Standardization of Human Diploid Fibroblast Cultivation: Trypsinization Procedure

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Human embryonic diploid lung fibroblasts were used to examine the influence of the trypsinizing procedure on the growth and adsorption of these cells. The best buffer for trypsinizing these cells was Hanks balanced salt solution containing 0.5% lactalbumin hydrolysate. Trypsinizing cells at 4°C gave better growth results than trypsinization at higher temperatures. The presence of antibiotics in the trypsin buffer increased the longevity of the cells. Cells initially trypsinized from tissue in phosphate-buffered saline without Ca++ or Mg++ and 0.33 M sucrose plus 10^{-8} M Mg^{++} gave rise to better subsequent growth and adsorption than cells from tissue trypsinized in other buffers.

The most common technique for suspending cultured cells from the glass surface on which they grow has been the application of trypsin solutions. The techniques have largely been developed empirically, and, although they are effective, they always induce some form of damage to the cells (4, 6, 10, 11). Such damage may have important consequences on the subsequent growth of such cells as human embryonic diploid lung fibroblasts (HEDLF). Standardizing the growth of these cells has become an important issue with regard to their use as a medium for human viral vaccine production. Since trypsinizing and passing these cells is one of the most critical times in the in vitro manipulation of these cells, this process was investigated to obtain optimum and standard conditions for this procedure.

MATERIALS AND METHODS

Cells. The strains of HEDLF cells used for this experiment were grown out from human embryonic lung tissue by a procedure similar to that used by Hayflick and Moorhead (2). One strain was used for all but one of the experiments reported here, and the growth characteristics of these cells corresponded to those expected of human diploid fibroblasts (2, 3).

Cultivation technique. Eagle's medium (1) was used throughout these experiments supplemented with 10% calf serum, 4 mM L-glutamine, 1 mM Na pyruvate, 0.05% Na bicarbonate, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. The same batch of medium was used throughout these experiments but three to four batches of calf serum were required.

The cells were grown in Roux bottles with a growth surface of 264 cm². A 150-ml amount of medium per bottle was used. The cultures were observed daily with a Leitz inverted microscope, and when a culture was confluent the cells were trypsinized in 50 ml of 0.25% trypsin (1:300, Nutritional Biochemicals Corp., Cleveland, Ohio) made in the buffers described in the text. The suspended cells were centrifuged at about 1,000 × g for 15 min and were counted with a hemocytometer, and 10⁶ cells were added to new culture bottles.

Estimate of the number of cells attached to glass. After overnight incubation of the culture the number of cells which attached to the glass was measured by a procedure described earlier (J. Litwin, Eur. Symp. Poliomyelitis Allied Dis., 12th, in press.). Fifteen fields-of-view distributed evenly over the growth surface were selected at random. The attached cells in each field-of-view were counted with a Leitz inverted microscope, and the total number of attached cells per bottle was estimated by multiplying the average number of cells per field-of-view by a factor derived from the area of the bottle divided by the area of the field-of-view.

Trypsin. Four different batches of trypsin (1:300, Nutritional Biochemicals Corp.) were tested in the following way. Trypsin solutions (0.25%) were made in phosphate-buffered saline (PBS) without Ca++ or Mg++. The pH was adjusted to about 7.3 with 1 N NaOH, and the solutions were sterilized by Seitz filtration. The cells from four cultures of HEDLF made from the same parent culture were suspended, each with trypsin from a different batch. The cells were counted and passed by the usual procedure, and after overnight incubation the number of attached cells was measured. When these cultures were confluent again, the cells were suspended by trypsin from the same batch that was used the first time, and the cells were passed as before. This procedure was repeated for a total of five passages. The culture

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series which showed the best cell growth and highest per cent of cells attached to the glass was determined, and the batch of trypsin used for suspending these cells was purchased in large quantities. Most experiments reported in this paper were performed with this trypsin batch.

It was found that one of the trypsin batches occasionally caused considerable damage to diploid and HeLa cells, sometimes rendering the cells difficult to sediment in the centrifuge at about 1,000 X g. The cell viability of these trypsinized preparations was reduced, and the survivors grew at a slower rate than cells exposed to other trypsin batches. This damage was most pronounced in diploid cells older than 40 cell divisions.

RESULTS

The effect of buffer on cell trypsinization. Trypsin solutions (0.25%) were made in the following buffers: PBS, Hanks balanced salt solution (BSS), 0.33 m sucrose plus 10^{-4} m MgCl_2, complete Eagle's medium without calf serum, complete Eagle's medium with 4× amino acid and vitamin concentration without calf serum, and 0.5% lactic albumin hydrolysate (LAH) in Hanks BSS.

Replicate cultures of HEDLF were made from the same parent culture, and from that point each culture series was trypsinized with only one of the trypsin preparations. One culture series, however, was trypsinized with a different preparation with each passage.

The growth and longevity of cells trypsinized in PBS, Hanks BSS, sucrose and Eagle's medium with 4× amino acids and vitamins were identical (Fig. 1 and 2). Cells trypsinized in Eagle's medium (Fig. 2) showed the same growth rate as the others until about 55 cell divisions. Then the cells began to grow slower, but they eventually reached a slightly higher number of cell divisions (71) than most of the others (67). The culture series, trypsinized with different preparations with each passage (Fig. 1), stopped growing at an earlier time (63 cell divisions) than the others, and the growth results were slightly more erratic. Cells trypsinized in LAH (Fig. 2) gave rise to the best growth, increasing at a constant growth rate to about 72 cell divisions before senescence.

The percentage of cells that attached to the

![Graph 1](image1.png)  
**Fig. 1.** The effect of trypsin buffer on the growth of human embryonic diploid lung fibroblast cells. Trypsin solutions (0.25%) were made in the following buffers and used to suspend diploid cells from the glass surface. (p) PBS, (h) Hanks BSS, (s) 0.33 m sucrose plus 10^{-4} m Mg^{2+}, and (v) a different trypsin buffer was used at each passage.

![Graph 2](image2.png)  
**Fig. 2.** The effect of trypsin buffer on the growth of human embryonic diploid lung fibroblast cells. Trypsin solutions (0.25%) were made in the following media and used to suspend diploid cells from the glass surface. (e) Complete Eagle's medium without calf serum, (X) complete Eagle's medium with 4× amino acids and vitamin concentrations but without calf serum, and (I) 0.5% lactic albumin hydrolysate in Hanks BSS.
The attachment of cells suspended in PBS trypsin was not as good as those cells suspended in other trypsin buffers. The best and least variable results occurred with Eagle's medium, Eagle's medium plus 4× amino acids and vitamins, and LAH. In most cases the adsorption was high for the first 40 cell divisions, and then the results became highly erratic.

The effect of trypsinization temperature. Culture series were trypsinized at 40, 37, 30, 22, and 4 °C in 0.25% trypsin in Hanks buffer, pH 7.3 (Fig. 4). The cells trypsinized at 40 °C showed a marked reduction in longevity although the growth rate was initially similar to the other cultures. Cells trypsinized at 37 to 22 °C showed identical growth rates and longevity. The 22 °C culture was lost accidentally after the ninth passage. Cells trypsinized at 4 °C had the same growth rate as the others but a much greater longevity.

The time required for all the cells to be suspended at 4 °C was frequently only 5 to 10 min longer than at 37 °C. Cells could be suspended at 4 °C also in PBS buffer or heat-inactivated trypsin in PBS but only after 1 to 4 hr of incubation in the refrigerator and with vigorous shaking. The cells which were suspended in both of these media showed good adsorption but poor growth when passed to a new culture.

The effect of trypsinization temperature on cell adsorption can be seen in Fig. 5. The best adsorption values were obtained with cells trypsinized at 4 °C. Cells trypsinized at 22 °C also gave good results. Between 30 and 37 °C the values were similar but not as good as at lower temperatures. Trypsinization at 40 °C had an adverse effect on the ability of cells to attach to glass.

The effect of initial trypsinization of lung tissue on subsequent cell growth. Lung tissue fragments from a human embryo were divided into approximately equal portions. They were trypsinized under identical conditions with 0.25% trypsin in PBS, Hanks BSS, sucrose, Eagle's medium, and distilled water. The last trypsinization was done in error but a few cells survived and slowly grew out. After this initial trypsinization all the cultures were trypsinized with 0.25% trypsin in Hanks buffer, at pH 7.3 (Fig. 6).
The cells originally trypsinized in distilled water had a lower growth rate than the other cultures and a shorter longevity.

Cells originally trypsinized in PBS and sucrose grew almost identically and became senescent after about 57 cell divisions.

The cells originally trypsinized in Hanks BSS grew well for the first 60 days (40 cell divisions), and then the growth slowed markedly and the cells stopped growing after about 48 cell divisions.

The cells originally trypsinized in Eagle's medium also grew well but became senescent after 51 cell divisions.

**The effect of antibiotics in the trypsin solution of cell growth.** The reports of Ludovici et al. (8, 9) suggested that some antibiotics and amino acids in the trypsin solution may profoundly influence the cell population. This possibility was tested with a HEDLF strain different from that used in the other experiments reported in this paper. One culture series was treated with 0.5% trypsin preparation in Hanks BSS containing 100 units/ml of penicillin, 100 μg/ml of streptomycin, 1 μg/ml of fungizone, and 1 μg/ml of aureomycin. A control culture series was treated with 0.5% trypsin in Hanks BSS without antibiotics (Fig. 7). The growth medium contained penicillin and streptomycin also, but it did not contain fungizone or aureomycin. Although the resulting growth rates were the same, the control cells stopped growing and degenerated long before the culture series treated
with antibiotic trypsin. No signs of cell alteration or population changes were observed, but the conditions employed in this experiment were not the same as used by Ludovici et al. (8, 9).

**DISCUSSION**

The suspension of cells from the glass surface represents an interruption of the growth process and exposes the cells to manipulations which can be damaging. The usual procedure of treating cells with trypsin has been demonstrated to be a source of cell damage (4, 6, 10, 11), a contributing factor in cell alteration (8, 9), and a possible cause of chromosome abnormalities (5). The whole procedure may introduce artifacts which affect cell growth. For systematic studies with diploid cells and the production of as large a cell population as possible for vaccine production, it is important that this procedure be standardized. One factor of great importance is the selection of a batch of trypsin which is nontoxic and shows good activity.

It has been observed that the type of buffer in which the trypsin is dissolved is of some importance (Fig. 1, 2, and 3). The best growth and adsorption was obtained when media such as Eagle's or LAH were used as the trypsin buffer. Cells trypsinized in PBS without Ca²⁺ or Mg²⁺ gave poor adsorption values, implying that more cell damage occurred than with other buffers, even though the resulting growth was good.

The slightly worse growth results obtained when different buffers containing trypsin from the same batch were used with each passage (Fig. 1 and 3) illustrate the importance of treating these cells consistently during their in vitro life time. Perhaps the buffers influence the type and extent of damage the cell receives during trypsinization. As the cells age above 40 cell divisions, they may be less capable of adjusting to these different types of damage.

The temperature of trypsinization which yielded the best growth results was 4 C (Fig. 4), probably because at this low temperature cell damage was kept to a minimum. The readiness with which cells could be suspended at 4 C was surprising because the rate of enzyme activity should have been greatly reduced. The possibility exists that the enzyme was present in such a large excess that all or most of the substrate became saturated with enzyme from the beginning, and the cells were suspended at a rate almost independent of temperature. It is possible that a study of the trypsin-temperature kinetics on diploid cells may yield some information about the relative quantitative change in cell attachment to glass as the cells age in vitro. It was observed also that the lower the trypsinization temperature the higher was the subsequent cell adsorption (Fig. 5).

The results obtained with cells derived from tissue trypsinized in different buffers and treated identically thereafter (Fig. 6) suggest that cell damage experienced at this primary stage may greatly influence the subsequent growth and life expectancy of these cells. This point is particularly illustrated by the poor growth response of cells derived from tissue originally trypsinized in distilled water, although this treatment may have selected a population of slower growing cells with a short lifetime. Similarly, past experience has shown that cells in passage which encounter a toxic substance or damaging situation and then recover frequently do not grow as fast or as long in subsequent passages as similar cells which never experience these situations. The shorter longevity of the cells from tissue originally trypsinized in Eagle's medium or Hanks BSS and the better adsorption of cells from tissue originally trypsinized in PBS cannot be explained at present.

The prolonged growth of cells suspended with trypsin containing antibiotics (Fig. 7) suggests that these substances may have some subtle effect on cell physiology. It has been found that gentamicin in the culture medium increased the growth potential of diploid cells (7), and if penicillin and streptomycin were removed from the medium the growth rate of diploid cells was reduced (unpublished results). It is possible that the antibiotics may have greater activity on cells when mixed with trypsin since the enzyme makes cells more permeable (11).

At present, suspension of cells by enzyme treatment and passage to new culture bottles represent the best means of handling diploid cells. Any attempt to standardize the diploid cell culturing system for virus vaccine production should include standardization of the trypsin, the buffers, and the procedures used for suspending cells from the glass surface.

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