Colonial Study of Cells from the Human Female Genitourinary Tract*

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SUMMARY

Cells from human cervix, vagina, Fallopian tube, and kidney tissue, fetal or adult, were obtained by tryptic digestion and maintained in vitro by serial massive-inoculum passage for a few passages. Colony-forming capacity of cells of early passages was examined directly and by colony-to-colony passage. Viability of cells so revealed was low and not sustained by selection of viable cells. In colonial populations different colony forms were seen according to cell form, orientation, and susceptibility to tryptic dispersion. One colony form seen commonly with cervical cultures was “fibroblast-like,” but no colonies were analogously “epithelial-like.” Colony formation was affected variably by use of mixed serum supplement (bovine fetal and human adult), individual donor source of human serum, basal medium in relation to source of cells, and cell attachment conditions in relation to cell source. When optimum serum supplement and basal medium were employed, effect of medium pH (or bicarbonate concentration) was seen, but an optimum could not be established within narrow limits. The chief conclusion is that, with appropriate medium, colonial culture can be applied to a study of cells from the non-neoplastic human female genitourinary tract.

Cultivability of human cells obtained by tissue biopsy, as shown by Hsu and Kellogg (16) and the potentiality of colonial culture (Puck et al. [30]) allow use of in vitro culture not only as a source of cells but as a method for quantitative characterization of human cells as they exist in tissue in health and disease. Appreciation of the significance of cultural properties of cells of abnormal origin requires knowledge of cells of non-pathological origin for comparison. In relation to this need, interest of one of us (G. T. M. C.) in human cervical cancer (5) led to a study of cells in culture from the human female genitourinary tract. This report describes analysis of colony generation by cells in early-passage culture, morphology of cells and colonies, and effects of some cultural conditions on establishment of cells on glass and their multiplication into colonies.

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MATERIALS AND METHODS

Media.—Media employed included (a) two phosphite-buffered salt solutions (K1R, K1D) for washing and diluting of cells; (b) a phosphite-buffered glucose-supplemented salt solution (K2G); (c) a basal medium (KBM) buffered with 20 mm phosphite at pH 7.3 and containing fructose, glucose, Eagle vitamins, lactalbumin hydrolysate, and other components (40); and (d) an enriched medium of KBM mixed 1:1 with Difco Medium 199 (26). Basal medium contained 50 μg/ml neomycin sulfate. Tests with a human cell strain had shown that 10 μg/ml of this antibiotic in our medium reduced relative plating efficiency about 50 per cent. Plating efficiency with adult cervical cells was not affected by 10 μg/ml of neomycin, as prescribed by others (16). Purchased bovine fetal serum and human adult serum from a blood bank were sterilized by sintered-glass filtration and stored frozen. Enzymes (trypsin 1-300, Nutritional Biochemicals Corp., and U.S.P. pancreatin, Parke, Davis & Co.) were used in 0.2 per cent (w/v) concentration for tissue treatment and 0.1 per cent or less for dispersal of cultivated cells.

Cell sources.—Samples of nonpathological hu-
Fetal tissue made available by therapeutic abortion of human adult cervix, vagina, and Fallopian tube were obtained as biopsy specimens, tissue from reparative surgery or tissue from hysterectomy. Human fetal tissue made available by therapeutic abortion represented the 16th to 26th week of gestation and was not associated with known fetal pathology. Specimens obtained in neomycin-supplemented salt solution were washed twice, minced, rewarshed, suspended in enzyme solution, and incubated at 37°C with agitation. Colony generation tests indicated that 1 hour or less of incubation was sufficient for maximum yield of viable cells and that trypsin or pancreatin were somewhat more efficient dispersing agents than lipase. From these results, trypsin was used to disperse tissue, and trypsin or pancreatin to disperse cultures. Dispersed cells were pipetted, diluted with 5 per cent (v/v) bovine fetal serum in diluting solution, and, if from tissue, sedimented at low speed and rediluted. Cells were counted at first with a hemocytometer and later with a Coulter electronic counter.

Culture technic.—Stock cultures of cells from tissue were prepared by inoculating 60-mm. Pyrex dishes heavily with cell suspension, replacing medium after 48 hours of incubation to remove unattached cells, and replacing medium completely on days 4, 7, and 10. New cultures were started from at least 10⁴ cells if available, on day 11. Replicate stock cultures provided inocula for most experiments. Test cultures were prepared from well dispersed, counted cells inoculated into dishes containing 5 ml. of medium prewarmed and equilibrated with incubator atmosphere (2.5 per cent carbon dioxide in humidified air). Medium pH was adjusted by addition of sodium bicarbonate as described (40). For colony growth, cultures usually were incubated for 12–14 days at 36.5°C, without medium change. Cell colonies were isolated for dispersal with aid of a dish-orienting device at the stage of an inverted microscope, and greased glass cylinders. Experiments were done with duplicated cultures. Culture medium pH was estimated immediately on removal of cultures from the incubator, by visual comparison with stable pH indicators and sensitive flowmeters.

Observations.—Unstained cells in cultures were counted by microscopic scanning. Cells were stained with modified Wright stain for final colony counts; macroscopically visible groups of cells were counted as colonies unless otherwise noted. Chromosome counts were made by J. Clausen according to a described procedure (4).

RESULTS

Colony and cell morphology, and chromosome number.—Sampled cells were from fetal or adult vagina, cervix, Fallopian tube, and kidney and therefore presumably represented varied mixtures of cells of epithelial and fibroblastic origin. In primary or following culture no colonies were seen of cells resembling those in the epithelial-like sheets of cells commonly seen to migrate from explants of epithelial tissue. All cells established on glass were more or less stretched, rather than polygonal. Colonies of cells from the various tissues included three types differentiable on the basis of cell shape, cytoplasmic areal size, and arrangement of cells. All colonies were classified as "macrocytic": cells were relatively large as stretched, did not accumulate in heaps, and showed some degree of growth orientation in most instances. Colonies from adult cervix or vagina represented the first type: cells were elongated and aligned (Fig. 1), so that macroscopically the colonies appeared "feathery" (Fig. 14). Increase in cell density within such colonies resulted in accentuation of cell alignment and compression, to give a whorly appearance. Colonies from fetal kidney tissue represented the second type: cells were shorter in stretched length, and fusiform to triangular in shape. In some colonies cells stained densely and were arranged loosely without marked orientation (Fig. 2). In other colonies lighter-staining cytoplasm was apparent, some alignment was evident, and increase in cell density within the colony produced a peculiar overlayering (Fig. 9). Colonies of the third type were seen in cultures of fetal Fallopian tube, which also yielded colonies resembling kidney colonies in loose or lacy arrangement of cells (Fig. 4). The typifying colonies were distinct in circularity and exhibition of relatively entire border (Fig. 13). With staining, colonial structure was disrupted by cell retraction. Staining revealed for some of the cells a fan shape or the appearance of a flipped-over fried egg (Figs. 5, 6). The colony types were distinguishable secondarily by reaction to application of trypsin. Colonies of the first type (Fig. 14) were refractory to tryptic dispersal. The other colony types were affected by trypsin more or less quickly at room or incubator temperature. An illustrative extreme difference in reaction to trypsin has been seen with colonies from fetal testicular tissue (Figs. 7, 8); the colonies described here varied similarly but less. With serial passage of stock cultures, particularly of adult cervical tissue cells, progressive increase in stretched areal...
size of cells was seen. Larger (in this sense) cells were seen first at the outer border of colonies (Fig. 9). In later-passage cultures "megacytic" colonies were seen (Fig. 10), and, finally, cells appearing singly or in small groups were so large as to be visible macroscopically (Figs. 11, 12). Numbers of cells in megacytic colonies or cell groups always were small.

Representative counts of chromosomes per cell from cultures of the tissues studied indicated persistence of some cells with 46 chromosomes (Table 1). Chromosome form was predominantly metacentric or submetacentric.

Analysis of colony formation.—Initial experiments with cells dispersed directly from adult cervix and Fallopian tube gave plating efficiencies (colonies per 100 inoculated cells) of 0.1 or less. To initial failure of dispersed cells to become attached to glass was a possible but not major factor in low incidence of colony formation (Charts 1, 2). The low incidence of cells able to generate colonies large enough to be finally scored as such reflected failure of cells to survive continued incubation and progressively to achieve successive divisions. The sampled cells represented a spectrum of viability (ability to multiply) and not a simple mixture of completely inviable and completely (or indefinitely) viable cells. Use of larger inocula increased the incidence of "successful" growth centers, but these generated monolayers with continued incubation, rather than large colonies surrounded by single surviving cells.

This implication of a continuous rather than discrete distribution of viability in the studied populations was analyzed further, by deliberate selection for viability. From colonial cultures 10 large colonies were removed and the constituent cells dispersed. The cells from each colony were inoculated into a separate dish and grown into new colonies with growth medium at pH 7.3. or 7.8. This procedure was repeated serially, to segregate "lines" of cells progressively separated from their original relatives by division and progressively selected for capacity to generate large colonies. Such colonies were estimated to include 10⁸ or more cells. This segregative selection did not yield sustained increase in viability (Table 2).

With cultures of adult cervical cells, detailed analysis of viability was achieved as the limit of measurement of viability was approached. Serial passages 5, 8, and 9 of the stock culture showed plating efficiencies of 31, 9, and 2 per cent, respectively. At passage 9, incidence of colony generation was not dependent on medium volume within fivefold variation, or inoculum density within eightfold

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**TABLE 1**

**CHROMOSOME COUNTS FOR CELLS IN EARLY-PASSAGE CULTURE FROM NORMAL HUMAN GENITOURINARY TRACT TISSUE**

<table>
<thead>
<tr>
<th>Source no.</th>
<th>Donor</th>
<th>Tissue</th>
<th>Culture passage</th>
<th>Chromosome count range</th>
<th>No. cells with 46 chromosomes/no. cells examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Adult</td>
<td>Cervix</td>
<td>5</td>
<td>45–82</td>
<td>10/22</td>
</tr>
<tr>
<td>2</td>
<td>Adult</td>
<td>Cervix</td>
<td>7</td>
<td>40–75</td>
<td>2/7</td>
</tr>
<tr>
<td>3</td>
<td>Adult</td>
<td>Fallopian tube</td>
<td>2</td>
<td>41–&gt;100</td>
<td>8/21</td>
</tr>
<tr>
<td>4</td>
<td>Fetal</td>
<td>Fallopian tube</td>
<td>4</td>
<td>45–47</td>
<td>16/28</td>
</tr>
<tr>
<td>5</td>
<td>Fetal</td>
<td>Fallopian tube</td>
<td>5</td>
<td>46–47</td>
<td>1/2</td>
</tr>
<tr>
<td>6</td>
<td>Fetal</td>
<td>Vagina</td>
<td>0</td>
<td>45–80</td>
<td>12/14</td>
</tr>
<tr>
<td>7</td>
<td>Fetal</td>
<td>Vagina</td>
<td>8</td>
<td>41–&gt;100</td>
<td>4/2/15</td>
</tr>
<tr>
<td>8</td>
<td>Fetal</td>
<td>Kidney</td>
<td>1</td>
<td>45–77</td>
<td>1*3</td>
</tr>
</tbody>
</table>

* Actual counts = 45–47 ± 2.
CHART 1.—Multiplication of human adult cells of the female genitourinary tract in early-passage culture after dispersion from tissue with trypsin. Cultures were grown in KBM medium with 10 per cent each of human adult and bovine fetal serum, replaced after 48 hours and at 3-day intervals. Dishes were scanned on days 2, 5, and 8. Number of cells per growth center (single cell or cell group) was counted, and counts were ranked and grouped.

CHART 2.—Multiplication of human fetal cells of the female genitourinary tract in early-passage culture after dispersion from tissue with trypsin, analyzed as in Chart 1.
variation (Table 3). Representing passage 10, three colonies of a colonial culture of passage 9 (Fig. 15) were each dispersed and the constituent cells propagated. As illustrated for the progeny of one colony (Fig. 16), none of the sampled cells generated a scorable colony, although many of the cells survived and enlarged until macroscopically visible. This survival of cells descending from viable cells, rather than disappearance, confirmed loss of viability as ability to multiply.

Effect of medium serum supplement on colony

TABLE 2
INHERITANCE OF VIABILITY IN COLONIALLY SEGREGATED LINES OF CELLS IN EARLY-PASSAGE CULTURE FROM NORMAL HUMAN GENITOURINARY TRACT TISSUES

<table>
<thead>
<tr>
<th>Tissue source</th>
<th>Culture passage</th>
<th>Colonial passage*</th>
<th>pH 7.3</th>
<th>pH 7.8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Fetal Fallopian tube†</td>
<td>2</td>
<td>1</td>
<td>100‡</td>
<td>36‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Adult Fallopian tube</td>
<td>3</td>
<td>1</td>
<td>38</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fetal vagina</td>
<td>2</td>
<td>1</td>
<td>87</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fetal kidney</td>
<td>2</td>
<td>1</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Colonial passage was done by growing dispersed cells from single selected colonies of the parental passage, and serially selecting and propagating colonies from colony progeny; cultures were grown in KBM medium with 10 per cent each of human adult and bovine fetal serum.
† Fetal tissues were obtained from single fetus.
‡ Number of macroscopically visible progeny colonies after 10 days of incubation.

generation.—Initial experiments suggested that colony formation by cells from the studied tissues was dependent on use of mixed serum supplement in medium, and a differential effect of culture medium pH (or bicarbonate concentration). Variability of human adult serum used in the mixed supplement was shown with fetal vaginal cells. Samples of serum from 45 blood-bank donors each were combined in 10 per cent concentration with 10 per cent of previously tested bovine fetal serum in KBM medium at pH 7.3. Cultures were incubated for 14 days without medium change. "Feathery" colonies (the first type previously described) and "dense" colonies (all other types) were counted independently, to distinguish any difference in response. Results (Chart 3) showed that individual sera varied considerably in enhancement of colonial growth, that sera yielding the most colonies yielded the largest number of large colonies, and that the variability of sera differed little with respect to the categories of colonies. Only one large colony appeared among three colonies counted in four control cultures grown only with the bovine fetal serum supplement. Selected human adult serum was essential to relatively good plating of the test cells.

Further experiments with pooled selected hu-
human adult serum and bovine fetal serum confirmed the importance of the mixed supplement as a factor in colony generation, at either low or high medium pH (or bicarbonate concentration). With adult cervical cells, concentrations of from 10 to 30 per cent human adult or bovine fetal serum alone in KBM medium yielded only a few small colonies. The most and largest colonies were produced with 20 or 10 per cent of human adult serum mixed with 10 or 5 per cent bovine fetal serum. Response to the serum supplement varied with the origin of cells; with fetal kidney and vaginal cells in KBM medium, only the kidney cells produced large colonies and only with the mixed serum supplement (Table 4).

The basal medium was another factor in colony generation. With fetal kidney cells, combination of the mixed serum supplement with KBM was sufficient for formation of large colonies, although enrichment with medium 199 increased the number of such colonies (Table 5). In contrast, the enriched medium was necessary for generation of large colonies by fetal vaginal cells. Actual colony counts (Tables 4, 5) and appearance of illustrative colonial cultures (Figs. 17, 18) revealed an interesting feature of cellular response to medium variation. Even in inadequate media, some colonies and an occasional large colony were produced, to suggest cellular heterogeneity. This suggestion was reinforced by the extreme variation in colony counts for duplicate cultures sometimes seen with single serum supplements (Table 4) and not evident with mixed serum supplement (Tables 5–7). Lability of the growth-enhancing activity of the human adult serum did not appear to be a factor in variation in cellular response. With adult cervical cells in mixed serum medium, colony counts were not reduced with samples of human serum stored 41 days at 37° C. or room tempera-

**TABLE 4**

**EFFECT OF SERUM CONTENT OF MEDIUM ON COLONY GENERATION BY CELLS FROM FETAL HUMAN FEMALE KIDNEY AND VAGINA**

<table>
<thead>
<tr>
<th>Per Cent (v/v) Serum in Medium: Human/Bovine Fetal</th>
<th>Colonies Per Dish of Cells From</th>
<th>Kidney 1*</th>
<th>Vagina 4</th>
<th>Vagina 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>0/5</td>
<td>0, 0/0, 0, 0</td>
<td>0, 0/0, 0</td>
<td>0, 0/0, 0</td>
<td></td>
</tr>
<tr>
<td>0/10</td>
<td>0, 0/0, 0</td>
<td>0, 0/0, 0</td>
<td>0, 0/0, 0</td>
<td></td>
</tr>
<tr>
<td>0/20</td>
<td>0, 0/0, 0</td>
<td>0, 0/0, 0</td>
<td>0, 0/0, 0</td>
<td></td>
</tr>
<tr>
<td>0/30</td>
<td>0, 0/0, 0</td>
<td>0, 0/0, 0</td>
<td>0, 0/0, 0</td>
<td></td>
</tr>
<tr>
<td>0/40</td>
<td>0, 0/0, 0</td>
<td>0, 0/0, 0</td>
<td>0, 0/0, 0</td>
<td></td>
</tr>
<tr>
<td>10/0</td>
<td>0, 0/0, 0</td>
<td>0, 0/0, 0</td>
<td>0, 0/0, 0</td>
<td></td>
</tr>
<tr>
<td>20/0</td>
<td>0, 0/0, 0</td>
<td>0, 0/0, 0</td>
<td>0, 0/0, 0</td>
<td></td>
</tr>
<tr>
<td>10/10</td>
<td>20, 3/4, 31</td>
<td>0, 0/0, 0</td>
<td>0, 0/0, 0</td>
<td></td>
</tr>
</tbody>
</table>

* Stock passage.

† Colonies 1 mm. or more in diameter per dish/total of all colonies per dish, visible by low magnification, for duplicate cultures; pancreatinized cells were grown at pH 7.5 in KBM medium. Inocula per dish were 1,000 for kidney, 1,070 for vagina 4, and 1,000 cells for vagina 7.

**CHART 3.**—Correlation between numbers of large (diameter >1 mm.) colonies and total (all macroscopically visible) colonies of passage 2 human fetal vaginal cells after 14 days of growth in KBM medium supplemented with 10 per cent unselected bovine fetal serum and 10 per cent of human serum samples from 84 individual adult donors. Each circle represents counts for a single culture. Large colonies were counted independently as "dense" (more "epithelial-like") or "feathery" (more "fibroblast-like"). Human serum samples associated with yield of less than five colonies are counted by the boxed "12." Four control cultures grown with only 10 per cent bovine fetal serum supplement exhibited 0 feathery colonies, 1 dense colony, and 0, 0, 0, and 3 total colonies.

**CHART 4.**—Correlation between numbers of large (diameter >1 mm.) colonies and total (all macroscopically visible) colonies of passage 2 human fetal vaginal cells after 14 days of growth in KBM medium supplemented with 10 per cent unselected bovine fetal serum and 10 per cent of human serum samples from 84 individual adult donors. Each circle represents counts for a single culture. Large colonies were counted independently as "dense" (more "epithelial-like") or "feathery" (more "fibroblast-like"). Human serum samples associated with yield of less than five colonies are counted by the boxed "12." Four control cultures grown with only 10 per cent bovine fetal serum supplement exhibited 0 feathery colonies, 1 dense colony, and 0, 0, 0, and 3 total colonies.
ture compared with 4°C and -20°C. Serum stored at the higher temperatures produced an effect manifest as some reduction in colony size, but not extreme variation in size.

Effect of bovine fetal serum on cell establishment in culture.—Colony-generation analysis and colony-to-colony passage with cells from different tissues had shown a continuous rather than discrete distribution of viability. This variation in viability could not be attributed to differences in cytologic origin of cells that were heritable. Since large colonies could appear in inadequate media, however, it was possible that cytologic origin did add to the basic variation in viability of the cells. Distinction of colony forms was not objectively reliable enough to resolve this point. Variable effect of serum supplement in relation to tissue origin of cells was examined. "Establishment" of cells in culture was differentiated from multiplication, as attachment of cells to glass coupled with viability as well as physical attachment of cells. Establishment was measured by incubating inoculated cells for 24 hours in medium of itself insufficient for colony formation.

<table>
<thead>
<tr>
<th>TABLE 5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EFFECT OF BASAL MEDIUM ON COLONY GENERATION BY CELLS FROM FETAL HUMAN FEMALE KIDNEY AND VAGINA</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CELLS AND PARENTAL PASSAGE NO.</th>
<th>KSG</th>
<th>KKB</th>
<th>KBM/199</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney 1</td>
<td>1.2/15, 16</td>
<td>25.58/84.74</td>
<td>49.52/77.81</td>
</tr>
<tr>
<td>Vagina 4</td>
<td>0.0/5, 6</td>
<td>0.0/16.40</td>
<td>42.48/79.82</td>
</tr>
<tr>
<td>Vagina 7</td>
<td>0.0/4, 4</td>
<td>0.0/14.17</td>
<td>88.11/22.6</td>
</tr>
</tbody>
</table>

* Medium at pH 7.5 was supplemented with 10 per cent each of human adult and bovine fetal serum. KSG = glucose-supplemented balanced salt solution; KKB = salt solution + fructose, glucose, pyruvate, oxalacetate, Eagle vitamins, and lactalbumin hydrolysate; KBM/199 = KBM + medium 199 in equal volumes.

† Colonies per dish 1 mm. or greater/total colonies per dish for duplicate cultures inoculated with about 1,000 cells and incubated without medium change.

‡ Not done: total colonies were too many for accurate count.

origin of cells was examined. "Establishment" of cells in culture was differentiated from multiplication, as attachment of cells to glass coupled with retention of colony-generation capacity during incubation in medium of itself insufficient for colony formation. Initial observations suggested that bovine fetal serum in medium promoted such survival as well as physical attachment of cells. Establishment was measured by incubating inoculated cells for 24 hours in medium with varied content of bovine fetal serum (least being 0.2 per cent as carry-over from diluting medium) at varied pH and replacing establishment medium with mixed serum growth medium to reveal viability. With fetal vaginal cells, adult Fallopian tube cells, and adult cervical cells, effect of bovine fetal serum on establishment varied, and some variation in effect of medium pH (or bicarbonate concentration) was suggested (Chart 4). A control culture of continuously cultivable microcytic cells with known response showed the expected discrete effect of serum concentration, for comparison (40).

**TABLE 6**

**EFFECT OF GROWTH-MEDIUM PH ON COLONY GENERATION BY GLASS-ATTACHED CELLS OF ADULT HUMAN FEMALE CERVIX AND VAGINA**

<table>
<thead>
<tr>
<th>CELLS AND PASSAGE NO.</th>
<th>PH</th>
<th>Colonies per dish</th>
<th>Controls at PH 7.4†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vagina 4</td>
<td>7.1</td>
<td>155,280</td>
<td>161,156</td>
</tr>
<tr>
<td>Vagina 5</td>
<td>7.2</td>
<td>199,218</td>
<td>198,113</td>
</tr>
<tr>
<td>Vagina 7</td>
<td>7.4</td>
<td>196,123</td>
<td>196,123</td>
</tr>
</tbody>
</table>

* Macroscopically visible colonies per dish, for duplicate dishes of about 1,000 cells incubated 44 hours for attachment and 14 days for growth. Attachment medium = 10 per cent bovine fetal serum in KBM/199; growth medium = KBM/199 + 10 per cent each human adult and bovine fetal serum.

† Colonies per dish for control cells inoculated directly into unchanged growth medium. Both test and control medium pH were determined by variation in the amount of sodium bicarbonate added.

**TABLE 7**

**EFFECT OF ESTABLISHMENT-MEDIUM PH* AND SERUM CONTENT ON ATTACHMENT AND VIABILITY OF SEVENTH-PASSAGE ADULT HUMAN VAGINA**

<table>
<thead>
<tr>
<th>PER CENT (v/v)</th>
<th>SERUM, HUMAN/BOVINE FETAL</th>
<th>COLONIES PER DISH FROM CELLS AT PH†</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.1</td>
<td>7.3</td>
<td>7.8</td>
</tr>
<tr>
<td>0/0</td>
<td>165,159</td>
<td>281,197</td>
</tr>
<tr>
<td>10/0</td>
<td>203,212</td>
<td>226,196</td>
</tr>
<tr>
<td>0/10</td>
<td>270,315</td>
<td>292,290</td>
</tr>
<tr>
<td>10/10</td>
<td>229,292</td>
<td>196,253</td>
</tr>
</tbody>
</table>

* pH was varied by the amount of bicarbonate added.

† About 1,000 trypsinized cells were inoculated into KBM/199 medium with indicated pH and serum content, and incubated 44 hours; establishment medium was replaced with mixed serum growth medium at pH 7.1 and incubation continued.

‡ Many of these colonies showed central retraction.

§ Cultures with growth medium at indicated pH were inoculated directly and incubated without medium change.

With adult cervical cells of the first stock passage from tissue, colonial cultures grown in KBM/199 medium with mixed serum supplement yielded plating efficiencies approaching 30 per cent, with or without prior incubation in establishment
CHART 4.—Effect of medium pH (or bicarbonate concentration) and bovine fetal serum content on attachment to glass and retention of viability by cells incubated at 36.5°C. for 24 hours in KBM medium. Established cells were grown into colonies in medium with mixed serums at pH 7.3. For comparison, cells of a colonially purified line of a continuously propagable strain with known attachment properties also were tested (upper left).
medium with bovine fetal serum. Similar plating efficiencies were obtained with adult vaginal cells. Earlier experiments with mixed serum supplement and directly inoculated cultures had suggested a difference between cervical and vaginal cultures with respect to range of medium pH promoting colony generation. The establishment experiments indicated pH 7.3 as adequate for this phase of colony generation. Adult cervical cells were established with bovine fetal serum medium at pH 7.4, then grown with mixed serum medium at varied pH. These cultures were compared with directly inoculated cultures in mixed serum medium at pH 7.4. Variation in plating efficiency with growth medium pH was not great, and test and control cultures at pH 7.4 showed similar plating efficiencies (Table 6). Under similar conditions response of adult vaginal cells differed in both respects. With vaginal cells in later passage, varied combinations of pH, bovine fetal, and human adult serum yielded good and reproducible preservation of viability during establishment, but no optimum pH between 7.1 and 7.8 (Table 7). If cellular heterogeneity was a factor in variation in colony formation as discussed earlier, its influence was minimized when serum supplements, basal medium, and medium pH were combined to yield increased plating efficiency.

**DISCUSSION**

The single most important factor in successful colonial culture of cells from the human female genitourinary tract as studied here was the use of a mixed serum supplement in the medium. Results with this medium showed that, regardless of tissue origin, cells were heterogeneous with respect to capacity for sustained multiplication. This capacity appeared finite. The nature of any superimposed cellular heterogeneity that might account for observed lessened sensitivity to medium variation with increased plating efficiency was not indicated. Although effects of medium pH were observed, for instance, optimum pH levels for colony formation could not be defined uniquely between 7.1 and 7.8 with medium giving good plating efficiency by cells of different origin. This range corresponds to that found optimum for growth of human strain cells from the human female diploid tissue origin by Mackenzie et al. (24), who dissociated pH effects from effects of bicarbonate variation.

Our incidence of colony formation in primary and following cultures of cells of non-neoplastic origin was of the order reported by Puck et al. (30) and Krooth and Tjio (19) suggests indefinite cultivability of euploid human cells, whereas that of Hayflick and Moorhead (13) is in opposition, in agreement with earlier findings of Swim and Parker (39). Ferguson and Wansbrough (8) reported long-term culture of human diploid cells without establishing a limit of cultivation. Technics employed in these studies differed: for example, Puck et al. (30) emphasized critical selection of serum as a factor in cultivability, whereas Hayflick and Moorhead (13) did not consider serum a crucial influence.

Evaluation of cultural sampling of tissue-cell populations raises the question of recognition of cell types. The report by Konigsberg (18) on formation of primitive muscle fibers by fibroblastic colonies shows that colonial culture is not incompatible with distinctive cell behavior, but it is clear from the studies by Sato et al. (37) and Holtzer et al. (15) that culture can be a highly selective or alternative procedure. Composition of cultures with serial propagation thus may be a critical factor in behavior. Differentiation of cells by colonial morphology could aid desirable monitoring of culture composition, but more exploration is needed. Cell culturists usually describe cultures as epithelial-like or fibroblast-like (38) in reference to features connoted by these terms from histology. Whether the epithelial-like morphology of many cell strains reflects epithelial origin or an effect of aneuploidy (30) is not clear. Distinctive variations in colonial morphology have been described for cell strains, as for instance by Murphy et al. (28), on the bases of cell form and orientation. We have used the "micracytic-macrocytic-megacytic" terminology similarly based (32) to describe animal-cell colonies, to avoid possible misuse of meaningful histologic terms. As yet we have not seen micracytic colonies in any cultures recently derived from non-neoplastic tissue. Our cervical-cell macrocytic colony form (which was feathery, highly oriented, and trypsin-refractory) resembles in appearance the "fibroblast" type already described (29). The work of Castor et al. (2) with cells of this general form from various tissues suggests that the "fibroblast" designation may be significant in terms of common synthetic function, as does demonstration of the fiber-forming capacity of the prototypic L-strain fibroblasts (25). The increase in stretched size of cells accompanying loss of viability, which we call "megacytic" degeneration, was seen by Moser (27) and by Zaroff et al. (42) in their colonial cultures. Some features of the progression resemble those of Phase III of fibroblastic cultures as described by Hayflick and Moorhead (19). This outcome of loss of viability
was important to us in providing positive evidence of loss of ability to multiply, as would not have been provided by mere absence of colonies or cells.

The question of the propagability of cells of non-neoplastic origin is of interest in relation to cancer research because it may permit approach to understanding of some bases of the controlled versus the uncontrolled quality of normal compared with malignant multiplication of cells. Significance of the question depends on interpretation of "viability" of cells, denoting ability to multiply. Usually viability of cell cultures is described in terms of cell yield, number of serial transfers achieved, or period of maintenance of cultures by growth. To compare monolayer culture growth with colonial growth, it is helpful to consider that a single colony 1 mm. in diameter may represent cellular replication on the order of 1,000-fold, or about ten cell generations (31). Three serial colony-to-colony passages achieved in less than 2 months of cultivation may represent a potential gain of one thousand million cells from one cell. By rough calculation, the cellular equivalent of an entire adult man could be generated from an ovum by less than 50 cell generations (10⁸ gm/man x 10⁸ cells/gm = 10¹³ = 2⁴³ cells/man). Since non-neoplastic cell population growth serves to replace rather than accumulate cells, tissue cell viability is best described by the turnover time or time needed for population renewal (21). With a 24-hour generation time, 0.1 per cent of cells able to achieve 10 successive generations, and tenfold decline in incidence of viability with such achievement, complete renewal of the viable component of a population would require about 10 days. This value is compatible with our findings and with estimates of tissue-cell viability obtained by other means (21). The need to consider "viability" in terms of both capacity to multiply and capacity to sustain multiplication stemming from any given viable cell is suggested by the possible alternative states of cells in tissue. Many cells may exist in prolonged interphase, from which new cells emerge by division (20). If differentiation represents variation in phenotypic expression of common genetic endowment (11), the interphase component of a tissue population (as opposed to the differentiated and, therefore, inviable component) may represent a mixture of cells with varied physiologic capacities. Influence of physiologic capacity on viability measured by colony formation was shown by Lockart and Eagle (23) with respect to relation between medium volume and rate of metabolite synthesis. Parabiotic growth of inositol-dependent and -independent cells illustrates the possibility of contribution of cellular heterogeneity to population viability (7). As discussed by Sanford et al. (33), and verified by critical cloning (34) and colonial culture (31), dependence of animal-cell viability on direct intercellular association can be ruled out as a factor in viability, as it was questioned early in the history of cell culture. The limitation of cellular viability shown in the present study was characterized by cellular ability to form colonies that contained cells heterogeneous with respect to viability. Such limitation could not be attributed to physiologic heterogeneity in the sense discussed above (7, 23) because inadequate rates of synthesis would have prevented colony formation, and existence of mixture of wholly capable with incapable cells should have yielded highly viable cells with selective passage. With the adult cervical cells shown heterogeneous in viability, colony formation was not dependent on medium volume or inoculum density, as could be expected. The fact that improvement in medium increased both the absolute number of colonies and the number of large colonies, instead of increasing the size of a constant number of colonies to greater or lesser degree, suggests the possibility of cellular heterogeneity in another respect. Swim and Parker (39) observed limited cultivability of human fibroblastic cultures from various tissues including uterine. From comprehensive study of medium components, these authors concluded that medium deficiency in the ordinary sense was not the limiting factor; they suggested that progressive loss of viability might reflect failure of medium to maintain proportions of variedly physiologically competent and mutually supportive cells as donated by tissue to original cultures. Likewise rejecting simple medium inadequacy, Hayflick and Moorhead (13) in contrast postulated asynchronous duplication of a self-replicating factor essential for cell survival, distributed randomly to cells at division. Limitation of viability was shown here by use of colony-to-colony passage to reduce pre-existing cellular heterogeneity that might be heritable, and to dilute out pre-existing cellular reserves of essential metabolites by generation of new cells. Our results do not rule out the mechanism suggested by Swim and Parker (39) but suggest that disturbance of mutually supportive heterogeneity, if present, is manifest by random distribution of an essential factor. It is difficult otherwise to explain continued formation of colonies with declining frequency. Lack of unequivocally discrete differences in colony morphology of tissue-derived populations independent of staining prevented study of the mechanism suggested by Swim and Parker (39), by use of controlled mixed cultures. What
was needed for this purpose was media extending the limited viability of different cell types while selecting for them; mixed serum medium supplemented with medium 199 increased viability but reduced variation.

Since selection of human adult serum was essential to the demonstrable need for both human adult and bovine fetal serum for colony generation, this need cannot be attributed to unique properties of these sera. Other experience suggests that the mixed serum supplement provides factors that may be provided by either serum alone, if less frequently occurring samples are selected. More than one worker has found bovine fetal serum a useful medium supplement by itself (1). An extensive literature testifies to both a role of serum in cell growth and a number of possibilities with regard to the nature of the role (3, 6, 9, 10, 12, 14, 17, 22, 36). Since serum protein is produced by animal cells, prolongation of the cultivability of euploid cells by critical selection of serum (30), or increasing incidence of viability with selected serum supplements as reported here, does not settle the question of viability of non-neoplastic cells or their heterogeneity. We may be substituting serum for materials elaborated by appropriate mixtures of cells in tissue. Interaction among variedly competent cells of aneuploid or potentially neoplastic nature may also be a factor in their cultivability, although repeated cloning of such culturally established cells (35, 41) suggests the presence of heritably viable and, therefore, potentially infinitely propagable cells.

REFERENCES


Figs. 1—6.—Morphology of human adult or fetal cells in colonies after 12—15 days of incubation at 36.5°C. in medium containing adult bovine cell serum; colonies stained with modified Wright stain, X74.

Fig. 1.—Adult vaginal cells, passage 4.

Fig. 2.—Fetal kidney cells, passage 2.

Fig. 3.—Fetal kidney cells, passage 2.

Fig. 4.—Fetal Fallopian tube cells, passage 5.

Fig. 5.—Fetal Fallopian tube cells, passage 5; cell at left resembles form of flipped-over fried egg.

Fig. 6.—Fetal Fallopian tube cells, passage 5, from same culture as cells in Fig. 5; cells exhibit fan shape with membranous borders.
FIGS. 7, 8.—Human fetal testicular cells in washed colonies under 0.1 per cent trypsin, after 10 minutes at 36.5° C.; unstained, X74. These cells illustrate extremes of differential response to trypsin seen in lesser degree with colonies of female cells. Cells of one colony form rounded quickly (Fig. 7); cells of other colony form were refractory (Fig. 8).

FIGS. 9–12.—Human adult cervical cells, passages 5–9, in colonies grown 12–15 days in mixed serum medium; colonies stained with modified Wright stain, X74. Photographs show progressive changes in cell morphology with passage and loss of viability, including peripheral decrease in degree of alignment within colony (Fig. 9), increase in stretched areal size (Fig. 10), decrease in number of cells in “colonies” (Fig. 11), and final appearance of very large cells (megacytes) visible macroscopically (Fig. 12).
Figs. 13—18.—Colonial cultures grown 12–15 days at 36.5° C. in mixed serum medium; colonies stained with modified Wright stain, X1.3–1.5.

Figs. 13, 14.—Extremes of colony form varying from round, entire edged (Fig. 13, fetal Fallopian tube cells) to irregular, feathery edged (Fig. 14, adult cervical cells). These are forms of colonies of cells illustrated, respectively, in Figs. 5, 6, and 9.

Figs. 15, 16.—Colony-forming capacity of human adult cervical cells after serial passage. Passage 9 culture (Fig. 15) was inoculated with about 1,000 cells and incubated 14 days. Each of three colonies removed from culture, dispersed and incubated 14 days yielded only megacytes as illustrated by culture of one colony (Fig. 16).

Figs. 17, 18.—Form of variation in cell viability in varied medium. Cultures shown were grown from equal inocula incubated in 10 per cent each of human adult and bovine fetal serum in KBM/199 (Fig. 17) or salt solution K2G (Fig. 18). Some colonies including two large colonies are present in inadequate medium, and large colonies are about as large as largest in more adequate medium.
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