High-Yield Method for Dispersing Simian Kidneys for Cell Cultures

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A technique for dispersion of animal tissue cells is described. The proposed technique is based on the concomitant use of trypsin and disodium ethylenediaminetetraacetate (EDTA). The use of the two dispersing agents (trypsin and disodium EDTA) markedly enhances cell yield as compared with the standard cell dispersion methods. Moreover, significant reduction in the amount of time required for complete tissue dispersal, presence of a very low number of nonviable cells, less cell clumping, and more uniform monolayer formation upon cultivation compare favorably with the results usually obtained with the standard trypsinization technique.

Primary monolayer simian kidney cell cultures are widely used as substrates for replication of viruses, production of vaccines, and as bioassay systems for detection of viruses. The expense involved in the procurement, maintenance, and screening of animals makes it highly desirable to recover maximum cell yields from each processed kidney.

Introduction of trypsin by Rous and Jones (14) for dispersion of plasma clot cell cultures was followed by numerous other enzymatic techniques for preparation of cell suspensions. In practice, most enzymatic procedures are variants of a method proposed by Dulbecco (4), by Dulbecco and Vogt (5), or its modification by Youngner (17). Apart from the enzymes, various chelating agents were employed to effect cell dispersion. The later category includes disodium ethylenediaminetetraacetate (EDTA; reference 16), a compound widely used for the dispersion of cell culture monolayers. It was suggested that concomitant use of trypsin and disodium EDTA may significantly exceed the cell yields commonly obtained when either of these dispersing agents is used singly (12). The purpose of this communication is to describe a technique for a concomitant application of trypsin and disodium EDTA to obtain high yield dispersion of simian and rabbit kidneys as well as embryonic tissues.

MATERIALS AND METHODS

Source of tissue. Rhesus and vervet monkey kidneys were excised from animals weighing approximately 2 kg; the rabbit kidneys were obtained in a similar manner by using animals of different ages. After exsanguination of the animals which had been anesthetized with sodium pentothal, the kidneys were removed and placed in containers with Hanks balanced salt solution (BSS), 0.5% lactalbumin hydrolysate (w/v), 2% calf serum (v/v), 200 units of penicillin, and 200 μg of streptomycin per ml. Rat, mouse, and hamster embryos of different gestation stages were also used.

Stock trypsin solution (20×). One hundred grams of trypsin (1:250, Difco) was added to 2 liters of warm (37 C) calcium- and magnesium-free Dulbecco's BSS. The suspension was stirred at 37 C in a 3-liter Ehrlenmeyer flask with a 6-cm Teflon-coated magnetic bar. Maximum speed was maintained for 3 to 4 hr until the dispersion was almost clear. The solution was then sterilized by filtration through a membrane filter (Millipore Corp., Bedford, Mass.), with GS prefiter 0.45- and 0.22-μm filter pad assembled together as a unit. To eliminate excess of Triton X-100 from the pads, the sterile membrane filter system was flushed with 2 liters or more of double distilled water at 90 C before use (1). Washing is unnecessary when filters free from Triton X-100 are used. The sterile trypsin solution was kept frozen (15 to 90 days) at −15 C until used. Any precipitation that appeared upon thawing disappeared when the solution was warmed to 37 C.

Stock disodium EDTA solution (400×). An 8-g amount of disodium EDTA (ACSS0311 Fisher) was dissolved in 100 ml of calcium- and magnesium-free Dulbecco's BSS and sterilized by passing through a 0.22-μm membrane filter (Millipore Corp.).

The solution to be used for dispersing tissues was prepared immediately before use. Fifty milliliters of 5% stock trypsin and 2 ml of 8% stock disodium EDTA solutions were added to 800 ml of calcium- and magnesium-free Dulbecco's BSS. This gave a
final concentration of 0.25% trypsin (w/v) and 0.02% disodium EDTA (w/v) solution at pH 7.2, with an osmolarity of 373 milliosmols.

Growth media. Growth medium was Hanks BSS containing 0.5% lactalbumin hydrolysate (w/v; 10). Calf serum (2%) was added to grow rhesus cells, whereas 5% calf serum was added to grow vervet and all other cells. The media were also sterilized with membrane filters as described.

Dispersion procedure. The capsule and calyces of the kidneys were removed and discarded. The remaining renal tissue was minced, weighed, and placed in a flat-bottomed trypsinization flask containing a Teflon-coated magnetic bar, as described by Rappaport (13). Minced embryo tissues were processed similarly. Approximately 125 ml of warm (37°C) dispersing solution was added for each 10 g of tissue. The flask was then placed on a magnetic stirrer and the tissue suspension was vigorously stirred, just below the foaming speed. Fifteen-minute intervals were maintained between decanting of the dispersed cells. Dispersion continued until all parenchymal tissue was exhausted. The whole process was carried out at room temperature. The supernatant was discarded when it contained many red blood cells.

At the end of each dispersion and before decanting, the undisplaced pieces of tissue were allowed to settle in the flask for about 1 min. Supernatant fluid was then passed through a double layer of cheesecloth into a 200-ml round-bottomed centrifuge bottle containing 100 ml of Hanks BSS, 0.5% lactalbumin hydrolysate (w/v) with 2% calf serum (v/v). After each interval, the amount of dispersing mixture was reduced by 5 to 10% to maintain a fairly constant proportion of tissue to the dispersing agents. The suspension was centrifuged as soon as available at room temperature for 10 to 20 min at 90 × g. Centrifugation was done in an International Centrifuge (model V, size 2) with a no. 256 head and 250-ml cups.

Immediately after centrifugation, the supernatant was discarded and the sedimented cells were dispersed in growth medium by vigorous pipetting. The cells obtained during each dispersion interval were pooled into approximately 300 ml of growth media per pair of kidneys and kept in suspension, at room temperature, by gentle agitation with a magnetic stirrer.

Cell counts and viability assays were performed by using the trypan blue exclusion method (9). When a cell clump was present, only those cells which could be individually identified within the clump were counted. Viable and nonviable cells were counted and the percentage of stained nonviable cells was determined. Two chambers of the Spencer hemocytometer with an area of 18 mm² were counted for each sample. After counting, the cell suspension was diluted with the growth media appropriate to obtain the desired cell concentration and seeded by means of a Cornwall syringe.

When different dispersing methods were used for comparison, the minced kidney tissue was divided into samples of equal weight, each to be treated with one of the dispersing agents in the manner just described.

RESULTS

Table 1 records the cell yields obtained in 10 experiments when samples of rhesus monkey kidneys were treated (i) with trypsin; (ii) with trypsin-disodium EDTA by the technique described above; and (iii) with trypsin as in (i), but using the method of Youngner (17), in which a final centrifugation of all the cells in a conical graduate tube was made to obtain the total packed-cell volume. After this, the cells were suspended and counted as described above.

Results reported in the literature with simian kidneys are shown in Table 2. Also shown are the results obtained in our laboratory during routine dispersion of a total of 3,300 g of simian kidney.

### Table 1. Cell yield with rhesus monkey kidneys

| Method of tissue dispersion | Cells per gram of tissue ± standard error | Amt of dead cells (%) ± standard error |
|-----------------------------|----------------------------------------|--------------------------------------|
| (i) Trypsin 0.25% in PBS   | 96 ± 15                                | 15 ± 9                               |
| (ii) Trypsin 0.25%; EDTA 0.002% in PBS | 146 ± 20                           | 8 ± 6                                |
| (iii) Trypsin 0.25% in PBS (Youngner) | 76 ± 15                                | 30 ± 10                              |

* Values to be multiplied by 10⁴.

### Table 2. Monkey kidney cell yields reported by different authors

| Author                  | Avg cell number per pair of kidneys* | No. of cells per gram of tissue* | Seeding cell/ml | Amt of cell suspension (ml per pair of kidneys) |
|-------------------------|--------------------------------------|---------------------------------|-----------------|-----------------------------------------------|
| Results of this study   | 1,310                                | 132                             | 0.3             | 4,400                                         |
| Ganchevici et al.       | 565.6                                | 56.5                            | 0.09            | 7,070                                         |
| Dobrova (2)             | 900                                  | 100                             | 0.3             | 3,000                                         |
| Wallis et al. (15)      | 705                                  | 83                              | 0.3             | 2,380                                         |
| Mironova et al.         | 1,017                                | 113.7                           | 0.3             | 3,400                                         |
| Rappaport (13)          | 900                                  | 100                             | 0.3             | 2,400                                         |
| Kammer (8)              | 1,470*                               | 90                              | 0.21            | 7,000                                         |

* Nine grams of tissue per pair of kidneys. Values to be multiplied by 10⁴.

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* Fifteen grams of tissue per pair of kidneys.
Fig. 1. Random field of 7-day culture fixed with 10% Formalin and stained with Giemsa. A 30-ml Falcon plastic flask was seeded with 5 ml of cell suspension of $75 \times 10^3$ cells per ml. $\times 178$.

Fig. 2. Random field of 7-day culture fixed with 10% Formalin and stained with Giemsa. A 30-ml Falcon plastic flask was seeded with 5 ml of cell suspension of $150 \times 10^3$ cells per ml. $\times 178$. 
kidney tissues for over a 2-year period. No significant differences in the rhesus and vervet renal tissue cell yields were found: $131 \times 10^4$ cells/g and $133 \times 10^4$ cells/g, respectively, for rhesus and vervet kidneys. The average values for rhesus and vervet cell yields are reported in Table 2.

Figures 1 to 3 show microphotographs of cultures seeded with different concentrations of cell suspensions. In all cases, a complete sheet containing $6 \times 10^5$ cells/cm² was obtained in 7 days. With an inoculum of $28 \times 10^3$ cells/cm² ($300 \times 10^3$ cells/ml), a confluent cell sheet was obtained in 5 days.

Six experiments performed with rabbit kidneys gave the following results. When young rabbits were used (3 g per pair of kidneys), yields of $75 \times 10^4$ cells per g and $120 \times 10^4$ cells per g were obtained with trypsin and with trypsin-disodium EDTA, respectively. However, when adult rabbits were used (6 g per pair of kidneys), the respective yields were $37 \times 10^4$ and $76 \times 10^4$ cells per g of tissue. No viable cells were obtained when disodium EDTA was used alone.

The time required for the tissue processing with the trypsin-disodium EDTA method was 40% less than with trypsin alone. For instance, to disperse three pairs of simian kidneys by using trypsin alone, it was necessary to perform 8 to 10 dispersion cycles. With the trypsin-disodium EDTA method, the complete dispersion was obtained in only four or five cycles.

Marked increases in the speed of tissue dispersion and cell yield were obtained also with embryonic tissue, although no quantitative assays were performed.

**DISCUSSION**

It appears that the cell yields obtained with the proposed trypsin-disodium EDTA cell dispersion method were far in excess of the cell yields reported by other workers (Table 2).

The cell yield values obtained during routine dispersions (Table 2) were somewhat lower than the corresponding cell yields in 10 controlled experiments (Table 1, ii). This discrepancy in the results may be attributed to the less exacting conditions of routinely conducted dispersions.

The increase of cell yields obtained with the trypsin-disodium EDTA method was not due to systematic errors in the count, as reflected by the fact that diluting the cell suspension to as low as $7 \times 10^4$ cells per ml of confluent cell monolayers were still obtained in 7 days. Apart from the increase in the cell yield, fewer dead cells and cell clumps were noted. These data compare favorably
with the results obtained with the method of Youngner. Decreases in the percentage of dead cells could most likely be attributed to: (i) reduction in the duration of the dispersion procedure, or (ii) the elimination of the cell centrifugation in a conical tube for determination of the total cell volume. This centrifugation kills almost 20% of the cells (Table 1, iii), as compared when this centrifugation is omitted (Table 1, i).

Cell cultures routinely prepared with the trypsin-disodium EDTA technique have been in use for over 2 years by different laboratories of Division of Biologics Standards, mainly for viral studies. No detectable differences were noted as compared with cells prepared with other methods. The cell monolayers prepared with trypsin-disodium EDTA dispersion have less debris and a more uniform cell layer, consequently making it easier to observe cytopathogenic effects.

It was observed that young rabbits gave a much higher cell yield than the kidneys from older animals. These results are in agreement with the data by Dosser et al. (3) for simian kidneys.

No attempt is made to explain the mechanism of increased cell yield obtained with the trypsin-disodium EDTA method. In spite of the fact that disodium EDTA has been used for dispersing monolayer cultures of established cell lines, we were not able to disperse renal tissues with disodium EDTA alone, and a few cells obtained were dead, most likely due to the extended time required to effect even partial dispersion. This appears to be in agreement with the results obtained by Easty and Mutolo (6), who likewise were unable to disperse liver tissue and ascites tumor islands with disodium EDTA alone.

It is concluded that the proposed technique offers definite advantages in insuring high yield dispersion of renal tissue.

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