Transformation of Cells from the Normal Human Amnion into Established Strains*

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Previous communications from this laboratory have reported the cultivation and poliovirus infection of cells from the normal human amnion (11), the cytological changes associated with poliovirus infection (3), and the continuous cultivation of a strain of cells (5). The present paper describes the transformation of cells from four normal human amnions into seven established, rapidly growing strains. These observations were made over a period of 8-14 months.

MATERIALS AND METHODS

The amniotic membranes were prepared and the cells cultivated by procedures developed in this laboratory and already reported (4). Technics for poliovirus assay and cytological studies have been described (2).

All primary cultures were prepared in petri plates, incubated at 37°C., and exposed to a constant atmosphere of approximately 5 per cent CO₂ in air.

Medium 199, Earle's balanced salt solution plus 0.5 per cent lactalbumin and 0.1 per cent yeast extract (LY), Eagle's medium, Tyrode's solution (containing 4 × the usual amount of glucose), and trypsin solution (0.25 per cent) were prepared in this laboratory. The chick embryonic extract (EE) was made from homogenized 10-day-old embryos, diluted with an equal volume of Tyrode's solution. The extract was centrifuged and stored at −60°C. All media and solutions, including media containing EE, were sterilized by Selas filtration (#03 porosity) to insure the absence of microbial contaminants as well as any viable cells from the EE. Penicillin (100 units / ml) and streptomycin (50 μg/ml) were added to the media. Pooled human serum was purchased at the Courtland Laboratories, Los Angeles; ox serum, lamb serum, and beef amniotic fluid (BAF) were collected at the San Francisco slaughter house; horse serum was obtained from Cutter Laboratories, Berkeley.

RESULTS

Preliminary studies on the growth and maintenance of amnion cells showed that the most consistent results were obtained with media containing 20 per cent human serum and 199, with or without the addition of EE. These studies also showed that the total cell population did not increase, even after the first transfer, although the cells remained in a healthy and viable condition for a study period of 1 month. These observations suggested: (a) that less favorable conditions (lowering the serum content of the media) might force adaptation of the cells which apparently had no challenge to proliferate, or (b) that a large cell population (transferring the cultures plate for plate) might permit a selective process to develop.

To explore these possibilities, four experiments were performed under identical culturing conditions in which:

1. Cells from a freshly collected membrane were used for each experiment.
2. The primary cultures were prepared in petri plates seeded with approximately 2 X 10⁶ cells in 5 ml culture medium.
3. Twelve culture plates were made for each of the following six culture media: (a) 20 per cent human serum, 4 per cent EE, 76 per cent medium 199; (b) 20 per cent human serum, no EE, 80 per cent medium 199; (c) 10 per cent human serum, 2 per cent EE, 88 per cent medium 199; (d) 10 per cent human serum, no EE, 90 per cent medium 199; (e) 5 per cent human serum, 1 per cent EE, 94 per cent medium 199; (f) 5 per cent human serum, no EE, 95 per cent medium 199.
4. Six cultures from each medium group were infected with poliovirus Type II (MEF-1) at the end of the 1st week to establish their viral susceptibility.
5. The six remaining cultures in each medium

* Aided by the American Cancer Society Institutional Grant 48H.
† U.S. Public Health Service Research Fellow of the National Cancer Institute.

Received for publication July 1, 1957.
group were transferred weekly; the cells were removed with 0.25 per cent trypsin solution and reseeded plate for plate (1:1) in their respective culture media.

There were four distinct stages of cellular development. The first stage was common to cultures in each medium group, but only those cultures prepared with 20 per cent human serum and 4 per cent EE underwent one or more subsequent stages. The fourth stage was marked by transformation of the cells into established strains with altered characteristics.

Stage I; stable population (Fig. 1).—Cells in each medium group remained equally healthy and viable after the weekly plate for plate transfers. The cultures maintained a constant cell population. The cell sheet appeared uniformly confluent, although dead or floating cells were always present in the medium.

The primary cultures from each medium group were susceptible to poliovirus infection and produced virus, as measured by standard plaque assays in the range obtained with HeLa cells (2, 3).

The primary cultures displayed a loose mosaic pattern of uniformly epithelial cells of varying sizes, interspersed with an occasional fibroblast. The cytoplasm was finely granular, the cell membrane was often indistinct, but no syncytium was noted. The nuclei were round or oval and contained one to three large, irregularly shaped nucleoli. Mitotic cells were rare or completely absent. Some binucleated cells were observed in all cultures. Gross examination of Feulgen-stained cultures disclosed a marked degree of polyploidy.

This morphological picture was retained by all cultures in each medium group for a period of about 2 months (seven to eight transfers). At this time the cells with medium containing 20 per cent human serum and 4 per cent EE altered abruptly and radically to fibroblast-like cells and entered Stage II. However, cells in the other five medium groups showed no cellular alterations, and two additional transfers were performed before the cell sheet underwent a marked sloughing. The few surviving cultures were discarded at the end of the 8th month.

Stage II; growing population (Fig. 2).—The fibroblast cells were cultured with the same medium, 20 per cent human serum and 4 per cent EE. They grew luxuriantly and were subcultured weekly at a 1:5 dilution into tubes, flasks, and bottles.

The cultures formed a dense network of spindle-shaped, predominantly mononucleated cells of varying sizes, interspersed with many mitotic forms. The cytoplasm was finely granular, the nuclei were oval and contained one or two well-defined, large, irregular nucleoli. The cells were capable of poliovirus infection and produced virus as measured by standard plaque assay in the range obtained with HeLa cells.

The cultures maintained the same rate of growth (based on weekly 1:5 dilution) and cellular morphology for a period of 2–2½ months (eight to ten transfers). The growth phase ended almost as abruptly as it had begun, the cells failed to proliferate after subculturing, mitotic forms became rare, and the cells entered Stage III.

A few fibroblast cultures were prepared at the beginning of Stage II with medium containing 20 per cent human serum and 80 per cent 199 (the embryonic extract was omitted). They were subcultured 3 times before the lag phase set in, and they entered Stage III several weeks earlier than the other cultures.

Stage III; decreasing population (Fig. 3).—Cells from the various culture containers were pooled into Earle's T60 flasks (3:1 concentration) in an effort to maintain a large population. No mitotic forms were seen, many of the cells died, and within a period of 3 weeks about 75 per cent of the cultures were lost.

The cells appeared more and more enlarged and thinly spread, extending 60–200 μ. The clear cytoplasm displayed a reticulum of delicate, parallel fibers, and a fine granular halo surrounded the nuclear area. Although no mitoses were noted, some cells developed ten to thirty nuclei, morphologically similar to those in the primary amnion cells. However, certain of these cells contained an occasional nucleus filled with small, dense aggregates of nucleolar material.

New media combinations were prepared for the cells. The serum content was raised and lowered (40 per cent, 10 per cent, 5 per cent); beef amniotic fluid was used at various concentrations in the human serum-199 media (10 per cent, 20 per cent, 40 per cent, 80 per cent); LY and Eagle's medium were combined with human serum (20 per cent); media containing 199 were fortified with additional glucose to give a final concentration of 0.5 per cent; the EE was increased to 8 per cent. Although these media maintained the cells, they failed to stimulate any active response. The lag period persisted for 2–3 months, whether the cells were carefully nursed with frequent washing and media replacement or neglected and allowed to remain for weeks without any attention. Radical alterations in cellular morphology and growth rate occurred at this time, and the cells entered Stage IV.

Stage IV; establishment of cell strains (Fig. 4).—The newly formed, altered cells were first observed...
in small colonies of eight to twenty cells, one or two in a culture flask. Within a month four out of six cultures from the first membrane had transformed, and one out of six from the second membrane. The remaining cultures from both of these membranes, as well as cultures from two subsequent membranes, have not altered. The transformations occurred at approximately the same culturing intervals, irrespective of the medium with which the cultures were prepared, as recorded in Table 1.

The colonies of altered cells were always found adjacent to large, polynucleated cells. These large cells were extremely motile, the cytoplasmic outlines changed constantly, and there was a marked shifting of the nuclei from a compact group, often eccentrically located, to a loosely aligned arrangement, unevenly distributed throughout the cell. which grew only on the surface of the flask in a tight mosaic; A4 contained somewhat elongated cells which grew in a loose mosaic; A5 exhibited more heterogeneity than the other strains, forming either tight or loose mosaics, and contained many multinucleated and giant cells; A5 consisted of flattened, very clear cells arranged in a loose mosaic.

The cytological and growth properties of the newly established strains were studied, and the five cell lines were compared, simultaneously, with primary amnion, the parent cell line; strain FL, derived from human amnion cells (5); and strain HeLa, a long established laboratory standard.

Cytological properties.—All eight cell lines had epithelial-like cells of varying sizes, with one or more round or oval nuclei. Gross examination of Feulgen and toluidine blue-stained cultures indicated that all cell lines were heteroploid. The cytological properties of the five sublines were similar, but they differed in one or more aspects from those of primary amnion, FL, and HeLa, as shown in Table 2.

<table>
<thead>
<tr>
<th>Amnion no.</th>
<th>Transfer Strain no.</th>
<th>Medium*</th>
<th>Weeks in cultivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A185†</td>
<td>21</td>
<td>A1</td>
<td>20 per cent human serum, 4 per cent EE</td>
</tr>
<tr>
<td>A2</td>
<td>21</td>
<td>A2</td>
<td>20 per cent human serum, 4 per cent EE</td>
</tr>
<tr>
<td>A3</td>
<td>23</td>
<td>A3</td>
<td>20 per cent human serum, 4 per cent EE</td>
</tr>
<tr>
<td>A4</td>
<td>11 †</td>
<td>A4</td>
<td>20 per cent human serum, 4 per cent EE</td>
</tr>
<tr>
<td>A203 †</td>
<td>14</td>
<td>A5</td>
<td>20 per cent human serum, 4 per cent EE</td>
</tr>
</tbody>
</table>

* 199 was added to make 100 per cent.
† Amniotic membranes are numbered consecutively upon arrival in this laboratory.
‡ Culture prepared without EE as described under Stage II.

Microscopic examinations were made at 2- to 3-hour intervals, and the marked area with its newly formed colony and large cell was described and drawn. These observations indicated that nuclei containing aggregates of small, coarse nucleolar material moved toward peripheral, tongue-like protoplasmic extensions and appeared to nip a small portion of the cytoplasm as they ruptured from the cell.

The newly formed cells showed a high incidence of mitosis, they grew vigorously, and within 2 weeks the cells had overgrown the flasks. Subcultures were performed, and the "strains" were easily established. All strains were cultured in 20 per cent human serum and 199 unless otherwise indicated. Although all the cultures displayed a heterogeneous assortment of cells, each strain presented a distinctive morphological picture. A1 formed a small, tight mosaic on the surface of the flask, as well as profusely growing clusters of cells suspended in the medium; A2 had larger cells...
cells from each of the four stages, and from FL and HeLa cells. The averages from four sets of measurements of 100 randomly selected cells are shown in Chart 1.

Primary amnion cultures displayed cells of relatively uniform size and distribution. Subcultured cells from the two intermediate stages showed an increase in the number and size of large cells. Cells from the newly established strains, as well as from strains FL and HeLa, were more similar in size and distribution to the cells from the primary cultures.

Effect of heterologous sera.—The effects of various sera were studied by preparing cultures from each strain with 20 per cent human, horse, or ox serum, and 199. The cells were subcultured weekly for ten transfers at a 1:10 dilution. Primary amnion was not included. All cell lines grew well but displayed different morphological pictures, as shown in Table 3.

Effect of storage.—Culture tubes from all cell lines, containing approximately $2 \times 10^6$ cells in 5 ml. culture medium (20 per cent human serum 199), were stored at 4°C, and weekly aliquots of 0.5 ml. were taken to seed duplicate tubes for a period of 4 consecutive weeks. Primary amnion did not withstand even the 1st week of storage. The seven other cell lines were capable of proliferation even at the end of 4 weeks. The percentage survival was highest with strain HeLa, and the cultures exhibited a heterogeneous population of cells. Strain FL had a higher percentage survival than the new sublines, but it and the other strains showed a more homogeneous cell population, suggesting that a selective process had occurred.

Additional membranes studied.—

a) Cultures prepared with embryonic extract:
they have remained for the last 8 months, enlarged, often multinucleated and dormant.

b) Cultures prepared without embryonic extract: Transformed cells appeared in cultures from two membranes in which the cells did not pass through Stages II and III. Cells from membrane A369 (A6) were cultured with 20 per cent human serum and 199 in a diphtheria toxin bottle and were transferred 3 times in the next 2 months, at a 1:1 dilution. There was a decrease in the cell population, as described for cultures prepared without EE, and the cells were transferred changed once a week, and the cells remained healthy and viable. They became increasingly granular over a period of 3 months but showed no other alterations in either morphology or growth rate. At this time two rounded macroscopic colonies were observed in one culture plate. Microscopic examination disclosed that these colonies were composed of transformed amnion cells. They were heterogeneous and increased rapidly in number. The cells were transferred, after 1 week, at a 1:4 dilution, and two cultures were prepared with 20 per cent lamb serum and LY, and two

<p>| TABLE 3 |
| EFFECT OF HETEROLOGOUS SERA |
| (See Figures 11-18) |</p>
<table>
<thead>
<tr>
<th><strong>Cell line</strong></th>
<th>Human serum</th>
<th>Horse serum</th>
<th>Ox serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>Loose or tight mosaic.</td>
<td>Clumped, tight mosaic.</td>
<td>Loose mosaic.</td>
</tr>
<tr>
<td>A2</td>
<td>Elliptical, well defined, clear, fine granules, compact.</td>
<td>Elliptical, well defined, granular, compact, small.</td>
<td>Flattened, elongated, well defined, clear, thin cell sheet.</td>
</tr>
<tr>
<td>A5*</td>
<td>Amoeboidal, poorly defined, coarse granules, spread.</td>
<td>Clumped, tight mosaic.</td>
<td>Tight mosaic.</td>
</tr>
<tr>
<td>HeLa</td>
<td>Loose mosaic.</td>
<td>Amoeboidal, poorly defined, coarse granules, spread.</td>
<td>Small, cuboidal, very granular, heavy cell sheet.</td>
</tr>
</tbody>
</table>

* A5 grew very slowly with ox serum and was transferred at a 1:5 dilution.

| TABLE 4 |
| STAGES OF CELLULAR GROWTH IN VARIOUS MEDIA |
| **Amnion** | **Medium A** Stages | **Medium B** Stages | **Media C, D, E, F** Stages | **Medium LY Lamb** Stages |
| (no.) | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| A185 | × | × | × | × | × | × | × |
| A206 | × | × | × | × | × | × | × |
| A213 | × | × | × | × | × | × | × |
| A234 | × | × | × | × | × | × | × |
| A305 | × | × | × | × | × | × | × |
| A369 | × | × | × | × | × | × | × |
| A400 | × | × | × | × | × | × | × |

* A5 grew very slowly with ox serum and was transferred at a 1:5 dilution. The cells were not subcultured, and the medium was changed twice weekly, and no gross alterations were observed in morphology or growth rate during the following 6 weeks, when transformation occurred abruptly in one culture plate. The newly formed cells gave rise to a heterogeneous population, similar in many aspects to the A185 strains. The remaining cultures showed no alterations during the next month.

Cells from another membrane (A400, A7) were cultured with 20 per cent lamb serum and LY to test the effect of nonhuman serum on the maintenance of amnion cells preparatory to virus isolation studies. The 36 petri plate cultures were fluid-
ured by standard plaque assays, in the range obtained with HeLa cells.

DISCUSSION

Workers in the tissue culture field have achieved cellular outgrowth from a wide variety of normal and malignant primary explants. For the most part such cells have undergone a number of serial transfers but are incapable of unlimited survival. The cultures enter a lag phase and display an absence of mitotic configurations, a complete indifference to any new stimuli, and an ever decreasing cell population. Occasionally, however, a small colony of “altered” cells appears quite spontaneously in a culture flask. These colonies show a high incidence of mitosis, grow very rapidly, and when subcultured will produce a hardy, established “strain.” Such strains, whether of normal or malignant origin, are composed of predominantly heteroploid cells (6–8).

Hsu (6) offers this postulation for such phenomena: “Heteroploids are more able to adapt to the in vitro condition than the diploids and tetraploids. Perhaps after a number of generations of subculturing, the heteroploids, originally in the minority, supplant other cell types and become the new stem line.”

Beatty (1) reported that amnion tissue in situ displayed variations or “inconstancy” in chromosome numbers. Our gross examinations of Feulgen-stained primary amnion cells in vitro showed a marked degree of polyploidy.

Petry and Damminger (9), in an extensive study of the structure of the human amnion, in situ, found that the different sections of the basal membrane were composed of columnar, cuboidal, or squamous epithelium, and that the membrane was backed with a delicate network of fibroblasts. In their examination of 15,000 cells they found wide variations in cellular and nuclear volumes and an absence of the metaphase and anaphase stages of mitosis. These authors suggested that the amnion cells divided amitotically.

Our observations of amnion cells, in vitro, showed that primary cultures contained epithelial cells of varying sizes, interspersed with an occasional fibroblast, and that mitotic configurations were very rare. Efforts to study the stages of mitosis and the origin of the mitotic cells (epithelial or fibroblastic) were unsuccessful, owing to the absence of dividing cells in cultures prepared for these purposes. Certainly optimal conditions were not achieved to promote an increase in the cell population. Although dead cells were always present in the amnion cultures, the number of viable cells remained constant. We are unable to explain whether the replacement of the cells was effected by mitosis, at such a low rate that it was not observed, or by amitosis.

Westwood, MacPherson, and Titmuss (10) achieved transformation of cells from human fetal liver, rabbit embryonic kidney, monkey kidney, and monkey testis into established strains in a time range of 26–85 days. In our studies, transformation of amnion cells to established cell lines was first observed after 8 months of cultivation, during which time the cultures maintained a stable rather than a multiplying population. The rate of cellular multiplication may be associated with the ability of cells to transform into established cell strains and may explain the variations in the time at which transformation occurs.

The influence of the culture media must also be considered. We found that amnion cells transformed into established cell strains at different time intervals with various media. Cultures from two membranes, prepared without embryonic extract, in media containing homologous and heterologous sera, yielded cells with altered characteristics in 3–4 months (A6–7). There were no gross morphological variations to herald such phenomena. These newly formed cells appeared spontaneously, suggesting the selection or mutation of a cell type within the cultures. Fogh and Lund (5) found that amnion cells, cultured without embryonic extract, transformed into an established strain (FL) after 8 months.

However, cultures prepared with embryonic extract underwent four clearly distinguishable stages of cellular development and gave rise to established cell strains in 6 months (A1–5). Various combinations of human serum, embryonic extract, and 199 were tested on parallel cultures from each of six membranes. All cultures prepared with 20 per cent serum and 4 per cent EE passed through one or two intermediate stages, but, of these cultures, cells from only two membranes transformed into established cell lines. It is suggested that the cul-

Fig. 1.—Stage I: Primary amnion cells, ×470, hematoxylin and eosin.

Fig. 2.—Stage II: Fibroblast-like cells, ×470, hematoxylin and eosin.

Photographs by Mr. Victor Durand.
Fig. 3.—Stage III: Polynucleated cell with parallel fibers, $\times$270, hematoxylin and eosin.

Fig. 4.—Stage IV: New cell strain with enlarged dormant cells. Note change in nuclei. $\times$270, hematoxylin and eosin.

Photographs by Mr. Victor Durand.
Fig. 5.—Strain A4, X100, hematoxylin and eosin.
Fig. 6.—Strain A1, X100, hematoxylin and eosin.
Fig. 7.—Strain A2, X100, hematoxylin and eosin.
Fig. 8.—Strain A5, X100, hematoxylin and eosin.
Fig. 9.—Strain FL, X100, hematoxylin and eosin.
Fig. 10.—Strain HeLa, X100, hematoxylin and eosin.
FIG. 11.—Strain HeLa, human serum, ×152, unstained.
FIG. 12.—Strain A1, human serum, ×152, unstained.
FIG. 13.—Strain FL, human serum, ×152, unstained.
FIG. 14.—Strain A5, human serum, ×152, unstained.
FIG. 15.—Strain FL, horse serum, ×152, unstained.
FIG. 16.—Strain A1, horse serum, ×152, unstained.
FIG. 17.—Strain HeLa, ox serum, ×152, unstained.
FIG. 18.—Strain A5, ox serum, ×152, unstained.
Photographs by Mr. Victor Durand.
ture medium may affect the selection of a specific cell type as well as the time of transformation.

Amnion cultures prepared with 20 per cent human serum, 4 per cent EE, and 199, which had retained a constant population of apparently epithelial-like cells for a period of 2 months, altered radically after the seventh or eighth transfer and displayed actively growing fibroblast-like cells. It is possible that these cells were the outgrowth of the occasional fibroblasts observed in primary cultures and that they were able to supplant the epithelial cells through favorable culturing conditions: the few original cells were saved by plate-to-plate transfers, the medium was possibly "conditioned" by the epithelial cells, and the embryonic extract stimulated their proliferation. Some epithelial cells probably remained in these cultures but were masked by the dense network of fibroblasts. The sudden end of this phase was marked by a 75 per cent decrease in the cell population.

The reticular pattern and spindle shape of the cells were replaced by scattered, loosely arranged patches of enlarged, flattened, nondividing cells, which were criss-crossed with delicate fibers and polynucleated. Some cells developed ten to 30 nuclei, morphologically similar to those of the primary cells. Certain of these, however, contained numerous, small, irregular aggregates of nucleolar material. Such nuclei appeared to shift toward the cell boundary, into protoplasmic extensions, possibly of their own making, and erupt from the cell with a small portion of cytoplasm. Newly formed cells, which gave rise to established strains, were found adjacent to such large, polynucleated cells. We have not confirmed these observations and conjectures with time-lapse photography, nor have we determined whether the transformed cells are of epithelial or fibroblastic origin.

It is suggested that the amnion cell may provide an experimental means to study the transition phase in the establishment of a cell strain.

**SUMMARY**

Human amnion cells in vitro did not increase in number over a period of 2-3 months but maintained a stable population, morphologically similar to the primary cultures.

Some of these cells were capable of transforming into rapidly growing, altered cell lines. Seven cell strains were derived from cultures of four of the eight amniotic membranes used in these studies. Transformation occurred with various media and at various time intervals. In cultures prepared without embryonic extract, newly formed cells appeared spontaneously after 8-10 months, whereas in cultures containing embryonic extract the cells passed through two clearly distinguishable intermediate stages, yielding altered cells after 6 months.

It was not determined whether the transformed cells were of epithelial or fibroblastic origin.

It is suggested: (a) The culture medium may influence the time at which transformation occurs and the selection of a specific cell type within a culture. (b) The amnion cell may provide a means to study the transition phase in the establishment of a cell strain.

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Cancer Res 1957;17:1047-1053.

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