Preparation and Cultivation of Primary Human Amnion Cells*

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Human amnion tissue is readily available to most laboratories as a source of cells for tissue culture studies. It has proved to be a useful tool in the field of virus research, and it offers important potentials in the field of cancer research as a source of normal human cells easily obtained in primary cultivation. Many investigators have been concerned with the problem of malignant properties of cells in serial cultivation. Recent investigations from this laboratory (3, 7) have described the development of strains of amnion cells in serial cultivation. The comparison of the biological properties of such strains of amnion cells with their prototype cell in primary cultivation may lead to some understanding of the origin and development of cells in serial cultivation and of the development of possible malignant properties during continued cultivation.

A preliminary report (8) from this laboratory first described the cultivation and poliovirus infection of human amnion cells from the membranes from ten normal deliveries. We have continued to explore ways to simplify and improve the preparation of human amnion cells, to increase the volume of cultures for virus assays (2) and virus-cell studies (1), and to utilize the cells as a source of normal human tissue for the comparison of the biological characteristics of primary and established strains of human cells in tissue culture (7). We describe in this publication an improved and modified procedure for the primary cultivation of human amnion cells based on experience during the last year with over 400 membranes. This procedure allows a high degree of flexibility in the collection of the membranes, their storage and trypsinization, and in the culture and maintenance of the cells before experimentation. Other laboratories have also modified amnion preparation to meet their specific requirements (4–6).

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MATERIALS AND METHODS

Collection.—Membranes were used routinely from uncomplicated vaginal deliveries, and they yielded good cultures of cells when collected with the following precautions: No medication was used in the vagina before delivery, and the sterile basin, instruments, and rubber gloves were free of detergents and disinfectants. If possible, it is advisable to manipulate the afterbirth with instruments rather than rubber gloves. The basin was held directly beneath the vagina to avoid possible contamination.

Two methods of collection were used: (*a*) The entire afterbirth was transferred directly into a sterile 2-liter beaker and covered immediately with sterile gauze and paper. The beaker was kept at room temperature and brought within 8 hours to the laboratory where the membranes were dissected. (*b*) A member of the hospital obstetrical staff cut the membranes from the placenta and dropped them into a wide-mouthed, cotton-stoppered 250-ml. Erlenmeyer flask containing 75 ml. of sterile phosphate buffer with 100 units of penicillin and 100 μg streptomycin/ml. Such membranes were kept at room temperature in the hospital up to 12 hours after delivery.

Dissection, washing, and storage.—In the laboratory, the membranes were placed in a sterile basin, and the amnion was teased from the chorion with sterile, bent-tip forceps. The amnion was transferred to a petri dish (150 mm. in diameter) and washed in several changes of phosphate buffer to remove as much blood, mucus, and extraneous material as possible. After washing, the intact amnion was (*a*) trypsinized immediately, (*b*) stored at room temperature for as long as 24 hours in phosphate buffer, or (*c*) placed overnight at room temperature in 0.1 per cent trypsin phosphate buffer solution. Tyrode's solution (4 × glucose) was used most frequently for collection, washing,

storage, and trypsin dilution, although no apparent differences were observed when Dulbecco's or Hanks' phosphate buffer was used.

Trypsinization and centrifugation.—

1. When the intact amnion was to be trypsinized immediately, it was spread out in a large petri plate containing 50 ml. of 0.25 per cent trypsin (Difco, 1:250) in phosphate buffer solution. Much residual blood and debris settled to the bottom of the dish, and after 1 hour the membrane was transferred to a 500–750-ml. Erlenmeyer flask containing 100–200 ml. of 0.25 per cent trypsin solution. The flask was either left at room temperature or incubated at 37° C. It was shaken either continuously on a rotary mechanical shaker (100 r.p.m.) or manually at several intervals. When the flask was at room temperature and shaken manually, the cells were liberated in 3–4 hours. When it was shaken mechanically or incubated at 37° C., the cells were liberated within 2–3 hours.

2. When the membranes were stored at room temperature in a 500-ml. Erlenmeyer flask containing 100 ml. phosphate buffer, the buffer was decanted and replaced with 0.25 per cent trypsin solution. Within an hour, the flask was shaken, and the trypsin which contained blood and debris was discarded and replaced. The flask was then shaken either manually or mechanically and was either left at room temperature or incubated at 37° C. The cells were liberated in 2–4 hours.

3. When membranes were stored overnight in a 500-ml. Erlenmeyer flask containing 100 ml. of 0.1 per cent trypsin solution, the flask was not shaken before the solution was discarded and replaced with 0.1 per cent or 0.25 per cent trypsin solution. After the fresh trypsin solution was added, the cells were sometimes released immediately upon shaking or within 1 or 2 hours.

Digestion was considered complete when the trypsin solution became clouded with cells and the membrane appeared transparent. The suspension of liberated cells was decanted into 100–250-ml. centrifuge tubes. The membrane was washed with several changes of buffer or with trypsin buffer solution to collect any remaining cells. The tubes were centrifuged at 1,000 r.p.m. (International Centrifuge No. 1) for 10 minutes. The sedimented cells were resuspended in buffer, pooled into 15-ml. tubes, and recentrifuged at 600 r.p.m. for 5 minutes to determine the total cell pack. A single amniotic membrane yielded a 1–3-ml. cell pack (2–6 $\times$ 10^8 cells, hemocytometer counts).

Dilution of cell pack.—The cells were examined microscopically before they were diluted with the various growth media discussed subsequently. Well rounded, optically clear cells which appeared singly or in small packets yielded the best cultures in the shortest period of time. Occasionally, membranes yielded cells which were angular and highly granular. Since a very low percentage of such cells adhered to the glass and since growth to a confluent sheet required 4–6 weeks, these preparations were discarded.

The cell pack was diluted 1:180 with growth medium (approximately 1 $\times$ 10^6 cells/ml). Petri plates (60 mm. in diameter) were seeded with 5 ml., diphtheria toxin bottles (Corning # 590, 2-liter) with 100 ml., milk dilution bottles with 10 ml. (Corning # 615), and culture tubes ($\frac{5}{8}$″ $\times$ 6″) with 1-ml. aliquots of the cell suspension.

Culturing and maintenance of primary and secondary cultures.—All cultures were incubated at 37° C. The petri plates were exposed to a humidified atmosphere containing approximately 5 per cent CO_2 and the medium was changed at 3–4-day intervals. A confluent sheet of cells was attained in 5–10 days. In bottle and tube cultures, growth was comparatively slower than in petri plates. The medium was changed every 7 days unless there was a marked drop in the pH. The cells formed a confluent sheet in 7–14 days. Cultures could be held in good condition for 2–3 weeks, with routine changes of the medium.

Secondary cultures were prepared from cells grown in diphtheria toxin bottles. The medium was removed and replaced with 50 ml. of versene (Disodium versenate, Versenes, Inc.; 1:5,000 in Ca- and Mg-free phosphate buffer). After 15 minutes, 50 ml. of 0.25 per cent trypsin solution was added to each flask for an additional 15 minutes. The cell suspension was centrifuged at 600 r.p.m. for 5 minutes, and the cell pellet was resuspended in growth medium. Petri plates were seeded with 2–3 $\times$ 10^6 cells, which formed a confluent sheet in 48 hours and which remained in good condition for 2–3 weeks.

RESULTS

Growth media.[1]—Studies were made of the effects of various media containing homologous and heterologous sera (20 per cent) with and without the addition of 2 per cent chick embryo extract (EE) on the comparative growth of cells from the same amnion. The results shown in Table 1 indicate that Parker's medium 199 with or without EE supported growth more frequently than did Earle's balanced salt solution to which 0.5 per cent

[1] Phosphate buffers, media, chick embryo extract, and monkey serum were prepared in this laboratory. Pooled human serum was purchased from Courtland Laboratories, Los Angeles; ox serum and lamb serum were obtained from a San Francisco slaughter house; horse serum was obtained from Cutter Laboratories, Berkeley.

lactalbumin hydrolysate and 0.1 per cent yeast extract were added (LY), with or without the addition of EE. Human and lamb sera gave equally good results, whereas ox and horse sera were less effective.

In five similar experiments, cells from the same preparation were grown in medium 199 with 5 per cent, 10 per cent, and 20 per cent human serum with and without the addition of chick embryo extract. Growth of primary and secondary cultures was equally good in each of these media.

The record of the routine preparation of 371 membranes during a 10-month period is shown in Table 2. If the contaminated preparations are disregarded, a comparison of the growth-promoting properties of the media shows that over 80 per cent of the membranes yielded good cultures in medium 199 + human serum with or without EE. The media composed of LY + human serum, 199 + horse serum, 199 + ox serum, and Earle's lactalbumin + ox serum supported growth in about 50 per cent of the preparations.

TABLE 1

COMPARISON OF VARIOUS MEDIA ON THE GROWTH OF AMNION CELLS

Number of experiments showing good growth out of six experiments performed in each case.

	Serum†			
Medium*	Human	Ox	Horse	Lamb
199	6	3	3	6
199+EE	6	4	4	6
LY	3	1	1	1
LY+EE	3	0	0	0

* 199 = Parker's medium 199.
EE = 2 per cent chick embryo extract.
LY = Earle's balanced salt solution containing 0.5 per cent lactalbumin and 0.1 per cent yeast extract.

† Twenty per cent serum was use.

DISCUSSION

Human amnion cells have been cultivated successfully in this laboratory for the last 2 years. Sporadic contaminations with yeast and bacteria have occurred. During two separate periods of time, membranes yielded angular, granulated cells which grew poorly or not at all. This coincided with the use of detergents in the delivery room. The cells appeared to be very sensitive to such agents, and every effort should be made to avoid the use of detergents and disinfectants in connection with basins, instruments, and gloves used in the collection of the membranes. Out of a total of 450 membranes which have been processed, there were five which, for some unknown reason, yielded no cells.

Studies of different media were designed to determine optimal growth conditions of the cells. Medium 199 plus 20 per cent human serum with or without the addition of chick embryo extract gave the most consistent results and was adopted as a standard. The animal sera available to us gave variable results from lot to lot.

It was also important to determine whether the culture conditions influenced the susceptibility of the cells to virus infection and the ability of the cell sheet to be maintained under the agar overlay.

TABLE 2

RECORD OF GROWTH OF AMNION CELLS DURING A 10-MONTH PERIOD

	No. of membranes			
Medium*	Total	Growth	No growth	Contaminated
Human serum+199	9	7	2	
" + "+EE	204	133	29	42
" +EL	4	3	1	
" +LY	38	14	14	10
Ox serum+199	19	9	9	1
" +EL	58	20	17	21
" +LY	7	3	4	
Horse serum+199	17	6	5	6
" +LY	2	1	1	
Monkey serum+EL	7	3	2	2
Lamb serum+199	2	1	1	
Puck's medium	4	3		1
Total:	371	203	85	83

* Twenty per cent serum was used in all media.
199 = Parker's medium 199.
EE = 2 per cent chick embryo extract.
EL = Earle's balanced salt solution containing 0.5 per cent lactalbumin.
LY = Earle's balanced salt solution containing 0.5 per cent lactalbumin and 0.1 per cent yeast extract.

Preliminary experiments with poliovirus showed that routine plaque assays on cultures of different ages (5 days to 2 weeks), cultures of primary and secondary cells, and cultures grown on different media (confluent plates from experiments shown in Table 1) gave comparable results. Although cells grew equally well in varying percentages of human serum, those grown in 5 per cent serum did not withstand the agar overlay satisfactorily.

Medium with the addition of EE has been used extensively in this laboratory, since it was found advantageous in experiments in progress on the serial cultivation of human amnion cells (7).

Preparations of amnion cells vary in the total yield of cells, the subsequent growth, and cellular morphology. The growth varies in that cells from some membranes grow in almost any medium, whereas others will grow only in a selected few media. Most of the membranes yielded epithelial cells of uniform size but with varying percentages (0.1–1.0 per cent) of giant cells. Occasionally, cul-

tures contained elongated, fibroblastic cells with interspersed patches of epithelial cells.

Human amnion tissue is being used as a source of material for the detection, primary isolation, diagnoses, assay, growth, and production of viruses. The amnion offers a relatively large source of normal human tissue which is routinely available and which can be prepared in primary cultivation directly on glass. The flexibility in the methods of preparation and in the growth media makes the applicability of the tissue widespread in the many biological fields in which tissue culture is employed.

SUMMARY

A modified and flexible procedure is reported for the preparation and cultivation of cells from the normal human amnion. Disinfectants and detergents were found detrimental when used for rinsing the vessels, gloves, and instruments used in collection, and they were avoided. The amnion could be stored at room temperature up to 36 hours after delivery. The intact amniotic membrane was digested with trypsin-buffer solution, and only one extraction was necessary. Petri plate cultures required an atmosphere of approximately 5 per cent CO_2. Confluent cultures in petri plates, tubes, and bottles were maintained for several weeks with routine changes of the medium. Although the cells grew in a variety of media, more consistent results were obtained with Parker's 199 + 20 per cent human serum with or without 2 per cent chick embryo extract than with other synthetic media plus human or animal sera.

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