhibit to a certain extent the cells that are more sensitive to cell-cycle inhibition due to the proximity of other cells and favour the population less sensitive to cell crowding.

Multiple subcultivations at 1:1 split ratios would cause, in the cell lines capable of initial increased DNA synthesis at higher inocula (CM19, Fig. 8), the selection of a cellular subset less sensitive to cell crowding (Table 2). Moreover, this difference is maintained after several splits at low density since cultures derived from the 1:1 group perform many more doublings than cultures derived from the 1:2 group (13·3 and 7·2, respectively). In this respect, it is pertinent to mention that although post-confluent human embryonic fibroblastic lung cultures cannot increase their density when they are subcultivated at a 1:1 split ratio (Macieira-Coelho, 1967*a*); nevertheless, they are actively stimulated by serial medium changes at resting phase and this response is due to the repeated division of the same cell population (Garcia-Giralt *et al.* 1970). This suggests that in human fibroblast populations there is a fraction of cells that is less sensitive to cell-cycle inhibition due to crowding, which could be less stringent in some postnatal fibroblast populations. The finding of two populations with different growth characteristics could be interpreted in terms of the commitment theory of aging, which considers two cell populations: one of potentially immortal cells and the other of cells committed to senescence and produced with a fixed probability at each division of the former (Holliday, Huschtscha, Tarrant & Kirkwood, 1977).

Changes in other patterns of growth in adult human fibroblasts have been correlated with certain pathological conditions (Miller & Todaro, 1969; Azzarone, Pedullà & Romanzi, 1976; Pfeffer, Lipkin, Stutman & Kopelovitch, 1976; Smith[1] *et al.*

1976; Vincent & Huang, 1976; Goldstein, Littlefield & Soeldner, 1977), and one might consider if the findings described above are also related to some unknown characteristics of the donor.

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