Standardization of Human Diploid Cell Cultivation

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Human embryonic diploid lung fibroblasts grown in Eagle's medium were exposed continually to a variety of environmental conditions over a large number of passages to observe how these conditions affected the growth and longevity of these cells in vitro. The cells grew well at temperatures between 34 and 37°C and some cells could be adapted to grow at 40°C. Very limited growth occurred at 30 to 31°C; however, confluent monolayers of cells could be maintained for months at 30°C and still give rise to actively growing cultures. Increasing the amino acid concentration in Eagle's medium or the calf serum concentration above 10% had no effect on the growth rate or longevity. One per cent calf serum could not support prolonged active growth. Trypsin concentrations between 1 and 0.1% and crystalline trypsin at 50 μg/ml showed no influence on cell growth. Ethylenediaminetetraacetic acid treatment and scraping, however, destroyed many of the cells, and the survivors grew poorly. The clonal morphology varied with age. Young cells frequently gave rise to densely packed clones, whereas older cells gave rise to clones with widely scattered cells. The cloning efficiency was high when the cells were young but decreased rapidly with successive passage. It was relatively constant from the 7th to 20th passage at about 15%.

Human diploid cells have assumed considerable importance in recent years as a potential medium for viral vaccines (5, 7; for a collection of papers dealing with many aspects of diploid cell uses and cultivation, see reference 24). However, to utilize these cells most efficiently, a critical analysis and standardization of the tissue culture techniques are required. The unique growth characteristics of diploid cells make them an excellent laboratory model for such studies.

The general growth characteristics of human embryonic lung fibroblasts have been described by Hayflick (4, 6) and others (13, 16, 17, 21, 24, 26), and various changes have been noted as these cells go through their different growth phases. The present report explores the extent of environmental conditions imposed upon these cells by in vitro growth affect their growth rate and longevity.

MATERIAL AND METHODS

Medium. Eagle's medium (1) was used throughout these experiments, usually supplemented with 10% calf serum, 1 mM sodium pyruvate, 4 mM L-glutamine, and a final concentration of about 0.05% sodium bicarbonate. Additional bicarbonate was added to the cultures as the pH dropped. Penicillin and streptomycin were added at a concentration of 100 units/ml and 100 μg/ml, respectively. The calf serum was no heat-inactivated.

Routine passage of cells. In most experiments, 3 × 10^6 cells were seeded into Roux bottles with a growth surface of about 264 cm² together with 150 ml of medium per bottle. The cells were passed either at predetermined intervals of 2, 3, or 4 days or when microscopic observation showed that the cells had grown to a confluent monolayer. The cells were suspended with 50 ml of 0.25% trypsin (1:300, Nutritional Biochemicals Corp.) in Hanks buffer adjusted to about pH 7.8 by the addition of 1 ml of a 2% tris(hydroxymethyl)aminomethane (Tris) solution. The suspended cells were centrifuged, resuspended in 10 ml of Eagle's medium plus 10% calf serum, and counted in a hemocytometer.

For cells which required long incubation periods between passages, the medium was changed every 3 or 4 days. Occasional tests for mycoplasma were made, especially at the terminal stages of growth, but all were negative.

Cloning. The cells were cloned at each passage in 5-cm plastic petri dishes (Nunc, Denmark) containing 5 ml of Eagle's medium plus 20% calf serum, 1 mM sodium pyruvate, 4 mM L-glutamine and 0.01% sodium bicarbonate. The plates were incubated in a 5% CO₂, 95% air atmosphere for 2 weeks without a change of medium or adjustment of pH and with the minimum amount of handling and movement. The clones were stained with 0.1% crystal violet in 25% ethanol, and all clones visible to the unaided eye were counted. The smallest of such colonies usually con-
tained 8 to 10 cells. The clones were scored by only one person throughout all of the experiments.

**Source of cells.** Lung (Lu) tissue was obtained from human embryos aborted in about the third month of gestation. The primary cells from the tissues were prepared for cultivation by a procedure similar to that described by Hayflick and Moorhead (6). The cell strains from different embryos are distinguished by letters A, B, C, etc.

In some experiments a cloned culture of HeLa cells was used to compare its growth with the diploid cells.

**Preservation of cells.** The primary tissue fragments and cells from the various passages were frozen slowly and stored in liquid nitrogen. The cells were suspended to a concentration of $2 \times 10^5$ to $5 \times 10^6$ cells per ml in Eagle's medium plus 10% calf serum and 5% dimethyl sulfoxide. After about 1 hr at 4°C, the cell suspension was frozen at a rate of about 1°C per minute by manually adding dry ice to an alcohol bath on a magnetic stirrer. When the temperature reached $-30$°C the ampoules were put directly into the liquid nitrogen freezer.

When frozen cells were used to start a culture, the ampoule was placed directly in a water bath (37°C) and thawed rapidly. The cells from one ampoule were seeded immediately into a large culture bottle. The dimethyl sulfoxide which is toxic to growing cells at a concentration of about 1% was diluted well below its toxic limit and had no apparent effect on the growth of these cells. The medium was changed on the 3rd or 4th day of incubation.

**Chromosome preparations.** Cells were grown on cover slips in Leighton tubes. After 2 to 3 days of incubation when many cells in mitosis were observed, the cover slip was incubated in Hanks balanced salt solution, diluted 1:5 with distilled water, and brought to about pH 8.0 with 2% Tris, for 10 to 15 min at 37°C. The cells were fixed in Carnoy's fixative and stored in 95% ethanol. They were air dried directly from the alcohol, stained with orcein (2% orcein in 50% acetic acid), washed, alcohol dehydrated, air dried, and mounted on slides.

**RESULTS**

Chromosome counts gave a value of 46 expected of human diploid cells. Approximately 3% of the cells were tetraploid. About 1% of the cells in mitosis had some form of chromosome abnormality, but this proportion did not appear to vary with the age of the cells.

**Comparison of different cell strains in routine passage.** Figure 1 illustrates the rate of division

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**FIG. 1.** Rate of cell division of several strains of human embryonic lung fibroblasts (Lu) compared to a culture of HeLa cells. The diploid cells yielded about two to three cell divisions per passage. Symbols: (□) HeLa; (●) Lu (M) experiment started passage 2, ended passage 18, contaminated; (△) Lu (L) experiment started passage 2, ended passage 31 after reaching senescence; (○), broken line Lu (A) experiment started passage 4, ended passage 38, contaminated; (△) Lu (J) experiment started passage 4, ended passage 28, contaminated; (○), solid line Lu (D) experiment started started passage 2, ended passage 22, contaminated.

**FIG. 2.** Effect of incubation temperature on cell growth. Symbols: (△) 40°C, passage 2 to 16, contaminated after 15 cell divisions; (○) 37°C, passage 2 to 14, contaminated after 29 cell divisions; (△) 34°C, passage 2 to 21, contaminated after 48 cell divisions; (●) 33°C, passage 9 to 11, contaminated after 7 cell divisions.
of various diploid cell strains and a HeLa cell culture in routine passage. Usually about one to two cell divisions occurred when the cultures were passed at intervals of 2 or 3 days. However, three to four cell divisions per passage occurred when the cell layer was allowed to become confluent. Most of the strains appeared to fall within the range of 40 to 60 cell divisions established by Hayflick and Moorhead (6). However, one cell strain, Lu (D), had a much slower growth rate than the others and appeared to reach senescence earlier.

Effect of temperature on cell growth. Figure 2 demonstrates that a temperature range of 34 to 37°C gave similar growth results. A few passages at 33°C suggested that the growth rate was similar to that at 34°C. At 31°C, the cells were capable of dividing only two or three times before all growth ceased. After about 3 weeks of incubation at 31°C under these conditions, most of the cells lost their ability to grow out again when placed at 37°C. However, diploid cell cultures grown at 37°C to a confluent monolayer could be stored at 30 to 31°C for at least 3 months with frequent changes of medium and still retained the ability to grow when subcultured again at 37°C.

HeLa cells were almost completely unstable to storage at 30 to 31°C and degenerated over 1 to 2 weeks of incubation. They could not be maintained in passage at this temperature or as dense monolayers. After 2 weeks at 30°C, a few cells survived; of these, very few were capable of growing when incubated at 37°C again. However, the progeny of these survivors showed no increased resistance to storage at 30°C.

After a long lag period at 40°C, some diploid cells arose which were capable of growing at this temperature at a rate slightly less than the parent culture at 37°C. Examination of other properties of these high-temperature-adapted cells was prevented when this culture became contaminated and was lost.

Effect of calf serum. The rate of cell growth in 20, 10, and 1% calf serum were compared over a series of passages (Fig. 3). The addition of 0.1% 15- and 400-centipoise methyl cellulose (Methocel, Dow Chemical Co., Midland, Mich.) to medium containing 1% calf serum had no effect on cell growth for the first 30 days, after which the growth with 15-centipoise methyl cellulose was much slower than with 400 or the control.

There is considerable variation from batch to
batch of calf serum and some have been found toxic. One batch of serum which seemed to induce a slightly different morphological response in diploid cells was obtained in large amounts and used throughout the passage series of a cell strain. Other cells were grown in medium containing serum from batches which varied from passage to passage. The results from the cells grown under the two different conditions (Fig. 4) showed that the constant or variable serum sources had no influence on the growth rate of these cells as far as the 14th passage (23 cell divisions). About this time the growth in the constant serum medium stopped, and the cells eventually died out after a long maintenance period. Growth of cells in variable serum medium continued in the usual pattern. Although different batches of Eagle's medium were used over the course of this experiment, the same batch was used for both cultures when the medium was changed or the cells were passed.

Effect of amino acid concentration. The amino acid concentration is one of the most common factors varied in Eagle's medium by different investigators. The effect on the growth of lung cells passed continuously in media with various amino acid concentrations can be seen in Fig. 5. Fourfold, twofold, and the standard onefold amino acid concentration in Eagle's medium (1) gave identical growth rates. Reducing the amino acid concentration below onefold caused a marked reduction in the growth rate.

Effect of various techniques for suspending cells. Suspending cells from the growth surface prior to passing on to a new culture is one of the most critical times in the in vitro life history of these cells. Figure 6 illustrates the effect of various techniques for suspending cells: the use of crystalline trypsin, 0.02% ethylenediaminetetraacetic acid (EDTA), scraping, and an alternative use of trypsin and EDTA.

Crystalline trypsin was used at a concentration of 50 μg/ml and was diluted in phosphate-buffered saline (PBS) without Mg²⁺ or Ca²⁺ to avoid excessive clumping of the cells. The pH was adjusted to about 7.8 by the addition of a 2% Tris solution. Suspending cells with crude or crystalline trypsin gave similar good results.

Those cultures treated with a combination of trypsin and EDTA were first exposed to cold
0.25% trypsin for 1 min. The trypsin was discarded and 0.02% EDTA was added. The culture was incubated at 37 C until the cells either came off or could be shaken off. Another passage series in which PBS was substituted for EDTA (data not shown) always yielded fewer cells than that obtained with EDTA.

The harmful effect of EDTA and scraping on these cells is quite apparent in Fig. 6; cells treated first with trypsin and then with EDTA grew for only a short time longer before growth appeared to cease.

The effect of varying the concentration of trypsin and prolonged incubation at 37 C in the presence of trypsin can be seen in Fig. 7. Suspending cells with either 1 or 0.25% trypsin gave similar growth results. Even a 4-hr incubation in 0.25% trypsin at 37 C with each passage did not appear to affect the growth rate of these cells for at least nine passages, after which the cell growth appeared to stop.

Suspending cells with 0.1% trypsin gave highly inconsistent results for the first nine passages, probably due to clumping and loss of many cells on the glass surface. This fault was eliminated by using PBS without Mg++ or Ca++ (pH 7.8) to dilute the trypsin instead of Hanks buffer which was used for the higher trypsin concentrations. After this change, results became consistent with the 0.25% trypsin control.

Variation in cloning efficiency and clonal morphology. Figure 8 illustrates the variation in cloning efficiency with diploid cell age. The values are the average of a large number of experiments. The cloning efficiency of cells 5- to 15-cell-divisions-old is usually high. After about 15 cell divisions, this value drops sharply to between 10 and 20% and remains relatively constant at this level until the cells approach senescence. After about 40 cell divisions, the cloning efficiency frequently was 1% or less.

A very marked change in clonal morphology was observed as the cell culture aged (Fig. 9). During the early passages when cell growth was most vigorous, a high proportion of clones were relatively large (between 0.5 and almost 1 cm) in diameter and contained densely packed cells. Clones in the same size range, or smaller, but with less densely packed cells were observed also.

As the cell cultures were passed, the frequency of the dense clones decreased and the loosely packed clones predominated. Between the 10th and 15th passages (about 30 divisions), most of the former clones had disappeared; the spacing between the cells as well as the size of the individual cells in these clones increased. This change in clonal morphology was a consistent and reproducible phenomenon which has occurred without exception in every cell strain examined.

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**Fig. 7.** Growth response of diploid cells suspended with different concentrations of trypsin. Symbols: (△) 0.1% trypsin, passages 2 to 28, contaminated after 46 cell divisions; (○) 0.25% trypsin, passages 2 to 10, contaminated after 21 cell divisions; (×) 1% trypsin, passages 2 to 16, accidentally destroyed after 36 cell divisions; (●) 0.25% trypsin and incubated in trypsin at 37 C for 4 hr, passages 2 to 9, stopped growing after 24 cell divisions.

**Fig. 8.** Variation of the average cloning efficiency with cell age.
FIG. 9. Typical clones formed from various strains of human diploid lung fibroblasts at different passages in their life history. About the 20th passage, clones are barely distinguishable and in older cultures only individual cells can be seen. (a) Passage 6, (b) passage 12, (c) passage 18, and (d) passage 22.

DISCUSSION

The importance of human diploid cells as a possible medium for virus vaccine production and also as a system for studying cell aging makes it necessary to standardize the cell culture techniques. Most of these techniques have been developed empirically and they vary in different laboratories. The following information forms a preliminary basis for more detailed experiments to be reported later.

It is apparent that cells from different human embryos may have widely different growth rates and longevity (Fig. 1.) Cells which have a low growth rate and longevity of no more than 30 to 40 cell divisions in vitro may reflect some basic deficiency or the presence of an infectious agent. It may be suggested that only cell strains having a growth potential of at least 60 cell divisions be used as a possible virus vaccine medium.

The inoculum size for our experiments was usually $3 \times 10^6$ cells per bottle or approximately $10^4$ cells per cm². It is assumed that this inoculum is similar to that ($10^4$ cells per ml) used by Hayflick (4). Since all of the cells settle onto the growth surface, it may be better to state the cell inoculum in relation to area. Such units can be interpreted better by other workers and may be translated to any type of flask or surface culture system regardless of the volume of medium used. Higher inocula reduced the lag phase of growth but produced no other benefit in terms of a higher cell yield, whereas lower inocula resulted in a longer lag phase but would eventually produce comparable cell densities. Inocula as low as 200 cells per cm² gave confluent monolayers within 2 weeks of incubation when the cells were no older than about 20 cell divisions. Beyond this age, such low inocula gave increasingly poor results. The cell yield varied from as high as $50 \times 10^4$ to $60 \times 10^4$ cells per bottle (approximately $2 \times 10^4$ cells per cm²) between the 5th and 10th passage (after about 20 to 30 cell divisions) to values close to or less than the inoculum at senescence.

The rate of growth appeared independent of temperature between 34 and 37 C (Fig. 2). The cut-off temperature for growth is probably be-
between 31 and 33°C. No cells could be adapted to grow at 30 or 31°C. After about two to three cell divisions at 30°C, all divisions ceased, and the cells eventually became morphologically similar to senescent cells. After about 1 month of incubation, most of the surviving cells had lost the ability to grow when placed back at 37°C. However, confluent monolayers of cells grown at 37°C could be stored at 30°C for as long as 3 months without any apparent influence on the growth ability of these cells. The ability to store confluent monolayers at low temperatures may be related to the fact that relatively few cell divisions occurred in such cultures. Such a technique may be useful for storing large masses of cells which can be used on very little notice.

The serum used in the culture medium was the least defined substance present and subject to considerable variation. Some sera have been found to contain various proportions of inhibitors of mitotic activity (20), cell attachment, (19), and cell growth (11, 15, 22, 25). These effects can be observed within a few hours or days of incubation. The results in Fig. 4 show a type of inhibition, which is not well recognized, in which normal growth was obtained for 60 days of serial cultivation before the cells stopped growing. Either some substance was accumulated by the cells to an inhibitory level or it became active only when the cells passed a certain age. Thus, along with the other types of inhibition noted, there may be present in serum substances which limit the growth potential of diploid cells. Standardization of serum added to tissue culture medium may require prolonged testing over many serial passages, especially with old cells.

Increasing the amino acid concentration suggested by Eagle (1) did not produce any significant increase in the growth rate or in longevity (Fig. 5); however, reducing the concentration decreased the growth rate markedly. These data infer that the limited life expectancy of diploid cells is not due to an amino acid deficiency in agreement with a similar suggestion by Hayflick (4). The possibility that the nonessential amino acids which had been found of advantage to cloned cells growing at low densities (11) may influence the growth potential of diploid cells is under investigation.

Preliminary observations on the primary outgrowth of cells from human embryonic lung tissue has suggested that concentrations of amino acids higher that onefold slowed down this growth.

The techniques for suspending cells from the growth surface are well known to cause cell damage (8, 10, 14, 18). However, alteration of cells (12) or chromosome damage (9) caused by trypsin has never been observed in our laboratory. The effect caused by EDTA and scraping (Fig. 6) may be related to the observation that cells suspended by these procedures were in the same extended form they had on the growth surface and much cell debris and damaged cells were observed in these suspensions. Scraping was also found to be very damaging to cells by Magee et al. (14). The poor growth results with these cells suggest that they were very easily damaged during manipulation. Since trypsinized cells were suspended mostly as spheres, it is reasonable to assume that they are stable in this form. The present data suggest that cells suspended by 0.25% trypsin were resistant to prolonged incubation in this enzyme at 37°C, but eventually cell longevity was limited (Fig. 7).

At the concentrations used, the activity of the crystalline and crude trypsin preparations for suspending cells from the glass surface (Fig. 6) appeared similar. The lowest concentration of crystalline trypsin capable of suspending diploid cells was about 10 μg/ml, whereas the crude trypsin was partially active at a concentration of 100 μg/ml. The growth rates of cells suspended with as high as 1% crude trypsin or as little as 0.1% trypsin (Fig. 7) were similar, but, when using 0.1% or lower trypsin concentrations, it was necessary to use a buffer without Ca²⁺ or Mg²⁺.

The lack of effect over a 10-fold range of trypsin concentration is difficult to understand in view of the reported cytotoxic effects of trypsin (9, 10, 14). It is possible that much of the damage introduced is reversible as suggested by Hebb and Chu (8).

There was considerable variation in the cloning efficiency from passage to passage. This variation seemed to have no correlation with cell growth in mass culture, perhaps because the conditions which influence cells at low densities may not be important to cells growing at high densities (2, 3, 11). The cloning efficiency of cells obtained from primary cultures was usually low. Thereafter, the cloning efficiency can be high for the next five to seven passages (about 15 cell divisions), and then it drops and remains relatively low (Fig. 8). The factors which we found to be important in cloning diploid fibroblasts were similar to those described by Puck et al. (22, 23). Of greatest importance was the physiological state of the culture trypsinized and the pH of the cloning culture, especially during the first few days of the culture.

A clear difference in clonal morphology with aging appeared (Fig. 9). Young cell populations from the same culture gave rise to at least two morphologically distinct fibroblast clones: (i) with densely packed cells and (ii) with widely separated cells. A similar heterogeneity in human
fibroblast populations was reported by Papayannopoulou and Martin (21).

Hayflick (4) had shown that the growth potential from cloned cells was the same as the parent culture. Our experience with growing cloned cells suggests that only cells from the densely packed clones can be grown to large populations (a confluent culture in a Roux bottle). We have been unsuccessful in growing cells from the clones with widely scattered clones to large populations even though they were grown from very young cultures.

As the cells in culture aged, the densely packed clones disappeared and those with widely separated cells predominated. This change always occurred with age and may be a property worth considering as another criterion for controlling diploid cells used for human viral vaccines.

Tracing the complete life history of diploid cells in vitro represents a sensitive laboratory system for systematically studying the influence of many factors on the growth rate and longevity of these cells. Such studies were designed to help establish a better defined and standardized cell cultivation system for viral vaccine production and the investigation of cell aging.

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LITERATURE CITED